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EXERCISE PHYSIOLOGY APPLIED TO THE EVALUATION OF EXERCISE TOLERANCE IN PATIENTS WITH LATE-ONSET POMPE DISEASE

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ABSTRACT

Pompe disease is a rare inherited metabolic disorder due to the deficiency of the lysosomal enzyme alfa glucosidase, with consequent abnormal glycogen accumulation in muscle fibres. The late-onset form (LOPD) is characterised by a progressive motor and respiratory impairment. The specific enzyme replacement therapy (ERT), has been proved to stabilise muscle functions, but its effect after many years of treatment seems to be limited, especially for those patients who are already compromised at baseline. Exercise intolerance is one of the first clinical hallmarks affecting LOPD patients' quality of life. Functional evaluation of exercise tolerance is a recently tested method to assess clinical LOPD outcome. Before the introduction of ERT, the positive effects of diet and exercise training had already been demonstrated in LOPD, but the effect of these interventions on patients treated with ERT is still debated and poorly known.

This thesis is articulated into 3 studies, the first investigate the long term (more than 5 years) effects of ERT on a cohort of patients with the juvenile form of LOPD. The second investigates the acute effects of ERT on the main variables linked to exercise tolerance, dividing patients by clinical severity and level of habitual physical activity, and comparing results obtained from exercise tolerance test with those from standard six minutes walking test (6MWT). The third study is still ongoing, and investigates if a specific program of exercise training, alone or combined to hyperproteic diet, can ameliorate exercise tolerance in LOPD patients chronically treated with ERT.

For patients' clinical evaluation the following parameters were mainly used: serum muscle enzymes (AST, ALT, CPK, LDH), muscle strength (by dynamometer), motor function (6MWT, Walton Scale), neurologic functional tests, pulmonary function tests (standard spirometry, Maximal Inspiratory Pressure, Maximal Expiratory Pressure).

The functional evaluation of exercise tolerance was conducted on a cycle-ergometer and a specific exercise protocol adapted to patients was developed. The main variable of exercise tolerance assessed were: maximal aerobic power; gas exchange threshold; kinetics of pulmonary gas exchange during constant work-rate exercises; skeletal muscle fractional O2 extraction by near-infrared spectroscopy; heart rate, pulmonary ventilation and gas exchange ratio during constant work-rate exercise; rates of perceived exertion; time to exhaustion.

The results of these studies demonstrate: 1. Patients with the juvenile form of Pompe disease show a stabilization of motor and respiratory functions after 5 years of regular treatment with ERT. Patients

who started therapy in an asymptomatic phase were still free of symptoms after 6 years of follow up, suggesting that ERT is more efficient if started at early stages of the disease. 2. The exercise tolerance test is not acutely influenced by ERT, its main utility in clinical practice is in the long term follow up of patients, in which it seems to be more sensible than the 6MWT. The habitual physical activity seems to improve the exercise tolerance, regardless the disease severity. 3. Aerobic exercise training may ameliorate skeletal muscle force, maximal aerobic capacity and peripheral oxygen extraction in LOPD patients on chronic ERT treatment.

GENERAL INTRODUCTION

POMPE DISEASE

Pompe disease, or glycogen storage disease type II, is an inherited metabolic disorder due to the deficiency of the enzyme acid alpha glucosidase (GAA), which normally breaks down the glycogen inside the lysosomes of various cellular types. Since the GAA is the only way for glycogen degradation inside the lysosomes, a GAA deficiency brings to glycogen accumulation inside these organelles, especially in muscle fibers of skeletal, respiratory and cardiac muscles. The consequence is a progressive invalidating disease [*HirschHorn R et al 2001*].

In clinical practice Pompe disease has been differentiated in two main subtypes: the classic infantile form, rapidly progressive with cardiac involvement, and the late-onset form, more slowly progressive and free from cardiac involvement. But patients can present a wide variability in the clinical spectrum, between these extremes [*van den Hout HM et al 2003; Hagemans ML et al 2005*].

The disease was first described in 1932 by dr J.C. Pompe, who reported a clinical case of a 7 years old patient with progressive muscle weakness and hypertrophic cardiomyopathy, with evidence of massive glycogen accumulation inside the skeletal muscle cells and the cardiac tissue [*Pompe JC 1932*].

Pompe disease is considered a rare disorder, with an estimated incidence varying from 1 in 14.000 to 1 in 300.000 live births, depending on the form, the geographic location and ethnicity. In the caucasian population the estimated incidence is 1/138.000 for the infantile form and 1/57.000 for the late onset form [*Ausems MG et al 1999*].

Molecular genetics

Pompe disease is a genetic disease, inherited in an autosomal recessive manner.

The gene encoding for the GAA (*GAA gene*) is located in the long arm of the 17 chromosome (17q25.2-q25.3). It contains 20 exons and 19 introns, with a total length of 28 Kb [*Tzall S et al 1991*].

The acid alpha glucosidase is a lysosomal enzyme, which normally catalyses the hydrolysis of the 1-4 and 1-6 links of the glucose molecules inside the glycogen, leading the release of the glucose subunits. This enzyme works at a pH between 4.0-5.0. The expression of the GAA is present in all body cells, but it is prevalent in muscle tissue, especially in the striated muscle [*Auricchio F et al* 1968].

The cDNA of the GAA codify for a precursor peptide of 952 amino acids, with a molecular mass of 105.3 kDa, which underwent important structural post-translational modifications. First, in the reticular endothelium, the precursor is glycosylated with high mannose olygosaccharides, subsequently, in the Golgi apparatus, there is a phosphorylation of the mannose residues forming 6 phosphate mannose (M6P). In that way there is the formation of an inactive precursor of 110 kDa. Then there are two proteolysis at the amino-terminal end, finally forming a protein of 70 and 76 kDa, which is the active form of the enzyme (with an affinity for glycogen increased of 10 times compared to the precursor). The final protein leaves the Golgi complex in a vesicle which delivers its content to early/late endosomes and lysosomes, utilizing the M6P receptor. Once inside the late endosomes, the receptor-ligand complexes dissociate due to the low pH in these vesicles, and the enzyme is delivered to the lysosome, whereas the receptors is recycled back [*Wisselaar Ha et al 1993; Kornfeld et al 1992*].

Now a day, around 300 mutations and 80 polymorphisms have been identified in the GAA gene, the majority are point and private mutations [*http://www.pompecenter.nl*].

In patients with the classical infantile form, there is a wide mutation spectrum, all severe. In this group of patients, the most frequent mutation is a deletion: c.525delT, with an allele frequency of 12% in the Italian population. This mutation causes the deletion of a pair of bases in the exon 2, resulting in a premature stop codon.

The most frequent mutation founded in the late-onset form involves the first intron of the GAA gene: c.-32-13T>G. This is a splicing mutation causing a defecting elimination of the exon 2. Since a certain amount of correct mRNA is still produced, the residual GAA activity leads to a clinical

moderate phenotype (thus, this mutation is not present in the classical infantile form) [*Huie Ml et al 1994*]. In a study performed in an Italian cohort of 40 patients with late-onset Pompe disease, the c.-32-13T > G was confirmed as the most frequent mutation, present as compound heterozygote in 85% of the patients, with an allele frequency of 42.3%. The c.-32-13T > G was associated with the c.2237G > A (p.W746X) in nine of the 40 patients [*Montalvo AL et al 2006*].

Although some genotype/phenotype correlations have been described in Pompe disease, it must be pointed out that clinical presentation could be very different even among patients of the same family [*Montalvo AL et al 2006*].

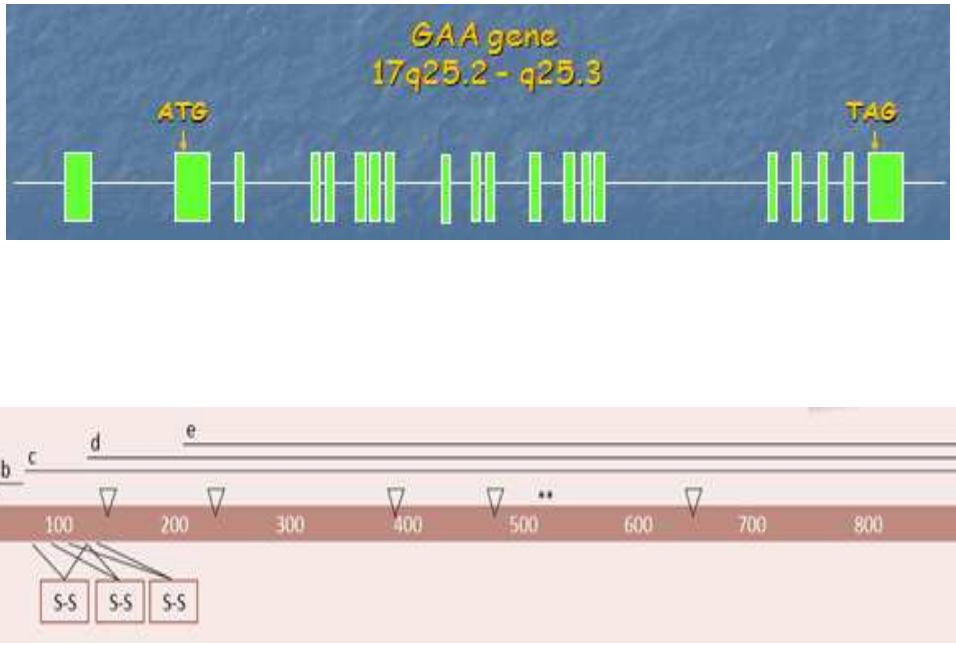

Figure 1. GAA gene and protein structure of GAA

Pathophysiology

In Pompe disease, the metabolic defect consist in the lack of function of the GAA enzyme, which works inside the lysosomes breaking down the lysosomal glycogen. This enzymatic defect leads to lysosomal glycogen accumulation in cells. Nevertheless, the glycogen accumulation is not the only mechanism of cell damage in Pompe disease, recent studies evidenced the key role of different pathways such as oxidative stress, mithocondrial dysfunction, accumulation of toxic potentially undegradable material and, most importantly, aberrant authofagy, which destroy the normal architecture of muscle cells and deeply contribute to muscle damage.

Authophagy is the natural, regulated, destructive mechanism of the cell that disassembles unnecessary or dysfunctional components, allowing the orderly degradation and recycling of cellular components. Three authofagic pathways have been described: microautophagy (direct), chaperone mediated authophagy, and macroauthophagy, most relevant for Pompe disease, which supplies aminoacid and energy sources under starvation by self digestion of cellular component [*Huang J et al 2007*]. Studies performed on Pompe muscle biopsies with the electron microscopy evidenced pathological autophagic abnormalities, with increase in the number of authophagosomes indicating an upregulation of authophagy and defect in the authophagosome-lysosome fusion. Thus, in Pompe disease there is a profound disorder of the intracellular recycling system, which contribute to the muscle weakness and to the incomplete response to therapy [*Raben N et al 2007].*

Moreover, muscle biopsies of the majority of patients with different forms of Pompe disease showed lysosomes containing large irregurarly shaped autofluorence inclusions identify as lipofuscin. Gradual intralysosomal accumulation of lipofuscin is characteristic of cellular oxidative damage and aging. Since lysosome and authophagy are involved in mitochondrial degradation, the reduced degradative capacity of lysosomes due to the progressive deposition of lipofuscin in these organelles leads to a decrease in the authophagic turnover of damaged mithocondria, which results in increased production of oxygen reactive species, perpetuating the production of lipofuscin. Furthermore, intralysosomal accumulation of lipofuscin affects the trafficking of the newly sintetised lysosomal enzymes, down regulating more the already impaired degradating capacity of lysosomes, in a sort of “vicious circle” [*Lim JA et al 2014*].

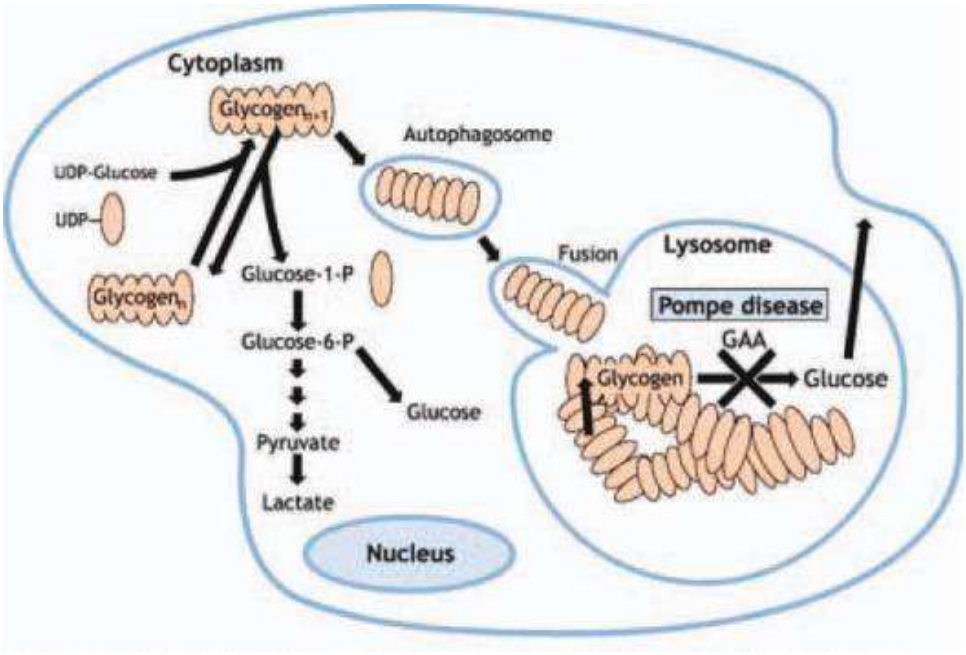

Figure 2. Primary metabolic defect in cells with Pompe disease. The lack of function of GAA brings glycogen accumulation in cells [from *Cupler EJ et al 2012*].

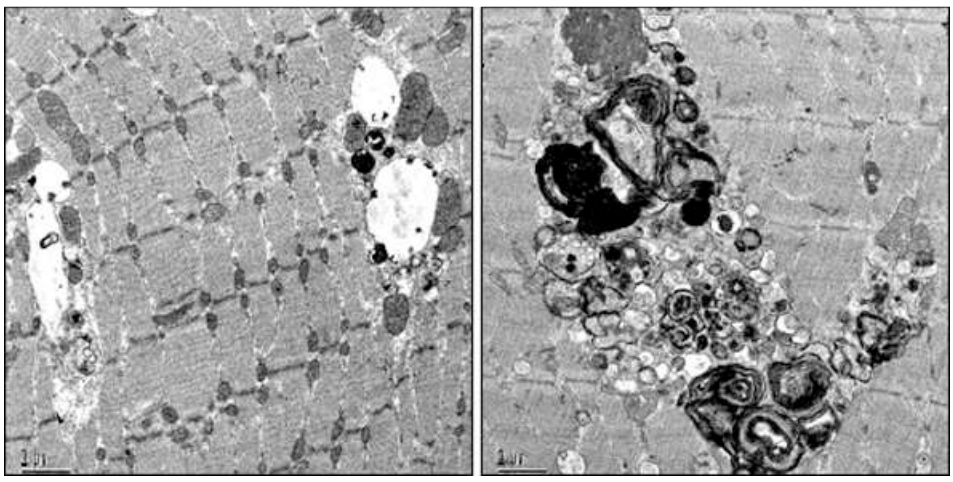

Figure 3. Electron microscopy of muscle fibers from a knockout mouse with Pompe disease evidencing the presence of autophagic buildup [from *Raben N et al 2007*].

Clinical Forms

Pompe disease presents as a spectrum, ranging from the severe classical infantile form to the milder late on-set form.

The infantile onset form can be divided in classical infantile and non classical infantile. The late onset form present after the 1 year of life and can be divided in juvenile form and adult form, depending on the age at onset [*van der Ploeg AT et al 2008*].

The classical infantile form usually presents within the first months of life with severe hypotonia (floppy infant), respiratory insufficiency and cardiomegaly due to hypertrophic cardiomyopathy. Other clinical features can be hepatomegaly, enlarged tongue, and lack of the achievement of the major motor milestones. If untreated, it leads to death within the first year of life, consequently to cardiopulmonary failure or aspiration pneumonia.

The non classical infantile form present before 2 years of life, has no or mild cardiomyopathy and is characterized by progressive hypotonia and motor delay or regression, with variable outcome [*van den Hout HM et al 2003*].

The late onset forms are characterized by a slowly progressive disease, usually without cardiac involvement.

The juvenile forms develops in the 1^{st} or 2^{nd} decade of life and are characterized by predominant skeletal muscle dysfunction, with motor and respiratory impairment.

The adult forms develops in the 3^{rd} or 4^{th} decade and affect the trunk and proximal limb muscles, mimicking limb girdle muscle dystrophies. The diaphragm muscle is often involved, leading to a respiratory insufficiency.

In the natural history of the late onset forms the myopathy and respiratory insufficiency deteriorate gradually, and patients often become dependent on ventilator and wheelchair [*Hagemans ML et al 2005*].

The major cause of death in adults is respiratory insufficiency. Rarely death occurs after strokes related to intracranial aneurysms or artheriopathy do to the accumulation of glycogen in the vascular smooth-muscle cells [*Laforet et al 2008*].

Diagnosis

The diagnostic process may start from the clinical evaluation, or from a genetic test in case of familiar screening, or from the neonatal screening, where available.

Clinical Suspicious:

In the classical infantile form the clinical presentation is quite characteristic, and consist in early onset (first months of life) of severe hypotonia, with babies having the classical aspect of the floppy infant, respiratory distress, abnormal electrocardiogram (short PR interval, high QRS complex voltage and arrhythmias) and cardiac imaging (X ray of the thorax or echocardiography) showing marked cardiac hypertrophy. Further characteristics may be motor delay or regression, hepatomegaly, macroglossia, skeletal abnormalities such as scoliosis, joint restriction, osteopenia with fractures, coclear involvement with deafness, and cerebral involvement with delayed myelination, anterior horn neurons involvement and aneurisysms. The laboratory blood exams usually show increased muscle enzyme: creatine chinase (CK), aspartate aminotransferase (AST), alanine aminotranferase (ALT), lactate dehydrogenase [*Kishnani PS et al 2006*].

As regards the late onset forms the clinical manifestations can be subtle, therefore the diagnosis may be significantly delayed.

The signs that should bring a physician to the suspect of a juvenile form is a patient with an age between 2 and 18 years presenting with muscular weakness, or motor abnormalities, without a cardiac involvement, with possibly recurrent respiratory infections. The blood tests show an increase in CK (>2 fold), AST, ALT and LDH.

Increased CK may occasionally be found in juvenile patients, in the absence of other clinical manifestations. In that case the exams should be repeated 3 times, and if consistently high further diagnostic evaluation to investigate a possible underling Pompe disease should be performed.

The main clinical features of the adult forms are the consequences of the skeletal muscle involvement and of the respiratory muscle involvement and includes: proximal lower limbs and paraspinal muscle weekness, exercise intolerance, back pain, respiratory distress, sleep apnoea, exertional dyspnea, recurrent respiratory infections. Respiratory disturbances can cause headache and somnolence. In some cases the disease presents suddenly with a severe acute respiratory failure, in these cases the prognosis is usually poorer. Again the laboratory tests shows an increase in CK (>2 fold), AST, ALT and LDH [*Raben N et al 2002*].

As regards instrumental exams electromyography and nerve conduction studies are not specific but can help in the diagnosis, showing myophatic features.

When skeletal muscle involvement is prominent, muscle biopsy can be useful, showing acid phosphatase positive cytoplasmatic vacuoles and increased glycogen content during periodic acid Schiff stain. Nevertheless morphologic changes are variable and some patients may show a confounding normal biopsy. This can be explained by the fact that glycogen storage may be different in different fibers.

Thus, the diagnosis of Pompe disease should always be confirmed by enzymatic essays or molecular analysis.

The differential diagnosis should include other muscular disorders such as muscular dystrophies, inflammatory myopathies, and other congenital storage disorders such as Danon disease, and glycogen storage disease type V [*Bembi B et al 2008*].

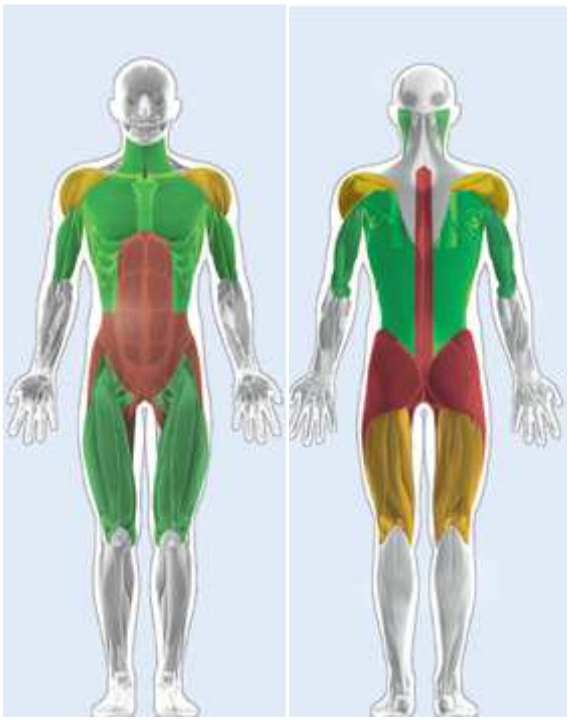

Figure 4. Muscle involvement in Late onset Pompe disease

Red: severe; yellow: moderate; green: mild; gray: null or minimal.

The enzyme assay

Enzyme GAA activity can be detected on different tissues: skeletal muscle (from muscle biopsy), skin fibroblast (from skin biopsy) or leucocytes (from peripheral blood samples). A pathological GAA level in any of these tissues is sufficient for the diagnosis of Pompe disease.

GAA enzyme activity is tested at an acid pH (3.7-4.4) using natural or synthetic substrates.

In the classic infantile form the GAA residual activity is usually undetectable (less than 1% of normal controls, in skin fibroblasts), while in the late onset forms it is usually reduced, but detectable (4-40% of normal controls, in skin fibroblasts) [*Raben N et al 2002*].

The molecular analysis

The analysis of the GAA gene is important to confirm the diagnosis of Pompe disease, especially in patients with inconclusive clinical and biochemical studies. The detection of the patient's specific mutation also allow a genetic test in eventual siblings and other close relatives. Furthermore it is important to perform molecular analysis because some genotype-phenotype correlation are known (see the molecular genetics above section) [*Kroos M et al 2008*].

Neonatal screening

In some country, but not in Italy, the neonatal screening for Pompe disease is recently became available. It consist in detecting enzyme assay on dried blood spot (DBS) on filter paper. If the results of the DBS indicates a severe reduction of GAA activity, the diagnosis should be confirmed by the gold standard tests (enzyme assay on leucocytes or fibroblast and genetic test). The aim of the neonatal screening is to early identify patients with the classical infantile form, to be able to start therapy as soon as possible. Unfortunately the neonatal screening deals with ethical issues, since it can identify also the late-onset forms, which can be asymptomatic until the second, four or six decade of life [*Umapathysivan K et al. 2001*].

Enzyme replacement therapy

The rationale for the enzyme replacement therapy (ERT) for lysosomal disorders, is to substitute the lacking endogenous enzyme injecting a recombinant enzyme which resemble the endogenous one, utilizing the ability of cells to internalize lysosomal enzymes through surface receptors.

The enzyme replacement therapy for Pompe disease consist in a recombinant human GAA produced from Chinese Hamster Ovary (CHO) cells (rhGAA; alglucosidase alpha, Myozyme®, Genzyme corporation, Framingham, MA). rhGAA is a protein of 110kDa, which is homologous to the endogenous GAA precursor, and contains M6P groups that enable the rhGAA to bind the M6P receptor on the cell surface and being internalized. Once inside the cells, the rhGAA, like the endogenous precursor, is clivated to the fully mature forms of 76 and 70 kDa.

The drug received broad label marketing approval in Europe in 2006, and lately in the US. This was the first specific approved therapy for Pompe disease, and is still the only commercially available treatment for Pompe disease worldwide.

The standard dosage approved for the drug is 20 mg/kg e.v. every two weeks, which is significantly higher compared to other ERT for other lysosomal storage disorders. The time of infusion is around 4 hours, if the drug is well tolerated.

Since the rhGAA is a protein, the main risk for the patient on treatment is to develop an immune reaction, which could be life-threatening especially for those patient with null endogenous GAA production (classic infantile forms called CRIM negative). Allergic reactions IgE mediated, ranging from mild urticaria to anaphylactic reaction, may also occur in the late-onset forms. Furthermore inhibitory IgG antibodies, which limits the efficacy of the drug, may be detected after many years of treatment. If the patient doesn't develop an antibody reaction against the recombinant protein, the side effects are usually mild and include fever, cough, nausea and abdominal pain. Even thought rhGAA is generally well tolerate, the ERT implies frequent intravenous infusions all lifelong, which can comprehensively compromise patient's quality of life [*Angelini et al 2012*].

As regards effectiveness of the drug, the initial assumption was that early treatment, initiated before irreversible compromising of muscle fibers, would reverse the glycogen accumulation and cure the disease. Unfortunately the clinical experience showed that while the cardiac muscle responded extremely well to therapy, the skeletal muscle did not. In fact both patients with the childhood and adult forms experience limited clinical benefit on the motor and respiratory symtomps, with partial functional improvement evidenced especially in the first years of treatment, and disease progression

thereafter [*Kishnani PS et al 2007*; *Strothotte S et al 2010*; *Bembi B et al 2010*]. The reason is probably in the poor uptake of the drug by the peripheral muscles, likely due to the low number of M6P receptors present on the plasma membrane of skeletal muscle cells.

Since the cardiac hypertrophy well responds to therapy, prolonging patients' survival, the indication is to start therapy as soon as possible in the classical infantile forms [*Nicolino M et al 2009*], while the timing of initiation of the therapy in the late onset forms is still debated.

The limitation of the current available therapy stimulate research on more effective treatments.

Follow up

A patient with Pompe disease should be followed up by a multidisciplinary team including a metabolic physician, a neurologist, a pneumologist, a physiotherapist and specialized nurses with experience in enzyme replacement therapy.

The regular follow up at the Regional Coordinator Center of Rare diseases of the Academic Hospital of Udine includes periodic visits (every 6 or 12 months, depending on the clinical severity of patients) with the following evaluations:

- general evaluation
- complete neurological evaluation
- blood exams, including blood count, renal and liver function, protein profile, glycemia, lipid profile and muscle enzymes: CK, LDH, AST and ALT
- electrocardiogram
- pulmonary function tests (spirometry with evaluation of the Forced Vital Capacity-FVC in sitting and supine positions, maximal inspiratory pressure -MIP, maximal exipiratory pressure MEP)
- 6 minutes walking test (6MWT)
- muscle imaging (every one or two years)
- DEXA body scan (every two years), to detect body composition

Other exams are performed depending on patient's clinical status, and include polisomnography to detect eventual nocturnal oxygen desaturations, and blood gas analysis (for those with respiratory insufficiency).

The 6MWT is a simple and reproducible test, which measures the distance a patient is able to walk over a total of 6 minutes on a flat surface. Patients are asked to walk as far as possible, but the individual is allowed to rest if needed. The principal parameters measured with the 6MWT are the total distance walked in meters, the peripheral oxygen saturation at baseline and end of the test, the heart rate, and the scoring at the BORG scale, which consist in two types of scores indicating, from 0 to 10, the subjective level of respiratory and muscle fatigue. The 6MWT is widely used in clinical practice to assess exercise capacity, since it does not require special equipments. Nevertheless this test has several limits, not providing information of function of the different organs and systems involved in exercise, or about its limiting factors [*ATS statement 2002*].

Muscle imaging usually consist in magnetic resonance (MRI) of the trunk and the thigh muscles. The imaging may evidence muscle abnormalities consisting in fatty degeneration and decreased muscle trophism, typically with a symmetric involvement. On MRI the fatty degeneration is seen as hyperintensity on T1-weighted images (figure 5) [*Bembi et al 2008*].

An extensive MRI study on patients with late onset Pompe disease have shown that there is a correlation between imaging findings and clinical severity. The adductor magnus, semimembranosus and semitendinosus muscles are involved in the initial stadium of the disease. Lately, the long head of the biceps femoris, the vastus intermedius, vastus medialis and, to a lesser degree, the vastus lateralis are involved with selective sparing of the short head of the biceps femoris, the sartorius, rectus, gracilis, and of the peripheral portion of the vastus lateralis.

Repeating MRI imaging every 1 or 2 years is therefore useful in evaluating disease progression and the treatment response.

MRI could also be useful in the initial differential diagnosis, in fact unless some feature of the MRI imaging in Pompe disease are in common with other myophaties, there are two patterns that are characteristic of Pompe disease: the selective sparing of the short head of the biceps, despite the severe involvement of the long head, and the relative sparing of the tensor fasciae latae, despite the severe involvement of the other pelvic muscles [*Pichiecchio A et al 2004*].

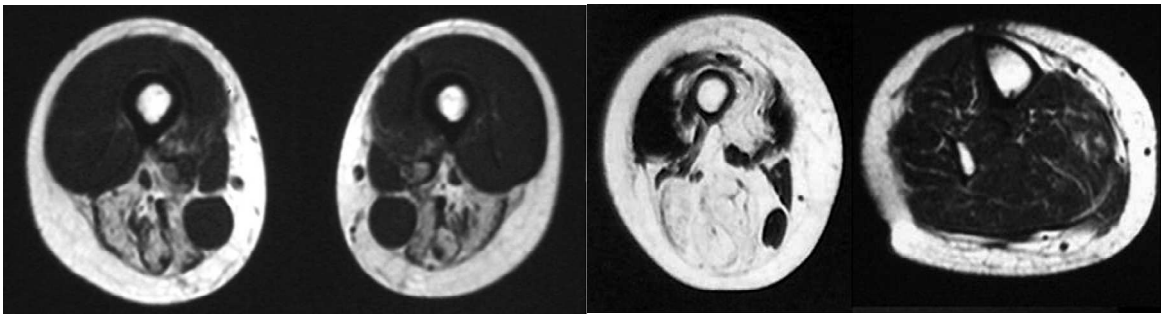

Figure 5. Magnetic resonance imaging of the thigh showing two stages of muscle fatty infiltration (moderate left, severe right), seen as hyperintensity on T1-weighted axial images, in two patients with late onset Pompe disease [from *Bembi et al. 2008*].

EXERCISE PHYSIOLOGY: PARAMETERS RELATED TO EXERCISE TOLERANCE

Aerobic exercises, such as running or cycling, require a strict integration of multiple systems of the body, including the respiratory, cardiovascular, and locomotor systems. During physical exercise, adequate interactions among these systems are required to transport an adequate amount of oxygen and nutrients to the exercising muscles as well as to remove the metabolically produced carbon dioxide (CO2) from the exercising muscles, to maintain homeostasis. Each of these systems plays an important role in exercise tolerance. The respiratory system is a ventilatory pump, moving oxygen from the atmosphere to the alveoli and carbon dioxide from the alveoli to the atmosphere. It must also provide an effective means of exchanging oxygen and carbon dioxide across the thin alveolar walls. The cardiovascular system is responsible for pumping oxygenated blood to the exercising muscles as well as returning oxygen-poor and carbon dioxide-rich blood to the gasexchanging surfaces of the lungs. The locomotor system, and particularly the muscles, must extract oxygen from the blood, generate adenosine triphosphate (ATP) in the mitochondria, and contract with force sufficient to support the intended activity. These three systems do not work independently, but in a highly coordinated manner. The most significant interdependence is the delivery of oxygen to the working muscles. The lungs must efficiently oxygenate blood returning from the venous system, and the left heart must then distribute this oxygenated blood to skeletal, cardiac, and respiratory muscles in proportion to the amount of work being done by the individual muscles. All of this coordination must occur in proportion to the amount of work being performed, whether it is mild, moderate, or extreme exercise.

Pathology in any of these important systems can lead to limitations in an individual's exercise tolerance. In patients with cardiomyopathy, for example, delivery of oxygen to the exercising muscles is insufficient to support mitochondrial ATP generation and, as a result, muscle contraction. Similarly, in patients with severe chronic obstructive pulmonary disease, altered respiratory system mechanics impair ventilation and the patient cannot eliminate CO2 being produced in the exercising muscles. As described above, patients with late onset Pompe disease may have both the respiratory and the locomotor system involved, with a double component for the exercise tolerance impairment.

VO2max

VO2max is the maximal capacity of the pulmonary and cardiovascular system to take up and transport oxygen to the exercising muscles and of the exercising muscles to extract and utilize oxygen from the blood during progressive exercise. It is one of the most useful parameters to assess an individual's capacity to perform sustained aerobic exercise, describing how much oxygen is being used by the tissues per minute.

VO2max is the product of the maximal cardiac output and the maximal arteriovenous oxygen difference (Fick equation):

$$\dot{Q} = \frac{\dot{V}O_2}{CaO_2 - C\bar{v}O_2},$$

where Q indicates the cardiac output, CaO2 indicates arterial oxygen content, and CvO2 indicates the mixed venous oxygen content. Rearranging this equation we see that:

$$\dot{V}O_2 = \dot{Q} \times (CaO_2 - C\bar{v}O_2)$$

This tells us that oxygen consumption is a function of cardiac output and the arteriovenous oxygen content difference.

Recall that:

$$CaO_2 = [(1.39 \times Hb \times SaO_2) + (0.003 \times PaO_2)]$$

And

$$C\bar{v}O_2 = [(1.39 \times Hb \times S\bar{v}O_2) + (0.003 \times P\bar{v}O_2)],$$

where Hb = hemoglobin concentration, PaO2 = partial pressure of oxygen in arterial blood, PvO2 = partial pressure of oxygen in mixed venous blood, SaO2 = arterial oxygen saturation, and SvO2 = mixed venous oxygen saturation. Oxygen consumption is therefore dependent on the hemoglobin concentration, the arterial partial pressure and saturation of oxygen (reflecting the adequacy of the ventilatory pump and gas exchange), and the mixed venous saturation and partial pressure of oxygen (reflecting the ability of the tissues to extract and utilize oxygen).

Therefore, VO2max provides information about many of the systems that are necessary to generate sustained, vigorous exercise; the higher the VO2max is, the more effective are all of these systems at performing their tasks and the greater is the person's exercise capacity. Maximum oxygen

consumption will vary from individual to individual. Whereas the VO2max for an average 30-year-old person might be 35–40 ml/kg/minute, an elite cyclist or cross-country skier might have a VO2max of 85 ml/kg/minute. Patients with a cardiomyopathy, on the other hand, may have a VO2max as low as 15 ml/kg/minute or less, severely limiting the capacity to perform normal activities of daily living. Maximum oxygen consumption declines with age, although that decline may be substantially delayed in physically active subjects.

Anaerobic threshold

During incremental exercise, ventilation increases to deliver oxygen to the alveoli and eliminate CO2. The anaerobic (or ventilator) threshold is the point in which the linear increase of ventilation in relation to workload changes its slope. The anaerobic threshold is related to the point at which anaerobic metabolism increases in exercising muscles to sustain work when aerobic metabolic capacity can no longer meet the physiologic demands, and the body shifts to anaerobic metabolism as an additional source of energy. The determination of the anaerobic threshold is a noninvasive, reliable, and reproducible diagnostic/prognostic marker that is based on ventilatory dynamics as exercise intensity progresses. In healthy untrained subjects the anaerobic threshold usually occurs at approximately 45% to 65% of peak VO2, and at a relatively lower percentage of peak VO2 among subjects with a reduced exercise capacity. The anaerobic threshold provides information at a submaximal level of exercise intensity, therefore it does not require a physiologically maximal exercise effort). Indeed, it is useful as a parameter on which to base an exercise prescription for patients with an maximal exercise performance, exercise intolerance.

O2 cost of exercise

In general, there is a linear relationship between increasing VO2 and the work rate (watts) achieved. The slope of this relationship reflects the ability of exercising muscle to extract O2 and to aerobically generate ATP. In general, a reduction (10 mL/min/w) throughout the exercise test or an acute flattening at a given point during exercise in the ΔVO2/ΔWR relationship suggests the possibility of a problem in O2 transport. General reductions may be seen in heart and lung disease, and disease in peripheral arterial function and/or mitochondrial myopathy, in which there are alterations in the cellular pathways involved in O2 utilization. Furthermore, a pattern of initial rise of the ΔVO2/ΔWR during exercise followed by abrupt flattening may reflect the onset of ischemia-induced left ventricular dysfunction in patients with coronary heart disease. Because consistent and accurate quantification of workload during treadmill testing is complicated by underlying variability during treadmill protocols (eg, body weight and handrail holding), assessment of the ΔVO2/ΔWR relationship is typically confined to exercise tests using a cycle ergometer.
[*Wasserman K et al 2005*].

VO2 Kinetics

The study of VO2 kinetics is the study of the dynamic VO2 response to exercise and its subsequent recovery. When starting a movement or a dynamic exercise, such as cycling or running, the energetic requirements of the contracting muscles increase immediately with the first contraction in what is called a 'square-wave' fashion. Regardless the term "squere-wave", the response demonstrates considerable inertia and, depending on the health or fitness of the individual and the exercise intensity, may take from 2 to 15 or more minutes to achieve the steady-state values.
In more details, at the onset of constant workrate exercise, there are 3 subsequent phases:

Phase I: an early rapid increase of VO2, that typically start within the first breath.

Phase II: a rapid exponential increase of VO2

Phase III: steady-state

Phase I represents the O2 exchange associated with the initial elevation of cardiac output, and thus pulmonary blood flow. Phase II reflects the arrival at the lung of venous blood draining the exercising muscles. Consequently, the pulmonary kinetics in Phase II widely reflects the kinetics of O2 consumption in the exercising muscles, although there is a temporal gap between events at the muscle and those recorded at the lung. For moderate intensity exercise, the onset of Phase III

corresponds to the point at which cardiac output plateaus and venous O2 content reach their nadir. At higher exercise intensities, the attainment of a steady state might be delayed or absent.

In the transition from rest, or unloaded exercise, to a work rate with a requirement below the anaerobic threshold, the vertical distance between the actual at a given moment and that required in the steady state represents the energy requirement that must be met from energy stores within the muscle. These stores consist principally of energy released through phosphocreatine hydrolysis and anaerobic glycolysis, with a small contribution from O2 stores (myoglobin, venous blood). The total O2 equivalent of that amount of energy is termed the O2 deficit. Thus, for a given VO2 the faster is the response, the smaller is the O2 deficit that will be incurred. In contrast, extremely unfit or unhealthy individuals will have a very slow response and will incur a high O2 deficit and thus a greater degree of intracellular perturbation (increased lactic acid, decreased phosphocreatine). Slow kinetics mandate a greater depletion of intramuscular phosphocreatine, and a greater rate of glycogenolysis leading to greater accumulation of lactate and protons and a greater utilization of the limited intramuscular glycogen reserves, all factors which predispose to a reduced exercise tolerance [*Grassi B et al 1996*].

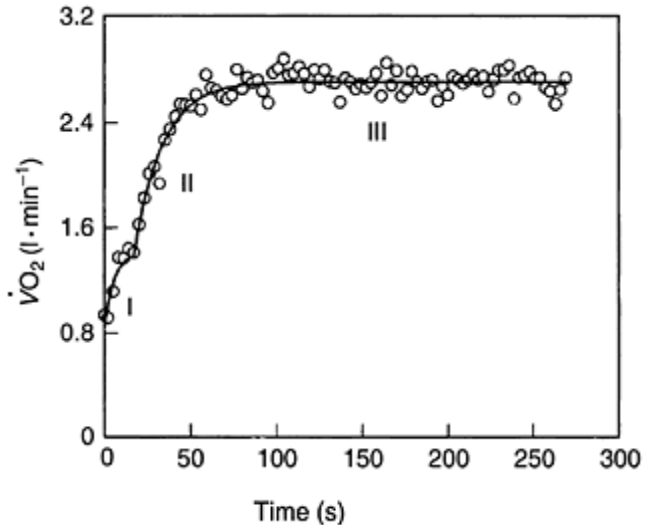

Figure 6. PhaseI, II and III of VO2 kinetic. Actual breath-by-breath alveolar VO2 response across the transient from unloaded cycling to a moderate work rate of a representative subject [data from *Grassi B et al 1996*].

Skeletal muscle fractional oxygen extraction

The fractional peripheral oxygen extraction indicates the balance between O2 delivery and muscle O2 consumption, and can be detected utilizing an instrument called Near Infrared Spectroscopy (NIRS). This is a non invasive toll used since 1977 in experiments investigating muscle oxidative metabolism in pathophysiology.

The physical principles of NIRS is based on the absorption of light NIR by hemoglobin in small arterioles, capillaries and venules. The instrument's light (700–1000 nm) penetrates skin, subcutaneous fat/skull, and underlying muscle, and is either absorbed or scattered within the tissue. Based on the Beer-Lambert Law, photons migrate successfully through tissue regions with the least absorbance. In the smallest vessels, minimal light absorption allows for multiple complete passage of photons along their path through tissue and therefore changes in chromophore concentrations can be detected from the light absorption. In contrast, light emitted into larger vessels (arteries and veins) is almost completely absorbed since the molar quantity of blood is so comparatively large. The degree of attenuation of NIR light in tissue is due to 3 factors:

1) O2-dependent absorption from chromophores of variable concentration (hemoglobin Hb, myoglobin Mb), and cytochrome oxidase;
2) absorption from chromophores of fixed concentration (skin melanine)
3) light scattering.

Thus, the oxygen saturation of the investigated tissue can be estimated.

The instrument provides separate measurements of changes in deoxygenated Hb and Mb concentrations, as well as oxygenated Hb and Mb concentrations. The fractional peripheral oxygen extraction can be calculated as concentration changes of deoxygenated Hb+Mb (Δ[deoxy(Hb+Mb)]). This parameter, with respect to an initial value arbitrary set equal to zero, is considered a peripheral oxygenation index since it is relatively intensive to changes in blood volume [*Grassi B et al 2007*].

Today, NIRS is extensively utilized to study circulatory and muscle metabolic pathologies in several situations. Distinct abnormalities in tissue oxygenation have been detected with NIRS in patients with peripheral vascular disease [*McCully KK et al 1994*]. Pronounced muscle O2 desaturation during exercise and delayed recovery after exercise have been observed in heart failure patients with impaired cardiac output and muscle blood flow [*Hanada A et al 2000*]. Moreover, abnormalities in muscle oxidative metabolism have been detected with NIRS in patients with metabolic myopathies such as Mc Ardle disease and mitochondrial disorders [*Grassi B et al 2007*].

Finally, in patients with Pompe disease oxygen changes in a superficial portion of the *vastus lateralis* muscle were evaluated during exercise, both before starting the enzyme replacement

therapy and after one year of treatment, demonstrating an increase in Δ[deoxy(Hb+Mb)] after therapy, index of a potential better peripheral oxygenation, however this data did not reach the statistical significance (but it must be considered that only 4 patients were included in this study) [*Marzorati et al 2012*].

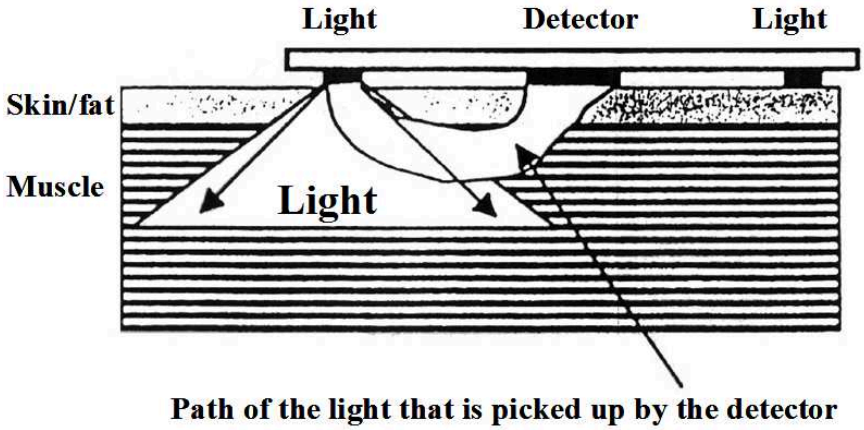

Figure 7. Physical functioning of NIRS. From *Mc Cully and Hamaoka 2000.*

AIM OF THE THESIS

My PhD work derives from a collaboration between the Regional Coordinator Centre for Rare Diseases of the Academic Hospital of Udine, and the laboratory of Exercise Physiology of the Department of Biomedical Sciences and Technologies of the University of Udine, and it is focused on late onset Pompe disease.

The Centre for Rare Diseases of Udine has the peculiarity of following a great number of patients with Pompe disease as for a single centre (22 patients), with a longtime experience with the enzyme replacement therapy (ERT) for this disease, as many patients, at present, have been treated for more than 5 years.
In literature, reports evaluating the long term (>3 years) effects of ERT in the late onset forms of Pompe disease (LOPD), and particularly in juvenile forms, are still limited.
As for clinical experience of the above Centre, there seems to be two categories of patients with LOPD: those who were diagnosed at very early stages of the disease, who started therapy having poor symptoms, who respond well to ERT, and those who were diagnosed at later stages, in which the therapy usually shows a positive effect within the first years of treatment, and then the disease may stabilize or even decline.

The laboratory of Human Physiology of Udine as an extensive experience in the functional evaluation of exercise tolerance in physiological and pathological conditions (*Grassi B et al 2007, Grassi B et al 2009, Salvadego D et al 2010, Salvadego D et al 2011*). Preliminary collaborative studies with the Neuromuscular Unit of the I.R.C.C.S Carlo Besta, conducted in a small number of patients with late onset Pompe disease (LOPD), have demonstrated the positive effect of ERT on exercise tolerance (*Marzorati et al 2012*).
Exercise functional tests were utilized in this thesis as a new toll to assess clinical outcome in LOPD patients.

Since ERT seems to have a limited effect in LOPD patients, new treatment strategies should be found in association to this therapy. Our proposal is that a personalized exercise training and a hyperproteic diet could improve clinical outcome in LOPD patients long term treated with ERT.

This thesis is articulated in 3 studies with the following objectives:

1) describe the clinical follow up of patients with the juvenile form of the disease long term treated with ERT
2) assess if ERT may have an acute effect on the main physiological and biochemical variables associated to exercise tolerance
3) evaluate if a specific program of exercise training, alone or combined to a specific diet, can ameliorate exercise tolerance and muscle function in LOPD patients long term treated with ERT.

STUDY I

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Enzyme replacement therapy in juvenile glycogenosis type II: a longitudinal study

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Abstract

Glycogenosis type II, a genetic muscle-wasting disorder, results in a spectrum of clinical phenotypes. Enzyme replacement therapy is effective in the infantile form of the disease, while little is known about its effectiveness in lateonset disease, especially in juvenile patients. The purpose of this retrospective cohort study was to assess the long-term effects of enzyme replacement therapy (ERT) in juvenile glycogenosis type II (GSDII). Eight Italian juvenile GSDII patients, receiving biweekly infusions of 20 mg/kg recombinant human α-glucosidase for at least 72 months, were enrolled (median age at therapy start was 11.8 years). Six-minute walk test (6MWT) and forced vital capacity (FVC), measured in upright position, were chosen as the principal outcome measures. Global motor disability (modified Walton scale (WS)), muscle enzymes levels [creatine phosphokinase (CK), lactate dehydrogenase (LDH), aspartate transaminase (AST), alanine transaminase (ALT)] and body mass index (BMI) were also analysed both at baseline (therapy start) and annually afterwards. At baseline, most patients (six out of eight) did not show muscle function impairment (WS≤2). The performance at 6MWT showed a slight improvement during follow-up as well as FVC. Muscle enzymes levels. showed a clear decrease after the 1st year of treatment while remained stable afterwards. An overall decrease in BMI was also observed during follow-up, although at the individual level, trends were variable. Conclusion: ERT is effective in stabilising

both motor and lung functions in juvenile patients with GSDII, possibly slowing down the rate of disease progression. Randomised controlled trials are needed to understand whether early treatment allows juvenile patients to reach adulthood with a more beneficial residual muscular function than untreated patients.

Keywords Enzyme replacement therapy . Glycogen storage disease type II . Pulmonary function . Exercise tolerance

Abbreviations

ERT Enzyme replacement therapy
GSDII Glycogenosis type II
GAA Acid α-glucosidase
LOTS Late-onset treatment study
FVC Forced vital capacity
6MWT Six-minute walk test
CK Creatine phosphokinase
Pt Patient

Introduction

Glycogenosis type II (GSDII; OMIM 232300) is a rare autosomal recessive myopathy caused by the deficiency of the lysosomal enzyme acid α-glucosidase (GAA), which is involved in glycogen metabolism [10, 14]. As a result, glycogen accumulates in lysosomes of several cell types, particularly in skeletal muscle fibres. The storage leads to a progressive disarrangement of tissue architecture and, consequently, to muscular atrophy and loss of function.

Diagnosis of GSDII is a two-step process, the first being the formulation of a clinical suspicion while the second the

measurement of GAA activity in peripheral blood cells, cultured fibroblasts or muscle biopsies [7]. If GAA activity is absent or deficient, diagnosis is confirmed. The molecular analysis of the GAA gene is also recommended, particularly

in patients whose clinical, morphologic and biochemical features are inconclusive [2, 7, 37], and plays an important role in genetic counselling. Moreover, it could be useful to predict the development of antibodies to exogenous enzyme, thus identifying children at risk for a poor response to enzyme replacement therapy (ERT) [6]. GSDII encompasses a broad spectrum of clinical phenotypes, according to age at onset, severity and organ involvement [7, 22]. Nevertheless, patients are usually classified as having the infantile (classic and non-classic forms) or the lateonset form(juvenile or adult). In the classical infantile disease, the main clinical features are cardiomyopathy and muscular hypotonia, rapidly leading to respiratory failure and death within the 1st year of life, if untreated [16, 21, 29], while the non-classic infantile form is characterised by a slower progressive cardiac and pulmonary involvement that generally leads to death during later infancy and early childhood [7, 24]. In late-onset GSDII the rate of disease progression is slower,

cardiomyopathy is usually absent, and the prevalent manifestations involve both skeletal and respiratory muscles, causing a progressive limb-girdle myopathy and a variable degree of respiratory impairment [12, 19, 36, 38]. Most of the untreated late-onset patients become wheelchair and ventilator dependent, and respiratory failure represents the leading cause of death [12, 13, 36]. Until the late 1990s, no specific therapies were available for patients affected by GSDII, other than supportive care [23]. In 2006, ERT using recombinant human GAAwas introduced and approved for all GSDII patients, on the basis of open-label studies showing improvements in survival, cardiac function

and motor outcomes in the classic infantile form [1, 15, 17, 30, 31]. So far, studies on the effectiveness of ERT in late-onset phenotypes [3, 8, 25, 27, 28, 33, 34] demonstrate positive but variable long-term effects. The late-onset treatment study (LOTS) [34] and the observational studies published by Angelini [3] and Strothotte [25] provided evidence on ERT clinical effectiveness in

improving functional exercise capacity and stabilising lung volumes over 18-, 12- and 36-month periods, respectively. However, much uncertainty still exists over the use of ERT in the paediatric clinical practice. In particular, the long-term effects on juvenile GSDII were not much studied so far [8, 28], and the correct timing for treatment start is still controversial. Focusing on juvenile GSDII patients, the purpose of this retrospective cohort study was to critically evaluate the long-term effects of ERT on muscle enzymes levels, motor performance and respiratory function over a long-term intravenous treatment (up to 7 years of follow-up).

Patients and methods

retrospective cohort study was conducted in 2013 on a group of eight Italian juvenile GSDII patients receiving biweekly

infusions of 20 mg/kg recombinant human α- glucosidase and followed-up at the Regional Coordinator Centre for Rare Diseases, University Hospital "Santa Maria della Misericordia", Udine. The study was approved by the local ethics committee, and patients were enrolled after informed consent was obtained from them or their parents. To be included in the study, patients needed to have a confirmed diagnosis of GSDII, defined as presenting with deficient GAA enzyme activity and/or GAA gene pathogenic mutations. GAA activity was measured in lymphocytes, cultured skin fibroblasts or muscle biopsies and was reported as a percentage of the activity recorded in healthy controls. A trained paediatrician reviewed all clinical documentations and gathered data on pretreatment demographic and clinical history (age at onset, age and disease duration at first infusion, use of ambulation devices, need for respiratory support). Information on height, weight, muscle enzymes [creatine phosphokinase (CK), lactate dehydrogenase (LDH), alanine transaminase (ALT), aspartate transaminase (AST)], sixminute walk test (6MWT), forced vital capacity (FVC) and global motor disability during follow-up was collected as well. All parameters were evaluated at baseline (T0) and annually afterwards. Body mass index (BMI) was calculated with the standard formula [Weight (in kilogrammes)]/[Height in metres)×Height (in metres)], and the corresponding Z scores were computed using age- and gender-specific paediatric

reference values [18]. Six-minute walk test was performed according to the American Thoracic Society guidelines [4]. Patients rated their dyspnoea and overall fatigue at baseline and at the end of the exercise using the Borg scale [9]. The maximum distance walked was recorded in metres, while Z scores were computed using paediatric age- and genderspecific reference values [11]. Pulmonary function tests were performed in the upright position, according to current guidelines [20]. Forced vital capacity was expressed as a percentage of the predicted value (FVC); the maximum value of

three reproducible measurements was used for analysis. Global motor disability was assessed through the modified Walton scale (WS; from 0=normal to 7=wheelchair bound) [23, 35]. Data were analysed, and graphics were plotted using the statistical package Stata (Stata Statistical Software: Release 11.0, 2009. StataCorp LP, College Station, TX, USA).

Results

Eight unrelated juvenile GSDII patients (five males and three females, born between 1991 and 1999) were enrolled in March 2013. Seven of them were already included in a study on late-onset GSDII published in 2010 [8]. Their characteristics at baseline (therapy start) are reported in Tables 1 and 2. In six patients, the first sign of disease was a CK increase, incidentally observed when a blood test was performed for different reasons (e.g. after detection of increased AST and ALT during a gastroenteritis), while the clinical onset was characterised by walking abnormalities for patient (Pt) 2 and by difficulty in climbing stairs for Pt 3. In all patients, the diagnosis was made within 1 year from clinical onset. The GAA enzyme activity at diagnosis ranged between 3 and 10.6 % of normal values. All patients showed the splicing mutation c-32-13 T>Gon one allele; differentmutations were found in the second allele in five patients, while the second mutation remained unknown in three. However, in two of them, GAA mRNA analysis showed that the second allele was not expressed, suggesting that the unknown allele may harbour an unidentified mutation in the non-coding regions of the GAA gene that prevent the formation of a stable mRNA. Most patients (n=6) started being treated shortly after the availability of ERT as a treatment for all GSDII forms, while two (Pts 6 and 7) started therapy after a couple of years. Overall, the age at therapy start ranged from 7.2 to 15 years. At therapy start, all patients showed a pathological elevation of CK (median 914 UI/L; range 366–1305), LDH (median 813 UI/L; range 520–1,561), ALT (median 120 UI/L; range 83–322) and AST (median 132 UI/L; range 79–283). Six patients did not show a significant muscle function impairment at baseline (WS scores of ≤2), while two (Pts 2 and 4) showed worse WS scores (2.5 and 7, respectively), one of them (Pt 2) already being wheelchair bound. The performance at 6MWT was variable, with a Z score ranging between −11.41 and 0.28. Finally, three patients had a decreased pulmonary function (restrictive spirometry pattern). Among them, patients 2 and 4 showed a severe impairment (FVC of <40 % predicted) and already required a ventilatory support. Data on a 6-year follow-up were available for all patients except one (Pt 6), who started therapy at the end of 2008 and whose length of follow-up was 4 years. This patient developed a brain tumour (pinealoblastoma) in 2012, and his follow-up visit was delayed due to surgery and hospitalisation. Patients 2 and 3 have been followed-up for 7 years. One patient

developed an allergic reaction (skin rash) following the second infusion. This led him to be pretreated with intravenous antihistamine drugs before the following infusions, without further problems. No adverse effects were reported in all the other patients throughout the entire follow-up period.

During the follow-up, muscle enzymes showed a sharp decrease after 1–2 years from therapy start and a stabilisation
afterwards (Fig. 1). However, CK levels decreased to normal values (expressed as inferior to 170 UI/L) only in one patient (Pt 7). Global motor disability (modified Walton scale) remained unchanged in all patients. When the performance at 6MWT was examined, two groups (Pts 2 and 4 vs others) were identified, according to disease severity. A slight improvement was clear in both, irrespective of their performance at therapy start (Fig. 2), although only in three patients that Z scores were normal at the end of follow-up. When the respiratory and muscular fatigue experienced after the test was analysed (Borg scale), an improvement was observed at T5 (5 years after ERT start) in five patients (Table 2). The same two groups could be identified when the FVC was studied as well. In both, a similar trend was seen, patients being quite stable in time, irrespective of their baseline pulmonary function (Table 2, Fig. 3). However, in Pts 2 and 4, a gradual reduction of ventilatory support was possible after therapy start. This led Pt 4 to stop non-invasive ventilation after 2 years, although starting it again 4 years later due to a moderate hypercapnia (pCO2 52 mmHg). Finally, BMI Z score remained negative or decreased to negative values in most patients (six out of eight) (Fig. 4).

Table 1 Characteristics of the study patients at therapy start

Patient	Birth year	Gender	Genotype	α-Glucosidase activity (%)	First signs or symptoms	Age at first signs or symptoms (years, months)	Age at diagnosis (years, months)	Age at therapy start (years, months)	Symptoms at therapy start	Scoliosis	Respiratory support	Wheelchair
1	1991	M	[c.-32-13 T>G; unknown (*n*=0)]	8.0	↑ CK incidentally detected	1 year and 6 months	2 years and 5 months	15 years	Myalgia after exercise	Yes	No	No
2	1993	M	[c.-32-13 T>G; c.2237G>A (p.W746X)]	3.8	Walking abnormalities	1 year and 11 months	1 year and 11 months	12 years and 7 months	Severe proximal hyposthenia, respiratory insufficiency	Yes	Yes	Yes
3	1993	M	[c.-32-13 T>G; unknown (*n*=0)]	10.6	Difficulty in climbing stairs	2 years	2 years and 2 months	12 years and 4 months	Mild proximal hyposthenia, easy fatigability	No	No	No
4	1994	F	[c.692+1G>C (*n*=0); c.1645G>C (p.G549R)]	3.3	↑ CK incidentally detected	4 years and 6 months	5 years and 2 months	11 years and 11 months	Respiratory failure after pneumonia, moderate proximal hyposthenia	Yes	Yes[a]	No
5	1995	M	[c.-32-13 T>G; c.2481+102_2646+31del (p.G828_N882del)]	4.8	↑ CK incidentally detected	2 years and 7 months	3 years	10 years and 4 months	Easy fatigability	No	No	No
6	1997	M	[c.-32-13 T>G; unknown]	5.0	↑ CK incidentally detected	9 years	9 years and 7 months	11 years and 7 months	None	No	No	No
7	1998	F	[c.-32-13 T>G; c.1465G>A (p.D489N)]	3.0	↑ CK incidentally detected	8 years	8 years and 11 months	9 years and 7 months	Myalgia after exercise	Yes	No	No
8	1999	F	[c.-32-13 T>G; c.307 T>G (p.C103G)]	7.1	↑ CK incidentally detected	6 months	1 year	7 years and 2 months	None	Yes	No	No

CK creatine kinase

[a] Invasive ventilation

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6	Patient 7	Patient 8
FVC %								
T0	113	27	138	14[a]	88	83	75	93
T5	102	24	119	30	87	91[b]	91	111
6-min walk test								
T0								
Metres	643	104[c]	617	15[a]	580	690	636	572
Z score	−0.73	−7.95	−1.08	−11.41	−1.51	0.28	−0.46	−0.02
T5								
Metres	713	353	821	510	830	782[b]	641	600
Z score	−0.21	−6.09	1.55	−3.12	1.77	1.13[b]	−0.43	−1.24
Borg scale								
T0								
Respiratory								
Pre	0	0	1	3[a]	1	0	0	1
Post	2	9	3	9[a]	3	0	3	3
Muscular								
Pre	0	0	1	2[a]	2	0	0	1
Post	3	9	5	9[a]	5	0	4	4
T5								
Respiratory								
Pre	0	0	1	0	0	0[b]	0	0
Post	0	4	3	1	0	0[b]	2	1
Muscular								
Pre	0	0	0	0	0	0[b]	0	1
Post	0	6	6	2	0	0[b]	1	3
Modified Walton scale								
T0	0	7	2	2.5	0	0	0	0
T5	0	7	2	2.5	0	0[b]	0	0

Table 2. Pulmonary function, exercise capacity and global motor disability at baseline (therapy start) and after 5 years of treatment.

FVC% forced vital capacity expressed as a percentage of the predicted value, *T0* ERT start, *T5* 5 years after ERT start.

[a] Tacheostomized at T0

[b] Four years after ERT start

[c] Six-minute walk test was stopped after 1 min and 30 s

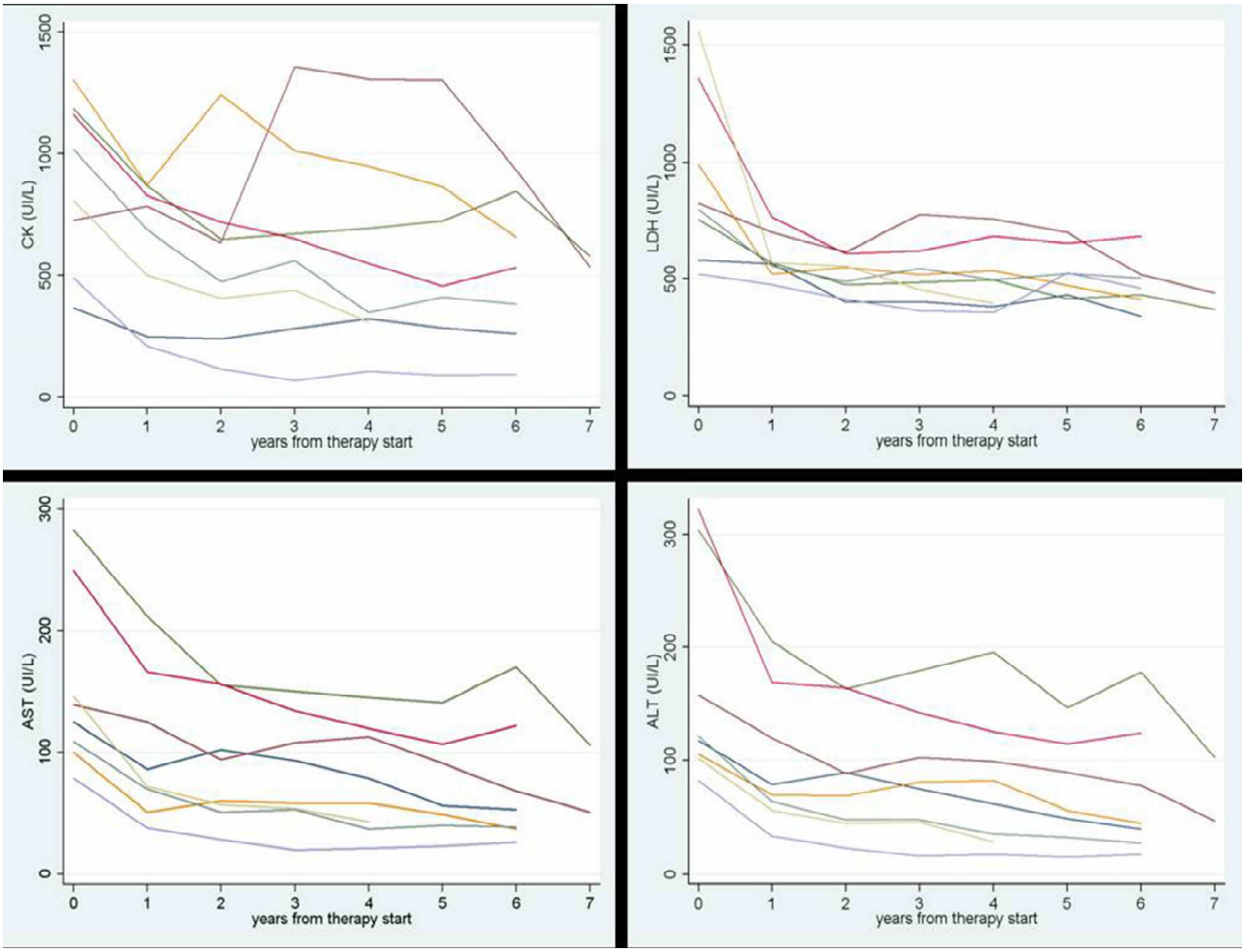

Fig. 1 Muscle enzymes levels from baseline (therapy start) to the end of follow-up. CK creatine phosphokinase, LDH lactate dehydrogenase, AST aspartate phosphatase, ALTalanine aminotransferase.

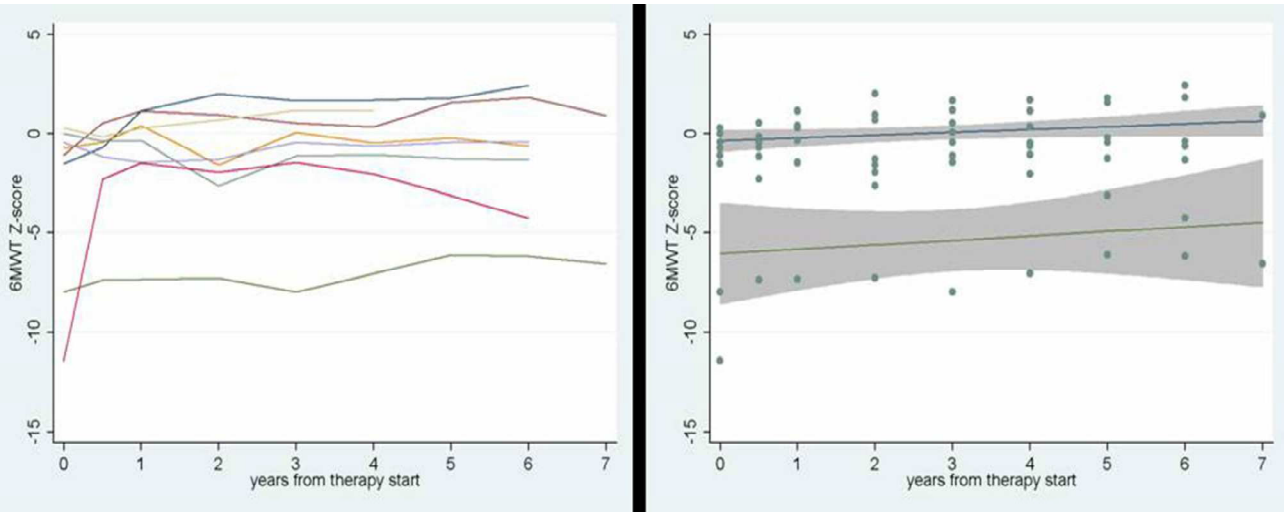

Fig. 2 Six-minute walk test (6MWT) Z score from baseline (therapy start) to the end of follow-up (individual curves and linear fitted values).

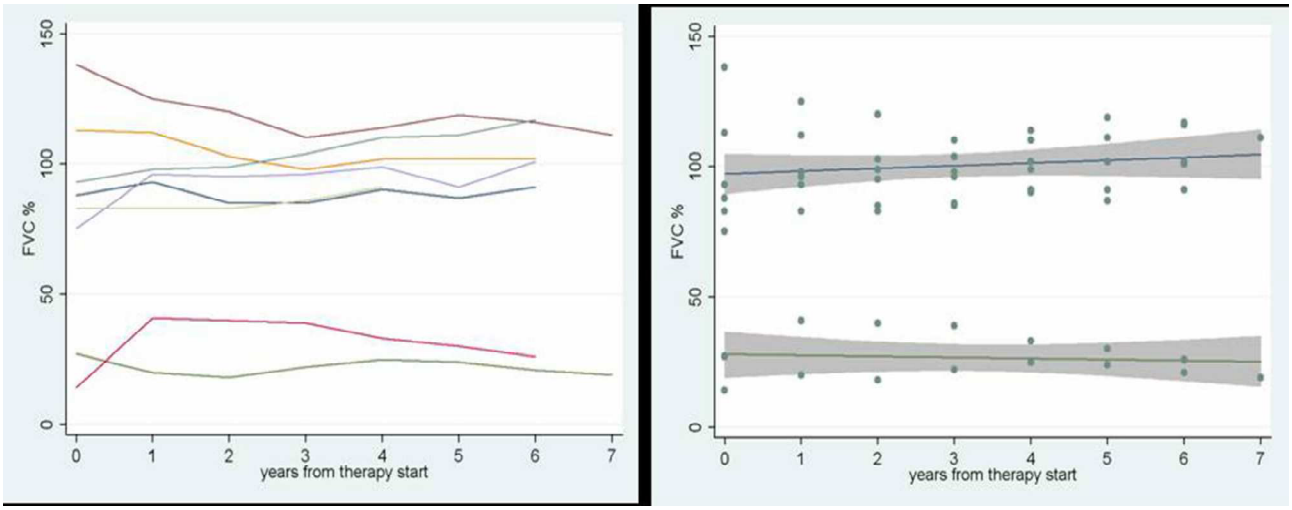

Fig. 3 Forced vital capacity (FVC) from baseline (therapy start) to the end of follow-up (individual curves and linear fitted values). FVC values are expressed as percentage predicted for height, age and gender using standard reference values.

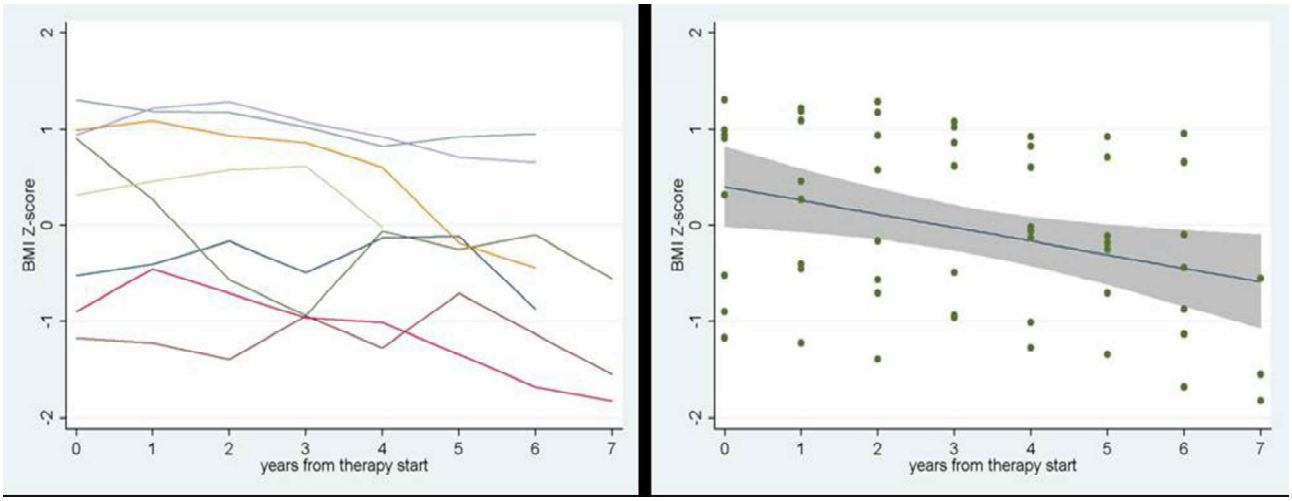

Fig. 4 Body mass index (BMI) Z score from baseline (therapy start) to the end of follow-up (individual curves and linear fitted values).

Discussion

It is well known that ERT is effective in infants with GSDII [1, 15, 17, 30, 31], while experience in late-onset disease is still limited, particularly in juvenile patients [8, 28]. However, due to the differences in the clinicalmanifestation of these forms, a diversified investigation seems to be worthwhile. This retrospective cohort study aimed to describe the longterm effects of ERT in eight Italian juvenile GSDII patients, starting therapy during childhood and being treated for at least 72 months. Although most patients did not show muscle function impairment before therapy start, muscle enzymes were pathologically high in all of them, and the introduction of ERT resulted in an initial decrease and a subsequent stabilisation. This trend is consistent with previously published studies [8, 25]. Moreover, the improvement in CK concentrations as an effect of ERT is important, in the light of the possible role of CK as a sensitive marker of muscle damage [5]. Performances at 6MWT, variable at therapy start, slightly improved in seven out of eight patients during the follow-up. In fact, patients were able to walk longer distances, and in several cases, the respiratory and muscular fatigue after the test was milder, irrespective of the distance walked. A strength worth to be mentioned is that the metres walked at the 6MWT were normalised for age and gender. This adjustment is particularly important when children are investigated in a longitudinal fashion, since it allows a comparison with peers of the same age and gender, in order to prevent misinterpreting stability or increase in the metres walked as stabilisation or improvement, when it could even represent worsening. The pulmonary function remained quite stable in time in all the patients, whether they were impaired at baseline or not. This apparent absence of effect should be read in the light of the results of previous studies on late-onset untreated patients, describing a worsening in lung function and exercise tolerance [32, 38] and, especially, a loss of predicted FVC, ranging between 1.6 % per year [32] and 2.2 % in 18 months [34]. Moreover, our results are consistent with the improvement of functional exercise capacity and stabilisation of lung volumes already described in previous studies [3, 8, 25]. However, Van der Ploeg et al. suggested that the effect on both motor and pulmonary functions is larger in

patients with higher residual muscular function at baseline, while in our patients, a similar trend is observed in all, irrespective of their baseline performance [34]. This may be explained by the difference between the two study groups, since Van der Ploeg described a cohort mainly represented by late-onset GSDII patients, mostly starting ERT over the third decade of life, while our patients received an early treatment during childhood and had, therefore, a shorter disease length. The BMI trends during the follow-up were variable at the individual level, but an overall

decrease could be observed, to be ascribed either to a weight loss or to a weight gain not appropriate for height. However, BMI—although adjusted for age and gender—does not allow to distinguish whether the muscle or fat tissues were lost; thus, opposite explanations can be suggested. First, patients with a better muscle function could practice more physical activity and, consequently, lose fat. On the other hand, patients finding exercise too tiring could practice less and, consequently, lose muscle. Therefore, it would be useful to evaluate body composition in GSDII patients, in order to understand the underlying mechanism of this decrease. In this study, unfortunately, body composition (as measured by dual X-ray absorbimetry total body scan) was not systematically assessed. In this paper, we describe five patients who started treatment during childhood, despite being asymptomatic. Interestingly, none of them developed muscle or respiratory symptoms after 6 years of follow-up, all showed a decrease in muscle enzymes, and one normalised. These data suggest that early ERT initiation may prevent or, at least, delay the onset of GSDII symptoms. However, we do not know how the natural history of these patients would have been, in the absence of treatment. Treating these patients before the clinical onset is not a widespread therapeutic decision, and the optimal timing for therapy start in juvenile GSDII patients is still a debated issue. However, since ERT seems to be more successful when administered prior to irreversible muscle damage [34], it is reasonable to consider that ERT should be started before the frank clinical onset of the disease. In fact, ERT seems to be more efficient on preserved than on damaged muscle, as suggested by the characterisation of pre- and post-ERT histopathology in infantile forms, provided by Thurberg et al. [26]. In their research, some features, such as low glycogen levels, mild ultrastructural damage, high proportion of type I fibres and young age at ERT start, were associated with a good histological response to ERT. Therefore, an early treatment could allow young patients to have a slower deterioration in their muscular function. However, the correct timing for therapy start in juvenile GSDII patients is still controversial, since no randomised controlled trials were performed on this subject, and only small observational retrospective studies are available in the literature, preventing from drawing strong conclusions. Our study itself suffers from these limitations, analysing data from only eight heterogeneous patients in a retrospective fashion. Therefore, there is the crucial need for appropriately conducted research able to compare the effectiveness of different timings for ERT start. These studies should be experimental, multi-centric and sufficiently large to achieve the power to detect clinically relevant differences.

In conclusion, our results suggest that ERT is effective in stabilising both motor and lung functions in juvenile patients with GSDII. However, well-conducted randomised controlled trials are

necessary to understand whether an early treatment allows juvenile patients to reach adulthood with a more beneficial residual muscular function than untreated patients.

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STUDY II

Investigation on acute effects of enzyme replacement therapy and influence of clinical severity and physical activity on physiological variables related to exercise tolerance in late onset Pompe patients.

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Abstract

Introduction: Late Onset Pompe disease (LOPD) is a progressive neuromuscular disorder in which impaired exercise tolerance is one of the clinical hallmarks. The specific enzyme replacement therapy (ERT) is administered to LOPD patients every two weeks. In clinical practice several LOPD patients refer a subjective improvement of exercise tolerance during the days immediately following the ERT infusion, and a gradual decrease during the 2 weeks interval between the infusions. In this study we investigated the acute effects of ERT on the main physiological variables associated with exercise tolerance in LOPD patients chronically treated with ERT. Furthermore we assessed whether the clinical severity of the disease and the habitual physical activity of patients would affect the investigated variables.

Patient and Methods: the day before (B) and the day after (A) ERT injection, 11 patients with LOPD treated with ERT for more than 2 years, performed on a cycle ergometer a costant work rate exercise followed by an incremental exercise to voluntary exhaustion. Oxygen uptake (VO2), heart rate, ratings of perceived exertion, peripheral fractional O_2 extraction and alpha glucosidase (GAA) activity on blood were determined in B and A.

The disease severity was characterized by neurological examination, Walton scale, 6 minutes walking test (6MWT), and pulmonary function tests.

Results: no significant difference in the main physiological variables related to exercise tolerance were found in A vs B, despite a significant increase in blood GAA activity. VO2 peak was significantly higher in patients with mild disease, while no differences in VO_2 peak were observed

between patients with only skeletal muscle impairment and patients with both skeletal and respiratory muscle impairment. VO2 peak were progressively lower as a function of decreasing habitual physical activity. Distance walked at 6MWT was significantly higher than VO2 peak expressed as percentage of normal values.

Conclusions: the exercise tolerance test is not acutely affected by ERT administration. The peripheral muscle component is prominent in determining the VO2 peak decrease, rather than the respiratory component in LOPD patients. The habitual physical activity improves the exercise tolerance, regardless the disease severity. The exercise tolerance test might be more sensible then the 6MWT in patients with mild LOPD.

Background

In total, twenty-two patients with late onset Pompe disease (LOPD) are regularly followed at the Regional Coordinator Centre for Rare Diseases of the Academic Medical Centre Hospital of Udine. Among them, 16 are long term treated with ERT (more than 2 years of therapy). Everyone receive regular ERT injection at a dosage of 20mg/kg i.v., every 2 weeks, as for standard therapy.

In clinical practice, many patients report a subjective improvement of general well-being and reduced fatigability during the days immediately following the ERT infusion, and a gradual loose of this positive effects during the two weeks interval between the infusions. This phenomenon could be attributed to acute effects of ERT on exercise tolerance.

Exercise intolerance is a key clinical manifestation of LOPD which can be caused by multiple factors including respiratory and peripheral muscle function alterations (*Muller Felber W et al 2007; Alejaldre A et al 2012).* As in many other pathological conditions (*Maltais et al.2014; Poole et al.2012; Salvadego et al.2010*), also in LOPD patients exercise intolerance may represent an important cause of the patients' decreased perception of general well-being. The incremental exercise tolerance test, up to voluntary exhaustion, is a validated method to evaluate exercise (in)tolerance in several physiological and pathological conditions among which metabolic myopathies (*Grassi B et al 2007; Grassi B et al. 2009*). In 2012 Marzorati et al. demonstrated the positive effect of ERT on some physiological variables associated with exercise tolerance in 4 patients with LOPD, after 1 year of therapy, and proposed this approach for the patients' clinical assessment in their follow up (*Marzorati M. et al* 2012). Subsequently a few other studies have utilized protocols of incremental exercise tests, with the determination of cardiovascular, pulmonary gas exchange and metabolic variables, to evaluate exercise (in)tolerance in LOPD patients (*Van den Berg L* et al 2015, *Crescimanno G et al* 2015). However, the 6 minutes walking test (6MWT) remains the most commonly utilized method to estimate the patients' exercise capacity in clinical practice and clinical trials (*Bembi B et al* 2010*; Anderson LJ et al* 2014).

Aims

The first aim of this study was to investigate the acute effects of ERT on the main physiological variables associated with exercise tolerance in LOPD patients chronically treated with ERT. The second aim was to assess whether the clinical severity of the disease would affect some of the investigated variables. The third aim was to assess whether the habitual physical activity of patients could influence the investigated variables.

Patients and methods

Eleven out of twenty-two patients with LOPD followed at the Regional Coordinator Centre for Rare Diseases of the Academic Medical Centre Hospital of Udine, Italy, were enrolled in this study. Inclusion criteria were: confirmed diagnosis by enzyme assay and/or genetic testing; chronic (at least 2 years) ERT at a standard dosage of 20 mg/kg every 2 weeks; willingness to participate to the study. Exclusion criteria were: wheelchair bound; severe cardiac disease (assessed by electrocardiogram and echocardiography) or respiratory assistance 24h/day. All patients recruited, or their parents when under 18 years of age, gave informed consent. The study was approved by the local institutional ethics committee. All procedures were in accordance with the recommendation found in Declaration of Helsinki (2000).

Clinical history was collected from clinical records. Before performing the study tests, patients were asked to indicate how they usually felt, in terms of general subjective well-being and fatigability the day before and the day after the ERT infusion. Habitual physical activity was evaluated by a questionnaire.

Full neurological examination, six minutes walking test (6MWT) and pulmonary function testing (PFT) in the sitting position were performed by utilizing standard methods within 3 days from the study. Distance walked at 6MWT and forced vital capacity (FVC) at PFT were calculated as a % of predicted values (*Enright PL et al 1998; Laslo G et al 2006*). Patients were classified as having a respiratory involvement if FVC was <90% and/or if they were treated with non-invasive nocturnal ventilation (NIV). Global motor disability was assessed by the modified Walton scale (WS) (*Slonim Ae et al 2006*).

At the study time, 24h before (BEFORE) and 24h after (AFTER) the ERT infusion, the patients underwent to blood samples collection, and then performed a modified exercise test (see below) to the limit of tolerance on a cycle ergometer. In all patients ERT was administered intravenously at the usual dosage and at the scheduled day (14 days after the previous injection).

GAA activity was measured in lymphocytes using the fluorogenic substrate 4-methylumbelliferyl-α-Dglucopyranoside (Glycosynth, Cheshire, England). Protein concentration of the samples was determined by the Lowry method. Enzymatic activity was expressed as nanomoles of substrate hydrolyzed per milligram of total protein per hour. All assays were done in triplicate.

Serum levels of muscle enzymes (creatine phosphokinase CK, lactate dehydrogenase LDH, alanine transaminase ALT, and aspartate transaminase AST) were measured by utilizing standard kits.

Exercise protocol

Exercise tolerance testing was performed at the Exercise Physiology Laboratory of the University of Udine. Experiments were conducted under medical supervision, and patients were continuously monitored by 12-lead electrocardiography (ECG). A mechanically braked cycle ergometer (Monark Ergomedic 839E) was utilized. Pedalling frequency was digitally displayed to the subjects, who were asked to keep a constant cadence throughout the test, between 60 and 70 rpm. Each patient had chosen his preferred cadence during practice trials, and this cadence was maintained during each repetition. Patients were allowed time to gain familiarity with the researchers and the experimental set-up, were carefully instructed about the procedures and were familiarized with the protocol using short practice runs before starting the study.

A specific functional evaluation test was utilized. The patients performed a low-intensity constant work rate (CWR) exercise for 8 minutes or until voluntary exhaustion, identified as the inability to maintain the desired cadence, despite verbal encouragement. In the patients who completed the CWR exercise, the test was immediately followed (no recovery period) by an incremental exercise up to voluntary exhaustion (Figure 1). During the CWR exercise work rate was selected according to the patient's physical fitness level, estimated during preliminary practice runs (22.8 ± 15.6 W). During the incremental exercise, work rate was increased by 5-10 W every minute.

Pulmonary ventilation ($\dot{V}E$), tidal volume (VT), respiratory frequency (fR), O_2 uptake ($\dot{V}O_2$) and CO_2 output ($\dot{V}CO_2$) were determined on a breath-by-breath basis by means of a metabolic unit (Quark b^2, Cosmed, Italy). Expiratory flow measurements were performed by a turbine flow meter calibrated before each experiment by a 3 L syringe at three different flow rates. Calibration of O_2 and CO_2 analysers was performed before each experiment by utilizing gas mixtures of known composition. The gas exchange ratio (R) was calculated as $\dot{V}CO_2/\dot{V}O_2$. Arterial Oxigen saturation (SAO2) was determined by pulse-oximetry.

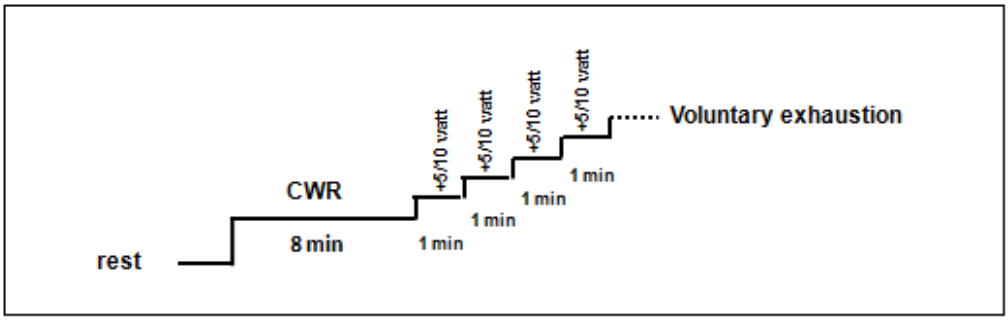


Figure 1 Exercise tolerance test protocol adapted to late onset Pompe patients.

Heart rate (HR) was determined by ECG. Ratings of perceived exertion (RPE) was obtained at rest and at every minute during exercise by using the Borg's modified CR10 scale (Borg 1998).

Considering that only one CWR bout could be carried out, a formal $\dot{V}O_2$ kinetics analysis was not performed (*Lamarra et al. 1987*). Mean $\dot{V}O_2$ values were calculated during the last 30 seconds of every minute of exercise. The O_2 cost of exercise was calculated during the last minute of the CWR exercise as $\Delta\dot{V}O_2 / \Delta$ work rate. One of the eleven subjects was only able to perform an unloaded pedalling and thus was excluded from the analysis of the O_2 cost. For all variables (see below), values determined at voluntary exhaustion were considered "peak" values.

Muscle oxygenation profiles of the *vastus lateralis* during exercise were evaluated by near-infrared spectroscopy (NIRS) (*Ferrari et al. 2011*). NIRS measurements in muscle tissue have been shown to be well correlated with local venous O_2 saturation (*Boushel et al., 2001; Wüst et al., 2014; Vogiatzis et al., 2014*). A portable NIR continuous-wave instrument (PortaMon, Artinis, The Netherlands) was utilized in this study (*Salvadego et al. 2011; Salvadego et al. 2013*). The instrument measures micromolar (μM) changes in oxygenated haemoglobin (Hb)+myoglobin (Mb) concentrations (Δ[oxy(Hb+Mb)]), and in deoxygenated [Hb + Mb] (Δ[deoxy(Hb+Mb)]), with respect to an initial value arbitrarily set equal to zero and obtained during the resting condition preceding the test. The sum of the two variables (Δ[total(Hb+Mb)]) is related to changes in the total Hb volume in the muscle region of interest (*Ferrari et al 2011*). In contracting muscle, Δ[deoxy(Hb+Mb)] is relatively insensitive to changes in blood volume and has been considered an estimate of skeletal muscle fractional O_2 extraction (ratio between O_2 consumption [$\dot{V}O_2$] and O_2 delivery [$\dot{Q}O_2$]) (*Ferreira et al., 2007; Grassi 2005; Kowalchuk et al., 2002).* A "physiological calibration" of Δ[deoxy(Hb+Mb)] values was performed by obtaining a transient ischemia of the limb after the exercise period: data obtained during exercise were expressed as a percentage of the values of maximal muscle deoxygenation obtained by pressure cuff inflation (at 300-350 mm Hg), carried out at the inguinal crease of the thigh for a few minutes, until Δ[deoxy(Hb+Mb)] increase reached a plateau.

Statistical analysis

Results were expressed as mean values ± standard deviation (x ± SD). The statistical significance of differences between means was checked by paired two-sided *Student's* t-test. The level of significance was set at $p<0.05$. Statistical analyses were carried out by utilizing commercially available software packages (Prism 5.0, GraphPad, USA; Statistical Package Social Sciences 15.0, SPSS Inc., USA).

Results

Eleven Caucasian patients with LOPD (6 females and 5 males; age 30.9 ± 15.1 years) participated to the study; their anthropometric and clinical characteristics are summarized in Table 1. Two patients (pt 2 and 8) were brothers, the others were unrelated.

Five out of eleven patients reported an acute improvement on their subjective wellbeing after ERT, one referred a worsening and 5/11 declared no difference.

Enzyme activity was significantly higher in AFTER vs BEFORE (Figure 2), whereas no differences were found between serum muscle enzymes levels in AFTER vs BEFORE: CK 446 ± 143 mmol/l vs 427 ± 211 mmol/l, LDH 474 ± 110 mmol/l vs 487 ± 122 mmol/l, AST 52 ± 32 U/l vs 51 ± 32 U/l and ALT 49 ± 37 U/l vs 47 ± 35 U/l.

Nine out of eleven patients were able to complete the 8-min CWR cycling test. Two patients (pt 5 and 9) reached exhaustion earlier (after 5 and 7 min, respectively). One patient (pt 1) completed the CWR test, but could not perform the incremental test. Therefore, 8 out of 11 patients were able to perform the incremental test both in BEFORE and in AFTER. Data were analyzed during the last minute of the CWR exercise and at voluntary exhaustion.

The exercise test was well tolerated, and no side effects occurred during and after the exercise.

Arterial oxygenation did not decrease below 96% in all patients.

All ventilatory, gas exchange, cardiovascular, and the VO2 cost of cycling did not differ in AFTER *vs* BEFORE (Table 2 and 3, Figure 3 and 4).

Data on peripheral fractional O2 extraction, obtained by NIRS did not differ in AFTER vs BEFORE both during constant work rate exercise and at peak (Figure 5)

Since no significant differences were observed in AFTER vs BEFORE, for further analyses only the data obtained in BEFORE were considered.

Comparing the main data obtained in our cohort from the 6MWT and from the exercise tolerance test, the walking distance reached during the 6MWT, expressed as percentage of predicted values resulted significantly higher than the $V'O_2$ peak (Figure 6).

Finally, data were further analysed dividing patients in three classes on the bases of the disease severity: 1. No *apparent symptoms*: Walton scale score 0, no respiratory involvement (N group: including pt 2,4,6,10,11); 2. *Skeletal muscle dysfunction*: Walton scale score ≥ 2, no respiratory involvement (M group: including pt 1,3,7); 3. *Skeletal and respiratory muscle dysfunctions*: Walton scale score ≥1 plus respiratory involvement (M+R group: including pt 5,8,9).

$V'O_2$ peak was then analysed with respect of the 3 groups of increasing disease severity, and the results were significantly lower in M and in M+R vs. N, whereas $V'O_2$ peak values in M and M+R

were comparable, both showing very low values (Figure 7), data expressed as a percentage of the values predicted for age- and sex-matched healthy normally active subjects (Wasserman et al., 1999).

Moreover, patients were divided in other 3 categories, depending on their level of habitual physical activity: 1. *None*: sedentary daily living (pt 1,5,7,8); 2. *Moderate:* active daily living (pt 3,4,6,9); 3. *High:* active daily living + regular exercise training. For 5 out of 11 patients there was no corrispondance between habitual physical activity level and degree of disease severity (Figure 8).

$V'O_2$ peak values, again expressed as a % of those predicted for age- and sex-matched healthy normally active controls, were progressively lower as a function of decreasing habitual physical activity (Figure 9).

The values of O_2 cost of cycling, obtained as $\Delta \dot{V} O_2/\Delta$ work rate during the CWR exercise, from the cohort of patients with LOPD evaluated in this study were markedly higher compared to values reported previously in normal subjects and in patient populations affected by other types of metabolic myopathies (mitochondrial disorders and Mc Ardle disease) (*Grassi et al 2009*) (Figure 10).

patient code	age years	gender	genotype c-/c-	height m	weight kg	BMI kg/cm2	years on ERT	WS	NIV	FVC %	subjective wellbeing
1	46	F	-32-13T>G/ 2481+102_2646+31del	1,64	50	18,6	2	3	no	108	better in A
2	47	M	-32-13T>G/ 2646_2646+1delTG	1,9	112	31,0	4	0	no	112	unchanged
3	19	M	-32-13T>G/ unknown	1,78	60	18,9	7	2	no	107	unchanged
4	17	M	-32-13T>G/ 2481+102_2646+31del	1,88	66	18,7	7	0	no	91	better in A
5	43	F	-32-13T>G/ 2237G>A	1,68	80	28,3	7	6	yes	64	better in A
6	21	M	-32-13T>G/ unknown	1,74	71	23,5	7	0	no	99	unchanged
7	43	F	N.A.	1,66	70	25,4	2	3	no	126	better in B
8	53	M	-3213T>G/ 2646_2646+1delTG	1,85	106	31,0	7	3	yes	55	better in A
9	20	F	692+1G>C/ 1645G>C	1,57	41	16,6	8	3	yes	22	better in A
10	15	F	-32-13T>G)/ 307T>G	1,6	59	23,0	8	0	no	106	unchanged
11	16	F	-32-13T>G)/ 1465G>A	1,62	57	21,7	7	0	no	102	unchanged
mean	30,9			1,7	70,2	23,3	6,0			90,2	
SD	15,1			0,1	21,9	5,1	2,2			30,7	

Table 1. Anthropometric and clinical characteristics of patients
BMI: body max index; ERT: enzyme replacement therapy; WS: Walton scale score; NIV: nocturnal non invasiv ventilation; FVC: forced vital capacity.
A: the day after the ERT injection, B: the day before the ERT injection

PARAMETER	before ERT	after ERT
	mean± SD	*mean ± SD*
WR watt	25 ± 12,8	25 ± 12,8
V'E L/min	26,2 ± 4,6	28,2 ± 4,5
fR breath/min	27,9 ± 10,9	27,2 ± 11,0
VT L/min	1,1 ± 0,4	1,2 ± 0,6
V'O2 ml/min	879,3 ± 195,5	907,8 ± 200,0
V'O2 ml/min/kg	13,3 ± 3,2	13,7 ± 3,4
V'CO2 ml/min	865,4 ± 163,7	900,7 ± 163,8
R	0,99 ± 0,08	1,00 ± 0.07
PETO2 mmHg	103,4 ± 4,9	104,1 ± 5,1
PETCO2 mmHg	38,6 ± 2,9	38,5 ± 2,5
HR beat/min	119,8 ± 21,8	122,2 ± 15,2
RPE	2,7 ± 2,7	2,9 ± 3,3
V'O2 cost ml/min/watt	22,0 ± 5,2	23,4 ± 6,4
Δ[deoxy(Hb+Mb)] (% of ischemia)	25,2 ± 15,4	28,1 ± 19,1

Table 2. Values of the main investigated paramenters during the contant-work rate exercise before and after ERT

Means ± SD of the main physiological variables determined during the last minute of the costant work rate cycle ergometer exercise. WR: work rate; V'E: pulmonary ventilation; fR: respiratory frequency; VT: tidal volume; V'O2: O2 uptake; V'CO2: CO2 output; R: respiratory gas-exchange ratio, PETO2: O2 end-tidal pressure; PETCO2: CO2 end-tidal pressure; HR: heart rate; RPE: ratings of perceived exertion; V'O2 cost: Δ V'O2/Δwork rate.

Δ[deoxy(Hb+Mb)]: NIRS-derived changes in deoxygenated Hb+Mb concentrations at the vastus lateralis muscle, expressed as a percentage of the maximal values obtained during a transient limb ischemia.

PARAMETER	before ERT *mean ± SD*	after ERT *mean ± SD*
WRpeak watt	74,5 ± 55,1	77,3 ± 59,0
V'Epeak L/min	44,7 ± 19,4	44,7 ± 19,2
fRpeak breath/min	32,3 ± 10,2	31,2 ± 11,6
VTpeak L/min	1,5 ± 0,7	1,6 ± 0,8
V'O2peak ml/min	1371,8 ± 672,6	1393,2 ± 654,2
V'O2peak/BM ml/min/kg	19,9 ± 7,3	20,4 ± 8,3
V'CO2peak ml/min	1498,4 ± 686,4	1563,6 ± 767,1
Rpeak	1,09 ± 0,08	1,10 ± 0,11
PETO2peak mmHg	106,9 ± 5,3	106,3 ± 7,4
PETCO2peak mmHg	38,6 ± 4,4	39,1 ± 5,3
HRpeak beat/min	146,4 ± 21,6	150,1 ± 22,3
RPEpeak	5,7 ± 2,3	5,8 ± 2,9
Time to exhaustion min	13,2 ± 4,8	13,5 ± 5,1
Δ[deoxy(Hb+Mb)]peak (% of ischemia)	53,9 ± 21,3	56,3 ± 23,0

Table 3. Peak values of the main investigated parameters before and after ERT

Means ± SD of the main physiological variables determined at peak (voluntary exhaustion). WR: work rate; V'E: pulmonary ventilation; fR: respiratory frequency; VT: tidal volume; V'O2: O2 uptake; V'CO2: CO2 output; R: respiratory gas-exchange ratio, PETO2: O2 end-tidal pressure; PETCO2: CO2 end-tidal pressure; HR: heart rate; RPE: ratings of perceived exertion.

Δ[deoxy(Hb+Mb)]: NIRS-derived changes in deoxygenated Hb+Mb concentrations at the vastus lateralis muscle, expressed as a percentage of the maximal values obtained during a transient limb ischemia.

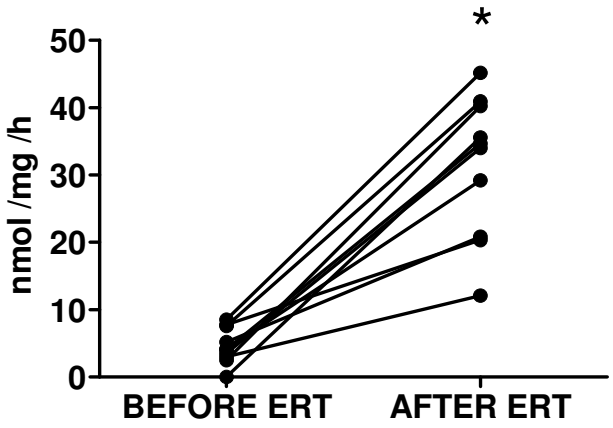


Figure 2. GAA activity on leukocites. Data obtained from blood samples of all patients.
* $p<0.05$
The figure show a significantly higher GAA activity (measured on leucocytes) in after vs before.

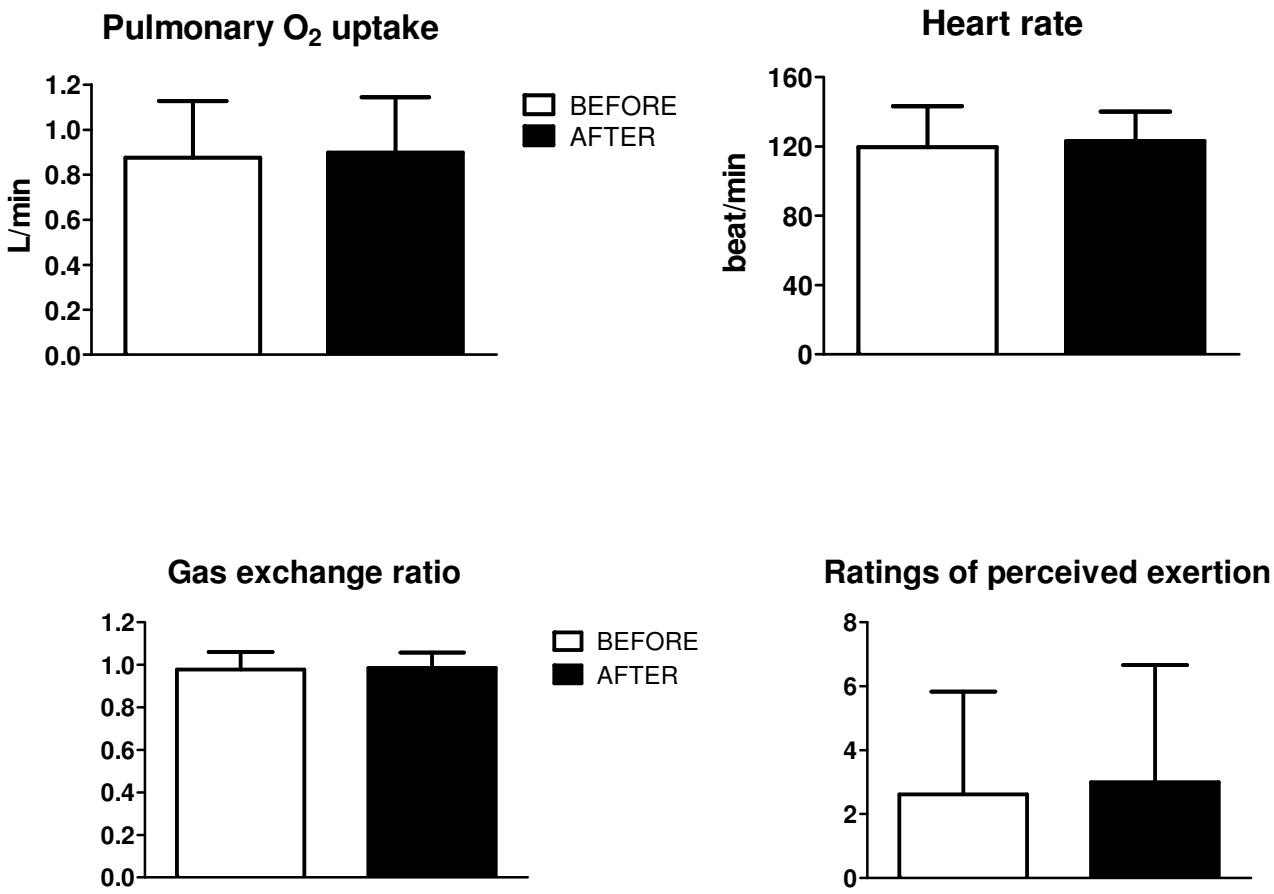


Figure 3: main measured variables during constant work rate exercise, before and after ERT.

The figure show no difference in pulmonary O2 uptake, heart rate, gas exchange ratio and ratings of perceived exertion in after vs before.

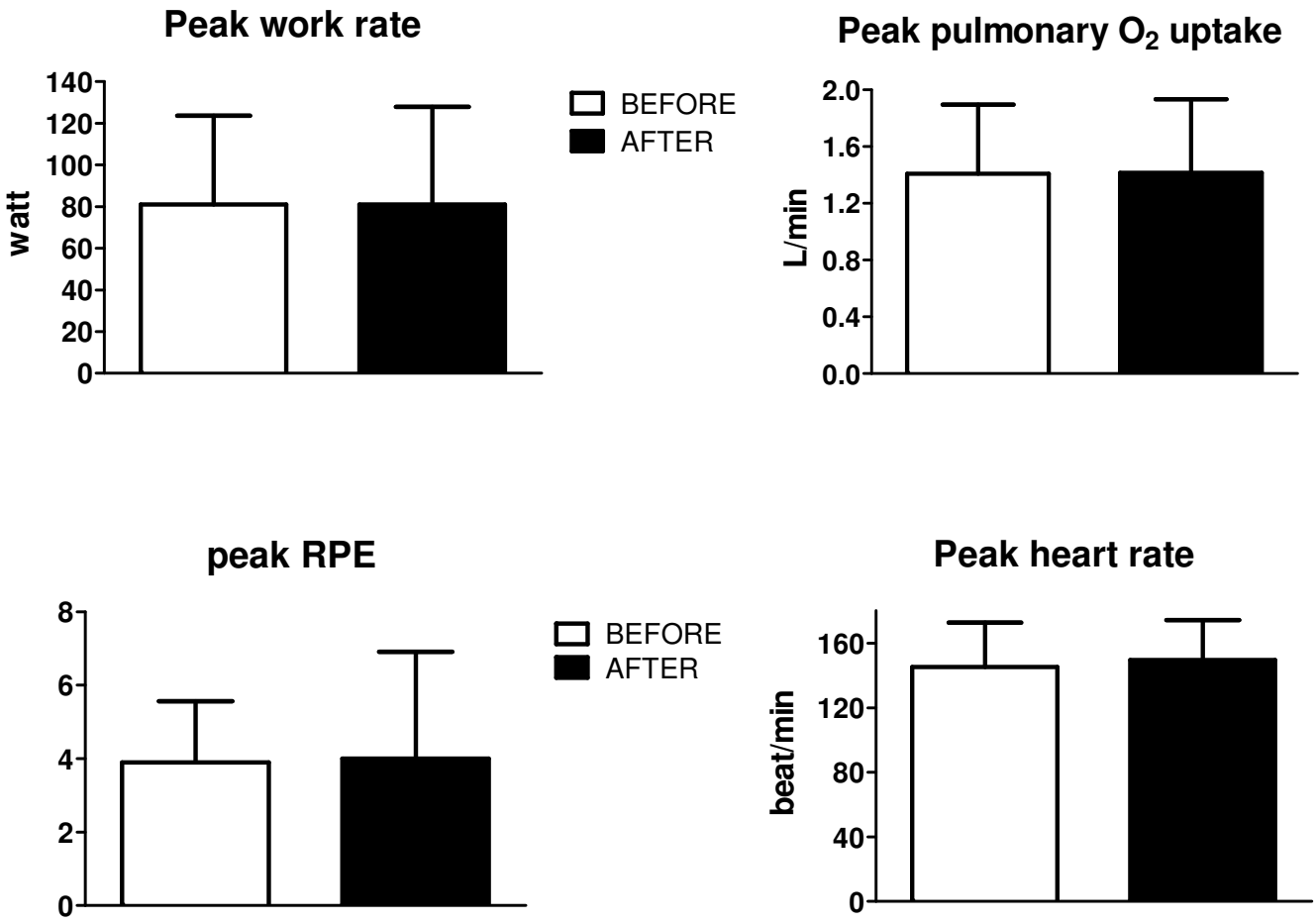


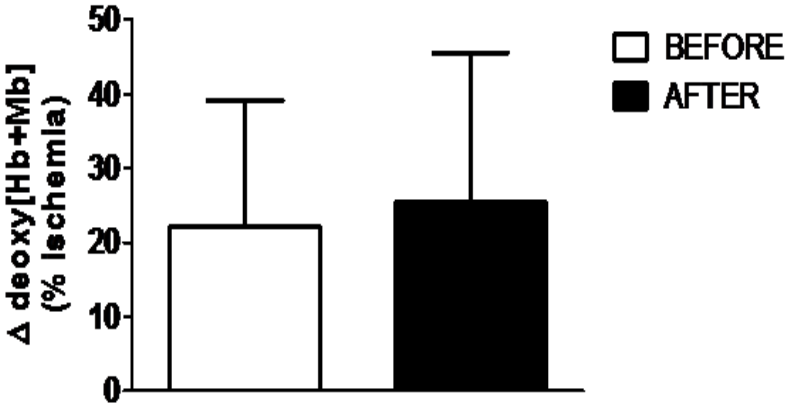
Figure 4. main measured variables at peak, before and after ERT.

The figure show no difference in peak work rate, peak pulmonary O2 uptake (peak VO2), peak rate of perceived exertion (RPE), peak heart rate, in after vs before.

Patients with Pompe disease included in this study show levels of peak VO2 notably low (normal values around 3L/min).

CONSTANT WORK RATE EXERCISE

Vastus lateralis fractional O2 extraction



INCREMENTAL EXERCISE

Vastus lateralis peak fractional O2 extraction

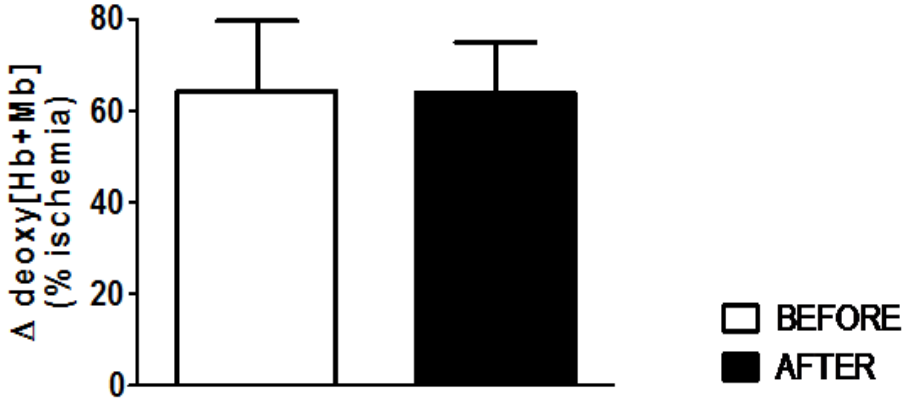


Figure 5. Fractional O2 extraction at constant work rate exercise and at peak before and after ERT.

Data obtained by near infrared spectroscopy (NIRS). The figure show no difference in fractional O2 extraction, both during constant work rate exercise and at peak in after vs before.

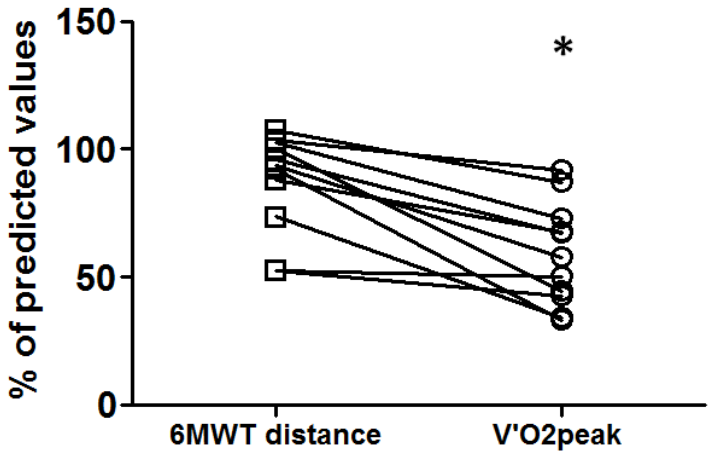


Figure 6. Relationship between 6-min walking distance and peak pulmonary V'O_2

* p<0.05

The figure show a significant reduction of VO2 peak measured by the exercise tolerance test, compared to the distance walked at the 6 minutes walking test (6MWT), with data expressed as percentage of predicted normal values.

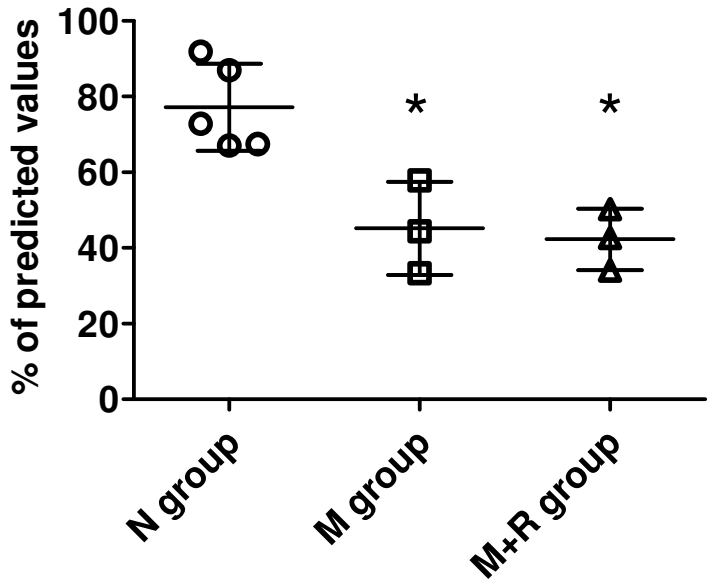


Figure 7. Peak pulmonary V'O_2 as a function of disease severity

N: no apparent symptoms ; M: skeletal muscle dysfunction ; M+R: skeletal and respiratory muscle dysfunctions.

*: $p<0.05$ vs. N group

patient code	WS score	6MWT distance %	NIV	FVC %	Phisical activity	
1	3	100	no	108	none	NC
2	0	108	no	112	active daily living + 2h swimming/week	
3	2	93	no	107	1h walking/day	
4	0	96	no	91	1h walking/day	NC
5	6	53	yes	64	none	
6	0	88	no	99	1h cycling/day	NC
7	3	92	no	126	none	NC
8	3	74	yes	55	none	
9	3	53	yes	22	30 min cycling/day	NC
10	0	104	no	106	active daily living + 4h dancing/week	
11	0	103	no	102	active daily living + 4h dancing/week	

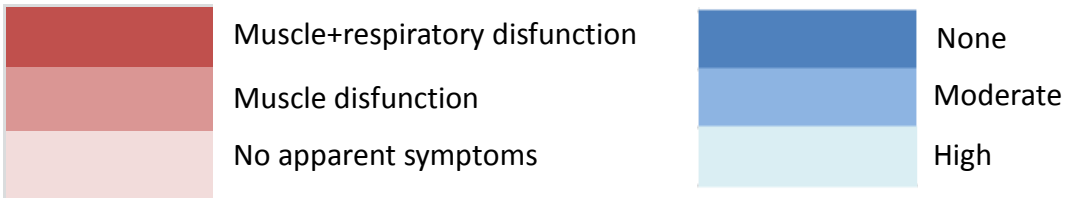


Figure 8. Lack of correspondence between disease severity and level of habitual physical activity in our cohort

NC= no correspondence, 5/11 patients

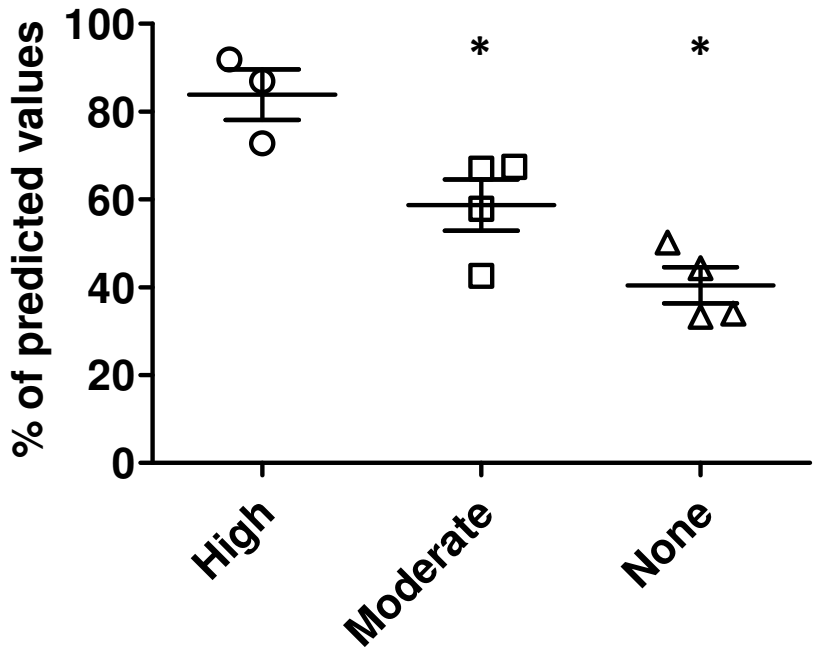


Figure 9. Peak pulmonary $V'O_2$ as a function of habitual physical activity

High: active daily living + regular exercise training; Moderate: active daily living; None: sedentary daily living.

*: $p<0.05$ vs. High group

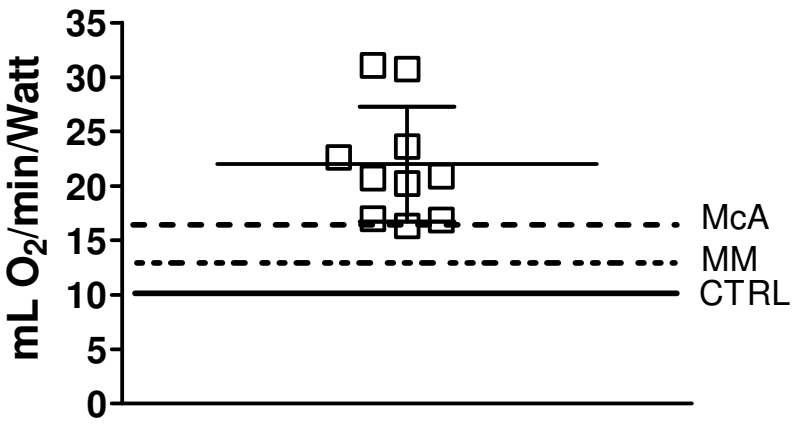


Figure 10. O_2 cost of cycling during CWR in LOPD

Lines denote the mean values of the O_2 cost of cycling previously determined in healthy subjects (CTRL), patients with mitochondrial myopathies (MM), and patiens with McArdle's disease (McA) (from Grassi et al., 2009).

Discussion

In patients with LOPD, as in patients affected by other neuromuscular disorders, exercise intolerance is one of the clinical hallmarks of the disease, and can significantly affect the patients' quality of life (Müller-Felber W et al 2007). Enzyme replacement therapy (usually carried out by an intravenous infusion every 2 weeks) has been demonstrated to ameliorate exercise tolerance evaluated after one year of treatment (*Marzorati et al. 2012*), but its effects after an acute infusion have never been investigated before.

Empirically, some LOPD patients refer a subjective improvement of exercise tolerance during the days immediately following the ERT infusion, and a gradual decrease during the 2 weeks interval between the infusions.

In the present study a novel and specific protocol of exercise test was utilized, composed of two consecutive parts (see Methods). In the first part the patients performed for 8 minutes a light-intensity CWR exercise. Analysis of physiological variables during the last minute of the exercise allowed an objective evaluation of exercise tolerance also in the patients who, as a consequence of early fatigue (a frequent finding in LOPD patients) could not continue to perform the subsequent incremental portion of the protocol. More specifically, the observation of lower R, lower RPE, lower HR, lower O_2 cost, and greater time to exhaustion would indicate an improved exercise tolerance. However, no differences were observed for any of these variables in AFTER *vs* BEFORE. The same conclusion can be drawn also for the more "traditional" peak values, determined during the incremental portion of the test. During both parts of the protocol no differences were observed, in AFTER *vs.* BEFORE, for the NIRS-obtained variable which we utilized to estimate skeletal muscle fractional O_2 extraction.

Thus, our results do not confirm an acute effect of ERT on physiological variables related to exercise tolerance, and do not confirm a decreased exercise tolerance during the 2 weeks between interventions, despite 6/11 patients reported a difference in their subjective wellbeing. The absence of any acute effect on exercise tolerance occurred despite a significant increase in GAA activity in blood in AFTER vs BEFORE. A note of caution is however necessary in this respect, since GAA was determined in blood, but not in skeletal muscle. Determination of GAA in muscle would need a muscle biopsy, which could not be carried out in the present study for ethical reasons.

The lacking of an acute effect of ERT on the main variables of exercise tolerance can be explained by the pathophysiology of muscle damage in LOPD patients, and the mechanism of action of the ERT in muscle cells. In these patients there is an impairment of lysosomal glycogenolysis, linked to the GAA defect, while the cytoplasmic breakdown of glycogen by myophosphorylase is unaffected. During exercise muscle cells utilize cytoplasmic glycogen as a source of energy, while the

lysosomal glycogen is preferentially utilized at rest (*McArdle et al 2007*; *Preisler N et al 2012*). The ERT helps the cell to break down lysosomal glycogen, resembling the endogenous GAA enzyme, but does not act on cytoplasmic glycogenolysis. This may explain the absence of acute effects of the drug on exercise tolerance. The positive effects of ERT in LOPD patients would then be substantially related to the long term effects of the drug on the chronic alteration of muscle cells structure secondary to the disease (Marzorati et al 2012), and therefore they cannot be seen after a single administration. The effect on subjective wellbeing after an acute ERT administration, reported by some patients, can be linked to other factors, which cannot be detected with an exercise tolerance test. Is it possible to hypothesize a role of ERT in acutely reducing the inflammation likely associated with Pompe disease. In fact lysosomal dysfunction and its consequent abnormal autophagy can trigger a pro-inflammatory status (Ryter SW et al 2014), and muscular inflammation has been described in autoptic specimens of LOPD patients (Hobson Web LD et al 2012). However, although a positive effect of ERT on inflammation has been demonstrated in other lysosomal storage disorders (Ko Y wet al 2015), it has never been investigated in LOPD patients, and further studies are needed to confirm this hypothesis. It must be also pointed out that not all patients in our cohort (5/11) reported an acute improvement on their subjective wellbeing after ERT, one of them referred a worsening and 5/11 declared no difference, therefore we cannot exclude a psychological effect.

A practical consequence to the absence of changes of the main physiological variables related to exercise tolerance with an acute ERT administration is that this type of functional evaluation can be performed in the patients' follow-up independently from the timing of the ERT infusion. Moreover, the study highlights the importance of using this functional evaluation test for assessing the patient's outcome in a long term. Nowadays the 6MWT is a widely used method to assess muscular function during the progression and the treatment of LOPD, however, considering the minimal differences of the distance covered among LOPD patients, alternative outcome measures should be found (Lachmann et al 2013). Interestingly, our data suggest that the functional evaluation test might be more sensitive in evaluating exercise limitations in patients with LOPD than the standard 6MWT. In fact in our cohort, the distance covered during the 6MWT was closer to the predicted values referred to healthy subjects than the V'O2 peak values measured during the exercise tolerance test, which resulted compromised even in those patients with apparently no symptoms. Our data are in contrast with a recent study by Crescimanno G et al, in which the two tests showed a similar overall degree of exercise impairment in a cohort of 8 LOPD patients (Crescimanno G et al 2015). Nevertheless, the population included in that study was older and more clinically compromised than patients included in the present study. Therefore, we suggest the use of the

exercise tolerance test in the clinical practice to detect early changes in patients with LOPD, especially in those with a mild disease.

In the present study interesting insights, in our opinion, may also derive from the evaluation of $V'O_2$ peak after the subdivision of patients in different categories on the basis of the clinical severity of the disease (Figure 4). As it could be expected, patients who were apparently free of symptoms had a higher $V'O_2$ peak, while lower $V'O_2$peak values were observed in patients with skeletal muscle impairments and in patients with both skeletal muscle and respiratory impairments, with no differences between the two subpopulations. Similar findings were previously observed in patients affected by other chronic diseases characterized by both pulmonary and skeletal muscle alterations (Maltais et al., 2014; Salvadego et al., 2015) and in healthy subjects exposed to prolonged muscle inactivity and hypoxia (Salvadego et al., 2011; Salvadego et al., 2016).
Moreover, patients grouped for habitual physical activity level showed significant differences in their performances: decrease in *moderate* vs *high* and *none* vs *high*, and a trend to decrease, even if not statistically significant, in *none* vs *moderate.* These data are important considering that in 5 out of 11 patients there was not correspondence between the degree of their clinical severity and the level of their physical activity since some patients who could potentially perform more exercise had a sedentary lifestyle and some patients more clinically compromised used to perform regular exercise, as well as possible for their clinical conditions. Thus, we can postulate that increasing habitual physical activity, with adequate training, could improve the exercise tolerance even in patients who already show motor and respiratory impairment. This is in accordance with previous studies who propose regular physical activity to ameliorate the clinical status in patients with LOPD (*Slonim AE* et al 2007; *Van den Berg L* et al 2015).

In this study, LOPD patients also showed markedly higher values of the O_2 cost of cycling compared to healthy subjects and to patients affected by other forms of muscle disorders (Figure 5) which directly involve the metabolic pathways (Grassi et al., 2009). Overall these findings suggest that in LOPD patients, the disease-related muscle damage and the resulting muscle dysfunction can play a key role in limiting oxidative performance and exercise tolerance.

This study suffers from several limitations. One of the limitations of the incremental exercise test in LOPD patients, as in other patients with neuromuscular disorders, is represented by the fact that profound muscle weakness or muscle pain may preclude the performance of an effective test. For

that reason we utilized a modified protocol of exercise tolerance test adapted to LOPD patients, where the incremental part was preceded by a light-intensity constant work rate exercise.

A further limitation of this study lies in the fact that after subdividing the patients into different subgroups the number in each subgroup became very small. Further larger studies are needed to confirm our insights.

Conclusions

In conclusion, in patients with LOPD under chronic treatment with ERT the administration of the drug does not have any acute significant effect on several physiological variables related to exercise tolerance. No changes of these variables were observed during the 2 weeks period between infusions. On the contrary, we suggest to perform exercise tolerance test to assess the outcome of LOPD patients in the long term, especially in patients with a mild disease to detect early changes in clinical status.

Study III

Exercise tolerance in patients with late onset Pompe disease on enzyme replacement therapy: effect of exercise training and hyperproteic diet

This study won a 3 years grant from the "ministero della salute Italiana" through "bando della ricerca finalizzata, giovani ricercatori 2011-2012" and, at present, is still ongoing.
In this thesis I report the preliminary results.

Abstract

Background: Late onset Pompe disease (LOPD) is a progressive neuromuscular disorder which can lead to important disability and impaired quality of life. The effect of the specific enzyme replacement therapy (ERT) in the long term is limited. Functional evaluation of exercise tolerance is a recent tested method to evaluate patient's clinical outcome. Before the introduction of ERT, the positive effects of diet and exercise training had been demonstrated in LOPD, but the effect of these interventions on patients treated with ERT is still debated and poorly known. The aim of this study is to evaluate if exercise training, alone or in combination with diet therapy, can improve exercise tolerance, motor functions and quality of life in LOPD patients chronically treated with ERT.

Patients and methods: a cross-over study was designed. LOPD patients were selected from two different clinical units in Udine and Milan. Participants were assigned to a sequence of two treatments (exercise alone; exercise and diet) and one control period, each lasting 26 weeks, followed by a 13-week wash-out. Exercise tolerance test, muscular strength, 6-minute walking test, serum muscular enzymes (AST, ALT, CPK, LDH), pulmonary function tests, body composition and quality of life (SF36 questionnaire) are evaluated before and after each period.

Preliminary Results: The study is ongoing. At present 12 patients have been enrolled. Only three patients have completed 2 intervention periods, 2 patients have completed one intervention period, all the others are under the first intervention period. For those who completed at least one intervention period, exercise training at home was feasible and safe. The compliance was higher for exercise therapy compared to diet therapy. Exercise improved inferior and superior arm force, VO2 max and peripheral oxygen extraction.

Conclusions: our preliminary data indicates a positive effect of exercise training in LOPD patients under chronic ERT, nevertheless we should wait the end of the study to draw proper conclusions.

Background

The late-onset phenotype of Pompe disease (LOPD) is characterized by lysosomal accumulation of glycogen in skeletal and respiratory muscles, resulting in progressive weakness, motor dysfunctions and respiratory distress. The consequence in the long term can be invalidating, since the muscle damage in LOPD can progressively lead patients to severe motor limitations, with need of using a wheelchair, and to respiratory insufficiency with necessity of a respiratory support [*Winkel LP et al2005*]. Comprehensibly, the quality of life of this patients can be impaired. Exercise intolerance appears early in the natural history of the disease, and is one of the first clinical hallmarks affecting patients' quality of life [*Mazorati M et al 2012*]. In 2006, the enzyme replacement therapy (ERT) with recombinant alfa-glucosidase became available, and showed to be effective on motor and respiratory functions of LOPD patients [*Van der Ploeg A et al 2010; de Vries JM et al 2012*], and to improve their quality of life [*Güngör D et al 2016*]. However, different authors observed that the positive effects of ERT are evident in the first years of treatment but seem to stabilize or even decline afterwards [*Bembi B et al 2010 Regnery C et al 2012*]. Before the introduction of ERT, the positive effects of diet and exercise training had already been demonstrated in LOPD [*Slonim AE et al 2006*] but the effect of these interventions on patients treated with ERT is still debated and poorly known.

Hour hypothesis is that exercise training, alone or in combination with hyperproteic diet, could improve exercise tolerance, motor function, muscle strength and consequently quality of life in LOPD patients on chronic ERT treatment. The ratio comes from cell pathology. In fact, at muscle fibre level the aerobic exercise could modify the energetic metabolism, increasing the use of fatty acids as an alternative source of energy, thus reducing proteolysis, autophagy and muscle destruction [*Slonim AE et al 2006*]. Moreover, exercise training could counteract the general deconditioning typical of chronic diseases as well as the chronic inflammatory condition associated with inactivity [*Pedersen BK et al 2011*]. Positive effects of exercise training have been already demonstrated in a study on Pompe mutant mice, in which animals under treadmill training improved aerobic capacity, strength and motor functions [*Nilsson MI et al 2012*].

The hyperproteic-low carbohydrate diet could reduce glycogen deposition in muscle cells and increase the intracellular protein synthesis, thereby reducing glycogen accumulation, proteolysis, muscle autophagy and damage [*Slonim AE et al 2006*]. Since diet and exercise act on different mechanisms, hour hypothesis is that their combined effect could be synergic. The expected improvement in the muscular functions could translate in an amelioration in patients' everyday life activities and quality of life.

The primary aim of this study is to evaluate the effects of an individualized exercise training program (alone or combined with a hyperproteic diet), on exercise tolerance, muscle functions and quality of life in LOPD patients chronically treated with ERT.
The secondary aim is to evaluate the compliance with the proposed intensive life-style intervention.

Patients and methods

This study belongs to a collaboration between 3 different Units: The Regional Coordinator Centre for Rare Diseases of the Academic Hospital of Udine, the Exercise Physiology Laboratory of the University of Udine and the Neuromuscular Unit of the Carlo Besta Institute in Milan.
Different professional figures are involved, including metabolic physicians expert in Pompe disease, neurologists, physiologist with experience in exercise functional studies, dietician and nurses.

Patients were recruited from the 2 different clinical centres in Udine and Milan.
Inclusion criteria were:

1. age ≥18 years,
2. diagnosis of LOPD confirmed by enzymatic test and/or genetic analysis,
3. enzyme replacement therapy for at least two years.

Exclusion criteria were:

1. significant cardiovascular disease,
2. wheelchair bound
3. severe respiratory insufficiency;
4. pregnancy,

Patients who already performed regular physical activity or were on diet therapy were also excluded.
The study was approved by the local Ethical Committee both in Udine and in Milan.
Informed consent was obtained from all patients before starting study tests.

The study was designed as a partially blinded crossover study. Blinding is applied to researcher working at laboratory assays, instrumental testing and data analysis.
Participants were assigned to a sequence of two treatments (exercise alone; exercise and diet) and one control period (free diet, no exercise training), each period lasting 26 weeks and followed by a 13-week wash-out.

Patients continue ERT during all the study period, at the usual dosage (20 mg/kg/b.w.).

Aerobic exercise (performed at home) consist of a 4 sessions/week, 1 hour/session, personalized training program. Each session includes: warm-up, stretching and balance exercises (10-15 minutes); strength training with very moderate loads of the main muscle groups carried out by elastic bands (15 minutes); a constant work-rate exercise on a cycle ergometer at 60% of the patient's maximal heart rate (HR) (30-40 min). Cycle ergometer with a HR-meter was shipped to each patient's domicile. The HR-meter allows the downloading of data on a personal computer. Hyperproteic diet is tailored by a specialist dietician to each patient according to the usual calorie intake, and is composed of 25-30% protein, 30-35% carbohydrate, and 35-40% fat.

The effectiveness of interventions is evaluated measuring exercise tolerance (maximal aerobic power; gas exchange threshold; kinetics of pulmonary gas exchange during constant work-rate exercises; skeletal muscle fractional O2 extraction by near-infrared spectroscopy; HR, pulmonary ventilation and gas exchange ratio during constant work-rate exercise; rates of perceived exertion; time to exhaustion), muscular strength (as measured by dynamometer), motor function (6-minute walking test [6MWT], WS), neurologic functional tests, Walton scale score, serum muscular enzymes (AST, ALT, CPK, LDH), pulmonary function (standard spirometry, Maximal Inspiratory Pressure, Maximal Expiratory Pressure [MIP e MEP]), body mass index, body composition (by impedentiometry), before and after each period.

Neurologic functional tests includes GSGC scores (Gait, Stairs, Gower, Chair).

Quality of life is evaluated using the validated, standardized Italian version of SF36 questionnaire, administered both before and after each intervention.

To check for compliance, patients are asked to complete a 3-day food diary every month, and a day by day physical activity diary during the period of intervention including the exercise. Moreover data from HR-meter during the treatment periods are collected.

Low compliance risk is minimized through periodical contacts, by phone or e-mail, between participant patients and the study staff.

Statistical analysis

Since few patients at present have completed more than one intervention period, no statistical analysis was possible for this thesis. Preliminary data are shown as mean ± standard deviation (SD) when stated.

At the end of the study, after checking for the absence of a carry-over effect (ANOVA; graphical assessment), a paired statistical analysis will be performed using a mixed-effects linear model to

account for the repeated measurements that yield period, sequence, and possible carryover effects and to model the various sources of intra-patient and inter-patient variability.

Preliminary results

At present, 12 patients have been enrolled in this study.

Six patients, 3 males and 3 females, all caucasian, have been enrolled in Udine. One more female patient will be enrolled in the study in the next future. Three patients have completed 2 intervention periods, 2 patients have completed one intervention period, one patient started the first intervention period recently.

Other 6 patients have been enrolled in Milan and have completed one intervention period (data not available).

General characteristics of patients enrolled in Udine are summarized in table 1 and 2.

patient code	gender m/f	age at diagnosis	age at study entry	genotype allele 1	genotype allele 2
UD01	M	3	20	c.-32-13T>G	c.2481+102_2646+31del
UD02	M	41	49	c.-32-13T>G	c.2646_2646+1delTG
UD03	F	29	50	c.-32-13T>G	c.2481+102_2646+31del
UD04	M	35	57	c.-32-13T>G	c.2646_2646+1delTG
UD05	F	25	46	c.-32-13T>G	c.2237G>A
UD06	F	29	42	not assessed	not assessed

patient code	years on ERT	comorbidities	echocardiography	ventilatory support
UD01	9	none	normal	none
UD02	6	hypertension	mild left ventricular hypertrophy	none
UD03	6	osteoporosis	normal	none
UD04	9	hypertension	aortic root ectasia	NIV
UD05	9	asthma; hypertension	mild mitral valve regurgitation	NIV
UD06	8	none	mild mitral valve regurgitation	NIV

Table 1 and 2. general characteristics of patients enrolled in Udine

NIV: non invasive nocturnal ventilation

All patients had the genotype c.-32-13T>G on one allele, which is characteristic of patients with the late-onset form of Pompe disease (table 1). One patient (patient 1) have the juvenile form of the disease, the others have the adult form.

Unless patients with late onset Pompe disease are generally considered free of cardiac symptoms, performing echocardiography we could evidence that 4 out of 6 patients had a cardiac abnormality (table 2), even though mild. Of note, 3 patients suffers from arterial hypertension, and take chronically medications for it. Three out of 6 patients need the use of respiratory support (non invasive ventilation) during night-time.

The study tests were well tolerated in all cases and, for those patients who already performed the exercise at home, no adverse events were reported.

Patient 1 and patient 3 completed the *control period* and the *exercise period*, patient 2 completed the *control period* and the *exercise + diet* period, patient 4 completed only the *control period,* patient 5 completed only the *exercise + diet* period*,* patient 6 just started the *exercise* period.

The compliance with the proposed interventions was very high as for the aerobic exercise, while more difficulties were referred in following the diet, especially as regards patient 5. All patients referred better subjective wellbeing and less fatigability after the *exercise* period, they also refer a difficulty in stopping the exercise for the 3 months of washout after the training period, but were adherent to it.

Not a specific trend was recognised as for values of CK in blood, distance walked at the six minutes walking test and vital capacity at spirometry, before and after each intervention period.

Patients who performed the *exercise* period (1 and 3) had in increase in the legs and arm extensor muscles force, after the training program at home (figure 1a and 1b).

Data obtained by NIRS showed an improvement in peripheral oxygen extraction, measured as Δ[deoxy(Hb+Mb)], both after the exercise period for patients 1 and 3 and after the *exercise + diet* period for patient 2, while there was no difference before and after the *control* period (figure 2a, 2b, 2c).

As regards the VO2 max, a slight increase was observed after the *exercise* period, while not after the *exercise + diet* period (figure 3a, 3b and 3c).

No evident changes were observed in BMI or in body composition in terms of lean body mass or fat mass, as measured by impedentiometry.

Figure 1a. Force of the knee extensor muscles before and after the *exercise* period

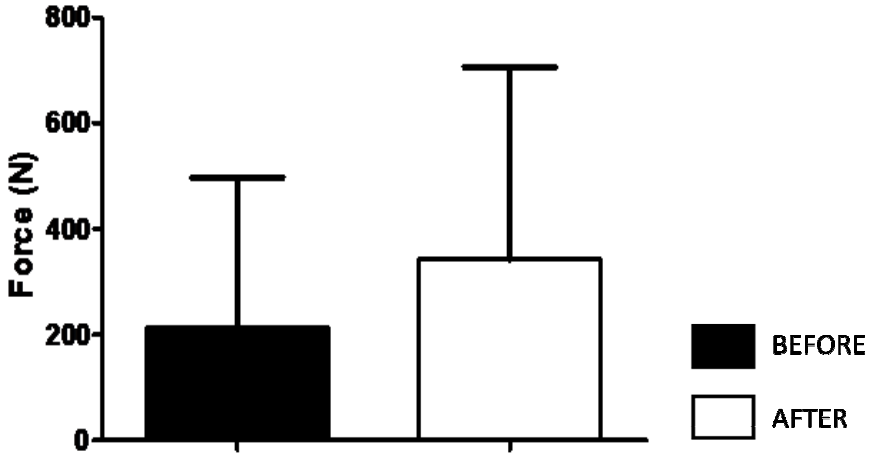
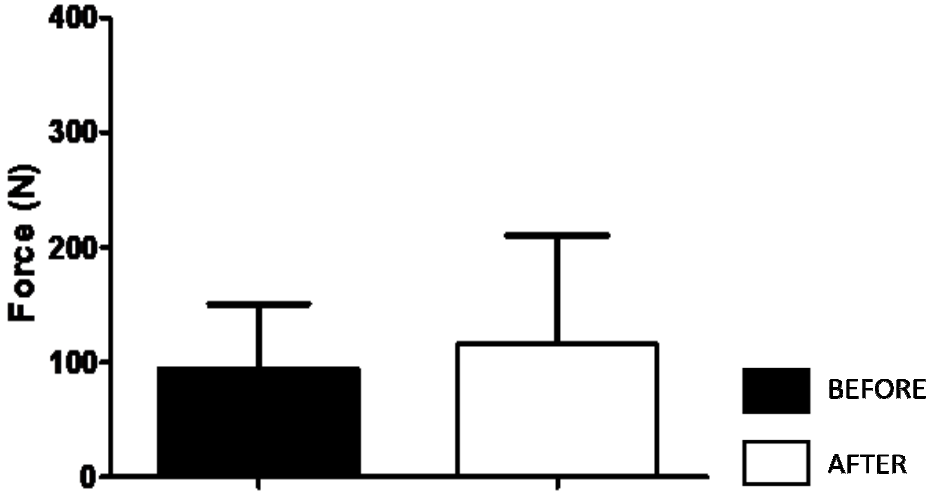


Figure 1b force of the elbow extensor muscles before and after the *exercise* period



Data are expressed as mean ± SD

Figure 2a vastus lateralis peak fractional O2 extraction before and after the *control* period

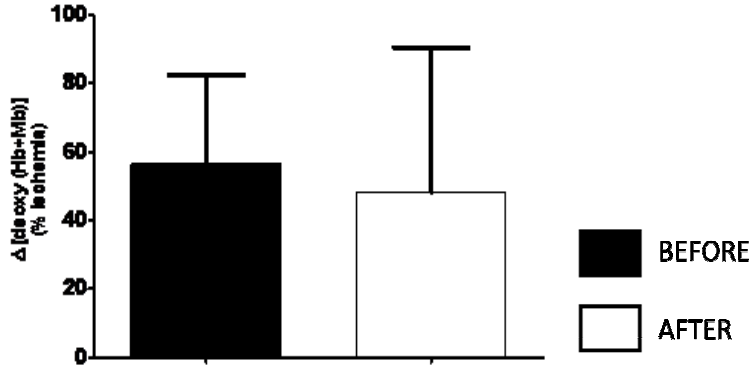


Figure 2b v. lateralis peak fractional O2 extraction before and after the *exercise* period

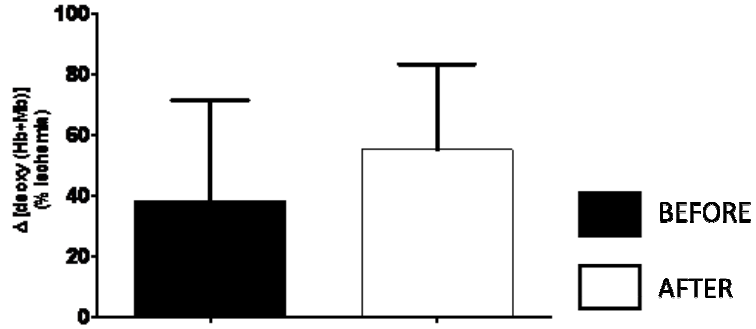
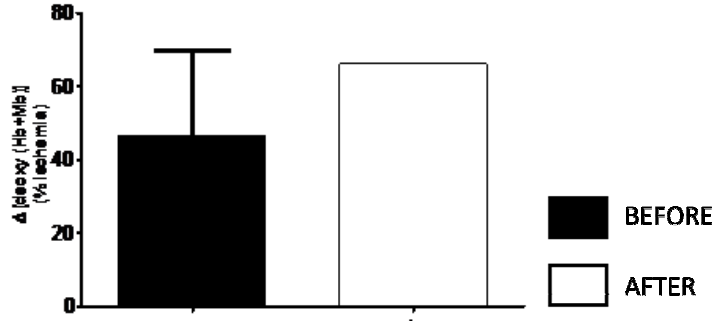


Figure 2b v. lateralis peak fractional O2 extraction before and after the *exercise + diet* period



Data are expressed as mean ± SD

Figure 3a. VO2 max before and after the *control* period

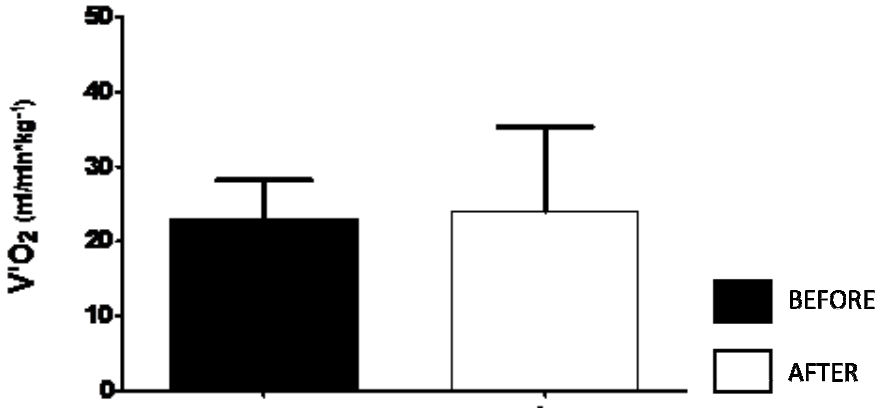


Figure 3b. VO2 max before and after the *exercise* period

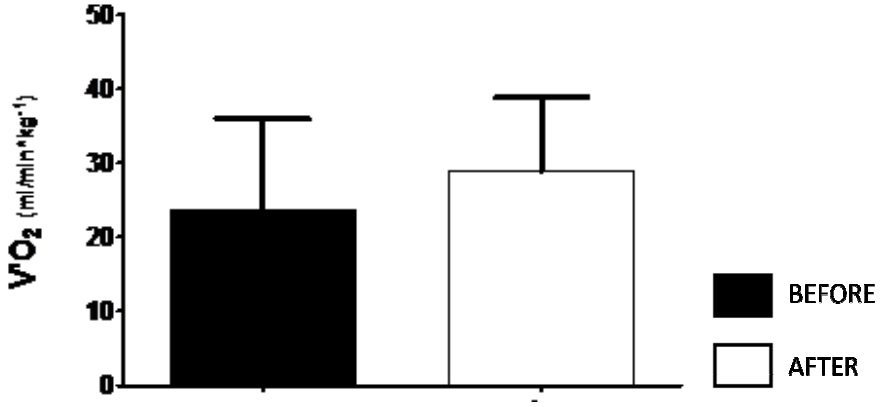
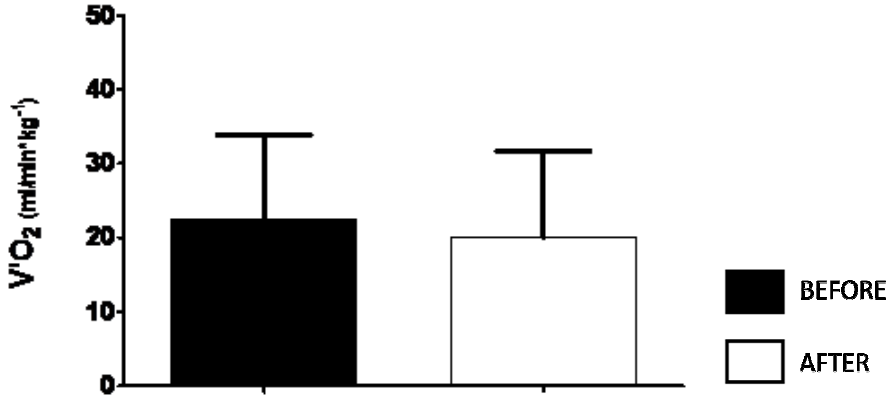


Figure 3c. VO2 max before and after the *exercise* + *diet* period



Data are expressed as mean ± SD

Discussion

As far as we know, this is the first study investigating the effect of an hyperproteic diet and a personalized aerobic exercise on patient with Pompe disease long term treated with enzyme replacement therapy. When the ERT was still not available, many clinician use to prescribe diet and exercise to patients, following the data by Slonim et al, which published the results of a 10 years retrospective study on 34 patients with LOPD treated with what they called NET (nutrition and exercise therapy) [*Slonim AE et al 2007*]. Slonim first noted that the degree of glycogen deposition in Pompe pathology was not proportional to the severity of the myopathy, realizing that there was an increase in proteolysis contributing to the muscle damage. That was confirmed by a rapid fall in plasma aminoacid levels following a protein load ingestion in a child with Pompe disease, that was successfully treated thereafter with hyperproteic diet, improving muscle function and exercise tolerance [*Slonim Ae et al 1983*]. Subsequently he proposed to treat patients with the NET, formulated to decrease glycogen deposition, counteract proteolysis and promote fatty acid utilization in muscles. The hyperproteic diet consisted in 25-30% protein, 30-35% carbohydrate, and 35-40% fat, the same utilized in our study. Patients on NET also took supplements of the aminoacid L-Alanine, which was not utilized in this study since further studies failed to demonstrate the clinical efficacy of this supplementation [*Mundy HR et al 2006*]. Exercise of NET consisted in a treadmill program daily for 45-50 min, followed by aerobic upper-body exercise for 10-15 min. We choose exercise on a cycle-ergometer to allow also the participation of patients who had ambulatory difficulties, and proposed only for 4 sessions a week, to be more compatible with everyday life, and increase compliance. Among the patients on NET, 26 were considered in good compliance with the proposed interventions and 8 were considered not compliant. Rate of deterioration (as measured by Walton Scale) on NET was compared with each patient's rate of deterioration before starting the NET, evidencing that the more compliant patients responded far better than those who became less active and did not follow the diet [*Slonim Ae et al 2007*].

After the advent of ERT for Pompe disease only few studies have been published investigating the effects of exercise on patients already on therapy, and no studies investigate the effect of the hypeproteic diet associated to exercise.

In 2011 Terzis G et al. reported the effects of a 20-week exercise training in 5 LOPD patients receiving ERT. A significant increase in muscular strength (15-50% at various body parts) and distance walked at the 6-minute walking test (203.8 ± 177 m before vs. 248.2 ± 184 m after) was observed after training, suggesting a positive effect on muscular function and functional capacity. In this study total and lower extremities lean body mass did not change significantly [*Terzis G et al 2011*]. The same group investigated the possibility to increase the effectiveness of the ERT, in

patients already on regular exercise training, if the exercise training was performed during enzyme infusion for 6 months, but the results did not show a further improvement [*Terzis G et al 2012*].

More recently, Van der Berg et al performed a larger study, including 25 patients with LOPD who performed a 12 weeks exercise training, continuing regularly ERT infusions. The prescribed exercise consisted in 3 sessions a week of a training combining aerobic, resistance and core stability exercises, utilizing a cycloergometer and other gym instruments such as chest press, biceps curl, leg press and leg curl After 12 weeks of training VO2 peak, maximum workload capacity and ventilator threshold improved significantly, maximum heart rate and forced vital capacity did not change. There was also an increase in strength of the hip flexors and the shoulder abductors. Functional neurological tests evidenced an improvement in time to climb four steps and time to rise from supine to a standing position. The distance walked at the 6 minutes walking test increased by 6%. Two patients experienced significant increase of CK (up to 10125 U/l in one case) with muscle pain and fatigue during the first weeks of training, but continued training with the resolution of symptoms. There were no changes in lean body mass or in fat mass. This study was very important to demonstrate that the composed training program can be safe for patients with Pompe disease and can improve muscle functions in patients already on enzyme replacement therapy [*Van der berg LE et al 2015*].

Finally, Krase et al. reported the clinical case of a patient on ERT who performed regular aerobic exercise, who voluntary withdraw the ERT, continuing only the exercise for 3 years. As reported by authors, muscle strength decreased 1 year after withdrawing from ERT, whereas it was restored during the last 2 years of exercise *per se*. FVC decreased during ERT *per se*, recovered with ERT + exercise and slightly decreased with exercise *per se*. Six minutes walking distance was increased during the first 12 months after initiation of ERT and remained unaltered thereafter [*Krase et al 2016*].

Although our data are only partial and the study is still ongoing, some general consideration can be elicited from our preliminary data. First of all, our proposed aerobic exercise can be feasible and well tolerated even in patients who have already a respiratory insufficiency, needing a respiratory support during night time. Moreover, the exercise at a cycloergometer is more practicable than the exercise at the treadmill for patients with LOPD who often have walking difficulties. For the same reason, evaluation of exercise tolerance in these patients is more appropriate with a cycloergometer test.

Our patients founded exercise easy to practice at home and referred beneficial effect on their subjective wellbeing. Our preliminary data suggest a positive effect of exercise especially on muscle strength of selected muscles, a better exercise tolerance in terms of increased VO2 max and

a better peripheral muscle oxygenation, as evidenced by the data of the NIRS. As regards the association between exercise and diet we did not see better results after the *exercise+diet* intervention period compared to before, but it must be pointed out that only two patients have already completed the *exercise+diet* period, one of which was completely not compliant with the proposed diet. Previous studies, performed before the advent of ERT, evidenced that only 25% of patients showed improvement in muscle or respiratory functions after a high iperproteic diet, but the compliance with the diet was very poor in most studies. Compliance following the introduction of a high-protein diet is often poor due to the large quantities of protein necessary, and a perceived risk of weight gain [*Bodamer OA et al 1997*]. With the aim to improve the adherence to the proposed diet for the next patients who will face the *exercise + diet* period, we will increase the number of contacts with the dietician and try to maximally personalize the proposed nutrition scheme, to be more feasible in everyday life.

Interesting insights are waited by the end of our study. Compared to the study of Van der Berg et al. our population of LOPD patients appears generally more clinically severe at baseline, so it will be interesting at the end of the study to see if we can see beneficial results of exercise even in more severely affected patients. Moreover, this is the first time that the effect of hyperproteic diet associated to exercise is evaluated in a cohort of patients already long term treated with ERT.

Late onset Pompe disease in advanced stages causes significant disability with great impact for the health care system. At present, ERT with human recombinant alfa-glucosidase is the only available treatment for LOPD, but its effectiveness seems to be limited and a dosage augmentation is not feasible because of its high costs. Exercise training and diet therapy could be helpful in increasing ERT clinical efficacy, improving patients' muscle function, reducing their disability, and ameliorating their quality of life. These effects may result in a reduction of patients' demand of assistance and in a lower cost for the health system.

Conclusions

Our preliminary data suggest a beneficial effect of exercise training performed at home in patients with late onset Pompe disease on chronic ERT. However, we should attend the end of the study to perform statistical analysis and draw proper conclusions.

CONCLUDING REMARKS

Our studies, focused on late onset Pompe disease, improve the current knowledge on clinical long term outcome of patients under enzyme replacement therapy (ERT), confirm the utility of functional evaluation of exercise tolerance in patients' follow up, and propose dietetic and physical activity interventions as addictive therapy to improve the effects of the ERT.

In particular we have observed:

1) after 5 years of regular treatment with ERT, patients with the juvenile form of Pompe disease show an improvement or even a normalization (for those who were diagnosed in an asymptomatic phase) of muscle enzymes, and stabilize motor and respiratory functions.
   Patients who started therapy in an asymptomatic phase are still free of symptoms after 6 years of follow up.
   Although these findings should be confirmed by studies conducted on larger cohorts, our results confirm the hypothesis that the ERT is more efficient if started at early stages of the disease.

2) Functional evaluation of exercise tolerance on a cycle-ergometer is a safe and useful toll to assess clinical outcome of LOPD patients, which can be used also in patients with walking difficulties and patients with mild or moderate respiratory insufficiency. The peripheral muscle component is prominent in determining the VO2 peak decrease, rather than the respiratory component in LOPD patients.
   ERT do not have an acute effect on this test, so it can be performed regardless the ERT timing.
   Compared to the standard and widely used six minutes walking test, exercise tolerance test on a cycle-ergometer seems to be more sensible in detecting exercise intolerance in LOPD patients, thus we suggest to use it especially in the long term follow up of patients with mild form of the disease, to detect early changes.
   Patients who regularly perform physical activity show better performances at the exercise tolerance test, regardless the severity of the disease, confirming the potential beneficial effect of prescribing exercise in LOPD patients.

3) Although only preliminary results are available, a personalized aerobic exercise training performed at home seems to ameliorate skeletal muscle force, maximal aerobic capacity and peripheral muscle oxygen extraction in LOPD patients chronically treated with ERT. Adherence to diet therapy seems more difficult than compliance with exercise training. Interesting results are waited by the end of this study.

In the present thesis the functional evaluation of exercise tolerance was utilized as a new instrument to clinically evaluate LOPD patients' outcome. The reported studies, in particular study II e III, can be considered an example of translational medicine, in which methods and procedures developed at a research level are applied to patients with the aim to improve their follow up and treatment. Moreover, these studies can be considered as a model for utilizing exercise functional tests in the assessment of different neuromuscular disorders in future studies.

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