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General Introduction

Pompe Disease

Pompe disease (PD, OMIM 232300) is an inborn metabolic disorder caused by the functional deficiency of the acid lysosomal α-glucosidase (GAA, acid maltase, E.C.3.2.1.20), the enzyme hydrolyzing α-1,4, and α-1,6-glucosidic bonds in glycogen and belonging to family GH31 of the carbohydrate active enzyme (CAZy) classification (www.cazy.org; {Lombard, 2014}).

GAA deficiency results in glycogen accumulation in lysosomes and in secondary cellular damage, with mechanisms not fully understood {van der Ploeg, 2008} {Shea, 2009} {Parenti, 2011} {Parenti, 2014}. In PD, muscles are particularly vulnerable to glycogen storage, and disease manifestations are predominantly related to the involvement of cardiac and skeletal muscles.

The GAA gene, localized in 17q25.2-q25.3, consists of 20 exons spread over 28 kb of genomic sequence and encodes for a protein of 952 amino acids with a predicted molecular mass of 110 kDa. {Hoefsloot et al, 1990} The 110 kDa GAA protein synthesized in the endoplasmic reticulum is a precursor polypeptide, which undergoes N-glycan processing in the Golgi apparatus, and is proteolytically processed in the lysosomes into active isoforms of 76 and 70 kDa, through an intermediate molecular form of 95 kDa. {Hoefsloot et al, 1990}.

So far more than 500 different GAA gene variations have been identified (The Human Gene Mutation Database, HGMD and Pompe Center, https://digitalinsights.qiagen.com/products-overview/clinical-insights-portfolio/human-gene-mutation-database/) and the type of mutation correlates in most cases with the residual enzyme activity. GAA activity may range from complete deficiency (<1%) in the severe forms, to partial (up to 30%) deficiency in milder forms. PD is often due to missense mutations in GAA gene that causes the synthesis of a misfolded enzyme protein.

The disease is classified into distinct categories according to the severity of symptoms: an early onset "classical" form, early onset "not classical" or intermediate form, and the attenuated late onset juvenile and adult forms. The "classic" infantile-onset form represents the worst spectrum, with a severe hypertrophic cardiomyopathy typical ECG pattern within 12 months of age, generalized hypotonia and a rapidly progressive course. When untreated, classic infantile PD patients die by the end of the first year. {Van den Hout et al, 2003; Kishnani et al, 2006}.

The late onset is within 12 months of age without cardiomyopathy or later than 12 months of age.

In the late-onset, slowly progressive juvenile and adult-onset forms symptoms related to skeletal muscle dysfunction, resulting in both mobility and respiratory problems, are the primary manifestations. {Hagemans et al, 2005}.

The age at onset, the rate of disease progression and the sequence of respiratory and skeletal muscle involvement vary substantially among different patients. The clinical variability makes diagnosis difficult, and, in several cases, the final diagnosis is made many years after the start of symptoms.

The estimated incidence of PD has been reported to vary between 1:40,000 and 1:146,000. {Van der Ploeg et al, 2008; Semplicini et al, 2018} Newborn screening programs implemented in some countries are changing this figure, with incidence rates of approximately 1: 17,000-1:27,000. {Scott et al, 2013; Bodamer et al, 2017; Chien et al, 2019}.

Since early 2006, enzyme replacement therapy (ERT) with recombinant human α-glucosidase has been approved and is currently considered the standard of care for the treatment of PD, improving survival and function, or stabilizing the disease course {Kishnani, 2007} {Strothotte, 2010} {van der Ploeg, 2010} {McIntosh, 2018}.

These enzyme precursors, manufactured in eukaryotic cells systems (chinese hamster ovary cells), are administered periodically to patients by an intravenous route. They bind to the mannose 6- phosphate receptor, which is present on the cell surface of cardiomyocytes and skeletal muscle cells, and they are internalized into the myocyte and transported via endocytosis to the lysosome. Inside the lysosome, rhGAA breaks down the accumulated glycogen into glucose. {Reuser et al, 1984; Van der Ploeg et al,1991}.

But limitations of ERT are also known. Not all patients respond equally well to treatment (some patients experience little clinical benefit or show signs of disease progression {Schoser, 2007}) and skeletal muscle - one of the major sites of disease and an important target of therapy - is more refractory to treatment than other tissues.

It is not totally clear why this happens but probably several factors concur in limiting therapeutic success of ERT, including the age at start of treatment {Chien, 2009} {Kishnani, 2009}, the immunological status of patients {Kishnani, 2010}, the possible instability at neutral pH of the recombinant enzyme during the transit to lysosomes {Shen, 2008}, the relative deficiency of the cation-independent mannose-6-phosphate receptor in muscle cells {Wenk, 1991} {Koeberl, 2011}.

For all these reasons alternative treatments for Pompe disease would be highly desirable.

Aim of the thesis

The general aim of my thesis is to investigate innovative technologies to identify new pharmacological approaches for Pompe disease (PD).

In particular, the purpose of my research project is to evaluate the effect of pharmacological chaperones in improving the stability and efficacy of ERT and to test the efficacy of a new human recombinant enzyme, genetically modified, produced in rice (Oryza sativa).

This project has been conducted thanks to the collaboration between three institutions: The Department of Translational Medical Sciences DISMET in Naples, the Center for Rare Diseases Helios Dr. Horst Schmidt of Wiesbaden (in Germany) and a Biotechnology Company, Transactiva Molecular Farming of Udine. DISMET has a long-lasting experience in therapy with pharmacological chaperones that has led to telling the therapeutic approach to the first clinical experiences. The Center for Rare Diseases Helios in Wiesbaden offers the best opportunities for accessing and selecting samples needed to complete the study.

Transactiva has developed a technological platform to purify human recombinant proteins in plants, including this new recombinant enzyme for Pompe disease.

The characterization of the cellular mechanisms underlying the different ERT response has the potential to lead to the development of individualized and

personalized therapeutic protocols in the different PD patients, with consequent optimization of the efficacy of the therapies and improvement of the patients' quality of life.

PART 1

Chapter 1

Pompe Disease: understanding the distribution of patients, disease severity, diagnostic pathways, availability of standard care and new treatments, treatment response as well as social aspects like quality-of-life evaluation. A questionnaire provided to all ERNs.

Introduction

The awareness of rare diseases, also in Pompe disease, the diagnostic options, the follow up as well as standard of care and treatment options including new treatment strategies varies from country to country, also inside Europe. At the moment, there are no clear reports creating a figure of the situation in Europe.

MetabERN is the European network for rare diseases and aims to connect the most specialized centers of Europe to promote prevention, accelerate diagnosis and improve standards of care for patients living with inherited metabolic disease. The network is divided in 7 disorder groups and it is organized in 8 work packages.

The "Center for Rare Diseases" of Wiesbaden, in Germany, where I spent 6 months, was one of the main centers of reference for lysosomal diseases and it was the coordinating center of the MetabERN. During my stay I had the opportunity to directly access the metabERN Databases and to gather information needed for my research program.

As of July 2018, 659 Pompe patients - divided in 222 pediatric and 457 adult patients, were followed by 69 HCPs in 18 European countries inside of the MetabERN. {MetabERN Databases. July 2018}. This is the largest cohort of Pompe patients known so far in Europe and representing about 40% of the Pompe patients included in registries run by Companies.

Aim of the project

The purpose of this project has been to map the distribution of Pompe patients in different European countries and analyze the medical and social conditions and needs of these patients in these countries.

I have generated survey monkey questionnaire to collect and analyze data to identify patients eligible for new treatments.

Data could be useful to identify the difference of management of the Pompe patients, if any, in Europe implement the management of the disease, highlight the burden of the disease, and possibly help in the choice of the therapy.

Material and Methods

A questionnaire, based on the current literature concerning diagnostic guidelines, medical follow up and treatment options and QoL analysis in Pompe disease has been created to be administered to the treating physician of in each HCP member of MetabERN.

The questionnaire is intended to collect data about:

- Number of patients in different centers,
- Disease phenotype
- Availability of Diagnostic options and pathways,
- Characteristic of the Medical follow up
- Standard of care and compliance with national and international guidelines
- Availability of new treatment options,
- Response to treatment,
- Compliance to treatment
- Cost of treatment
- Inclusion into a registry
- Contact/affiliation to Patient Association

The questionnaire could be administered to all MetabERN HCPs through a secure web-interface.

PD Survey

1. *Details about the center (HCP)*

Country ____

Institution ____

Address ____

Contact(s) ____

2. *Patients*

How many Pompe patients have been followed in your center? ________

- Number of **patients currently in follow-up** ________
- Number of **historical patients** (no longer in follow-up) ________

Patients currently in follow-up:

Males (total number) _______

- infantile-onset (onset 0-12 mo) with cardiomyopathy _______

- infantile-onset (onset 0-12 mo) without cardiomyopathy _______

- late onset (onset > 12 mo) _______

Females (total number) _______

- infantile-onset (onset 0-12 mo) with cardiomyopathy _______

- infantile-onset (onset 0-12 mo) without cardiomyopathy _______

- late onset (onset > 12 mo) _______

(Infantile-onset Pompe disease (IOPD): individuals with onset before age 12 months with cardiomyopathy. Typically, onset is at the median age of 4 months with hypotonia, generalized muscle weakness, feeding difficulties, failure to thrive, respiratory distress.
Late-onset Pompe disease (LOPD): individuals with onset before age 12 months without cardiomyopathy and all individuals with onset after age 12 months. It is characterized by proximal muscle weakness and respiratory insufficiency.)

How many patients present other cases in family?

Affected siblings? ________

Affected cousins? ________

Affected relatives? ________

3. Diagnosis and patient characterization

What tests are in use for diagnosis in your HCP?

	Available at your HCP	Available elsewhere (indicate where)
Hex 4 assay	_____	________________________
GAA screening test in DBS	_____	________________________
GAA assay in:		
- Peripheral lymphocytes	_____	________________________
- Cultured fibroblasts	_____	________________________
- Muscle Biopsy	_____	________________________
- Muscle histology	_____	________________________
GAA gene sequencing	_____	________________________
CRIM status	_____	________________________

Has CRIM status been tested in your patients? Yes ______ No _______

If yes, through:

- ☐ Acid alpha-glucosidase protein quantitation performed by antibody-based method in cultured fibroblasts
- ☐ Molecular genetic testing to determinate if the pathogenic variants result in total absence of enzyme activity.

How many patients are CRIM +? _______

How many patients are CRIM -? _______

Have you ever had asymptomatic CKemia patients? Yes ______ No _______

If yes, please explain.

4. *Age at diagnosis*

What is the age-range at diagnosis in your cohort? _____________

What is the median age at diagnosis in your cohort? _____________

Can you provide an estimate of the delay between age

at onset of manifestations and age at diagnosis? ______________

Point 4 is optional. We understand that answering may require time and effort, but information on this point may be very useful for the purpose of this questionnaire.

5. Therapy and patient care

How many patients are on ERT treatment? _______

- How many patients are on therapy with Myozyme? _______
- How many patients are on therapy with Lumizyme? _______

What is the standard ERT dosing used in your centers?

- infantile-onset patients ________
- late-onset patients ________

Are there deviations from standard dosing? Why?

____

____

Is the ERT available in you center? Yes ______ No _______

Do you refer patients to other centers? Yes ______ No _______

Is for some of your patients difficult to reach your center? Yes ______ No _______

How do you manage access of patient that live far from your hospital?

____

____

Do you have a specialized center in metabolic disease? ________

Is the assistance offered by your center multidisciplinary? ________

- Does your center offer psychological support? ________
- Does your center offer physiotherapy support? ________
- How many patients are doing physiotherapy? ________

Do your patients only take hospital therapy or even home treatment?

Hospital care only _______

Both hospital and home care _______

Home care only _______

Comment

____

____

____

How is ERT reimbursed in your HCP/country?

- National Health System ________

- Insurance ________

- other, specify

____

Are your patients compliant with therapy?

- ☐ totally
- ☐ partially

Estimate % of non-compliant patients ____%

What type of tests are performed to monitor patients?

- ☐ Chest radiography
- ☐ ECG Electrocardiography
- ☐ Echocardiography
- ☐ 6-min walk test
- ☐ EMG of paraspinal muscles
- ☐ Periodic sleep evaluation (with capnography and pulse oximetry)
- ☐ Bone densitometry
- ☐ Hearing evaluation
- ☐ other

Comment

____

____

____

Did some patient interrupt ERT? No _____ Yes _____

If yes, how many patients? ______

Why? (indicate numbers)

Immune response _____

Concurrent diseases _____

Non-compliance _____

Lack of reimbursement / access to ERT _____

Other (specify)

____

Were there any disease progression developments after the interruption of ERT?

No _______ Yes, _______

please explain.

____

____

Are there patients with feeding difficulties? No _______ Yes, _______

please explain.

____

____

How many patients are ventilator dependent?

____

How many patients already needed respiratory support at start of ERT?

____

How many patients are invasively ventilated?

____

How many patients require ventilation during part of the day?

____

How many patients need a wheelchair?

____

How many patients are totally wheelchair dependent?

____

6. Patient organizations

Are patients informed about patient organizations and their importance?

Are your patients in contact with patient organizations?

No _______ Yes, _______

please specify.

____

How is collaboration between patient organizations and health professionals supported?

____

____

Are questionnaires provided to your patients?

No _______ Yes, _______

please specify.

FSS ☐ (fatigue severity scale)

☐ IPA/Erasmus MC

Other ________

Are you aware of Pompe patient registers?

No _______ Yes _______

Are your patients included in a registry?

No _______ Yes _______

If yes, which one?

____

____

Has your center even taken part in a sharing and/or interchange of patients?

No _______ Yes _______

If yes, specify?

____

____

Do you have patients who have taken part in clinical trials?

Yes:

How many patients? _______

No:

Are you interested in participating in clinical trials?

Yes _______

No _______

Have you ever pondered the idea to include some patients in a transplant list?

No

Yes, please explain.

Conclusions

The numerosity as for 659 Pompe patients who are in the follow up of the MetabERN represents a huge number which might contribute to increase the knowledge about the situation at demographic, medical and social levels in EU. In 2015, the worldwide Pompe registry (Genzyme) has enrolled 1409 Pompe patients (Genzyme Report 2015). So, the MetabERN is covering almost 40% of the worldwide registered Pompe patients.

This questionnaire made available to the metabERN and the data generated by this study are an important tool to better define all the potential differences existing in EU regarding the management of a rare metabolic disease.

This could help to identify the special needs of Pompe patients, offering the opportunity to design a document to attract attention from stakeholders to design a strategy for the best treatment of the patients.

Chapter 2

“Pompe Desease Juvenile-onset: Clinical picture and genotype-phenotype correlation”

Introduction

Different mutations, as a different residual activity of the GAA in Pompe disease lead to different phenotypic forms of the pathology, with clinical manifestations of different severity, age of onset and different involvement of the organs.

As a general rule, earlier is the onset of symptoms so much fast is the progression of disease. Thus, the two general classifications – Infantile onset Pompe Diseae (IOPD) and Late Onset Pompe Disease (LOPD) – tend to be clinically useful in determining prognosis and treatment options {PD – Gene reviews}.

In LOPD, progression of skeletal muscle involvement is slower than in the infantile forms and eventually involves the diaphragm and accessory respiratory muscles {Winkel et al 2005}.

LOPD can manifest at various ages with muscle weakness and respiratory insufficiency. It may present from the first decade to as late as the seventh decade of life with progressive proximal muscle weakness which can lead to the inability to ambulate and to early respiratory failure, rarely manifest cardiomyopathy and, in any case, is less severe than the infantile forms.

Despite this, there are a number of "non-classical" or "intermediate" clinical phenotypes, expression of a phenotypic continuum that varies from the most severe to the attenuated forms.

Juvenile Onset pompe Disease (JOPD) is a subcategory of LOPD, with signs and symptoms manifesting between 2 and 18 years old.

With these nonclassical presentations, there is a spectrum of disease severity and progression, and therefore clinical heterogeneity {Dasouki et al.; 2014} {Van Capelle et al.; 2016}.

Although LOPD has been divided into childhood-, juvenile-, and adult-onset disease, many individuals with adult-onset disease recall symptoms beginning in childhood and, thus, late onset is often the preferred term for those presenting after age 12 months {Laforêt et al 2000}.

For this reason, diagnoses are not infrequently delayed, up to several years from first symptoms.

The classification is therefore still unclear and there are few data on patients with juvenile form in the literature.

The time frame in which the disease progresses from first symptoms to various disease specific events, for example difficulties in climbing stairs, rising from a chair, need of walking aids and wheelchair dependency, could inadequately be extracted from the literature {Winkel et al 2005}.

Aim of the Project

During my period in Germany, I also had the opportunity to collaborate with the team of Prof. Hahn of the University Hospital of Giessen, on a study about a subcategory of Late Onset Pompe Disease, the juvenile form, concerning how difficult could be to interpret the symptoms and get to the right diagnosis as soon as possible to start the enzyme replacement therapy.

The aim of this work is to show how difficult could be to interpret the symptoms and get to the right diagnosis as soon as possible to start the enzyme replacement therapy to counteract the progression of the disease.

Results

Data from 8 specialized centers of the country were collected, retrospectively using questionnaires, for a total number of 27 patients (15 males and 12 females).

We collected the following data: age, age of onset of early symptoms and age at the diagnosis; genetics, laboratory values at diagnosis, presence of muscular weakness and/or muscular pain; speech problems; pulmonary functions, sleep apnea, ventilatory support, cardiomyopathy, heart rhythm disorder, frequency of pneumonia, infections, contractures.

Clinical picture

Of 22 patients, for 11 of them the age at onset of the first symptoms was within the first year of life (Fig.1).

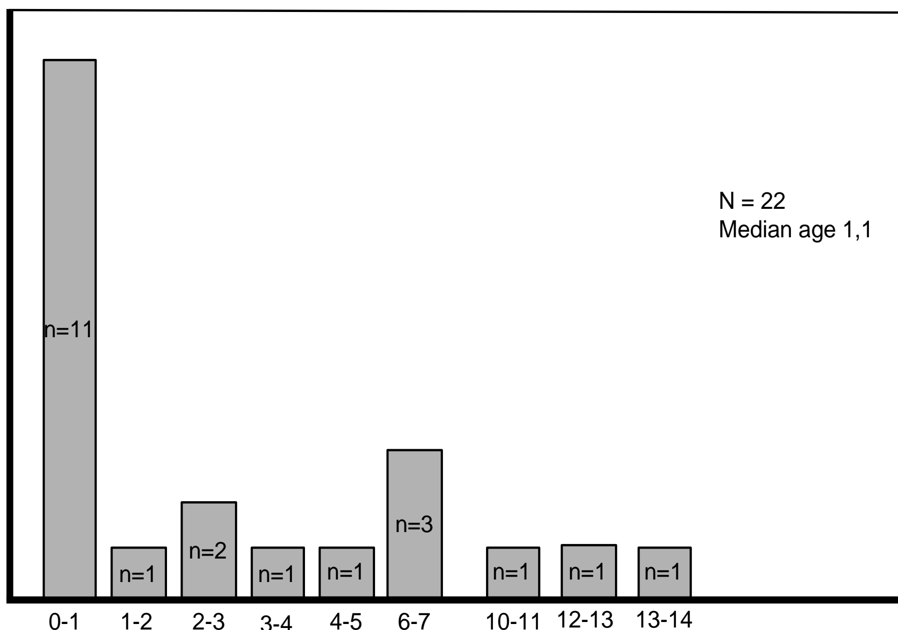


Fig.1 Patients' age at onset of the first symptoms. 5 patients were asymptomatic.

Of 27 patients, 3.7% had speech disorders, none showed hearing impairment; dysphagia was not detected in any of them.

14.8% needed ventilation, 11.1% had sleep apnea. 11.1% already use a wheelchair. No arrhythmia or any disturbance of the heart rhythm was found for anyone. 11.1% had cardiomyopathy and 25.9% muscle and scoliosis contractures (Fig.2).

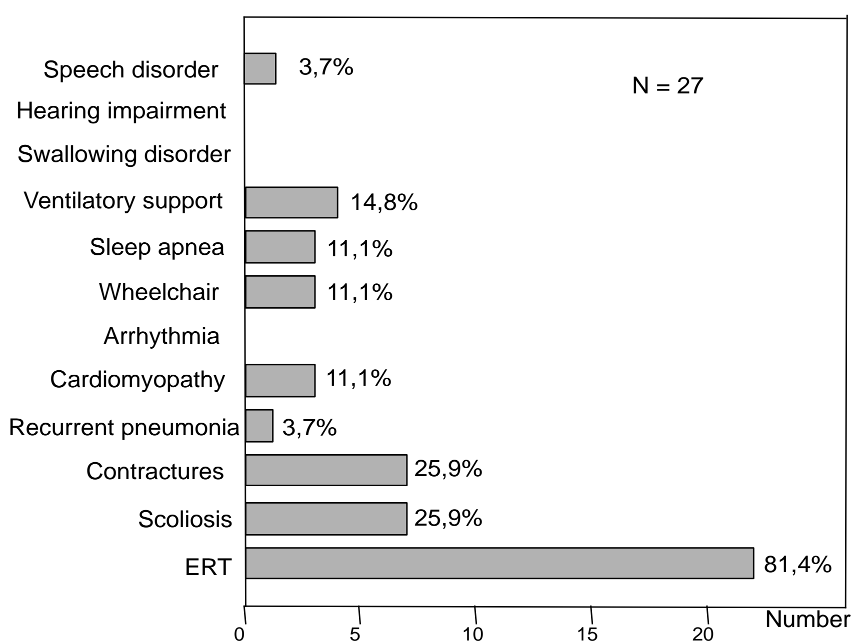


Fig.2 Data collected from 8 specialized centers of Germany, for a total number of 27 patients (15 males and 12 females).

Genotype-phenotype correlation

The c.32-13T> G was found to be the most frequent mutation, present in heterozygosis in 22 patients, and in one patient was found to be present in homozygosity. c.1051delG; IVS16+102_IVS17+31del; c.525delT; c.1548G>A; c.1822C>T mutations produce a very severe phenotypic spectrum.

The pathogenic variant p. Glu 176ArgfsTer45 (c.525delT) is especially common among the Dutch. It results in negligible GAA enzyme activity and must be considered one of the more severe alterations {PD – Gene reviews}. The same mutation can be observed in both infantile and late onset patients even if with different incidence; for instance, in two different groups of Italian patients, the c.525delT was observed in 13,8% in cases with the infantile form {Pittis et al.; 2007} but also in 3,8% of cases of late onset disease.
{Pittis et al.; 2008}.
c.701C>A; c.1396G>T; c.1561G>A; c.2746G>T mutations produce a potentially less severe phenotype spectrum. The c.510C> T mutation results to be non-pathogenic.

The c.1655t> C mutation was found in homozygosity in only one case and the resulting phenotype was classified as potentially less severe (Table 1).

Mutation (n=26)	Effect	Comment	Number	%
c.32-13T>G Intron 1	pot. mild		22	49%
c.1817A>G	not described yet		3 (Siblings)	6,7%
c.1051delG	Very severe	Frameshift	2	4,4%
c.1655T>C	pot. less severe		2 (homozygot)	4,4%
c..2799ins466bp (IVS18-3C>G)	not described yet		2 (homozygot)	4,4%
IVS16+102_IVS17+31del	Very severe	lost 55 AS	2 (Siblings)	4,4%
c.307T>G	pot. less severe		1	2,2%
c.510C>T	SNP, non pathogenic		1	2,2%
c.525delT	Very severe	Frameshift	1	2,2%
c.701C>A	pot. less severe		1	2,2%
c.982del7	not described yet	Frameshift	1	2,2%
c.1128_1129delinsC	Very severe	Frameshift	1	2,2%
c.1143delC	not described yet	Frameshift	1	2,2%
c.1370C>T	pot. mild		1	2,2%
c.1396G>T	pot. less severe		1	2,2%
c.1447G>A	Less severe		1	2,2%
c.1548G>A	Very severe	Stop-Mutation	1	2,2%
c.1548G>T	not described yet		1	2,2%
c.1561G>A	pot. less severe		1	2,2%
c.1754G>A	Non pathogenic		1	2,2%
c.1822C>T	Very severe	Stop-Mutation	1	2,2%
c.1933G>A	pot. less severe		1	2,2%
c.2214G>A	Very severe	Stop-Mutation	1	2,2%
c.2481+102_2646+31del	not described yet		1	2,2%
c.2702T>A	pot. less severe		1	2,2%
c.2746G>T	pot. less severe		1	2,2%

Tab.1 The c.32-13T> G is present in heterozygosis in 22 patients, with residual enzyme activity, enough to prevent the onset of severe phenotype.

The c.32-13T> G mutation was found associated with a second mutation classified as very severe (c.2214G> A; c.1051delG;; c.1822C> T; c.1548G> A; IVS16 + 102_IVS17 + 31del c.525delT) in 8 patients, while in half of the cases this mutation was associated with a second mutation classified as potentially less severe (c.1561G> A; p.E521K; c.2746G> T, p.Val916Phe; .1396G> T; c.701C> A; p.Thr234Lys) (Fig.3).

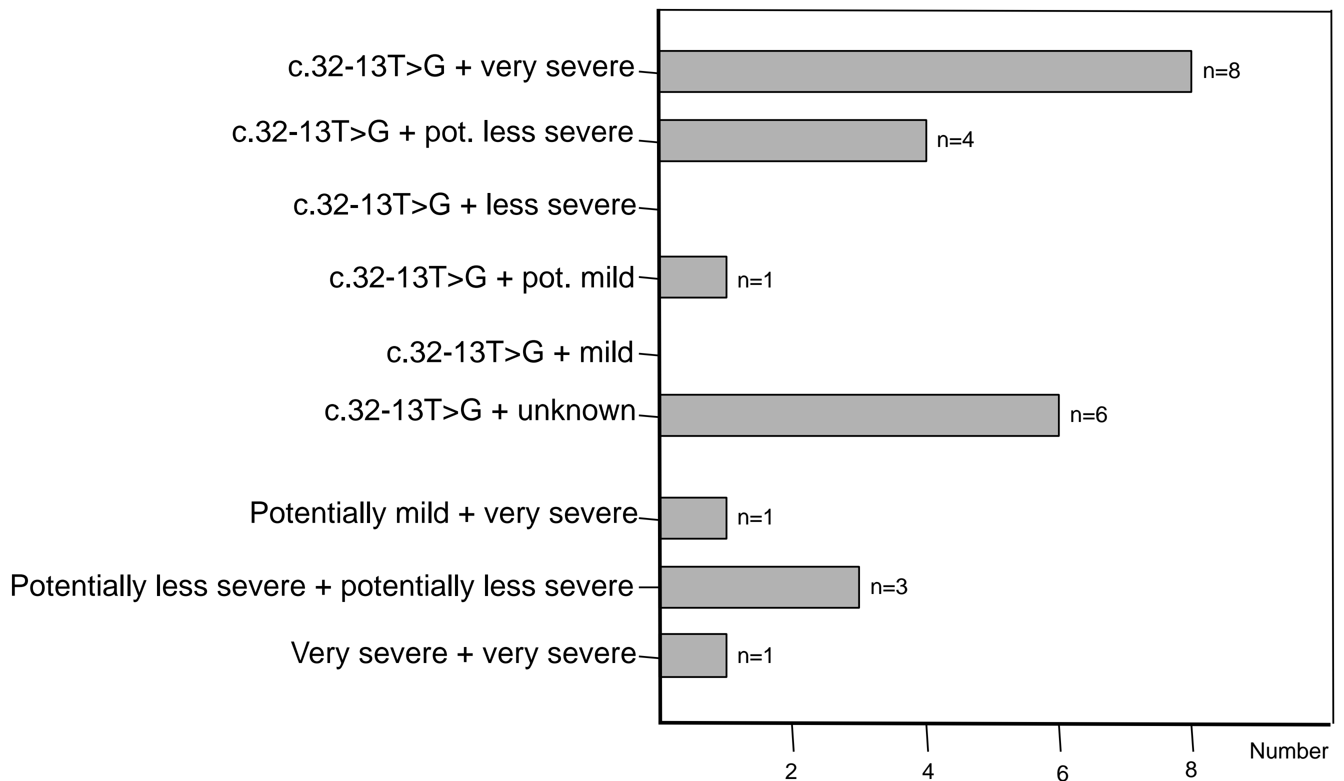


Fig.3 Association of c.32-13T> G mutation to other very severe or potentially less severe mutations.

We have seen that where the c.32-13T> G mutation was associated with a mutation classified as very severe early symptoms appear for the most part within the first year of age, even if out of a total of 8 patients, 2 were asymptomatic.

When the c.32-13T> G mutation was associated with a mutation classified as potentially less severe the peak appears to be between 1 and 5 years of age. Whereas the c.32-13T> G mutation is associated with a second one classified as potentially mild (1 case) or mutations whose effect is unknown (6 cases) the onset of the early symptoms is less homogeneous, even here the main incidence seems to be included within the first year of life (Fig.4).

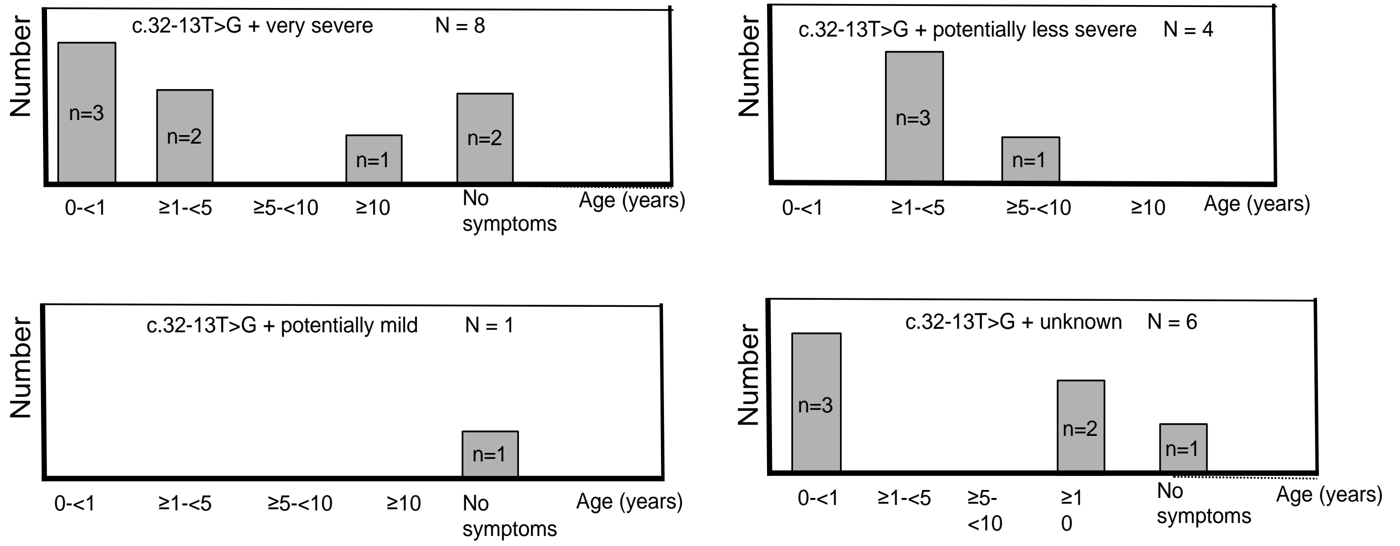


Fig.4 Different onset of symptoms related to various mutation correlations.

Conclusions

Pompe disease is a rare condition that affects about 1: 40.000 people. The infantile-onset form may be easier to detect, because the peculiar symptoms indicate a specific diagnosis, but in its juvenile form this disease can be difficult to diagnose because symptoms appear gradually, and they could be confused with other diseases. Many signs and symptoms are not unequivocal and there is therefore the possibility to think initially of more common conditions.

Biological indicators of non-classic Pompe disease are for example increased creatinine kinase, LDH levels in blood, but normal levels do not exclude the disease {Winkel et al 2005}.

CK is a muscle enzyme involved in converting phosphocreatine and adenosine diphosphate to creatine and adenosine triphosphate to generate energy crucial for muscle function. When there is muscle injury and sarcolemma disruption, leakage of CK into the blood stream can occur, resulting in an elevation of sCK. An elevation in sCK can be associated with no symptoms or minor symptoms such as nonspecific cramps, myalgia, or fatigue {Chan et al.; 2017}.

the CK (creatinine kinase) levels are very high in the case of muscle damage, but they are high also in many other muscle disorders.

A diagnosis is particularly important for long-term management and prognosis. Clinically, Pompe disease encompasses a continuous spectrum of phenotypes, and the mutation profile is very heterogeneous and, although

some mutations are common in different ethics groups, most mutations are present in single individuals or small number of families. {Dardis et al.; 2013}.

Finding relationships between genotypic characteristics and clinical manifestations, to highlight some indicators that can help to predict the development of the disease is extremely important.

Approximately 400 mutations have been reported for the GAA gene, and mainly they are point mutations.

The intronic mutation c.-32-13T>G is present in 40-70% of the alleles in patients with the LO form of the disease and it is the most frequent (49%) found in the cohort of patients' subject of the present study.

It leads to the synthesis of three different aberrant splicing variants in which GAA exon 2 is partially or completely spliced out in addition to retaining small amounts of normal spliced mRNA. The consequence is that patients carrying this mutation express some residual enzyme activity, which may be enough to prevent the onset of severe phenotype and could explain the LO origin of the disease {Dardis et al.; 2013}. Severe mutations in heterozygosity with "mild" mutation led to an LO form of the disease.

What is clear is that for the most part the presence in heterozygosis of the c.-32-13T mutation> G leads to a very severe phenotype. Only in half of the cases the phenotype turns out to be potentially less severe.

In conclusion, is clear that the clinical picture is very variable but, in 50% of patients the early symptoms occur in the first few years of life.

The genotype-phenotype correlation is extremely difficult but patients without c.32-13T> G are more often symptomatic, have an early onset of symptoms and the achievement of motor milestones is less timely.

But, when this mutation is present, the second mutation appears to have no influence on the phenotype.

Extremely important is also to consider that some clinical differences among patients (as age of onset or presence of pain) can be related to their genotypes; So, have a clear general map of all the mutations and being able to get a connection with the various phenotypic spectra is the necessary basis to be able to identify the disease as quickly as possible.

The ability to correctly differentiate Pompe disease from other disorders is critical to minimizing diagnostic delays and optimizing patient outcomes.

Early diagnosis is crucial as treatment significantly improves these patients' long- term outcomes.

PART 2

Chapter 3

Innovative technologies for Pompe Disease Therapy: a new human recombinant enzyme produced in rice (Oryza sativa).

Introduction

The only treatment approved for PD at the moment is the enzyme replacement therapy with recombinant human GAA (rh-GAA). Although effective on some aspects of the disease, ERT has important limitations. First, the response to this therapy is quiet variable in different patients and some tissues such as skeletal muscles appear to be refractory to therapy. In previous studies we have observed alterations of the location of the mannose-6-phosphate receptor in PD patients' cells and variable uptake of the recombinant enzyme, with an apparent correlation with the degree of disease severity {Cardone et al, Pathogenetics, 2008}. Furthermore, the recombinant enzymes appear to be unstable both in vitro and in vivo (in the mouse model of PD and in treated patients). Finally, ERT is extremely expensive. The treatment of an individual patient can in fact cost up to hundreds of thousands of euros per year {Wyatt et al, 2012}.

An expression vector has been developed by Transactiva that drives the production of recombinant proteins in the seed endosperm at levels suitable for industrial use. The recombinant proteins produced in this way can be easily and economically isolated through the industrial processing of the seed.

Aim of the Project

Aim of this project is to investigate the efficacy of this new human recombinant enzyme genetically modified, produced in rice (Oryza sativa) R-rh- GAA.

In particular:

- Its characterization
- The study of cellular bases of the variable response to ERT
- The evaluation of the effect of pharmacological chaperones in improving the stability and efficacy of R-rh-GAA.

Results

The studies are performed in fibroblasts of patients with different clinical forms of PD and with a different response to enzyme replacement therapy (Table 1).

Patient ID	Mutation allele 1	Mutation allele 2	Phenotype
PD 1	**p.L552P**	**p.P79Rfs*12**	infantile-onset classic
PD 2	**p.R375L**	**p.B755Sfs*41**	infantile-onset classic
PD 3	**p.L552P**	**p.L552P**	Infantile-onset atypical

Table 1. PD patients carrying different mutations and with early-onset phenotypes.

Preliminary Data: Characterization of R-rh-GAA in vitro

First, I performed a concentration curve of R-rh-GAA in comparison with rh-GAA (Myozyme) in a cell-free system, to understand how this enzyme acted and if it was comparable to Myozyme. Both enzymes have got comparable activity (Fig. 1).

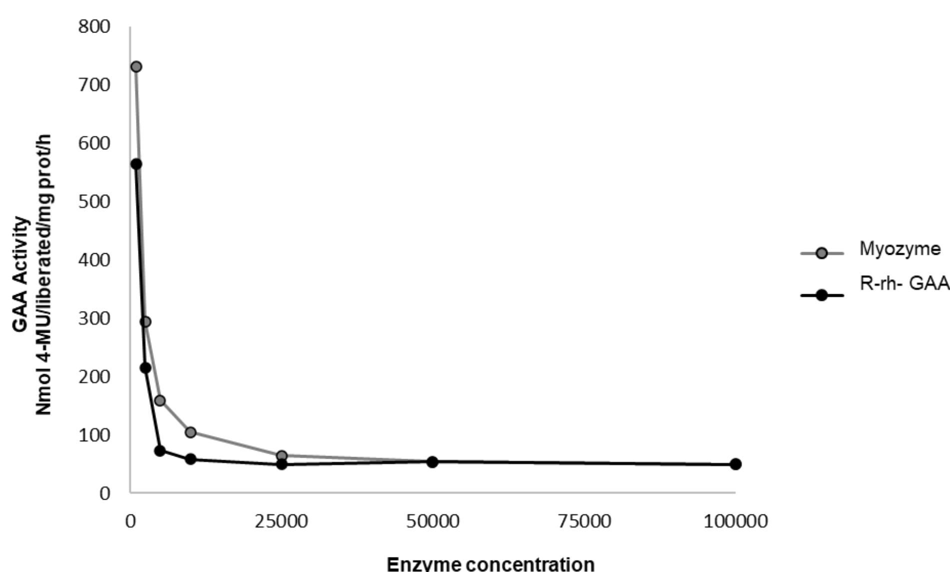
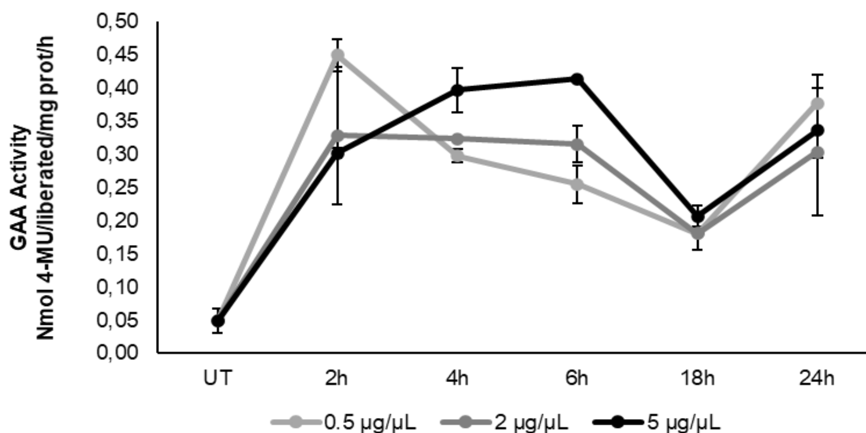


Fig 1. Comparison curve of rh-GAA and R-rh-GAA activity in a cell-free system. GAA enzymatic assay: The activities are expressed as nmoles 4-Methylumbelliferone liberated/mg protein/h.

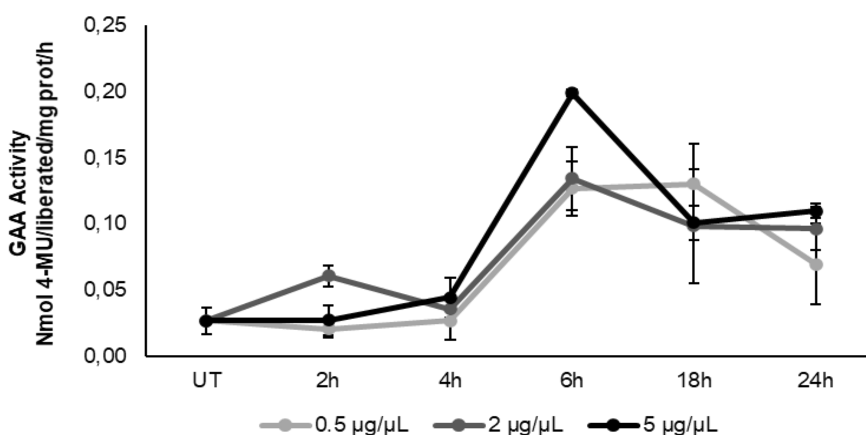
Then, fibroblasts from 2 PD Patients were incubated in the presence of 0.5 up to 5 μg/μL R-rh-GAA.

The results were compared to those obtained in untreated cells.

The enhancing effect of R-rh-GAA in this range of concentrations is modest and with significant effects on endogenous residual activity in the cells from patients 1 and 3 (Fig. 2).



PD1



PD3

Fig 2. GAA activity in PD patient fibroblasts treated with different concentrations of R-rh-GAA at different time points: cells were incubated with the enzyme and harvest after 2h,4h,6h,18h and 24h. The enzymatic activity was measured, and results were compared to those obtained in untreated (UT) cells.

Prolonged exposure to the enzyme, up to 72h and increasing concentrations of the enzyme (50μM Fig.3a and 100μM Fig.3b), show a negligible effect on the ability of the enzyme to correct GAA activity.

Some effect is visible treating cells with 100μg/μL for 48h (Fig.3b).

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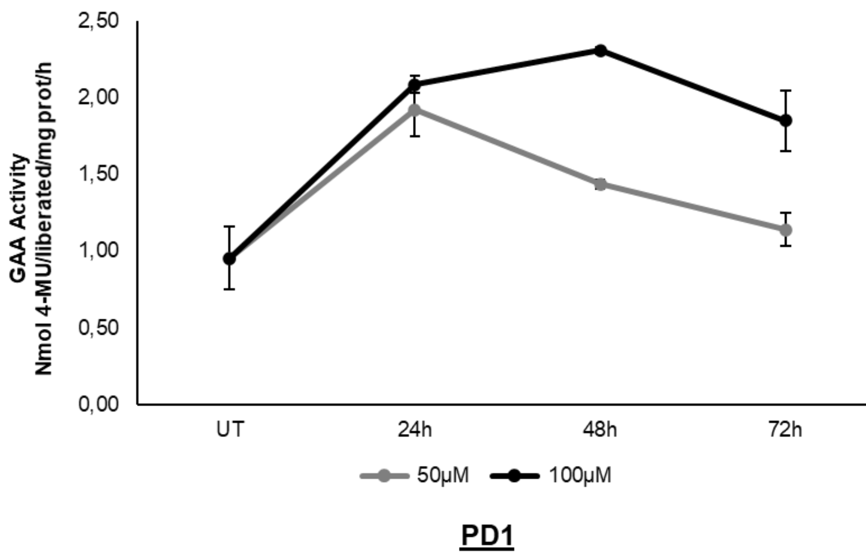

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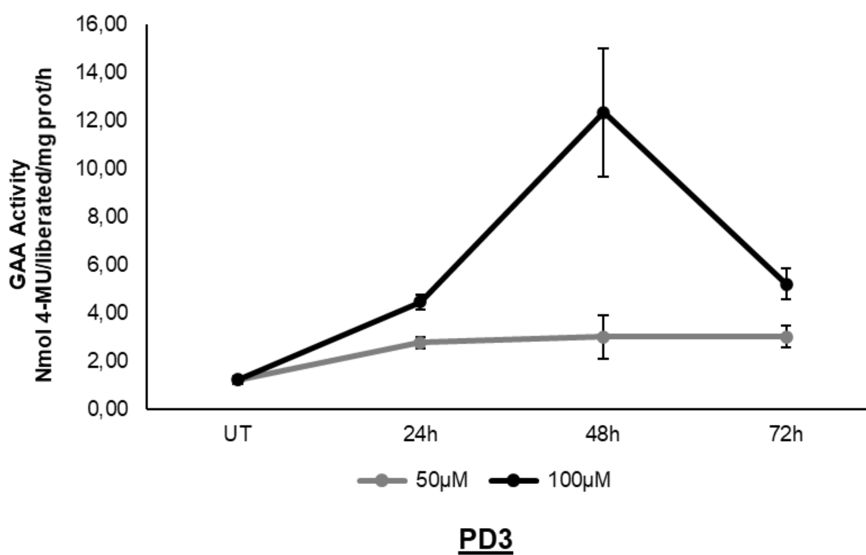

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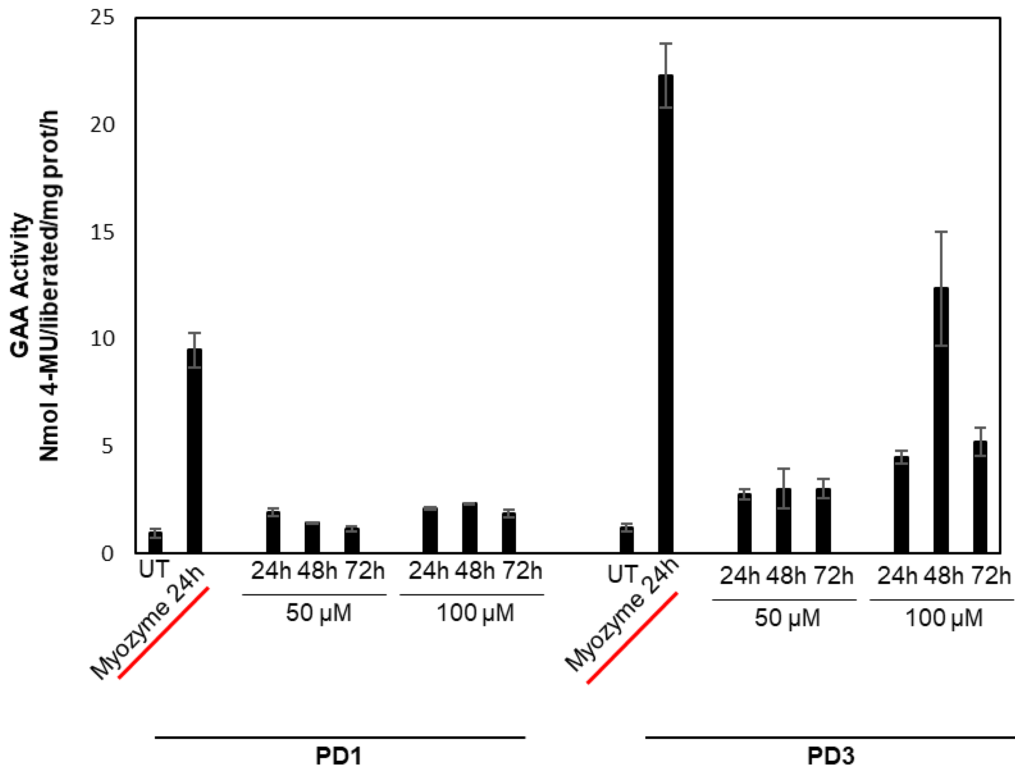
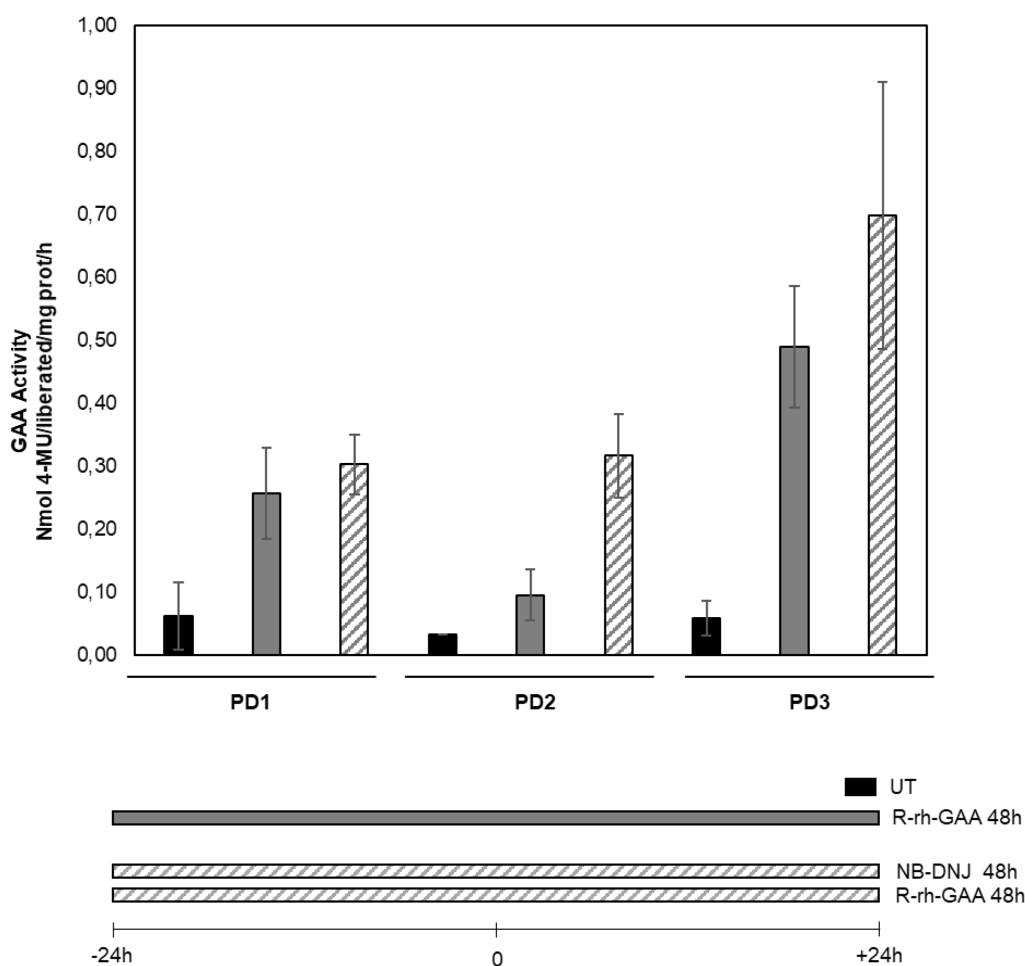


Fig.3 PD patients Fibroblasts treated with 50µM (Fig. 3a) and 100µM (Fig. 3b) of R-rh-GAA up to 72h.Treatment with 100µM for 24/48h shows some significant effect on residual activity in both patients. The effect of R-rh-GAA remains negligible if compared to myozyme (fig.3c).

I also tested the effect, on the activity of R-rh-GAA, of a pharmacological chaperone already approved and used for Pompe Disease, NB-DNJ.

I performed the experiments treating cells, always fibroblasts of 3 PD patients, for 48h, and I compared the enzymatic activity of R-rh-GAA alone and in co-administration with NB-DNJ (Fig. 4a). There is an increase due to the combined effect with chaperone but, despite this, the enzymatic activity of R-rh-GAA remains poor when compared to Myozyme (Fig. 4b).

a



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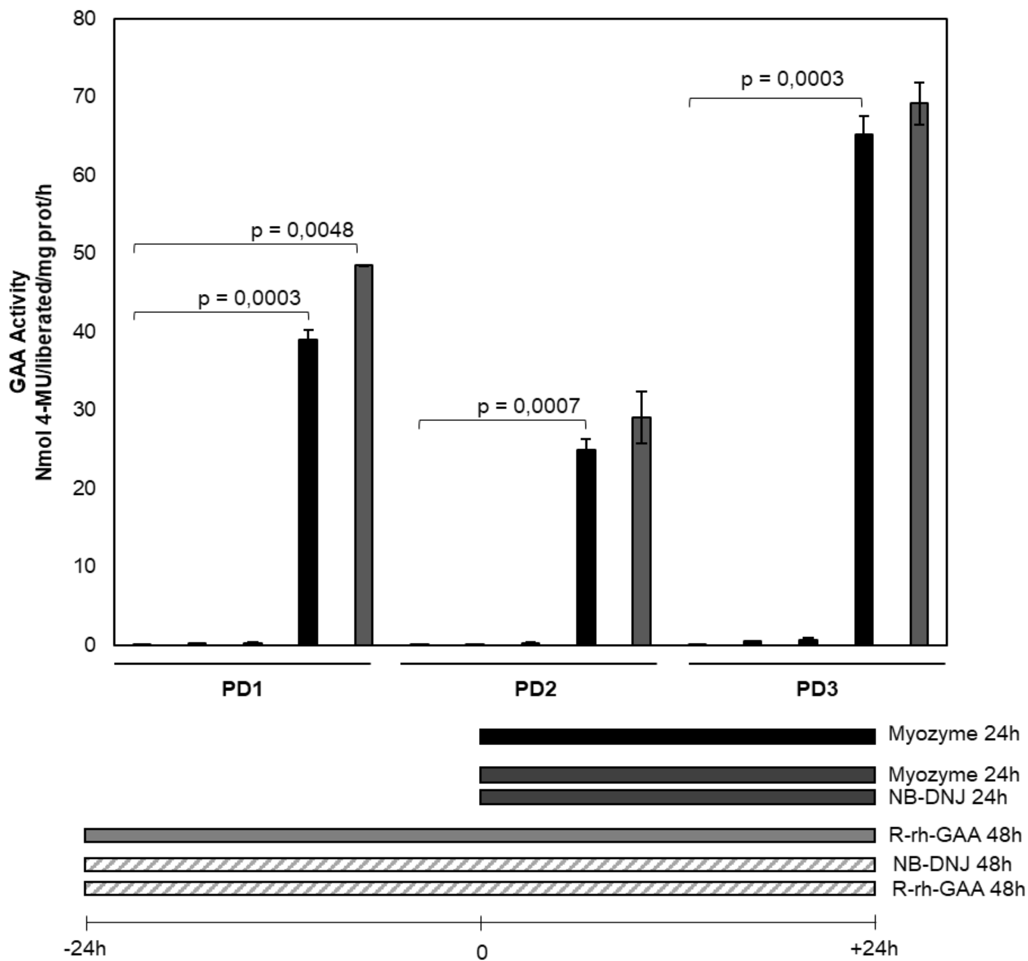


Fig.4 Treatment with 20μM NB-DNJ combined to 100μm R-rh-GAA for 48h since it was seen previously to be the most effective combination (Fig. 4a).

In Fig. 4b the same experiment represented in the presence of treatment with Myozyme, where the differences in efficacy are clear and evident.

Conclusions

Transactiva is a leader in the field of genetic engineering, with the production of genetically modified plants that respect the strictest directives in terms of eco-compatibility and disposal of plant and organic waste in general.

The identification of innovative solutions to the problems that the ERT currently presents is particularly urgent, first because it is fundamental to protect and increase the well-being of patients.

A significant alternative to the currently available therapies, represented by the development of an ERT based on a human GAA produced in plants, also had the purpose of leading to a substantial reduction of the costs related to the ERT, to lead to competitive advantages for the country, starting with a reduction of the financial burden for the National Health System.

Just the platform developed by Transactiva to produce lysosomal enzymes is exceptionally competitive with respect to current standards.

According to the preliminary results obtained, however, this new recombinant enzyme has not shown the expected efficacy. Probably we must investigate more deeply the interactions involved in cellular system, there may be an alteration in glycosylation, which can interfere with activity.

So that pursue with the in vivo experiments on the mouse model has been made unworkable, mainly for ethical reasons, in accordance with the European directives for the use of laboratory animals, to guarantee the respect of the requirements well-being of animals.

Chapter 4

Pharmacological allosteric chaperone of the human lysosomal α-glucosidase.

Introduction

Therapy with pharmacological chaperones has also been proposed as a complementary approach to ERT to try to overcome the strong limitations of this therapy. The combination of pharmacological chaperones and ERT has been translated into clinical application over the past 10 years.

Pharmacological chaperone therapy exploits small-molecule ligands that may bind directly to the defective enzymes, templating the protein folding on the most stable conformation(s) and preventing their recognition and disposal by the ERAD {Fan, 2008} {Parenti, 2009}.

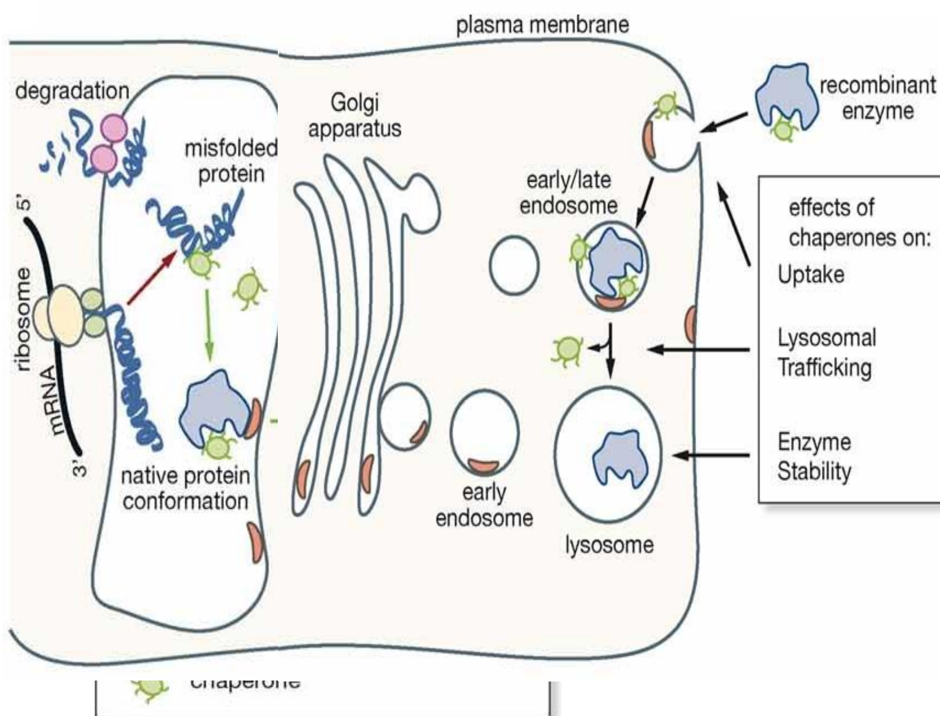
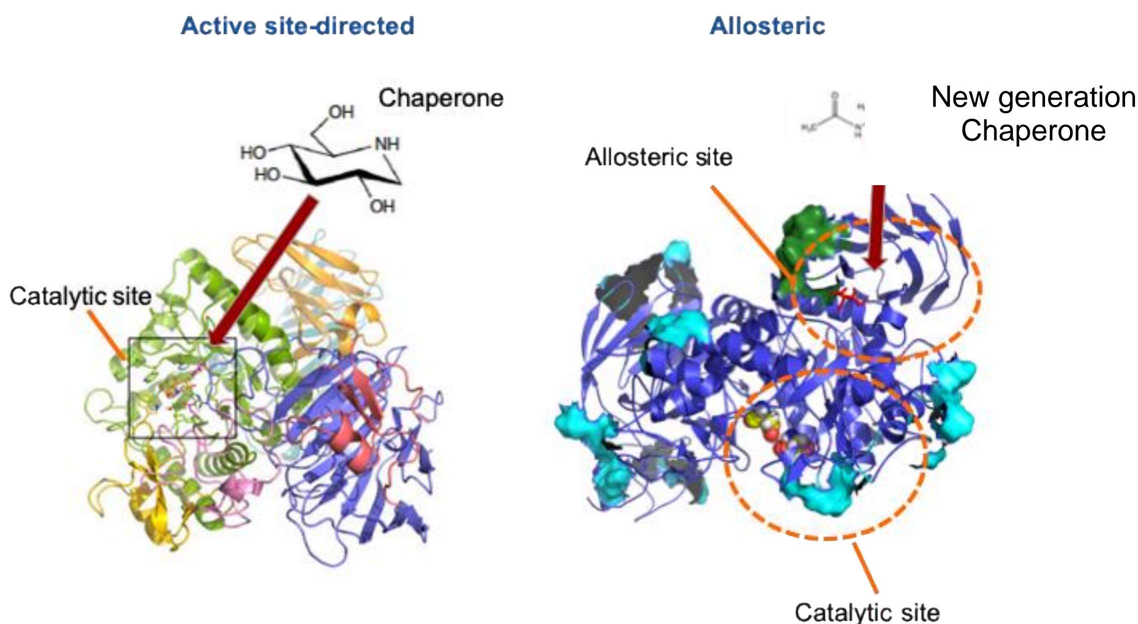


Fig.1 PC are small-molecule ligands that increase stability of mutated proteins and prevent their degradation.

Compared to ERT, small-molecule chaperones have important advantages in terms of biodistribution, oral availability, reduced impact on patients' quality of life.

Most chaperones proposed or used for the treatment of lysosomal storage diseases (LSD) are reversible competitive inhibitors of the target enzymes. Previous studies have shown that deoxynojirimycin (DNJ), N-butyl-deoxynojirimycin (NB-DNJ) and 1-deoxy-galactonojiirimycin (DGJ), may also potentiate the effects of the enzymes used for ERT in Pompe {Porto, 2009} and Fabry diseases. These active sites directed pharmacological chaperones (PCs) interfere with the activity of these enzymes {Valenzano, 2011} {Parenti, 2014}.

The paradox that an inhibitor can increase the enzymatic activity is explained as therapeutic levels can be reached at sub-inhibitory intracellular concentrations or because the PC inhibitor may be displaced from the active site by the high concentrations of the natural substrate accumulated in the lysosome or because of the acidic conditions within the organelle.



Adapted from Porto C, et al. *Mol Ther*, 2012; 20:2201-2211

Fig.2 Active site directed PCs interfere with the activity of enzymes.

Most of chaperones until now identified are reversible competitive inhibitors, as they bind the active site of the target enzyme.

An ideal chaperone should be able to protect the enzymes from degradation without interfering with its activity, be largely bioavailable in tissues and organs, reach therapeutic levels in cellular compartments where its action is required, show high specificity for the target enzyme with negligible effects on other enzymes, and have a good safety profile.

By a preliminary screening - conducted in collaboration with the biochemical research group of Prof Marco Moracci at Cnr, in Naples – of molecules already approved as pharmaceutical drugs and/or nutraceuticals that can be rapidly introduced in therapeutic treatments without the need of long and expensive clinical trials, L-Carnitine, a conditionally essential micronutrient and nutraceutical {Rebouche, 2004}, was identified as a possible target.

Aim of the project

Aim of this project is to investigate the mechanisms of action of novel chaperones, in particular:

- To characterize this putative allosteric chaperone L-Carnitine
- To study its effect on the uptake, maturation, and intracellular trafficking of recombinant enzymes.

Results

Effect of L-Carnitine on rh-GAA in cell-free system

To test L-Carnitine (L-CAR) on GAA, Roberta Iacono, of Prof Moracci's group, analyzed its effect on the pH stability of the enzyme as already performed in previous studies on lysosomal enzymes {Shen, 2008} {Porto, 2012}. In particular, she analyzed rh-GAA stability incubating the enzyme at different pHs and assaying the residual activity on 100 mM 4-nitrophenyl-α-D-glucopyranoside (4NP-Glc) in 100 mM sodium acetate buffer, pH 4.0 in which rh-GAA is stable for up to 24 hours. Instead, at pHs, either acidic (pH 3.0) or neutral (pH 7.0), lower and higher, respectively, when compared to the lysosomal compartment, the enzyme halved its activity in about 5 hours {Porto, 2012}.

L-CAR, already at the concentration of 10 mM, rescued the activity of rh-GAA on 4NP-Glc after 5h of incubation at pH 7.4 (Figure 1a).

Interestingly, L-CAR increased in dose-dependent manner also the structural stability of rh-GAA as analyzed by Differential Scanning Fluorimetry (DSF) (Figure 1b). The variations of the melting temperature (ΔT_m) increased by 2.4±0.1°C at 2 mM of L-CAR concentration (Figure 1c).

The dissociation constant of L-CAR for rh-GAA was measured by DSF (Figure 1d). L-CAR showed a K_D similar to that of the allosteric chaperone NAC (9.16 ± 1.02 mM and 11.57 ± 0.74 mM, respectively) {Roig-Zamboni, 2017}.

As expected for molecules that do not bind to the rh-GAA active site, these values are higher than the typical K_i of 3.4 ΔM exhibited by active-site directed molecular chaperones such as DNJ inhibitor {Porto, 2012}.

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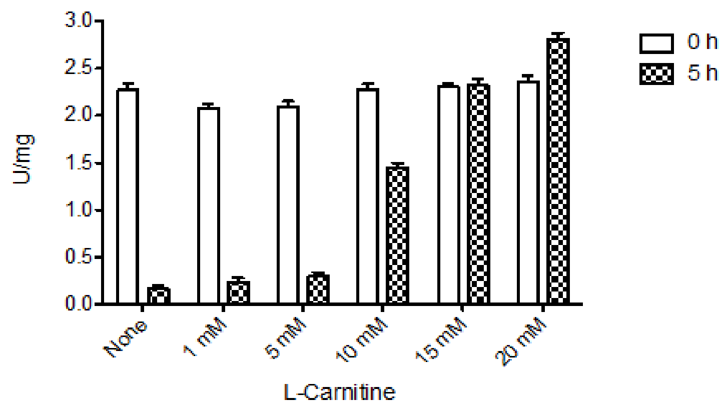

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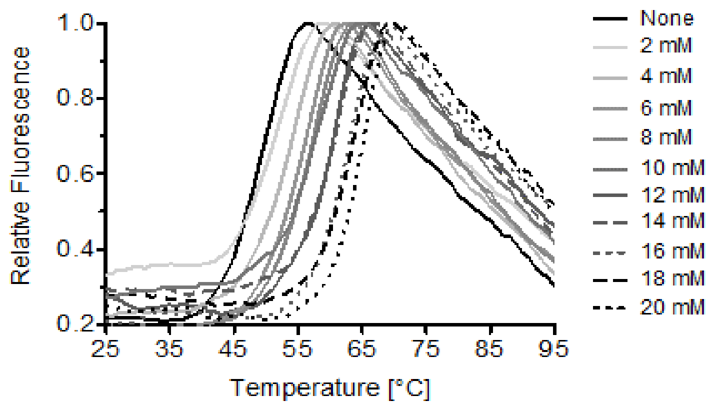

c

	Tm	DTm [°C]
None	48.6 ± 0.03	-
2 mM	51.0 ± 0.1	2.4 ± 0.1
4 mM	52.8 ± 0.1	4.2 ± 0.1
6 mM	54.7 ± 0.1	6.1 ± 0.1
8 mM	56.0 ± 0.2	7.4 ± 0.2
10 mM	58.6 ± 0.2	10 ± 0.2

	Tm	ΔTm [°C]
None	48.6 ± 0.03	-
12 mM	59.3 ± 0.1	10.7 ± 0.1
14 mM	59.9 ± 0.2	11.3 ± 0.2
16 mM	61.8 ± 0.4	13.2 ± 0.4
18 mM	62.8 ± 0.1	14.2 ± 0.1
20 mM	64.1 ± 0.1	15.5 ± 0.1

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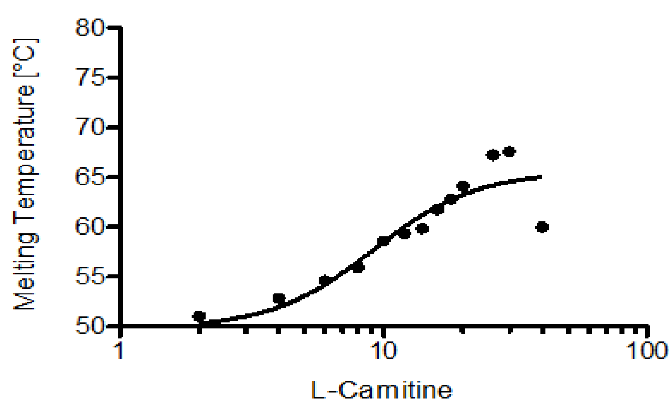


Figure 1. Comparison of the effect of L-carnitine on the stability of rh-GAA. L-CAR at various concentrations was incubated with rh-GAA and the enzymatic activity was measured after 5 h of incubation at pH 7.4 (Fig. 1a). To test the effect of L-CAR on the structural stability of rh-GAA L-CAR was incubated with rh-GAA at ten concentrations (from 2 to 20 mM) (Fig. 1b). Changes in the fluorescence of SYPRO Orange were monitored by DSF as a function of temperature at pH 7.4. (Fig. 1c). T_ms were calculated according to Niesen et al., 2007 {Niesen, 2007}. The standard deviations for each melting temperature were calculated from three replicates (Fig. 1d). *Determination of the K_D rhGAA-L-CAR by DSF.* For the determination of the dissociation constant (K_D) of L-CAR experimental data were best fitted according to a simple cooperative model equation reported in {Vivoli, 2014}.

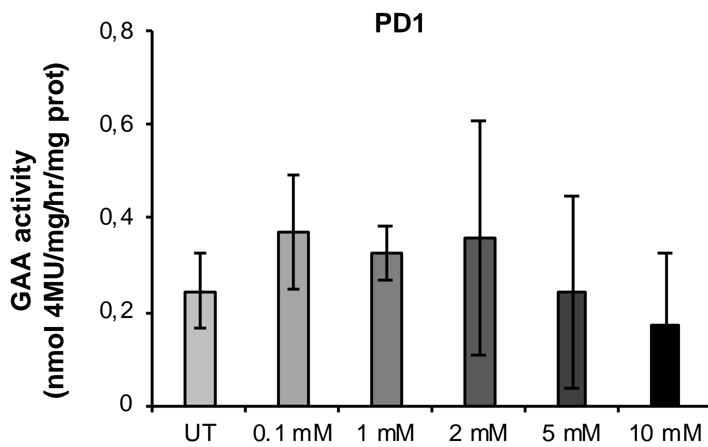
Dose effect of L-Carnitine on residual activity of Acid α-glucosidase in cultured PD patient cells

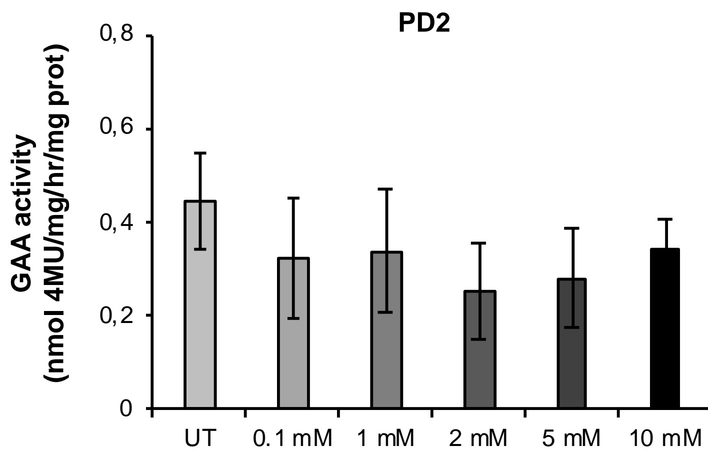
I investigate the effect of L-Carnitine on mutant GAA activity in cultured fibroblasts from three PD patients carrying different mutations and with early-onset phenotypes (Table 1).

Fibroblasts were incubated in the presence of 0.1 to 10 mM L-CAR for 24 hours and the results were compared to those obtained in untreated cells. The chaperone had negligible and non-significant effects on endogenous residual activity in the cells form patients 1 and 2, while significant enhancing effects were seen in patient 3, homozygous for the p.L552P mutation, that had been already reported to be responsive to the active site-directed chaperones DNJ and NB-DNJ {Parenti et al, 2007} (Figure 2). Significant increments in activity were observed in a range of L-CAR concentrations between 1 and 10mM, with a 2.8-fold increase at 2 mM.

Patient ID	Mutation allele 1	Mutation allele 2	Phenotype
PD 1	**p.L552P**	**p.P79Rfs*12**	infantile-onset classic
PD 2	**p.R375L**	**p.B755Sfs*41**	infantile-onset classic
PD 3	**p.L552P**	**p.L552P**	Infantile-onset atypical

Table 1. PD patients carrying different mutations and with early-onset phenotypes.





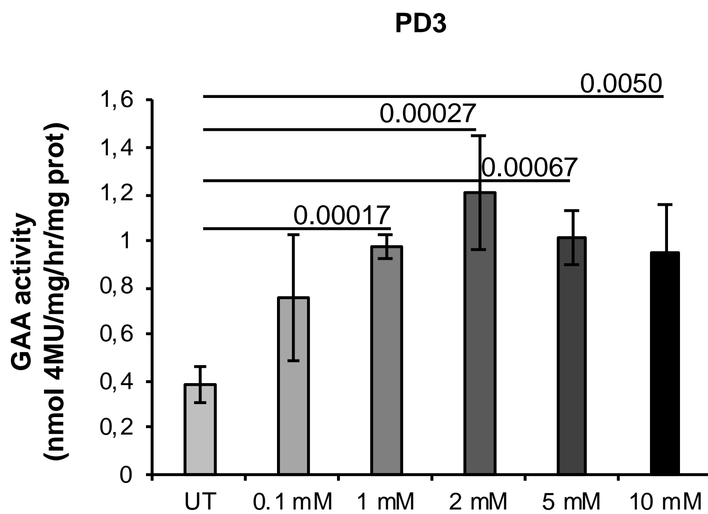


Fig2. Effect of L-CAR on the residual activity of mutated GAA in fibroblasts. Fibroblasts derived from three PD patients were treated for 24h with variable L-Carnitine concentrations before being harvested and used for GAA assay. The chaperone has significant effects on endogenous residual activity in the cells from patient 3.

The activities are expressed as nmoles 4-Methylumbelliferone liberated/mg protein/h.

Enhancing effect of L-Carnitine on correction of GAA activity by the recombinant enzyme rh-GAA used for ERT.

It has been previously shown that active site-directed chaperones also enhance the wild-type recombinant enzymes used for ERT in PD and Fabry disease {Porto, 2009}, with a synergistic effect. In PD fibroblasts the imino-sugar NB-DNJ enhanced rh-GAA efficacy by approximately 1.3 to 2-fold. An enhancing effect on correction of GAA activity by rh-GAA and enzyme processing was also demonstrated with the allosteric NAC {Porto, 2012}.

I tested whether the allosteric pharmacological chaperone L-Carnitine also shows a similar effect in combination with ERT in the three cell lines indicated above. I first studied the optimal conditions to evaluate this effect. I compared a protocol based on pre-incubation of cells with L-CAR for 24 hours, followed by co-incubation of L-CAR and rh-GAA for additional 24 hours, with a protocol based on co-incubation of L-CAR and rh-GAA for 24 hours (Figure 3a).

The results of both protocols were compared with those obtained in cells treated with rh-GAA alone. The second treatment protocol gave the best results and was selected to evaluate the optimal L-CAR concentration for rh-GAA enhancement. With the co-dosing of rh-GAA and L-CAR (1-20 mM) GAA activity enhancements were observed at 5,10- and 20-mM L-CAR concentrations (Figure 3b). The highest and statistically significant enhancements were obtained at 10 and 20 mM. Higher L-CAR concentrations (up to 50 mM) were toxic for fibroblasts (not shown).

I selected the concentration of 10 mM for further experiments, as this concentration appeared to combine efficacy and safety for cells.

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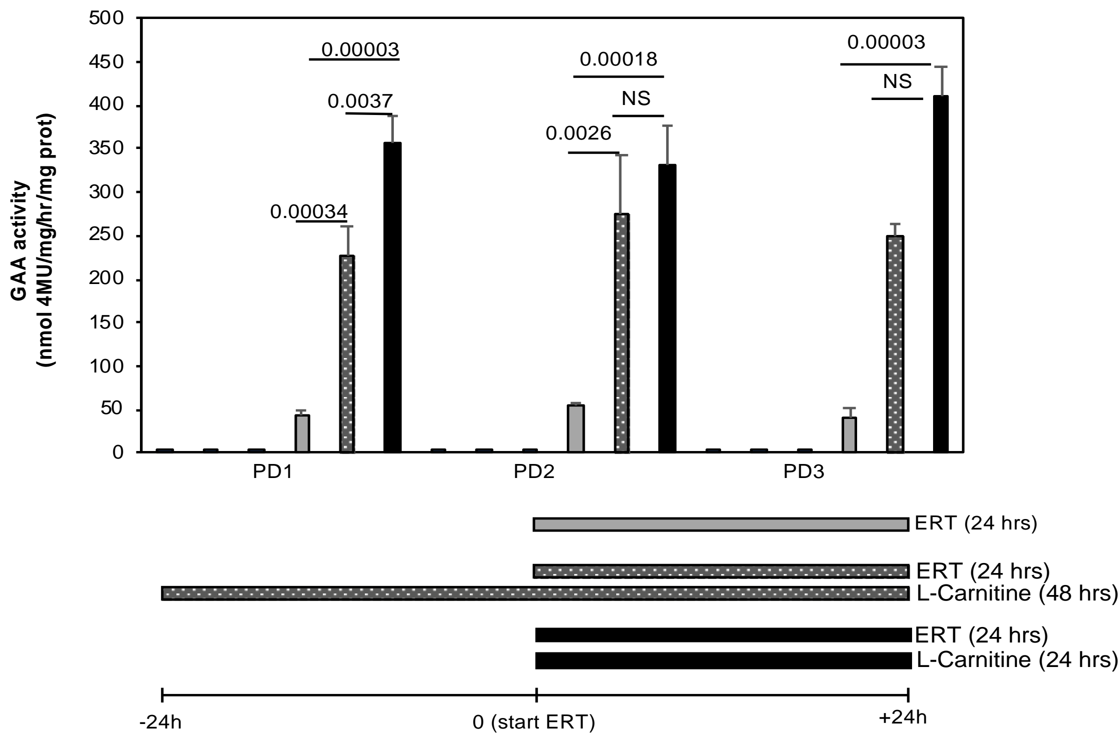

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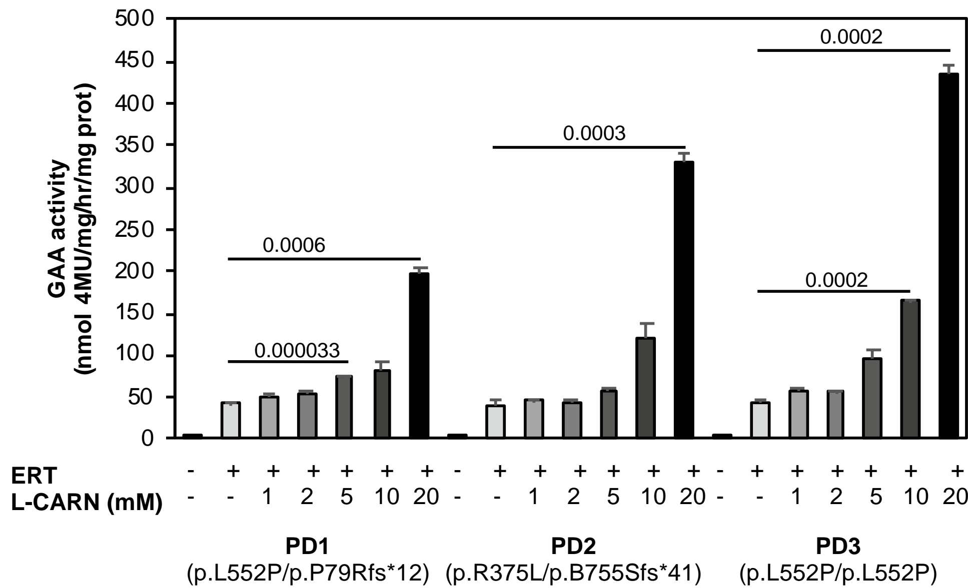

Fig. 3 Effect of L-Carnitine on rh-GAA. PD Fibroblasts incubated with 50µM rh-GAA in the absence and in presence of 10 mM L- CAR at different timings: rh-GAA alone for 24h (light grey bars), L-CAR for 48h of which the last 24h plus rh-GAA (dark grey bars) and rh-GAA plus L-CAR at the same time for 24h in total (black bars) (Figure 3a). Same fibroblasts treated with rh-GAA plus variable L-CAR concentrations (1mM, 2mM, 5mM, 10mM, 20mM) for 24h (Figure 3b).

L-Carnitine improves rh-GAA enzyme processing, and it increases GAA activity progressively over time.

For enzyme replacement therapy rh-GAA is provided by the manufacturer as a 110 kDa precursor. Once internalized by cells through the mannose-6-phosphate receptor and the endocytic pathways, the enzyme is converted into an intermediate of 95 kDa and the active molecular isoforms of 76 and 70 kDa. I investigated the effect of L-Carnitine on rh-GAA processing in PD1 and PD2 fibroblasts.

Cells were incubated for 24 hours with rh-GAA alone or with rh-GAA in combination with 10 mM L-Carnitine. In the cells treated with the combination of rh-GAA and L-Carnitine the amount of the 70-76 kDa mature GAA active peptides was dramatically improved (figure 4).

The corresponding GAA activities measured in PD1 and PD2 cells confirmed the enhancing effect of L-CAR and were in line with those observed in previous experiments.

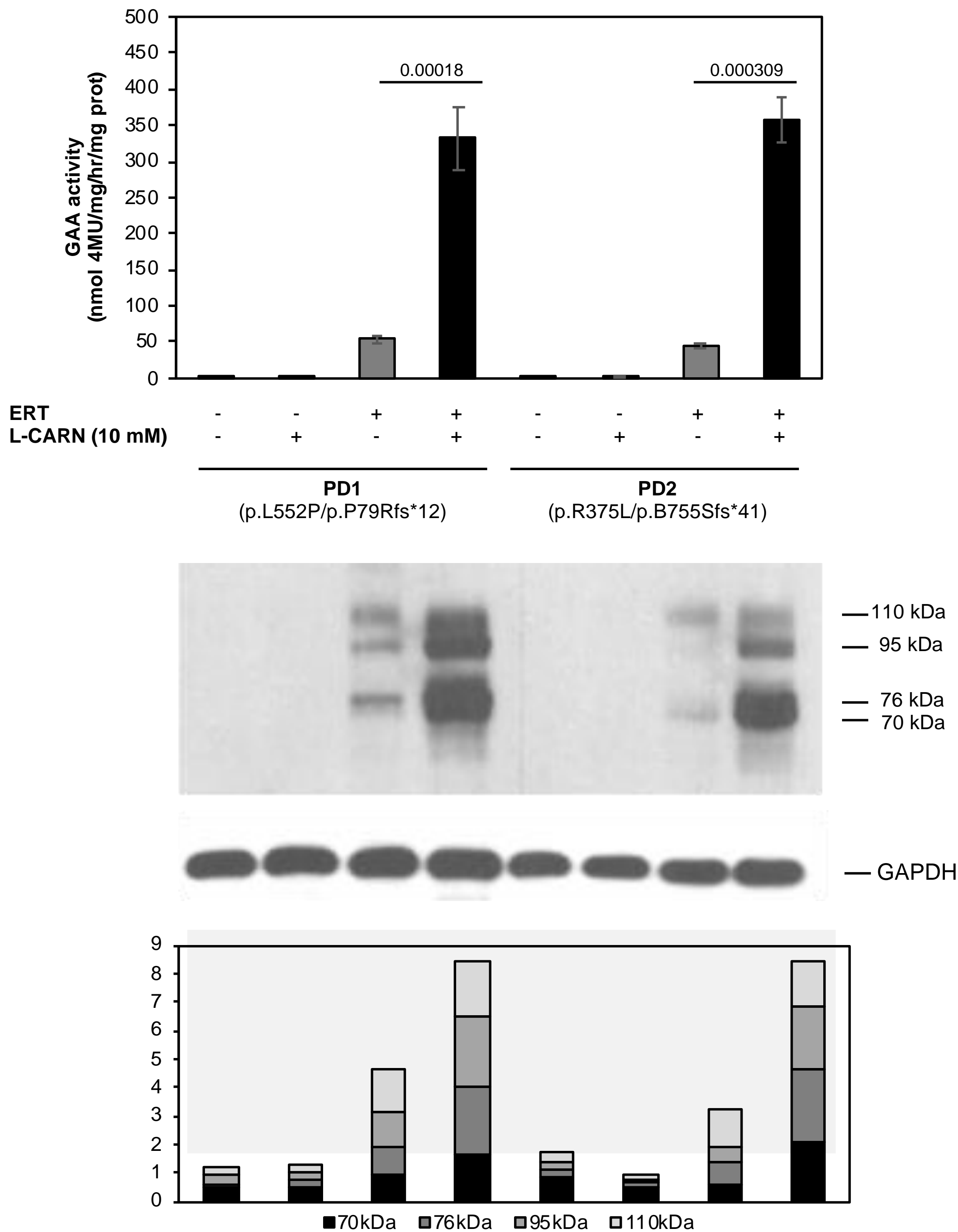

Fig 4. Effect of L-Carnitine on rh-GAA processing in PD fibroblasts. Cells were incubated for 24 hours with rhGAA alone or with rhGAA in combination with 10 mM L-CAR. In the cells treated with the combination of rhGAA and L-CAR the amount of the 70-76 kDa mature GAA active peptides was dramatically improved, as indicted by a quantitative analysis by western blot. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is the loading control. The increase of GAA activity confirms the enhancing effect of L-CAR**.**

I also looked at the kinetics of GAA enhancements at different time-points in PD fibroblasts treated with rh-GAA alone or in combination with 10 mM L-CAR. GAA activity increased progressively over time and an enhancing effect of co-incubation with L-CAR was already detectable at 2 hours and became progressively more pronounced up to 24 hours (Figure 5, left). The amounts and the processing of rh-GAA, analyzed by western blot, also improved over time (Figure 5, right).

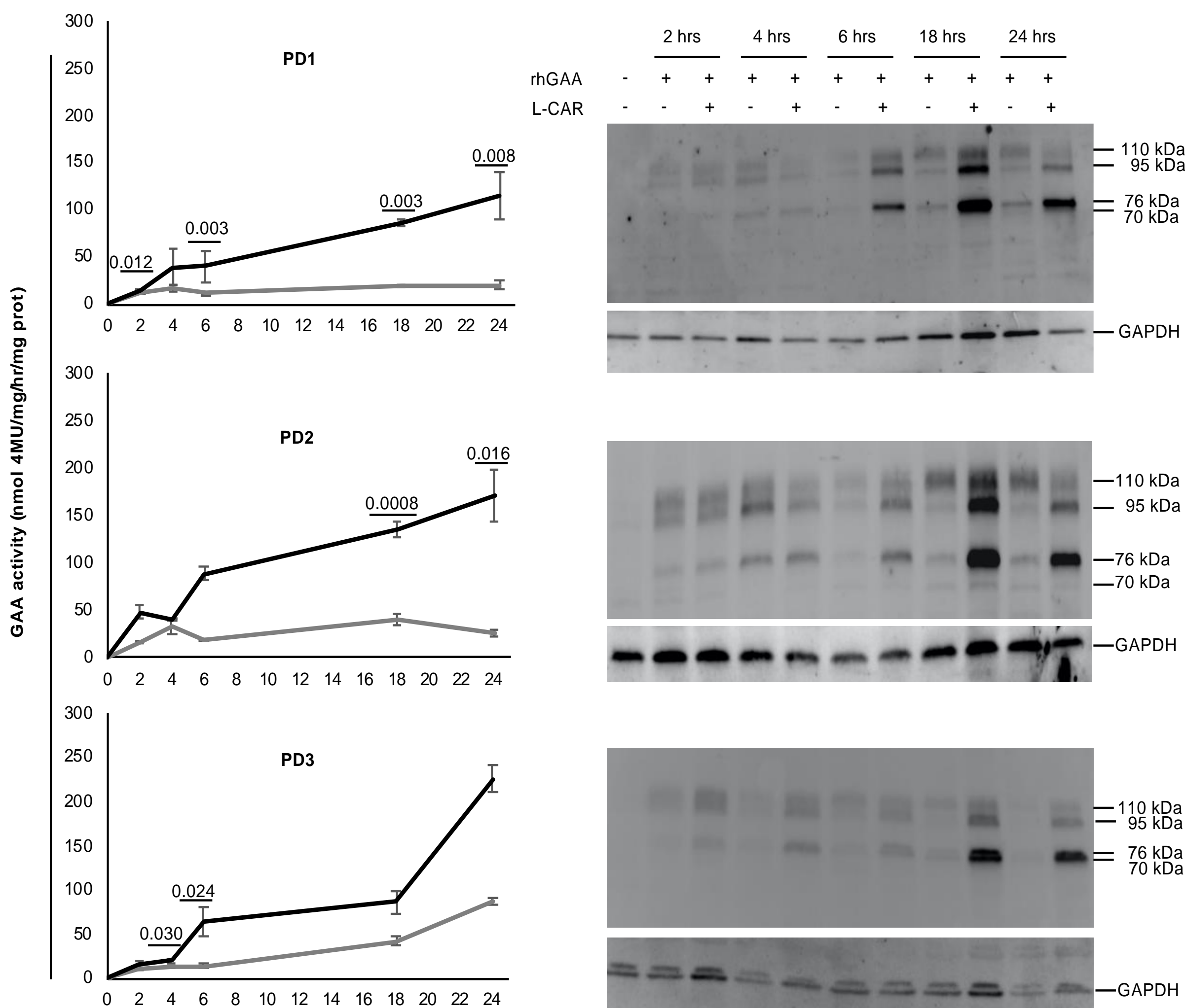

Fig. 5 rh-GAA + L-CAR time course. PD Fibroblasts were incubated with 50µM rh-GAA in the absence (grey lines) and in presence (black lines) of 10 mM L-carnitine for 2h, 4h, 6h, 18h and 24h. Substantial enhancement of rh-GAA correction by L-Carnitine is seen after 18-24h of incubation (left). Western blot analysis confirms these results (right).

Improvements of lysosomal trafficking of rh-GAA after treatment with L-Carnitine

I looked at the effects of rh-GAA and L-CAR co-dosing on lysosomal trafficking of the recombinant enzyme. The cells were incubated under the conditions selected in the previous experiments, and co-localization of rh-GAA with Lamp2 was analyzed by confocal immune-fluorescence microscopy. In all three cells lines the co-localization was improved (Figure 6). Mostly in PD2 the chaperone improved rh-GAA localization to lysosomes.

This result is confirmed by a quantitative analysis of total GAA signal and of GAA signal co-localized with Lamp2 performed by ImageJ Software.

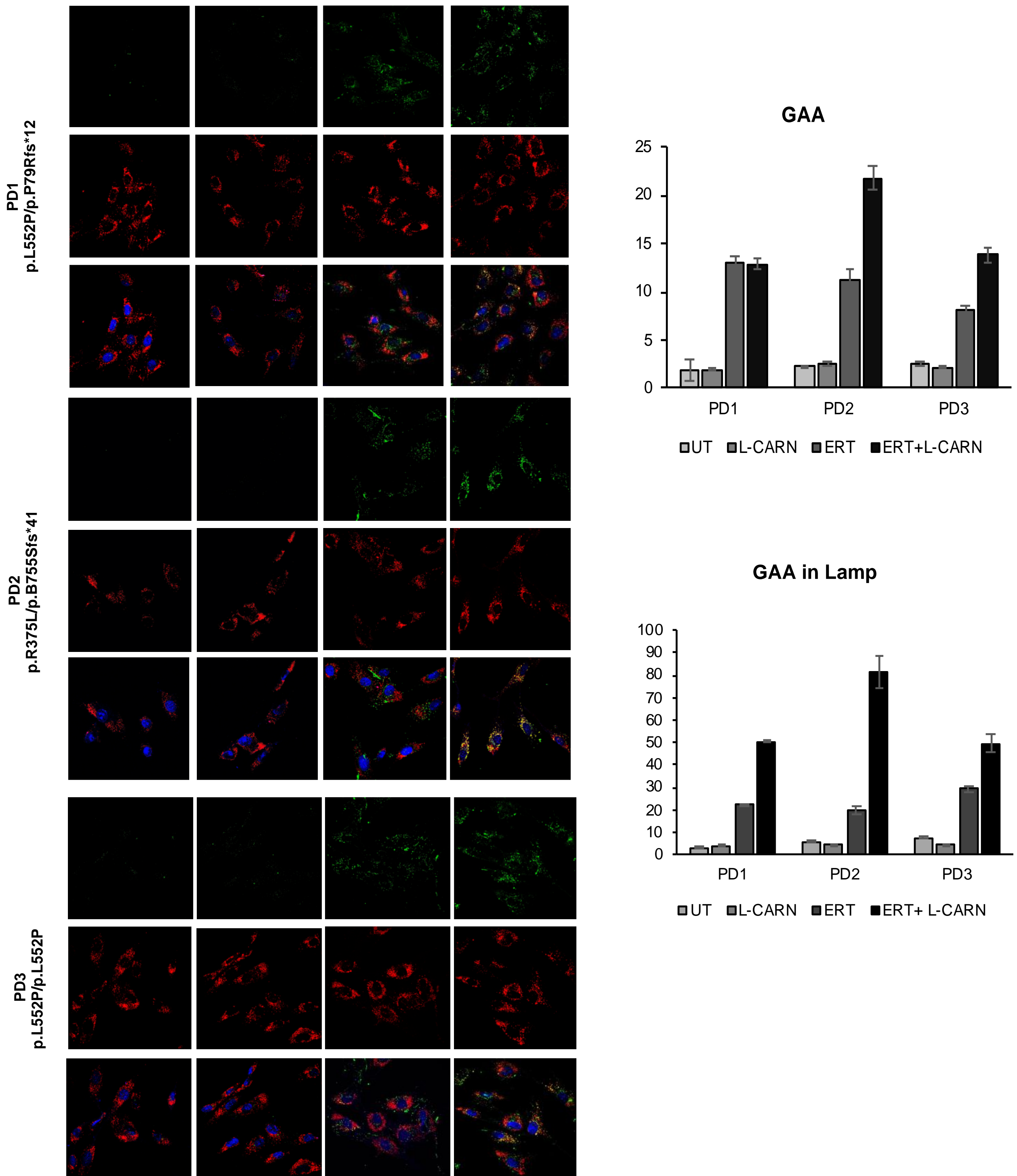

Fig 6. Confocal analysis of immunolocalization of rh-GAA (green) and the lysosomal marker Lamp2 (red) in PD cells treated with rh-GAA ± 10mM L-Carnitine. The images represent the results obtained after 24h of incubation. It is shown the merged images of double staining, with magnification x63. Quantitative analysis of rh-GAA and Lamp2 colocalization confirm this data.

Conclusions

The problems connected to the inhibitory activity of Pharmacological chaperones (PCs) currently used in clinics for Lysosomal Storage Disease can be addressed by the identification of novel allosteric chaperones, that, not binding to the active site of the enzyme, are non-inhibitory and can be potentially more effective than active site directed PCs. Another limitation of inhibitors acting as PCs is that they are effective in rescuing only some disease-causing missense mutations, mainly located in the catalytic enzyme domains, and are thus potentially effective only in a limited number of patients.

In this study is shown that L-Carnitine can stabilize GAA without interfering with its activity. In cell-free assays, these PCs prevented the loss of GAA activity at pH 7.0 and increased the enzyme thermal stability in a concentration dependent manner.

The crucial experiment demonstrating the efficacy of L-CAR on Pompe Disease was the correction of the enzyme defect in patient’s fibroblasts at a greater extent than that observed with NB-DNJ. When the recombinant enzyme was administered to patient's fibroblasts in combination with L-CAR, the lysosomal trafficking, the maturation, and the intracellular activity of the enzymes increased concretely.

The use of L-Carnitine as drug for the treatment of Pompe Disease is particularly attractive. L-Carnitine is involved in fatty acid metabolism and synthesized mainly in the liver and kidneys from the essential amino acids

lysine and methionine as ultimate precursors to form trimethyl lysine. L-CAR is not toxic at the concentration normally administrated and its use is approved as nutraceutical.

The synergy between L-Carnitine and ERT demonstrated here may be translated into improved clinical efficacy of ERT, as described for Pompe and Fabry diseases {Shen, 2008} {Porto, 2009 }. It is worth noting that, while the activity enhancement of endogenous defective enzymes by chaperones in most cases resulted in minor changes in terms of residual activity, likely leading to a modest impact on patients' outcome, the synergy between ERT and L-CAR based PCT has the potential to determine remarkable increases of specific activity, independently of mutations affecting individual patients.

Materials and methods

Fibroblast cultures

Human PD fibroblasts were already available at the cell Bank of the Department of Translational Medical Sciences (DISMET), Section of Pediatrics, Federico II University, Naples. They are derived from skin biopsies after obtaining the informed consent of patients.

All cell lines were grown at 37°C with 5% CO_2 in Dulbecco's modified Eagle's medium (Invitrogen, NY, USA), supplemented with 20% fetal bovine serum (Invitrogen, NY, USA), 2 mM/L glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin.

Reagents

Rh-GAA (Algucosidase α, Myozyme). As source of enzyme, I used the residual amounts of the reconstituted recombinant enzyme prepared for the treatment of PD patients at the Department of Translational Medical Sciences of the University of Naples, 'Federico II'. Myozyme is supplied by Sanofy Genzyme.

R-rh-GAA human recombinant enzyme, genetically modified, produced in rice (Oryza sativa) in Buffer solution 20mM Phosphate pH 6.2 plus 2% Mannitol was developed, produced, and supplied by Transactiva Molecular Farming.

L-Carnitine Hydrochloride was purchased by Sigma-Aldrich.

NB-DNJ (N-Butyldeoxynojirimyc-HCl) was purchased by Santa Cruz Biotechnology.

Cell lysates

Cells were plated at a density of $3x10^4$ cells/cm^2 in DMEM + 20% FBS + 2 mM L-glutamine and 100 U/ml Pen/Strep at 37°C. The cells were washed with PBS and detached by trypsinization, and centrifuged at 10.000 rpm for 10 minutes at 4°C.

The cell pellets were resuspended in H_2O and disrupted by 5 cycles of freezing and thawing. Lysates were centrifuged at 13.000 rpm for 10 min at 4°C. Protein concentration was measured in according to Lowry method.

Incubation of fibroblasts with recombinant enzymes and GAA assay

To study the rh-GAA & R-rh-GAA uptake and correction of GAA activity in PD fibroblasts, the cells were incubated with 50 μM rh-GAA for 24 h, 100μM R-rh-GAA for 48 h, in the absence or in the presence of 10 mM L-Carnitine and / or 4 mM NB-DNJ. Untreated cells were used for comparison.

GAA activity was assayed by using the fluorogenic substrate 4-methylumbelliferyl-alpha-D-glucopyranoside (4MU) (Sigma-Aldrich) according to a published procedure {Porto, 2009}.

Cell homogenates were incubated with the fluorogenic substrate (2 mM) in 0.2 M acetate buffer, pH 4.0, for 60 minutes in incubation mixtures of 20 μl. The reaction was stopped by adding 1 mL of glycine-carbonate buffer 0,5M pH 10.7. Fluorescence was read at 365 nm (excitation) and 450 nm (emission) on a Promega GloMax Multidetection system fluorometer.

Western blot analysis

Fibroblast extracts (20 µg protein) denatured by heat (5 minutes at 95°C) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10% polyacrylamide) and proteins were transferred to NC membranes. The membrane was incubated with blocking solution (0,1% TBS (Tris Buffered Saline) Tween-20, 5% not fat dried Milk) for 1 h. The membrane was then incubated with the primary anti-GAA antibody (PRIMM 1:2000 diluted in blocking solution) overnight at 4°C, anti-GAPDH antibody (1:2000 diluted in blocking solution) washed three times with 0.1% TBS tween-20 and incubated with appropriate secondary antibodies 1:5000 diluted in blocking solution. Immunoreactive proteins were detected by chemiluminescence. Quantitative analysis of band intensity was performed using ImageJ software.

Immunofluorescence analysis and confocal microscopy

For immunofluorescence studies, cells (human fibroblasts) grown on coverslips were fixed using methanol (5 minutes at -20°C to study the colocalization GAA-LAMP2), permeabilized using 1% PBS (phosphate buffered saline) – Triton 0,1% and blocked with 0.05% saponin, 1% BSA diluted in 1% PBS at room temperature for 1 h. The cells were incubated with the primary antibodies anti-GAA rabbit polyclonal antibody (PRIMM) and anti-LAMP2 mouse monoclonal antibody overnight at 4°C diluted in blocking solution, washed with 1% PBS and then incubated with appropriate auto

fluorescent secondary antibodies (anti-rabbit or anti-mouse antibodies conjugated to Alexa Fluor 488 or 596) and DAPI (4′,6-diamidino-2-phenylindole, Invitrogen) in 0.05% saponin, 3% BSA, 1% PBS. Samples were then washed, mounted with mowiol and examined with a Zeiss LSM700 confocal microscope. Colocalization and quantitative analysis were performed with Fiji (ImageJ) software.

Chapter 5

MicroRNA as Biomarkers in Pompe Disease

Introduction

Although extraordinarily effective in some patients and on some aspects of the disease (most notably survival and cardiomyopathy in infantile patients; motor and respiratory function in late-onset forms), in other patients ERT results in minor effects in specific muscles with signs of continuing disease progression. {Prater, 2012}.

The response of patients to Enzyme replacement therapy (ERT) with recombinant human GAA is highly variable.

A major issue in the monitoring of disease stage and therapeutic efficacy is the availability of objective and reliable tests that are not influenced by inter and intra-investigator variance. Currently, clinical tests (muscle strength, muscle function, patient-reported outcomes) are in common use. {Angelini, 2012}.

The aim is to identify new reliable biomarkers to monitor disease progression and efficacy of therapies, and to obtain information on the pathophysiology of Pompe Disease.

MicroRNAs are short non-coding RNAs that regulate gene expression. After processing, in their mature form they can bind and inhibit their target genes.

Recently, it has been seen that microRNAs can be released into the circulation and found in different micro vesicles, such as exosomes, necrotic cells and so on. They can be extracted from biological fluids, so they are easily detectable without invasive procedures.

Circulating microRNAs have already been proposed as biomarkers in different fields and in muscular diseases.

In addition, they can be used as targets for new therapies.

We explored the possibility to use microRNAs (miRNAs) as disease markers in Pompe Disease.

Results of this study have been published in the attached paper.

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microRNAs as biomarkers in Pompe disease

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Purpose: We studied microRNAs as potential biomarkers for Pompe disease.

Methods: We analyzed microRNA expression by small RNA-seq in tissues from the disease murine model at two different ages (3 and 9 months), and in plasma from Pompe patients.

Results: In the mouse model we found 211 microRNAs that were differentially expressed in gastrocnemii and 66 in heart, with a different pattern of expression at different ages. In a preliminary analysis in plasma from six patients 55 microRNAs were differentially expressed. Sixteen of these microRNAs were common to those dysregulated in mouse tissues. These microRNAs are known to modulate the expression of genes involved in relevant pathways for Pompe disease pathophysiology (autophagy, muscle regeneration, muscle atrophy). One of these microRNAs, miR-133a, was selected for further quantitative real-time polymerase chain reaction analysis in plasma samples from 52 patients, obtained from seven Italian and Dutch biobanks. miR-133a levels were significantly higher in Pompe disease patients than in controls and correlated with phenotype severity, with higher levels in infantile compared with late-onset patients. In three infantile patients miR-133a decreased after start of enzyme replacement therapy and evidence of clinical improvement.

Conclusion: Circulating microRNAs may represent additional biomarkers of Pompe disease severity and of response to therapy.

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Keywords: Pompe disease; microRNAs; miR-133a; Enzyme replacement therapy; Next-generation sequencing

INTRODUCTION

Pompe disease (glycogenosis type 2, OMIM 232300, ORPHA365, ICD-10 E74.0) is a metabolic myopathy caused by pathogenic variants of the *GAA* gene and deficiency of acid α-glucosidase (GAA), an enzyme involved in the lysosomal breakdown of glycogen.[1] The primary pathological hallmarks of Pompe disease are generalized glycogen storage, most prominent in heart and skeletal muscles, and accumulation of autophagic material in skeletal muscle fibers.[2]

Pompe disease is typically characterized by broad clinical variability, with a phenotypic continuum that ranges from infantile-onset forms, characterized by cardiomyopathy and rapidly progressive course, to late-onset phenotypes associated with attenuated course and predominant involvement of skeletal muscles. Central nervous system involvement and motor neuron dysfunction are emerging as additional features of Pompe disease that may contribute to cognitive decline in infantile-onset forms[3] and to respiratory insufficiency.[4]

Also patients' response to enzyme replacement therapy (ERT) with recombinant human GAA (rhGAA) is highly variable. Although extraordinarily effective in some patients and on some aspects of the disease (most notably survival and cardiomyopathy in infantile patients; motor and respiratory function in late-onset forms), in other patients ERT results in minor effects in specific muscles with signs of continuing disease progression.[5]

Due to this variability, assessing patient status and response to ERT is a critical issue in the management of Pompe disease. In this respect, a major challenge is the need for reliable, measurable, and objective disease markers that are not

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influenced by inter- and intrainvestigator variance. Particularly, the degree of muscle involvement remains difficult to assess. Currently, clinical tests (muscle strength, muscle function, patient-reported outcomes) are in common use to this purpose.[6] Although several of these tests have been specifically validated for Pompe disease,[7–9] they still present some caveats. In particular, some of them appear to be specific for subsets of patients and require specific medical skills and collaboration by patients. Key factors in evaluating the reliability of tests to assess muscular involvement in Pompe disease are their clinical relevance and the minimal clinically important difference,[9] which for some of these tests needs to be further established. Biochemical or imaging-based tests include the evaluation of the glucose tetrasaccharide (GLC4)[10] in plasma and urine, muscle ultrasound,[11] muscle nuclear magnetic resonance .[12] However, the clinical relevance of the latter tests also requires further assessment.

We explored the possibility to use microRNAs (miRNAs) as disease markers in Pompe disease. miRNAs are small noncoding RNAs that regulate gene expression by targeting messenger RNAs. miRNAs are able to concurrently target multiple effectors of pathways in the context of a functional gene network, thereby finely regulating multiple cellular functions involved in disease development and progression.[13–15] In several other disease conditions, including muscular dystrophies,[16] the analysis of miRNA expression has led to the discovery of altered pathways in response to disease,[17] and has highlighted potential targets of therapeutic intervention. Because miRNAs are variably dysregulated in these disorders, their expression profile may represent a potential biomarker in diagnostic and prognostic applications.

In this study, we comprehensively analyzed by next-generation sequencing (NGS)-based procedures the expression of miRNAs in muscle and heart of a Pompe disease murine model, and in patients' plasma, with the aim to identify tools to assess patient clinical conditions and response to treatments.

MATERIALS AND METHODS

A Gaa-/- knockout Pompe disease mouse model obtained by insertion of neo into the *Gaa* gene exon 6 (ref. [18]) was purchased from Charles River Laboratories (Wilmington, MA), and was maintained at the Cardarelli Hospital's Animal Facility (Naples, Italy).

Animal studies were performed according to the EU Directive 86/609, regarding the protection of animals used for experimental purposes, and according to Institutional Animal Care and Use Committee (IACUC) guidelines for the care and use of animals in research. The study was approved by the Italian Ministry of Health, IACUC n. 523/2015-PR (06/11/2015). Every procedure on the mice was performed with the aim of ensuring that discomfort, distress, pain, and injury would be minimal. Mice were euthanized following ketamine xylazine anesthesia.

miRNAs expression profiles were analyzed by small RNA-seq in the Pompe disease mouse model (Gaa-/-)[18] in comparison with wild-type animals. We analyzed two of the main disease target tissues, i.e., heart and gastrocnemius muscle, from Pompe disease and age-matched wild-type mice. The tissues were collected at different time-points (3 and 9 months) that reflect different stages of disease progression. After small-RNA sequencing, a bioinformatic analysis was carried out to assess the reliability and statistical relevance of data (Supplementary Fig. S1). The threshold value for significance used to define up-regulation or down-regulation of miRNAs was a false discovery rate (FDR) lower than 0.05.

Plasma samples from 52 Pompe disease patients were already available and stored at −80 °C at the biobanks of the seven collaborating centers (Department of Translational Medical Sciences Federico II University, Naples; Department of Neurosciences, Federico II University, Naples; Department of Neurosciences, University of Messina; Bambino Gesù Hospital in Rome; Centre for Rare Disease, Udine; Department of Neurosciences, University of Turin; Center for Lysosomal and Metabolic Diseases; and Department of Pediatrics, Erasmus University Medical Center, Rotterdam). In all patients the diagnosis was confirmed by enzymatic analysis and *GAA* gene sequencing. Plasma had been obtained according to standard procedures during periodic follow-up admissions to the respective hospitals. Patients (or their legal guardians) had signed an informed consent agreeing that the samples would be stored and used for possible future research purposes.

Samples from age-matched controls were analyzed for comparison. Pediatric control samples derived from residual and unused amounts of plasma collected for routine chemistry in patients undergoing minor urologic surgical procedures (phimosis, hypospadias), and did not require additional medical procedures. Patients or their legal guardians consented to the use of these samples for research purposes. Juvenile and adult control samples were obtained by healthy volunteers, who consented to the use of their blood for research.

Each patient code is composed of a two-letter code identifying the city of the collaborating center, and a progressive number assigned at the time of arrival at our lab.

Total RNA extraction preserving miRNA fraction

Total RNA, including small RNAs, was extracted using the miRNeasy Kit (Qiagen) according to the manufacturer's instructions. RNA was quantified using a NanoDrop ND-8000 spectrophotometer (NanoDrop Technologies) and the integrity was evaluated using an RNA 6000 Nano chip on a Bioanalyzer (Agilent Technologies). Only samples with an RNA integrity number (RIN) >8.0 were used for library preparation.

Small RNA-seq analysis in tissues

Small RNA libraries were constructed using a Truseq small RNA sample preparation kit (Illumina) following the manufacturer's protocol. Using multiplexing, we combined

up to 12 samples into a single lane to obtain sufficient coverage. Equal volumes of the samples that constituted each library were pooled together immediately prior to gel purification and the 147–157 bp products from the pooled indexes were purified from a 6% polyacrylamide gel (Invitrogen). Libraries have been quality-checked using a DNA 1000 chip on a Bioanalyzer (Agilent Technologies) and quantified using the Qubit® 2.0 Fluorometer (Invitrogen). The sequencing was carried out by the NGS Core Facility at TIGEM, Naples. Cluster generation was performed on a Flow Cell v3 (TruSeq SR Cluster Kit v3; Illumina) using cBOT and sequencing was performed on the Illumina HiSeq1000 platform, according to the manufacturer's protocol. Each library was loaded at a concentration of 10 pM, which we had previously established as optimal.

Small RNA-seq analysis in plasma

For plasma preparation EDTA was used as anticoagulant, both in patients and in mouse samples. Processing of samples from Pompe disease and control sets were conducted simultaneously to minimize batch effect. Prior to RNA extraction, a *C. elegans*-specific synthetic exogenous miRNA (ce-miR-39) was spiked in the samples as control for the extraction efficiency. RNA was isolated using the miRNeasy Kit (Qiagen) and reverse-transcription polymerase chain reaction (RT-PCR) was performed using miScript System (Qiagen). RNA recovery was assessed by comparing the Ct values (obtained with the assay targeting the synthetic miRNA) with a standard curve of the synthetic miRNA generated independently of the RNA purification procedure. After assessment of RNA recovery, equal amounts based on starting volume (3 μl) were used for the preparation of small RNA libraries, as described for tissues. NextSeq 500/550 High Output Kit v2 (75 cycles) Cd. FC-404-2005 was used and sequencing was performed on the Illumina NextSeq 500 platform, according to the manufacturer's protocol. Each library was loaded at a concentration of 1.8 pM, previously established as optimal.

Bioinformatics analysis

To identify differentially expressed miRNAs (DE-miRNAs) across samples, the reads were trimmed to remove adapter sequences and low-quality ends, and reads mapping to contaminating sequences (e.g., ribosomal RNA, phIX control) were filtered out. The filtered reads of tissue samples were aligned to mouse mature miRNAs (miRBase Release 20), while filtered reads of plasma samples were aligned to human mature miRNAs using CASAVA software (Illumina). The comparative analysis of miRNA levels across samples was performed with edgeR,[19] a statistical package based on generalized linear models, suitable for multifactorial experiments.

Gene Ontology and KEGG pathway enrichment analysis of miRNA targets, predicted by TargetsScan,[20] was performed using the goana and kegga functions of Limma,[21] with FDR <0.05 as threshold for significant enrichment.

Quantitative real-time polymerase chain reaction (qRT-PCR) of miRNA

Expression of mature miRNAs was assayed using Taqman Advanced MicroRNA Assay (Applied Biosystems) specific for miRNA selected for validation. qRT-PCR was performed by using an Applied Biosystems 7900 Fast-Real-time PCR System and a TaqMan Fast Advanced Master Mix. Primers for selected miRNAs were obtained from the TaqMan Advanced miRNA Assays. Samples were run in duplicate. Single TaqMan microRNA assays were performed according to manufacturer's instructions (Applied Biosystems). Differences in miRNAs expression, expressed as fold changes, were calculated using the 2-ΔΔCt method. In plasma samples, to calculate ΔCts values, the average of two normalizers was used: spike in ce-miR-39 and endogenous stable miRNA miR-93 (ref. [22]). Next, to calculate ΔΔCt values an average of five controls, each of them analyzed in three independent experiments, was used. miR-16 was used as endogenous normalizer in tissue samples.

In vivo experiments in the Pompe disease mouse model

Pompe disease mice received a single high-dose (100 mg/kg) injection of Myozyme into the retroorbital vein. Tissues (heart, gastrocnemius) were collected 48 h after the injection and miR-133 was analyzed by qRT-PCR as indicated.

The results obtained were compared with those obtained in control animals injected with equivalent volumes of saline. Four animals for each group were analyzed.

Statistical analysis

Group-wise comparisons (Fig. 4**a**) were performed by two-way analysis of variance (ANOVA) with Tukey post hoc test. A Student's *t* test was used for the statistical analysis of the data shown in Fig. 4**d** and Supplementary Fig. S2.

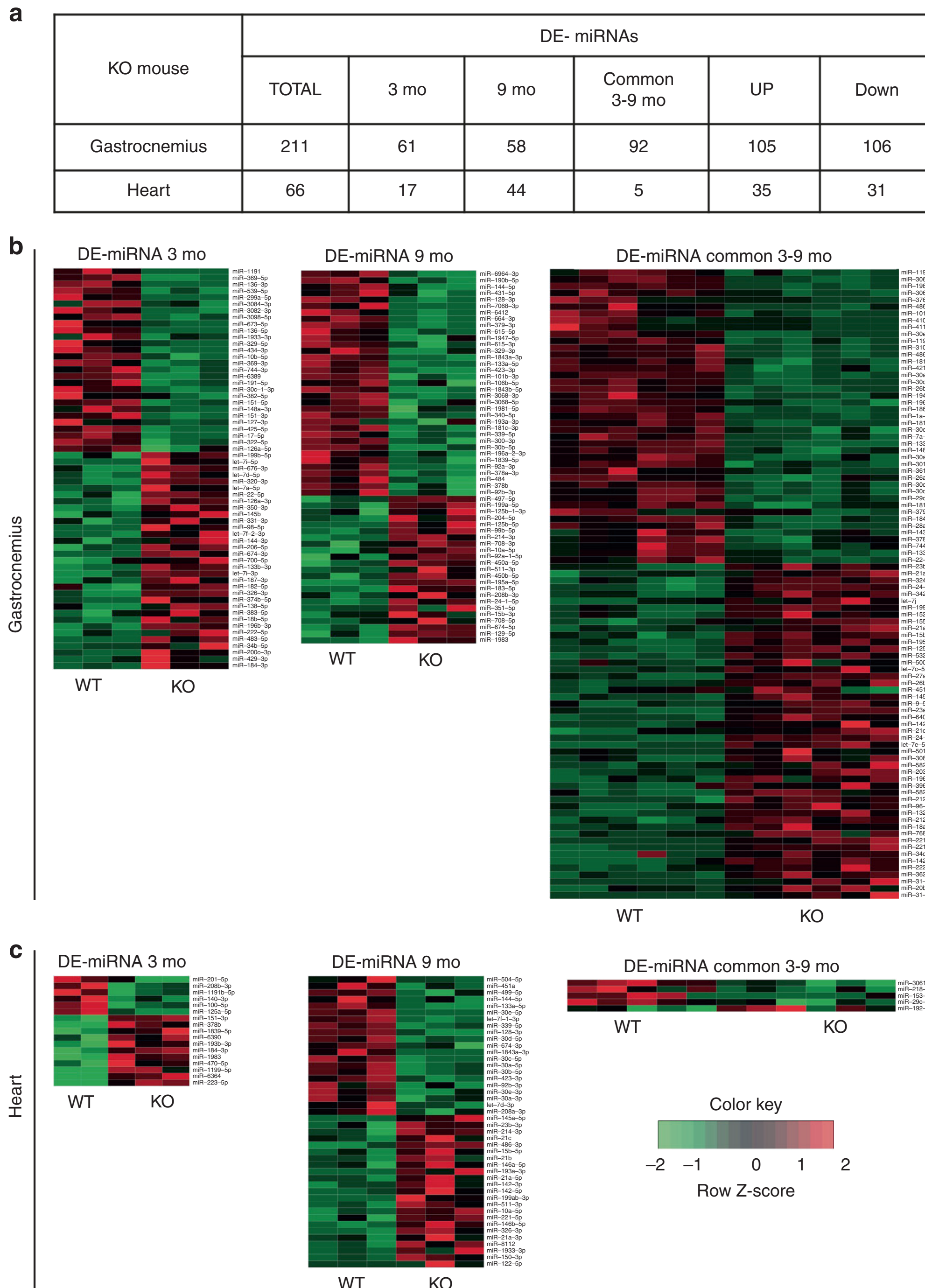
RESULTS

Global analysis of miRNA expression profiles in the skeletal muscle and heart of the Pompe disease mouse model

The small RNA-seq analysis in tissues from the Pompe disease mouse model generated a list of 277 miRNAs that were differentially expressed (DE-miRNA) in the two tissues examined (gastrocnemius and heart) with statistical significance, compared with control mice. The complete list and the results of the sequencing are provided in Supplementary Table S1. Figure 1 provides a summary of the results obtained in the two tissues examined and at the different time-points. The patterns of miRNA dysregulation varied depending on age, indicating changes related to disease progression, and tissues, suggesting tissue-specific involvement of different pathways. The DE-miRNA were either up-regulated or down-regulated.

Pompe disease patients show differential expression of circulating miRNAs

We then looked at plasma samples from patients affected by Pompe disease. We decided to perform a small RNA-seq



a

KO mouse	DE- miRNAs					
	TOTAL	3 mo	9 mo	Common 3-9 mo	UP	Down
Gastrocnemius	211	61	58	92	105	106
Heart	66	17	44	5	35	31

Fig. 1 Results of small-RNA-seq analysis in Pompe mouse model (knockout). a Summary of number of the differentially expressed microRNA (DE-miRNA) up- and down-regulated in gastrocnemius and heart at 3 and 9 months. **b** Heatmap of the DE-miRNAs identified in mice gastrocnemius at 3 and 9 months or common in both ages. **c** Heatmap of the DE-miRNAs identified in mice heart at 3 and 9 months or common in both ages. The colors indicate up-regulation (red) or down-regulation (green) of each DE-miRNA. KO knockout, WT wild-type

analysis on a representative subgroup of patients covering both infantile-onset (IOPD) and late-onset (LOPD) cases. We first ran a pilot small RNA-seq analysis in plasma samples from 6 patients (NA1, NA3, NA4, NA8, RD2, TO1) (3 IOPD, 3 LOPD), selected from 52 patients (information on the patients is provided in Supplementary Table S2) whose plasma samples were already available at the biobanks of the clinical centers involved in this study. Patients, all under ERT treatment, were selected for this preliminary analysis based on their phenotypes, current clinical condition, and age to have relatively homogeneous groups representative of both Pompe disease forms, and based on blood sampling time with respect to therapy (at least 10 days after the previous enzyme replacement infusion). The results obtained in IOPD patients

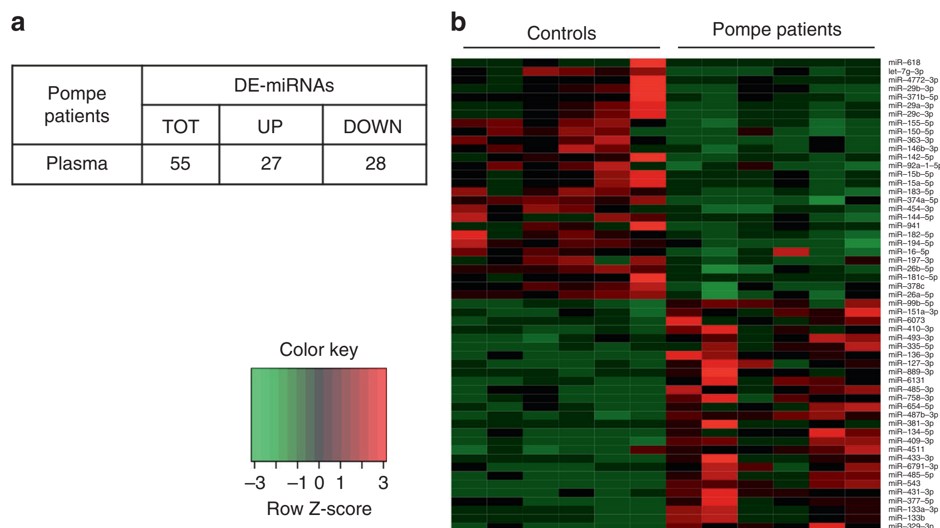


Fig. 2 Results of small-RNA-seq analysis in Pompe disease patient plasma. (**a**) Summary of the number of differentially expressed microRNA (DE-miRNA) up- and down-regulated. (**b**) Heatmap of the 55 DE-miRNAs up-regulated (red) or down-regulated (green)

Table 1 miRNAs dysregulated both in Pompe mice tissues and in patient plasma

DE-miRNAs	Pompe patients	Pompe KO mouse	
	Plasma	Gastrocnemius	Heart
miR-127-3p	↑	↓	
miR-136-3p	↑	↓	
miR-182-5p	↓	↑	
miR-133a-3p	↑	↓	
miR-142-5p	↓	↑	↑
miR-155-5p	↓	↑	
miR-15b-5p	↓	↑	↑
miR-26a-5p	↓	↓	
miR-26b-5p	↓	↓	
miR-29c-3p	↓	↓	↓
miR-410-3p	↓	↓	
miR-144-5p	↓	↓	↓
miR-183-5p	↓	↑	
miR-329-3p	↑	↓	
miR-92a-1-5p	↓	↑	
miR-99b-5p	↑	↑	

The arrow's direction indicates the up- or down-regulation
DE-miRNA differentially expressed microRNA, *KO* knockout

were compared with control samples obtained from age-matched pediatric patients, while the results obtained in LOPD were compared with those of age-matched healthy volunteers.

We found 55 miRNAs that were differentially expressed with an FDR <0.05 (the complete list is provided in Supplementary Table 3) and that were either up- or down-regulated (Fig. 2). Sixteen miRNAs were differentially expressed both in tissues from Pompe disease mice and in patient plasma (Table 1). For the majority of these miRNAs the pattern of expression was different in the two species. However, this was not surprising, considering the different sources of biological samples and patient clinical heterogeneity.

The DE-miRNAs in Pompe disease are involved in pathways that are potentially relevant for the disease pathophysiology

A literature analysis indicates that the 16 DE-miRNAs that are dysregulated both in mouse tissues and in patients' plasma have been linked, as expected, to multiple function and pathways. The majority of them are reported to be involved in cancer-related processes, likely due to the preponderant number of studies in the literature that are focused on the role of miRNAs in cancer.

Interestingly, some DE-miRNAs are involved in pathways that are of potential relevance for Pompe disease pathophysiology, such as muscle cell proliferation, regeneration and differentiation (miR-127, miR-133, miR-136, miR-142, miR-329), autophagy (miR-26, miR-29c, miR-142, miR-183), apoptosis (miR15b, miR-26, miR-29c, miR-136, miR-144, miR-410), endoplasmic reticulum/oxidative stress (miR15b, miR-99, miR-133, miR-155, miR-144, miR-329), muscle metabolism and insulin response (miR-26, miR-29c, miR-182), inflammation (miR-142, miR-155, miR-410), fibrosis (miR-29c, miR-410), cardiomyopathies (miR-26, miR-133, miR-182, miR-410), and atrophy (miR-182). The finding of dysregulation of miRNAs involved in the process of atrophy may also be compatible both with primary muscle atrophy and with denervation atrophy.

To gather more information on the processes in which the DE-miRNAs can be functionally involved, we carried out Gene Ontology and KEGG pathway enrichment analysis using as queries the list of their predicted targets identified through TargetScan.[20] The most recurrent target genes in the most frequently enriched KEGG pathways (genes that are predicted target of at least four DE-miRNAs) are shown in Fig. 3.

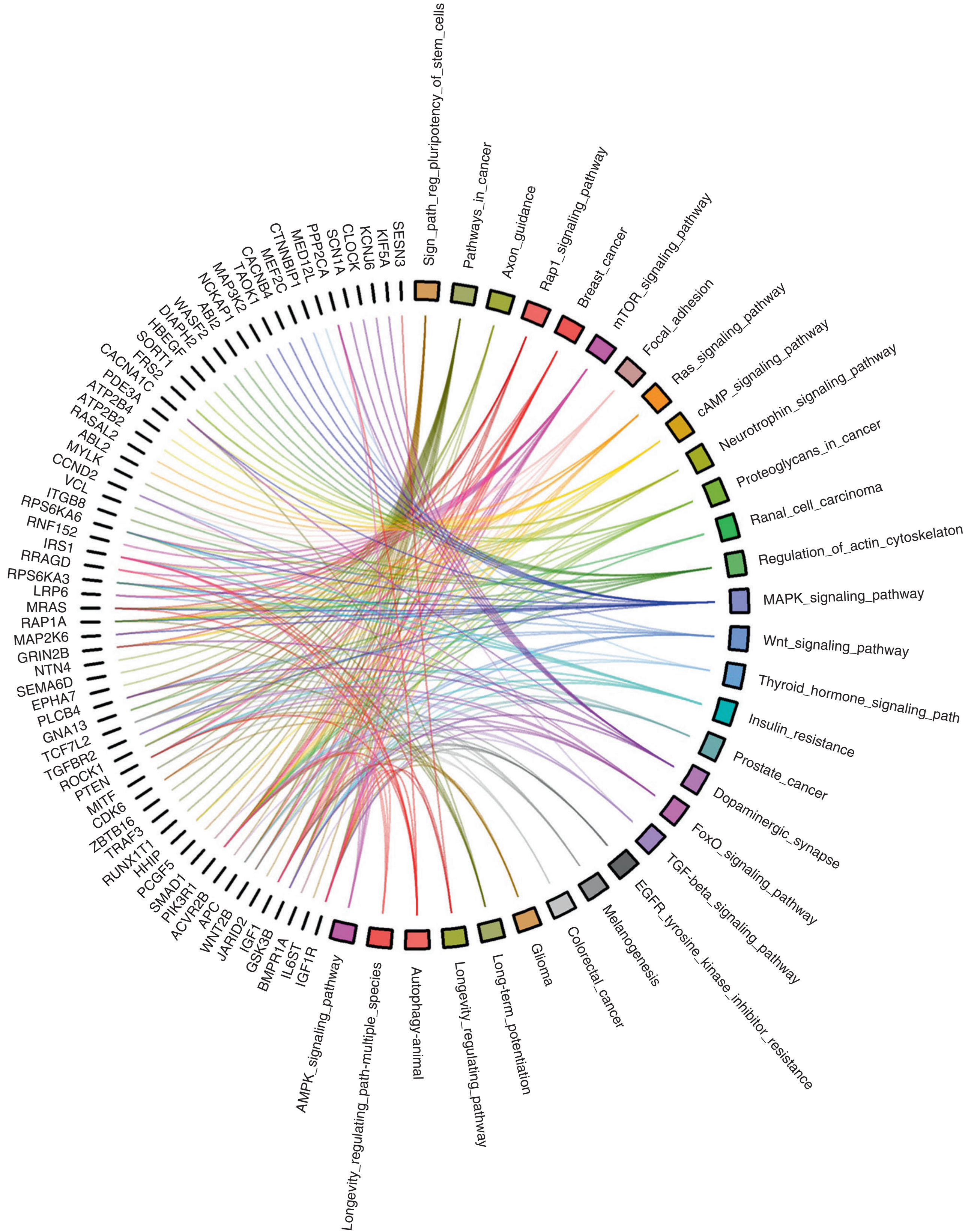


Fig. 3 Bioinformatic analysis of differentially expressed microRNA (DE-miRNA) targets. Circos plots of the most frequent pathways (right from top to bottom) and most frequent genes (left from bottom to top) predicted to be targeted by the 16 common DE-miRNAs

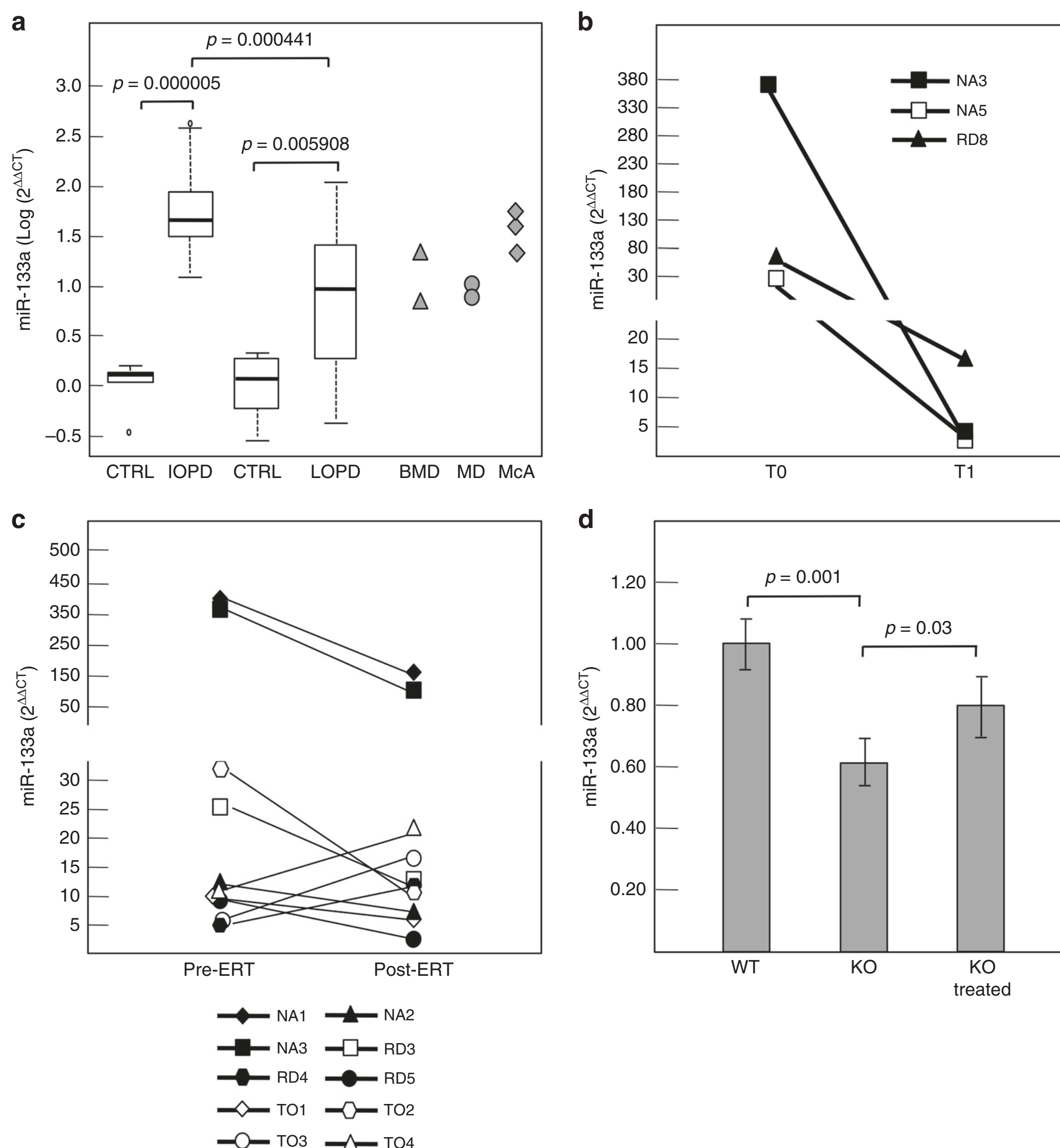


Fig. 4 Expression level of circulating miR-133a and effect of therapy. a In infantile-onset Pompe disease (IOPD) patients miR-133a levels were significantly ($p = 0.000005$) higher (12.44- to 414.96-fold) than those of age-matched controls. IOPD patients had significantly higher levels compared with late-onset Pompe disease (LOPD) patients ($p = 0.000441$). In LOPD patients miR-133a was also significantly increased ($p = 0.005908$) compared with age-matched controls. *P* analysis of variance (ANOVA) = 0.03662. miR-133a was also elevated in patients with other myopathies, including Becker muscular dystrophy (BMD, gray triangles), Steinert myotonic dystrophy (MD, gray circles), McArdle muscle (McA, gray diamonds). **b** Expression level of miR-133a in plasma from 3 IOPD. For each patient, miR-133a was analyzed before first enzyme replacement therapy (ERT) infusion (T0) and after 3 years of treatment (NA3), 7 months (NA5), and 3 years and 3 months (RD8) (T1), and after near-complete correction of clinical manifestations. **c** Expression level of miR-133a in plasma from 10 PD patients pre-ERT infusion and 24 h post-ERT. **d** Effect of a single high-dose infusion of rhGAA (100 mg/kg). Expression of miR-133a was analyzed in gastrocnemii from 9-month-old Pompe disease mice, compared with wild-type age-matched animals and 48 h after rhGAA infusion. KO knockout, WT wild-type

As a result, we found that a number of DE-miRNAs and their target genes are involved in processes that are potentially relevant for Pompe disease pathophysiology (Fig. 3), like MAPK, FoxO, mTOR, AMPK and insulin signaling pathways, ubiquitin-mediated proteolysis, cardiac hypertrophy, fibrosis, muscle atrophy and regeneration, autophagy, regulation of stem cells pluripotency, and myogenesis. We also found some DE-miRNAs that are implicated in other pathways, such as oxidative stress and inflammation, that have occasionally been associated with Pompe disease.[23, 24] Some DE-miRNAs are involved in more than one of these pathways, which suggests that they play a significant role in Pompe disease pathophysiology, and in an intricate interplay between secondary cellular events triggered in response to glycogen storage.

miR-133a levels correlate with Pompe disease clinical forms

We selected one of the miRNAs, miR-133a, that were differentially expressed both in Pompe disease mice and in

patients for further validation and confirmation by qRT-PCR. The analysis was performed in patients' plasma from all 52 patients. Information on these patients is provided in Supplementary Table S2. Ten of the patients were affected by IOPD, whereas 42 had LOPD.

miR-133a has been reported to be a member of the myogenic miRNAs (so-called Myo-miRs);[25] is expressed in the skeletal and cardiac muscles of mammals, birds and zebrafish; and is dysregulated in cardiac hypertrophy and other cardiac disorders,[26] and in muscular dystrophies.[16, 27]

qRT-PCR analysis showed minor variations of miR-133a levels with respect to age in controls (Fig. **4a**), with slightly higher levels in younger individuals. Conversely, quantitative analysis confirmed that miR-133a is up-regulated in Pompe disease patients (Fig. **4a**), as already observed by small RNA-seq. In IOPD patients miR-133a levels were significantly ($p = 0.000005$) higher (12.44 to 414.96-fold) than those of age-matched controls and did not overlap with controls. IOPD patients had the highest levels of miR-133a, which were significantly higher than those observed in LOPD patients ($p = 0.000441$). In LOPD patients miR-133a was also significantly increased ($p = 0.005908$) compared with age-matched controls, but with lower levels, with a broader distribution of values, and with some overlap between controls and LOPD patients.

As positive control, we performed the analysis in samples from two patients affected by Becker muscular dystrophy. In addition, we analyzed samples from patients with other myopathies, including three patients with McArdle glycogenosis and two patients with Steinert myotonic dystrophy. Compared with age-matched controls all these patients showed increased miR-133a levels (Fig. **4a**).

We also measured miR-133a levels in plasma from the murine Pompe disease model. MiR-133a was modestly elevated only in older mice (15 months of age) (Supplementary Fig. S2). Considering that the phenotype observed in the knockout mouse is relatively mild (with the exception of the presence of cardiomyopathy) and allows for near normal survival and fertility, these data are not surprising.

ERT affects circulating miR-133a levels in Pompe disease

We then tested whether miR-133a levels are influenced by ERT with rhGAA. Only for 3 IOPD patients (NA3, NA5, RD8) were stored plasma samples available, which had been obtained before starting ERT (T0), at the age of 9, 2 and 3 months respectively. Before starting ERT all patients showed the full clinical picture of IOPD, with hypertrophic cardiomyopathy and severe hypotonia. In the pre-ERT samples miR-133a levels were high. After 3 years of treatment (NA3), 7 months (NA5), and 3 years and 3 months (RD8), respectively, a good clinical response to ERT had already been achieved in all patients, with near-complete correction of cardiac hypertrophy as assessed by cardiac ultrasound scan, and adequate motor function for age. At that time plasma miR-133a levels were substantially decreased in all patients (Fig. **4b**), suggesting a correlation between miRNA levels and the effects of treatment.

We also analyzed the effects of single ERT infusions in ten patients, for which samples before and 24 h after an ERT infusion were available in our laboratories (Fig. **4c**). One of these samples (NA3) had been obtained after the first ERT infusion and pretreatment levels corresponded to those shown in Fig. **4b**. All other patients had already been treated for variable periods. All patients displayed up-regulation of miR-133a before ERT with respect to the mean of control samples. In seven patients the level of miR-133a decreased after ERT, with most evident decreases in the patients with the highest miR-133a preinfusion levels.

We also tested the effect of a single infusion of rhGAA in the Pompe disease mouse model. As previously shown, in the Pompe mouse miR-133a is down-regulated in gastrocnemius, while in heart it is normally expressed. We gave a single injection of rhGAA at a high dose (100 mg/kg) to obtain detectable changes in miR-133a expression. Forty-eight hours after the injection, tissues were collected and analyzed by qRT-PCR. In gastrocnemius miR-133a levels returned to values that are close to those measured in wild-type animals (Fig. **4d**), while no changes were observed in heart (not shown).

DISCUSSION

In this study we have evaluated whether miRNA profiles may represent potential biomarkers for Pompe disease. Small RNA-seq was selected as a tool to analyze miRNA expression because this approach is well suited for large-scale quantitative analysis of nucleotide sequences.

The design of the study, with an initial step in which we first performed the small RNA-seq analysis in the Pompe disease mouse model and subsequently in a selected number of patients, represented a compromise between the potential of NGS and the costs of this methodology. An advantage of using the animal model was the possibility to run our preliminary analysis in a homogenous sample, with a common genetic background and uniform disease progression, and to look at the tissues that are most affected by disease pathology. This initial approach allowed us to identify and select a restricted number of miRNAs that are dysregulated in Pompe disease. These miRNAs were subjected to a bioinformatic analysis to gather information on their possible role in Pompe disease pathophysiology.

The DE-miRNAs that we selected in this way were (1) dysregulated in Pompe disease (both in mice tissues and in patients' plasma); (2) potentially relevant for the disease pathophysiology according to the bioinformatic and literature analysis; and (3) measurable in samples, like plasma, that are readily available in Pompe disease patients and in general require minimally invasive procedures.

One of these miRNAs was further validated in the whole cohort of our patients by qRT-PCR. miR-133a was particularly attractive, because it had already been proposed as a biomarker for Duchenne/Becker muscular dystrophy,[16] and

because it has been shown to be involved in processes including muscle regeneration, atrophy, and inflammation.

Both in IOPD and in LOPD patients miR-133a was significantly elevated. However, the levels observed in IOPD were significantly higher compared with those measured in patients with attenuated phenotypes, suggesting a correlation between miR-133a levels and the clinical forms of the disease. This difference was not due to an age-related effect, because in age-matched controls miR-133a levels were only modestly higher than those obtained at later ages.

While in IOPD patients, who show a more homogeneous phenotype,[28–30] we consistently found increased miR-133a levels, the results in LOPD showed marked variability. It was thus impossible, in a relatively limited number of patients, to establish clear correlations between individual parameters (such as age, diseases stage, clinical scores, motor performance, need for ventilator support). It is reasonable to think that in LOPD patients, who show an extremely broad rage of phenotypes,[31] several factors (muscle mass, fibrosis, in addition to age, disease severity, etc.) concomitantly contribute to the expression of miR-133a and to its plasma levels. We believe that studies in larger cohorts of patients, based on the concerted effort of international consortia and on the collaboration of the leading centers in the follow-up of Pompe disease patients, may further validate the results of our study, and address possible correlations between miRNA levels and LOPD patient status, response to therapy, or outcome.

An important finding of our study is that miR-133a levels showed a trend toward decrease in response to ERT. Samples before the start of treatment were only available for three patients. In all of them we found a clear decrease of miR-133a, concomitant to overt clinical improvement. miR-133a levels also decreased in seven of ten patients even after a single injection after ERT treatment. In addition, we found normalization of miR-133a in gastrocnemii from Pompe disease animals after single injection of high-dose rhGAA. Our results suggest that miR-133a may represent an adjunct marker of ERT effects, to be used in combination with clinical and biochemical measures.

The availability of a broad range of reliable tests appears particularly important for the development of guidelines for patient monitoring, for ERT inclusion and exclusion criteria that are currently being defined in some countries,[32] to correlate different ERT regimens and patient response to treatment, and to optimize and personalize treatment protocols. In addition, disease markers that provide information on Pompe disease pathophysiology may allow for identification of dysregulated pathways, new therapeutic targets, and, possibly, for development of novel therapeutic strategies.

We chose to use one DE-miRNA as a measurable marker in our cohort of Pompe disease patients. However, should the costs of NGS become more affordable (as is indeed expected in the coming years, thanks to the development of second-generation technologies and more flexible instruments), this approach may be proposed to collect a large body of data that may be even more informative in patient follow-up and allow for the identification of a specific "signature" of Pompe disease, and of disease progression, outcome, or response to therapies.

ELECTRONIC SUPPLEMENTARY MATERIAL

The online version of this article (https://doi.org/10.1038/s41436-018-0103-8) contains supplementary material, which is available to authorized users.

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DISCLOSURE

The authors declare that they have no conflicts of interest.

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Conclusions

Insufficient delivery of the therapeutic enzyme to target tissues, with incomplete correction of pathology in skeletal muscles, has been extensively documented in PD animal models and patients.

We explored the possibility to use microRNAs (miRNAs) as disease markers in Pompe Disease.

In our work we found:

- 198 miRNAs differentially expressed with statistical significance in gastrocnemius and 66 DE-miRNAs in heart, in the mouse model.
- 55 miRNAs differentially expressed in patients' plasma.

One of these microRNAs, miR-133a, was selected for further quantitative real-time polymerase chain reaction analysis in plasma samples from patients, obtained from seven Italian and Dutch biobanks. miR-133a levels were significantly higher in PD patients than in controls and correlated with phenotype severity, with higher levels in infantile compared with late-onset patients. In three infantile patients miR-133a decreased after starting enzyme replacement therapy and evidence of clinical improvement. Expanding the cohort of patients and identifying more miRNAs we could establish possible relationships between miRNAs and ERT or disease progression.

Chapter 6

Pathogenesis of Mucopolysaccharidoses, an Update

Introduction

The Mucopolysaccharidoses (MPSs) are lysosomal storage diseases, due to deficiencies of enzymes involved in the breakdown of Glycosaminoglycans (GAGs).

GAGs are a heterogeneous family of highly sulphated, complex, linear polysaccharides that are composed of repeating disaccharide units and are present in every mammalian tissue.

The degradation of GAGs is performed by lysosomal hydrolases, which act sequentially; thus, a deficiency of one of these activities results in a block of further degradation of GAGs. GAGs have a variety of important biological roles. For a long time, they were thought to be exclusively constituents of extracellular matrix and membranes, and to be mainly involved in cell hydration and structural scaffolding.

However, recent evidence indicates that GAGs also play a key role in cell signalling and modulate several biochemical processes that are fundamental for cell biology, including regulation of cell growth and proliferation, promotion of cell adhesion, anticoagulation, wound repair, and others.

The characterization of this cascade of secondary cellular events is critical to better understand the pathophysiology of MPS clinical manifestations. In

addition, some of these pathways may represent novel therapeutic targets and allow for the development of new therapies for these disorders.

Results of this study have been published in the attached Review.

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Review

Pathogenesis of Mucopolysaccharidoses, an Update

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Abstract: The recent advancements in the knowledge of lysosomal biology and function have translated into an improved understanding of the pathophysiology of mucopolysaccharidoses (MPSs). The concept that MPS manifestations are direct consequences of lysosomal engorgement with undegraded glycosaminoglycans (GAGs) has been challenged by new information on the multiple biological roles of GAGs and by a new vision of the lysosome as a signaling hub involved in many critical cellular functions. MPS pathophysiology is now seen as the result of a complex cascade of secondary events that lead to dysfunction of several cellular processes and pathways, such as abnormal composition of membranes and its impact on vesicle fusion and trafficking; secondary storage of substrates; impairment of autophagy; impaired mitochondrial function and oxidative stress; dysregulation of signaling pathways. The characterization of this cascade of secondary cellular events is critical to better understand the pathophysiology of MPS clinical manifestations. In addition, some of these pathways may represent novel therapeutic targets and allow for the development of new therapies for these disorders.

Keywords: mucopolysaccharidoses; LSDs; GAGs; autophagy; lysosomal storage disorders

1. Introduction

The knowledge on the pathophysiology of mucopolysaccharidoses (MPSs) has evolved over a long period of more than a century. The first MPSs were initially identified as distinct clinical entities [1–5] based on the description of their peculiar phenotypes and on the characterization of pathological findings, while the biochemistry, the biology, and the molecular bases of all these disorders continued to remain unknown for several decades.

The discovery of lysosomes and the characterization of their role in cell biology and human disease marked a decisive turn in the history of MPSs [6]. Like for many other lysosomal storage diseases (LSDs), the pathophysiology of this class of disorders was ascribed to a block in the degradative function of lysosomes [7]. Accordingly, MPSs manifestations were viewed as direct consequences of lysosomal engorgement with undegraded mucopolysaccharides, also referred to as glycosaminoglycans (GAGs).

Nowadays, with the changing vision of lysosomal biology, there is a renewed interest in the pathophysiology of LSDs, including MPSs. A large number of studies have challenged the traditional concept of lysosomes as exclusively degradative organelles, and LSDs are now viewed as disorders that simultaneously affect multiple cellular pathways and signaling cascades, each contributing to disease pathophysiology and to clinical manifestations.

2. Mucopolysaccharidoses (MPSs), Glycosaminoglycans (GAGs), and Lysosome Biology

The MPSs are lysosomal storage diseases, due to deficiencies of enzymes involved in the breakdown of GAGs. GAGs are a heterogeneous family of highly sulfated, complex, linear polysaccharides that are composed of repeating disaccharide units and are present in every mammalian tissue [8]. The degradation of GAGs is performed by lysosomal hydrolases, either exoglycosidases that cleave the sugar residue at the end of the oligosaccharide chains, or sulfatases that remove sulfate from specific sugar residues [9]. These enzymes act sequentially; thus, a deficiency of one of these activities results in a block of further degradation of GAGs.

Different lines of research have changed our view of MPSs pathophysiology in recent years. Significant advances derived from studies on GAG functions in cell biology. GAGs have a variety of important biological roles. For a long time, they were thought to be exclusively constituents of extracellular matrix and membranes, and to be mainly involved in cell hydration and structural scaffolding. However, recent evidence indicates that GAGs also play a key role in cell signaling and modulate several biochemical processes that are fundamental for cell biology, including regulation of cell growth and proliferation, promotion of cell adhesion, anticoagulation, wound repair, and others [10]. In particular, extracellular GAGs represent reservoirs and co-receptors of different signaling molecules.

Additional and critical information on the pathophysiology of MPS was gathered thanks to studies that clarified the role of lysosomes in cell biology. The degradative function committed to the turnover of cellular constituents was long considered the primary task of lysosomes. This function was seen as a "house-keeping" process that was constitutively active in cells. Recent studies have challenged these assumptions and have provided incontrovertible evidence that the lysosomal compartment is part of a complex pathway, the autophagic-lysosomal pathway (ALP), and that lysosomal biogenesis and the activation of this pathway are tuned in adaptation to environmental stimuli, through the phosphorylation status and subcellular localization of transcription factor EB (TFEB) [11,12]. These activities are mainly regulated through the function of the multiprotein complex mammalian target of rapamycin complex 1 (mTORC1), localized at the cytosolic surface of the limiting membrane of the lysosome. mTORC1 is a kinase active on several substrates, including transcription factor EB (TFEB), that regulates ALP activation and recycling of cellular components, such as lipid stores [13]. Calcium signaling is also involved in regulating TFEB nuclear translocation and activation of autophagy [14]. Thanks to this characterization of lysosomal function and biology, lysosomes are now viewed as signaling hubs involved in many critical cellular processes, such as nutrient-sensing and regulation of metabolism, secretion, vesicle and membrane trafficking, growth, adaptive immunity, and others [11,15,16].

3. Lysosomal Storage and Secondary Disruption of Cellular Pathways

Given GAGs role in cell biology and in signaling pathways, it is not surprising that mutations causing defects in the degradation of these molecules have highly debilitating consequences, with multisystemic involvement and variable association of somatic, neurological, hematologic, and ocular symptoms.

In addition, according to the newest information on lysosomal biology and on their central role in many cellular functions, multiple and diverse events are now emerging as important players in the pathogenesis of MPSs. Specifically, these events include storage of secondary substrates unrelated to the defective enzyme; abnormal composition of membranes and aberrant fusion and intracellular trafficking of vesicles; impairment of autophagy; mitochondrial dysfunction and oxidative stress; dysregulation of signaling pathways and activation of inflammation; abnormalities of calcium homeostasis and signaling [17–20] (Figure 1). It is likely that these factors or processes influence severity of symptoms and clinical manifestations, although, as this field is still largely unexplored, it is difficult to establish clear correlations between secondary cellular impairments and disease phenotypes [21].

Figure 1. The pathogenetic cascade of mucopolysaccharidoses (MPSs). Multiple pathogenetic events in MPSs, including primary storage of glycosaminoglycans (GAGs) and secondary disrupted pathways: secondary storage of different substrates and abnormal membranes composition; impaired fusion and vesicles trafficking; impairment of autophagy; mitochondrial dysfunction and oxidative stress; dysregulation of signaling pathways and activation of inflammation; impaired calcium homeostasis and signaling.

3.1. Secondary Storage

In several LSDs, including MPSs, secondary storage of substrates that are not explained by the primary lysosomal defect has been consistently documented. The types of secondarily stored compounds are highly heterogeneous and include glycosphingolipids, phospholipids, and cholesterol [22–27].

Secondary storage has been described both in patients affected by MPSs and in animal models from different species. A comprehensive analysis of brain cortex tissues from post-mortem autopsy samples in patients affected with MPS I, II, IIIA, IIIC, and IIID was performed by biochemical analytical procedures (high performance liquid chromatography) and by histochemical staining of fixed tissues. This analysis revealed both changes in GAG composition (increase of heparan sulfate, decrease of keratan sulfate) and accumulation of secondary substrates including GM2 and GM3 gangliosides and lactosylceramide [28].

Similar studies were also done in animal models. Morphological and histological studies in huntaway dogs, a canine model of MPS IIIA, showed variably stained storage granules within neurons, including some that stained for gangliosides. On ultrastructural analysis, these granules contained both dense granular materials interpreted as GAGs, and a variety of multilamellar bodies interpreted as ganglioside accumulation [24].

In a feline model of MPS VI, abnormal lysosomal inclusions were pleiomorphic and resembled the zebra bodies and dense core inclusions typical of MPSs, while others appeared as membranous storage bodies characteristic of gangliosidoses. Pyramidal neurons showed positive staining for GM2 and GM3 gangliosides. In addition, positive filipin staining indicated storage of unesterified cholesterol [29]. As gangliosides can influence dendritogenesis during development, it is possible to speculate that accumulation of these compounds can trigger the changes in dendrite and axon morphology seen in MPS patients, leading to synaptic dysfunction, neuronal cell death in the brain, and neurodegeneration [30].

Secondary storage is not only confined to lysosomes but may contribute to accumulation in other compartments of toxic storage materials, including aggregated-prone proteins, alpha-synuclein, and

damaged mitochondria [31] associated with common neurodegenerative disorders, such as Alzheimer's, Parkinson's, and Huntington's disease. Alpha-synuclein accumulation has been consistently found in a variety of LSDs, including MPSs, and suggests correlations between alpha-synuclein aggregation toxicity and LSDs pathophysiology [32–37]. In MPS IIIA, a link between lysosomal dysfunction and presynaptic maintenance appeared to be mediated by a concurrent loss of α-synuclein and cysteine string protein α (CSPα) at nerve terminals. The relative loss of α-synuclein function by its abnormal autophagy was proposed as a contributing factor to neuronal degeneration [35].

The mechanisms leading to secondary storage are not clear. In principle, secondary storage may derive from inhibition by primary substrates of other lysosomal enzymes, from modification of the lysosomal environment, such as pH changes, or from impairment of vesicle trafficking through the endosomal/lysosomal system and the autophagic pathway [23,38,39].

While in the past secondary storage was typically considered a nonspecific and insignificant pathological feature of MPSs, newer studies support a substantial role of secondary storage as a major determinant in the pathophysiology of these disorders.

3.2. Abnormal Composition of Membranes and Aberrant Intracellular Trafficking

An important consequence of secondary storage is its effect intracellular vesicle trafficking. Specifically, some substrates, such as cholesterol and other lipids, have been thought to play a role in altering membrane composition and jamming the endolysosomal system [23,38,39]. For example, the effect on vesicle trafficking has been studied in detail in the animal models of MPS type IIIA and of Multiple Sulfatase Deficiency, a peculiar disorder in which sulfatases, including those implicated in the breakdown of GAGs, are simultaneously deficient due to a defective post-translational modification of a cysteine at the catalytic site of these enzymes [40,41]. In the animal models of both diseases secondary cholesterol storage in endo-lysosomal membranes induces critical changes in the biochemistry and organization of lysosomal membranes. Abnormalities of the lipid composition of membranes impact on the function of a family of proteins, named soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs), which are crucial components of the cellular membrane fusion machinery and are responsible for mediating membrane fusion processes in cells. Thus, SNAREs dysfunction leads to an impaired ability of lysosomal membranes to fuse with other membranes of other vesicles, such as endosomes and autophagosomes [42].

The defective trafficking of vesicles has several deleterious consequences and has been thought to have a role in the development of neuropathology in MPSs. For example, in cultured adrenal chromaffin cells from in MPS type IIIA, evidence of impaired exocytosis was observed [43]. It has been speculated that if these abnormalities also occur in central nervous system neurons, they may lead to a reduction in neurotransmitter release and explain some aspects of the MPS type IIIA neurological phenotype.

In addition, recent data indicate that alpha-synuclein is a key chaperone assisting synaptic vesicle recycling and transmission at presynaptic terminals [44,45] by sustaining the function of the specific set of SNARE proteins involved in the synaptic vesicle trafficking [46,47], and that in the MPS type IIIA mouse model SNAREs dysfunction impairs synaptic vesicle recycling and neurotransmission, contributing to neurodegeneration [35].

3.3. Abnormal Autophagy

An important and deleterious effect of lysosomal storage and of defective vesicle trafficking is the impairment of the ALP and a block or reduction of the autophagic flux. Autophagy is an evolutionary conserved catabolic process that allows lysosomal delivery of intracellular components destined to degradation and turn over. A functional ALP is critical for many cell functions and for cell survival; thus, an impairment of this process may have disastrous consequences. This pathway has been shown to be affected in several LSDs, including MPSs, [33,48,49]. Original studies on the impairment of autophagy in LSDs were performed in animal models of an MPS IIIA and of multiple sulfatase deficiency. In both disorders, accumulation of immature autophagosomes, decreased co-localization

of lysosomal and autophagic markers, increased ubiquitin levels and p62/SQSTM1-positive puncta, increased the number of mitochondria in different areas of brains and in neurons was interpreted as the consequence of defective autophagosome-lysosome fusion [33]. Evidence suggesting that a block of autophagy contributes to the MPS IIIA phenotype has been obtained using an MPS IIIA animal model in Drosophila. These MPS IIIA flies showed reduced ability to climb, indicating neurological impairment. Knockdown of Atg18 and Atg1, both essential components of the autophagic pathway, resulted in further worsening of performance in the climbing assay [50], suggesting a role of autophagy in the pathophysiology of the disease. Autophagy was shown to be also impaired in several other MPS animal models, including MPS II [51], MPS IIIC [52], MPS VI [53], and MPS VII [54]. Changes in expression of autophagy-related genes, coding for Atg1 and Atg18 proteins, were recently reported in MPS IIIA [50]. Another intriguing link between autophagy impairment and MPS has been discovered recently [55]. Mutations in the *VPS33A* gene, encoding a protein (VPS33A) that is involved in autophagy, resulted in an MPS-like disorder characterized by high levels of heparan sulfate in plasma and urine of patients, and in a phenotype sharing similarities with those of MPSs.

The impairment of the autophagic flux has been recognized as important pathogenetic factor for neurodegeneration in lysosomal storage diseases, including MPSs [27,55]. In neurons, basal levels of autophagy are essential for neuronal function and survival, since they prevent toxic proteins from reaching harmful concentrations and contribute to the degradation of aged or damaged organelles, such as mitochondria [56,57]. A recent study in the mouse model of MPS IIIA has shown that restoration of the ALP was associated with reduced neuroinflammation and amelioration of memory deficits [37].

In addition, impairment of lysosome/autophagy pathway affects extracellular matrix formation and skeletal development and growth in MPSs [58,59].

Deregulation of mTORC1 signaling arrests bone growth in lysosomal storage disorders. In the mouse model of MPS VII lysosomal dysfunction induced a constitutive activation of mTORC1 in chondrocytes. As a consequence, chondrocytes fail to properly secrete collagens, the main components of the cartilage extracellular matrix. Rescue of the autophagy flux resulted in restored collagen levels in cartilage and ameliorated the bone phenotype [54].

3.4. Mitochondrial Dysfunction

A primary function of autophagy is to mediate mitochondrial turnover [60]. This selective form of autophagy is known as mitophagy and its importance in preserving functional integrity of mitochondria has been increasingly recognized in the past few years. Thus, it is not surprising that disorders characterized by an impairment of autophagy are associated with mitochondrial dysfunction, and that mitochondrial dysfunction contributes to the pathophysiology of these disorders.

Perturbations in mitochondrial function and homeostasis caused by impaired autophagy have been recognized in several LSDs, including some MPSs [61], and have been proposed as one of the mechanisms underlying neurodegeneration [25,62–64]. Studies in an MPS IIIB mice have shown increased amounts of the small mitochondrial protein subunit C of the mitochondrial ATP synthase in specific brain regions, including the entorhinal and the somatosensory cortex. This finding was already evident at 1 month of age and was shown to increase with time [65]. Similar findings were observed post-mortem in the cerebral limbic system and central gray matter of the mid brain and pons in a patient with Hurler-Scheie MPS I [66].

Pathological findings have been described in detail in the murine model of MPS IIIC, in which accumulation of pleomorphic, swollen mitochondria containing disorganized or reduced cristae, was observed in neurons in all parts of the brain. These abnormalities appeared to be progressive. Some neurons containing swollen mitochondria were already detectable at 5 months of age, while by the age of 12 months mitochondrial damage was seen in the majority of neurons [52,63].

3.5. Oxidative Stress

Increased oxidative stress and enhanced susceptibility of cells to mitochondria-mediated apoptotic insults are obvious consequences of defects in mitophagy and mitochondrial dysfunction. Elevation of reactive oxygen species (ROS) and accumulation of damaged mitochondria has been observed in some MPSs [67].

Some studies performed in animal models have linked oxidative stress to MPSs [68,69]. These studies showed the presence of oxidative stress already in the early stages of disease progression in the murine model of MPS IIIB. Other studies demonstrated an oxidative imbalance in animal models of MPS I [70] and MPS IIIA [71]. Elevated oxidative stress has also been documented in blood samples from patients affected by MPS type I [72] and MPS II [73]. In these patients, oxidative damage to proteins and lipids increased catalase activity and reduced total antioxidant status were found. Interestingly, oxidative stress may be directly linked and explain the activation of inflammation and the abnormalities of autophagy [74]. Studies in an MPS IIIB murine model suggest that oxidative stress is not a consequence, but a cause of neuroinflammation, since it is present at a very early stage in the brain [75].

3.6. Alteration of Signaling Pathways

Non-physiologic activation of signaling cascades is an important and intriguing aspect of MPS pathophysiology that in recent years is attracting growing interest. Indeed, aberrant signaling may be directly implicated in the pathophysiology of some of the most prominent and most debilitating clinical manifestations of these disorders, such as pain, physical disability, neurodegeneration, skeletal abnormalities, and heart involvement.

Several factors contribute to signaling dysregulation. One of them is the synthesis of aberrant GAGs that interferes with normal GAG interactions with different receptors, such as the fibroblast growth factors (FGFs), and with morphogens such as those implicated in neurogenesis, axonal guidance, and synaptogenesis. In addition, several of the secondary events triggered by storage (impaired autophagy, mitochondrial dysfunction and oxidative stress abnormal trafficking of vesicles, membranes, and membrane proteins) may affect the internalization and trafficking of signaling molecules [76]. Neurodegeneration and skeletal involvement are important examples of MPSs clinical manifestations that may be linked to altered signaling (Figure 2).

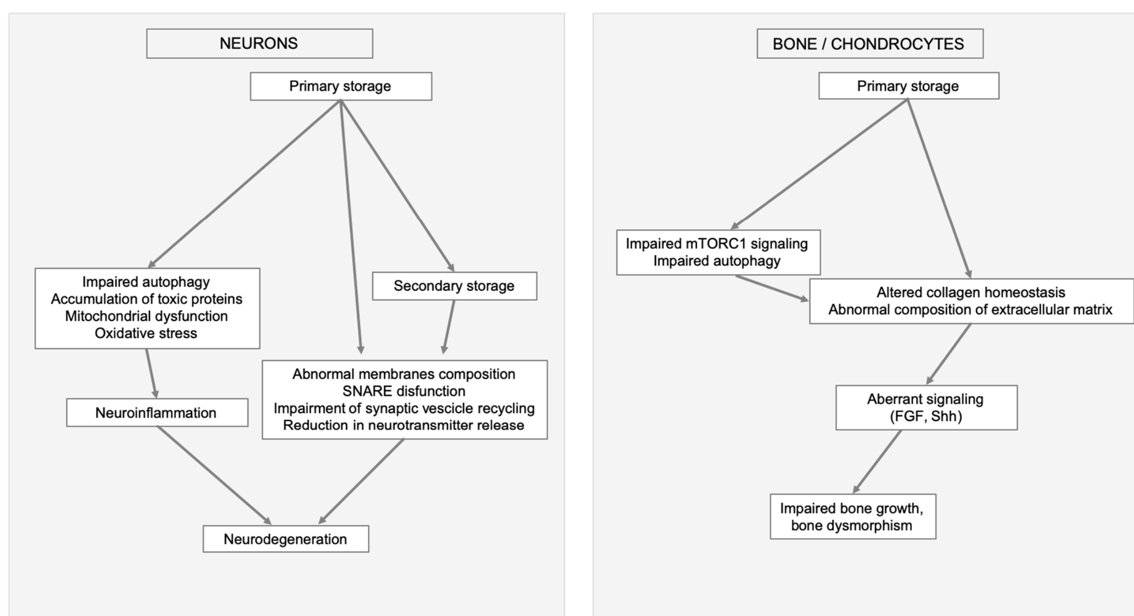

Figure 2. Examples of MPSs clinical manifestation triggered by primary storage and linked to secondary cellular impairments and altered signaling. In neurons and brain (left) impaired autophagy, mitochondrial dysfunction, oxidative stress, and secondary storage cause neuroinflammation, abnormal membranes composition, soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNARE) disfunction, impairment of synaptic vesicle recycling, and reduction in neurotransmitter release ultimately leading to neurodegeneration. In bone (right) secondary events, like aberrant mammalian target of rapamycin complex 1 (mTORC1) signaling, impaired autophagy, altered collagen homeostasis, abnormal composition of extracellular matrix e aberrant signaling, result in impaired bone growth and bone dysmorphism.

Severe neuroinflammation is a consistent finding in MPS animal models and may be a factor implicated in the progression of neurodegeneration. Accumulation of GAG-related oligosaccharides in microglia, likely released by lysosomal exocytosis, are thought to induce inflammation in the brain by activating toll-like receptor (TLR) receptors of microglia cells, and to induce release of inflammatory cytokines. Evidence of neuroinflammation in MPSs has been obtained both in vitro and in vivo. In vitro lipopolysaccharide (LPS)-TLR4 activated microglia has been shown to express and secrete inflammatory cytokines and chemokines such as tumor necrosis factor-α (TNFα), interleukin-1β (IL-1β), interleukin-6 (IL-6), and macrophage inflammatory chemochine ligand-3 (CCL3) [77]. In in vivo studies, an increase of activated astrocytes and microglial cells has been observed in the brain, in the cortical area, and in the spinal cord of several murine models of MPSs, such as MPS I, IIIA, IIIB, and IIIC [63,77,78]. In brains from the MPS IIIB mouse, a massive upregulation and activation of astrocytes and microglia, and secretion of inflammatory cytokines and other proteins related to immunity and macrophage function has been described early in disease progression [79]. The same mouse model showed activation TLR4/myeloid differentiation primary response 88 (MyD88) pathway in the brain [80]. In brains from the mouse model of MPS VII, a gene expression profile analysis revealed up-regulation of genes related to the immune system and inflammation. The patterns of gene expression dysregulation appeared specific for different brain regions, suggesting that specific brain regions may be more vulnerable to activation of inflammation than others [81].

The characterization of the molecular pathways underlying neuroinflammation in MPSs has possible therapeutic implications. The TLR4-TNFa pathway has been recognized as a potential therapeutic target. Preclinical studies with pentosan-sulfate, an FDA-approved drug with anti-inflammatory and pro-chondrogenic properties, showed clinical improvements in MPS VI rats and in MPS I dogs, with a reduction of pro-inflammatory cytokines in tissues and in the cerebrospinal

fluid [82–84]. A pilot clinical study based on weekly pentosan-sulfate injections for 12 weeks in three male Japanese patients with attenuated MPS II resulted in decreased inflammatory cytokines macrophage migration inhibitory factor (MIF) and TNF-α [85].

Aberrant signaling also plays an important role in the pathophysiology of bone and skeletal involvement in MPSs. In this case excess of extracellular, rather than intracellular GAGs, appear to be most significant. In fact, chondrogenesis, the earliest phase of skeletal formation, is mostly controlled by cellular interactions between the extracellular matrix (of which GAGs are important components) and differentiation factors, other signaling molecules and transcription factors [86]. Recent studies point to a role of autophagy as quality control pathway of collagen, another important component of extracellular matrix. These studies suggest that an impairment of autophagy leads to a collagen proteostatic defects, thus providing a possible mechanism implicated in skeletal defects in LSDs [59].

Much attention has been paid to fibroblast growth factors (FGFs) signaling pathway. FGFs are a cytokine family that modulates cell growth, migration, differentiation, and neuroectodermal development [87]. Abnormally accumulated GAGs and defective proteoglycan desulfation have been shown to affect FGF2-heparan sulfate interactions and FGF signaling in the murine model of Multiple Sulphatase Deficiency. [33] and in multipotent adult progenitor cells derived from MPS I patients [88]. Exogenous and endogenous GAGs were also shown to modulate the bone morphogenetic protein-4 (BMP-4) signaling activity in MPS I cells [88]. Dysregulated FGF2 signaling was found in MPS I chondrocytes, together with altered GAG, FGF2, and Indian hedgehog distribution in growth plates from MPS I mice [89]. In two different MPS II animal models, D. rerio and M. musculus the FGF pathway activity was shown to be impaired during early stages of bone development. In both models, the FGF signaling deregulation anticipated a slow but progressive defect in bone differentiation [90]. Abnormally accumulated GAGs and defective proteoglycan desulfation have been shown to alter FGF2-heparan sulfate interactions and fibroblast FGF signaling pathway also in the murine model of multiple sulfatase deficiency [33].

Studies performed in the MPS VII canine model showed failed initiation of secondary ossification in vertebrae and long bones at the appropriate postnatal developmental stage and suggested dysregulation of signaling pathways modulating bone development and ossification. Epiphyseal chondrocytes showed abnormal persistence of Sox9 protein and were unable to successfully transition from proliferation to hypertrophy [91]. Targeted gene expression profiling showed differential expression of a number of genes involved in pathways important for the regulation of endochondral ossification. Osteoactivin (GPNMB) was the top upregulated gene. In addition, elements of key osteogenic pathways such as Wnt/β-catenin and BMP signaling were not upregulated in MPS VII during critical developmental window suggesting that these bone formation pathways are not activated [92].

Proteomic studies in the murine MPS I model revealed significant decreases in key structural and signaling extracellular matrix proteins, such as biglycan, fibromodulin, PRELP, type I collagen, lactotransferrin, and SERPINF1. Genome-wide expression analysis in the same mouse model identified several significantly dysregulated mRNAs (Adamts12, Aspn, Chad, Col2a1, Col9a1, Hapln4, Lum, Matn1, Mmp3, Ogn, Omd, P4ha2, Prelp, and Rab32) [93].

It has also been suggested that in MPS II perturbations of GAG catabolism may affect morphogens release and activity, such as sonic hedgehog (Shh) distribution and signaling. Uncleared GAGs may interfere extracellularly with Shh binding to Patched, therefore blocking Shh pathway transduction [51]. Dysregulation of the Shh and Wnt/β-catenin signaling has been linked to aberrant heart development and atrioventricular valve formation in a zebrafish mode of this disorder [94].

Impaired calcium homeostasis and signaling has been demonstrated in some LSDs, such as Niemann-Pick disease type C [95,96]. These abnormalities are of particular interest as calcium signaling is an essential process in cells and is maintained by the concerted action of channels, pumps, transporters, and receptors that maintain intracellular calcium stores. In recent years, lysosomes have emerged as a major intracellular calcium storage organelle, with an increasing role in triggering

or modulating cellular functions such as endocytosis, calcium release from cellular organelles and autophagy. Evidence of disruption of calcium and proton homeostasis was also shown in MPS I [97].

4. Conclusions

The characterization of the cellular processes that are involved in the pathophysiology of lysosomal diseases has important implications for the treatment of LSDs, including MPSs. Some of the pathways that are dysregulated in these disorders may be pharmacologically or genetically manipulated and may represent novel and promising therapeutic targets. It is reasonable to think that future research will focus on these aspects, possibly developing complementary strategies to improve the outcome of traditional therapies aimed at restoring the function of defective enzymes.

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Abbreviations

MPS	Mucopolysaccharidosis
GAGs	Glycosaminoglycans
LSD	Lysosomal Storage Disease
ALP	Autophagic-lysosomal pathway
TFEB	Transcription factor EB
mTOR	Mammalian receptor of rapamycin
SNARE	N-ethylmaleimide-sensitive factor attachment protein receptors
LAMP1	Lysosomal-associated membrane protein 1
LC3	Microtubule-associated proteins 1A/1B light chain 3B
ROS	Reactive oxygen species
FGF	Fibroblast growth factor
TLR	Toll-like receptor
LPS	Lipopolysaccharide
TNF-α	Tumor necrosis factor-α
IL	Interleukin
CCL3	Chemochine ligand-3
MyD88	Myeloid differentiation primary response 88
MIF	Macrophage migration inhibitory factor
BMP-4	Bone morphogenetic protein-4
GPNMB	Transmembrane glycoprotein NMB
SHH	Sonic hedgehog

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Conclusions

Given GAGs role in cell biology and in signalling pathways, it is not surprising that mutations causing defects in the degradation of these molecules have highly debilitating consequences.

An important consequence of secondary abnormalities is its effect on intracellular vesicle trafficking.

The defective trafficking of vesicles has several deleterious consequences and has been thought to have a role in the development of neuropathology in MPSs.

Moreover, an important and deleterious effect of defective vesicle trafficking is a block or reduction of the autophagic flux. Autophagy is an evolutionary conserved catabolic process that allows lysosomal delivery of intracellular components destined to degradation and turn over.

The impairment of the autophagic flux has been recognized as important pathogenetic factor for neurodegeneration in lysosomal storage diseases, including MPSs.

A primary function of autophagy is to mediate mitochondrial turnover. This selective form of autophagy is known as mitophagy, and it is extremely important in preserving functional integrity of mitochondria.

Thus, it is not surprising that disorders characterized by an impairment of autophagy are associated with mitochondrial dysfunction, and that mitochondrial dysfunction contributes to the pathophysiology of these disorders.

Increased oxidative stress and enhanced susceptibility of cells to mitochondria-mediated apoptotic insults are obvious consequences of defects in mitophagy and mitochondrial dysfunction. Elevation of reactive oxygen species (ROS) and accumulation of damaged mitochondria has been observed in some MPSs.

Interestingly, oxidative stress may be directly linked and explain the activation of inflammation and the abnormalities of autophagy.

Aberrant signalling also plays an important role in the pathophysiology of bone and skeletal involvement in MPSs.

In conclusion, the characterization of the cellular processes that are involved in the pathophysiology of lysosomal diseases has important implications for the treatment of LSDs, including MPSs. Some of the pathways that are dysregulated in these disorders may be pharmacologically or genetically manipulated and may represent novel and promising therapeutic targets.

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