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Neuromuscular Imaging and Phenotypical Variation in Pompe Disease

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**Neuromuscular Imaging and Phenotypical Variation
in Pompe Disease**

Neuromusculaire beeldvorming en fenotypische variatie
bij de ziekte van Pompe

Proefschrift

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Stephan Constantinus Antonius Wens
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Chapter 1

General introduction and scope of this thesis

Pompe disease (OMIM 232300) is an inheritable lysosomal storage disorder and was first described by J.C. Pompe in 1932.[1] It is caused by a deficiency of the enzyme acid α-glucosidase (GAA) and leads to glycogen accumulation in various body tissues, predominantly in skeletal muscle.[2, 3] In the Netherlands, the incidence of Pompe disease is 1 per 40,000 births per year.[4] Dutch patients diagnosed with Pompe disease are referred to the 'Center of Lysosomal and Metabolic Diseases' at Erasmus MC University Medical Center, which is the national centre of expertise for this orphan disease. Over the years we have clinically evaluated close to 200 Pompe patients and followed a cohort of over 400 patients worldwide using questionnaires. This has provided us a unique opportunity to study features of Pompe disease in a relatively large group of patients. This introductory chapter outlines the etiologic, pathophysiological, clinical, diagnostic and therapeutic aspects of this rare disorder in relation to the focus of research of this thesis.

ETIOLOGY AND PATHOPHYSIOLOGY

Pompe disease, also known as acid maltase deficiency and glycogen storage disorder type II, is an autosomal recessive disorder caused by mutations in the *GAA* gene (OMIM 606800). This gene is located on chromosome 17 in the region q25.2-q25.3. Because of the autosomal recessive inheritance, both alleles of the *GAA* gene need to carry a pathogenic mutation to develop Pompe disease. Mutations can either have an effect on the biosynthesis, posttranslational modification or intracellular transport of the α-glucosidase protein.[5] To a certain extent, the *GAA* genotype determines the phenotype: a heteroallelic combination of two severe mutations generally results in a complete enzyme deficiency and causes the classic infantile

form of Pompe disease. If one of the two mutations is not fully deleterious some residual enzyme activity remains, resulting in the non-classic or late-onset form of Pompe disease manifesting at any time during childhood or adulthood.[5, 6] Under normal conditions the information on the *GAA* gene is copied onto messenger RNA (mRNA). This mRNA is transported to the cytoplasm and imported into the endoplasmic reticulum where it is translated to a protein and transported into the Golgi complex (110 kDa precursor form of GAA). During this transport, the protein undergoes multiple posttranslational modifications before it attains its full activity as GAA in the lysosomes (76 and 70 kDa mature forms).[7] Lysosomes contain multiple hydrolytic enzymes that are able to break down different macromolecules. Over 40 different lyososmal hydrolases have been identified and one of these hydrolases is GAA.[8] This enzyme is needed for the degradation of glycogen into glucose molecules in the lysosomes. Due to the GAA deficiency in patients with Pompe disease, glycogen accumulates in the lysosomes, eventually resulting in the loss of integrity of the muscle fibers leading to clinical symptoms of muscle weakness.[9]

CLINICAL HETEROGENEITY

Pompe disease has a broad clinical spectrum, as it ranges from severely affected patients with the classic infantile form to mildly affected patients with the non-classic or late-onset form of the disease (Figure 1). In this thesis the term 'non-classic Pompe disease' is used for patients who develop symptoms in childhood or adulthood.[10] Patients with classic infantile Pompe disease have a complete GAA deficiency and present with generalized hypotonia and a characteristic hypertrophic cardiomyopathy soon after birth. If these patients are not being treated with enzyme replacement therapye (ERT) they will not reach major motor milestones such as sitting and rolling over. Eventually, untreated patients will die in the first year of life.[3, 11, 12]

The major part of this thesis concerns patients with non-classic Pompe disease. These patients experience their first symptoms in childhood or adulthood and have a slowly progressive course of the disease.[10, 13, 14]
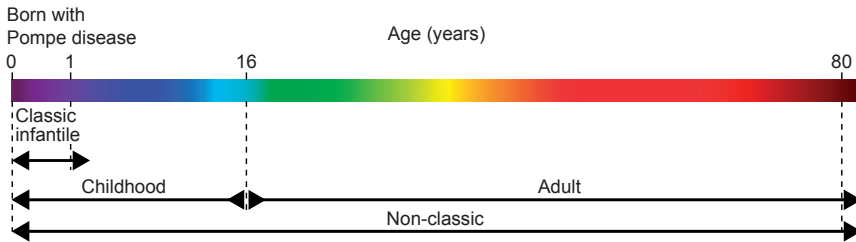
Figure 1 **The clinical spectrum of Pompe disease**

This figure was adapted from the publication written by Güngör and Reuser entitled 'How to describe the clinical spectrum in Pompe disease?' in the *American Journal of Medical Genetics* 2013, by permission of John Wiley and Sons.

Figure 2 **Muscle weakness in adults with Pompe disease**
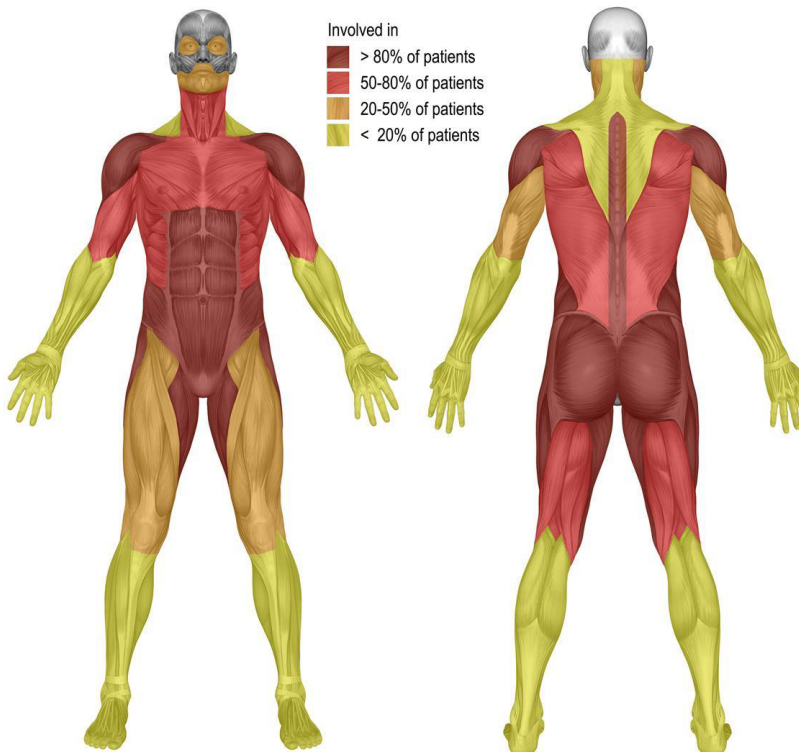
This figure was adapted from the publication written by van der Beek et al. entitled 'Clinical features and predictors for disease natural progression in adults with Pompe disease: a nationwide prospective observational study' in *Orphanet Journal of Rare Diseases* 2012, by permission of BioMed Central.

Limb girdle muscle weakness is the presenting symptom in most patients, leading to difficulties in walking, climbing stairs, rising from a chair and lifting objects (Figure 2). Other less common features that can be present are scapular winging, ptosis and bulbar weakness.[15-19] Without treatment the majority of patients become wheelchair dependent and ventilator dependent during the course of the disease.[14, 18] Particularly in patients with non-classic Pompe disease there is a large clinical heterogeneity. Even in patients sharing the same *GAA* genotype and haplotype, the phenotype varies widely regarding to age of symptom onset and rate of disease progression.[20, 21] This indicates that modifying factors might play a role in determining the course of the disease; e.g. modulating environmental factors, such as exercise and nutrition therapy, have been described to play such a role.[22, 23] In addition, genetic background factors such as polymorphisms in the ACE and ACTN3 gene have been reported to influence the clinical picture in patients with Pompe disease.[24-26] To gain a better understanding of genotype-phenotype correlations and potential influence of genetic background factors, we studied phenotypical variation in 22 families with Pompe disease with the same genotype, all living in the Netherlands (**Chapter 2**). We hypothesized that siblings with Pompe disease with the same *GAA* genotype are genetically more identical that non-related patients and therefore are predisposed to develop more similar phenotypes.

LABORATORY DIAGNOSTICS

A clinical suspicion of Pompe disease can be confirmed by determining the enzyme activity of GAA. GAA activity can be measured with the artificial substrate 4-methylumbelliferyl-alpha-D-glucopyranoside (4-MU) or the natural substrate glycogen. Determination of GAA enzyme activity in fibroblasts is considered as gold standard test, however, since it takes approximately six weeks to culture fibroblasts, GAA activity is generally measured in leukocytes. GAA activity in leukocytes can only be measured reliably in the presence of acarbose. Without acarbose neutral α-glucosidases interfere with the test results. Other possibilities are measuring GAA activity in dried blood spots or muscle tissue.[3, 27, 28] Dried blood

spots can only be used for screening purposes, not for making a definitive diagnosis of Pompe disease.

Patients with classic infantile Pompe disease have less than 2% residual enzyme activity in fibroblasts, while patients with non-classic Pompe disease have 5-25% residual enzyme activity. The vast majority of adult patients (>90%) carry the common splice site mutation c.-32-13T>G (IVS1).[3, 5, 21, 28, 29] In our cohort of approximately 100 adult patients, there were four adults with unexpectedly low residual GAA activities in the range of patients with classic infantile Pompe disease. None of these patients had the IVS1 mutation. In one of the studies described in this thesis we aimed to understand the discrepancy between low fibroblast enzyme activity, the slow progression of the disease and the genotype-phenotype correlation in three of these patients (**Chapter 3**).

Over 95% of patients with Pompe disease have increased creatine kinase (CK) levels in their blood. Increased CK levels, together with increased levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT), indicate skeletal muscle damage.[30, 31] These parameters are sensitive, but not specific for Pompe disease. Because AST and ALT can also be elevated in patients with liver diseases, some patients with Pompe disease are initially misdiagnosed as having a liver disorder. Other biomarkers of muscular damage are myoglobulin, lactate dehydrogenase and troponin.[30] Skeletal muscle-specific and cardiac muscle-specific subtypes of troponin T and troponin I do exist and are expressed from closely related genes.[32, 33] Although cardiac troponin T (cTnT) is an important biomarker in patients with acute myocardial infarction (AMI), increased plasma concentrations of cTnT have been observed in a small percentage of the general population due to hypertension, diabetes or chronic kidney disease.[34-37] In one of our adult patients with Pompe disease we found increased plasma concentrations of cTnT at the time she complained of non-specific chest pain. However, a comprehensive cardiac evaluation did not reveal any abnormalities. This is likely compatible with the previously described absence of cardiac involvement in patients with non-classic Pompe disease.[38, 39] In one of our studies we determined if increased cTnT levels are observed in the Pompe population and we investigated whether such an increase could be the result of skeletal muscle damage and not myocardial damage (**Chapter 4**).

IMAGING

Pompe disease also affects the respiratory muscles leading to a reduced pulmonary function. Patients may develop nocturnal hypoventilation presenting with symptoms of daytime somnolence and morning headache. These patients require nocturnal or continuous mechanical ventilator support.[14, 18, 40-42] The leading cause of death in patients with Pompe disease is respiratory failure.[43, 44] Therefore, it is extremely important to monitor pulmonary function, including functional vital capacity (FVC), forced expiratory volume in one second (FEV_1), maximum static inspiratory (MIP) and expiratory (MEP) pressures.[45, 46] Preferentially, spirometry should be performed in sitting and supine position, since there is a prominent reduction of the pulmonary function in supine position (i.e. postural drop) in most patients (Figure 3).[18, 40, 41] Weakness of the diaphragm is considered to be the major cause of this postural drop in Pompe disease.[41, 47] Although standard pulmonary function tests may be indicative for diaphragmatic weakness, they provide too little insight in dynamics of the diaphragm. It has also been found that the effects of ERT on pulmonary function are less pronounced compared to the effects on muscle strength; in 35% of the patients FVC in supine position deteriorates.[42, 48-50] It has been hypothesized that ERT in Pompe patients with progressive respiratory insufficiency might have been started too late to repair muscle damage of the diaphragm. To obtain insight into the function and strength of the respiratory muscles, in particular the diaphragm, we performed a pilot study in ten adult Pompe patients using magnetic resonance imaging (MRI) in combination with spirometry (**Chapter 5**). This technique makes it possible to directly assess the contribution of individual respiratory muscles during the breathing cycle, which is difficult with pulmonary function tests.[51-54]

Imaging procedures such as computed tomography (CT) or MRI are not part of the diagnostic procedure in Pompe disease, however, these assessments can provide important information. Several studies have been published about muscle MRI in which the distribution and severity of affected muscles in patients with non-classic Pompe disease is described.[43, 55-57] Recently, it has been shown that MRI also can be used to study the effects of enzyme replacement therapy (ERT) on individual muscles.[58] In patients with classic infantile Pompe disease muscle MRI has not been studied

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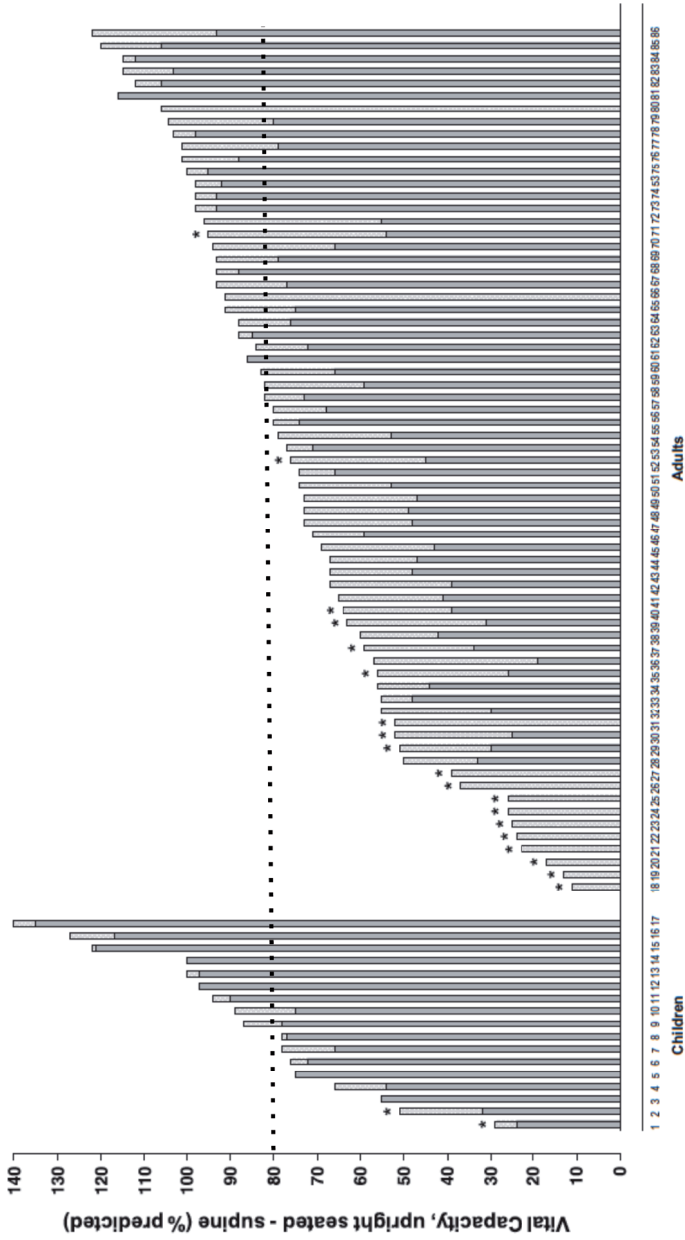
Figure 3 Vital capacity in sitting and supine position in children and adults with Pompe disease

This figure was adapted from the publication written by van der Beek et al. entitled 'Rate of progression and predictive factors for pulmonary outcome in children and adults with Pompe disease' in *Molecular Genetics and Metabolism* 2011, by permission of Elsevier. Vital capacity in upright seated position compared to vital capacity in supine position in 17 children and 69 adults with Pompe disease. The light gray bars represent vital capacity in upright position; the dark gray bars represent vital capacity in supine position. Asterisks identify patients using ventilatory support.

before. We performed a pilot study in a small group of patients with classic infantile Pompe disease to investigate whether muscle MRI potentially can be a useful tool to study the extent of muscle damage of the upper legs (**Chapter 6**).

Apart from glycogen accumulation in skeletal and respiratory muscles, it has been shown in several morphological studies and autopsy reports that glycogen also accumulates in smooth muscle fibers and in the endothelial layer of arteries.[9, 59, 60] In some reports it has been hypothesized that glycogen accumulation in smooth muscle tissue of arteries leads to vascular abnormalities such as aneurysms.[59, 61-65] Increased aortic stiffness, which is an independent risk factor for cardiovascular disease and mortality, was earlier demonstrated by our group in a study of 17 patients with non-classic Pompe disease using transthoracic Doppler echocardiography, a technique to measure aortic stiffness indirectly.[66-68] Nowadays, tonometry of the carotid-femoral pulse wave velocity (cfPWV) is considered to be the gold standard test to directly measure arterial stiffness.[69] To determine whether structural changes in the arteries of patients with Pompe disease lead to functional abnormalities, we measured arterial stiffness and blood pressure in 84 patients with non-classic Pompe disease and compared the results with 179 age and gender-matched volunteers (**Chapter 7**).

TREATMENT

Pompe disease is the first treatable genetic muscle disease. In 2006, ERT with recombinant human acid α-glucosidase (rhGAA) (Myozyme®, Genzyme) was approved in the Netherlands for all Pompe patients. ERT is administered intravenously once every two weeks. RhGAA binds to the mannose 6-phosphate receptor, which is present on the cell surface of cardiomyocytes and skeletal muscle cells. The enzyme is then internalized into the myocyte and transported via endocytosis to the lysosome. Inside the lysosome, rhGAA breaks down the accumulated glycogen into glucose.[70-72] The first studies on the effects of ERT in patients with classic infantile Pompe disease started in 1999 at our center. Over the years it has been shown that patients with classic infantile Pompe disease have an improved survival, a decrease of the hypertrophic cardiomyopathy to

a normal shaped and functioning heart, improved motor functioning and increased ventilator-free survival.[3, 73-75] The first results of ERT in a large international group of 90 patients with non-classic or late-onset Pompe disease, including 22 patients of our center, originated from a randomized, multicenter, placebo-controlled trial, which was published in 2010. After 1.5 years, patients receiving ERT had an improved walking distance and stabilization of the pulmonary function.[42] Several studies have also shown similar results with an improvement in walking distance and stabilization of muscle strength and pulmonary function after a treatment duration of 6-54 months. [49, 50, 76-80] Recently, it has been demonstrated that ERT also has a significant positive effect on survival in patients with non-classic Pompe disease, with a hazard ratio of 0.41. It was estimated that eight years of treatment with ERT would result in one extra year of life.[81]

Since not all patients with Pompe disease respond equally well on ERT, it is important to study additional strategies to improve muscle strength and daily functioning. It has been shown that exercise training has a positive effect on muscle strength and endurance in patients with different neuro-muscular diseases.[82] There is some evidence that exercise training could be beneficial for patients with Pompe disease.[23, 83] In one study it has even been shown that patients receiving ERT had better functional outcomes when they were specifically subjected to concomitant physiotherapy.[50] Exercise studies in Pompe disease are limited and comprised only small groups of patients and the exercise programs differed between patients. We explored with a prospective design whether 23 patients with non-classic Pompe disease receiving ERT benefit from a standard exercise program (**Chapter 8**).

SCOPE OF THE THESIS

The aims of the studies described in this thesis were as follows:

Clinical heterogeneity, genotype-phenotype correlations and the role of modifiers
1. To study the phenotypical variation in families with non-classic Pompe disease and to determine to what extent siblings and families with the

same *GAA* genotype have the same clinical presentation and course of the disease, and the potential role of genetic background factors.

2. To understand the exceptionally low GAA activity measured in fibroblasts of three adult patients with Pompe disease, in relation to the slow progressive course of the disease and the genotype.

3. To determine if a standardized exercise program can modify endurance, muscle strength and muscle function in adults with Pompe disease receiving ERT.

Laboratory diagnostics and imaging

4. To determine whether increased levels of cTnT in patients with Pompe disease are an early indicator of subclinical myocardial damage or can be attributed to skeletal muscle damage.

5. To obtain insight into the function and strength of the respiratory muscles, in particular the diaphragm, in ten adult patients using MRI in combination with spirometry.

6. To assess whether muscle MRI can be a useful tool to investigate the extent of muscle damage in classic infantile Pompe disease.

7. To investigate whether patients with non-classic Pompe disease have increased arterial stiffness using various techniques including tonometry of the cfPWV.

OUTLINE OF THE THESIS

Chapter 1 provides a general introduction about Pompe disease. In **Chapter 2** the phenotypical variation in families with the non-classic form of Pompe disease is described. In **Chapter 3** three adult patients with Pompe disease having a very low GAA activity in fibroblasts are discussed. **Chapter 4** focuses on the increased plasma levels of cTnT and the source of this elevation. In **Chapter 5** the use of MRI is described to explore dynamic respiratory function in adult Pompe patients. The usefulness of muscle MRI in classic infantile Pompe disease is studied in **Chapter 6**. **Chapter 7** focuses on arterial stiffness measured with cfPWV in patients with non-classic Pompe disease. The effects of exercise training on muscle strength,

muscle function and exercise tolerance are described in **Chapter 8**. Finally, the general discussion (**Chapter 9**) summarizes the main findings and discusses the clinical implementation and future perspectives.

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Chapter 2

Phenotypical variation within 22 families with Pompe disease

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ABSTRACT

Introduction Pompe disease has a broad clinical spectrum, in which the phenotype is partially explained by the genotype. The aim of this study was to describe phenotypical variation among siblings with non-classic Pompe disease. We hypothesized that siblings and families with the same genotype share more similar phenotypes than the total population of non-classic Pompe patients, and that this might reveal genotype-phenotype correlations.

Methods We identified all Dutch families in which two or three siblings were diagnosed with Pompe disease and described genotype, acid α-glucosidase activity, age at symptom onset, presenting symptoms, specific clinical features, mobility and ventilator dependency.

Results We identified 22 families comprising two or three siblings. All carried the most common mutation c.-32-13T>G in combination with another pathogenic mutation. The median age at symptom onset was 33 years (range 1-62 years). Within sibships symptom onset was either in childhood or in adulthood. The median variation in symptom onset between siblings was nine years (range 0-31 years). Presenting symptoms were similar across siblings in 14 out of 22 families. Limb girdle weakness was most frequently reported. In some families ptosis or bulbar weakness were present in all siblings. A large variation in disease severity (based on wheelchair/ventilator dependency) was observed in 11 families. This variation did not always result from a difference in duration of the disease since a third of the less affected siblings had a longer course of the disease. Enzyme activity could not explain this variation either. In most families male patients were more severely affected. Finally, symptom onset varied substantially in twelve families despite the same *GAA* genotype.

Conclusion In most families with non-classic Pompe disease siblings share a similar phenotype regarding symptom onset, presenting symptoms and specific clinical features. However, in some families the course and severity of disease varied substantially. This phenotypical variation was also observed in families with identical *GAA* genotypes. The commonalities and differences indicate that besides genotype, other factors such as

epigenetic and environmental effects influence the clinical presentation and disease course.

INTRODUCTION

Pompe disease (OMIM 232300: acid maltase deficiency or glycogen storage disease type II) is an autosomal recessive disorder caused by a deficiency of acid α-glucosidase which leads to accumulation of glycogen in various tissues. The deficiency is caused by mutations in the acid α-glucosidase (*GAA*) gene (OMIM 606800).[1-7] The combination of mutations in the *GAA* gene determines the phenotype to a certain extent.[8,9] A heteroallelic combination of two severe mutations that results in a complete enzyme deficiency causes classic infantile Pompe disease. Soon after birth these infants present with generalized hypotonia, hypertrophic cardiomyopathy, feeding difficulties and respiratory problems. If not treated they usually die before the age of one year.[4,10,11] If one of the two mutations is not fully deleterious and some residual enzyme activity remains, the disease can manifest at anytime during childhood or adulthood. This is referred to as non-classic Pompe disease and patients develop slowly progressive limb girdle weakness and respiratory problems.[12,13] Thus, the *GAA* genotype is the first level at which clinical heterogeneity arises.

In the Netherlands, about 95% of adult patients with Pompe disease and 68% of affected children under 18 years have the mild and common mutation c.-32-13T>G in one *GAA* allele combined with a far more severe mutation in the other allele. c.-32-13T>G is a so called 'leaky splice' mutation, producing only 5-15% of the normal amount of messenger RNA and resulting in a proportional amount of structurally and functionally normal acid α-glucosidase.[8,14] The phenotype of patients with one c.-32-13T>G mutation combined with a fully deleterious mutation varies widely in age of symptom onset and rate of disease progression despite the similar *GAA* genotype and haplotype.[14,15] Since siblings with Pompe disease are genetically more identical than non-relatives, we assumed that they would develop more similar phenotypes. Some phenotypical variation within affected families has been described in case reports.[16-21]

The aims of our study were to describe the phenotypical variation in a large group of families, to determine to what extent the disease in siblings and families with the same *GAA* genotype had the same clinical presentation and followed the same course, and to identify possible genotype-phenotype correlations.

METHODS

Study population

In the Netherlands, all patients diagnosed with Pompe disease are referred to Erasmus MC University Medical Centre, which is the national centre of expertise for this orphan disease. For our study we identified all families with non-classic Pompe disease in which two or three siblings were affected. We used the definition of non-classic Pompe disease as described in a previously published letter.[13] Patients with non-classic Pompe disease have onset of symptoms in childhood or adulthood. Onset of symptoms can also be in the first year of life, however, these patients do not have persisting and progressive cardiac hypertrophy such as in classic infantile Pompe disease. In literature, non-classic Pompe disease is also referred as late-onset Pompe disease, although onset of disease can also be in childhood. All patients were seen between October 2004 and December 2012. The Medical Ethical Committee of Erasmus University Medical Center approved the study protocols.

Enzyme and mutation analyses

Enzyme activity in fibroblasts was measured with 4-methylumbelliferyl-α-D-glucopyranoside (4-MU) as substrate.[14] *GAA* sequence analyses were performed using genomic DNA isolated from white blood cells as previously described.[22,23]

Clinical features

At the first visit, a thorough medical history was obtained. Patients were asked at what age they first experienced symptoms and what the nature of these symptoms was. The duration of the disease was calculated from the

onset of symptoms to the current age of the patient. Distribution of weakness and specific symptoms such as ptosis, bulbar weakness and scapular winging were recorded. Where applicable, the age at start of wheelchair or ventilator use was recorded.

Statistical analyses

The data were analysed using SPSS 20. Descriptive statistics were used for all calculations and data are presented as medians with ranges. The following parameters were assessed whether they had an influence on the phenotypical variation in and/or between families: disease duration, gender, genotype, enzyme activity in fibroblasts and co-morbidity.

RESULTS

Study population

Among a total of 126 patients with Pompe disease cared for at our centre, we identified 23 families with two or three affected siblings. Because in one family a patient was already wheelchair dependent due to other causes, we did not include this family in our analyses. The common c.-32-13T>G mutation was encountered in all families; in 12 families in combination with c.525delT (r.0), and in 10 families with another equally detrimental second mutation.[24] Four patients from different families died before they were referred to our centre and these patients were not included because of lack of data. The remaining 22 families comprised 50 patients. The median age at first visit was 49 years (range 0-72 years). None of the patients was being treated with enzyme replacement therapy (ERT) at this time. The characteristics of the study population are presented in Table 1.

Table 1 Patient characteristics[a]

	Total
Patients	50
Gender: males (%)	24 (48)
Median age at symptom onset in years (range)[b]	33 (1-62)
Median age at diagnosis in years (range)[c]	39 (0-72)
Current median age in years (range)	53 (5-79)
Mobility (%)	
• ambulant	30 (60)
• walking aids	6 (12)
• wheelchair dependent	14 (28)
Ventilator dependent (%)	14 (28)
α-Glucosidase activity in fibroblasts (nmol MU/mg.h)[d]	13 (8-18)
Families	22
Families with genotype (%)	
• c.-32-13T>G / c.525delT (r.0)	12 (55)
• c.-32-13T>G / c.378_379del (p.Cys127LeufsX18)	2 (9)
• c.-32-13T>G / c.925G>A (p.Gly309Arg)	2 (9)
• c.-32-13T>G / other pathogenic mutation	6 (27)

[a] Data are numbers with percentages or medians with ranges. [b] The median age at onset of symptoms is calculated for 47 patients because three patients are asymptomatic. [c] One patient was diagnosed at birth because she had a brother with Pompe disease, but she was asymptomatic. [d] Control range 40-180 nmol MU/mg.h.

Symptom onset and clinical features

Figure 1 gives an overview of the disease course of each family: from symptom onset, to the age of diagnosis and the age siblings became wheelchair or ventilator dependent. The youngest patient developed symptoms in the first year of life, while the oldest patient developed symptoms at the age of 62 years. The median difference in symptom onset was nine years between siblings (range 0-31 years). All siblings within a family developed first symptoms either in childhood (4/22) or in adulthood (18/22). The age of diagnosis varied between 0 and 72 years (median 39 years). The median delay between symptom onset and diagnosis was four years (range 0-43 years) for all patients. The median time between diagnosis of the first sibling and of the second or third family member was one year (range 0-19 years).
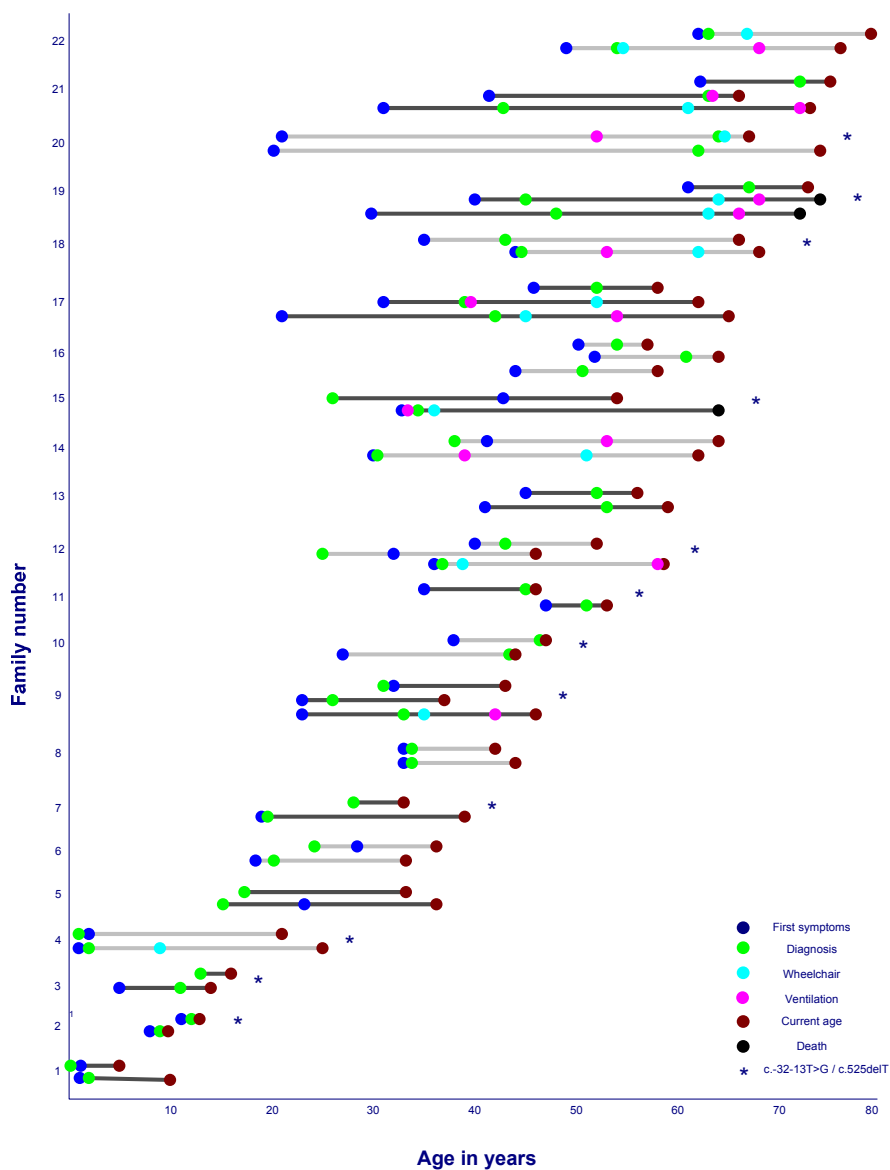
Figure 1 Course of the disease in 22 families with non-classic Pompe disease

Families 1-4 are childhood-onset patients and families 5-22 are adult-onset patients. Each dot represents an event during the course of the disease. Families highlighted with * share the same *GAA* genotype.

Table 2   Clinical characteristics within 22 families[a]

	Total of families (%)
Presenting symptoms in all siblings (%)	
• skeletal muscle weakness[b]	13 (59)
• skeletal muscle + bulbar weakness	1 (5)
• variation in first symptoms[c]	8 (36)
Clinical features (%)	
• all siblings had a ptosis	4 (18)
• all siblings had bulbar weakness	2 (9)
• all siblings had scapular winging	1 (5)
Mobility (%)	
• All siblings were ambulant	11 (50)
• One or two siblings were wheelchair dependent[d]	10 (45)
• All siblings were wheelchair dependent	1 (5)
Ventilation (%)	
• None of the siblings was ventilator dependent	12 (54)
• One or two siblings were ventilator dependent[d]	9 (41)
• All siblings were ventilator dependent	1 (5)

[a] Data are numbers with percentages. [b] Limb girdle weakness, delayed motor development. [c] In three families one sibling was still asymptomatic. [d] In five families consisting of three siblings, one or two siblings were wheelchair or ventilator dependent, while the other sibling was not.

Table 2 shows the similarities and differences in clinical characteristics within families. In 13 families all siblings presented with skeletal muscle weakness (i.e. limb girdle weakness or delayed motor development). In one family all three siblings presented with a combination of limb girdle weakness and bulbar weakness. Bulbar weakness comprised dysarthria, dysphagia and chewing difficulties. In the eight remaining families there was a variation in the nature of first symptoms, including three families with a pre-symptomatic sibling (only enzyme and genetic diagnosis, no clinical signs). Specific clinical features seen throughout the course of disease such as ptosis, bulbar weakness and scapular winging were clustered within some families.

Severity of the disease and possible influencing factors

As shown in Table 2, in approximately 50% of the families there was at least one wheelchair or ventilator dependent patient. The majority of these wheelchair and/or ventilator dependent patients (93% and 86%) had a sibling who was ambulant and not using ventilator support at the same age that they became dependent themselves. In one family co-morbidity

played a role in clinical variability between siblings, as one sibling acquired kyphoscoliosis after vertebral spondylitis and became wheelchair dependent (Figure 1, family 22). We have added a movie file that illustrates the marked differences in disease course within some sibships (see Additional file 1).

In a subgroup of 11 families representing the largest variation in disease severity, with at least one wheelchair or ventilator dependent sibling and one ambulant and ventilator independent sibling, we compared the characteristics of the siblings. The most severely affected sibling had the longest duration of the disease in 64% of these families. Males were more severely affected than females in seven families (64%). In the majority of families (60%), the patient with the highest enzyme activity in fibroblasts was more severely affected.

Twelve families shared genotype c.-32-13T>G / c.525delT (r.0). The onset of symptoms varied substantially between these families with the same set of *GAA* mutations (Figure 1); e.g. families 2-4 had symptom onset in childhood, while in families 11, 12, 15, 18 and 19 the first symptoms presented later in adulthood.

DISCUSSION

This is the first study that describes phenotypical commonalities and variation in a large cohort of families representing 50 patients with non-classic Pompe disease. We confirmed the presence of phenotypical commonalities within sibships, including: symptom onset either in childhood or in adulthood, similar presenting symptoms (in 70% of families), and the occurrence of particular symptoms such as ptosis and bulbar weakness. However, the rate of disease progression varied substantially within some families and also between families with the same *GAA* genotype.

Infants with classic infantile Pompe disease show a consistent phenotype, also within sibships, while the clinical spectrum of non-classic Pompe disease is much more variable regarding onset and progression of the disease.[3,6-8,12] Since siblings share the same set of *GAA* mutations and are genetically more related than non-relatives, we expected to find less variation

in phenotype within sibships compared to the overall Pompe population. In our 22 Dutch families the presenting symptoms were often the same. As limb girdle weakness is known to be the most frequently occuring initial symptom in Pompe disease, it is not surprising this was found frequently in all siblings of a family.[3,5-7,12] Other symptoms also seemed to cluster within families. Remarkably, bulbar weakness was a presenting symptom in all three siblings of one family. Bulbar weakness has been reported in approximately 25% of adult Pompe patients, but rarely as first symptom (1-2%).[6,12,25] It was also found in all siblings of a second family but later on in the course of the disease. The same applies to ptosis.[26-28] Ptosis has been reported in 23% of the Dutch adults with Pompe disease.[12] In our cohort of families we found the same percentage. In four families ptosis was present in all siblings. All carried the c-32-13T>G mutation, but the second *GAA* mutation differed between the families. The same applied to the two families with bulbar weakness, suggesting a role for (epi)genetic factors in the clustering of symptoms within families.

We also found differences in phenotype within the same family. The median difference in symptom onset between siblings was nine years, with extremes of 20 to 31 years in three families. Since time of symptom onset was based on patients reporting their own history, there may be a recall bias leaving some uncertainty in the differences between time of symptom onset between siblings. However, it is inevitable that large differences will occur within some families. The majority of wheelchair and/or ventilator dependent patients had an ambulant or non-ventilated sibling, while they had already been using these supportive measures when they were the same age. The duration of the disease (time from symptom onset) could not always explain this difference, since a third of the patients were wheelchair or ventilator dependent and needed these resources at an earlier stage of their disease than their ambulant or non-ventilated sibling

We looked for possible factors explaining the observed phenotypical variation between siblings. Siblings who had the disease for longer were often more severely affected. However, this does not explain why these siblings developed symptoms at a younger age and why their disease progressed more rapidly. Gender could play a role since twice as many males as females were more severely affected. Earlier studies on the natural course

of Pompe disease also show male gender to be a predictive factor for a more severe respiratory status.[12,29] GAA activity did not explain differences in phenotype, since in only three families did the most severely affected patient have the lowest amount of enzyme activity in fibroblasts. This is in accordance with previously published data on Pompe patients, describing the absence of a correlation between the clinical course and residual activity in patients with the c-32-13T>G/null genotype.[5] Other co-morbidities contributed to phenotypical differences in only one family.

The substantial differences in age of symptom onset observed in the 12 families with an identical *GAA* genotype (c.-32-13T>G / c.525delT (r.0)) suggest that other factors such as variability in genetic background, modifying genes or environmental factors are likely to play a role.[5,8,14,16,20,30] An example of a potential modifying gene is the angiotensin-converting enzyme gene, which has been described as playing a role in modulating phenotype and prognosis in Pompe disease.[31,32] This and other modifying genes might also explain the clear differences between families with the same *GAA* genotype, and the clustering of symptoms such as ptosis and bulbar weakness in certain families.

CONCLUSION

This study in families with non-classic Pompe disease showed that onset of symptoms within a family appeared to be either in childhood or adulthood, presenting symptoms of siblings were often similar and some specific clinical features clustered in certain families. However, the course and severity of disease can vary substantially within some families and between families with the same *GAA* genotype. This phenotypical consistency and variation within sibships indicates that other factors such as epigenetic and environmental effects influence the course of clinical disease. Additional studies are needed to identify these factors and to determine which prognostic factors will predict the disease course.

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Chapter 3

Remarkably low fibroblast acid α-glucosidase activity in three adults with Pompe disease

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ABSTRACT

Introduction Most adults with Pompe disease are compound heterozygotes in which one acid α-glucosidase (*GAA*) allele harbors the c.-32-13T>G mutation, causing partial loss of GAA, and the other allele harbors a fully deleterious mutation. The fibroblast GAA activity in these patients is usually between 5% and 25% of the average in healthy individuals. In some adult patients, however, the fibroblast GAA activity is much lower and is in the range that is normally observed in classic infantile Pompe disease. We investigated the genotype-phenotype correlation in three such adult patients and measured the GAA activity as well as the glycogen content in muscle and fibroblasts in order to better understand the clinical course.

Methods DNA was sequenced and GAA activity and glycogen content were measured in leukocytes, fibroblasts and muscle. Muscle biopsies were microscopically analyzed and the biosynthesis of GAA in fibroblasts was analyzed by immunoblotting. GAA activity and glycogen content in fibroblasts and muscle tissue in healthy controls, adult patients with Pompe disease and classic infantile patients were compared with those of the three index patients.

Results One patient had genotype c.525delT / c.671G>A (r.0 / p.Arg224Gln). Two affected brothers had genotype c.569G>A / c.1447G>A (p.Arg190His / p.Gly483Arg). In all three cases the GAA activity and the glycogen content in fibroblasts were within the same range as in classic infantile Pompe disease, but the activity and glycogen content in muscle were both within the adult range. In fibroblasts, the first step of GAA synthesis appeared unaffected but lysosomal forms of GAA were not detectable with immunoblotting.

Conclusion Some adult patients with mutations other than c.-32-13T>G can have very low GAA activity in fibroblasts but express higher activity in muscle and store less glycogen in muscle than patients with classic infantile Pompe disease. This might explain why these patients have a slowly progressive course of Pompe disease.

INTRODUCTION

Pompe disease (OMIM 232300: acid maltase deficiency or glycogen storage disease type II) is an autosomal recessive metabolic disorder caused by the deficiency of acid α-glucosidase (GAA) and is characterized by progressive accumulation of glycogen in many tissues.[1-4] The deficiency results from pathogenic mutations in the *GAA* gene (MIM 606800). The clinical spectrum is broad, as it ranges from severely affected classic infantile patients presenting in the first months of life with generalized muscle weakness and cardiac hypertrophy, to patients whose first symptoms appear in late adulthood and usually consist of slowly progressive limb girdle weakness and decreased respiratory function.[1-3,5-7] Most adult patients harbor the common c.-32-13T>G mutation in one *GAA* allele and a more severe mutation in the other. c.-32-13T>G reduces the fidelity of mRNA splicing and also reduces the GAA activity in fibroblasts to 5-25 % of normal (4.2-20 nmol/mg.h compared to 45-180 nmol/mg.h average normal).[8-10] Classic infantile patients have two severe mutations, which results in an enzyme activity in fibroblasts of less than 1-2 % of average normal.[3,4,7] Besides measuring GAA activity in fibroblasts, which is considered to be the gold standard test, it is also possible to measure the activity in leukocytes, dried blood spot or muscle tissue.[3,4,11]

  In the Netherlands all patients with Pompe disease are referred to Erasmus MC University Medical Center, which has been designated the expertise center for this orphan disease. We screened our database and found that 4 of the 106 adults in our patient cohort have a low GAA activity in fibroblasts within the range of classic infantile patients. None of these four patients have the common c.-32-13T>G mutation but other pathogenic mutations, as described previously.[12-15] We described one of the patients earlier.[16] In this paper we present a study of the other three adult patients in which we compared the GAA activity and the glycogen content in muscle tissue with that in fibroblasts. We aimed to understand the discrepancy between low fibroblast GAA activity and the slow progression of the disease.

METHODS

Patients and controls

The three index patients were referred to Erasmus MC University Medical Center in Rotterdam between 2007 and 2010 and thereafter were followed up every 3-6 months. As control for GAA activity in muscle, muscle biopsies from patients with a suspected myopathy were used. Informed consent was obtained from the patients and the controls. The study protocols were approved by the Medical Ethical Committee at Erasmus MC University Medical Center.

Clinical parameters and laboratory testing

Muscle strength was measured with the Medical Research Council grading scale (MRC). Pulmonary function was measured with spirometry in sitting and supine position (if possible). The muscle biopsy was taken from the quadriceps muscle. The muscle sections were fixed in 4% glutaraldehyde and thereafter embedded in glycolmetacrylate (GMA) and stained with periodic acid-Schiff (PAS). Acid phosphatase staining was performed on muscle cryosections. Serum creatine kinase (CK) was determined.

Enzyme analysis and glycogen content

The GAA activity in leukocytes was measured with glycogen as substrate in the presence of acarbose to inhibit the glucoamylase activity (Glyc+ACAB) in accordance with previously described procedures.[17,18] The GAA activity in fibroblasts and muscle was measured with 4-methylumbelliferyl-α-D-glucopyranoside (4-MU) and/or glycogen as substrates.[8] The glycogen content of cultured skin fibroblasts was measured after 4 weeks of sustained confluence and the glycogen content of muscle biopsies was measured after storage at -80 ºC. The assay encompasses the measurement of liberated glucose after incubating cell and tissue lysates with amyloglucosidase and amylase.[19,20] For comparison we measured the enzyme activity and the glycogen content in fibroblasts and muscle of patients with classic infantile Pompe disease, in samples from patients with juvenile and adult Pompe disease who have the c.-32-13T>G mutation, and in samples from healthy controls.

Molecular analysis

GAA sequence analysis was performed on genomic DNA isolated from white blood cells using routine procedures.[21,22] The effect of novel missense mutations was investigated by transient expression of site-directed muta- genized *GAA* cDNA constructs in HEK293T cells. Cells and media were harvested 72 hours after transfection. The synthesis and posttranslational modification of GAA in transfected cells and in fibroblasts of the patients was investigated by SDS-PAGE followed by immunoblotting as previously described.[9]

RESULTS

Patients

Patient 1 is a 51-year-old Caucasian male who had been referred to our hospital in March 2007. He had been diagnosed with Pompe disease at the age of 26 years because of slowly progressive limb girdle weakness. At the age of 45 years he became wheelchair dependent. One year later he was admitted to the hospital with an acute respiratory distress syndrome. He subsequently became ventilator dependent. Neurological examination revealed facial weakness and severe symmetric limb girdle weakness: arms MRC grade 2 and legs MRC grade 1. CK was 256 U/l (normal value <170 U/l in men). Pulmonary function testing revealed a forced vital capacity (FVC) in sitting position of 0.43 L (9% of normal). The GAA activity in leukocytes was 0.6 nmol/mg.h measured with Glyc+ACAB as a substrate (control range 40-250 nmol/mg.h), and in fibroblasts was 0.5 nmol/mg.h measured with 4-MU-α-D-glucopyranoside (control range 45-180 nmol/ mg.h). The muscle biopsy showed irregular hypertrophic muscle fibers with some glycogen accumulation as detected by PAS staining (Figure 1D). DNA analysis showed heterozygosity for c.525delT (r.0) and the novel mis- sense mutation c.671G>A (p.Arg224Gln). The biochemical assessment of the muscle biopsies of all three patients is described in paragraph 3.2.

The second patient, a 25-year-old Caucasian male (patient 2), had been referred in July 2009. Since the age of 15 years he had found it difficult to lift heavy objects and a few years later he became unable to run, due to
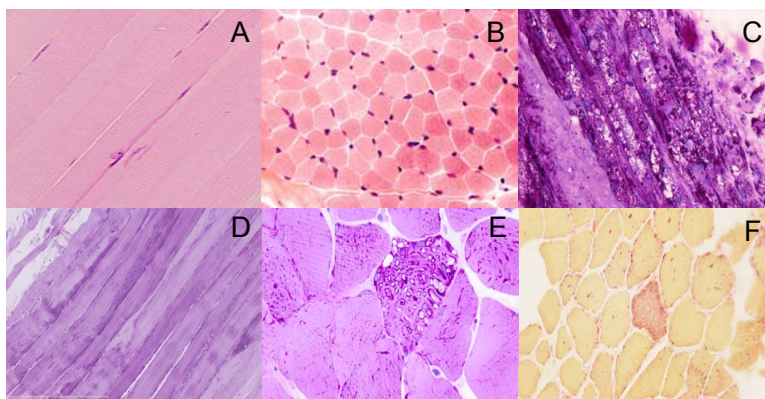
Figure 1 Lysosomal pathology in muscle biopsies

Panels A (longitudinal) and B (cross-sectional) show muscle biopsies of a healthy individual stained with hematoxylin and eosin (HE). Panels C, D (both longitudinal) and E (cross-sectional) are stained with PAS to show lysosomal glycogen storage in a patient with classic infantile Pompe disease (C) and in patients 1 and 2 (D and E). Panel F (cross-sectional) shows the muscle biopsy of patient 1 stained with acid phosphatase.

progressive limb girdle weakness. Neurological examination revealed limb girdle weakness MRC grade 4 of the upper extremities and MRC grade 3 of the lower extremities. CK was 1304 U/l. FVC in sitting position was 4.42 L (75% of normal) and in supine position 3.85 L (65% of normal). The GAA activity in leukocytes was 0.0 nmol/mg.h measured with Glyc+ACAB as substrate and in fibroblasts was 1.0 nmol/mg.h measured with 4-MU-α-D-glucopyranoside. The muscle biopsy showed a vacuolar myopathy and glycogen accumulation in 5-10% of the muscle fibers as detected by PAS staining (Figure 1E). DNA analysis demonstrated heterozygosity for c.569G>A and c.1447G>A (p.Arg190His / p.Gly483Arg).

The third patient, a 23-year-old Caucasian male (patient 3) and brother of patient 2, was referred to our hospital in November 2010. At that time he was already wheelchair dependent because of a meningomyelocele, tethered cord and hydrocephalus for which he had been operated multiple times. His clinical condition had been deteriorating for five years: it was becoming increasingly difficult for him to transfer from his wheelchair to the toilet or to his car. Neurological examination revealed proximal weakness of the upper extremities (MRC grade 4) and a pre-existing weakness with sensory

disturbances of the lower extremities. Because of the family history the patient was also tested for Pompe disease. The GAA activity in leukocytes was 4.0 nmol/mg.h measured with Glyc+ACAB as substrate; in fibroblasts it was 0.8 nmol/mg.h measured with 4-MU-α-D-glucopyranoside. He had the same genotype as his brother, c.569G>A and c.1447G>A (p.Arg190His / p.Gly483Arg). Serum CK was 735 U/l. Pulmonary function testing revealed an FVC in sitting position of 2.93 L (57% of normal) and an FVC in supine position of 2.46 L (48% of normal). His muscle biopsy showed a vacuolar myopathy and glycogen accumulation in 50% of the muscle fibers as detected by PAS staining (not shown).

Enzyme activities and glycogen content in patients' samples

Table 1 provides an overview of the GAA activities that we measured in leukocytes, fibroblasts and muscle tissue, and the glycogen content of fibroblasts and muscle tissue from the three index patients and from patients with classic infantile, juvenile and adult Pompe disease and healthy controls. The enzyme activity in leukocytes, measured with Glyc+ACAB as substrate, was deficient in the three index patients as well as in the all other patients with Pompe disease. In fibroblasts of the three index patients the enzyme activity was within the range of activities that is usually measured in classic infantile Pompe disease and in severe childhood cases. Using the natural substrate glycogen, GAA activity of the three index patients was equally low and far closer to the activities usually measured in infantile and childhood cases than in adult cases of Pompe disease. By contrast, the enzyme activity in muscle of the three index cases was clearly above the range of activities typically measured in classic infantile Pompe disease.[23] Patients 1 and 3 had activities that were even above the adult range. The glycogen content of the fibroblasts of the index cases was as high as in classic infantile Pompe disease, but the glycogen content of muscle was within the range of juvenile and adult patients with Pompe disease and far below the median content in classic infantile patients (1839 μg/mg).

Table 1 GAA activity, glycogen content and *GAA* mutations in patients with Pompe disease and healthy controls

	Patient 1	Patient 2	Patient 3	Classic infantile	Juvenile/adult	Control
*Activity in leukocytes (Glycogen)	0.6	0.0	4.0	0-3.5	0-10	40-250
Activity in fibroblasts (4-MU)	0.5	1.0	0.8	0-2.3 (n=59)	4.2-20 (n=65)	45-180 (n=218)
Activity in fibroblasts (Glycogen)	5.7	4.2	4.8	6.0	184.8	388-1370 (n=72)
Activity in muscle (4-MU)	5.4 (9.0)	1.1 (2.7)	4.9 (7.5)	0-1 (n=8)	0.6-2.6 (n=7)	8-40 (n=40)
#[Glycogen] fibroblasts	188	126	190	145-211 (n=2)	21-25 (n=2)	27
[Glycogen] muscle	168	145	331	122-3120 median = 1839 (n=8)	86-409 median = 218 (n=7)	30-180 median = 88 (n=40)
Allele 1	c.525delT	c.569G>A	c.569G>A	2 severe mutations	c.-32-13T>G +	no mutations
Allele 2	c.671G>A	c.1447G>A	c.1447G>A		severe 2nd mutation	

*The GAA activity in leukocytes, fibroblasts and muscle was measured using glycogen (nmol glucose/mg.h) and /or 4-MU-α-D-glucopyranoside (nmol MU/mg.h). GAA activity in muscle was measured twice (in brackets the 2nd measurement).

#The glycogen concentration in fibroblasts and muscle is expressed in µg/mg.

Molecular analysis and immunoblotting

Like other lysosomal glycoproteins, GAA is cotranslationally imported into the endoplasmic reticulum and undergoes a series of posttranslational modification events before it attains its full activity in the lysosomes. Proteolytic events can be visualized by immunoblotting and show a stepwise conversion of a 110 kDa precursor form, via a 95 kDa intermediate form, to a mature enzyme of 76 kDa. Lanes 5-7 in figure 2 depict the synthesis of GAA in fibroblasts of the three index patients: the 110 kDa precursor is made in normal quantity but processed forms are lacking. By contrast, adult patients carrying the most common c.-32-13T>G mutation (lane 2) have reduced enzyme synthesis, as demonstrated by significantly reduced expression of the 110 kDa precursor, but normal processing. As illustrated in lane 3, a selected young adolescent patient with Pompe disease with *GAA* genotype c.861C>T / c.925G>A (p.Pro287Pro / p.Gly309Arg) had normal synthesis but impaired maturation of GAA, resulting in weak signals of

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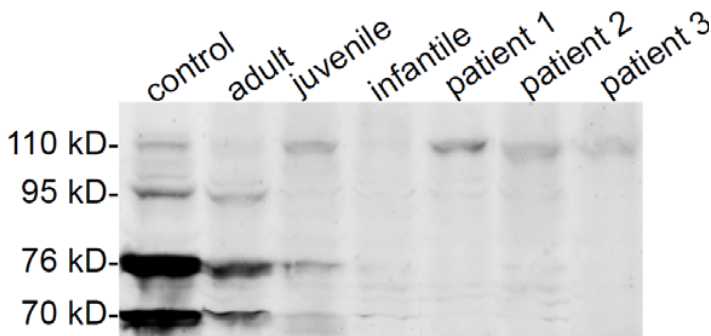
Figure 2   Western blot assay of GAA in fibroblasts

Four distinct forms of GAA were separated by SDS-PAGE. These were revealed by immunoblotting using rabbit anti human GAA antibodies. Equal amounts of protein were loaded in each lane. The lanes are labeled as follows: control: healthy individual; adult: affected adult with genotype c.-32-13T>G / c.1293_1312del (p.Gln443Aspfs*15); juvenile: affected adolescent with genotype c.861C>T / c.925G>A (p.Pro287Pro / p.Gly309Arg); patient 1 with genotype c.525delT / c.671G>A (r.0 / p.Arg224Gln) and patients 2 and 3 with genotype c.569G>A / c.1447G>A (p.Arg190His / p.Gly483Arg) are the patients described in this study.

76 and 70 kDa. The patient with classic infantile Pompe disease (lane 4) with genotype c.525delT / c.525delT (r.0 / r.0), does not demonstrate any synthesis of GAA.

Upon DNA analysis patient 1 turned out to be compound heterozygote for the known pathogenic mutation c.525delT (r.0) and the novel missense mutation c.671G>A (p.Arg224Gln). Whereas c.525delT prevents the synthesis of GAA, c.671G>A (p.Arg224Gln) seems to hamper the posttranslational modification of GAA and thereby causes substantial loss of mature enzyme and associated loss of activity (Figure 2). In a transient expression system of GAA the residual activity of this novel mutation was 1.5% of average normal. The two affected brothers were identified as compound heterozygotes for c.569G>A (p.Arg190His) and c.1447G>A (p.Gly483Arg). Both these mutations are described as less severe mutations in the June 25th, 2012 version of the Pompe disease mutation database at www.pompecenter.nl. The mutation c.1447G>A (p.Gly483Arg) has previously been described in a patient with late-onset Pompe disease in combination with c.-32-3C>A, which is also listed as a less severe mutation.[24] When we analyzed the effect of the missense mutations of the two brothers in a transient expression system, both mutations sustained a low level of GAA activity: 20% for c.569G>A (p.Arg190His) and 3% for c.1447G>A (p.Gly483Arg).

DISCUSSION

Patients with Pompe disease whose symptoms appear in the second to third decade of life or even later, usually have substantially more residual GAA activity in cultured fibroblasts than patients with classic infantile Pompe disease who manifest symptoms within the first 3 months of life.[3] The three adult patients that we described fit into the category of exceptional cases of adult Pompe disease with very low GAA activity in fibroblasts.[12-16] Whereas the activity in fibroblasts, either measured with 4-MU or glycogen as substrates, usually discriminates very well between classic infantile and more slowly progressive forms of Pompe disease, it seems that in these particular cases the activity in fibroblasts does not reflect the activity in muscle. In two of the three cases this is very clear, as the GAA activity

in muscle is within or even above the range measured in adult Pompe disease, but in all three cases the activity in fibroblasts is within the range that is characteristic for classic infantile Pompe disease. In the third case, the activity in muscle is also higher than in fibroblasts but the difference is less pronounced. We did not measure the GAA activity in muscle with glycogen as substrate, because the assay does not discriminate between classic infantile Pompe disease and adult Pompe disease due to the high background signals.

In line with the view that lysosomal glycogen storage is primarily determined by the degree of enzyme deficiency, we were able to demonstrate glycogen storage in the cultured fibroblasts of the three index patients in which the activity was as low as in cells of patients with classic infantile Pompe disease. By contrast, the storage of glycogen in muscle tissue of the three index patients was no different from that usually found in patients with a slowly progressive disease, and the GAA activity was accordingly higher. As shown in Table 1 there is an overlap between the ranges for glycogen content in muscle in all forms of Pompe disease and even healthy controls. However, the values measured in the three index patients are far closer to the median glycogen content in juvenile/adult patients than in classic infantile Pompe disease. Thus, the supposed correlation between enzyme activity and glycogen storage holds, but the activity in fibroblasts does not represent the activity in muscle in these particular cases.

The unbalance between the GAA activities in muscle *versus* fibroblasts is probably related to the *GAA* genotype of the patients. It is notable that none of the adult patients with unexpected low activity in fibroblasts, including the thirteen cases recently reported by Bali et al.[12], carry the most common mutation c.-32-13T>G which is frequently associated with a later onset and slowly progressive phenotype.[13-16] It is also remarkable that in all these exceptional cases, close to normal synthesis of the 110 kDa precursor form can be demonstrated in the fibroblasts by immunoblotting, but that processed forms of 95 kDa and 76 kDa are virtually lacking. Thus, an explanation for the different GAA activities in fibroblasts and muscle tissue could be that the posttranslational modification or the stability of these mutant forms of GAA would be less compromised in muscle than in fibroblasts due to cell-type-specific differences in processing and lysosomal

transport events. In the past we found indications of cell-type-specific pro-cessing in a fourth case of adult onset Pompe disease with unusually low enzyme activity in fibroblasts.[15] Unfortunately, in the three cases described here there was not enough muscle biopsy sample left for us to be able to use western blotting to confirm this hypothesis about differential process-ing. However, the scarcity of glycogen storage in the muscle tissue sections together with the modest muscle cell damage support the hypothesis that the higher activity in muscle than in fibroblasts delays the age of onset and slows disease progression.

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Chapter 4

Elevated plasma cardiac troponin T levels due to skeletal muscle damage in Pompe disease

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ABSTRACT

Introduction Elevated plasma cardiac troponin T (cTnT) levels in patients with neuromuscular disorders may erroneously lead to the diagnosis of acute myocardial infarction (AMI) or myocardial injury.

Methods and results In 122 patients with Pompe disease, the relationship between cTnT, cardiac troponin I (cTnI), creatine kinase (CK), creatine kinase myocardial band (CK-MB) levels and skeletal muscle damage was assessed. ECG and echocardiography were used to evaluate possible cardiac disease. Patients were divided into classic infantile, childhood onset and adult onset patients. cTnT levels were elevated in 82% of patients (median 27 ng/L, normal values <14 ng/L). cTnI levels were normal in all patients, while CK-MB levels were increased in 59% of patients. cTnT levels correlated with CK levels in all three subgroups (P<.001). None of the abnormal ECGs recorded in 21 patients were indicative of AMI, and there were no differences in cTnT levels between patients with and without (n=90) abnormalities on ECG (median 28 ng/L in both groups). The median left ventricular mass index measured with echocardiography was normal in all three subgroups. cTnT mRNA expression was not detectable in controls but was strongly induced in Pompe patients. cTnT protein was identified by mass spectrometry in patient-derived skeletal muscle tissue.

Conclusions Elevated plasma cTnT levels in patients with Pompe disease are associated with skeletal muscle damage rather than acute myocardial injury. Increased cTnT levels in Pompe disease and likely other neuromuscular disorders, should be interpreted with caution to avoid unnecessary cardiac interventions.

INTRODUCTION

Troponin T (TnT), troponin I (TnI) and troponin C form the troponin complex involved in muscle contraction. After depolarization TnT binds tropomyosin resulting in interaction between actin and myosin and subsequent muscle contraction.[1-3] Skeletal muscle-specific and cardiac muscle-specific forms of TnT and TnI are expressed from closely related genes.[2, 4] Cardiac troponins (cTn) are important biomarkers in patients with acute myocardial infarction (AMI), because they are released into the circulation after ischemic damage to cardiomyocytes.[5, 6] However, increased concentrations of cardiac troponin T (cTnT) have been observed in the general population and in several patient groups including those with heart failure and end-stage renal failure but without AMI.[6-8] Recently, an elevation of plasma cTnT in the absence of cardiac troponin I (cTnI) in a number of neuromuscular diseases has been shown.[9] It has been suggested that diseased skeletal muscle tissue is an alternative source for elevated cTnT levels in certain neuromuscular conditions and dialysis patients.[3, 10-13] However, due to similarities between members of the troponin family, the presence of cTnT in tissue samples has been the subject of debate due to potential cross reactivity of antibodies used in immunoblot or ELISA assays.

Pompe disease is a neuromuscular metabolic disorder with an overall disease incidence at approximately 1:40,000 live births. The disease is caused by mutations in the acid α-glucosidase (*GAA*) gene (MIM 606800), which encodes a lysosomal enzyme responsible for glycogen degradation.[14] Dysfunctional or low levels of GAA result in glycogen accumulation affecting mainly skeletal muscle tissue. Diagnosis of Pompe disease is based on GAA enzymatic activity measurements. Patients with classic infantile Pompe disease have a residual enzyme activity of less than 1% leading to a severe form of the disease. They present shortly after birth with muscle weakness and a hypertrophic cardiomyopathy with progressive cardiac failure, which results in death within one year of age if left untreated.[15, 16] Enzyme replacement therapy (ERT) can be life-saving for classic infantile patients and the cardiomyopathy responds well to ERT.[14, 17] Patients with non-classic or late onset Pompe disease have a residual enzyme activity of up to 20% and show slower disease progression. They present during childhood or adulthood with limb girdle weakness and reduced pulmo-

nary function.[18] In these patients ERT positively alters the natural course by increasing muscle strength and stabilizing pulmonary function.[19, 20] The heart is generally not affected in non-classic Pompe disease.[21, 22] Increased cTnT concentrations were found in one of our adult patients who reported atypical chest pain. However, a comprehensive cardiac evaluation failed to reveal cardiac abnormalities. This discrepancy between elevated cTnT concentrations and absence of AMI prompted us to investigate the presence of increased cTnT levels in more detail in a large cohort of Pompe patients.

METHODS

Study population

In the Netherlands, all patients with Pompe disease are referred to the Center for Lysosomal and Metabolic Diseases of the Erasmus University Medical Center Rotterdam. In total 122 patients at our center participated in this study. The patients were subdivided into three groups. The first group comprised 14 patients with classic infantile Pompe disease who had developed symptoms in the first year of life and had a hypertrophic cardiomyopathy. All these patients started treatment with ERT before the first year of life. Plasma samples analysed for cTnT in this study were taken after ERT, when the left ventricular hypertrophy (LVH) was resolved in ten out of 14 patients. The other patients had non-classic or late onset Pompe disease: group two comprised 13 patients with symptom onset before the age of 18 years (childhood onset), and group three comprised 95 patients with symptom onset after the age of 18 years (adult onset).[23] Diagnosis was confirmed by a deficiency of GAA in leukocytes or fibroblasts and mutation analysis. The Medical Ethics Committee at Erasmus University Medical Center approved the study protocol. All patients provided written informed consent.

Laboratory testing

cTnT levels were measured in heparin plasma samples using a 5th generation highly sensitive assay of Roche Diagnostics; 99th-percentile 14 ng/L, limit of detection (LoD) 5 ng/L.[24] In seven adult patients cTnT levels were

measured before the start of ERT and up to two years after treatment. Plasma cTnI levels were measured using the Access AccuTnI assay of Beckmann Coulter with a 99th-percentile cut-off at 0.04 μg/L and LoD of 0.01 μg/L.[25] Creatine kinase (CK) and creatine kinase myocardial band (CK-MB) levels were measured using Roche Diagnostic assays. As reference intervals for CK we used in children (2-13 years) <230 U/L, male adolescents (13-17 years) <270 U/L, female adolescents (13-17 years) <123 U/L, male adults (>17 years) <200 U/L, and female adults (>17 years) <170 U/L. The sex-specific 99th-percentiles for CK-MB levels were 7.6 μg/L in male patients and 4.7 μg/L in female patients.[26]

ECG and echocardiography

Standard 12-lead ECG recordings were examined for signs of ischemia, LVH and rhythm or conduction abnormalities. Additionally, 13 patients with the classic infantile form underwent echocardiography within one month after the plasma samples were taken (Sonos 5500 or 7500 ultrasound system, Philips, Best, The Netherlands), as well as 48 patients with non-classic Pompe disease with a suspicion of LVH based on the ECG, or who already underwent this procedure during a previous study.[21] The left ventricular mass index (LVMI) was calculated and indexed by body surface area.[27] As reference values for children and young adults we used previously published data.[28] Additionally, the left ventricular end diastolic dimension (LVID ED), the left ventricular end systolic dimension (LVID ES), interventricular septum (IVS), left ventricular posterior wall (LVPW) and fractional shortening (FS) were measured.[27, 29]

Human tissue biopsies

Skeletal muscle biopsies of Pompe patients were taken from the quadriceps muscle before the start of ERT. Human adult heart and liver biopsies were obtained from the Department of Pathology of Erasmus MC. Biopsies from the quadriceps muscle of healthy controls were obtained from Maastricht University Medical Center.

Expression of cTnT

Tissue biopsies were homogenized in RLT buffer (Qiagen) at 4°C using a PRO200 disruptor. After 2 times extraction using phenol/chlorophorm/

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isoamylalcohol (Sigma P3803), total RNA was isolated using the RNeasy Mini Kit with DNase I digestion to remove genomic DNA (Qiagen, Cat. No 74106). cDNA was synthesized using the iScript cDNA synthesis kit (Bio-Rad). cTnT mRNA expression was quantified using quantitative PCR with the iTaq Universersal SYBR Green Supermix (Bio-Rad) and 10 pmol/ul forward and reverse primers as described.[30] Primers annealed to separate exons to ensure that only cDNA was amplified rather than genomic DNA. Two primer sets were used for cTnT mRNA: the C-terminal primer set amplified the region encoding the C-terminal part of the cTnT protein that is common between different splice forms; the N-terminal cTnT primer set amplified the region encoding the N-terminal part of the cTnT protein that is known to be differentially spliced. The identity of cTnT qPCR products was determined by cloning the PCR fragment into a plasmid vector using topo cloning (Life Technologies) followed by sequence analysis on an AB3130 Genetic Analyzer (Applied Biosystems, Hitachi). Primer sequences are listed in Supplemental Table 1.

Mass spectrometry

Muscle biopsies were homogenized in 100 ml 4x loading buffer (NuPAGE LDS sample Buffer, Lifetechnologies, NP0007) in a 2 ml Eppendorf tube filled with stainless steel beads (Qiagen, Cat. No. 69989) using a TissueLyser II (Qiagen, Cat. No. 85300) operated for 2.5 minutes at maximum power at 4°C. Beads were removed, homogenates were heated for 10 minutes at 70°C, cleared by centrifugation and separated on a 4-12% bis-tris gel using MOPS running buffer according to the manufacturer's instructions (Invitrogen). 1D SDS-PAGE gel lanes were cut into 1-mm slices using an automatic gel slicer and subjected to in-gel reduction with dithiothreitol, alkylation with chloroacetamide and digestion with trypsin (Promega, sequencing grade). Nanoflow LC-MS/MS was performed on an ACQUITY UPLC system (Waters) coupled to either an Orbitrap Fusion Tribrid mass spectrometer (Thermo) operating in positive ion mode. Tryptic peptide separation was performed on a BEH C18 column (1.7 μm, 75 μm x 250 mm) at 10,000 psi using a linear gradient from 0 to 80% B (A=0.1% formic acid; B=80% (v/v) acetonitrile, 0.1% formic acid) in 70 minutes and at a constant flow rate of 200 nl/min. The column eluent was directly sprayed into the ESI source of the mass spectrometer. Mass spectra were acquired in continuum

mode and fragmentation of the peptides was performed in data-dependent mode. The MS raw data were analyzed using the MaxQuant software (version 1.5.2.8).[31] A false discovery rate of 0.01 for proteins and peptides and a minimum peptide length of 6 amino acids were required. The Andromeda search engine was used to search the MS/MS spectra against the Uniprot database (taxonomy: *Homo sapiens*, release Feb 2015) concatenated with the reversed versions of all sequences. A maximum of two missed cleavages were allowed and enzyme specificity was set to trypsin. Further modifications were cysteine carbamidomethylation (fixed) as well as protein N-terminal acetylation, methionine oxidation and phosphor (STY) (variable).

Statistical analysis

The data were analysed using SPSS 21. Data are presented as medians with interquartile ranges (IQR) or numbers with percentages. We calculated the proportions of Pompe patients with increased cTnT, cTnI, CK and CK-MB levels relative to the 99th-percentiles of these parameters in the general population and the corresponding 95% confidence intervals. To compare differences in cTnT levels regarding sex, use of ERT, wheelchair use and hypertension, the Mann-Whitney Test was used. Median differences of cTnT levels before and after two years of ERT were compared using the Wilcoxon signed rank test for paired samples. To calculate the relationship between cTnT and CK levels, age and disease duration, the Spearman's correlation coefficient rho was used. A p value lower than 0.05 was considered statistically significant.

RESULTS

Study population

The characteristics of 122 Pompe patients are summarized in Table 1. All patients with the classic infantile form and the majority of patients with non-classic Pompe disease received ERT at a median period of five years. None of the patients had renal failure, while 39% of the adult patients had hypertension.

Plasma parameters and cardiac evaluation

One adult patient experienced exercise-induced chest pain, but cardiac evaluation did not reveal any abnormalities. Nevertheless, the cTnT level of this patient was elevated (36 ng/L, normally <14 ng/L) and remained stable throughout the next day. None of the other patients reported cardiac symptoms indicative of AMI. This event triggered the current study to evaluate plasma and other parameters to monitor cardiac function in a large number of patients. The results are summarized in Table 2 and on an

Table 1 Patient characteristics

	Total group	Classic infantile	Childhood onset	Adult onset
No. (%) of patients	122 (100)	14 (11)	13 (11)	95 (78)
No. (%) of males	65 (53)	8 (57)	9 (69)	48 (51)
Age, median (IQR*), years	48 (31-62)	5 (2-10)	14 (10-21)	55 (44-64)
Disease duration, median (IQR), years	14 (8-22)	5 (2-10)	9 (4-12)	16 (12-25)
No. (%) of patients on ERT†	102 (84)	14 (100)	11 (85)	77 (81)
Duration of ERT, median (IQR), years	5 (4-6)	5 (2-10)	6 (3-8)	5 (4-6)
No. (%) of wheelchair dependent patients	33 (27)	5 (36)	0 (0)	28 (29)
No. (%) of ventilator dependent patients	35 (29)	2 (21)	0 (0)	33 (35)
Systolic blood pressure, median (IQR), mmHg	127 (112-146)	99 (95-107)	109 (102-119)	133 (120-150)
Diastolic blood pressure, median (IQR), mmHg	76 (67-87)	57 (54-61)	63 (60-67)	79 (74-89)

* IQR = interquartile range

† ERT = enzyme replacement therapy

individual basis in Supplemental Table 2.

When compared to the 99th-percentile, plasma cTnT levels were elevated in 82% (95% confidence interval (CI) 75-88%) of all Pompe patients. cTnT levels were elevated in 79% (95% CI 56-95%) of patients with classic infantile Pompe disease, 39% (95% CI 16-64%) of childhood onset patients and 88% (95% CI 82-94%) of adult onset patients. When using the sex-specific 99th-percentile limits, cTnT levels were still elevated in 77% (95% CI 66-86%) of male patients and 88% (95% CI 78-95%) of female patients. In only one patient, who was asymptomatic, the cTnT level was below the LoD. Next, other parameters of cardiac abnormalities including cTnI and CK-

Table 2 Plasma parameters and cardiac evaluation

	Total group (n=122)	Classic infantile (n=14)	Childhood onset (n=13)	Adult onset (n=95)
cTnT levels, median (IQR), ng/L*	27 (18-39)	22 (14-31)	9 (6-39)	29 (21-40)
No. (%) of patients with cTnT >14 ng/L	100 (82)	11 (79)	5 (38)	84 (88)
cTnI levels, median (IQR), µg/L†	<0.01 (<0.01-<0.01)	<0.01 (<0.01-<0.01)	<0.01 (<0.01-<0.01)	<0.01 (<0.01-<0.01)
CK levels, median (IQR), U/L‡	358 (198-682)	779 (372-1165)	677 (350-839)	326 (183-530)
No. (%) of patients with CK >ULN	95 (78)	13 (93)	12 (92)	70 (74)
CK-MB levels, median (IQR), µg/L§	7.4 (4.5-11.5)	11.2 (8.4-14.3)	4.5 (3.2-21.3)	7.1 (4.6-10.8)
No. (%) of patients with CK-MB >99th-percentile	72 (59)	10 (71)	5 (38)	57 (60)
ECG, No.				
Normal	90	11	10	69
Abnormal	21	2	2	17
Ischemic changes	0	0	0	0
Left ventricular hypertrophy	7	1	0	6
Rhythm or conduction disturbance	14	1	2	11
Missing	11	1	1	9
Echocardiography, No.	61	13	13	35
Left ventricular mass index (IQR), g/m²‖	74 (63-87)	74 (66-93)	66 (58-82)	78 (59-88)
Left ventricular end diastolic dimension (IQR), mm#	45 (40-50)	36 (28-41)	47 (42-57)	46 (41-51)
Left ventricular end systolic dimension (IQR), mm**	28 (25-34)	23 (18-25)	31 (27-36)	30 (27-34)
Interventricular septum (IQR), mm††	8 (7-10)	8 (7-10)	7 (6-8)	9 (8-10)
Left ventricular posterior wall (IQR), mm††	9 (7-10)	6 (6-9)	7 (6-8)	9 (9-10)
Fractional shortening (IQR), %‡#	34 (31-41)	37 (33-43)	34 (33-40)	33 (29-40)

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*   99th-percentile <14 ng/L in the general adult population, LoD 5 ng/L

†   99th-percentile <0.04 µg/L in the general adult population, LoD 0.01 µg/L

‡   References ranges in our hospital: children (2-13 years) <230 U/L, male adolescents (13-17 years) <270 U/L, female adolescents (13-17 years) <123 U/L, male adults (>17 years) <200 U/L, female adults (>17 years) <170 U/L. Upper limit of normal (ULN)

§   99th-percentile <4.7 µg/L in females and <7.6 µg/L in males

‖   Reference ranges left ventricular mass index (LVMI): in children the LVMI depends on body surface, in adult females it ranges from 43-95 g/m^2 and in adult males from 49-115 g/m^2

Reference ranges left ventricular end diastolic dimension (LVID ED): in children the LVID ED depends on weight, in adults it ranges from 37-55 mm

**   Reference ranges left ventricular end systolic dimension (LVID ES): in children the LVID ES depends on weight, in adults it ranges from 20-40 mm

††   Reference ranges interventricular septum (IVS) and left ventricular posterior wall (LVPW): in children the IVS and LVPW depend on weight, in adults they range from 6-11 mm

Reference ranges fractional shortening (FS): an FS below 25% was considered as insufficient shortening of the ventricle for every group

MB levels were assessed. cTnI values were below the 99th-percentile in all patients. When compared to the sex-specific 99th-percentile limits, CK-MB levels were elevated in 54% (95% CI 40-68%) of male patients and 66% (95% CI 54-78%) of female patients.

Additionally, ECG and echocardiography were assessed. In the adult on-set patient population, 73% of tested patients showed normal ECGs, while 89% of tested patients showed a normal LVMI assessed by echocardiography. Similar outcomes were seen for childhood onset patients (77% with normal ECGs and 100% with a normal LVMI). In classic infantile patients 79% of tested patients showed normal ECGs and 69% showed a normal LVMI. The other parameters that were measured by echocardiography are shown in Table 2. Overall, 87% of tested patients showed a normal LVMI, 98% showed normal values of LVID ED and LVID ES, 82% showed a normal IVS, 95% showed normal LVPW, and finally 97% showed normal percentages of FS.

To determine to what extent cardiac abnormalities could cause elevated plasma cTnT, values were compared between patients with and without cardiac abnormalities. In adult onset patients, cTnT values did not differ between patients with normal and abnormal ECGs (31 versus 30 ng/L, P=.24). The four adult patients with LVH on echocardiography showed cTnT levels of 7, 14, and twice 19 ng/L, while 28 out of 31 adult onset patients without LVH contained values above 19 ng/L. Rhythm or conduction abnormalities without LVH were detected in two childhood onset patients, with cTnT levels of 21 and 30 ng/L. However, three other childhood onset patients without abnormalities contained strongly elevated cTnT levels of 48, 50, and 138 ng/L. One classic infantile patient showed a conductance disturbance on ECG (cTnT 28 ng/L), and four other patients (numbers 7, 8, 9 and 12 in Suppl. Table 2) showed an increased LVMI assessed by echocardiography. Patients 7 and 12 had just started with ERT and showed elevated cTnT levels, while patients 8 and 9 suffered from long-term LVH with elevated cTnT levels. Other patients in this group without detectable cardiac abnormalities contained strongly elevated cTnT levels ranging from 20 to 59 ng/L. Taken together, the cardiac abnormalities observed in a subset of Pompe patients could not explain the elevated cTnT levels in the

majority of patients. This suggested an alternative source of elevated cTnT in Pompe patients independent of myocardial muscle damage.

Plasma cTnT levels correlate with CK levels

Most patients with limb girdle muscle weakness, including Pompe disease, have increased CK levels resulting from skeletal muscle damage.[20, 32] Figure 1 shows that CK and cTnT levels correlated in all three subgroups (P<.001). In adult patients a slight negative correlation was found between cTnT levels and age (rho=-0.29, P=.005). cTnT levels did not differ when adult patients were grouped according to sex (P=.10), treatment with ERT (P=.29), wheelchair dependency (P=.57) or hypertension (P=.76). These findings suggested that similar to CK, cTnT is released from damaged skeletal muscle into the circulation in Pompe patients.

Expression of cTnT in skeletal muscle tissue of Pompe patients

To determine whether cTnT is expressed in skeletal muscle tissue, real-time quantitative polymerase chain reaction (RT-qPCR) analysis was performed. Primers were used that recognize a C-terminal sequence present in all known isoforms. Cloning of the RT-qPCR product followed by sequence analysis confirmed the identity of the product (Suppl. Figure 1A, B). Strong expression of cTnT was detected in normal heart tissue, while cTnT was not expressed in the skeletal muscle biopsies taken from healthy controls of several ages (Figure 2). In contrast, two out of three skeletal muscle biopsies taken from adult Pompe patients with elevated circulating cTnT showed strong expression of cTnT mRNA. The cardiac-specific marker MYBPC3 was expressed in heart but not in the Pompe skeletal muscle biopsies. Surprisingly, albumin, which was highly expressed in liver tissue, was expressed in the skeletal muscle biopsy of Pompe patient 1, which was the same biopsy that failed to express cTnT. We speculate that this biopsy may have contained adipose tissue, known to express albumin.

cTnT is known to be alternatively spliced. Notably, isoform 6 is expressed in normal adult heart, isoforms 1, 7, and 8 are expressed in foetal heart, and isoform 7 is also expressed in diseased heart.[33] To determine which cTnT isoform is expressed in skeletal muscle of Pompe patients, N-terminal primers that flank the region of alternative splicing were used in RT-qPCR.
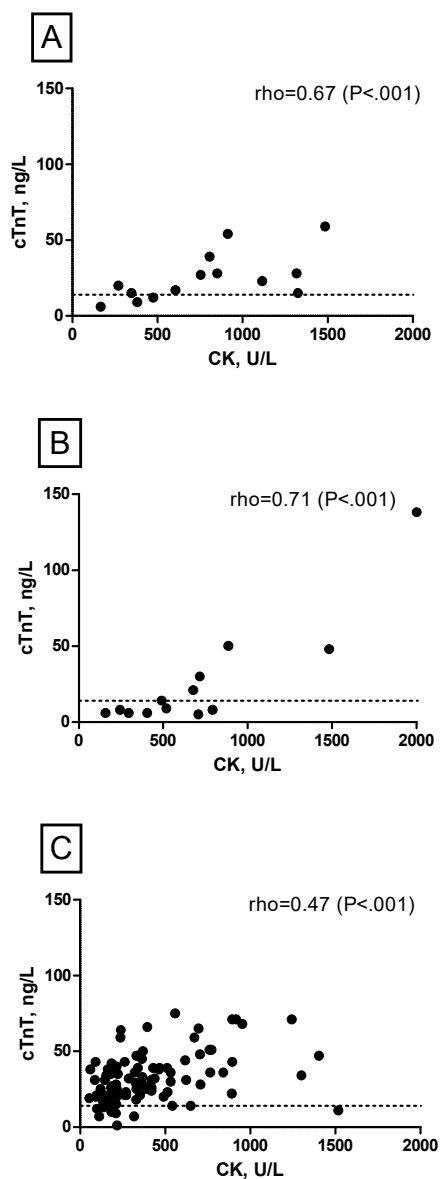
Figure 1 Plasma cTnT and CK levels are correlated.

Spearman correlation analysis was performed for (A) 14 classic infantile patients, (B) 13 childhood onset patients, and (C) 95 adult patients. Corresponding p-values are indicated. The dashed line represents the 99th-percentile cut-off at 14 ng/L for cTnT.
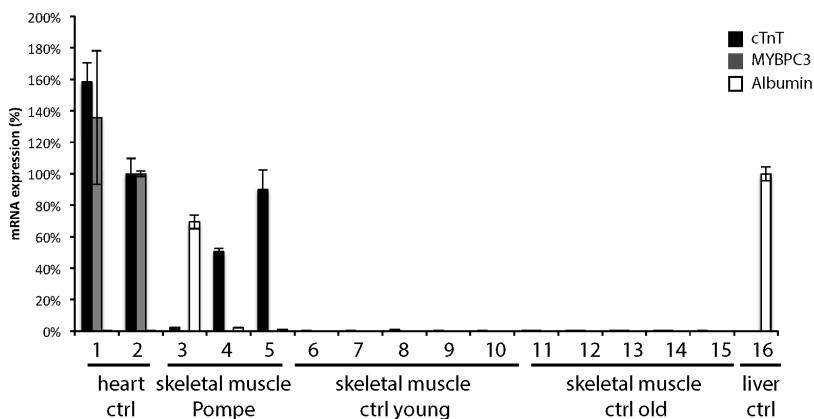
Figure 2 **cTnT mRNA expression is elevated in skeletal muscle of Pompe patients.**

Tissue biopsies were analyzed using RT-qPCR for expression of cTnT (using C-terminal primers), MYPBC3 (cardiac muscle-specific marker) and albumin in heart, skeletal (sk) muscle and liver. Sixteen individuals are shown: 2 controls (heart biopsy), 3 Pompe patients (skeletal muscle biopsy), 5 young controls (Ctrl) (skeletal muscle biopsy), 5 old controls (skeletal muscle biopsy), 1 control (liver biopsy). Plasma cTnT levels of Pompe patients ranged from 51-68 ng/l and ages at sampling ranged from 31-59 years. Age groups of controls are indicated, in which "young" indicates a range of 18-20 years, and "old" a range of 69-80 years. Liver served as positive control for albumin and was not assessed for MYPBC3 and cTnT expression. Values are normalized for b-actin and are expressed relative to one positive control (heart 2 for cTnT and MYPBC3; liver for albumin). Data indicate means +/- SD of three technical replicates.

Agarose gel electrophoresis showed a single product of 129 nt for RT-qPCR of normal cardiac and Pompe-derived skeletal muscle biopsies (data not shown). Cloning and sequencing revealed that the Pompe skeletal muscle product was isoform 6 (Suppl. Figure 1B), which is the cTnT isoform expressed in healthy heart.

To determine whether the presence of cTnT mRNA also leads to cTnT protein expression in skeletal muscle from Pompe patients, mass spectrometry was used. In an unbiased proteomic screen, the cTnT-specific tryptic peptide DLNELQALIEAHFENR was unambiguously identified amongst many other peptides specific for fast and slow skeletal muscle troponin (Suppl. Figure 2). In conclusion, Pompe patients have elevated mRNA expression of cTnT in skeletal muscle that is translated into cTnT protein,

consistent with the possibility of leakage of cTnT protein from skeletal muscle into the circulation as a result of muscle damage.

Effect of ERT on plasma cTnT levels

ERT has been shown to improve skeletal muscle function in adult Pompe patients. To determine the effect of ERT on cTnT levels, seven adult Pompe patients were tested just before start of ERT, after one and three months of treatment, and after two years. In six patients, cTnT levels decreased after start of ERT (Figure 3A). Interestingly, CK levels closely followed cTnT levels in these patients (Figure 3B). At group level, median decreases of cTnT and CK during two years of ERT were 8 ng/L (P=.03) and 136 U/L (P=.02), respectively (Suppl. Figure 3).

DISCUSSION

We found that plasma cTnT but not cTnI levels were increased in the majority of patients with Pompe disease, and that cTnT levels correlated with CK levels. Elevated cTnT levels could not be explained by cardiac abnormalities. cTnT was expressed in skeletal muscle biopsies taken from
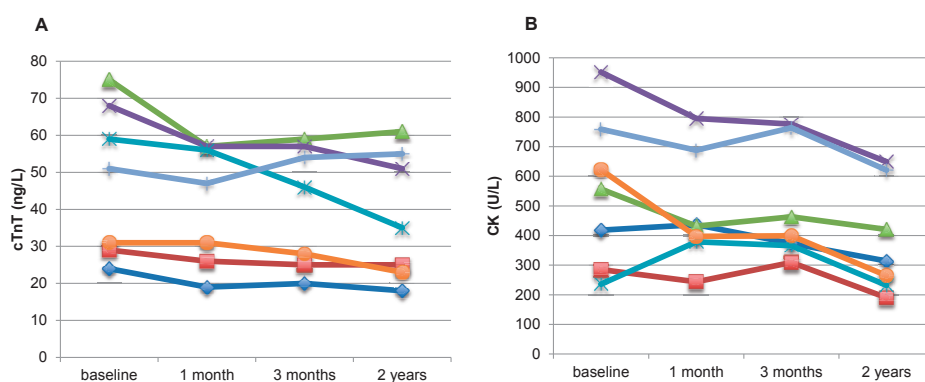
Figure 3 **Effects of ERT on plasma cTnT and CK levels.**

Seven adult Pompe patients with elevated plasma cTnT (A) and CK (B) levels at baseline and after start of ERT. Individual patients are indicated by a single color.

Pompe patients, but not healthy individuals. We conclude that increased plasma cTnT levels in Pompe patients are most likely the result of skeletal muscle damage rather than myocardial damage.

Cardiac troponins are the preferred biomarkers for detection of AMI. According to an expert consensus document for the universal definition of myocardial infarction, AMI should be diagnosed if a rise and/or fall of cardiac biomarkers is detected (preferably cTnT or cTnI), with at least one value above the 99th-percentile cutoff value. These changes need to be accompanied by symptoms of ischemia, ECG changes, imaging evidence of recent loss of viable myocardium or new regional wall motion abnormalities, and/or identification of an intracoronary thrombus by angiography or autopsy.[5] The next generation of high-sensitivity assays for detecting markers of myocardial damage in blood has improved the early detection of AMI. However, by increasing the sensitivity for detection of AMI, the specificity of detection has decreased leading to more false-positive results caused by pathologies potentially not related to AMI.[6, 8]

Increased plasma concentrations of cTnT have been described in individual cases and small sample size reports of patients with various myopathies.[9, 11-13, 34] In some skeletal muscle myopathies such as Duchenne muscular dystrophy, the heart is known to be affected, which might explain increased cTnT levels.[35] Hypertrophic cardiomyopathy is a characteristic feature in patients with classic infantile Pompe disease (but not in the childhood and adult forms of Pompe disease) and in most cases it is known to respond well to ERT.[17, 21, 22] In our study all classic infantile patients were already being treated with ERT (median five years, IQR 2-10 years), but in four patients LVH was not (yet) resolved at the time cTnT levels were measured. Patient 12 (Suppl. Table 2) had only received two infusions of ERT (cTnT 39 ng/L) and patient 7 was treated for six months (cTnT 20 ng/L). Both patients showed normalization of LVMI values upon prolonged ERT. Patient 9 (cTnT 27 ng/L) started with ERT at the age of seven months and was treated for 13 years. At the start of treatment she already had a severe hypertrophic cardiomyopathy, which persisted over the years. Patient 8 showed an increased LVMI after four years of ERT (cTnT 23 ng/L), but the LVMI was normalized after an additional two years of ERT. For these

patients, the source of plasma cTnT levels, being heart or skeletal muscle-derived, is unknown.

The high degree of similarity between members of the troponin family, especially between fast skeletal, slow skeletal, and cardiac troponin (http://www.uniprot.org/), complicates the identification of cTnT in adult muscle biopsies using single antibodies because of potential cross reactivity. Indeed, our initial experiments using three antibodies for cTnT yielded inconclusive results. Reports in the literature using antibody-based detection suggested that cTnT can be expressed in diseased skeletal muscle.[3, 10, 13, 36, 37] Here we used sequence-based techniques to exclude detection of troponin paralogs. This showed induced mRNA expression of the cTnT isoform 6 in skeletal muscle of Pompe patients, which is normally expressed in the heart of healthy adults, rather than re-expression of an embryonic splice form.[33, 38] Mass spectrometry analysis confirmed expression of cTnT at the protein level. Although the mass spectrometry method is not quantitative and it is difficult to predict the ability to detect certain peptides, a higher number of cTnT peptides was anticipated based on the high mRNA expression of cTnT in diseased skeletal muscle. This may indicate protein instability or a low efficacy of protein translation. A genome-wide mRNA expression study indicated elevated cTnT expression in skeletal muscle biopsies taken from untreated classic infantile Pompe patients, who have LVH.[39] cTnT expression in skeletal muscle from adult, childhood onset, and treated classic infantile Pompe patients, who lack a cardiac phenotype, has not been reported so far. Accumulating evidence indicates that cTnT expression in skeletal muscle is a general phenomenon in several neuromuscular diseases. Because these disorders can have various causes, it is likely that a more general signal such as muscle tissue injury rather than a disease-specific signal plays a role in induction of expression. Future experiments should elucidate how re-expression of cTnT in diseased skeletal muscle is regulated and whether it serves a biological function or is merely an aberrant side effect.

cTnI and CK-MB were measured as two other biomarkers for possible cardiac damage. It has been described that cTnI has a higher specificity for cardiac tissue than cTnT and that cTnI is not expressed in healthy or diseased skeletal muscle.[6, 13, 40] Indeed, we did not find increased cTnI levels

in our Pompe patients. Therefore, cTnI might be a better biomarker for AMI in Pompe disease and possibly also other neuromuscular disorders, as suggested previously.[9] CK-MB levels were increased in 59% of Pompe patients. Elevation of CK-MB in patients with neuromuscular diseases of various etiologies without evidence of acute myocardial injury has previously been described as a result of regeneration after skeletal muscle damage, providing a possible explanation why these levels are also elevated in Pompe patients.[3, 9, 41]

Our study has some limitations. First, although all findings point to skeletal muscle damage as being the cause of increased plasma cTnT levels in Pompe disease, we cannot fully rule out that there may have been subclinical myocardial damage in some patients. However, we consider all the evidence together sufficiently convincing to state that diseased skeletal muscle tissue is the source of elevated cTnT levels in patients with Pompe disease who do not have signs of cardiac involvement. Second, the reference values for cTnT measured with high-sensitivity assays are based on measurements in healthy adults, raising the question how these relate to values in childhood onset and infantile patients. Recent studies showed that cTnT levels measured with high-sensitivity assays are lowest in individuals younger than 18 years and that cTnT levels increase with age, suggesting that the number of classic infantile and childhood onset patients with elevated cTnT levels may even be underestimated in this study.[42] Another limitation is that we did not use the high-sensitivity troponin I assay, which is more sensitive than the assay we used. However, cTnI levels were very low in our patient population (median <0.01 μg/L), while the 99th-percentile limit in the general adult population is 0.04 μg/L, which makes it unlikely that the assay used here showed false negative outcomes. A final limitation of our study is that we did not use MRI to evaluate myocardial damage. We did not use this technique because a large proportion of the Pompe patients had a decreased forced vital capacity, particularly in supine position, making it difficult for them to remain in this position in the MRI for 30-60 minutes. Moreover, a recent study showed that there was no cardiac involvement on MRI in a small group of adult Pompe patients.[22]

CONCLUSIONS

In the majority of patients with Pompe disease plasma cTnT levels are increased due to skeletal muscle damage, while myocardial damage is unlikely. Not only in Pompe disease, but likely also in other neuromuscular diseases, it is important to take this in consideration and to avoid unnecessary and potentially harmful cardiac interventions.

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SUPPLEMENTAL MATERIAL

Supplemental Figure 1. Sequence analysis of cTnT mRNA isoforms.

A Sequence analysis of the RT-qPCR product from skeletal muscle of Pompe patients using C-terminal cTnT primers to verify that cTnT cDNA was amplified.

B Alignment of the PCR product from A) against cTnT isoform 6.

C Sequence analysis of RT-qPCR products of skeletal muscle of Pompe patients using N-terminal cTnT primers resulting in identification of the adult cTnT isoform 6.

D Alignment of the PCR product from C) against cTnT isoform 6.

Supplemental Figure 2. cTnT protein expression in skeletal muscle of a Pompe patient.

HCD fragmentation mass spectrum of the specific tryptic cTnT peptide identified from a skeletal muscle biopsy of Pompe patient 3 (see Figure 2). B and y fragment ions are indicated.
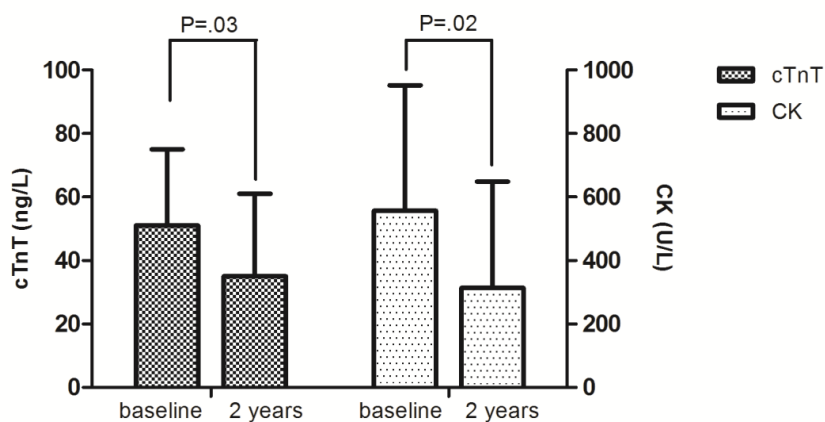
Supplemental Figure 3. Effects of ERT on plasma cTnT and CK levels.

The median differences of plasma cTnT and CK levels before and after two years of ERT in seven adult Pompe patients.

Supplemental Table 1. Primers used for RT-qPCR analysis.

Gene	Forward (5'-3')	Reverse (5'-3')	Product after amplification (bp)
Albumin	AGTGAGGTTGCTCATCGGTT	TGGACACTGCTGAAGATACTGA	93
b-Actin	GGCAATGTGAAAATGACCCTG	AGCAGACCTTCTTTGGCAGAT	101
MYBPC1*	CTCCCAGCTGTCCATCTTGT	GCCAGGTCCATCCATTTTCC	150
MYBPC3†	CCCATTGGCCTCTTCGTGAT	CTGCTCAGGTCCACCCATTT	146
C-terminal cTnT	AGACTGAGCGGGAAAAGAAGAAG	TGCCTCCAAGTTATAGATGCTCTG	131
N-terminal cTnT	AAGAGGTGGTGGAAGAGTACGAG	CTTCATCTTCTTCTGCCCTGGT	129

* MYBPC1 = skeletal muscle-specific marker

† MYBPC3 = cardiac muscle-specific marker

Supplemental Table 2. Individual plasma and cardiac parameters.

Patient	Phenotype	Gender	Age in years	ERT	SBP*	DBP†	cTnT‡	cTnI§	CK‖	CK-MB#	ECG**	LVMI††	LVID ED‡‡	LVID ES§§	IVS‖‖	LVPW##	FS***
1	classic infantile	female	2	yes	90	59	6	<0.01	166	3,7	normal	65	29	19	7	6	34
2	classic infantile	male	2	yes	93	56	9	<0.01	381	7,3	normal	64	31	19	6	5	39
3	classic infantile	female	2	yes	99	58	12	<0.01	474	11,5	normal	74	36	23	5	5	35
4	classic infantile	male	1	yes	107	62	15	<0.01	1324	10,7	normal	66	26	18	7	5	32
5	classic infantile	male	14	yes	-	-	15	<0.01	346	-	missing	-	-	-	-	-	-
6	classic infantile	female	8	yes	97	55	17	<0.01	605	9,5	normal	71	37	25	8	8	32
7	classic infantile	male	1	yes	98	54	20	<0.01	269	7,1	normal	83	23	14	8	7	41
8	classic infantile	male	4	yes	95	51	23	<0.01	1114	12,3	normal	101	41	25	9	6	38
9	classic infantile	male	14	yes	-	-	27	0.016	753	11	LVH	119	50	26	11	10	49
10	classic infantile	female	8	yes	112	54	28	<0.01	850	16,2	normal	85	44	25	10	9	44
11	classic infantile	female	14	yes	114	69	28	<0.01	1316	11,2	rhythm / conduction	68	40	25	8	7	37
12	classic infantile	female	1	yes	105	66	39	0.013	805	21,7	normal	199	26	18	12	9	31
13	classic infantile	male	5	yes	103	59	54	<0.01	912	17,4	normal	74	37	23	7	6	37
14	classic infantile	female	5	yes	95	51	59	<0.01	1484	11,9	normal	58	34	18	5	6	46
15	childhood onset	male	10	yes	113	56	5	<0.01	707	3,1	normal	69	46	31	7	7	34
16	childhood onset	male	5	yes	104	66	6	<0.01	158	4	missing	62	40	23	6	5	42
17	childhood onset	female	11	yes	108	66	6	<0.01	405	4	normal	76	47	27	9	7	42
18	childhood onset	female	14	yes	95	62	6	<0.01	295	3,3	normal	55	28	20	5	4	30
19	childhood onset	female	16	no	-	-	8	<0.01	792	5,8	normal	87	57	33	7	9	43
20	childhood onset	male	25	yes	120	60	8	<0.01	244	2,8	normal	109	56	38	9	8	32
21	childhood onset	male	10	yes	101	54	9	<0.01	518	4,5	normal	63	42	28	6	6	33
22	childhood onset	male	21	yes	140	90	14	<0.01	490	2,8	normal	92	57	37	7	8	35
23	childhood onset	male	10	yes	110	60	21	<0.01	677	11,2	rhythm / conduction	52	44	30	6	6	33

Supplemental Table 2. Individual plasma and cardiac parameters. (continued)

Patient	Phenotype	Gender	Age in years	ERT	SBP*	DBP†	cTnT‡	cTnI§	CK‖	CK-MB#	ECG**	LVMI††	LVID ED‡‡	LVID ES§§	IVS‖‖	LVPW##	FS***
24	childhood onset	female	13	yes	115	63	30	<0.01	716	10,5	rhythm / conduction	66	57	36	7	6	37
25	childhood onset	male	21	no	107	67	48	0.016	1482	31,3	normal	60	50	36	7	7	28
26	childhood onset	male	20	yes	100	60	50	<0.01	885	31,3	normal	42	41	27	6	6	34
27	childhood onset	male	20	yes	120	75	138	<0.01	2069	36,6	normal	66	54	34	8	7	38
28	adult onset	female	33	no	124	78	<5	<0.01	216	1,1	missing	-	-	-	-	-	-
29	adult onset	male	42	no	-	-	7	<0.01	316	3	normal	99	46	25	9	10	46
30	adult onset	female	54	no	120	96	7	<0.01	112	2,2	normal	77	43	29	9	10	33
31	adult onset	male	55	yes	109	65	9	<0.01	209	3,1	normal	66	45	27	9	8	40
32	adult onset	male	70	yes	159	108	10	<0.01	183	3,2	normal	-	-	-	-	-	-
33	adult onset	female	39	yes	121	74	11	<0.01	1516	8,6	normal	-	-	-	-	-	-
34	adult onset	male	56	yes	135	68	12	<0.01	98	2,8	normal	-	-	-	-	-	-
35	adult onset	male	66	yes	117	83	13	<0.01	178	4,2	rhythm / conduction	51	43	32	8	7	26
36	adult onset	male	68	yes	143	94	13	<0.01	136	3,3	normal	-	-	-	-	-	-
37	adult onset	male	44	yes	138	84	14	<0.01	540	9,7	normal	121	53	35	12	11	34
38	adult onset	male	44	yes	124	84	14	<0.01	648	8,7	normal	-	-	-	-	-	-
39	adult onset	female	67	yes	158	80	15	0.033	211	7	normal	-	-	-	-	-	-
40	adult onset	male	66	yes	163	94	17	<0.01	137	4,5	normal	-	-	-	-	-	-
41	adult onset	female	66	yes	143	91	17	<0.01	133	3,7	normal	-	-	-	-	-	-
42	adult onset	female	49	yes	115	75	18	<0.01	328	6,3	normal	-	-	-	-	-	-
43	adult onset	female	49	yes	132	76	18	<0.01	196	5,8	normal	-	-	-	-	-	-
44	adult onset	female	67	yes	117	67	18	<0.01	163	3,2	normal	-	-	-	-	-	-
45	adult onset	male	64	yes	164	96	19	<0.01	188	3,5	normal	98	53	36	9	9	32
46	adult onset	female	75	yes	127	75	19	<0.01	53	3	rhythm / conduction	132	58	44	13	11	24
47	adult onset	male	64	yes	169	81	20	<0.01	491	14,8	missing	81	51	30	8	10	41
48	adult onset	male	72	yes	144	87	20	<0.01	173	7	LVH	-	-	-	-	-	-

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Supplemental Table 2. Individual plasma and cardiac parameters. (continued)

Patient	Phenotype	Gender	Age in years	ERT	SBP[*]	DBP[†]	cTnT[‡]	cTnI[§]	CK[‖]	CK-MB[#]	ECG[**]	LVMI[‡]	LVID ED[##]	LVID ES[§§]	IVS[‖‖]	LVPW[##]	FS[***]
49	adult onset	male	40	yes	132	73	20	<0.01	214	5	normal	-	-	-	-	-	-
50	adult onset	male	42	yes	119	76	20	<0.01	92	1,8	rhythm / conduction	-	-	-	-	-	-
51	adult onset	female	55	yes	142	88	21	<0.01	167	10,8	normal	90	47	31	11	10	34
52	adult onset	female	43	yes	134	78	21	<0.01	261	7,5	normal	76	46	31	9	9	33
53	adult onset	female	60	yes	-	-	21	<0.01	352	6,9	normal	-	-	-	-	-	-
54	adult onset	male	57	yes	115	74	21	<0.01	271	6	normal	-	-	-	-	-	-
55	adult onset	female	64	yes	152	84	21	<0.01	138	5,6	rhythm / conduction	-	-	-	-	-	-
56	adult onset	female	56	yes	108	70	21	<0.01	109	5	missing	-	-	-	-	-	-
57	adult onset	male	69	yes	157	90	21	<0.01	97	4	normal	-	-	-	-	-	-
58	adult onset	female	53	yes	138	77	22	0,011	890	6,6	normal	-	-	-	-	-	-
59	adult onset	female	50	no	129	74	23	<0.01	513	9,3	normal	96	50	37	11	12	26
60	adult onset	female	62	yes	115	75	23	<0.01	224	4,9	rhythm / conduction	-	-	-	-	-	-
61	adult onset	female	66	yes	158	75	23	<0.01	268	4,1	normal	-	-	-	-	-	-
62	adult onset	female	57	no	130	80	24	<0.01	418	18	missing	54	38	27	8	8	29
63	adult onset	male	57	yes	155	97	24	<0.01	199	6,5	rhythm / conduction	-	-	-	-	-	-
64	adult onset	female	49	yes	103	70	25	<0.01	388	7,5	normal	70	41	30	11	8	27
65	adult onset	male	44	yes	108	64	25	<0.01	331	5,5	normal	-	-	-	-	-	-
66	adult onset	male	75	yes	201	94	25	<0.01	119	2,9	normal	-	-	-	-	-	-
67	adult onset	female	54	yes	166	100	26	<0.01	418	8	normal	-	-	-	-	-	-
68	adult onset	male	73	no	108	57	26	<0.01	180	6,8	missing	-	-	-	-	-	-
69	adult onset	male	67	yes	150	79	26	<0.01	213	6,5	rhythm / conduction	-	-	-	-	-	-
70	adult onset	male	56	yes	124	90	27	<0.01	381	7,4	normal	68	41	28	10	10	32
71	adult onset	female	65	yes	122	77	27	<0.01	188	5,9	normal	-	-	-	-	-	-

Supplemental Table 2. Individual plasma and cardiac parameters. (continued)

Patient	Phenotype	Gender	Age in years	ERT	SBP*	DBP†	cTnT‡	cTnI§	CK‖	CK-MB#	ECG**	LVMI††	LVID ED##	LVID ES§§	IVS‖‖	LVPW##	FS***
72	adult onset	male	62	yes	120	76	28	<0.01	350	9,9	normal	-	-	-	-	-	-
73	adult onset	female	39	yes	126	69	28	<0.01	211	8,6	normal	-	-	-	-	-	-
74	adult onset	female	46	yes	139	84	28	0.012	706	7,1	normal	-	-	-	-	-	-
75	adult onset	male	51	no	148	93	29	<0.01	326	5,8	missing	-	-	-	-	-	-
76	adult onset	female	59	yes	145	104	30	<0.01	531	14,2	rhythm / conduction	59	39	24	10	10	38
77	adult onset	female	44	no	110	63	31	<0.01	622	15,4	normal	-	-	-	-	-	-
78	adult onset	male	41	yes	137	93	31	<0.01	418	8,9	normal	-	-	-	-	-	-
79	adult onset	male	62	yes	116	76	31	<0.01	86	3,5	normal	-	-	-	-	-	-
80	adult onset	female	76	yes	146	87	31	<0.01	145	3,4	normal	-	-	-	-	-	-
81	adult onset	female	36	yes	97	55	32	<0.01	437	7,3	normal	-	-	-	-	-	-
82	adult onset	male	47	no	111	75	32	<0.01	285	4,6	normal	-	-	-	-	-	-
83	adult onset	female	57	no	130	78	33	<0.01	365	9,2	missing	-	-	-	-	-	-
84	adult onset	female	37	yes	149	83	34	<0.01	1299	15,9	normal	64	40	25	10	8	38
85	adult onset	male	63	yes	159	89	34	<0.01	153	3,6	normal	-	-	-	-	-	-
86	adult onset	female	58	yes	134	86	35	<0.01	220	7,1	normal	54	34	23	10	9	32
87	adult onset	female	66	yes	151	78	35	<0.01	179	6,2	normal	-	-	-	-	-	-
88	adult onset	female	59	yes	120	66	36	0.012	841	22,2	normal	82	51	28	8	9	45
89	adult onset	male	48	no	150	89	36	<0.01	764	18,4	normal	88	50	29	10	9	42
90	adult onset	male	44	yes	141	89	36	<0.01	530	11,5	normal	-	-	-	-	-	-
91	adult onset	female	58	yes	125	75	36	<0.01	320	10,3	normal	-	-	-	-	-	-
92	adult onset	female	57	yes	127	66	38	<0.01	463	12,9	normal	68	44	37	8	9	16
93	adult onset	male	79	yes	118	71	38	<0.01	59	4,2	normal	-	-	-	-	-	-
94	adult onset	male	63	yes	162	79	38	<0.01	162	3,7	normal	-	-	-	-	-	-
95	adult onset	female	48	yes	161	81	39	<0.01	466	13,2	normal	47	42	21	8	9	50
96	adult onset	female	41	yes	140	79	39	<0.01	511	12,3	normal	86	55	38	9	9	31
97	adult onset	male	72	yes	150	95	39	<0.01	428	12,3	missing	-	-	-	-	-	-

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Supplemental Table 2. Individual plasma and cardiac parameters. (continued)

Patient	Phenotype	Gender	Age in years	ERT	SBP*	DBP†	cTnT‡	cTnI§	CK‖	CKMB#	ECG**	LVMI††	LVID ED‡‡	LVID ES§§	IVS‖‖	LVPW##	FS***
98	adult onset	female	56	no	145	84	39	<0.01	338	8,3	normal	-	-	-	-	-	-
99	adult onset	male	71	yes	170	89	40	<0.01	214	6,9	normal	78	52	34	8	9	35
100	adult onset	male	63	yes	111	72	42	<0.01	184	4,7	normal	-	-	-	-	-	-
101	adult onset	female	34	yes	164	105	43	<0.01	893	10,4	normal	82	50	36	8	9	28
102	adult onset	male	50	yes	110	69	43	<0.01	262	6,9	rhythm / conduction	-	-	-	-	-	-
103	adult onset	male	68	yes	106	73	43	<0.01	91	2,8	normal	-	-	-	-	-	-
104	adult onset	male	43	yes	133	83	44	<0.01	616	9,4	normal	-	-	-	-	-	-
105	adult onset	female	34	yes	115	69	45	0.032	363	7,6	normal	58	41	29	7	10	29
106	adult onset	male	33	no	131	81	47	<0.01	1403	16,9	missing	78	48	31	9	10	35
107	adult onset	female	62	no	131	69	47	<0.01	329	13,2	normal	-	-	-	-	-	-
108	adult onset	male	54	yes	118	67	47	<0.01	337	10,5	LVH	-	-	-	-	-	-
109	adult onset	female	58	yes	121	74	48	<0.01	704	19,8	rhythm / conduction	83	46	32	10	10	30
110	adult onset	female	25	yes	126	79	50	<0.01	368	10,4	normal	-	-	-	-	-	-
111	adult onset	male	31	yes	97	53	51	<0.01	771	13,9	normal	65	37	27	8	9	27
112	adult onset	female	31	no	121	78	51	0.013	759	11,1	normal	-	-	-	-	-	-
113	adult onset	male	38	yes	150	89	59	0.013	670	22,9	LVH	86	54	24	10	9	56
114	adult onset	male	48	no	140	82	59	0.015	236	9,9	LVH	101	46	25	10	10	46
115	adult onset	male	41	yes	127	62	64	<0.01	238	6,3	normal	-	-	-	-	-	-
116	adult onset	female	48	yes	135	88	65	<0.01	695	28,8	normal	56	41	27	10	8	34
117	adult onset	male	38	yes	130	79	66	<0.01	394	8	normal	83	49	25	9	11	49
118	adult onset	male	59	no	154	107	68	<0.01	952	7,5	rhythm / conduction	-	-	-	-	-	-
119	adult onset	male	44	yes	147	89	71	0.018	1243	30,9	LVH	56	48	32	8	8	33
120	adult onset	male	46	yes	157	90	71	<0.01	893	22,6	normal	91	53	37	9	10	30
121	adult onset	female	27	yes	148	85	71	<0.01	915	17,2	LVH	59	46	34	8	7	26
122	adult onset	male	50	no	163	107	75	<0.01	557	18,7	normal	-	-	-	-	-	-

*   SBP = systolic blood pressure in mmHg

†   DBP = diastolic blood pressure in mmHg

‡   cTnT = cardiac troponin T: 99th percentile <14 ng/L in the general adult onset population, LoD 5 ng/L

§   cTnI = cardiac troponin I: 99th percentile <0.04 µg/L in the general adult population, LoD 0.01 µg/L

∥   CK = creatine kinase: references ranges in our hospital: children (2-13 years) <230 U/L, male adolescents (13-17 years) <270 U/L, female adolescents (13-17 years) <123 U/L, male adults (>17 years) <200 U/L, female adults (>17 years) <170 U/L

CK-MB = creatine kinase myocardial band: female adults <4.7 µg/L and male adults <7.6 µg/L

** rhythm and conduction disturbances included: prolonged PR-interval (n=1), prolonged QT-interval (n=1), right bundle branch block (n=8), left bundle branch block (n=1), pacemaker rhythm (n=1), sinus tachycardia (n=2)

†† LVMI = left ventricular mass index: reference range in children depends on body surface, in adult females it ranges from 43-95 g/m2 and in adult males from 49-115 g/m2

‡‡ LIVD ED = left ventricular end diastolic dimension: reference range in children depends on weight, in adults it ranges from 37-55 mm

§§ LIVD ES = left ventricular end systolic dimension: reference range in children depends on weight, in adults it ranges from 20-40 mm

∥∥ IVS = interventricular septum: reference range in children depends on weight, in adults it ranges from 6-11 mm

LVPW = left ventricular posterior wall: reference range in children depends on weight, in adults it ranges from 6-11 mm

*** FS = fractional shortening: an FS below 25% was considered as insufficient shortening of the ventricle for every group

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Chapter 5

Lung MRI and impairment of diaphragmatic function in Pompe disease

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ABSTRACT

Introduction Pompe disease is a progressive metabolic myopathy. Involvement of respiratory muscles leads to progressive pulmonary dysfunction, particularly in supine position. Diaphragmatic weakness is considered to be the most important component. Standard spirometry is to some extent indicative but provides too little insight into diaphragmatic dynamics. We used lung MRI to study diaphragmatic and chest-wall movements in Pompe disease.

Methods In ten adult Pompe patients and six volunteers, we acquired two static spirometer-controlled MRI scans during maximum inspiration and expiration. Images were manually segmented. After normalization for lung size, changes in lung dimensions between inspiration and expiration were used for analysis; normalization was based on the cranial-caudal length ratio (representing vertical diaphragmatic displacement), and the anterior-posterior and left-right length ratios (representing chest-wall movements due to thoracic muscles).

Results We observed striking dysfunction of the diaphragm in Pompe patients; in some patients the diaphragm did not show any displacement. Patients had smaller cranial-caudal length ratios than volunteers ($p<0.001$), indicating diaphragmatic weakness. This variable strongly correlated with forced vital capacity in supine position ($r=0.88$) and postural drop ($r=0.89$). While anterior-posterior length ratios also differed between patients and volunteers ($p=0.04$), left-right length ratios did not ($p=0.1$).

Conclusion MRI is an innovative tool to visualize diaphragmatic dynamics in Pompe patients and to study chest-wall and diaphragmatic movements in more detail. Our data indicate that diaphragmatic displacement may be severely disturbed in patients with Pompe disease.

INTRODUCTION

Pompe disease (OMIM 232300: acid maltase deficiency or glycogen storage disease type II) is an inherited progressive metabolic myopathy caused by acid α-glucosidase deficiency due to mutations in the acid α-glucosidase (*GAA*) gene (OMIM 606800).[1,2] Pulmonary dysfunction caused by progressive weakness of the respiratory muscles is a characteristic feature of the disease.[1,3,4] In patients with the classic infantile form cardiorespiratory failure leads to death within the first year of life.[5,6] In patients with late-onset or non-classic Pompe disease pulmonary dysfunction progresses more slowly. The first sign of respiratory involvement in these patients is decreased pulmonary function in supine position, eventually necessitating respiratory support during sleep. Patients in the end-stage of the disease require continuous respiratory support.[7-9] Weakness of the diaphragm–the main respiratory muscle–is considered to be the major cause of respiratory dysfunction in Pompe disease.[3,10] Although pulmonary function tests (PFTs) may be indicative of diaphragmatic weakness by showing a difference between forced vital capacity (FVC) in sitting and supine position–i.e. postural drop–or by a decreased mean inspiratory pressure (MIP), they provide too little insight in dynamics of the diaphragm.[11] More insight in the function of the diaphragm has become extra relevant since enzyme replacement therapy (ERT) has been available for Pompe disease. While several studies have shown that ERT has positive effects on skeletal muscle function by showing stabilization or improvement of muscle strength or the distance walked in six minutes, the effects on lung function especially in supine position seem to be less pronounced.[7,12-16] In an earlier study that compared the effects of ERT on pulmonary function in sitting and supine positions, we found that 15% of patients were therapy resistant when pulmonary function was measured in sitting position, and that 35% were therapy resistant when it was measured in supine position.[13] Recent MRI sequences and image analysis techniques make it possible to directly assess the individual contribution of respiratory muscles–including the diaphragm–during the breathing cycle.[17-22]

The aim of the current study was to determine whether MRI could be used as an innovative tool to gain greater insight into the function of the

diaphragm in Pompe disease, and to correlate these data with the results of PFTs.

METHODS

Study population

All patients with Pompe disease in the Netherlands are referred to Erasmus MC University Medical Centre Rotterdam. For this cross-sectional pilot study we selected ten adult patients with various degrees of respiratory dysfunction. As controls we included six age and gender-matched volunteers. Informed consent was obtained from all participants. The study protocol was approved by the Medical Ethical Committee at our hospital (Amendment 7 to protocol MEC-2007-103).

PFT

An MRI-compatible spirometer was used to standardize lung volumes and breathing movements during the MRI (MasterSceen Pneumo spirometer, CareFusion, Houten, the Netherlands). Before MRI, FVC and forced expiratory volume in one second (FEV_1) were measured according to ATS/ERS standards.[23,24] Spirometry parameters are expressed in percentages predicted. Postural drop (ΔFVC) was calculated as ($FVC_{sitting}$-FVC_{supine})/$FVC_{sitting}$ *100%. An ΔFVC of more than 25% is thought to reflect diaphragmatic weakness.[11,25] Before MRI, a Dwyer pressure gauge was used according to ATS/ERS standards to measure maximum static inspiratory (MIP) and expiratory pressures (MEP).[26] Results are expressed in kilopascal (kPa). The carbon dioxide (CO2) fraction in the expired gas was measured with a capnograph (ms-capno, Viasys Healthcare, Wurzberg, Germany) at maximum expiration. In the absence of ventilation irregularities, the expiratory CO2 approximates the arterial CO2 pressure. A daytime expiratory CO2 over 6.0 kPa suggests hypercapnia and chronic alveolar hypoventilation.[27]

MRI and imaging analysis

Scanning was performed with a 3T GE Signa 750 MRI (General Electric Healthcare, Milwaukee, USA) using the whole-body coil for radio-frequency excitation and a 32-channel torso coil for signal reception. First, a 3-plane

localizer was performed during a maximum inspiratory movement (i.e. a five-second breath-hold scan); all subsequent volumes imaged were based on this localizer. Second, shimming was performed on this localizer, and shim settings were maintained throughout scanning. To evaluate changes in lung shape and volume, two static scans were acquired. These use two 12-second breath-hold scans covering the entire thoracic region acquired at end-inspiration and end-expiration in a 3D RF-spoiled gradient echo sequence with TR/TE=1/0.5 ms, flip angle 2°, sagittal volume acquisition with 3 mm slice thickness, 1.5 mm slice separation between slices and planar pixel resolution between 1.4x1.4 and 1.5x1.5 mm^2. Overall acquisition time per patient was 20 minutes.

Each lung was segmented manually at inspiration and expiration using 3D Slicer (http://www.slicer.org), with segmentation being performed every second slice in the axial plane.[28] A full 3D segmentation was reconstructed by interpolating the individual segmentation slices in the cranial-caudal axis. Using the 3D lung segmentations, the length and volume of each independent lung was estimated along the main axes of the MRI acquisition (cranial-caudal, anterior-posterior and left-right). To cope for variations in lung size due to inter-subject anatomical variations, each length at maximum inspiration was divided by the corresponding length at maximum expiration. Therefore the normalised value is a rate of length increase compared with the expiration point (e.g. a ratio of 1.2 would mean an increase of 20% in length). Because of the assumption that the chest-wall is responsible for changes in volume in the anterior-posterior and left-right directions, and the diaphragm expands the lung in the cranial-caudal directions, it is possible to study the contributions to volume changes of the chest-wall and the diaphragm individually.

Statistical analysis

Data were analyzed using SPSS version 21 (SPSS, Chicago, IL, USA) and are presented as medians with ranges, or as numbers with percentages. The Mann-Whitney test was used to analyze differences in PFT results and MRI findings between patients and volunteers. The Spearman's correlation coefficient (r) was used to calculate the relationship between PFT outcomes and MRI results in Pompe patients. A p-value <0.05 was considered statistically significant.

RESULTS

Study population

Table 1 shows the characteristics of the Pompe patients and the volunteers. All Pompe patients had an GAA deficiency and all patients carried the common mutation c.-32-13T>G in one *GAA* allele and a second pathogenic mutation in the second allele. In five patients this second pathogenic mutation was c.525delT, in two patients c.1548G>A, and the other three patients carried a different second mutation. None of the patients was currently smoking and two patients had smoked in the past. None of the patients or volunteers had co-morbidities that could influence the function of the diaphragm.

Table 1   Patient characteristics and PFT results in patients and volunteers

	Patients	Volunteers	P-value
Age (years)	46 (32-66)	43 (27-55)	0.25
Gender, (% males)	5 (50)	3 (50)	1.0
Height (cm)	178 (154-196)	177 (175-190)	0.39
Weight (kg)	73 (61-88)	85 (65-94)	0.13
BMI (kg/m^2)	23.4 (20.6-25.4)	24.9 (21-25.7)	0.18
Duration of the disease (years)	16 (9-30)	-	-
Duration of ERT (years)	5.5 (0-7)	-	-
Wheelchair dependent (%)	1 (10)	0 (0)	0.79
Ventilator dependent (%)	3 (30)	0 (0)	0.37
Pulmonary function test			
FVC$_{sitting}$ (%)	60 (45-84)	102 (92-111)	0.001
FVC$_{supine}$ (%)	43 (27-70)	102 (87-113)	0.001
ΔFVC (%)[a]	33 (11-44)	0 (0-10)	0.001
FEV$_{1\ sitting}$ (l/s)	59 (42-80)	98 (85-117)	0.001
FEV$_{1\ supine}$ (l/s)	40 (30-63)	91 (76-112)	0.001
MIP (kPa)	6.9 (3.9-8.3)	8.6 (6.4-11.8)	0.07
MEP (kPa)	10.0 (6.4-11.8)	12.5 (10.3-14.2)	0.02

Continuous variables are expressed as median and range, categorical variables as number and percentage. BMI = body mass index, ERT = enzyme replacement therapy, FVC = forced vital capacity, FEV$_1$ = forced expiratory volume in one second, MIP = maximum static inspiratory pressure, MEP = maximum static expiratory pressure.
[a] ΔFVC is calculated as (FVC$_{sitting}$-FVC$_{supine}$) / FVC$_{sitting}$ x 100%.

PFT

Table 1 shows PFT in sitting and supine positions. In both these positions, patients had lower median values for FVC and FEV_1 than healthy volunteers did (p=0.001). The median ΔFVC was higher in Pompe patients (p=0.001). The median MEP was lower in patients (10.0 kPa) than in volunteers (12.5 kPa, p=0.02). The median MIP showed a trend towards a lower median value for the patients (6.9 kPa) relative to the healthy volunteers (8.6 kPa, p=0.07). Three patients had a expiratory $CO2$ fraction over 6.0 kPa and two of these patients were ventilator dependent.

MRI

Figure 1 shows the line-up during the MRI. Participants were placed in supine position with the MRI-compatible spirometer positioned above them. Figure 2 shows coronal slices through the carina for the breath-hold scans with the corresponding color plots of a Pompe patient and a volunteer. In the Pompe patient there was hardly any displacement of the diaphragm. Additional file 1 in the online data supplement shows the color plots of each individual subject and additional files 2 and 3 demonstrate two examples of dynamic MRI scans in a healthy volunteer and a patient with Pompe disease.

Figure 3 shows the changes per individual in the three chest-cage directions between inspiration and expiration. In volunteers, the main contributor to the changes in lung volume was the diaphragm (white bars). In most

Figure 1   Line-up during the MRI

Patients were placed in supine position in the MRI scanner with an MRI-compatible spirometer positioned just above the head.
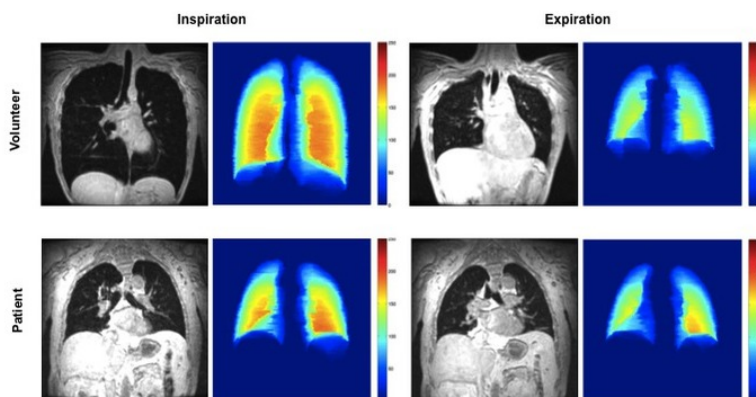
Figure 2   MR images and color maps at maximum inspiration and expiration

MR images during 12-second breath-holds in inspiration and expiration in a patient with Pompe disease and a healthy volunteer. The color maps represent the thickness of the segmentation in the anterior-posterior axis (red being the thickest and blue being the thinnest). Note the limited increase in vertical length in the Pompe patient relative to the increase in the healthy volunteer.
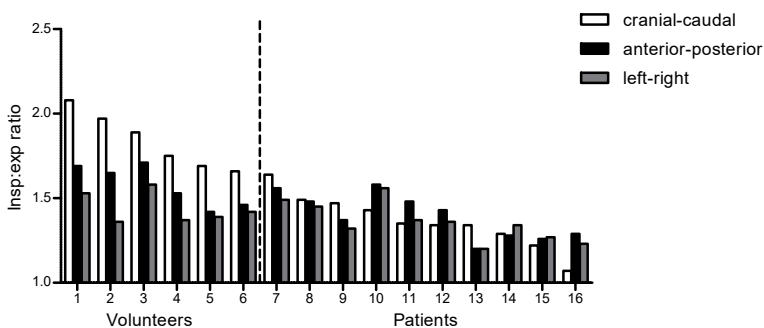

Figure 3   Ratios between inspiration and expiration in three directions for patients and volunteers measured with MRI

The length ratios between inspiration and expiration in the cranial-caudal direction (white bars), anterior-posterior direction (black bars) and left-right direction (grey bars) are shown for individual patients and volunteers. Volunteers are numbered 1 to 6 and patients 7 to 16. The length ratios are calculated by dividing the median length during inspiration by the median length during expiration for each axis.

Pompe patients, these changes were due mainly to the thoracic muscles (grey and black bars), but, as Figure 3 shows, these patients had a large variety in diaphragmatic and chest-wall movements. The median cranial-caudal length change, representing diaphragmatic displacement before normalization for lung size, was 82 mm (range 46-90 mm) in volunteers and 28 mm (range 5-49 mm) in patients (p=0.002). The median anterior-posterior length change was 37 mm in volunteers (range 25-42 mm) and 18 mm (range 13-31 mm) in patients (p=0.006); the median left-right length change was 24 mm in volunteers (range 21-34 mm) and 17 mm (10-26 mm) in patients (p=0.02).

Figure 4 shows the different length ratios after normalization for lung size. The cranial-caudal length ratio between inspiration and expiration (representing diaphragmatic displacement) was lower in Pompe patients (median 1.35, range 1.07-1.64) than in volunteers (median 1.82, range 1.66-2.08) (p=0.001). While the anterior-posterior length ratio was also lower in patients (median 1.40, range 1.20-1.58) than in volunteers (median 1.59, range 1.42-1.71) (p=0.04), the left-right length ratio did not differ significantly between patients (median 1.35, range 1.20-1.56) and volunteers (median 1.41, range 1.36-1.58) (p=0.1). In the three Pompe patients who were ventilator dependent the cranial-caudal length ratio was lower than in the other Pompe patients (median 1.22 versus 1.43, p=0.02). These Ventilator
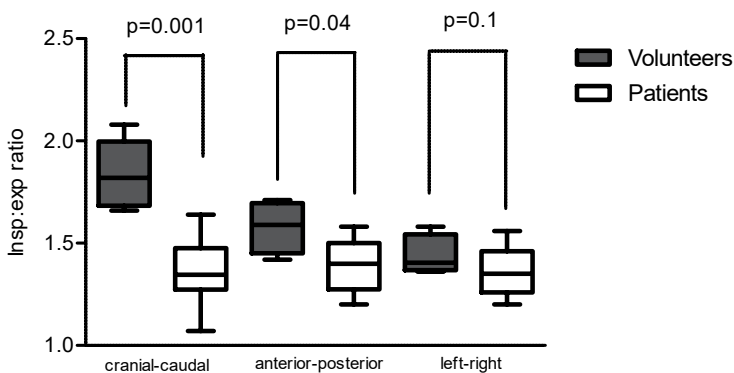
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Figure 4 Median ratios between inspiration and expiration in three directions for both groups

This figure shows the same ratios as Figure 2, but now for the groups of Pompe patients and volunteers. The box plots represent the median with the range. The Mann-Whitney test was used to calculate the difference in each direction between patients and volunteers.

dependent patients had a longer duration of the disease (median 29 years versus 15 years). There was no correlation between the cranial-caudal length ratio and the duration of ERT.

Correlation between PFT and MRI

As Figure 5 shows, ΔFVC and FVC supine were strongly correlated with the cranial-caudal length ratio (r=0.89 and r=0.88, p<0.001) in Pompe patients, but there were no correlation between MIP and the cranial-caudal length
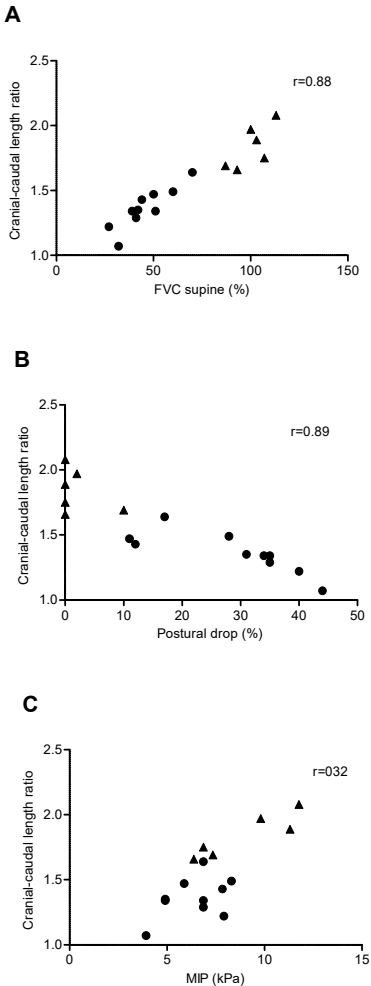

Figure 5 Correlation between cranial-caudal length ratios and FVC supine (A), postural drop (B) and MIP (C)

The dots represent patients and the triangles volunteers. Spearman's correlation coefficient (r) was used to calculate the correlation between the cranial-caudal length ratio versus FVC in supine position, the postural drop (ΔFVC) and MIP. As these calculations were performed only in the Pompe patients, the volunteers were excluded for these analyses. FVC = forced vital capacity, MIP = maximum static inspiratory pressure.

ratio (r=0.32, p=0.37), MEP and cranial-caudal length ratio (r=0.23, p=0.53), or FVC sitting and cranial-caudal length ratio (r=0.46, p=0.18). The only significant correlation regarding the anterior-posterior length ratio was with FVC supine (r=0.74, p=0.02).

DISCUSSION

Our study shows that MRI can be used as an innovative tool to gain greater insight into involvement of the diaphragm in Pompe disease. It was demonstrated that the diaphragmatic function is severely impaired and in some patients there was even hardly any displacement of the diaphragm. To a lesser extent, movement of the anterior chest-wall was reduced. Our results suggest that diaphragmatic displacement measured with MRI is strongly correlated with the postural drop and FVC in supine position measured with common spirometry.

Decreased pulmonary function is an important feature of Pompe disease. Ten or 15 years after onset, half of the adult patients with Pompe disease require ventilator assistance. The main cause of death in this group of patients is respiratory failure, a process in which dysfunction of the diaphragm is considered to play an important role.[8,9,29] Our MRI study suggests that the function of the diaphragm in Pompe disease is more impaired than that of the thoracic musculature. It is not clear how and why the diaphragm muscles are more severely affected than the other respiratory muscles. Our study supports a recent study describing atrophy of the diaphragm and reduced lung height on static MRI and computed tomography scans in patients with Pompe disease. In this latter study semi-quantitative scoring scales were used, and computed tomography was used to measure lung height in one direction.[30] In our study MRI was performed under spirometry control and lung-shape variations were quantified in three directions. This enabled us to show that the cranial-caudal movement related to diaphragmatic function in patients with Pompe disease is impaired more than the anterior-posterior motions of the anterior chest-wall. Similarly, the correlation we found between ΔFVC and FVC in supine position and our MRI results suggest that both these parameters might be used as an

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indirect tool for determining diaphragmatic function, with the advantage that MRI also visualizes diaphragmatic and chest-wall movements.

A striking finding in our study was that displacement of the diaphragm was extremely impaired in some of the patients, while still residual pulmonary function in supine position was measurable. This could have important consequences when therapy comes in place and might explain why pulmonary function, particularly in supine position, responds poorly to ERT in some of the Pompe patients. Therefore, more studies are required to investigate at what stage the diaphragm and other respiratory muscles become affected in Pompe disease; especially since it has been shown that response to ERT is better in patients who are less severely affected.[3,13,31] Another intriguing question is how MIP and MEP relate to diaphragmatic weakness. It has been hypothesized that these parameters might be better predictors for diaphragmatic weakness than FVC in supine position.[30] Our study implied a weak correlation between MIP or MEP and diaphragmatic displacement. A possible explanation could be that MIP reflects both the strength of the diaphragm and other inspiratory muscles, while the cranial-caudal length ratio only reflects diaphragmatic displacement. In an earlier study we found a positive correlation between FVC in upright position and MIP an MEP.[3] Larger studies are required to explore this relationship in more depth. Comparison of diaphragmatic involvement in patients with Pompe disease to those with other neuromuscular disorders such as Duchenne Muscular Dystrophy might provide insight whether onset and the extend of diaphragmatic involvement is disease specific.

A limitation of our pilot study is that we selected a relatively small number of adult Pompe patients with variable degrees of respiratory dysfunction (FVC in supine position: 27 to 70% of normal). This subset of patients may not be fully representative for the total group of Pompe patients. In subsequent studies also patients with normal or close to normal respiratory function need to be studied to get more insight at what stage of the disease the diaphragm becomes affected. The use of MRI to evaluate diaphragmatic and chest-wall movements has some limitations. Contraindications such as metal implants, invasive ventilation and claustrophobia make it impossible to scan certain patients. Moreover, patients need to be able to perform spirometry in supine position. In next studies it might also

be considered to include other techniques to measure lung and respiratory muscle function in addition to spirometry such as sniff nasal inspiratory pressures, transdiaphragmatic pressures or transdiaphragmatic twitch pressures.[32] Prigent et al. showed that transdiaphragmatic pressures and transdiaphragmatic twitch pressures correlated well with all spirometry volumes and non-invasive maximal pressures in adult patients with Pompe disease.[33] Whether transdiaphragmatic pressure measurements show a better correlation with the cranial-caudal length ratio measured with lung MRI than with spirometry data needs further investigation.

CONCLUSION

MRI appears to be an innovative tool to visualize diaphragmatic dynamics in Pompe patients and to study chest-wall and diaphragmatic movements in more detail. Our data indicate that diaphragmatic displacement can be very severely impaired in patients with Pompe disease and might explain why FVC responds poorly to ERT in some of the patients. As MRI adds detailed dynamic and structural information to data obtained by pulmonary function tests, particularly of the diaphragm, it may serve as a valuable tool in providing new insights in when the diaphragm starts to be involved in the disease process and on its responsiveness to therapy. It may also serve as a prognostic tool. More research is warranted to explore these topics.

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SUPPLEMENTARY FILES

Volunteers

Pompe patients

| Inspiration | Expiration | Inspiration | Expiration |
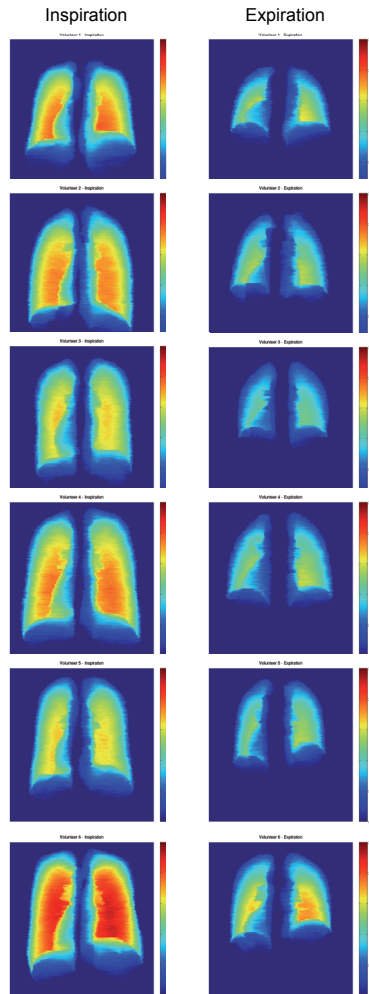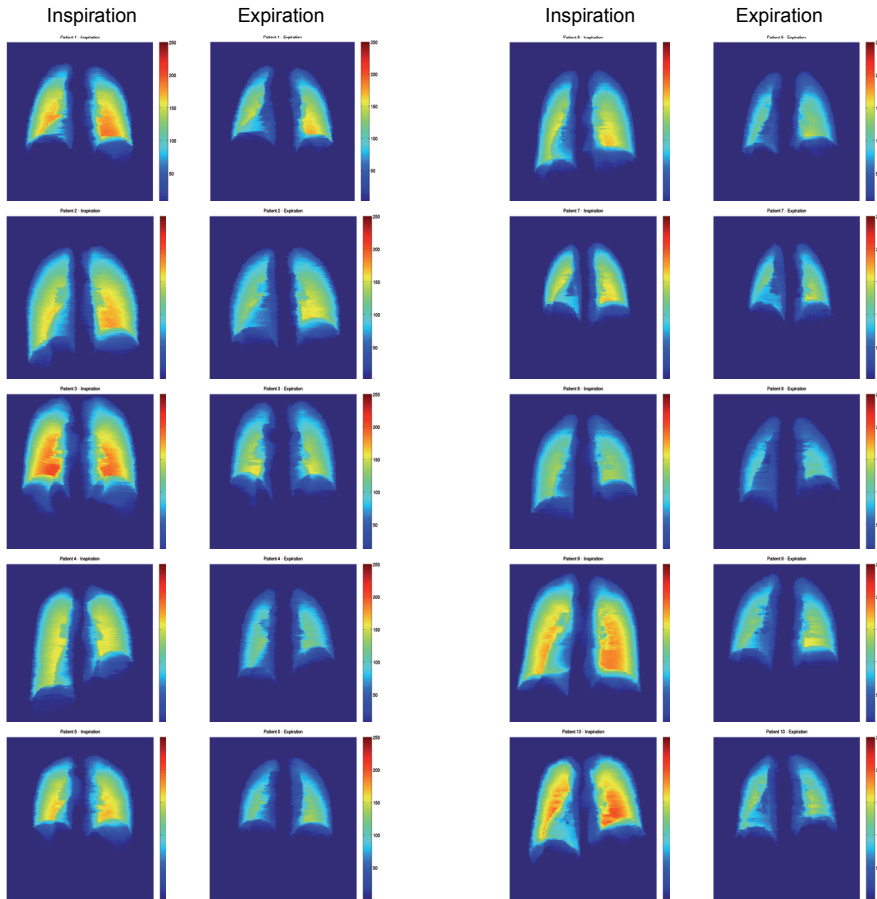
Additional file 1 Color maps made at maximum inspiration and expiration
The color maps represent the thickness of the segmentation in the anterior-posterior axis (red being the thickest and blue being the thinnest). Note the limited increase in vertical length in most Pompe patients (7 to 16) relative to that in the healthy volunteers (1 to 6).

Chapter 6

Muscle MRI in classic infantile Pompe disease

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ABSTRACT

Objectives Neuromuscular imaging techniques are helpful tools to create a better understanding of pathophysiological processes of neuromuscular diseases. MRI has been used to study skeletal muscle damage in patients with late-onset Pompe disease. We used this technique to investigate the upper leg muscles of patients with classic infantile Pompe disease.

Methods Five patients with classic infantile Pompe disease were included. MR images were compared with histopathology sections taken from the lateral vastus muscle. For comparison, an MRI was performed in a child without a neuromuscular disease.

Results The median age at the time the MRIs and biopsies were taken was four months and none of the infants was treated with enzyme replacement therapy yet. All infants had prominent muscle weakness and strikingly abnormal muscle histopathology. MR images showed hypertrophic muscles in four patients and no abnormalities in the fifth.

Conclusion The hypertrophic appearance of the muscles demonstrated using MRI in patients with classic infantile Pompe disease, is in accordance with the muscle firmness on clinical examination. Whether the hypertrophic appearance of muscles on MRI in classic infantile patients is related to glycogen accumulation as observed in muscle biopsies requires further investigation.

INTRODUCTION

Pompe disease (OMIM 232300: acid maltase deficiency or glycogen storage disease type II) is an inherited metabolic disorder with an incidence of 1:40,000 births per year. It is caused by mutations in the acid α-glucosidase gene (*GAA*). Deficiency of GAA leads to lysosomal accumulation of glycogen predominantly in muscle tissue.[1-4] The disease has a broad clinical spectrum, which is largely determined by residual GAA activity. In patients with the most severe classic infantile form, enzyme activity is virtually absent. These patients present shortly after birth with skeletal muscle weakness, which rapidly progresses to total paralysis within a few months. Patients eventually die from cardiorespiratory failure.[1,2] Milder forms of the disease may present at any age from infancy to late adulthood.[5] These patients have some residual enzyme activity, but usually no more than 30% of average normal activity.[4] Most often these patients present with limb girdle muscle weakness and respiratory insufficiency.[6,7] Since 2006, enzyme replacement therapy (ERT) has become available. This has improved survival and motor outcome significantly in infants, and more recently positive effects have also been demonstrated in adults.[4,8] These include improved distance walked on the six-minute walk test and stabilization or improvement of pulmonary function, muscle strength, and better survival.[9-13] However, there is a large variation in response between patients.

Neuromuscular imaging techniques may be helpful to create a better understanding of the pathophysiological process and the severity of muscle abnormalities. Computed tomography, ultrasound and magnetic resonance imaging (MRI) may all provide information on the distribution and severity of disease in the affected muscles. MRI provides the best soft tissue contrast and information on the shape, volume and tissue architecture of striated muscles.[14] Several cross-sectional studies have been published on muscle MRI findings in adult and juvenile patients with Pompe disease, but so far muscle MRI has not been used in classic infantile Pompe disease.[15-21]

To study whether muscle MRI can be a useful tool to investigate the extent of muscle damage in classic infantile Pompe disease, we performed MRI of the upper legs of five infants with Pompe disease and compared

the findings with the pathology found in muscle biopsy sections of the quadriceps muscles from these patients.

METHODS

Patients

Five patients with classic infantile Pompe disease participated in this study. They were diagnosed with Pompe disease between June 2009 and September 2012. Psychomotor development was assessed using the Alberta Infant Motor Scale (AIMS).[22] An MRI of an infant without Pompe disease was used as a control. Informed consent was obtained from all parents. The Medical Ethics Committee at Erasmus University Medical Center Rotterdam, The Netherlands, approved the study protocols.

Muscle imaging

MR examinations were performed using a 1.5-tesla (1.5T) GE imaging system (General Electric Healthcare, Milwaukee, USA) with an eight-channel receiving only phased-array cardiac coil. Examination areas ranged from the anterior inferior iliac spine to the knee. No intravenous contrast medium or sedative drugs were administered. The infants were placed in supine position and supported by a vacuum mattress. The MRI sequence protocol included axial and coronal images obtained with the following pulse sequences: T1- weighted fast spin-echo images without fat suppression (repetition time (TR) 500-600ms, echo time (TE) 14-23ms, slice thickness 6-8 mm); T2-weighted fast spin-echo images with fat suppression (TR 2500-5000ms, TE 64-85ms, echo train length: six to eight, slice thickness 3-5 mm); and coronal images using a Spin-Echo Inversion Recovery Weighted sequence (STIR) (TR 4500ms, TE 35ms, Inversion Time 155ms, echo train length eight, slice thickness 3-5 mm). In all sequences a matrix of 256 x 192 was used with a slice gap of 1-2 mm and a field of view (FOV) of 20-28 cm. The average scanning time was 25 minutes per patient.

Muscle biopsy

Needle biopsies were taken from the lateral vastus muscle as part of the standard procedure in patients with Pompe disease at our center. The muscle sections were fixed in 4% glutaraldehyde, embedded in glycol-metacrylate and stained with periodic acid-Schiff (PAS).[23]

RESULTS

Patients

Five patients with classic infantile Pompe disease were included in this study (four males and one female). All patients had generalized muscle weakness in the first months of life, and the characteristic hypertrophic cardiomyopathy. Except for patient 4, all other patients needed nasogastric tube feeding and supplemental oxygen. At clinical examination all five patients had a generalized hypotonia characterized by slipping through and head lag, and the AIMS score was below the 5th percentile (Table 1). In all patients except patient 2, the muscles had a firm texture on palpation,

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Table 1 **Patient characteristics**

Patient	Age at MRI	GAA activity	Mutation 1	Mutation 2	AIMS†
1	5	0	c.2104C>T	c.379-380delTG	6
2	4	0	c.2481+102_2646+31del	c.del525T	8
3	3	0	c.1209C>G	c.del525T	0
4	3	1.4	c.925G>A	c.2608C>T	5
5	5	0	c.del525T	c.del525T	4
Control	8	NA*	NA	NA	NA

*NA = not applicable. †AIMS = Alberta Infant Motor Scale, reference values in children aged three to four months are 12.6 and in children aged four to five months 17.9. The age at MRI is expressed in months. GAA activity was measured in leukocytes with glycogen as substrate in the presence of acarbose, and expressed as nmol/mg/h (normal range 30-160 nmol/mg/h).

especially the calves. None of the patients were treated with ERT at the time the MRIs and muscle biopsies were taken. Table 1 shows the age at time of the MRI, the GAA activity and *GAA* genotypes of all patients. An MRI of the upper leg of an eight month-old boy was used as a control.

Muscle imaging

The T1 and T2-weighted images did not show signal intensity changes in the muscles of the upper legs. In patients 1, 3, 4 and 5 the T1-weighted images showed symmetric hypertrophic muscles of the upper legs with less subcutaneous fat compared to those of the control patient (Figure 1). Patient 2 did not have hypertrophic muscles on MRI.
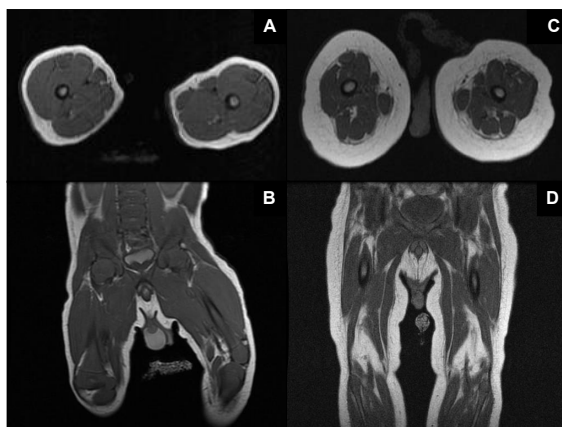
Figure 1   Muscle MRI of two classic infantile patients and a control patient

Image A (patient 1) is an axial T1-weighted image showing hypertrophic muscles of the upper legs and little subcutaneous fat. Image B (patient 3) is a coronal T1-weighted image demonstrating hypertrophic muscles. Images C (axial) and D (coronal) are T1-weighted images of the control patient.

Muscle biopsy

Four of the classic infantile patients underwent muscle biopsies. No biopsy was taken from the fifth patient since the parents did not give their consent for it (patient 3). In two patients the biopsy was taken within 24 hours of muscle imaging and in the other two patients one day and one week before muscle imaging, respectively. In all patients the muscle biopsy showed a vacuolar myopathy with irregular atrophic and hypertrophic muscle fibers,
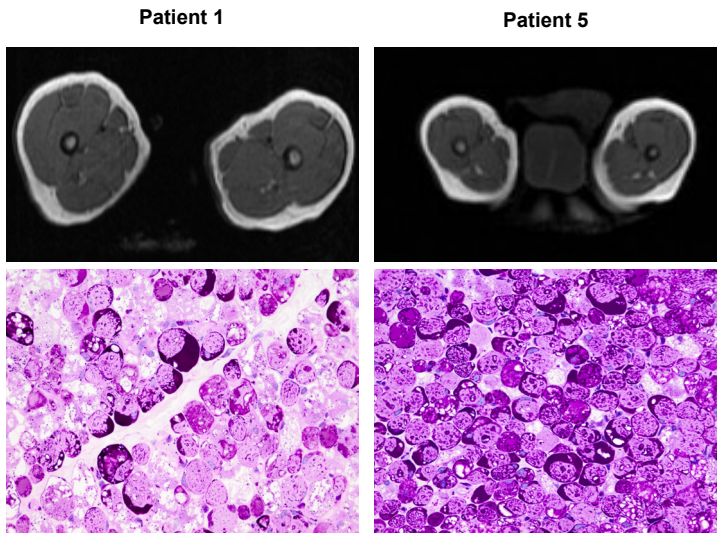
Figure 2   Muscle MRI and histopathology of two classic infantile patients

Muscle MRI of the upper legs and PAS stained biopsies of the lateral vastus muscle of two classic infantile patients (patients 1 and 5). The MR images show hypertrophic muscles and little subcutaneous fat and the muscle biopsies show severe PAS-positive vacuolar changes in almost all muscle fibers.

with positive PAS-material in almost all vacuoles (80-95%). Figure 2 shows the muscle biopsies and corresponding MR images of patients 1 and 5.

DISCUSSION

In this study we found that muscle MRI taken from the upper legs of patients with classic infantile Pompe disease showed almost no abnormalities except for some hypertrophy of the muscles on T1 and T2-weighted images. To the best of our knowledge this is the first study to describe muscle MRI findings in classic infantile Pompe patients. We were rather surprised to find only minor abnormalities on MRI in these patients, while the clinical status was quite advanced in most of them. At clinical examination the muscles had a firm constitution on palpation, which we considered most likely attributable to the severe glycogen accumulation that we observed

in muscle biopsies.[1,2] Interestingly, the only patient whose muscles did not have a firm texture on palpation also did not have hypertrophic muscles on the MR images.

In an earlier histopathological study five stages in the pathological process of classic infantile Pompe disease based on electron microscopy were distinguished.[24] Stage 1 was described as small glycogen-filled lysosomes between intact myofibrils, Stages 2 and 3 as stages with increased lysosomal glycogen with leakage to the cytoplasm and fragmentation of myofibrils and abnormal mitochondria. In Stage 4 most glycogen was cytoplasmatic, while the contractile elements were severely damaged. In Stage 5 - the end stage- there was complete loss of myofibrils, while the cells were bloated due to water influx. The muscle biopsies from our patients showed extensive glycogen accumulation contained in vacuoles in virtually all muscle fibers, and there was no obvious replacement of muscle tissue by fat or connective tissue. It seems likely that the hypertrophic aspect on MRI is due to glycogen accumulation and reactive increase of organelles, with severe myopathy signs and beginning of fibril dissolution as observed in the histopathology (resembling Stages 2-3 as described by Thurberg et al.).[24]

Muscle MRI has been described in more detail in adult patients with Pompe disease. In these patients MRI shows fatty infiltration particularly in the spine extensors, abdominal belt and scapular and pelvic girdle muscles.[16,19,25] Although the disease in these patients is considered to be slowly progressive and the process of glycogen accumulation to be spread over many years, it apparently results in considerable muscle atrophy, fat infiltration and replacement of muscle by connective tissue over longer periods of time as shown on MRI. In these adult patients glycogen storage may vary extensively between muscle fibers even within the same muscle. Also the extent of tissue damage between muscle groups varies greatly.[26-28] It remains an interesting topic for future research to study why some muscles are more severely affected than others and to investigate the effects of ERT by muscle MRI in adult patients. A recent study has shown that quantitative whole-body MRI is a feasible technique to use.[21]

A limitation of our study was that only the upper legs were scanned and that only T1 and T2-weighted sequences were used. This was due to limited scanning time and because we did not want to administer seda-

tive drugs considering the anaesthetic risks to these infants. Although it might have been more informative to perform a total body MRI, taking the homogeneous process of muscle destruction in infants into account we consider it unlikely that the results would have been completely different. Now patients are living longer due to ERT it will be certainly of interest to repeat MRI scans in long-term surviving patients with classic infantile Pompe disease. Other MRI techniques could also be of additional value to objectively quantify disease severity and progression in Pompe disease; these include C-NMR spectroscopy, T2 mapping and 3-point Dixon imaging.[29-33] It has been shown that C-NMR spectroscopy can demonstrate glycogen accumulation in muscle in patients with glycogenosis type III and in adult patients with Pompe disease, indicating that this technique could also be useful in infants with Pompe disease.[32,33]

In conclusion, muscle MRI in patients with classic infantile Pompe disease shows a hypertrophic appearance of the muscles, which is in accordance with the muscle firmness on clinical examination. Whether the hypertrophic appearance is related to glycogen accumulation alone as observed in muscle biopsies, and whether muscle MRI can be used as a marker to evaluate the effects of ERT requires further investigation.

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Chapter 7

Increased aortic stiffness and blood pressure in non-classic Pompe disease

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ABSTRACT

Vascular abnormalities and glycogen accumulation in vascular smooth muscle fibres have been described in Pompe disease. Using carotid-femoral pulse wave velocity (cfPWV), the gold standard methodology for determining aortic stiffness, we studied whether aortic stiffness is increased in patients with Pompe disease. Eighty-four adult Pompe patients and 179 age and gender-matched volunteers participated in this cross-sectional case-controlled study. Intima media thickness and the distensibility of the right common carotid artery were measured using a Duplex scanner. Aortic augmentation index, central pulse pressure, aortic reflexion time and cfPWV were assessed using the SphygmoCor® system. CfPWV was higher in patients than in volunteers (8.8 versus 7.4 m/s, $p < 0.001$). This difference was still present after adjustment for age, gender, mean arterial blood pressure (MAP), heart rate and diabetes mellitus ($p = 0.001$), and was shown by subgroup analysis to apply to the 40-59 year age group ($p = 0.004$) and 60+ years age group ($p = 0.01$), but not to younger age groups ($p = 0.99$). Except for a shorter aortic reflexion time ($p = 0.02$), indirect indicators of arterial stiffness did not differ between patients and volunteers. Relative to volunteers (20%), more Pompe patients had a history of hypertension (36%, $p = 0.005$), and the MAP was higher than in volunteers (100 versus 92 mmHg, $p < 0.001$). This study shows that patients with non-classic Pompe disease have increased aortic stiffness and blood pressure. Whether this is due to glycogen accumulation requires further investigation. To reduce the potential risk of cardiovascular diseases, we recommend that blood pressure and other common cardiovascular risk factors are monitored regularly.

INTRODUCTION

Pompe disease (OMIM 232300: acid maltase deficiency or glycogen storage disease type II) is an inheritable lysosomal storage disorder caused by a deficiency of acid α-glucosidase that leads to glycogen accumulation in various body tissues, predominantly skeletal, cardiac and smooth muscle.[1,2] Patients with the classic infantile form of the disease develop generalized hypotonia and a hypertrophic cardiomyopathy. Without treatment, these patients die in the first year of life due to cardiorespiratory failure.[3] In the non-classic form of the disease, which can present at any age, progressive muscle weakness is the predominant symptom. In general, there is no significant cardiac involvement.[4,5]

Over the last two decades, several reports have hypothesized that glycogen accumulation in the smooth muscle tissue of arteries leads to vascular abnormalities in non-classic Pompe disease, such as cerebral aneurysms, basilar artery dolichoectasia, carotid dissection and dilated arteriopathy of the thoracic aorta.[6-11] The first indication of increased aortic stiffness in non-classic Pompe disease was reported in a study on 17 Pompe patients that used transthoracic Doppler echocardiography, a non-invasive tool that measures aortic stiffness indirectly.[12] The presence of increased aortic stiffness may be relevant, since it is considered to be an independent risk factor for cardiovascular disease and mortality.[13,14] The emerging gold standard for measuring it directly and non-invasively is tonometry of the carotid-femoral pulse wave velocity (cfPWV).[15]

To determine whether increased arterial stiffness is a feature of non-classic Pompe disease, we used various techniques including tonometry of the cfPWV to investigate the structural and functional vascular properties of different vascular territories in a large group of patients with non-classic Pompe disease. We compared the results with those in age and gender-matched volunteers.

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MATERIALS AND METHODS

Study population

Between March 2012 and June 2013 we performed a cross-sectional single-centre case-controlled study in 84 patients with non-classic Pompe disease and 179 volunteers. All patients were 18 years or older. As the volunteers were matched for gender and age category, there were, per patient, at least two volunteers of the same gender and the same age category. The volunteers were either the patients' partners, or were employees or students of Erasmus University Medical Center, or otherwise visitors to it. Informed consent was obtained from all participants. The study protocol was approved by the Medical Ethical Committee at Erasmus University Medical Center

Cardiovascular risk factors

All participants filled out a questionnaire to assess cardiovascular risk factors, which included gender, date of birth, length, weight, medication use, and comorbidities such as hypertension, diabetes mellitus, hypercholesterolemia and cardiovascular disease (i.e. transient ischemic attack, stroke, myocardial infarction or rhythm disturbances). Body mass index (BMI), which was calculated as body weight divided by height squared, was expressed as kg/m^2. An automatic mercury cuff sphygmomanometer on the right arm was used to measure heart rate and blood pressure, including the mean arterial blood pressure (MAP), when the patient was in supine position after five minutes of rest in a quiet and heat-controlled room. Hypertension was defined as a systolic blood pressure (SBP) > 140 mmHg and/or diastolic blood pressure (DBP) > 90 mmHg.[16]

Vascular measurements

Aortic stiffness was measured using arterial tonometry from the right radial, right carotid and right femoral arteries using the SphygmoCor® device (Sphygmocor version 7.1, AtCor Medical, Sydney, Australia) according to previously described procedures.[17] Transit distance was assessed on the basis of body surface measurement from the carotid artery to the femoral artery: 80% of this distance was used as pulse-wave-travelled distance.[15] The primary outcome measure, cfPWV, was expressed in meters per

second (m/s). The secondary and indirect outcome parameters for arterial stiffness measured with pulse wave analysis (PWA) of the right radial artery were central pulse pressure (PPc), augmentation index (AIx) and aortic reflexion time (ATr). PPc was calculated as the difference between aortic systolic and diastolic pressure; AIx was calculated from the aortic pressure waveform obtained by applying a general transfer function to the radial pressure waveform. For the comparison between two groups, AIx was normalized to a heart rate of 75 beats per minute. ATr was calculated as the time interval between the foot of the pressure wave and the shoulder of the reflected wave.[18-20]

The distensibility and IMT of the right common carotid artery were measured two centimetres below the carotid bifurcation using a vessel-wall movement-detector system (Art.Lab, Esaote Europe, Maastricht, the Netherlands) according to previously described procedures.[17] The distensibility coefficient, a local measurement of carotid stiffness, was calculated as $(2\Delta D/D)/PP(10^{-3}/kPa)$.[21] in which D is the diameter during diastole, ΔD is the measured distensibility (systolic diameter – diastolic diameter), and PP is the aortic pulse pressure derived from radial applanation. Tonometry and ultrasonography were performed by two trained physicians (SW or EK).

Statistical analyses

Unless otherwise specified, data are presented as medians with interquartile ranges (IQR). Because cfPWV, PPc, AIx, ATr, distensibility and IMT were not normally distributed, the Mann-Whitney test was used to compare these outcomes between the total groups of patients and volunteers.

Linear regression analyses were used to study cfPWV separately. First, univariate regression analyses were performed. Next, the regression coefficients with a p-value less than 0.05 or variables that were considered clinically relevant were analyzed in a multivariate regression model using bootstrap estimation. The final multivariate model contained age, gender, MAP, heart rate and diabetes mellitus.

We also performed a stratified analysis between patients and volunteers in three age groups: 20-39, 40-59 and 60+. To determine whether cfPWV was influenced by disease duration, treatment with ERT, and wheelchair or ventilator dependency, we also analyzed cfPWV in separate multivariate

regression models using bootstrap estimation. The final model for these analyses included four variables: age, gender, MAP and heart rate. The data were analyzed using SPSS 20. A p-value of less than 0.05 was considered statistically significant.

RESULTS

Study population

Table 1 shows the characteristics of the two groups. In total, 84 patients with non-classic Pompe disease and 179 volunteers were included in the study. Twenty-six percent of the patients were wheelchair dependent, and 31% needed artificial ventilation. Eighty-two percent of the patients were treated with ERT and the median duration of treatment was 5 years (IQR 4-5). CfPWV could not be measured in nine patients, either because they could not lie in supine position due to respiratory failure, or because their use of accessory muscles made it difficult to visualize the carotid artery. Neither could cfPWV be measured in other eight volunteers due to temporary technical problems with the SphygmoCor® system.

Cardiovascular risk factors

In the past, more patients with Pompe disease than volunteers had been diagnosed with hypertension (p=0.005, Table 1). Seventy-seven per cent of the Pompe patients with an earlier diagnosis of hypertension used antihypertensive medication at the time of evaluation, and 89% in the group of volunteers. Despite treatment with antihypertensive medication, blood pressure continued to be high in 52% of the Pompe patients who were treated for hypertension and in 65% of the volunteers. As Table 2 shows, MAP was higher in patients than in volunteers (100 mmHg (IQR 90-111) versus 92 mmHg (IQR 84-103), p<0.001). Thirty-nine per cent of the patients met the criteria for hypertension (SBP > 140 mmHg and/or DBP > 90 mmHg), versus 20% of the volunteers (p=0.001). At group level, patients with Pompe disease had a higher heart rate (75 beats per minute (bpm) (IQR 68-83)) than the volunteers (65 bpm (IQR 59-71), p<0.001).

There were no differences in the occurrence of comorbidities such as diabetes mellitus or hypercholesterolemia. Seven patients with Pompe

Table 1   **Demographic and clinical characteristics**

	Pompe patients	Volunteers
Number of individuals	84	179
Age in years	54 (42-63)	54 (41-61)
Gender – male (%)	41 (49)	85 (48)
Body mass index in kg/m^2	24.7 (22.0-27.3)	24.0 (22.2-26.0)
Smoking status (%)		
• Never	36 (43)	107 (60)
• Past	41 (49)	53 (29)
• Current	7 (8)	19 (11)
Medical history		
• Hypertension (%)	30 (36)[#]	35 (20)
• Diabetes mellitus (%)	5 (6)	6 (3)
• Hypercholesterolemia (%)	8 (10)	18 (10)
• Cardiovascular disease (%)	7 (8)	9 (5)
Antihypertensive medication (%)	23 (77)	31 (89)
Disease duration in years	16 (12-23)	-
Patients receiving ERT (%)	69 (82)	-
Duration of ERT in years	5 (4-5)	-
Wheelchair use (%)	22 (26)	0 (0)
Ventilator use (%)	26 (31)	0 (0)

Continuous variables are given as medians with interquartile ranges (IQR), and categorical variables as numbers and percentages. [#] = In the past, more patients with Pompe disease than volunteers were diagnosed with hypertension (p-0.005).

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Table 2 Functional and structural hemodynamic parameters in patients and volunteers

	Pompe patients (n=84)	Volunteers (n=179)	P-value
Mean arterial pressure (mmHg)	100 (90-111)	92 (84-103)	<0.001
Heart rate (beats per minute)	75 (68-83)	65 (59-71)	<0.001
Diameter carotid artery (mm)	6.6 (6.1-7.3)	7.1 (6.5-7.8)	<0.001
Intima media thickness (µm)	582 (514-683)	592 (506-695)	0.68
Distensibility coefficient (10^{-3}/kPa)	2.9 (1.9-4.1)	2.7 (2.2-4.1)	0.72
Carotid-femoral pulse wave velocity (m/s)	8.8 (7.1-10.7)	7.4 (6.6-8.6)	<0.001
Central pulse pressure (mmHg)	38 (31-47)	36 (31-43)	0.30
Augmentation index (%)	21.7 (13.2-30.3)	19.4 (8.5-29.4)	0.23
Aortic reflexion time (ms)	140 (132-149)	144 (135-159)	0.02

Continuous variables are given as medians with interquartile ranges (IQR). The diameter of the carotid artery was measured in 179 volunteers and 84 patients; IMT was measured in 179 volunteers and 83 patients; the distensibility coefficient was measured in 176 volunteers and 80 patients; cfPWV was measured in 171 volunteers and 79 patients; and PPc, Aix and ATr were measured in 178 volunteers and 83 patients.

disease had a medical history of cardiovascular disease: four of them (three of whom female) had had a transient ischemic attack between the ages of 48 and 62 years; two male patients had developed atrial fibrillation at the ages of 37 and 64 years; and one female patient had had a myocardial infarction at the age of 74 years. Nine volunteers had a medical history of cardiovascular disease. This was not statistically different from the seven Pompe patients (p=0.30).

Aortic stiffness

As shown in Table 2, median cfPWV was 8.8 m/s in patients with Pompe disease (IQR 7.1-10.7) and 7.4 m/s in volunteers (IQR 6.6-8.6) (p<0.001). This difference was still present after adjustment for age, gender, MAP, heart rate and diabetes mellitus (p=0.001). Subgroup analysis showed that while it occurred in the 40-59 year age group (8.6 m/s in patients versus
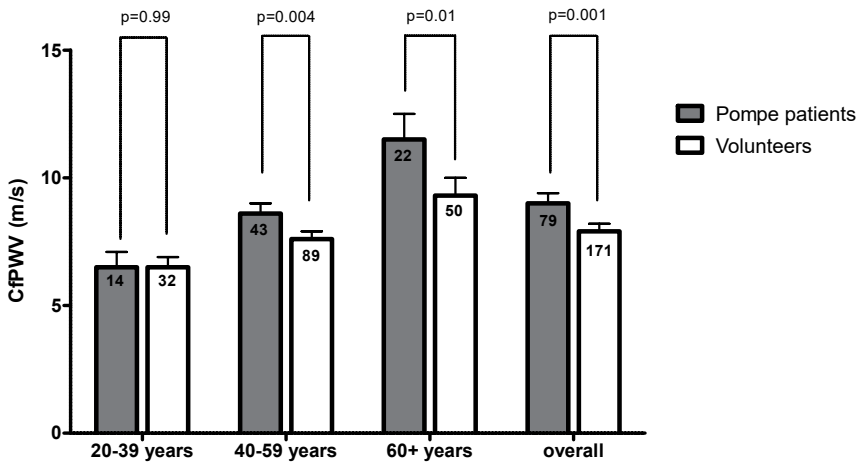
Figure 1   Mean values of cfPWV in patients and volunteers per age category

The boxplots represent the mean values and 95% CI of cfPWV per age category
after adjustment for age, gender, mean arterial blood pressure, heart rate and
diabetes mellitus. The number of patients and volunteers per age group are given in
the bars.

7.6 m/s in volunteers, p=0.004) and the 60+ years (11.5 versus 9.3 m/s, p=0.01), it did not occur in the 20-39 year age group (Figure 1). PPc and AIx did not differ significantly between patients and volunteers, while ATr was shorter in patients than in volunteers (p=0.02).

CfPWV did not differ between Pompe patients whose disease duration was shorter than 15 years (n=35) and those whose disease duration was longer than 15 years (n=44) (9.1 versus 9.4 m/s, p=0.51). Neither did it differ between patients treated with ERT (n=64) and treatment-naïve patients (n=15) (9.4 versus 8.6 m/s, p=0.17). Similarly, cfPWV did not differ between wheelchair dependent patients (n=12) and ambulant patients (n=62) (9.4 versus 9.2 m/s, p=0.75), or between ventilator dependent patients (n=18) and non-ventilated patients (n=56) (9.6 versus 9.1 m/s, p=0.42).

Carotid stiffness and IMT

Table 2 shows that there were no significant differences in distensibility or IMT between patients and volunteers. The diameter of the carotid artery was smaller in patients than in volunteers (6.6 mm (IQR 6.1-7.3) versus 7.1

mm (IQR 6.5-7.8), p<0.001). Subgroup analysis showed that the distensibility was lower in patients with Pompe disease aged between 20-39 years than in volunteers (p=0.03), but not in the other age-related subgroups.

Glycogen accumulation in vascular smooth muscle

Figure 2 shows the muscle biopsy of a 50-year-old male patient with Pompe disease who participated in this study and had no medical history of cardiovascular diseases. He developed the first symptoms at age 44, and now has severe limb girdle weakness and decreased pulmonary function in sitting and supine positions. The biopsy was stained with periodic acid-Schiff (PAS) and shows glycogen storage in skeletal muscle fibers and in vascular smooth muscle fibers.
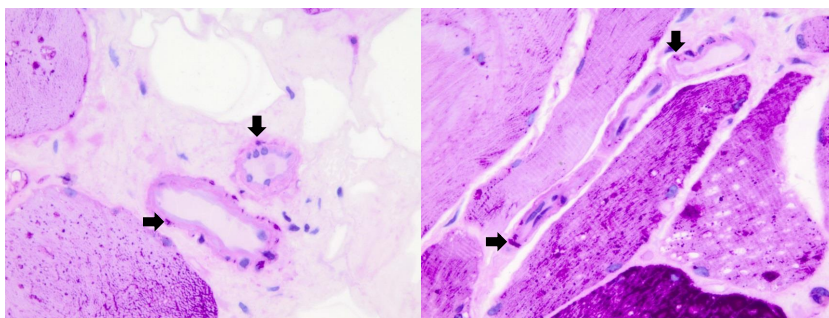

Figure 2   Glycogen accumulation in vascular smooth muscle fibres

Muscle biopsies of adult patients with non-classic Pompe disease stained with periodic acid-Schiff (PAS) to demonstrate glycogen storage in vascular smooth muscle fibres (black arrowheads). Original magnification: 630x.

DISCUSSION

We found that patients with the non-classic form of Pompe disease have increased aortic stiffness and blood pressure. These findings indicate clinical relevant involvement of the cardiovascular musculature in this group of Pompe patients.

   Glycogen accumulation in smooth muscle fibres and in the endothelial layer of arteries has been shown in morphological studies and autopsy reports of patients with Pompe disease.[8,22,23] Figure 2 shows glycogen storage

in vascular smooth muscle fibres in one of our adult patients with Pompe disease before start of ERT. While it is likely that the increased glycogen storage in smooth muscle fibres results in an increased arterial stiffness, it is also possible that glycogen accumulation in the endothelium damages the vascular wall, making it more vulnerable to atherosclerosis; in the presence of other cardiovascular risk factors, this process might increase arterial stiffness. As vascular stiffness has also been described as depending on the distribution of collagen and elastin, an increase in abnormal collagen and a decrease in normal elastin contributes to greater arterial stiffness.[20] During the course of the disease, patients with Pompe disease may develop striated muscle atrophy with some increase in connective tissue. If this process also occurred in the vascular smooth muscle fibers of Pompe patients, it would help to explain increased aortic stiffness.

Increased aortic stiffness has also been described in other lysosomal storage disorders such as Fabry disease and mucopolysaccharidosis type I (MPS I or Scheie syndrome). In Fabry disease, storage of glycosphingo-lipids takes place in vascular endothelial and smooth muscle cells, and in MPS I, accumulation of glycosaminoglycans in the connective tissue of the aortic wall impairs the formation of elastin and leads to changes in collagen formation.[24,25] It has been shown in Fabry disease that ERT can normalize aortic stiffness.[25] As most of the patients with Pompe disease enrolled in our cross-sectional study were treated with ERT, the difference in cfPWV between patients and volunteers might have been even more pronounced if it had been measured before the start of ERT. It would thus be interesting to establish whether aortic stiffness in Pompe patients decreases during ERT, and whether cfPWV can be used as a non-invasive tissue biomarker for disease progression and therapy response.

To the best of our knowledge, this is the first study to show that arterial blood pressure is increased in patients with Pompe disease. It is reasonable to assume that any structural changes in the blood vessels caused by the increased aortic stiffness would lead to higher blood pressure. Increased aortic stiffness and hypertension are both independent risk factors for cardiovascular disease and mortality.[13,14] It has been shown that patients with non-classic Pompe disease have improved survival when treated with ERT.[26] Since cardiovascular diseases are more common at a higher age,

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Pompe patients will be more likely to develop cardiovascular diseases, as they owe their higher age to ERT. In these patients it is therefore important to closely monitor risk factors for cardiovascular diseases such as hypertension, hypercholesterolemia, diabetes mellitus and smoking, and to treat them when indicated. Although exercise and training, dietary changes and pharmacological treatments have sometimes been shown to reduce arterial stiffness in healthy individuals and other patient groups,[27-29] it has not yet been investigated whether they are also beneficial in Pompe patients.

Although we expected Pompe disease to have a similar effect on the vascular structure of all arteries, and thereby to increase cfPWV and reduce distensibility, we found changes in carotid distensibility only in patients aged between 20-39 years. In those with high blood pressure and diabetes it has been reported that the aorta stiffened more than the carotid artery with age and with other cardiovascular risk factors such as increased body mass index and heart rate.[30] These findings may have been paralleled by our own finding in most of the Pompe patients whose blood pressure and heart rate were increased, who also had greater aortic stiffness without decreased distensibility.

A strength of our study is that we used cfPWV, which is currently the gold standard for measuring aortic stiffness directly and non-invasively in a relatively large group of Pompe patients. A previous study that found increased aortic stiffness in a small group of patients with Pompe disease used transthoracic echocardiography, an indirect way of measuring aortic stiffness.[12]

Our study has some limitations. First, due to physical or temporary technical problems, measurements of aortic stiffness were not available for all subjects. However, as most of the patient data that were missing in the group of Pompe patients were those for the elderly and more severely affected patients, this would probably have led to an underestimation of the real difference in aortic stiffness between Pompe patients and healthy volunteers. Second, as this was a cross-sectional study, we do not know about the progression of aortic stiffness over time, or about the effects of ERT on aortic stiffness. Additionally, rather than measuring glucose or cholesterol in blood samples, we used a questionnaire to establish whether patients had a history of hypercholesterolemia or diabetes mellitus.

In conclusion, patients with non-classic Pompe disease have increased aortic stiffness and blood pressure. To reduce the potential risk of cardio-vascular diseases, we recommend that blood pressure and other common cardiovascular risk factors are monitored closely. Prospective studies should investigate whether ERT reduces aortic stiffness, or whether early treatment even prevents it.

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Chapter 8

Safety and efficacy of exercise training in adults with Pompe disease: evalution of endurance, muscle strength and core stability before and after a 12 week training program

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ABSTRACT

Introduction Pompe disease is a proximal myopathy. We investigated whether exercise training is a safe and useful adjuvant therapy for adult Pompe patients, receiving enzyme replacement therapy.

Methods Training comprised 36 sessions of standardized aerobic, resistance and core stability exercises over 12 weeks. Before and after the training program, the primary outcome measures safety, endurance (aerobic exercise capacity and distance walked on the 6 min walk test) and muscle strength, and secondary outcome measures core stability, muscle function and body composition, were evaluated.

Results Of 25 patients enrolled, 23 successfully completed the training. Improvements in endurance were shown by increases in maximum workload capacity (110 W before to 122 W after training, [95% CI of the difference 6.0 to 19.7]), maximal oxygen uptake capacity (69.4% and 75.9% of normal, [2.5 to 10.4]), and maximum walking distance (6 min walk test: 492 meters and 508, [−4.4 to 27.7]). There were increases in muscle strength of the hip flexors (156.4 N to 180.7 N [1.6 to 13.6) and shoulder abductors (143.1 N to 150.7 N [13.2 to 35.2]). As an important finding in secondary outcome measures the number of patients who were able to perform the core stability exercises rose, as did the core stability balancing time (p<0.05, for all four exercises). Functional tests showed small reductions in the time needed to climb four steps (2.4 sec to 2.1, [−0.54 to −0.04]) and rise to standing position (5.8 sec to 4.8, [−2.0 to 0.0]), while time to run, the quick motor function test results and body composition remained unchanged.

Conclusions: Our study shows that a combination of aerobic, strength and core stability exercises is feasible, safe and beneficial to adults with Pompe disease.

INTRODUCTION

Pompe disease (glycogen storage disease type II, acid maltase deficiency) (OMIM # 232300) is a rare metabolic myopathy caused by glycogen accumulation resulting from deficiency of lysosomal acid α-glucosidase (GAA). It presents as a wide clinical spectrum, the most prominent symptoms in adults being proximal skeletal muscle weakness and respiratory problems.[1,2] Skeletal muscle weakness typically fits a pattern of limb girdle myopathy, with the abdominal and paraspinal muscles and the musculature of the hip being the most affected muscle groups.[3–5]

Enzyme replacement therapy (ERT) with recombinant human acid α-glucosidase (Myozyme/Lumizyme) was approved for the treatment of Pompe disease in 2006. In adults, ERT has been shown to elicit positive effects on skeletal muscle strength, walking distance, respiratory function and survival.[6–8] Patients' fitness and physical functioning may be further supported by treatments additional to ERT, such as exercise training. Although some recent studies suggest that exercise training may be beneficial, evidence is still limited.[9, 10]

A recent study on common clinical practice in the Netherlands showed that there is a lack of uniformity in the type of physical therapy training programs applied, and that physical therapists and patients all seek guidance and standardization.[11] We therefore aimed to determine whether a standardized and well structured exercise intervention program combining aerobic, resistance and core stability exercises was feasible and safe, and whether it added value to treatment with ERT alone. In a group of relatively mildly affected adult Pompe patients receiving ERT for more than a year, we evaluated the effects of such a regime on endurance, muscle strength and function, core stability, and body composition.

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METHODS

Patients

Patients were recruited at the Centre for Lysosomal and Metabolic Diseases, Erasmus MC University Medical Centre, Rotterdam, the Dutch national

referral centre for patients with Pompe disease. There were three inclusion criteria:

1. A confirmed diagnosis of Pompe disease measured by decreased GAA activity in leukocytes or fibroblasts, and mutation analysis;
2. Age > 17 years;
3. Treatment with ERT for at least 52 weeks

There were four exclusion criteria:

1. The use of walking aids or a wheelchair;
2. Ventilator dependency;
3. Concurrent medical conditions;
4. Participation in other exercise-training programs.

The study was approved by the Ethical Committee at Erasmus MC University Medical Centre. Informed consent was obtained from all patients.

Study design and intervention

Three times a week for 12 weeks, all patients followed a standardized training program that was provided under the supervision of physical therapists at carefully selected sports or fitness centres near the patients' homes. To ensure the uniformity of the program and its supervision, all therapists attended a one-day instruction program at Erasmus MC University Medical Centre. The training program is depicted in Fig. 1. The first training session was on-site supervised by one of the researchers from Erasmus MC (LvdB, MF), who subsequently attended each training site every two weeks to monitor proper conduct of the program.

Patients were randomly subdivided into two groups: group 1 (n = 13), which started the training program at week 1; and group 2 (n = 12), which started at week 13. The staggered start of training allowed us to investigate whether any improvement observed in the training period could also be attributed to ERT. Furthermore, the duration of the effect of the training program can be evaluated from the follow-up of patients in group 1 after they stop training in week 12.

To assess the effects of the program, each patient visited our centre (Erasmus MC) on two separate days in weeks 0, 12 and 24. The primary end-
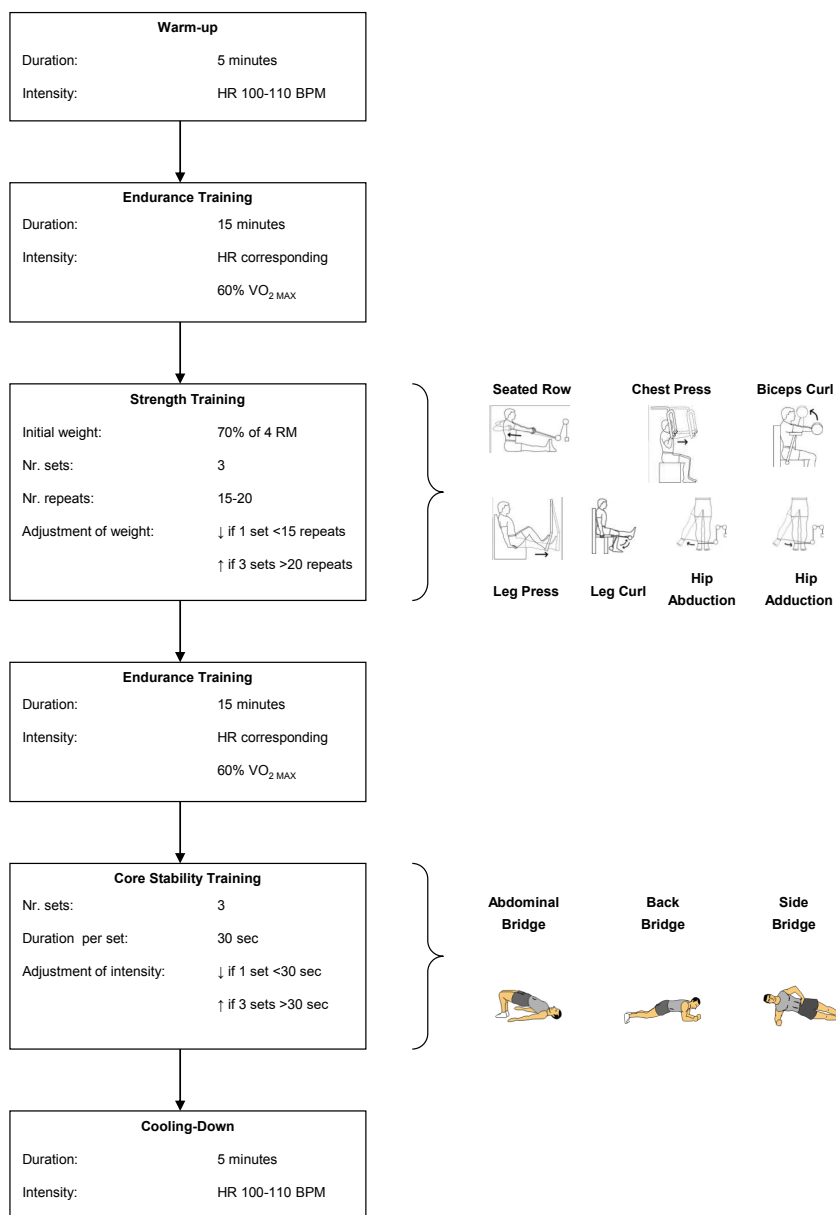
Figure 1 Flowchart for the standardized exercise-training regime combining aerobic, resistance and core stability exercises

points of this study were safety, endurance and muscle strength. Secondary endpoints were core stability, muscle function, and body composition.

Under the supervision of the physical therapist, training diaries were kept by all patients, who recorded the days on which they trained, the weight and number of repeats of resistance exercises, and the perceived level of exertion. To evaluate training progress and patients' motivation, patients were telephoned weekly.

Assessments

Safety: Plasma CK was measured every two weeks as a safety marker for exercise-induced muscle damage.[12] and patients were contacted every week to record potential side effects such as pain and fatigue.

Endurance: Aerobic (endurance) exercise capacity was determined using an incremental cycle ergometer. After 4 min of unloaded cycling on the cycloergometer (Jaeger ER 800; Erich Jaeger, Würzburg Germany) exercise intensity was increased progressively until exhaustion (i.e. ramp protocol), during continuous measurement of patients' heart rates and ventilator parameters using spiroergometry equipment (Oxycon Pro, Jaeger, Würzburg, Germany). The rate of increase was determined considering the patient' s functional capacities and ranged from 5– 20 Watts/minute. The duration of every individual test exceeded 6 min but did not take longer than 12 min. At exhaustion, the rating of exertional symptoms was assessed using the Borg scale (scale 6– 20).[13] Maximum workload capacity (WMAX) and peak oxygen uptake capacity (VO2 PEAK) were measured. The ventilatory threshold (VT) was assessed independently by two clinical exercise physiologists using the ventilatory equivalents method.[14] The test was considered to be maximal when one of the following criteria was met:
1. heart rate > 90 % of that predicted,
2. respiratory exchange ratio (RER) > 1.11, or
3. VO2 stabilized despite increased workload.

Walking distance on the 6-min walk test (6MWT) was evaluated according to the American Thoracic Society guidelines.

Core Stability: To assess the dynamic balance, which reflects core stability, one physical therapist (MF) measured time in balance (in seconds) for each of the four core stability exercises of the training program (Fig. 1).[15]

Muscle strength: Muscle strength was assessed by one investigator (SW) using Hand-held Dynamometry (HHD). Assessments were performed in a standardized manner, and sum scores were calculated as described previously.[6]

Muscle Function: Functional activity assessments comprised three timed tests: 10 meter running, climbing four steps, and rising from supine to standing positions[16] and the Quick Motor Function Test (QMFT), a test specifically designed and validated for Pompe patients.[17] The QMFT consists of 16 specific motor skills related to daily activities. A total score is achieved by summing the scores for each item (ranging from 0 "cannot perform" to 4 "can perform with no effort"), and is expressed as a percentage of the maximum score.

Body composition: Bone-mineral density (BMD) and body-composition measurements were performed conform DXA technology using a Lunar DPX densitometer and analyzed with Encore 2002 software (GE Lunar DPX, GE Health Care). Bone densitometry was performed in a standardized manner as described previously.[18] Body composition was described in terms of the mineral, lean and fat body mass (kilograms). The percentage of fat mass and, more specifically, android and gynoid fat, were calculated.

Statistical analysis

Patient characteristics were summarized using descriptive statistics. Data for the two groups were combined after verifying that there were no significant differences between outcome measures before the start of the training (group 1 – week 0; group 2 – week 12; student's t-test for normally distributed, and Mann–Whitney for not normally distributed data). Mean differences before and after the training were compared using the paired t-test for normally distributed data, and otherwise the Wilcoxon signed rank test for paired samples. For group 2, we also used these tests to compare the outcome measures before and after 12 weeks of ERT only (week 0 to 12). Significance level was set at $p<0.05$. Statistical analyses were performed using SPSS for Windows (release 17.0; SPSS, Inc., Chicago, IL).

8

RESULTS

Patients

A total of 25 patients fulfilled the inclusion criteria to participate in this study. Two patients did not complete the training program because they were insufficiently motivated. This left 23 patients, who successfully completed the study. Their ages ranged from 20 to 71 years (median of 46 years). They had been receiving ERT for 1 to 6 years with a median of 3 years (Table 1).

Table 1 Patient characteristics

	Group 1 (n=12)	Group 2 (n=11)	Total group (n=23)	p-value*
Male gender (%)	7 (58%)	5 (45%)	12 (52%)	0.54
Age in years (range)	45.4 (19.6-70.5)	46.6 (32.9-66.1)	46.0 (19.6-70.5)	0.85
Disease duration in years (range)	15.5 (8.1-28.1)	16.1 (6.0-32.1)	15.8 (6.0-32.1)	0.83
ERT duration in years (range)	3.3 (1.4-6.5)	3.0 (1.3-3.6)	3.1 (1.3-6.5)	0.96
Training sessions (max. 36)	33 (27-36)	32 (24-35)	32 (24-36)	0.70

ERT = enzyme replacement therapy
Group 1 trained in weeks 1-12 and group 2 trained in weeks 13-24
*For the difference between group 1 and 2 (chi-2 test for proportions and Wilcoxon signed rank test for continuous data)

Effect of ERT only

During the 12 weeks before training started, patients in group 2 (ERT only) underwent the same set of assessments as in the 12 weeks of training (ERT plus training). This enabled us to use group 2 to compare the effects of ERT only with the combined effects of ERT and training. During the first 12 weeks of ERT only, we detected no significant improvements in the main outcome measures.

Effect of training

Patients in the two randomly assigned groups were comparable in terms of age, gender, disease duration, time on ERT, number of training sessions completed, and baseline test results. This allowed us to analyse the effect of training in the total group of 23 patients.

Safety: During the first week of training, two patients had a high plasma CK level (10125 U/l and 6149 U/l), and also experienced muscle pain and fatigue. Over the following week, their CK-values dropped to their normal range, and the fatigue and pain disappeared. Both patients continued training. None of the other patients had pain, fatigue, or increases in plasma CK levels during the study period.

Endurance: All patients were able to complete the incremental cycle test without adverse events. One was excluded from the analysis because he did not reach the required maximum intensity defined in the method section. After 12 weeks of training WMAX, VO2peak and VT improved significantly (Table 2). VO2peak and VT increased both in absolute values and adjusted for body weight or as a percentage of normal values. The

Table 2 Aerobic fitness measured in an incremental cycle test and a 6-min walk test before and after 12 weeks of training

	Before training	After training	p-value**
	Mean ± SD	Mean ± SD	
Incremental cycle test (N=22*)			
Ventilatory threshold (VT)			
• Absolute values (l/min)	1.25 ± 0.36	1.38 ± 0.36	<0.01
• Adjusted for body weight (ml/min/kg)	16.7± 4.3	18.5 ± 4.7	<0.01
• VT/VO2 peak (%)	77.6 ± 12.1	78.3 ± 12.3	0.742
Data at exhaustion			
• Maximum workload (Wmax, Watt)	110 ± 52	122 ± 53	<0.01
• Maximum heart rate (bpm)	156 ± 25	161 ± 20	0.16
• Pulmonary ventilation (l/min)	59.6 ± 20.2	68.2 ± 21.0	<0.01
• Tidal volume (l)	1.85± 0.48	1.85± 0.44	0.91
• Gas exchange ratio	1.15± 0.09	1.14± 0.08	0.71
Peak oxygen uptake (VO2 peak)			
• Absolute values(l/min)	1.67 ± 0.62	1.82 ± 0.60	<0.01
• Adjusted for body weight (ml/min/kg)	22.1 ± 7.0	24.1 ± 7.1	<0.01
• As % of normal	69.4 ± 17.4	75.9 ± 18.0	<0.01
6-min walk test (N=22)			
Maximum walking distance (6MWT, m)	492 ± 89	508 ± 97	0.01
Pulmonary function test (N=23)			
Forced vital capacity (FVC, % of normal)	89.2 ± 12.6	90.0 ± 14.0	0.51

* One patient was excluded because he did not reach the required maximum intensity

** For the difference before and after training (paired samples t-test)

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ratio VT/VO2peak did not change, as both the numerator and the denominator increased. There were no significant differences between patients' maximum heart rates before and after 12 weeks of training, indicating that the results were truly based on an increase in fitness rather than on greater exertions by the patients towards the end of the training period. FVC did not change. Average walking distance on the 6MWT increased by 16 meters ([4.4-27.7], p=0.01).

Core stability: Figure 2 shows the results of the core stability tests. At the start of the program, many patients experienced difficulties in performing the core stability exercises, reporting problems with initiating movement and controlling balance. During the training program, the number of patients who were able to perform the exercises increased for three of the four exercises (from 17 to 21 for the abdominal bridge, 15 to 16 for the left side bridge and 13 to 16 for the right side bridge). The average time they were able to remain in balance improved for all four positions (by 58% for the back bridge, 229% for the left and 223% for the right side bridges, and 86% for the abdominal bridge; p<0.05).
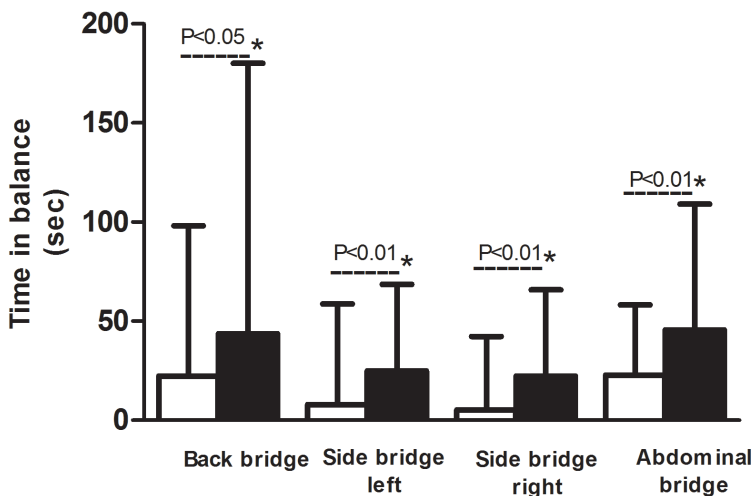

Figure 2 Effects of training on core stability tests

Time patients (N=23) were able to remain in balance for the four different core stability exercises before (white bars) and after training (black bars).

Table 3 Muscle strength measured by hand-held dynamometry (HHD) before and after 12 weeks of training

	Before training Mean ± SD	After training Mean ± SD	p-value*
HHD of individual muscle groups (Newton) (N=23)			
• Neck extensors	142.1±18.4	140.4±13.8	0.65
• Neck flexors	124.7±40.2	132.1±33.9	0.09
• Shoulder abductors	143.1±29.1	150.7±35.4	0.02
• Elbow flexors	226.4±41.5	230.8±42.3	0.42
• Elbow extensors	180.5±20.1	176.2±25.3	0.27
• Hip flexors	156.4±61.9	180.7±57.7	<0.01
• Hip abductors	159.4±58.3	158.0±68.1	0.75
• Knee extensors	189.2±34.1	193.0±31.8	0.27
• Knee flexors	121.2±57.2	122.6±56.7	0.51

* For the difference before and after training (paired samples t-test and the Wilcoxon signed-ranks test for paired data)

Muscle strength: Of the nine muscle groups tested, there were increases in the strength of the hip flexors and shoulder abductors (Table 3).

Muscle function: Twelve weeks of training significantly reduced the time taken by patients to climb four steps (on average 0.3 sec less, [−0.54 to −0.04], p=0.02 Table 4) and to rise from supine to a standing position (1 sec less, [−2.0 to 0.01], p=0.05). The QMFT sum score and the time to run 10 meters did not change.

Body composition: There were no changes in mineral bone mass (2.83 kg ± 0.58 before training vs. 2.82 kg ± 0.57 after training), in lean body

Table 4 Muscle function measured by the quantitative motor function test (QMFT) and timed tests before and after 12 weeks of training

	Before training Mean ± SD	After training Mean ± SD	p-value*
QMFT score (N=22)**	51 ± 8	51 ± 9	0.65
Timed Tests (N=22)**			
- 10 m running (sec)	4.97 ± 1.50	4.70 ± 1.34	0.16
- Climbing four steps (sec)[‡]	2.37 ± 0.80	2.08 ± 0.74	0.02
- Rising from supine to standing position (sec)[‡]	5.83 ± 4.25	4.83 ± 2.38	0.05

*For the difference before and after training (paired samples t-test and the Wilcoxon signed-ranks test for paired data[‡])

** QMFT score and timed test were not available for one patient

mass (42.53 kg ± 7.99 vs. 43.14 kg ± 8.28), or in fat mass (30.11 kg ± 9.23 vs. 29.29 kg ± 9.06). Likewise, there were no changes in bone mineral density, overall fat percentage, and android and gynoid fat percentages (results not shown).

Duration of training effect

After the initial 12 weeks of training group 1 was planned to discontinue training, but 11 of 13 patients chose to continue training with the same or slightly modified intensity. Therefore we were not able to assess how long the training effect maintained after withdrawal.

DISCUSSION

This study provides clinical evidence that a combination of aerobic, resistance and core stability training can be performed safely in patients with Pompe disease, and helps to improve endurance, core stability and muscle function.

Improved endurance was shown by improved aerobic fitness over 12 weeks of training. In addition to the 11% increase in maximum workload capacity, peak oxygen uptake and ventilatory threshold improved by 9% and 10%, and the 6MWT by 3%. The increase in peak oxygen uptake was relatively small compared to a number of studies in neuromuscular dystrophies and metabolic myopathies like McArdle disease, showing an increase ranging from 12-47 %.[19–22] In these studies, patients spent more time per week on endurance training, which might explain the larger increase. A second explanation might be the lower baseline VO2peak than in our study.

The baseline values on VO2peak we found in our study were slightly higher than those reported in two Italian studies of Pompe patients (range 15.1 – 26.4 ml/kg/min).[23, 24] These include 8 patients who were assessed prior to treatment (median 20.5 mg/kg/min), and one patient assessed during ERT (15.7 mg/kg/min), while all our patients had been on ERT for at least a year, which might explain the somewhat higher VO2peak values in our cohort. Nevertheless, our 6MWT results also suggest that our

cohort had a relatively good endurance capacity before the training (77% of normal expected). Finally, their ventilator threshold as a percentage of the peak oxygen uptake (78%) is relatively high for an untrained population, and comparable to that seen in the three Italian patients that reached the VT (five did not reach the VT in this study, while all our patients reached VT). Our patients thus had relatively good endurance and tolerance capacity before training, which is in agreement with the fact that mildly affected Pompe patients were selected for this study.

So far the 6MWT was used in clinical trials for Pompe disease to assess endurance capacity, but since most patients have walking difficulties it has been questioned whether the 6MWT fully reflects this.[25, 26] Our study indicates that the incremental cycle test offers a good alternative to test endurance capacity, providing insight into patients' aerobic capacity.

Core stability has not been trained previously in neuromuscular disorders presenting with limb girdle weakness. One possible reason may lie in the assumption that core stability exercises are not feasible for such patients. Indeed, on starting training, many patients had difficulty performing the exercises. During the program, however, they learned to activate the proper muscle groups and were able to remain in balance for longer. Our results thus indicate that core stability training is feasible and improves time in balance in patients with Pompe disease who are treated with ERT. Feedback from patients during the training also suggests that they perceived their improved core stability to facilitate daily activities. Further studies on how training influences patient reported outcomes such as quality of life are needed.

An increase in muscle strength was observed in two of the nine muscle groups tested: the hip flexors and shoulder abductors. We are not completely certain whether the increased strength of the hip flexors resulted from strength training, core stability exercises, or both. Core stability may support gains in muscle strength by improving proprioception and coordination.

The combined effects of the training program on endurance, core stability, and muscle strength also led to some functional improvement, with patients becoming able to climb four stairs and rise to a standing position faster.

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In our study all patients received ERT. It has been reported that the main incremental effects of ERT are observed during the first year. Therefore only patients who had received ERT more than 1 year were allowed to participate. The study had a staggered design with patients in group 2 starting after a period of no training allowing evaluation of the effect of ERT only, and patients in group 1 scheduled to stop training after 12 weeks with the intention to study how long the effect of training continued. During ERT only patients remained more or less stable indicating that training was the main driver behind the effects. We were not able to assess how long the training effect continued, since all patients in group 1, except two, chose to continue training after 12 weeks. Although patients' choice interfered with our study design, it also reflects the positive feedback they have given us on the program.

Compliance was high in our study. It is likely that the beneficial effects experienced, the supervision by physiotherapists and weekly telephone consultations contributed to this. We therefore recommend that the program be incorporated into regular supervised physiotherapy sessions.

Few studies have been conducted on exercise and training in Pompe disease. The largest so far was performed before ERT became available.[9] For a mean duration of four years, 26 patients participated in a combined nutrition and endurance exercise therapy program that led to improved muscle function as measured with the Walton score. More recently, a German observational study showed that the effect of ERT on walking distance was most pronounced in five patients who, incidentally, were also subjected to endurance training on a cycle ergometer during ERT infusions.[10] Two other studies, one in mice and one in five adult patients with Pompe disease, however, did not confirm a beneficial effect of endurance training while receiving ERT.[27, 28]

Previous exercise studies in Pompe disease mainly focused on endurance training. It has been envisaged that resistance training might lead to muscle damage, thereby aggravating muscle weakness.[29–31] Prior to our study, only Terzis et al. combined endurance with resistance training in five patients with Pompe disease receiving ERT. The combined results of these five patients showed that both muscular strength and walking distance improved.[28]

Before starting our study we carefully considered whether we should perform exercise-endurance training only, or a combination of different types of exercises. We chose the latter, because we not only wanted to improve endurance, but also target all affected muscles (resistance exercises), and ameliorate proprioception and the strength of those proximal muscles not targeted by resistance training (core stability exercises). Our decision to use a combined program was also driven by the fact that our patient population was not large enough to run three separate programs.

Although we cannot rule out the possibility that endurance training alone might have had a greater impact on endurance, we observed that the extra exercises had positive effects on core stability, and may also have improved muscle strength and function. Earlier studies in patients with inherited muscular myopathies did not include core stability exercises; our study shows them to be both safe and easy to learn. Patients in our study were mildly affected; those who are more severely affected may need slightly adjusted exercise-training programs but we recommend including similar components.

CONCLUSIONS

Our study shows that a combination of endurance, strength and core stability training is feasible and can be performed safely in patients with Pompe disease. Such training helps to improve endurance, muscle strength, muscle function and core stability. This training program thus seems to offer added value for Pompe patients to those of ERT.

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Chapter 9

General discussion

GENERAL DISCUSSION

During the last decade major developments have been made in treating rare genetic diseases, in particular lysosomal storage and muscular disorders. Pompe disease was the first treatable inheritable muscle disorder, and Erasmus MC played a major role in the development of this therapy. The first clinical trials with recombinant human acid α-glucosidase (rhGAA) in Pompe disease started in 1999. In 2006 the EMA and FDA approved enzyme replacement therapy (ERT) with rhGAA (Myozyme®, Genzyme), changing the perspectives of patients with Pompe disease. In the Netherlands, all patients are followed and treated at the 'Center of Lysosomal and Metabolic Diseases' at Erasmus MC University Medical Center, which provides a unique opportunity to study Pompe disease in a relatively large group of patients. Previous studies described the natural course of Pompe disease, the development of ERT and the effects of ERT in Dutch Pompe patients. The primary aims of this thesis were 1) to study genotype-phenotype correlations in patients with non-classic Pompe disease, 2) to investigate respiratory functioning in Pompe patients by performing dynamic MRI of the diaphragm, and 3) to examine arterial stiffness using carotid-femoral pulse wave velocity (cfPWV) in Pompe disease. The secondary aims were 1) to determine whether increased plasma cardiac troponin T (cTnT) levels in Pompe patients indicate cardiac involvement or skeletal muscle damage, and 2) to study the effects of exercise training in mildly affected patients being treated with ERT. This general discussion summarizes the main findings of our studies and future perspectives are discussed.

MAIN FINDINGS

- Siblings with Pompe disease often share a similar phenotype, however, within some families the course and severity of the disease varies substantially, indicating that other genetic and environmental factors play a role in determining the phenotype;
- Lung MRI, which is an innovative technique to visualize diaphragmatic dynamics, shows severely impaired diaphragmatic function in adult patients with Pompe disease;

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- Patients with Pompe disease have an increased arterial stiffness and blood pressure, which probably results in an increased risk for cardiovascular diseases;
- Elevated plasma cTnT levels in patients with Pompe disease are associated with skeletal muscle damage rather than myocardial damage;
- Exercise training is safe and improves endurance, core stability and physical functioning in mildly affected Pompe patients.

GENOTYPE-PHENOTYPE CORRELATION

In Pompe disease the phenotype is mainly determined by the *GAA* genotype. In general, a combination of two severe mutations results in a complete enzyme deficiency causing the classic infantile phenotype.[1-3] Patients with onset of symptoms in childhood or adulthood usually have one severe mutation and one less severe mutation, resulting in some residual enzyme activity.[3, 4] Until today more than 400 mutations have been described and a list is being tracked at www.pompecenter.nl. In this list the effect of a single mutation on the patients' phenotype is described, including a link to the reference. The most frequent detected mutation in patients with non-classic Pompe disease is c.-32-13T>G, which is a 'leaky splice' mutation leading to variable skipping and inclusion of exon 2. This results in 5-25% of structurally and functionally normal acid α-glucosidase (GAA).[4, 5] In patients with non-classic Pompe disease there can be a large variation in phenotype despite sharing the same *GAA* genotype; the age of onset of symptoms is variable, as well as the nature of first symptoms and the course of the disease.[5, 6] Since siblings share the same set of *GAA* mutations and are genetically more related than non-relatives, we expected to find less variation in phenotype in sibships with Pompe disease; i.e. the age of symptom onset, the course of the disease, presenting symptoms and some specific symptoms. In **chapter 2** we described that affected siblings in families with Pompe disease all developed symptoms either in childhood or in adulthood; siblings often had similar presenting symptoms (in 70%

of families); and siblings shared the occurrence of specific features such as ptosis and bulbar weakness. However, in some families a clear phenotypical variation was observed regarding the course and severity of the disease. Since these patients share the same *GAA* genotype, others factors must explain the differences between these patients, such as epigenetic and environmental effects. Genetic confounding factors may be found in genes related to composition and function of muscle fibers. It is known that polymorphisms in the ACE-gene can influence fiber type composition, biomechanical muscle properties and sport performances in humans.[7-9] Moreover, in other glycogen storage diseases such as McArdle disease it was shown that ACE-polymorphisms can affect the phenotype.[10, 11] Another gene polymorphism that is associated with muscle damage after extreme performances and to muscle function decline with age, is the ACTN3 null polymorphism (XX genotype).[12, 13] Both these polymorphisms have been reported to modify the phenotype in patients with Pompe disease sharing the same *GAA* genotype.[14-16] It was demonstrated in two relatively large groups (n=38 and 85) of patients with Pompe disease, that an earlier and more severe clinical picture is associated with the deletion/deletion (DD) genotype in the ACE-gene and the ACTN3 null polymorphism.[14, 16] In addition, it was published that Pompe patients with the DD genotype had a poor treatment outcome compared to patients with other ACE polymorphisms.[15] However, this was studied in a small group of patients (n=16). Besides differences in gene polymorphisms, environmental factors might also modify the clinical course; e.g. high-protein and low-carbohydrate nutrition and exercise training have been reported to slow down deterioration of muscle function in adult patients with Pompe disease.[17] To know whether these environmental factors and polymorphisms play a role in phenotypical variation in patients and siblings with Pompe disease, studies in larger groups of patients are needed.

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IMAGING AND LABORATORY DIAGNOSTICS

Lung MRI

Pulmonary dysfunction caused by progressive weakness of the respiratory muscles is a characteristic feature of Pompe disease, particularly in supine position.[3, 18, 19] It has been shown that in the majority of patients forced vital capacity (FVC) in sitting position stabilized, while FVC in supine position deteriorated in 35% of patients despite initiating ERT.[20, 21] In general, pulmonary function tests are being used to monitor different parameters such as FVC in sitting and supine position, postural drop (i.e. the difference between FVC in sitting and supine position expressed in percentages) and maximum static inspiratory (MIP) and expiratory (MEP) pressures. Postural drop and MIP are considered to represent diaphragmatic weakness, which is thought to be the major cause of respiratory dysfunction in Pompe disease.[19, 22] However, these parameters provide too little insight into the individual contribution of the diaphragm and thoracic musculature to respiratory dysfunction in Pompe disease. In **chapter 5** we showed that lung MRI is an innovative tool to visualize diaphragmatic and chest-wall movements directly and in more detail. We observed severe dysfunction of the diaphragm in adult patients with Pompe disease. In some patients there was hardly any displacement of the diaphragm, while the thoracic musculature was far less affected. Similar results were recently found in a group of 11 Pompe patients.[23] However, in this study only the right lung was evaluated in maximum inspiration and expiration, and spirometry was not used during the MRI. Lung MRI might be useful to monitor changes in diaphragmatic movements in response to ERT, and therefore be helpful to identify responders and non-responders. Moreover, it can also be useful in patients who have no or mild limb girdle weakness to evaluate the presence of diaphragmatic weakness, so ERT can be initiated in the early state of the disease before irreversible respiratory muscle dysfunction has occurred. Respiratory insufficiency is even reported as first symptom in a small percentage of patients with non-classic Pompe disease, which emphasizes the importance of monitoring pulmonary function properly.[19, 24-26] This year an extended study will start at our center using lung MRI in a larger group of adult Pompe patients, including both static and dynamic

3D MR images. This latter technique was recently studied as an extension of our MRI study and showed that diaphragm weakness and ribcage expansion can be studied even more accurately. This study showed that Pompe patients could compensate diaphragmatic weakness by increasing chest-wall movements in the early stages of the disease. As a result, in some patients standard pulmonary function tests were relatively normal, while MRI already showed decreased displacement of the diaphragm [Mogalle et al. submitted]. In our extended study we will not only use lung MRI to examine the diaphragm, but we also include ultrasound to measure diaphragm thickness at maximum inspiration and expiration. The advantage of ultrasound above MRI is that this technique is inexpensive, more widely available and can be performed at bedside. In addition, the function as well as the structure of the diaphragm can be visualized which is difficult with MRI.[27, 28] Possible disadvantages of ultrasound compared to MRI are that there might be more interobserver variability, only a small part of the diaphragm can be visualized, and chest-wall movements cannot be measured.

To date, there is no clear explanation why FVC in supine position, representing diaphragmatic functioning, deteriorates during ERT in a substantial proportion of patients with the non-classic form of Pompe disease. We suggest several hypotheses for this finding. First, the diaphragm in patients with Pompe disease might have lost regenerative capacity because it is already early affected in the course of the disease. Loss of regenerative capacity of the diaphragm could be the result of impaired satellite cell differentiation, which was recently described in skeletal muscle of patients with Pompe disease and in patients with other myopathies.[29, 30] Second, the diaphragm in humans might have lower concentrations of mannose 6-phosphate receptors compared to skeletal muscle, reducing the uptake of the enzyme administered during ERT. However, in mice it was shown that the diaphragm had more M6PR and responded better to therapy than skeletal muscles.[31] Third, there might be a shift from fast type II muscle fibres to slow type I fibres during the course of the disease, leading to less muscle strength of the diaphragm. This process was described in patients with COPD, in whom there is an increase in oxidative enzymes and a decrease in glycolytic activity, leading to muscle damage and replacement of muscle by connective tissue.[32, 33] Fourth, there might be an increased failure

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of productive autophagy in the diaphragm compared to skeletal muscle, making ERT less efficient. Autophagy is a regulated process in which cytoplasmatic components such as toxic proteins and damaged organelles are removed to keep muscle homeostasis. When this process is impaired, the autophagic buildup interrupts the architecture of muscle fibers, resulting in muscle weakness.[34, 35] Autophagic debris has been found in skeletal muscle fibers of patients with Pompe disease.[36-38] Another hypothesis is that the diaphragmatic weakness is not only caused by glycogen accumulation in the muscles of the diaphragm, but also by glycogen accumulation in the anterior horn neurons of the spinal cord.[39, 40] Since ERT does not cross the blood-brain-barrier, it cannot correct pathology in the central nervous system (CNS).[41] Glycogen accumulation has been demonstrated in the CNS of patients with classic infantile Pompe disease, but it is assumed that the residual GAA activity of 5-25% in patients with non-classic Pompe disease is sufficient to prevent glycogen storage in the CNS. To investigate whether the previously mentioned hypotheses are correct, histopathological studies of the diaphragm are required. However, taking a biopsy of the diaphragm is a high-risk intervention and we did not have the possibility yet to study the diaphragm of deceased Pompe patients in our cohort. In literature there are only few autopsy reports of patients with Pompe disease. In three of these reports the diaphragm was examined post-mortem and revealed severe glycogen accumulation in skeletal myocytes. In addition, the Schwann cells of small nerves within the diaphragm showed moderate glycogen accumulation.[42-44]

Many Pompe patients require assisted ventilation because ERT has limited effect on the pulmonary function in supine position. Therefore, alternative therapies could be beneficial for these patients such as diaphragm pacing, local gene therapy using hematopoietic stem cell transplantation (HSCT) and the use of induced pluripotent stem (iPS) cells.[45-47] Diaphragm pacing has been studied in patients with amyotrophic lateral sclerosis (ALS) and in patients with spinal cord injuries.[45] Although it was thought to be a promising technique, a recent publication showed that addition of diaphragm pacing to standard care with non-invasive ventilation was associated with decreased survival in patients with ALS.[48] Currently, an observational study is recruiting patients with Pompe disease to examine

the response to diaphragm pacing (www.clinicaltrials.gov: NCT02354651). The use of HSCT and iPS cells showed to be effective in mice with Pompe disease. HSCT using lentiviral vectors resulted in obvious glycogen clearance in several tissues, improving respiratory function and skeletal muscle strength.[46, 49, 50] In another study direct delivery of the recombinant adeno-associated virus *GAA* gene into the diaphragm of mice increased respiratory function.[51] At this moment, a Phase I/II safety study with adeno-associated virus-mediated *GAA* gene therapy is being performed in ventilator dependent children with Pompe disease (www.clinicaltrials.gov: NCT00976352). In these patients the recombinant adeno-associated virus-mediated *GAA* gene is delivered into the diaphragm. The first results of this trial indicate that the treatment was safe and may modestly improve ventilatory performance measures; i.e. increased tidal volumes and longer periods of unassisted breathing.[52] One of the latest developments is the use of iPS cells in Pompe disease. These cells can theoretically differentiate into any type of cell in the body. In one study iPS cells were generated from fibroblasts of mice with Pompe disease and subsequently differentiated into skeletal muscle cells.[47] Future studies need to show whether these invasive therapies are safe, effective and could be used in clinical practice in patients with Pompe disease. Lung MRI in combination with spirometry could be used to monitor the effects of these therapies on pulmonary function. A non-invasive therapy that could be useful is respiratory muscle strength training. This type of training has the potential to increase inspiratory and expiratory muscle strength.[53, 54] However, it has only been described in case reports, not in a larger group of patients with Pompe disease and definitely requires further evaluation.

Increased arterial stiffness and plasma cTnT levels

Cardiovascular involvement is a familiar feature in classic infantile Pompe disease.[1-3] In juvenile and adult Pompe patients this is still a topic of discussion, however, there are several reports about cardiovascular morbidity such as dilated thoracic arteriopathy and the occurrence of cerebral aneurysms.[55-58] These observations could be the result of glycogen accumulation in vascular smooth muscle fibers.[42, 43, 59] Increased aortic stiffness was found in 17 patients with non-classic Pompe disease using transthoracic Doppler

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echocardiography.[60] This is a technique to measure aortic stiffness indi-rectly, while nowadays tonometry of the carotid-femoral pulse wave veloc-ity (cfPWV) is considered to be the golden standard to directly measure arterial stiffness.[61] Using cfPWV we studied whether structural changes in the arteries of adult Pompe patients result in an increased arterial stiffness. We described in **chapter 7** that these patients indeed have an increased arterial stiffness, which is an indirect risk factor for cardiovascular disease and mortality.[62, 63] Additionally, Pompe patients had an increased blood pressure compared to age and gender-matched volunteers. We assume that glycogen accumulation in smooth muscle fibers resulted in increased arterial stiffness leading to hypertension, however, we did not examine this hypothesis. Other possibilities could be that glycogen accumulation in the endothelium damages the vascular wall making it more vulnerable to atherosclerosis, or that glycogen accumulation in vascular smooth muscle fibers impairs the formation of elastin leading to changes in collagen formation as in Fabry disease and mucopolysaccharidosis type 1.[64, 65] Our findings of increased arterial stiffness and hypertension in Pompe disease are important, because as a result of the introduction of ERT Pompe pa-tients have a prolonged survival, resulting in an increased lifetime risk on cardiovascular complications. Therefore, it is important to register whether cardiovascular events occur more often than in the past in Pompe patients and to treat and monitor other cardiovascular risk factors such as hypertension, hypercholesterolemia, diabetes, obesity and smoking. In patients with Fabry disease it has been demonstrated that arterial stiffness decreased during ERT.[65] Whether arterial stiffness decreases in patients with Pompe disease during ERT and if cfPWV can be used as a biomarker for disease progression or therapy response, requires further investigation.

There are several biomarkers for skeletal muscle damage, such as creatine kinase (CK), myoglobulin, lactate dehydrogenase and aminotransferases.[66] Troponin is also a known biomarker of muscular damage, from which vari-ous subtypes exist that are expressed from closely related genes.[66, 67] cTnT is an important biomarker in patients with acute myocardial infarction (AMI), however, increased plasma cTnT concentrations have also been observed in the general population.[68, 69] We found increased plasma cTnT levels in one of our adult Pompe patients who reported atypical chest pain, while

additional cardiac evaluation did not reveal any abnormalities. Generally, in patients with non-classic Pompe disease it is considered that there is no significant cardiac involvement.[70-72] Therefore, the question arose whether increased plasma cTnT levels in Pompe patients indicate cardiac involvement or skeletal muscle damage. We demonstrated in **chapter 4** that plasma cTnT levels were increased in the majority of patients with Pompe disease. In the absence of increased cTnI levels, a strong correlation with CK levels, normal cardiac evaluation in almost all patients, and RNA and protein expression of cTnT in skeletal muscle biopsies taken from Pompe patients, we assume that plasma cTnT levels were increased as a result of skeletal muscle damage and not due to myocardial damage. Our findings are supported by a recent paper which describes that adult Pompe patients do not have abnormalities on cardiac MRI.[72] In addition, increased cTnT levels were also demonstrated in other neuromuscular disorders and it was suggested that this is the result of re-expression of fetal isoforms of cTnT in regenerating skeletal muscle.[73-77] AMI should be diagnosed if a rise and/or fall of cTnT or cTnI is detected, with at least one value above the 99th percentile cutoff value.[78] It has been described that cTnI has a higher specificity for cardiac tissue than cTnT and cTnI has not been found in diseased or regenerating skeletal muscle.[68, 73, 79] Together with the fact that we found normal plasma cTnI levels in all Pompe patients, we suggest determining plasma cTnI instead of cTnT levels in patients with Pompe disease and other neuromuscular diseases when there is a suspicion of AMI.

Muscle MRI

Neuromuscular imaging techniques are helpful tools and they are increasingly being used to create a better understanding of pathophysiological processes of neuromuscular diseases, to monitor disease progression and to evaluate effects of therapy. These techniques, including CT, ultrasound and MRI, provide extra information in addition to the physical examination. Since CT exposes patients to ionizing radiation and ultrasound is too time consuming and does not have the possibility to study multiple muscles into detail, MRI has become the preferred neuromuscular imaging technique to use.[80-83] Several studies demonstrated that muscle MRI is useful in non-classic Pompe disease to study muscle involvement, monitor

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disease progression and treatment effects, even in patients who are Ventilator dependent.[84-87] We used MRI to examine the upper leg muscles of patients with classic infantile Pompe disease. In **chapter 6** we described that MRI showed a hypertrophic appearance of the muscles in these patients. We assume this is caused by glycogen accumulation as observed in muscle biopsies of patients with classic infantile Pompe disease, but we did not demonstrate this assumption. To examine this hypothesis other MRI sequences should be used in future studies; e.g. glycogen accumulation could be demonstrated using C-NMR spectroscopy.[88, 89] A major advantage of MRI is that muscles can be studied which are difficult to examine during neurological examination; e.g. tongue muscles, trunk and abdominal muscles.[84, 86, 90] Most studies showed that T1-weigthed sequences are suitable for assessing the pattern and severity of affected muscles in patients with non-classic Pompe disease, since these sequences detect both muscle atrophy and fatty replacement of muscle fibers.[84, 86, 90] Other MRI techniques, such as T2 mapping and 3-point Dixon imaging, showed to be objective quantitative methods to monitor disease activity in Duchenne muscular dystrophy.[91, 92] During a recently performed open-label multicenter study, 3-point Dixon imaging was used to measure changes in the degree of fatty infiltration in skeletal muscle of patients with non-classic Pompe disease (www.clinicaltrials.gov: NCT01288027). In another study it was demonstrated with proton-density fat-fraction (PDFF) whole-body MRI that the fat fraction of individual muscle groups in Pompe patients can be calculated and quantified.[93] This technique was even more sensitive for the detection of muscle abnormalities in some muscle groups than physical examination. Therefore, muscle MRI in Pompe disease could be used for several purposes in addition to the physical examination. First, it could be a feasible technique to monitor the effects of ERT.[94] Second, it could be used to study muscle involvement in clinically asymptomatic patients since it has been shown that muscles can already be affected in these patients before onset of symptoms has occurred.[89, 90, 95] This is important since it is known that the best responders on ERT are likely patients who are mildly affected.[96] Third, some studies describe that muscle MRI has the potential to shorten the diagnostic delay of approximately seven years in patients with non-classic Pompe disease by recognizing patterns of muscle involve-

ment, so treatment can be initiated in an early stage of the disease.[86, 90, 97] In Dutch neuromuscular centers, a guideline of the Dutch Neuromuscular Research Center (ISNO) is used for patients presenting with limb girdle weakness. This guideline suggests that GAA activity should be measured in any patient with limb girdle weakness having increased CK levels and without a clear family history of neuromuscular diseases. We expect that by using this guideline the diagnostic delay will decrease, whereby limiting the use of MRI for this purpose in the Netherlands.

THERAPIES

Enzyme replacement therapy

In 2006, when the use of ERT was approved in patients with Pompe disease, a clinical benefit was demonstrated only in patients with the classic infantile form of the disease. In these patients the hypertrophic cardiomyopathy decreased, patients reached motor milestones and had a prolonged survival.[98-100] The first results in a large group of patients with non-classic Pompe disease were published in 2010. In a randomized, placebo-controlled trial in 90 patients it was demonstrated that after 1.5 years of therapy patients gained an improved walking distance and the pulmonary function stabilized.[20] These results were supported by several other studies in which the duration of treatment fluctuated from 6-54 months.[101-106] In 2012 the effects of ERT after a median duration of two years in a group of 69 adult patients were published. This study showed that muscle strength increased and pulmonary function in sitting position stabilized. Patients who responded best were younger, more often female and were less severely affected before start of therapy.[96] In 2013 it was shown that adult patients also had an improved survival after receiving ERT.[107] Most studies showed that ERT positively alters the natural course in patients with non-classic Pompe disease on the relatively short-term, however, the long-term effects in larger groups of patients are unknown and currently topic of investigation. Pending on these results, researchers try to improve the effects of ERT in several ways: increasing the dose of rhGAA and frequency of dosing, increasing levels of M6P on rhGAA (www.clinicaltrials.gov: NCT01898364),

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using immunomodulation therapy to induce immune tolerance, and using adjuvant therapies to ERT such as clenbuterol (www.clinicaltrials.gov: NCT01942590), GAA fused to the glycosylation-independent lysosomal targeting (GILT) tag which contains a portion of insulin-like growth factor II (www.clinicaltrials.gov: NCT01924845) and chaperone therapy.[108-118]

Physical therapy and exercise training

In the past, clinicians were often afraid that exercise training in patients with neuromuscular disorders would result in progression of muscle weakness. However, moderate-intensity strength training and aerobic exercise training were shown to do no harm in patients with various neuromuscular disorders such as myotonic dystrophy type I, facioscapulohumeral muscular dystrophy, inflammatory myopathies and neuropathies.[119-121] Until recently there was little evidence that exercise training in Pompe disease would be beneficial. In a group of 34 Pompe patients receiving a combination of a high-protein/low-carbohydrate diet and submaximal aerobic training there was a positive effect on muscle strength and pulmonary function. Particularly patients who were fully compliant to the program stabilized or showed a slight improvement in muscle function.[17] Another study in five patients receiving ERT showed an improvement in muscle strength and endurance after completing a 20-week training program.[122] We showed that twelve weeks of exercise training indeed significantly improves muscle strength, endurance and physical functioning in mildly affected patients with Pompe disease who were treated with ERT (**chapter 8**). It also reduced fatigue and pain.[123] The training program that we used comprised aerobic training, strength training and core stability training three times a week. Patients performed the program safely. Since our study lasted twelve weeks and only mildly affected patients were included, future studies are needed to explore the effects of exercise training on a longer term and in a larger group of patients including the whole spectrum of the disease.

FUTURE PERSPECTIVES

Pompe disease is a rare disorder. To study the effects of ERT and any future therapy in a larger patient population, it would be best to collect patient data in a European or worldwide cohort. Other advantages of collaboration between multiple centers are that 1) genotype-phenotype correlations and the effect of polymorphisms and environmental factors can be studied more reliable, 2) the use of neuromuscular imaging techniques can be examined in a larger group of Pompe patients before and during ERT, and 3) more definite criteria for starting and stopping of ERT can be developed. We have already discussed the first two points earlier in this discussion. In the next section we will discuss our vision on how to develop starting and stopping criteria, and the importance of studying prognostic factors.

In patients with classic infantile Pompe disease who are diagnosed soon after birth, there is no discussion whether to start with ERT, since it was shown that without treatment these patients die in the first year of life.[1-3] It was also demonstrated that ERT should be started as soon as possible, since survival was best in patients receiving ERT before the age of six months.[124, 125] In patients with non-classic Pompe disease there is a broad clinical spectrum, making it more difficult to decide when to initiate treatment. However, it was shown that younger patients with mild limb girdle weakness and pulmonary dysfunction responded best to ERT, indicating that ERT in principle should be started as early as possible after onset of symptoms.[96, 126-128] An important issue is that ERT is a very expensive treatment and especially therefore, it should not be started too early in the course of the disease. On the other hand, ERT should also not be initiated too late once muscle weakness has become irreversible. The consensus at our center is that patients who have clinical signs of limb girdle weakness or respiratory dysfunction, which both could be mild, should start with ERT when they deteriorate over time. A difficult group of patients are those who are severely affected, i.e. requiring a wheelchair or ventilation, or patients who are deteriorating despite treatment with ERT. Nowadays, there are no clinical studies providing evidence whether these patients should start or continue with ERT, or treatment should be stopped. These are difficult ethical dilemmas. Recent meetings between experts within the

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European Pompe Center (EPOC) help to formulate these criteria. Currently, we recommend treating these patients for a period of two years and subsequently judging their responses to ERT. Treatment should be continued if patients improve, stabilize or deteriorate less than before start of ERT. This is consistent with current recommendations of other expert groups.[129-131] Finally, there are patients with Pompe disease who are still asymptomatic. At our center these patients are assessed once a year, including measuring muscle strength, pulmonary function, biomarkers of skeletal muscle damage and in the future lung and muscle MRI. Once there are clinical signs of disease activity, the possibility of starting with ERT is discussed with the patient. In general, this is also the consensus at other centers.[95, 129-131]

To better predict the effect of ERT in an individual patient before start of treatment, we are trying to develop prediction models that enable us to predict the individual response on muscle strength and pulmonary function. For this purpose, prognostic factors need to be determined in a larger group of patients; e.g. the influence of age, gender, genotype, polymorphisms and environmental factors. This project is a collaborative action between the European neuromuscular centers within EPOC. Hopefully these prediction models will be helpful to make these difficult decisions.

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Chapter 10

Summary | Samenvatting

SUMMARY

Pompe disease is a progressive metabolic myopathy with an autosomal recessive inheritance pattern. Mutations in the *GAA* gene result in a complete or partial deficiency of the lysosomal enzyme GAA that leads to glycogen accumulation in various tissues.

In **chapter 1** Pompe disease is introduced and different aspects of the disease are summarized such as the etiology, pathophysiology, diagnostics and treatment options. In addition, the broad clinical spectrum of this disease is described. At the one end of the spectrum there are patients with the classic infantile form of the disease. These patients have a complete GAA deficiency. They have a rapidly progressive course of the disease and without treatment these patients die in the first year of life. On the other end of the spectrum there are adult patients with the non-classic or late-onset form of the disease. These patients develop symptoms in adulthood and have a slowly progressive course of the disease.

The genotype-phenotype correlation is addressed in more detail in **chapter 2**. The *GAA* genotype is the first level at which clinical heterogeneity arises. Patients with the classic infantile form of the disease have two severe mutations in the *GAA* gene and patients with the non-classic form harbor one severe mutation and a second mutation that is not fully deleterious. The clinical phenotype in classic infantile patients is rather homogeneous, while in patients with non-classic Pompe disease there is a large variation in phenotype, even among patients with the same *GAA* genotype. Over 90% of adult patients and 50% of children with Pompe disease harbour the c.-32-13T>G mutation in combination with a severe mutation. In this group of patients there is no clear correlation between residual enzyme activity and phenotype. Probably, other factors such as environmental and

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genetic factors play an important role. To have a better understanding of the genotype-phenotype correlation in patients with non-classic Pompe disease we studied 22 Dutch families with two or more affected siblings. Siblings affected with Pompe disease all developed symptoms either in childhood or in adulthood. In addition, siblings often have similar presenting symptoms and in some families specific clinical features such as ptosis and bulbar weakness are present in all of them. However, within other families there is still a substantial variation in the course and severity of the disease, as also observed between families with the same *GAA* genotype. This phenotypical variation and consistency within families indicates that other genetic background or environmental factors than the *GAA* genotype influence the course of the disease. Additional studies are needed to identify these factors.

Chapter 3 focuses on the genotype-phenotype correlation in three adult patients with Pompe disease with residual GAA activity measured in fibroblasts in the range that is normally observed in classic infantile patients. None of these patients carried the common mutation c.-32-13T>G. To have a better understanding of the clinical course in these three patients enzyme activity was measured in leukocytes, fibroblasts and muscle tissue, and glycogen content was measured in fibroblasts and muscle tissue. In all three cases enzyme activity and glycogen content in fibroblasts were within the same range as in classic infantile Pompe disease, but the activity and glycogen content in muscle tissue were within the range as in non-classic Pompe disease. The higher enzyme activity and less glycogen storage in muscle might explain why these three patients have a slowly progressive course of the disease.

Chapter 4 describes the finding of increased plasma cardiac troponin T (cTnT) levels in 122 patients with Pompe disease. Troponin is a protein involved in muscle contraction. Both a skeletal muscle-specific and cardiac muscle-specific troponin form exist. Cardiac troponins are important biomarkers in patients with acute myocardial infarction, however, in patients with neuromuscular disorder such as Pompe disease these troponins can also arise from an alternative source. We demonstrated that in 82% of the Dutch Pompe patients cTnT was elevated and correlated well with creatine kinase levels, a marker of skeletal muscle damage. cTnI, which is a

specific for cardiac damage, was normal in all patients. Left ventricular hypertrophy is also associated with increased cTnT, however, this finding was present in only one patient with classic infantile Pompe disease as demonstrated by cardiac evaluation of most patients. RNA expression analysis demonstrated the presence of cTnT in skeletal muscle tissue taken from Pompe patients. We concluded that most patients with Pompe disease have elevated plasma cTnT levels reflecting skeletal muscle damage rather than myocardial damage.

Chapter 5 presents a study of the respiratory function using MRI. Patients with non-classic Pompe disease not only develop slowly progressive limb girdle weakness, but also respiratory insufficiency, most prominent in supine position. In this chapter we describe a pilot study in ten adult patients in whom the respiratory function is measured by lung MRI in combination with spirometry. This non-invasive technique allows to directly assess the contribution of the diaphragm and other respiratory muscles during the breathing cycle. MRI showed that most patients with Pompe disease have a severely impaired diaphragmatic function. In some of them there was hardly any movement of the diaphragm. This might indicate that the diaphragm is already involved early in the disease process. We hypothesize that lung MRI might be an useful tool in decision making when to start with ERT, and also to monitor treatment effects. Further studies will be performed in the near future.

In **chapter 6** the results of muscle MRI of the upper legs of patients with classic infantile Pompe disease are described. Five patients were included and MR images were compared to muscle histopathology sections taken from quadriceps muscle. MRI showed almost no abnormalities except for some hypertrophy of the upper leg muscles, while the clinical status was quite advanced in most patients and muscle biopsies were strikingly abnormal. Future investigation is required to study if the hypertrophic appearance of the muscles on MRI is related to glycogen accumulation only and if MRI is an effective technique to evaluate the effects of ERT in classic infantile Pompe disease.

Chapter 7 shows the results of a cross-sectional case-controlled study in 84 patients with non-classic Pompe disease in whom we investigated arterial stiffness. We performed this study since it has been shown that gly-

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cogen not only accumulates in skeletal muscle, but also in smooth muscle fibers and in the endothelial layer of arteries. We hypothesized that glycogen accumulation in these structures would result in increased arterial stiffness. Arterial stiffness is an independent risk factor for cardiovascular disease and mortality. Pompe patients did not only have an increased arterial stiffness, but also an increased blood pressure compared to age and gender-matched volunteers. To reduce the potential risk of cardiovascular disease, we recommend that blood pressure and other cardiovascular risk factors are closely monitored. The effects of ERT on arterial stiffness need to be investigated in future studies.

In **chapter 8** we focused on exercise training in patients with non-classic Pompe disease receiving ERT. The training program comprised 36 sessions of standardized aerobic, resistance and core stability exercises during 12 weeks. We studied whether a standardized and structured exercise intervention program has added value to ERT and also whether patients with a neuromuscular disorder such as Pompe disease can safely perform this type of exercises. Overall there was an improvement in endurance and core stability. In most patients muscle strength stabilized or slightly improved. We concluded that exercise training is safe and can serve as a beneficial adjuvant treatment in mildly affected patients with non-classic Pompe disease, when it is performed under the supervision of a trained physical therapist.

Finally, **chapter 9** summarizes the main findings, discusses the significance, and provides suggestions for future research.

SAMENVATTING

De ziekte van Pompe is een progressieve metabole myopathie en erft autosomaal recessief over. Mutaties in het *GAA* gen resulteren in een volledige of gedeeltelijke deficiëntie van het lysosomale enzym GAA. Deze deficiëntie leidt tot glycogeenstapeling in verschillende weefsels.

In **hoofdstuk 1** wordt de ziekte van Pompe geïntroduceerd en verschillende aspecten van de ziekte samengevat zoals de etiologie, pathofysiologie, diagnostiek en behandelingsopties. Daarnaast wordt het klinische spectrum van de ziekte beschreven. Aan het ene eind van het spectrum bevinden zich patiënten met de klassieke vorm van de ziekte van Pompe. Deze patiënten hebben een complete deficiëntie van het enzym GAA. De ziekte is snel progressief en zonder behandeling overlijden deze patiënten in het eerste levensjaar. Aan het andere eind van het spectrum bevinden zich volwassen patiënten met de niet-klassieke vorm van de ziekte van Pompe. Deze patiënten ontwikkelen symptomen tijdens de volwassenheid en hebben een langzaam progressief ziektebeloop.

De correlatie tussen genotype en fenotype wordt in **hoofdstuk 2** in meer detail behandeld. Het *GAA* genotype is het eerste niveau waarop klinische heterogeniteit ontstaat. Patiënten met de klassieke vorm van de ziekte van Pompe hebben twee ernstige mutaties in het *GAA* gen en het fenotype in deze groep is vrij homogeen. In patiënten met de niet-klassieke vorm is er een grotere variatie in fenotype, zelfs tussen patiënten met hetzelfde *GAA* genotype. Meer dan 90% van de volwassen patiënten en 50% van de kinderen met de ziekte van Pompe dragen de c.-32-13T>G mutatie bij zich in combinatie met een ernstige tweede mutatie. Aangezien er geen correlatie is tussen de resterende enzymactiviteit en het fenotype moeten er andere factoren zijn die een belangrijke rol spelen zoals genetische en omgevingsfactoren. Om de correlatie tussen genotype en fenotype verder te bestuderen hebben we 22 Nederlandse families met twee of meer aangedane broers of zussen onderzocht. In de meeste families hebben patiënten hetzelfde fenotype en ontwikkelen ze symptomen tijdens de jeugd of in de volwassenheid. Vaak presenteren ze zich met dezelfde eerste symptomen en binnen sommige families komen dezelfde klinische kenmerken voor zoals ptosis en bulbaire zwakte. Echter in een aantal families is er een evidente

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variatie qua beloop en ernst van de ziekte, zoals ook wordt gezien tussen families met hetzelfde *GAA* genotype. De variatie en overeenkomsten in fenotype binnen families wijzen erop dat andere genetische en omgevings-factoren naast het *GAA* genotype het ziektebeloop beïnvloeden. Hiervoor zijn aanvullende studies nodig om deze factoren te identificeren.

Hoofdstuk 3 beschrijft de correlatie tussen het genotype en fenotype in drie volwassen patiënten met de ziekte van Pompe met een enzymactiviteit in fibroblasten die normaal wordt gemeten in patiënten met de klassieke vorm. Geen van deze drie patiënten heeft de mutatie c.-32-13T>G welke het meest voorkomt onder volwassen patiënten. Om een beter inzicht te krijgen van het klinisch beloop in deze patiënten werd de enzymactiviteit en/of de hoeveelheid glycogeenstapeling gemeten in leukocyten, fibro-blasten en spierweefsel. In alle drie de gevallen was de enzymactiviteit en hoeveelheid glycogeenstapeling in fibroblasten in dezelfde range als in die van klassiek-infantiele patiënten met de ziekte van Pompe. Echter de enzymactiviteit en hoeveelheid glycogeenstapeling gemeten in spier-weefsel was in de range van patiënten met de niet-klassieke vorm van de ziekte, wat verklaart waarom deze drie patiënten een langzaam progressief beloop hebben.

In **hoofdstuk 4** wordt de bevinding van een verhoogd cardiaal troponine T (cTnT) in 122 patiënten met de ziekte van Pompe besproken. Tropo-nine is een eiwit dat is betrokken bij spiercontractie en ruwweg bestaan er twee vormen, namelijk skelet- en hartspier specifiek troponine. Car-diale troponines zijn belangrijke biomarkers in patiënten met een acuut myocardinfarct. Echter, in patiënten met neuromusculaire aandoeningen zoals de ziekte van Pompe zijn alternatieve oorzaken aannemelijk. 82% van de Nederlands patiënten met de ziekte van Pompe hebben een ver-hoogd cTnT, wat correleert met het creatine kinase (een biomarker voor skeletspierschade). Daarnaast werd gekeken naar cTnI, een specifieke biomarker voor hartspierschade, welke normaal was in alle patiënten. Tevens vond er een uitgebreide cardiale evaluatie plaats waarbij er slechts in één patiënt met de klassiek-infantiele vorm linker ventrikelhypertrofie werd gevonden als verklaring voor het verhoogde cTnT. Aanvullend werd RNA expressie van cTnT aangetoond in skeletspierweefsel van patiënten met de ziekte van Pompe. Wij concluderen dat het overgrote deel van de

patiënten met de ziekte van Pompe een verhoogd plasma cTnT heeft, wat het meest waarschijnlijk veroorzaakt wordt door skeletspierschade en niet hartspierschade.

Hoofdstuk 5 beschrijft een studie waarbij er gekeken is middels MRI naar de longfunctie van volwassen patiënten met de ziekte van Pompe. Deze patiënten ontwikkelen naast langzaam progressieve spierzwakte ook respiratoire insufficiëntie, het meest uitgesproken in liggende houding. In een pilot studie in tien volwassen patiënten hebben we een MRI van de longen verricht in combinatie met spirometrie. Door middel van deze non-invasieve techniek is het mogelijk om op een directe manier het diafragma en de overige ademhalingsspieren te bekijken. Bij Pompe patiënten toonde de MRI een ernstig verminderde functie van het diafragma en in sommige patiënten was er nauwelijks beweging van het diafragma. Dit kan erop wijzen dat het diafragma al vroeg betrokken is in het ziekteproces. Of MRI in de toekomst een bruikbare techniek is om te beslissen wanneer te starten met ERT en om de effecten van deze therapie te monitoren moet verder worden onderzocht in toekomstige studies.

Hoofdstuk 6 beschrijft de bevindingen van MRI van de bovenbeenspieren bij vijf klassiek-infantiele patienten met de ziekte van Pompe. De MRI beelden werden vergeleken met spierbiopten genomen uit de musculus quadriceps. De MRI's lieten nagenoeg geen afwijkingen zien behoudens enige hypertrofie van de bovenbeenspieren, terwijl de meeste patienten een ernstig klinisch beeld hadden en een zeer afwijkend spierbiopt. Toekomstig onderzoek moet uitwijzen of de hypertrofisch uitziende spieren op MRI het gevolg zijn van alleen glycogeenstapeling en of MRI van de spieren een effectieve techniek is om de effecten van ERT te evalueren in patienten met de klassiek-infantiele ziekte van Pompe.

In **hoofdstuk 7** worden de resultaten besproken van een cross-sectionele case-controlled studie naar de arteriële vaatstijfheid in 84 patiënten met de niet-klassieke vorm van de ziekte van Pompe. Deze studie werd verricht omdat er niet alleen glycogeenstapeling is in skeletspierweefsel, maar ook in gladspierweefsel en in de endotheellaag van arteriën. Wij veronderstelden dat glycogeenstapeling in deze structuren zou leiden tot een verhoogde arteriële vaatstijfheid; een onafhankelijke risicofactor voor cardiovasculaire ziekten en mortaliteit. Deze studie toont aan dat patiën-

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ten met de ziekte van Pompe naast een verhoogde vaatstijfheid ook een verhoogde bloeddruk hebben in vergelijking met voor leeftijd en geslacht gecorrigeerde vrijwilligers. Wij adviseren om de bloeddruk en andere cardiovasculaire risicofactoren goed te monitoren en eventueel te behandelen om zo het cardiovasculaire risicoprofiel te verlagen. Het effect van ERT op de arteriële vaatstijfheid dient verder te worden onderzocht in toekomstige studies.

Hoofdstuk 8 behandelt de effecten van een trainingsprogramma in patiënten met de niet-klassieke vorm van de ziekte van Pompe. Het programma bestond uit 36 sessies van gestandaardiseerde aerobics oefeningen, uithoudingstesten en rompstabiliteitsoefeningen. Wij hebben onderzocht of zo'n gestandaardiseerd en gestructureerd trainingsprogramma een toegevoegde waarde heeft naast ERT en of dit veilig kan worden uitgevoerd in patiënten met een neuromusculaire aandoening zoals de ziekte van Pompe. Na het trainingsprogramma was er een verbetering van het uithoudingsvermogen en de rompstabiliteit. In de meeste patiënten was er tevens een stabilisatie danwel lichte verbetering van de spierkracht. Uit het onderzoek blijkt dat dit type trainingsprogramma veilig en voordelig is naast ERT in mild aangedane patiënten met de ziekte van Pompe, mits het wordt uitgevoerd onder de begeleiding van een getrainde fysiotherapeut.

Het afsluitende **hoofdstuk 9** geeft een samenvatting van de belangrijkste bevindingen, bediscussieert het belang van deze bevindingen aan de hand van gegevens uit de literatuur, en bespreekt suggesties voor toekomstig onderzoek naar de ziekte van Pompe.

Chapter 11

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DANKWOORD

Na een periode van 5.5 jaar is het eindelijk zover, 'mijn' proefschrift ligt voor u. Ik heb het woord 'mijn' tussen haakjes gezet, omdat dit proefschrift niet tot stand zou zijn gekomen zonder de hulp van velen. Daarom wil ik nu van de gelegenheid gebruik maken om een aantal personen te bedanken voor de geboden mogelijkheden, medewerking en ondersteuning de afgelopen jaren om dit proefschrift te krijgen zoals het is geworden.

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Prof.dr. A.T. van der Ploeg, beste Ans. Als ik van iemand kan leren wat het is om hard te werken en voor patiënten door het vuur te gaan, dan ben jij het wel. Zonder jouw toewijding en energie zou het Centrum voor Ly-

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sosomale en Metabole Ziekten niet zo'n goed geoliede machine zijn zoals het nu is. Ik wil je bedanken dat ik deel uit mocht maken van het Pompe onderzoeksproject en ik hoop in de toekomst nog verder met je te kunnen samenwerken.

Dr. E. Brusse, beste Esther. Je bent zo'n beetje tegelijk met mij begonnen met je werkzaamheden binnen het Pompe team. Je hebt in korte tijd laten zien dat je een aanwinst voor het team. Het was voor mij erg fijn dat je copromotor werd, zodat sommige artikelen hierdoor sneller tot stand zijn gekomen dan dat zonder jou was gelukt. Bedankt en ik weet zeker dat ik nog veel van je zal leren tijdens mijn opleiding.

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Beste Yordi. Bedankt dat je mijn paranimf wil zijn. We kennen elkaar al vanaf de kleuterschool en hebben door de jaren heen nagenoeg dezelfde weg afgelegd qua opleiding. Wat begon met een veterstrikdiploma bij juffrouw Corrie op de Toverfluit, het krijgen van gele en rode kaarten bij juffrouw Corina op de Mariaschool, het zelf bier brouwen op het Markland College als afstudeeronderzoek, het afronden van de Geneeskunde opleiding in Rotterdam, maakt nu dat we ons 'bijna' medisch specialist mogen noemen. Ik denk dat we in de toekomst nog regelmatig onder het genot van een biertje anekdotes uit het verleden zullen aanhalen en wie weet eindigen we nog in hetzelfde ziekenhuis, jij als longarts en ik als neuroloog.

Beste Ralph. Ik ben trots dat je als grote broer paranimf wil zijn. We hebben totaal verschillende richtingen gekozen in ons werk, maar toch zijn er veel overeenkomsten. Hard werken, studeren in de avonden en weekenden (soms tot ongenoegen van onze vrouwen) en klaarstaan voor andere mensen. Hierdoor kunnen we af en toe ook lekker klagen over hoe druk het is en dat het soms zoeken is naar tijd voor leuke dingen met de kinderen. Hoe dan ook, het wekelijks avondje squashen houden we erin, want het is toch

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ABOUT THE AUTHOR

Stephan Wens was born on February 12th, 1984 in Breda, the Netherlands. He graduated at the Markland College in Oudenbosch in 2002. The same year he started studying Medicine at the Erasmus University Medical Center in Rotterdam and obtained his medical degree in 2008. From 2008 to 2009 he worked as a medical docter at the department of Neurology at the Amphia hospital in Breda. In April 2010 he started working as a resident at the Neurology department of the Erasmus University Medical Center in Rotterdam (head: Prof.dr. P.A.E. Sillevis Smitt). In November 2010 he started to work on his PhD thesis about Pompe disease at the Center for Lysosomal and Metabolic Diseases at the Erasmus University Medical Center in Rotterdam under the supervision of Prof.dr. P.A. van Doorn and Prof.dr. A.T. van der Ploeg. From November 2013 he is continuing his residency in Neurology at the Erasmus University Medical Center in Rotterdam, while finishing his PhD thesis. He married in 2011 with Bianca Wens-Roovers and they live in Oudenbosch, the Netherlands. They are the proud parents of Indy and Liam.

Stephan Wens
Oudenbosch, April 2016

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Wens SC, Kroos MA, de Vries JM, Hoogeveen-Westerveld M, Wijgerde MG, van Doorn PA, van der Ploeg AT, Reuser AJ. Remarkably low fibroblast acid α-glucosidase activity in three adults with Pompe disease. *Mol Genet Metab 2012; 107(3): 485-489.*

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Mogalle K, Perez-Rovira A, Ciet P, **Wens SC**, van Doorn PA, Tiddens HAWM, van der Ploeg AT, de Bruijne M. Automatic quantification of diaphragm mechanics in Pompe disease using dynamic 3D MRI. *Submitted.*

Güngor D, Kruijshaar ME, Plug I, Rizopoulos D, Kanters TA, **Wens SC,** Reuser AJ, van Doorn PA, van der Ploeg AT. Quality of life and participation in daily life activities of adults with Pompe disease receiving enzyme replacement therapy: 10 years of international follow-up. *J Inherit Metab Dis 2015;39(2):253-60*

Other publications

Klimek M, Hol JW, **Wens SC**, Heijmans-Antonissen C, Niehof S, Vincent AJ, Klein J, Zijlstra FJ. Inflammatory profile of awake function-controlled craniotomy and craniotomy under general anesthesia. *Mediators Inflamm 2009.*

SCA Wens en EACM Sanders. Twee patiënten met pijn, koorts en een progressieve tetraparese. *Tijdschr Neurol Neurochir 2010;111:245-51.*
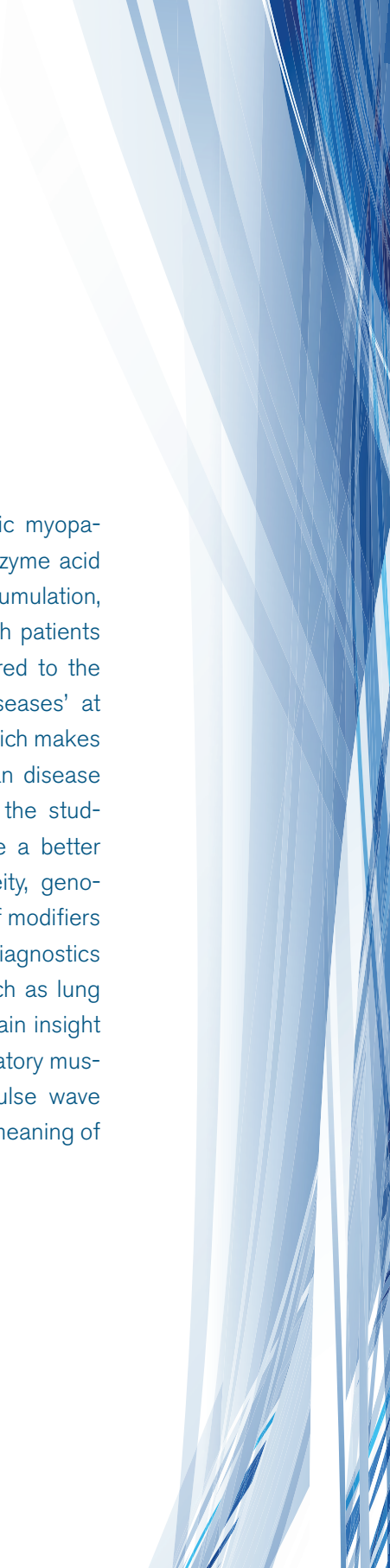
PHD PORTFOLIO

General courses	Year	Workload (ECTS)
- Short introduction course on statistics and survival analysis	2010	0.4
- Biostatistical methods II: Classical regression models	2010	4.3
- Basiscursus Regelgeving Klinisch Onderzoek (BROK)	2011	0.9
- Basic introduction course on SPSS	2011	0.6
- Biostatistical methods I: Basis principles	2011	5.7
- Principles of research in medicine	2011	0.7
- Biostatistics for clinicians	2011	0.7
- Biomedical English Writing and Communication	2012	3.0
- Repeated measurements	2013	1.4
Presentations	**Year**	**Workload (ECTS)**
- 16th Symposium of the World Muscle Society, Almancil, Portugal (poster presentation)	2011	0.5
- 5th Symposium Steps Forward in Pompe disease, Budapest, Hungary (oral and poster presentation)	2011	1.5
- Wetenschappelijke vergadering Nederlands Vereniging voor Neurologie, Nunspeet, the Netherlands (poster presentation)	2011-13	1.0
- 6th Symposium Steps Forward in Pompe disease, Berlin, Germany (poster presentation)	2012	0.5
- Research conference on Pompe disease, Boston, USA (oral presentation)	2012	1.0
- 10th and 11th Pompe disease expert day, Rotterdam, the Netherlands (oral presentation)	2012	2.0
- Research conference on Pompe disease, Rotterdam, the Netherlands (oral presentation)	2013	1.0
- Symposium Iberia LATAM Pompe disease, Madrid, Spain (oral presentation)	2013	1.0
Teaching	**Year**	**Workload (ECTS)**
- Lecture about neuromuscular imaging, Rotterdam, the Netherlands	2011	1.0
- Lecture about Pompe disease for medical students, Rotterdam, the Netherlands	2011	1.0
- Supervising Master's theses E. Kuperus, Rotterdam, the Netherlands	2012	6.0
- Supervising Master's theses A. Oskam, Rotterdam, the Netherlands	2012-13	6.0
- Lecture about Pompe disease for pathologists, Utrecht, the Netherlands	2013	1.0
- Lecture about Pompe disease for neurologists, Rotterdam, the Netherlands	2015	1.0
Other	**Year**	**Workload (ECTS)**
Study Coordinator EMBASSY trial, Rotterdam, the Netherlands	2011-13	6.0

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ABBREVIATIONS

4-MU	4-methylumbelliferyl-α-D-glucopyranoside
6MWT	Six-Minute Walk Test
ΔFVC	Postural drop in forced vital capacity from sitting to supine position
AAV	Adeno-associated viruses
ACTN3	Alpha-actinin-3
ACE	Angiotensin-converting enzyme
ACS	Acute coronary syndrome
AIMS	Alberta infant motor scale
Aix	Augmentation index
ALS	Amyotrophic lateral sclerosis
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
ATr	Aortic reflexion time
CI	Confidence interval
CfPWV	Carotid-femoral pulse wave velocity
CK	Creatine kinase
CK-MB	Creatine kinase myocardial band
CNS	Central nerve system
CT	Computed tomography
cTnT	Cardiac Troponin T
cTnI	Cardiac Troponin I
DBP	Diastolic blood pressure
DD	Deletion/deletion
DNA	Deoxyribonucleic acid
ECG	Electrocardiography
EMA	European medicines agency
EPOC	European Pompe Center
ERT	Enzyme replacement therapy
FDA	Food and drug administration
FEV_1	Forced expiratory volume in one second
FVC	Forced vital capacity
GAA	Gene encoding for acid α-glucosidase

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Gly + ACAB	Glycogen + acarbose
HE	Hematoxylin and eosin staining
HHD	Hand-held dynamometry
HSCT	Hematopoietic stem-cell transplantation
ICF	International classification of functioning, disability and health
IGF-2	Insulin growth factor 2
IMT	Intima media thickness
iPS	Induced pluripotent stem cell
IQR	Inter quartile range
ISNO	Interuniversitair Steunpunt Neuromusculair Onderzoek
IVS1	Common splice-site mutation in Pompe disease (c.-32-13T>G)
kDa	Kilodalton
kPa	Kilopascal
LVH	Left ventricular hypertrophy
LVMI	Left ventricular mass index
MAP	Mean arterial blood pressure
MCS	Mental component summary
MIP	Maximum static inspiratory pressure
MEP	Maximum static expiratory pressure
MRC	Medical research council grading scale
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
MU	4-methylumbelliferone
NA	Not applicable
OMIM	Online Mendelian Inheritance in Man
PAS	Periodic acid-Schiff staining
PCS	Physical component summary
PDFF	Proton-density fat-fraction
PFT	Pulmonary function testing
PPc	Central pulse pressure
PWA	Pulse wave analysis
QMFT	Quantitative motor function test
RhGAA	Recombinant human acid α-glucosidase

SBP	Systolic blood pressure
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TE	Echo time
TR	Repetition time
VO2max	Maximum oxygen uptake
WGM	Walter Gardner scal
Wmax	Maximal workload

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Pompe disease is a progressive metabolic myopathy. It is caused by a deficiency of the enzyme acid α-glucosidase and leads to glycogen accumulation, predominantly in skeletal muscle. All Dutch patients diagnosed with Pompe disease are referred to the 'Center of Lysosomal and Metabolic Diseases' at Erasmus MC University Medical Center, which makes it possible to study features of this orphan disease in a relatively large cohort. The aims of the studies described in this thesis were to have a better understanding of the clinical heterogeneity, genotype-phenotype correlations and the role of modifiers in Pompe disease. In addition, laboratory diagnostics and imaging techniques were studied, such as lung MRI in combination with spirometry to obtain insight into the function and strength of the respiratory muscles, tonometry of the carotid-femoral pulse wave velocity to study arterial stiffness, and the meaning of increased plasma cardiac TnT levels.