UNIVERSITÀ DI TORINO

Doctoral School in Life and Health Sciences Ph.D. in Biomedical Sciences and Oncology Department of Public Health and Pediatrics Sciences

PEDIATRICS CURRICULUM

Academic year 2020-2021 XXXII Ph.D. cycle

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The use of biomarkers, clinical assessments, and clinical history for a better approach to patients with complex diseases.

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Preface

This dissertation is a collection of several projects related to chronic diseases of genetic and metabolic etiology, that I had the opportunity to develop during my Ph.D. program from 2016 to date.

Lysosomal diseases in particular are an important model of preventive medicine in which laboratory tests, the use of biomarkers and the accurate analysis of clinical history, can change the diagnostic and therapeutic approach to the patient.

The following dissertation will address the importance of early diagnosis and timely treatment of patients suffering from hereditary metabolic diseases, before the onset of irreversible organ damage, and how the advent of newborn screening has been fundamental in changing the clinical history and outcome of these patients.

Newborn, family and selective screening programs, have redefined the epidemiology of metabolic diseases, making possible to diagnose more patients in a pre-clinical and asymptomatic stage of the disease.

For this purpose, it is necessary to develop biomarkers and evaluation scales that can guide the clinician in the early definition of the phenotype and of the prognosis for a proper planning of the follow-up and initiation of therapy.

Such analytical tools should be both highly sensitive and specific, minimally invasive and of low cost. They should allow to evaluate the course over time of the disease, the response to treatments and, as in the case of alpha-fetoprotein for Beckwith-Wiedemann syndrome, the onset of early injuries.

These topics will be analyzed through the articles published during my Ph.D.

Chapter I: Inborn error of metabolism treatment: focus on the different outcome to renal replacement therapy

Inborn errors of metabolism (IEMs) form a large class of genetic diseases, most of which are due to defects of single genes that code for enzymes that convert substrates into products[2]. Individually IEMs are rare, but collectively they represent a large and diverse group of diseases with a total prevalence reaching 40 per 100,000 births[3], even if the real prevalence varies considerably worldwide based on the racial and ethnic composition of the population[1].

They represent an important burden for the medical care systems in Western countries.

Although several approaches are used to treat patients with an IEM, they all have the common goal to overcome the primary enzyme defect through various therapeutic strategies such as substrate reduction, toxic substrate elimination, stimulation of alternative metabolic pathways or replacement of the deficient enzyme.

Understanding the molecular and biochemical etiologies of IEMs is essential for successful treatment.

Severe urea cycle defects (UCD), organic acidemias (OA) and maple syrup urine disease(MSUD) are life-threatening IEMs presenting in the first days of life with acute neurological deterioration. Common clinical features include feeding refusal, vomiting, hypotonia, abnormal movements, seizures, lethargy and coma[4].

Hyperammonemia is chiefly responsible for this picture in UCD and OA, whereas increased concentrations of leucine and related metabolites sustain neurotoxicity in MSUD.

Renal replacement therapy (RRT) is an emergency option in affected newborns, mostly performed as *ultima ratio*. We analyzed a 10-year experience using emergency RRT in newborns with UCD, OA and MSUD referring to our Department.

Early response to RRT was associated with survival irrespective of pre-treatment picture and it can be considered as a therapeutic approach even in huge neonatal metabolic decompensations.

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Part of this work has been published as:

F Porta, L Peruzzi, R Bonaudo, S Pieretti, M Busso, E Cocchi, A Conio, V Pagliardini and M Spada: "Differential response to renal replacement therapy in neonatal-onset inborn errors of metabolism" Nephrology 23 (2018) 957–961.

Background

After variable symptom-free intervals, newborns with severe urea cycle disorders (UCD), organic acidemias (OA) or maple syrup urine disease (MSUD) present with acute life-threatening neurological deterioration. Common clinical features include feeding refusal, vomiting, hypotonia, abnormal movements, seizures, lethargy and coma.[1] Hyperammonemia is chiefly responsible for this picture in UCD and OA, whereas increased concentrations of leucine and related metabolites sustain neurotoxicity in MSUD. Prompt correction of the acute metabolic derangement is mandatory in these disorders as the duration and severity of the metabolic decompensation have been related to both neurological outcome and prognosis quoad vitam.[2–4]

Besides supportive care and specific metabolic management, including pharmacological therapies available for selected disorders, the role of renal replacement therapy (RRT) is growing in the life-long emergency treatment of intoxication-type inborn errors of metabolism (IEM).[5–7]

Due to the rarity of IEM and/or the challenge of performing RRT in low-weight babies, however, single-centre experiences on the use of RRT in newborns with IEM may be precious to the optimization of this practice.

Here, we report our 10-year practice on this topic, pointing out the clinical and biochemical parameters associated with patients' short-term survival.

Patients and Methods

Patients' assessment and emergency management

From 1 January 2007 to 31 December 2016, 12 newborns with early-onset acute metabolic decompensation due to intoxication-type IEM were treated with RRT besides standard metabolic emergency management. All patients were transferred to the Pediatric Intensive Care Unit of our Department. A pediatric nephrologist was immediately alerted to avoid any delay in RRT. Front-line basic laboratory tests (compete blood count, blood gases, blood glucose, plasma ammonia, blood lactate, electrolytes, liver function and clotting test, creatine kinase, uric acid and urine analysis) addressed the metabolic suspect and the consistent emergency treatment. General supportive care included mechanical ventilation, circulatory support and correction of electrolyte imbalances. In all patients, a central venous catheter ensured hydration and protein-free energy supply (glucose 10 mg/kg per min).

Patients with suspected UCD were treated with a loading dose of sodium benzoate (250 mg/kg) or sodium benzoate + sodium phenylacetate (Ammonul, 250 mg/kg) and L-arginine (250 mg/kg) over 120 min and carglumic acid (100 mg/kg) according to accepted protocols.[1]

Patients with suspected OA were administered hydroxicobalamin (1 mg/day), carnitine (100 mg/kg per day) and biotin (10 mg/day). Plasma amino acids, acylcarnitines and urine organic acids were available within 24 h of the clinical onset. Seriate assessments of the Glasgow Coma Scale (GCS) and measurements of blood toxic metabolites (ammonia in UCD and OA; leucine in MSUD) were performed to monitor for treatment effectiveness.

RRT modalities

Renal replacement therapy was considered a first-line treatment. The RRT modalities used included continuous veno-venous hemodiafiltration (CVVHDF), continuous veno-venous haemodialysis (CVVHD) and peritoneal dialysis (PD). CVVHDF was performed with a continuous RRT machine equipped with neonatal blood lines and a polyethersulfone 0.2-m2 filter.

A dual lumen 5 French catheter was inserted into the femoral or subclavian vein. The circuit was pre-primed with warmed packed red blood cells (50 mL) and saline solution or 5% albumin in normal saline solution (50 mL); a heparin bolus (10 IU/kg) followed by continuous infusion (5–20 IU/kg per h) was administered according to the coagulation pattern.

The blood flow rate was set at 15–30 mL/min; the dialysate flow rate was set at 83 mL/min.

Fluid loss was adjusted to obtain a slightly positive fluid balance.

PD was used in combination with CVVHD in cases were rebound hyperammonemia was observed after the acute phase, except from one case where it was the first choice for clinical reasons.

PD was performed with Automated Peritoneal Dialysis (Home-Choice Baxter Deerfield, Illinois, U.S.), and started using fill volumes (20–30 mL/kg) with a swan neck curl neonatal catheter (38 cm) were used. Dwell times ranged from 15 to 20 min. Standard solutions with glucose concentrations of 1.36 or 2.27% were used.

Statistical analysis

Statistical analysis was performed with R: A Language and Environment for Statistical Computing (R Foundation for Statistical Computing, Vienna, Austria). The Shapiro–Wilk test was used to test the normality of data distribution. Differences between groups were established using the Student's t test or the Mann–Whitney U test. Statistical significance for all calculations was considered achieved when the two-tailed P value was less than 0.050. Data are presented as median and interquartile range or mean and standard deviation.

Results

Clinical, biochemical and molecular characteristics of the 12 newborns with IEM requiring RRT are summarized in Table 1.

Table 1 Main clinical and biochemical characteristics and molecular data of 12 patients with neonatal-onset inborn errors of metabolism (IEM) requiring emergency renal replacement therapy (RRT)

	Clinical features at onset					Metabolic features at onset					Genotype		Long-term outcome	
Patient	Sex	Birth weight (g)	Age (h)	GCS	Intubation	Amines	pH, BE	Ammonia (μmol/L)	Leucine (μmol/L)	Diagnosis	Gene	Allele 1	Allele 2	Age (last visit)	Picture
1	Male	1910	29	3	Yes	Yes	7.35, −5.2	1916	—	CPSd	CPS1	R814W	L1318X	10 years	Alive, LT
2	Male	1830	24	3	Yes	Yes	7.29, −6.4	1557	—	CPSd	CPS1	R814W	L1318X	4 months	Dead
3	Female	2400	38	3	Yes	Yes	7.31, −5.6	703	—	CPSd	CPS1	R721X	R1453Q	Neonate	Dead
4	Female	2300	48	3	Yes	Yes	7.34, −0.5	2722	—	CPSd	CPS1	Q1046X	T544 M	Neonate	Dead
5	Male	2900	36	3	Yes	Yes	7.27, −8.9	531	—	OTCd	OTC	R26W	—	Neonate	Dead
6	Male	2550	60	3	Yes	Yes	7.31, −8.0	754	—	OTCd	OTC	R320X	—	Neonate	Dead
7	Male	3500	39	3	Yes	Yes	7.40, −1.7	1770	—	OTCd	OTC	S164P	—	Neonate	Dead
8	Female	3300	90	3	Yes	Yes	7.32, −6.3	552	—	ASLd	ASL	A300S	G305R	3.5 years	Alive, LT
9	Male	2850	52	10	No	No	7.19, −21.3	348	—	MMA	MUT	N219Y	W309X	2 years	Alive, LT
10	Female	2760	61	10	No	No	7.24, −19.5	713	—	MMA	MUT	Q35X	5262 N	15 months	Alive
11	Female	3100	264	11	Yes	Yes	7.49, −0.3	—	4483	MSUD	BCKDHA	Q177X	Q177X	5.5 years	Alive, LT
12	Female	3365	168	11	No	No	7.40, −6.1	—	2950	MSUD	BCKDHB	Q267T	E330K	2 years	Alive, LT

ASLd, argininosuccinate lyase deficiency; BE, base excess; CPSd, carbamoyl phosphate synthetase deficiency; GCS, Glasgow coma scale; LT, liver transplanted MMA, methylmalonic acidemia (methylmalonyl-CoA mutase deficiency); MSUD, maple syrup urine disease (branched-chain α-ketoacid dehydrogenase deficiency); OTCd, ornithine transcarbamylase deficiency..

Hyperammonemic newborns, either affected by UCD (8 patients) or methylmalonic acidemia (MMA, 2 patients), required earlier RRT with respect to patients with MSUD (75 (65–102) vs 301 (192–410) h of life, $P < 0.01$).

The employed RRT techniques were CVVHDF (six patients), PD (one patient), CVVHD (two patients) and the combination of CVVHD and PD (three patients).

The time courses of ammonia and leucine before and during RRT are depicted in Figure 1.

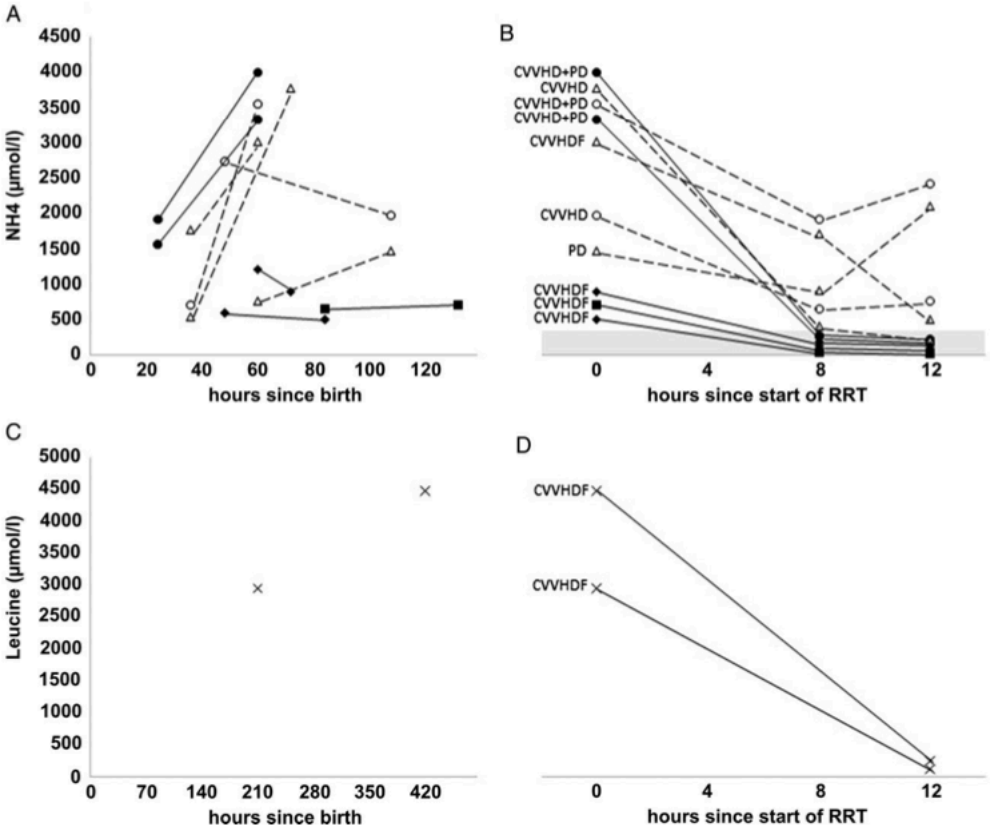

Fig. 1 Time course of ammonia (NH4) and leucine before (Panel A and C) and during renal replacement therapy (RRT) (Panel B and D) performed by continuous veno-venous hemodiafiltration (CVVHDF), continuous veno-venous haemodialysis (CVVHD) and/or peritoneal dialysis (PD) in 12 newborns affected by carbamoyl phosphate synthetase deficiency (circle), ornithine transcarbamylase deficiency (triangle), argininosuccinate lyase deficiency (square), methylmalonic acidemia (rhombus) and maple syrup urine disease (cross). Continuous and dotted lines represent survivors and non-survivors, respectively. The grey area in Panel B represents the NH4 range 0–300 µmol/L.

Median reduction of metabolites after 12 h of RRT was 65% in UCD, 80% in MMA and 95% in MSUD.

According to RRT modality, the same parameter was 88% using CVVHDF, 94% using CVVHD (with or without PD) and −42% using PD.

Of 12 patients, 7 survived the acute neonatal decompensation (5 treated with CVVHDF and 2 with the combination of CVVHD and PD).

All non-survivors were affected by mitochondrial UCD (two females with carbamoyl phosphate synthetase deficiency and three males with ornithine transcarbamylase deficiency), treated with CVVHD + PD (two patients), CVVHD (one patient), CVVHDF (one patient) or PD (one patient).

Among hyperammonemic patients, survivors and non-survivors showed similar birth weight (2670 (1883–3000) vs 2550 (2366–3100) g, P = 0.690), timing of onset (52 (27–71) vs 39 (37–52) h of life, P = 0.841), duration of intubation (96 (0–269) vs 76 (45–161) h, P = 0.917), ammonia at onset (1210 (577–1676) vs 754 (645–2087) µmol/L, P = 0.916) and at the start of RRT (758 (637–3545) vs 3010 (1805–3613) µmol/L, P = 0.426), age at RRT (76 (65–102) vs 74 (64–104) h, P = 0.999) and duration of coma before RRT (37 (29–40) vs 38 (26–51) h, P = 0.691).

All survivors showed plasma ammonia less than 300 μmol/L after 8 h of RRT and significantly lower ammonia concentration than non-survivors at the same time of treatment (183 (10–264) vs 909 (568–1770) μmol/L, $P < 0.01$).

Ammonia concentration after 12 h of RRT was also lower in survivors (136 (72–190) vs 753 (398–2200) μmol/L, $P = 0.015$).

All patients with MSUD survived the neonatal decompensation, with normalized leucine levels after 12 h of RRT (187.0 89.1 μmol/L; clearance 95 1%).

All survivors currently show normal or near-normal neurological development (48 39 months of age, five success- fully liver transplanted, one waiting for liver transplantation), except one patient with carbamoyl phosphate synthetase deficiency who died at 4 months of life because of sepsis (Table 1).

Discussion

Classic UCD, MMA and MSUD typically lead to early metabolic decompensation triggered by neonatal physiological catabolism.[8]

In the emergency setting, the occurrence of single hyperammonemia and/or associated with metabolic acidosis are the hallmarks of UCD and organic acidurias, respectively.

The peculiar odor of body fluids addresses the clinical diagnosis of MSUD as no abnormalities on front-line basic laboratory tests are generally detectable.

All these life- threatening disorders require prompt metabolic management aimed to stop protein catabolism and to clear toxic metabolites. For the latter purpose, RRT has been proposed since the late 70s for hyperammonemia and MSUD.[5,9]

During the last two decades, enormous technical and clinical improvements were achieved with different RRT methods, with the most published data on hyperammonemic patients, consistent with the favorable dialytic properties of ammonia.[10,11] As it is better tolerated in sick neonates, continuous RRT (mostly CVVHDF) was the most frequently employed method in the emergency treatment of critical newborns with inherited hyperammonemias or MSUD referred to our department.12 In our experience, CVVHDF was largely successful in newborns with neonatal-onset IEM, with more than 80% survival rate (five of six patients).

Due to the small population and the various aetiologies of acute neonatal metabolic decompensation, however, a comparative analysis of the effect of the different RRT on patients' survival could be misleading. Overall, ~60% of patients treated with RRT overcame the acute neonatal decompensation. All patients with MMA, argininosuccinate lyase deficiency and MSUD shared good prognosis, as well as half of the patients with carbamoyl phosphate synthetase deficiency.

Among hyperammonemic newborns, good early response to RRT warranted patients' survival irrespective of pre-treatment clinical or biochemical characteristics. Actually, all the survivors with

UCD and MMA showed ammonia concentrations below 300 μmol/L after 8 h of RRT. In particular, patients with MMA and cytosolic UCD (argininosuccinate lyase deficiency) presented the lowest ammonia concentrations at onset, readily normalized by RRT, whereas the higher ammonia concentrations at onset in survivors with mitochondrial UCD (carbamoyl phosphate synthetase deficiency) were rapidly and substantially reduced by RRT.

Given the high risk of life-threatening metabolic decompensations mostly triggered by intercurrent infections or protein overload, liver transplantation was indicated in all survivors and successfully performed in one patient with MMA, one patient with argininosuccinate lyase deficiency, one patient with carbamoyl phosphate synthetase deficiency and two patients with MSUD. In all patients, liver transplantation allowed correction or significant improvement of specific disease-related metabolic pictures, the stop of protein dietary restriction and the prevention of any metabolic decompensation until present.

On the contrary, higher ammonia levels after 8 h of RRT (>300 μmol/L) were observed in all non-survivors, likely due to extreme circulatory impairment. All these patients were affected by severe mitochondrial UCD, including ornithine transcarbamylase deficiency and carbamoyl phosphate synthetase deficiency. In particular, ornithine transcarbamylase deficiency was invariably lethal, consistent with its worse prognosis with respect to other UCD.[13]

Non-modifiable features, such as age at onset and peak ammonia levels, have previously been identified as predictors of outcome in UCD.[14,15] In our experience, the same variables were poorly predictive of short-term prognosis quoad vitam. Actually, we suggest that RRT treatment be considered in any newborn with intoxication-type IEM as survival and possibly the long-term outcome appear to be irrespective of the pre- treatment conditions but are mainly dependent on the rapid correction of the metabolic picture.

Moreover, given the severity of disorders with life-threatening onset in the neonatal period, we suggest that surviving patients could benefit from early liver transplantation,[16,17] virtually avoiding the risk of life-threatening metabolic decompensations during catabolic stress later in life.

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Chapter II: Newborn screening as an important diagnostic tool

Neonatal screenings represent an important secondary health intervention that allows the early diagnosis of a broad spectrum of congenital diseases, for which specific therapies are available. The promptly diagnosis before the onset of signs, symptoms and organ damage, can significantly improve the prognosis of the disease and the quality of life of patients, avoiding severe disabilities (mental and / or growth retardation, severe permanent damage) and, in some cases, even death.

In Italy, newborn screening is mandatory and offered for free to all newborns, in compliance with the Prime Ministerial Decree of January 12, 2017 (article 38, paragraph 2) on the new essential levels of assistance. It has been free and compulsory since 1992, and initially carried out for three diseases: congenital hypothyroidism, cystic fibrosis and phenylketonuria.

However, each region was allowed to autonomously expand the range of pathologies screened.

For this reason, in Piemonte, a form of expanded newborn screening was already present, comprising the three diseases mentioned above with the addition of the biotinidase deficiency, galactosemia and congenital adrenal hyperplasia syndrome.

In the 1990s, the development of laboratory technologies allowed to extend neonatal screening to a broad spectrum of congenital diseases, called Extended Neonatal Screening - SNE.

Italy is the European country with the most advanced neonatal screening policy, as defined in accordance with the Law 167/2016 and subsequent updates and implementing decrees.

The diseases researched are defined rare due to their low prevalence in the population (they affect no more than 5 per 10,000 inhabitants), but of great importance in public health for the burden of the disease.

The Italian SNE currently includes over 40 inherited metabolic diseases, and the 2019 Budget Law (Article 1 c. 544), which amended Law 167/2016, extended neonatal screening to genetic neuromuscular diseases, severe congenital immunodeficiencies and lysosomal storage disorders and established the periodic updating of the list of diseases to be screened.

Newborn screening consists in a non-invasive blood test, harvested between 48 and 72 hours of life of the baby, that can measure the concentration in the blood of specific metabolites.

In the presence of an alteration, additional diagnostic tests are required and the newborn must be referred to the clinical center to promptly start the specific treatment and follow-up.

Biotinidase deficiency is one of the mandatory diseases screened in accordance with the Prime Ministerial Decree of October 13, 2016.

Biotin is an essential vitamin that is obtained through the diet and also efficiently recycled for further use[1]. Biotin acts as a coenzyme for four carboxylation enzymes in the body: 3-methylcrontonyl-

CoA carboxylase (MCC), pyruvate carboxylase (PC), acetyl-CoA carboxylase (ACC) and propionyl-CoA carboxylase (PCC).

Biotinidase deficiency is an autosomal recessive enzyme deficiency of this recycling mechanism. It can be partial(10 to 30% of enzyme activity) in which patients have little or no symptoms; or profound(less than 30% of enzyme activity), that can lead to coma or death without treatment.

Treatment is very simple as patients need consistent and high doses of biotin administered. This simple treatment can reverse and prevent many symptoms of the disease like developmental delay, disability, and improve quality of life. This fact is a key reason for why this disorder is part of many newborn screening programs.

The first neonatal screening for biotinidase deficiency has been performed in 1985, and Piemonte has been the first Italian region, and one of the first European, to perform it since the early 1987 by the Regional Reference Center for Newborn Screening of Piemonte and Valle d'Aosta.

The data of a 30 years research experience in biotinidase deficiency screening and treatment of our center has been analyzed in the next article.

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Part of this work has been published as:

Porta F, Pagliardini V, Celestino I, Pavanello E, Pagliardini S, Guardamagna O, Ponzone A, Spada M. " Neonatal screening for biotinidase deficiency: A 30-year single center experience". Mol Genet Metab Rep., 2017 Sep 20;13:80-82.

Introduction

Biotinidase deficiency (OMIM 253260) is an autosomal recessive inborn error of metabolism leading to biotin shortage. As biotin is the essential cofactor of the four human carboxylases (namely propyonil-CoA carboxylase, methylcrotonyl-CoA carboxylase, acetyl-CoA carboxylase, and pyruvate carboxylase), its deficiency results in multiple carboxylase deficiency, a complex, life-threatening disorder impairing gluconeogenesis and organic acids metabolism.

Clinically, untreated biotinidase deficiency can present with variable neurological and dermatological signs, including seizures, hypotonia, feeding difficulties, developmental delay, ophthalmologic problems, hearing loss, ataxia, alopecia, and skin rash [1].

The vast majority of these symptoms improve with the administration of pharmacological doses of biotin, although late treatment is not usually fully effective in reversing neurological, ophthalmologic, and audiological sequelae [2].

After the discovery and characterization of biotinidase deficiency in 1983 [3], a rapid colorimetric method for measuring biotinidase activity on dried blood spots was developed [4]. This addressed the first neonatal screening for biotinidase deficiency in 1985 [5], allowing for the possibility of treatment anticipation and optimization of clinical outcomes.

At our department, the Regional Reference Center for Newborn Screening of Piemonte and Valle d'Aosta and the Regional Reference Center for diagnosis and treatment of inborn errors of metabolism, neonatal screening for biotinidase deficiency was incepted since the early 1987 and all identified patients have been followed at our clinic from the neonatal period to adulthood. Here we report our 30-year experience in this issue.

Methods

We reviewed the outcome of newborn screening for biotinidase deficiency performed at our department from January 1987 to December 2016 and the correspondent long-term clinical outcome. Newborn screening was performed using the previously described colorimetric assay to determine biotinidase activity [4].

Briefly, enzyme activity was screened in newborns by determining the amount of N- biotinyl-p-aminobenzoate (PAB) hydrolyzed to p-aminobenzoate on dried blood spots collected in the third day of life. The first tier test was performed by a semiquantitative colorimetric assay, distinguishing normal (purple colored) from biotinidase deficient (straw-colored) samples.

Newborns screened positive were recalled for re-determination of biotinidase activity on dried blood spot and, in case of confirmed abnormal results, referred to clinical evaluation and quantitative measurement of serum biotinidase activity.

Normal serum biotinidase activity, set on the basis of measurements in 120 healthy subjects, ranged from 3.1 to 6.7 nM PAB/min/ml. Profound and partial biotinidase deficiency were defined as < 10% and 10–30% of median serum enzyme activity, respectively.

Serum biotinidase activity was also assessed in heterozygous parents of patients with genotyped biotinidase deficiency.

Molecular analysis was performed by full gene sequencing in affected patients and by targeted mutation analysis in parents after informed consent. All detected patients were treated with biotin (10–20 mg/day) since the neonatal period and followed at our department up to now.

Results

Among 1,097,894 newborns screened, 461 were recalled, and 18 were identified as affected by biotinidase deficiency (incidence 1:61,000, false positive rate 0.04%, positive predictive value 3.9%, estimated cost per test 0.60 €).

All detected patients were born from non-consanguineous Italian parents. Patients' clinical, biochemical, and molecular characteristics are summarized in Table 1.

Table 1
Clinical, biochemical and molecular characteristics of 18 patients detected among 1,097,894 newborns screened for biotinidase deficiency.

Patient	Gender	Biotinidase activity		Genotype		Biotin treatment (mg/day)	Follow-up (years)	Clinical symptoms
		Serum activity*	% of median serum activity	Allele 1	Allele 2			
1	Female	0.8	17	NA	NA	10	30	No symptoms
2	Female	0	0	A171T/D444H	R211H	20	26	No symptoms
3	Female	0	0	Q456H	C186Y	20	26	No symptoms
4	Female	0	0	Q456H	E218D	20	25	No symptoms
5	Female	1.4	30	NA	NA	20	23	No symptoms
6	Male	0.6	13	A171T/D444H	D444H	20	22	No symptoms
7	Male	0	0	Q456H	E218D	20	21	No symptoms
8	Female	0	0	Q456H	G34S	20	18	No symptoms
9	Male	0	0	Q456H	G34S	20	16	No symptoms
10	Male	0	0	A171T/D444H	A171T/D444H	20	8	No symptoms
11	Male	0	0	C245Y	Q456H	20	7	No symptoms
12	Male	0	0	M399I**	Q456H	20	2	No symptoms
13	Male	0	0	C245Y	Q456H	20	2	No symptoms
14	Female	1.0	22	NA	NA	10	2	No symptoms
15	Male	1.2	24	NA	NA	10	2	No symptoms
16	Male	1.2	23	NA	NA	10	1	No symptoms
17	Female	1.2	25	NA	NA	10	1	No symptoms
18	Male	1.3	26	NA	NA	10	0.5	No symptoms

NA: not available.
* Normal value = 3.1–6.7 nM PAB/min/ml.
** Previously unreported mutation.

Ten patients were affected by profound biotinidase deficiency, with undetectable serum biotinidase activity. Of them, 8 (80%) harbored the missense mutation Q456H in compound heterozygosity. The

novel mutation M399I was identified in one patient with profound biotinidase deficiency (Table 1, patient 12).

The complex allele A171T/D444H in cis was the second most common molecular finding, either associated with profound and partial biotinidase deficiency depending on the second allele in trans (Table 1, patient 2, 6, and 10).

Eight patients were ascertained by 13–30% median serum biotinidase activity, consistent with partial biotinidase deficiency. Their serum biotinidase activity ranged from 0.6 to 1.4 nM PAB/min/ml.

Genotype was available in one patient with the partial form, harboring the protective D444H mutation (Table 1, patient 6).

Overall, 9 different mutations were identified in patients with biotinidase deficiency and confirmed in parents (Q456H, A171T/D444H in cis, E218D, G34S, C245Y, R211H, C186Y, M399I, D444H).

The in vivo biochemical effect assessed in heterozygous parents of patients with biotinidase deficiency is reported in Table 2.

Table 2
In vivo serum biotinidase activity in 16 heterozygous parents of patients with profound or partial biotinidase deficiency.

Number of subjects	Mutation	Range serum activity (nM PAB/min/ml)	% of median serum activity
5	Q456H	2.3–2.5	50–54
4	A171T/D444H	2.1–3.9	45–85
1	E218D	2.4	52
1	G34S	2.1	46
1	C245Y	3.6	78
1	R211H	3.3	72
1	C186Y	3.6	78
1	M399I	3.5	76
1	D444H	3.7	80

All identified patients were asymptomatic at first clinical evaluation in the neonatal period, when treatment with biotin was started. Clinical follow-up lasted 13.6 ± 10.8 years.

Compliance with biotin therapy was complete even on long-term follow-up. No adverse effects were reported. All patients underwent regular metabolic, ophthalmologic, and audiological evaluations revealing no signs or symptoms related to biotinidase deficiency.

Discussion

Shortly after the first pilot study of neonatal screening for biotinidase deficiency performed in Virginia in 1985, this condition was readily included in the screening program of our Region.

Our department, indeed, has been traditionally dedicated to the implementation of innovative screening procedures [6–8].

Also, a posteriori, biotinidase deficiency meets the major criteria for its inclusion in neonatal screening programs, being a severe disease if untreated, early detect- able by rapid and economical methods, and effectively treated by a simple and inexpensive therapy. During the last 30 years, the screening for biotinidase deficiency of over one million newborns allowed the identification of 18 patients at the pre-symptomatic stage. The combined incidence profound and partial biotinidase deficiency in our Region overlapped that reported worldwide [9], being comparable to that of many other disorders commonly included in neonatal screening programs [10,11].

A very low false positive rate characterized newborn screening for biotinidase deficiency at our clinic. In spite of the relative rarity of the disease, this parameter was even better than that advocated for expanded newborn screening programs by tandem mass spectrometry [12].

The long-lasting screening experience at our Center probably played a significant role on this achievement. On the other hand, the positive predictive value was low for this mass screening program; however, since this parameter is strictly dependent on the prevalence of the disease, this finding is not surprising. As for the estimated cost per test, newborn screening for biotinidase deficiency was cheaper than other single-disease screening programs performed at our Center, including galactosemia, cystic fibrosis, congenital adrenal hyperplasia, and hypothyroidism (around 2 € for each test).

From a molecular point of view, 80% of patients with profound biotinidase deficiency showed the same missense mutation Q456H in compound heterozygosity. This molecular finding was already identified as a common cause of biotinidase deficiency especially in patients with European ethnic backgrounds [13], in agreement of our findings.

Three patients with different biochemical phenotypes shared the same complex allele A171T/D444H in cis.

Consistently with previous observations, profound biotinidase deficiency resulted from homozygosity for this allele [14], as well as from its association with the R211H mutation in trans. On the other hand, the association of the allele A171T/D444H in cis and the protective mutation D444H in trans[15] resulted in partial biotinidase deficiency. In vivo biochemical data in heterozygous subjects are consistent with these findings.

From a clinical point of view, early biotin therapy allowed the full prevention of clinical symptoms in all detected patients with biotinidase deficiency. In particular, no adverse effects were registered and compliance to treatment was optimal even in patients on long-term follow-up (7 patients followed and treated for > 20 years).

These excellent outcomes are in stark contrast with those of patients with late diagnosis of biotinidase deficiency, suffering from irreversible neuro- logical damages if treated late and being even at risk of

death if left untreated [16]. If newborn screening is not performed, indeed, the clinical diagnosis of biotinidase deficiency is invariably arduous, as biotinidase deficiency can mimic atopic dermatitis and a wide range of neurological conditions, including neuromyelitis optica, optic atrophy, and myelopathies [17–23]. Despite these clinical evidences and the cost-effectiveness of newborn screening for biotinidase deficiency [24], however, the application of this preventive procedure is still not uniform in Europe (differently from the U.S.).

We hope that our long-lasting successful experience could promote the universal extension of this practice. Neurologists, moreover, should be aware of the opportunity of including biotinidase activity as an adjunct to the diagnostic work-up of unexplained central neurological disorders in patients not screened for biotinidase deficiency in the newborn period.

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Chapter III: The importance of biomarkers

A biomarker is a measurable indicator of some biological state or condition. Biomarkers are often measured and evaluated using blood, urine, or soft tissues[1] to examine normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention[2]. They can be used in disease screening, diagnosis, characterization, and monitoring as prognostic indicators, for developing individualized therapeutic interventions; for predicting and treating adverse drug reactions; for identifying cell types; and for pharmacodynamic and dose-response studies.

Good biomarkers should be measurable with little or no variability, should have a sizeable signal to noise ratio, and should change promptly and reliably in response to changes in the condition[3].

Beckwith–Wiedemann syndrome (BWS) is a pediatric overgrowth disorder involving a predisposition to tumor development. The clinical presentation is highly variable and it is a panethnic disorder with an estimated population incidence of 1 in 13700[4,5].

The incidence is equal in males and females with the notable exception of monozygotic twins that show a dramatic excess of females [4].

In addition to clinical heterogeneity, BWS also exhibits etiologic molecular heterogeneity. A variety of genetic and/or epi-genetic alterations in growth regulatory genes on chromosome 11p15.5 are associated with specific phenotype–epigenotype/genotype correlations including different recurrence risks. BWS usually occurs sporadically (85%), but familial transmission occurs in about 15% of cases[6].

Hepatoblastoma screening in BWS disease is currently based on measuring seric alpha-fetoprotein (αFP) every three months until the fourth birthday.

Frequent blood draws can be a burden for patients and their families so we have developed a less invasive alternative testing method based on measuring αFPs from dried blood spots (DBS), normally used for newborn screening.

This novel method shows consistent overlap with the traditional blood draws, thereby demonstrating its utility for hepatoblastoma screening in this setting and alleviating the burden of frequent blood draws.

This also may help increase patient compliance, reduces costs of health care screening and, due to the stability and easy execution of the sampling, it could theoretically be carried out independently by the patient at home, with subsequent sending of the sample by post, reducing the number of visits to the hospital.

The DBS-based method for the measurement of cancer biomarkers may also be applied to several other chronic diseases with increased risks of αFP-producing liver tumors.

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Part of this work has been published as:

Mussa A, Ciuffreda VP, Sauro P, Pagliardini V, Pagliardini S, Carli D, Kalish JM, Fagioli F, Pavanello E, Ferrero GB. "Longitudinal Monitoring of Alpha-Fetoprotein by Dried Blood Spot for Hepatoblastoma Screening in Beckwith-Wiedemann Syndrome". Cancers (Basel). 2019 Jan 14;11(1). pii: E86. doi: 10.3390/cancers11010086.

Introduction

The Beckwith–Wiedemann spectrum (BWSp) consists of the variable association of macroglossia, abdominal wall defects, organomegaly, ear pits/creases, facial nevus simplex, hyperinsulinemic hypoglycemia, lateralized overgrowth, and embryonal tumor predisposition [1–3]. BWSp includes the classical Beckwith–Wiedemann syndrome (BWS, OMIM #130650), the most common overgrowth and cancer predisposition disorder (1:10,500 live births) [4], and more subtle presentations with an 11p15.5 molecular anomaly, including Isolated Lateralized Overgrowth (ILO, OMIM #235000) [5]. The BWSp embryonal tumor predisposition in childhood includes an increased risk of developing hepatoblastoma (HB), which occurs in up to 3.5% of patients depending on the specific genetic anomaly [6,7]. HB typically occurs before 30 months of age with a peak of incidence at six months [8,9], and BWS represents the most relevant risk factor for HB, with a relative risk of 2280 times greater than in the general population [10]. The molecular subgroups of BWS patients with the highest risk for HB are those with paternal uniparental disomy (UPD) of chromosome 11 or genome-wide UPD [11–13]. Moreover, HB is the most common tumor diagnosed in BWSp children with the loss of methylation at imprinting control region 2, the molecular subgroup representing approximately 50% of BWSp patients [14].

HB usually grows rapidly; therefore, survival and prognosis are highly dependent on early diagnosis [8,9,15]. More than 95% of HB secrete the highly sensitive and specific tumor marker alpha-fetoprotein (αFP) [9,15], and measuring αFPs are used in diagnosis, follow-up, and relapse detection. BWSp patients are monitored for HB with αFPs every three months from birth to the fourth birthday [16–19]. Screening provides the opportunity for early detection and at earlier stages of the diagnosis, potentially allowing for less toxic therapies [20,21].

However, αFP screening in this population has been controversial for a number of reasons [6,7,14,22–24]. First, the variability in the physiologic decrease of normal serum αFP levels (from 105 U/mL magnitude at birth to concentrations steadily <10 U/mL by the age of 12–24 months) leads to challenges in the interpretation of αFP levels in early infancy [25,26]. Moreover, normal αFP values in BWS patients in the first year of life tend to be elevated compared with normal pediatric values [27]. Age-corrected reference values should be employed [25,26], and αFP trends, rather than reliance of the actual value compared to non-BWS norms, is a more accurate screening strategy [16,23,28].

Second, some health care providers consider the incidence of HB too low to warrant specific screening [2]: While recently the American Association for Cancer Research (AACR) Childhood Cancer Predisposition Workshop adopted a 1% risk threshold for surveillance and recommended αFP screening for all cases of BWSp [29], a consensus statement from the European Network of Human Congenital Imprinting Disorders (EUCID) judged a 5% threshold to be more appropriate for European healthcare systems and did not recommend αFP screening in these patients [2].

Lastly, the frequent blood draw schedule in early infancy is perceived as invasive, represents a burden for some children and families, and may cause compliance issues [22,23]. However, parents report being comforted and reassured by the screening [30]. For this reason, in a previous report we demonstrated the technical feasibility of αFP determination using dried capillary blood spots (DBS) [31]. Here we demonstrate the utility of DBS in parallel to the currently accepted practice of venous αFP sampling in a range of BWSp patients and normal controls. The method described in our previous report was introduced as a clinical practice pilot program in our institution and compared in parallel to the standard laboratory method. In this report, we describe our experience in the longitudinal monitoring of BWSp by the novel method, further supporting its utility in routine clinical practice.

Results

Measurements on plasma and DBS were closely correlated ($r2 = 0.999$, $p < 0.001$, Figure 1). Raw measurement data are provided in Supplementary Table S1.

The αFP measurements by the two methods showed consistency and largely overlapped across the wide range of physiological concentrations of the tumor marker (0.3–97,198.0 U/mL in plasma and from 0.1 to 97,889.0 U/mL on DBS).

In 202 cases, both methods measured αFP ≤10 U/mL, whereas in 51 cases, both measurements were >10 U/mL. In five cases, αFP was >10 U/mL in plasma and ≤10 U/mL on DBS: Patient 3 with 11.4 U/mL on plasma and 9.8 U/mL on DBS, Patient 4 with 11.5 U/mL on plasma and 4.1 U/mL was ≤10 U/mL (9.9 U/mL) and >10 U/mL on DBS (14.3 U/mL). All the paired measurements show differences between serum and DBS measurements within the coefficient of variation (CV%). Twenty-six patients and 20 controls had more than one αFP measurement; 22 patients and 123 controls had 1 paired measurement.

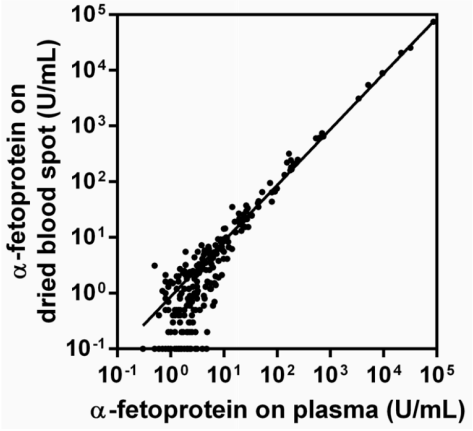

Figure 1. Correlation between α-fetoprotein (αPF) measured on plasma and on dried blood spot (DBS) ($r^2 = 0.999$, $p < 0.001$).

Of the 26 patients with a longitudinal assessment, 12 patients had αFP measurements >10 U/mL: Their αFP trend over time is displayed in Figure 2 to highlight the concordance between the two methods.

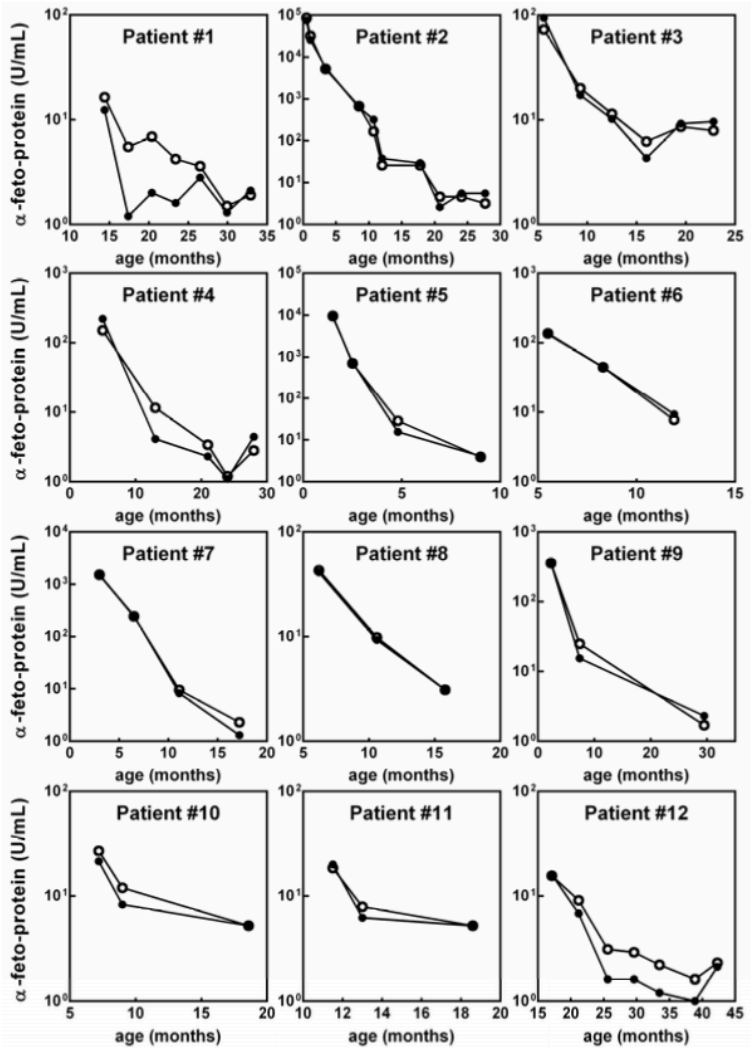

Figure 2. The longitudinal evaluation of alpha-fetoprotein (αFP) plasmatic concentration on dried blood spots (DBS, closed circles) overlapped that on standard laboratory method (open circles) in the 12 patients affected by cancer-predisposition syndromes with αFP concentrations >10 U/mL.

The remaining 14 patients were assessed longitudinally, and all had concordant DBS-plasma αFP <10 U/mL over time (not shown as values under 10 U/mL are considered within normal range).

Of the 22 patients tested with a single paired measurement, 19 had concordant DBS-plasma αFP values <10 U/mL and three had values >10 U/mL. In the latter group, Patient 29 showed 225.0 U/mL in and Patient 37 had 187.1 U/mL in plasma and 168.0 U/mL on DBS. The patient diagnosed with non-syndromic HB, a female aged 31 months, had an αFP of 583.5 U/mL measured by the traditional method and 601.0 U/mL on DBS.

Discussion

In this work, we demonstrate the feasibility of HB screening in overgrowth-cancer predisposition syndromes using DBS for αFP measurements. DBS, a novel method to measure αFP concentration showed consistent overlap with the traditional venous sampling method, showing reliability in the clinical setting of a tumor screening program. In the first two years of life—those in which the likelihood of developing HB is commonly higher in such conditions [9]—αFP concentrations decrease rapidly from a 106–105 to a <10 U/mL magnitude, with almost unpredictable and variable timing, making interpretations challenging. Although age-specific and gestational age-corrected normal αFP concentrations values are available [32], HB screening relies mostly on the longitudinal observation of repeated measurements of αFP concentrations rather than on the detection of a single measurement [28,29]. The DBS measurements overlapped the serum measurements across a wide range of physiologic concentrations and ages, demonstrating the utility of our methodology for longitudinal monitoring in both newborns and toddlers. Moreover, the range of measurements includes a 105 magnitude often observed in prematurity [32], which is common in the BWSp [33].

After the physiological decrease of αFP serum concentrations, a cutoff of >10 U/mL is commonly used to define normal values after 2 years of age. The measurement provided by the traditional and novel methods consistently matched across this diagnostic threshold, allowing, therefore, the determination of normal screens compared to abnormal screening tests. The few cases with discordant plasma and DBS measurements showed very tight fluctuations across the 10 U/mL threshold within the acceptable error range for these tests, and clinical management was not altered by these fluctuations.

Additionally, our patient who was ultimately diagnosed with a non-syndromic HB, showed that αFP levels determined by the two methods were highly consistent in the setting of a tumor diagnosis as well. Hence, we propose using DBS for HB screening and for patients presenting abnormal results suggesting a tumor diagnosis; further investigation with both conventional venous αFP testing and imaging studies should be used to confirm or exclude a HB diagnosis. Finally, the DBS technique alleviates the burden of frequent blood draws and is simple, efficient and low-cost, thus making the

routine measurement of αFP more practical. These aspects are crucial to improve patients' compliance with tumor surveillance recommendations for cancer predisposition syndromes. It has recently been shown that accurate knowledge about cancer risk and screening in the BWSp context decreases parents' worries about tumor development [30] and that the DBS method may also decrease children's anxiety related to HB screening. The DBS method is cheaper in terms of storage, transport, and handling even compared to other minimally invasive methods (i.e., finger poke in microtainer tube) that require that the samples remain in a liquid state [34]. DBS home sampling is associated with a reduction in costs both from a healthcare and from a societal perspective with patient costs abated nearly to zero and with a relevant decrease in costs related to the loss of productivity [35]. Other potential applications of the DBS method may come from this work and include screening of other conditions with increased risk of hepatocarcinoma or other αFP-secreting tumors as well as follow-up of patients treated for liver tumors. As screening for hepatocarcinoma in cirrhotic patients by repeated serum αFP measurement is feasible [36] and results in increased survival rates [28,37], monitoring in cirrhotic patients by DBS may represent a specific example of future applications.

Materials and Methods

Patients

Overall, 259 simultaneous plasma and DBS αFP measurements were performed in 171 children (mean age 38.7 ± 59.2 months, range 0–7.3 years, 88 males, 83 females). Of these, 116 paired measurements have been performed in 48 patients with syndromes with increased risk of HB for tumor screening (range 0–60 months): 39 were affected by BWSp/ILO (23 molecularly confirmed, 16 diagnosed clinically with negative molecular tests), 3 had macrocephaly-capillary malformation syndrome, 5 had undiagnosed likely syndromic overgrowth disorders, and one girl had an isolated HB. Of these, 31 measurements from 31 patients were previously reported in our preliminary report [31]. The remaining 143 paired measurements were performed in 123 children as controls: 27 were healthy children and 96 underwent blood tests for suspected conditions with no effect on plasmatic αFP concentration (20 suspected or well compensated thyroid disorders, 35 with recently healed infections, 12 serum lipids screening, 13 affected by phenylketonuria, 10 suspected iron deficient anemia, and 6 suspected precocious puberty). Besides studying the potential utility of the DBS method for longitudinal monitoring, we applied the novel method to patients admitted for suspected abdominal tumors. Twenty-three paired measurements were therefore performed in patients referred to our Division of Pediatric Oncology for suspected neoplasms and who had αFP measurement with the aim to identify potential αFP producing tumors. Among the last cases, the suspected diagnosis of neoplasm was excluded after the tests (including αFP and appropriate imaging) except for one female who was ultimately diagnosed with non-syndromic HB.

Study and Screening Protocol

Informed consent was obtained from parents using a study protocol approved by the Institutional Review Board of our University Hospital (IRB number CS/156/2014 Città della Salute e della Scienza di Torino, University of Torino, Italy). Our protocol for HB screening in overgrowth-cancer predisposition syndromes is consistent with that proposed by the American Association for Cancer Research [29] and is based on 3 months' abdominal ultrasound and simultaneous αFP measurements by standard laboratory methodology from birth up to the fourth birthday [17]. A negative αFP test is defined as an αFP measurement less than 10 U/mL (1 U/mL = 1.21 ng/mL) or declining with respect to previous measurement. The individual value is interpreted in the context of the αFP trend over time, with an expectation of declining values through infancy. In case of concentrations >10 U/mL, the results need to be interpreted on the basis of normal BWS values (which tend to be elevated over the first years of life compared with normal pediatric values), with age-specific and gestational age-corrected reference values provided in the literature [25] and with previous measurements performed in the same patient, if available. If the concentrations are less than the previous ones, then we consider the test negative and register the absolute value in order to perform subsequent comparisons. If we detect an αFP greater than the previous one, the test is referred to as positive, recent imaging is re-evaluated, and the patient is recalled for subsequent αFP remeasurements after a 6-week interval for rises greater than 50–100 U/mL [29]. In cases of significantly larger increases (greater than 1000 U/mL) or with further increase at the 6 weeks αFP remeasurement, second-step medical investigations are proposed (targeted liver US or MRI) [29].

Laboratory Assays

Paired αFP measurements were simultaneously performed on blood, obtained by venipuncture, and DBS, collected by heel-stick or by spotting single blood drops from a syringe directly onto standard filter paper employed for newborn screening. The DBS specimens were dried at room temperature, routinely stored in plastic bags at 4 ◦C and analyzed employing a 3.2 mm-diameter spotted filter paper punch containing approximately 3.4 µL of adsorbed blood. The serum αFP measurement kit (AutoDELFIA hAFP, Perkin Elmer, Waltham, Massachusetts) adapted to the DBS technique has been employed, as previously described [31]. The intra-assay CV% was evaluated for quality controls: For low concentrations (~10 U/mL), the CV%s were 3.02% for the plasma assay and 4.11% for the DBS one; for high concentrations quality controls (~70 U/mL), the CV%s were 3.05% and 3.22%, respectively.

Statistics

Data were analysed by GraphPad Prism 6.0 (GraphPad Software, Inc. La Jolla, CA, USA). Data distribution was assessed by the Shapiro–Wilk test and correlations tested by Pearson or Sperman methods, accordingly.

Conclusions

In conclusion, in this study we demonstrate that screening children with overgrowth disorders with HB predispositions can be performed by a novel simple method, which measures αPF on DBS. This novel technique may lead to increased adherence and reduced anxiety and cost.

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Chapter IV: Lysosomal storage disorders: how the advent of enzyme replacement therapy and early diagnosis changed the outcome and prognosis of patients in Pompe disease.

Lysosomal diseases are an extremely heterogeneous group of over fifty different genetically determined disorders, characterized by progressive accumulation of specific substrates, causing gradual cell and tissue damage. The severity and age of onset of the diseases depend in part, on the organs involved and the residual enzyme activity.

The first clinical manifestations may arise in the neonatal period or conversely in adulthood, with an extremely variable signs and symptoms spectrum; the genotype-phenotype correlation is known and demonstrable only for few mutations.

Pompe disease or type II glycogenosis (GSD II) (OMIM # 232300) is a rare lysosomal storage disorder caused by the defect of the enzyme alpha-1-4 acid glucosidase (GAA), causing accumulation of glycogen especially in cardiac and skeletal muscle cells, but also in other tissues such as smooth muscle and the central nervous system[1]. The accumulation of glycogen is followed by a dysfunction of the autophagic system with consequent cellular degeneration.

It is an autosomal recessive disease with prevalent chronic neuromuscular symptoms and a shortened life expectancy, especially in childhood forms.

The disease is classified, basing on the age of onset, in a classic neonatal form (Infantile Onset Pompe Disease, IOPD), which occurs early within 12 months of life, and Late Onset Pompe Disease (LOPD), that can occur at any age after the first year of life.

In IOPD the genetic mutations result in an almost complete absence of GAA. The clinical picture is characterized by severe and progressive hypotonia, hypertrophic cardiomyopathy, respiratory insufficiency, delay or regression in the acquisition and increase of creatine kinase (CK) levels. This disease, if not diagnosed and treated early, leads to death within the first-second year of life [2].

LOPD instead, is characterized by major involvement of muscle tissue, with little or no cardiac involvement. These patients manifest a progressive motor dysfunction with respiratory insufficiency, which is the most common cause of death [3].

The advent of enzyme replacement therapy (ERT) in 2006 dramatically changed the clinical picture of LOPD but especially of IOPD, with prolonged survival in about 60% of treated patients, generating a new phenotype: the IOPD long term survivors.

Treated IOPD have a completely different clinical phenotype from untreated patients, with prevalent neuromuscular involvement and improvement or normalization of cardiac damage. The knowledge derived from the observation of treated patients has allowed us to clearly observe that early treatment

correlates with a better outcome. Therefore, the early diagnosis is fundamental, to start ERT before irreversible organ damage.

This is why some Italian region include Pompe disease among those to be screened for newborns, and in Piemonte we are quickly acting to make it possible.

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Part of this work has been published as:

Parini R, De Lorenzo P, Dardis A, Burlina A, Cassio A, Cavarzere P, Concolino D, Della Casa R, Deodato F, Donati MA, Fiumara A, Gasperini S, Menni F, Pagliardini V, Sacchini M, Spada M, Taurisano R, Valsecchi MG, Di Rocco M, Bembi B. "Long term clinical history of an Italian cohort of infantile onset Pompe disease treated with enzyme replacement therapy". Orphanet J Rare Dis. 2018 Feb 8;13(1):32.

Background

Enzyme replacement therapy (ERT) with recombinant human acid α-glucosidase (rhGAA) for Infantile Onset Pompe Disease (IOPD) (OMIM #232300) became commercially available in 2006 on the basis of two pivotal multicentre studies [1, 2]. These first studies demonstrated the improvement of cardiomyopathy and a pro- longed survival in 26 treated infants and the progress of motor function milestones in some of them.

Further studies indicated that the age of ERT start was critical to obtain a better therapeutic result [3, 4]. Furthermore, data of literature showed an inverse correlation between the titer of anti-rhGAA IgG antibodies and the clinical outcome [5]. Patients with high titer were mainly cross-reactive immunologic material negative (CRIM- negative) and were not able to synthesize any kind of GAA protein, in contrast to CRIM-positive patients who produced a non-functional form of GAA [5]. In an at- tempt of suppressing anti-rhGAA IgG production, immunosuppression and ERT-naïve immunomodulation protocols have also been used in CRIM-negative patients with apparent success [6–9].

Recently, the efficacy of very early treatment (≤1 months of age) in CRIM-positive patients was re-ported in a number of studies from Taiwan, where a newborn screening program for Pompe disease has been performed since 2008 [10, 11]. By contrast a more variable and unpredictable outcome has been reported in CRIM-positive IOPD patients, who were diagnosed by clinical symptoms and received long-term ERT outside clinical trials[12–15]. Indeed, many patients have progressively lost the reached motor milestones and showed an impairment of respiratory function with the need of ventilation support [12–15]. Moreover, two studies on a limited number of CRIM-positive patients suggest that in the long-term some patients may probably benefit from a higher ERT dosage than presently recommended [16, 17].

Finally, a progressive white matter damage, presumably related to brain glycogen accumulation, has been shown in a number of patients [18–21]. This under-recognized problem adds up to other still unanswered questions regarding the optimal treatment approach for this rare disease.

In the present paper, we document the long-term outcome of 28 Italian IOPD patients treated with ERT with a median follow-up time of 6 years. The data reported here significantly contribute to improve the knowledge of long-term ERT outcomes of IOPD patients.

Methods

A retrospective multicentre observational study was de- signed to analyze the long-term clinical history of a cohort of IOPD treated with ERT. The study involved 13 Italian centres and enrolled overall 28 patients (15 females, 13 males) born in the period: February 2002– January 2013. Patients inclusion criteria were: a) con- firmed diagnosis of IOPD, based on clinical symptoms, enzyme and molecular analysis; b) to receive ERT. Collected clinical and functional data included: age at diagnosis, age at ERT start, signs and symptoms at dis- ease onset and during the follow-up visits; achieved motor functions; heart hypertrophy and ejection fraction normalization (yes/no/partially); respiratory function (need of ventilatory support); speech development and language intelligibility; hearing function; feeding impairment. Laboratory parameters included: transaminases, creatine kinase and IgG antibodies to rh-GAA; residual GAA activity (in lymphocytes, fibroblasts or muscle tis- sue); GAA mutation profile; CRIM status. Magnetic resonance imaging (MRI) data of the brain were analysed when available. All patients received alglucosidase-alfa treatment within the first 12 months of age (8 of them ≤3 months). Baseline ERT dosage was 20 mg/Kg/every other week (eow) in 26 patients, while the other 2 (patients: 27 and 28), who had participated in a clinical trial [2, 4] received 40 mg/Kg/eow. Due to poor clinical outcome or to infusion associated reactions (IAR), ERT dosage was modified in course of follow-up in 7 patients (Table 1).

Informed consent to data collection was obtained for all patients by parents or the legal representative.

Statistics

Overall survival (OS) was calculated as the time from birth to death for any cause; ventilator-free survival (VFS) as the time from birth to invasive ventilation or death. Observation periods were censored at the date of last contact when no event was observed. Patients were followed-up on ERT for a median time of 71 months (range1.9–134.3). OS and VFS curves were computed with the Cox regression model allowing for delayed entry, as all patients entered the study at ERT commencement. Analysis was performed overall and in subgroups defined by potential prognostic factors, such as CRIM status and age at start of ERT (≤ 3 months vs ≥ 3 months). Cumulative incidences of cardiac normalization and independent walking were calculated allowing for delayed entry, according to Andersen et al. [22]. All tests were two-sided. Ana- lyses were performed with SAS 9.2.

Results

Table 1 shows demographics, genetics and clinical data before ERT start as well as therapeutic management (ERT dosage and immunomodulation approach) and occurrence of infusion associated reactions (IAR) related to each patient.

Table 1 Genotypes, cross reactive immunological material (CRIM) status, ages of onset of symptoms, diagnosis and starting ERT, immunological data, Infusion associated reactions and ERT dosing of the 28 Pompe patients

Patient ID/gender	Genotype	Predicted mutations severity[a]	CRIM	Age of onset signs and symptoms[b]	Age diagnosis[b]	Age starting ERT[b]	Anti-rh GGA antibodies maximum titer	Immunomodulation	Severe IAR	ERT present or last dosing
1/M	NA	–	NA	1	1	2	NA	No	No	1
2/F	NA	–	NA	2	4	4	NA	No	Yes	1
3/F	c.[1833_1839del;1846G > T; 1847_1848insT]; [c1124G > T]	Very severe/potentially less severe	P(E)	1	5	5	NA	No	No	1
4/M	NA	–	NA	3	4	4	NA	No	No	1
5/F	c.[236_246 del]; [236_246 del]	Very severe	Neg (E)	1	1	2	1:25.600	No	Yes	1
6/F	c.[742delC];[c.896 T > C]	Very severe/potentially less severe	P(E)	4	6	10	NA	No	No	1
7/F	c.[2481 + 102_2646 + 31del]; [2481 + 102_2646 + 31del]	Very severe	Neg(E)	6	8	8	NA	No	No	1
8/F	c.[525delT];[c.670C > T]	Very severe/potentially less severe	P(E)	1	9	10	NA	No	No	1
9/M	c.[1497G > A];[1497G > A]	Very severe	Neg	birth	5 days	1	1:102.400	No	No	1
10/M	c.[1833_1839del;1846G > T; 1847_1848insT]; [1833_ 1839del;1846G > T; 1847_ 1848insT]	Very severe	Neg (E)	2	7	7	1:25.600	Yes 14 m [7]	Yes	1
11/M	c.[1942G > A]; [2646 + 2 T > A]	Potentially less severe/ very severe	P(E)	birth	12 days	19 days	NA	No	No	1
12/M	c.[236_246del]; [1655 T > C]	Very severe/potentially less severe	P(E)	2	3	4	NA	No	Yes	1
13/F	c.[236_246del];[1927G > A]	Very severe/potentially less severe	P	6	11	11	NA	No	No	5[c]
14/M	c.[236-246del]; [236-246del]	Very severe	Neg	4	4	4	1:204.800	Yes prophylactic and later 1 cycle therapeutic [8]	Yes	1
15/F	c.[955 + 1G > A]; [1438-2A > G]	Unknown/very severe	NA	birth	5 days	8 days	≤ 1:400	No	No	1
16/M	c.[525delT]; [2237G > A]	Very severe	Neg (E)	birth	3	4	1:102.400	Yes 4 cycles at 1–2–3-4 years [8]	Yes	6[c]
17/M	c.[1A > G];[1A > G]	Very severe	Neg(E)	2	6	6	_____NA	No	No	1
18/M	c.[784G > A]; [784G > A]	Potentially less severe	P	1	1.5	4	≤ 1:400	No	No	4[c]
19/M	c.[1802C > G]; [2800-1G > C]	Potentially less severe/ unknown	P(E)	10 days	3	3	≤ 1:400	No	No	2[c]
20/F	c.[1564C > G];[1564C > G]	Potentially less severe	P(E)	4	4	5	1:1.600	No	No	1
21/F	c.[930_932delGTT];[1927G > A]	Unknown/ Potentially less severe	P(E)	birth	19 days	1	NA	No	Yes	**7**[c]

Table 1 Genotypes, cross reactive immunological material (CRIM) status, ages of onset of symptoms, diagnosis and starting ERT, immunological data, Infusion associated reactions and ERT dosing of the 28 Pompe patients *(Continued)*

Patient ID/gender	Genotype	Predicted mutations severity[a]	CRIM	Age of onset signs and symptoms[b]	Age diagnosis[b]	Age starting ERT[b]	Anti-rh GGA antibodies maximum titer	Immunomodulation	Severe IAR	ERT present or last dosing
22/F	c. [1927G > A];[1927G > A]	Potentially less severe	P(E)	4	4	5	NA	No	No	1
23/F	c.[1933 G > A];[1933 G > A]	Potentially less severe	P(E)	birth	1	1	NA	No	No	1
24/F	c.[1933G > A]; [1564C > G]	Potentially less severe/ potentially less severe	P(E)	2	3	4	NA	No	No	1
25/F	c.[784G > A]; [1822C > T]	Potentially less severe/very severe	P	2	3	4	≤ 1:400	No	No	2[c]
26/F	c.[1064 T > C]; [2041-2A > C]	Potentially less severe/very severe	P(E)	3	5	8	≤ 1:400	No	No	3[c]
27/M	c.[1465G > A];[40_47del8]	potentially less severe/ unknown	P(E)	birth	3	4	≤ 1:400	No	No	2[d]
28/M	c.[2237G > A];[1655 T > C]	Very severe/potentially less severe	P(E)	2	4	9	1:3200	No	Yes	2[d]

[a]As reported in https://www.erasmusmc.nl/klinische_genetica/research/lijnen/pompe_center E = estimated on genotype [23]; ERT dosing: 1 = 20 mg/kg/14 days, 2 = 40 mg/kg/14 days, 3 = 20 mg/kg/10 days, 4 = 40 mg /kg/10 days, 5 = 20 mg/kg/7 days, 6 = 40 mg /kg/7 days, 7 = 15 mg/kg/7 days; F female; IAR infusion associated reaction; M male; N negative; NA not available; P positive

[b]months, unless differently indicated

[c]Patients in whom the dosage of ERT was modified in course of follow up due to poor clinical outcome or to infusion associated reactions. Dosing at the beginning of ERT = 20 mg/kg/14 days

[d] dosage of ERT received from the beginning of treatment

E = estimated on genotype [23]; ERT dosing: 1 = 20 mg/kg/14 days, 2 = 40 mg/kg/14 days, 3 = 20 mg/kg/10 days, 4 = 40mg /kg/10 days, 5 = 20 mg/kg/7 days, 6 = 40mg /kg/7 days, 7 = 15 mg/kg/7 days; F = female; IAR = Infusion Associated Reaction; M = male; N = negative; NA = not available

Median age at symptoms onset was 2 months (range 0–6), at diagnosis 3 months (range 5 days-11 months) and 4 months at ERT starting (range 8 days-11 months). The 17 patients who were alive at the end of data collection had been on ERT for a median of 71 months (range 25–134 months).

Residual GAA activity was less than 2% of the normal values for the reference laboratory in all patients (data not shown) and gene analysis was performed in 25 out of the 28 patients. CRIM status was tested on bloodspot or cultured fibroblasts by Western Blot analysis (lab. Great Ormond Street Hospital, London) in 5 patients and indirectly deduced from genotype [23] in other 19 patients. Seventeen of them (70.8%) were CRIM-positive and 7 (29.2%) CRIM-negative, while CRIM status was not available nor deducible in 4 patients.

Both at diagnosis and at ERT start all the patients showed: muscle weakness and hypotonia, increased serum CK (2 to 10 fold over the normal value) and severe hypertrophic cardiomyopathy, with increased thickness of septum and left ventricular wall. Eight out of the 28 patients (28.6%) had respiratory distress and one needed respiratory support (patient 16).

Follow-up

The data on clinical follow-up are reported in Table 2.

Table 2 Follow-up data of 28 IOPD patients treated with ERT

Patient ID/gender	CRIM	Survival (A or D)	Age last visit*	Cause of death	Asssisted ventilation (T or NIV or N)	Artificial nutrition (G or NG or N)	Motor achievements (none/HC/S/W)	Hearing deficit/ intelligible speech	Heart normalization	Contractures at last visit
1/M	NA	D	5 m	2	N	N	none	na/na	partially	N
2/F	NA	D	6 m	3	N	N	none	na/na	partially	N
3/F	P(E)	D	7 m	2	N	N	none	na/na	partially	Y
4/M	NA	D	13 m	2	N	N	none	na/na	partially	N
5/F	Neg(E)	D	15 m	3	N	N	none	na/na	Y	N
6/F	P(E)	D	15 m	2	N	N	none	na/na	partially	N
7/F	Neg(E)	D	18 m	2	N	N	none	na/na	partially	N
8/F	P(E)	D	19 m	2	T	N	none	na/na	partially	N
9/M	Neg	D	20 m	1	N	N	S	N/N	partially	N
10/M	Neg (E)	D	5.0	1	T	G	none	na/N	partially	Y
11/M	P(E)	D	6.8	1	N	N	W	N/Y	Y	Y
12/M	P(E)	A	2.5		N	N	W	N/Y	Y	N
13/M	P	A	3.0		T	G	None (previously S)	Y/N	partially	N
14/F	Neg	A	2.5		NIV (2y)	NG	S	Y/N	partially	N
15/F	NA	A	4.0		N	N	W	N/Y	Y	Y
16/M	Neg (E)	A	4.0		T (3y)	G	None (previously S)	N/N	partially	Y
17/M	Neg(E)	A	4,4		T	G	none	N/N	Y	N
18/M	P	A	4.5		NIV	N	None (previously S)	N/N	Y	N
19/M	P(E)	A	5,8		T (5y)	G	S	N/N	Y	Y
20F	P(E)	A	6.5		T	G	HC (previously S)	Y/N	partially	N
21/F	P(E)	A	6.0		T	G	S	Y/N	Y	Y
22/F	P(E)	A	7.0		T	G	S	N/Y	Y	Y
23/F	P(E)	A	7.0		N	N	W	Y/Y	Y	N
24/F	P(E)	A	9.75		N	N	W	Y/Y	Y	N
25/F	P	A	9. 16		T (7y)	N	S	N/Y	Y	Y
26/F	P(E)	A	10.9		NIV (8.5 y)	N	S (previously W)	N/Y	Y	Y
27/M	P(E)	A	11.5		N	N	W	Y/Y	Y	Y
28/M	P(E)	A	11.5		T	G	none	Y/N	Y	Y

*years unless differently indicated; A alive, D dead, E: estimated on genotype [23], F female, G gastrostomy, HC head control, M : male, m months, N no, na not assessed, NA: not available, Neg: negative, NG nasogastric tube, NIV non invasive ventilation, P: positive, S sitting independently, T tracheostomy, W walking independently, y:years, Y yes
Cause of death: 1- cardiorespiratory failure, 2- ERT withdrawn after failure to respond, 3- infusion-related reactions prevented ERT delivery

Nine patients (patients 1 to 9; 6 females, 3 males) died within the first 20 months of life (mean 13; median 15; range 5–20 months), 3 were CRIM positive (patients 3, 6, 8) and 3 CRIM negative (patients 5, 7, 9), while for 3 of them (patients 1, 2, 4) CRIM status was neither avail- able nor estimated on genotype.

Concerning the "long-term" follow-up group, 14 patients resulted CRIM-positive (patients 11–13, 18–28), 4 CRIM negative (patients 10, 14, 16, 17) and one neither examined nor deducible from genotype (patient 15). Patients 10 and 11 died at the age of 60 and 78 months respectively, due to respiratory failure secondary to pneumonia. The current median age of the 17 surviving patients at time of data acquisition was 6 years (range 2–11.5 years).

Ventilation

At the last clinical examination, out of the 19 long-term surviving patients only 6, 31%, (patients 11, 12, 15, 23, 24, 27) were free of any ventilation support, 3 patients, 15%, (patients 14, 18, 26), needed non-invasive ventilation (NIV) and 10 patients, 52%, (patients 10, 13, 16, 17, 19–22, 25, 28) were ventilated through tracheostomy. Figure 1a shows the OS and the VFS for the whole co- hort. Proportions of OS and VFS were 64.2% (SE 9.3) and 47.3% (SE 9.3) respectively at the age of 3 years and 58.8% (SE 9.8) and 31.8% (SE 8.6), respectively at the age of 6 years (individual data of survival and ventilation are reported in Table 2).

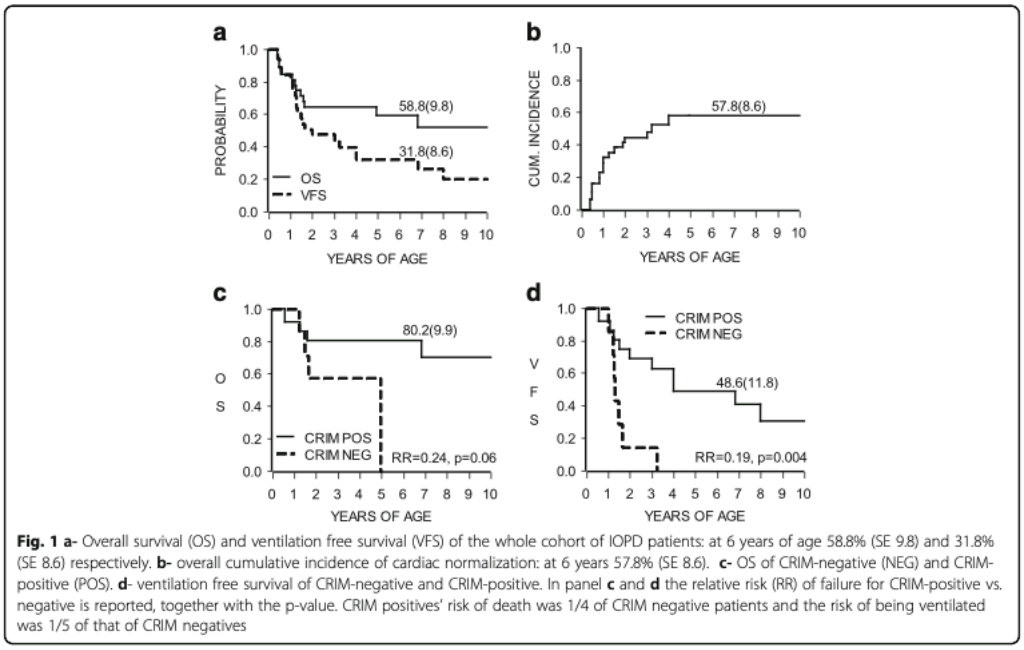

Fig. 1 a- Overall survival (OS) and ventilation free survival (VFS) of the whole cohort of IOPD patients: at 6 years of age 58.8% (SE 9.8) and 31.8% (SE 8.6) respectively. **b-** overall cumulative incidence of cardiac normalization: at 6 years 57.8% (SE 8.6). **c-** OS of CRIM-negative (NEG) and CRIM-positive (POS). **d**- ventilation free survival of CRIM-negative and CRIM-positive. In panel **c** and **d** the relative risk (RR) of failure for CRIM-positive vs. negative is reported, together with the p-value. CRIM positives' risk of death was 1/4 of CRIM negative patients and the risk of being ventilated was 1/5 of that of CRIM negatives

Feeding

Nine (32%) of the long-term surviving patients (patients 11, 12, 15, 18, 23–27) maintained autonomous feeding capacities, including 2 patients receiving ventilation sup- port (one tracheostomised). Nine patients (patients 10, 13, 16, 17, 19–22, 28) had gastrostomy and one (patient 14) was nourished by naso-gastric tube (Table 2).

Motor function

Only 7 (25%) patients (patients 11, 12, 15, 23, 24, 26, 27), reached independent ambulation at a median age of 16.5 months (range 12–19 months) and the overall cumulative incidence of independent ambulation was 23.7% (SE 7.6). All of them maintained the walking capacity except one (patient 26), who lost it at the age of 8.5 years due to progressive muscle weakness. The sitting position was achieved by 17/28 patients (60%; patients 9, 11–16, 18– 27), although 4 (patients 13, 16, 18, 20) subsequently lost this skill (Table 2). One patient (patient 9) belonged to the group of patients who died before 20 months of life and was CRIM-negative. Figure 2 shows for the whole group of patients the relation to OS and VFS of the best motor milestone achieved.

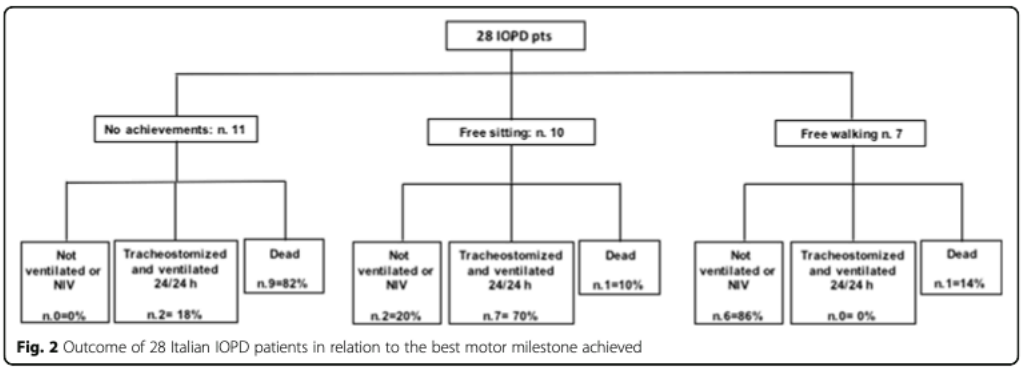

Fig. 2 Outcome of 28 Italian IOPD patients in relation to the best motor milestone achieved

At the time of last clinical evaluation, joint contractures (more frequent at lower limbs) were evident in 12 patients (Table 2). Facial muscle weakness and/or speech disorders and/or dysphagia were observed in all patients.

Nine patients (patients 11, 12, 15, 22–27) showed a hypernasal speech with intelligible language at an age ranging from 2.5 to 11.5 years. In the other patients, language was unintelligible due to reduced movement of lip and tongue and velopharyngeal incompetence. Swallowing function was not routinely studied.

Cardiac function

Heart parameters (septum with left ventricular wall thickness and ejection fraction) normalized in 15/28 patients (patients 5, 11, 12, 15, 17–19, 21–28) at a median age of 12 months (range 5–48 months) after a median treatment of 11 months (range 1–42 months). The other 13 patients (patients 1–4, 6–10, 13, 14, 16, 20) showed only a partial cardiac improvement (Table 2); of this group, 4 patients (patients 13, 14, 16, 20) were alive at a median age of 3.5 years (range 2.5–6 years) while 9 (patients 1–4, 6–10) died at a median age of 14 months (range 5–60 months). In Fig. 1b the estimated cumulative incidence of cardiac normalization at 6 years is re- ported: 57.8% (SE 8.6).

Hearing function

Nineteen patients were formally tested with administration of behavioral audiometry or evoked potentials: 11 had no hearing defect (patients 9, 11, 12, 15–19, 22, 25, 26) and 8 (patients 13, 14, 20,

21, 23, 24, 27, 28; all CRIM-positive) showed different degrees of hearing deficit (Table 2). Nine patients have never been formally tested but were reported to apparently hear sounds included in the vocal extension (80–1500 Hz).

Brain MRI abnormalities

Brain MRI was performed in 6 CRIM-positive subjects (patients 11, 15, 19, 20, 23, 27). In one patient (patient 27) it was repeated at 2, 3 and 6 years of age, whereas in the other 5 the exam was performed only once at the age of 6 years. Imaging data showed the presence of moderate periventricular white matter abnormalities (hypomyelination) in all of them. Additionally, patient 27 showed a progressive deterioration of MRI parameters (Fig. 3), associated with the worsening of cognitive performances (Wechsler scales IQ: WPPSI 85 at 3 years; 75 at 5 years 10 months; WISC III 73 at 8 years, 64 at 9 years, 50 at 11 years).

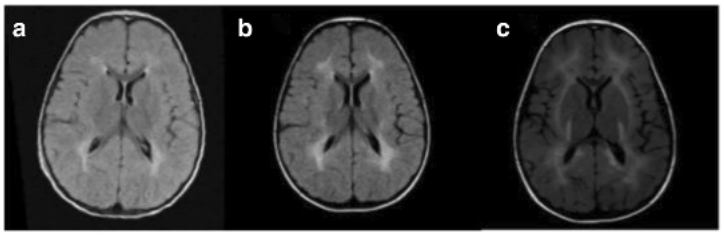

Fig. 3 Axial section T2 flair at the ages of 2 (**a**), 3 (**b**), 6 (**c**) years: white matter is progressively affected. In the last image the damage is extended to the posterior limb of the internal capsule and the subcortical white matter with U fibers. Basal ganglia are spared

Outcome according to CRIM status

Compared to the 7 CRIM-negative the 17 CRIM- positive patients showed a better outcome in terms of OS and VFS: risk of death in CRIM-positive patients was 1/4 compared to CRIM-negative patients (relative risk, RR = 0.24, 95% CI 0.05–1.10; p-value = 0.06) (Fig. 1c), while VFS risk (Fig. 1d) in CRIM-positive was 1/5 com- pared to CRIM-negative patients (RR 0.19, 95% confidence interval (CI) 0.06–0.59; p-value = 0.004). At the end of the study 13 CRIM-positive patients were alive at a median age of 7 years (range 2.5-11.5). Twelve out of 17 CRIM-positive patients (patients 11, 12, 18, 19, 21– 28) achieved normalization of heart function within 6 years of age, with a 6 year cumulative incidence of 74.2% (SE 9.4). On the contrary, only 2/7 (28%) CRIM- negative patients (patients 5 and 17) normalized their cardiac parameters.

Of the 7 infants who reached independent ambulation 6 were CRIM-positive (patients 11, 12, 23, 24, 27; 35% of CRIM-positive patients) and one had unknown CRIM status (patient 15) and very low-titer antibodies (≤1:400). No CRIM-negative patients achieved walking ability.

Outcome according to age at starting ERT

The effect of age at ERT starting (≤ 3 months and ≥3 months) on OS, VFS, normalization of cardiac parameters, and achievement of independent ambulation, was analysed on the whole cohort and no significant differences were found. Within the CRIM-positive sub- group, only 4 patients had started

ERT ≤ 3 months of age (patients 11, 19, 21, 23). This limited number pre- vented further investigations on the effect of an early start of ERT in the CRIM-positive subgroup.

Anti-rhGAA antibodies, adverse events, and immunomodulation treatment

Data concerning the titers of anti-rh-GAA antibodies were available in 13/28 (46%) patients (7 CRIM-positive: patients 18–20, 25–28; 5 CRIM-negative: patients 5, 9, 10, 14, 16; one unknown: patient 15) (Table 1). The 7 CRIM-positive patients and the unknown one had low- titer antibodies (≤ 1:3200), while the 5 CRIM-negative had intermediate to high antibody titers, with maximum values ranging from 1:25.600 to 1: 204.800.

Eight patients (3 CRIM-positive: patients 12, 21, 28; 4 CRIM-negative: patients 5, 10, 14, 16; one unknown: patient 2) experienced IAR (flushing, urticaria, bradycardia, respiratory distress).

Three of them (patients 12, 14, 28) were successfully treated with a premedication protocol including the use of corticosteroids or antihistaminics and decreased infusion rate. Two patients (16 and 21) who presented very severe reactions (massive urticaria and anaphylactic shock with glottis oedema respectively) followed a desensitization protocol. It initially provided a very diluted dose of ERT (1/10 or 1/20 of the recommended dilution administered in 24–48 h) and therefore a progressive increase in dosage and concentration of the enzyme over a period of 6 months. Other 2 CRIM-negative patients (2 and 5) never received any kind of desensitization treatment and ERT was interrupted. Patient 10 was immunomodulated at the time of IAR (see below).

Three CRIM-negative patients (patients 10,14,16) received a tolerance induction protocol, according to the de- scribed experiences [7, 8], 2 of them (patients 10, 16) after having developed an anti-rhGAA antibodies titer >1: 51.200 and one (patient 14) on a prophylactic basis, simultaneously with the first dose of rhGAA.

During follow-up period, all 3 patients showed a progressive loss of the motor, cardiac and ventilatory monitored functions. Patient 10 died at the age of 60 months after having received one cycle of immunomodulation. Due to periodic increase of anti-rhGAA antibodies, patient 16 has received 4 cycles of therapeutic immunosuppression [8] and has then changed the immunomodulation protocol [9]. Patient 14, who received prophylactic treatment, showed an increase of anti-rh-GAA to 1:204.800 after few months of ERT and received a cycle of therapeutic immunosuppression protocol [8]. One year after, antibodies titer increased again to 1:102.400 in concomitance to a very poor clinical condition (Table 2) and immunotherapy was not repeated.

Discussion

Our study presents the results of a retrospective analysis of the clinical outcome of 28 Italian IOPD patients

receiving ERT. The studied cohort included 17 CRIM- positive, 7 CRIM-negative and 4 not CRIM-defined patients who were followed for a median period of 6 years (range 2.5–11.5 years) in 13 Italian reference centres. To our knowledge this is the longest independent follow-up study in a heterogeneous group of IOPD patients. As re- ported in Table 2 and shown in Fig. 1, the survival data analysis confirmed the poorest prognosis of CRIM- negative patients, with only 4 surviving beyond 2 years of age (one of them, patient 10, died at 5 years), while at the end of the study 13 CRIM-positive children were alive at a median age of 7 years.

The Kaplan Meier survival rate of our patients was 64.2% (SE 9.3) at the age of 3 years and 58.8% (SE 9.8) at 6 years of age; the survival without invasive ventilation was 47.3% (SE 9.3) at 3 years and 31.8% (SE 8.6) at 6 years (Fig. 1). These results indicate a progressive worsening of the whole group of patients with age. They also are in agreement with those of other long-term ERT follow-up studies that reported a survival rate varying from 54% to 72% and a VFS of 35% to 40% [12, 13, 15]. In particular, they are quite similar to those reported by Kishnani et al. [3] in the phase III extension study at 3 years of age: rate of survival 72% and rate of survival without ventilation 49%.

Taking into account the achievement of developmental milestones, independent ambulation was reached by 25% of our patients, a percentage consistent with the data of 20 to 50% described by other authors [12, 14]. As expected and already observed by others [13] the ERT effects on motor development paralleled those on OS and VFS (Fig. 2). Also, the therapeutic response of monitored cardiac parameters (left ventricular wall thickness and ejection fraction), which normalized at a median age of 6 years in 57.8% of our patients (Fig. 1), was in agreement with the results observed by Hahn et al. and Broomfield et al. [13, 15]. In fact, they showed normalization of heart parameters in 52% and 73% of their cohorts, respectively. Similar percentages were obtained for the need of assisted feeding that was necessary in 52% of our patients, while in other long-term studies it varied from 40 to 65% [12, 15].

Analyzing the presence of hearing loss, we observed this complication in 57% of our patients, data consistent with literature results [24, 25]. In contrast with these observations, Hahn et al. described a hearing impairment in only 3 out of the 23 patients of their casuistry [13].

A worse therapeutic response to ERT of CRIM- negative patients has already been described by other long-term follow-up experiences [13–15]. In fact, Broomfield et al. [15] reports that only 33% of the CRIM-negative patients survived up to 42 months of life in comparison with a survival rate of 85% in the CRIM- positive group. Even less positive data emerged from the studies of Hahn et al. [13] and van Gelder et al. [14], who reported few CRIM-negative patients surviving until the age of 24 months.

Finally, in the retrospective review aimed to analyze the emerging phenotype of long-term survivals

(≥ 5 years of age), Prater et al. found that all CRIM-negative patients died before reaching the age of 5 years and therefore no CRIM-negative patient could be included in the study [26].

Our data are in line with these previous findings. In- deed, none of the 4 long-term surviving CRIM-negative patients maintained neither autonomous ventilator capacity nor oral feeding, nor reached independent walking or intelligible speech (Table 2).

The poorer prognosis of CRIM-negative patients has been mainly associated with the presence of elevated anti-rhGAA antibodies titers [5, 14, 27]. In our study, antibody testing was available only for 13/28 patients and also in our experience higher anti-rhGAA antibodies titers, varying from 1:25.600 to 1:204.800, were associated with the CRIM-negative status.

In an attempt to inhibit the development of antibodies in the CRIM-negative patients, immunotherapy was pro- posed by several authors [6–9]. These protocols demonstrated to be effective in reducing the antibody level and in inhibiting their production for long time. Further- more, immunomodulatory treatment started simultaneously with ERT showed to be more effective than immunosuppression in patients who had already developed a significant anti-rhGAA response [8, 9]. However only one patient of our study was treated with the preemptive protocol but without any apparent long-term efficacy in controlling the antibody response.

In agreement with the literature [12–15], we have con- firmed the better prognosis of CRIM-positive patients. However, ventilation, feeding and muscular function data showed clear clinical deterioration in at least 8 CRIM-positive patients, including those who had responded positively to an early ERT (Table 2). A similar long-term outcome, with a progressive impairment of pulmonary and muscle functions in CRIM-positive patients has been already described [12–15].

Recently, some authors suggested the efficacy of an in- creased ERT dosage in improving muscular outcome and reducing respiratory events that require hospitalization [16, 17]. Moreover, a dose-dependent effect of ERT in improving intracellular clearance and reducing the glycogen storage in skeletal and heart muscles has been described in vitro and in animal models [28–30].

The development of neonatal screening programs will probably modify the future of IOPD patients. The Taiwan experience showed that a very early diagnosis with a con- sequent pre-symptomatic ERT start, resulted in a better prognosis [10, 11]. In particular, Yang et al. [11] showed

that even few days of difference in therapy start (mean of 12 vs 21 days) may play a significant role in the clinical outcome. After one year of follow-up, they demonstrated a better improvement of biochemical parameters and functional tests in the very early treated CRIM-positive patients. These results, compared to those of CRIM- positive patients who begun therapy at symptoms appearance [12–15], suggest a positive impact of newborn screening programs for Pompe disease. However, an in- creasing number of observations have been recently published showing that early pre-

symptomatic treatment in CRIM-positive, low antibody titer patients does not completely prevent slow deterioration after the first years of life [10, 31, 32]. Moreover pre-symptomatic treatment does not always guarantee a positive outcome in a short- term, as shown by Schänzer et al. [33], who reported an extreme case of a CRIM-positive, low antibody titer IOPD infant, treated since the 3rd day of life with 40 mg/kg/ week who never achieved free sitting position and was tracheostomized and ventilated from 10 months on [33].

Prolonged follow-up studies are necessary. The retrospective-observational design of our study and the limited number of CRIM-positive patients receiving early ERT did not allow us to define any relation be- tween clinical outcome and age of therapy start.

Finally, an emerging finding in IOPD patients is the presence of periventricular white matter abnormalities [18–21]. We detected a pattern of hypomyelination in 6 children who underwent brain MRI. In one of them the exam was repeated 3 times during follow-up showing a progressive worsening of the lesions paralleling the development of a progressive cognitive impairment. A similar evolution has also been described by Ebbink et al. in other patients [19, 20]. These radiological abnormalities might be caused by neuronal glycogen storage [18] and the inability of the enzyme to cross the blood- brain barrier [28, 30] may justify the progressive cognitive and psychomotor worsening observed in these patients.

Conclusions

In conclusion, after 10 years of ERT we are aware of the long-term poor outcome of CRIM-negative patients but at the same time it has emerged that many CRIM- positive patients, in spite of an initial ERT positive response fail to improve or stabilize their clinical conditions. The development of neonatal screening programs, allowing a very early pre-symptomatic beginning of ERT could lead to a significant improvement of the clinical outcome. However, long-term studies are still lacking. Furthermore, to improve treatment efficacy, shared protocols of immunomodulation and ERT high dosage/frequent infusion trials in a wider number of patients, are needed.

Finally, brain involvement in Pompe disease remains an open issue not treatable by ERT in the current formulation and represents one of the main goals to be pursued by future research studies.

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Chapter V: Lysosomal storage disorders: how early diagnosis and early treatment can dramatically change the patient phenotype in Pompe disease

With conventional ERT, the clinical prognosis of IOPD is often unsatisfactory. About half of the patients treated with the recommended dosage (20 mg/kg every other week) require ventilatory support within the first years of life. The heterogeneous response to ERT has been related to different factors, including cross-reactive immunologic material (CRIM) status and age at ERT initiation.
The analysis of the state CRIM+ (Cross-Reactive Immunologic Material positive) and CRIM- (Cross-Reactive Immunologic Material negative) is important to choose the best treatment for the patient with IOPD[1] . CRIM- patients make no GAA protein and tend to develop sustained high antibody titers to ERT that make the treatment ineffective. However almost all Pompe disease patients develop antibodies against the ERT over time, but antibody titers are generally low for the majority of CRIM+ patients and they have typically a better clinical outcome.
The CRIM status can be determined with Western blot on tissue or inferred from the genotype, if already known.
Early treatment with a standard dosage of ERT improves clinical outcome and avoids mechanical ventilation in CRIM+ patients detected at newborn screening, not preventing persistent hyperCKemia and muscle weakness. Later treatment with higher dosages of ERT has shown to provide similar benefits in CRIM+ patients.
A recent study reported better outcomes in IOPD patients who received higher and more frequent doses of the drug (40 mg / kg / week), instead of currently 20 mg / kg every other week [2].
We described a case in which a patient receiving early higher dosage of alglucosidase alpha not only achieved normal neuromotor development, but also the full correction of biochemical markers of muscle damage until 3 years of age, an unmet target with the standard dosage. Nevertheless, speech delay was not prevented by this approach.

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Part of this work has been published as:

Spada M, Pagliardini V, Ricci F, Biamino E, Mongini T, Porta F. "Early higher dosage of alglucosidase alpha in classic Pompe disease". J Pediatr Endocrinol Metab. 2018 Dec 19;31(12):1343-1347. doi: 10.1515/jpem-2018-0336.

Background

Pompe disease (OMIM # 232300, glycogen storage type II) is an autosomal recessive metabolic myopathy caused by the deficient activity of the lysosomal acid α-glucosidase (GAA). The disease can present at any age with a wide rate of progression mainly dependent on the residual GAA activity [1], [2], [3]. Classic Pompe disease is characterized by a complete or near-complete GAA deficiency, leading to early massive glycogen storage in the cardiac, skeletal and smooth muscles. Clinically, classic Pompe disease presents in newborns or infants with hypertrophic cardiomyopathy, profound hypotonia and progressive respiratory failure generally leading to death within the first year of life [4]. In 2005, the first newborn screening pilot program for Pompe disease was successfully implemented [5], and in 2006 an etiologic treatment for Pompe disease with recombinant alglucosidase alpha became available. Enzyme replacement therapy (ERT) has been shown to ensure the main clinical benefits in Pompe disease [6], [7], [8]. Its effectiveness in the classic form, however, is often limited either in the short or long term [9]. Actually, half the patients treated with alglucosidase alpha do not survive ventilator-free beyond 3 years of age [10]. Many factors have been related to treatment ineffectiveness, including later age at diagnosis, worse clinical picture at the start of treatment, cross-reactive immunologic material (CRIM)-negative status and development of immune response against alglucosidase alpha (IgG anti-rhGAA) [11]. As for the latter, an immune tolerance protocol was proposed to prevent the formation of antibodies against the recombinant enzyme [12].

Recent observations suggested that higher doses of ERT could be advantageous in extending ventilator-free survival and improving the motor outcome in CRIM-positive patients with classic Pompe disease [13]. On the other hand, early treatment with a standard dosage of alglucosidase alpha warranted overlapping advantages in patients detected at newborn screening, although not in preventing muscle weakness and persistent sustained hyperCKemia [11].

Here, we report our 15-year single-center experience with different therapeutic regimens in classic Pompe disease, including the early use of a higher dosage of ERT.

Patients and methods

Classic Pompe disease was defined by a complete or near-complete GAA deficiency assessed on dried blood spots and a clinical onset within 6 months of life. This phenotype was confirmed by the molecular analysis of the GAA gene [14]. The CRIM status was assessed and related to the genotype,

as previously described [15]. Patients were treated with alglucosidase alpha (Myozyme, Sanofi Genzyme, Cambridge, MA, USA) and regularly followed up at our center. The employed dosages of alglucosidase alpha ranged from 20 mg/kg every other week to 40 mg/kg/week. Survival, ventilation-free survival, reduction in left ventricular mass index (LVMI), motor skills improvement and creatine kinase (CK) concentration were used to assess the treatment efficacy. LVMI was assessed by two-dimensional echocardiography [16]. Cardiac morphology and function were monitored by conventional echocardiography. Motor skills were evaluated using the Children's Hospital of Philadelphia Infant Test of Neuromuscular Disorders (CHOP-INTEND) [17]. The occurrence of IgG anti-rhGAA was monitored every 3 months using an enzyme-linked immunosorbent assay (Genzyme Antibody Testing Laboratories, Cambridge, MA, USA). Patients were given rituximab (375 mg/m2/week for 4 weeks) and methotrexate (0.4 mg/kg 3 times a week for 4 weeks) according to Messinger et al. for prophylactic immune tolerance induction [12]. The study was conducted according to the World Medical Association Declaration of Helsinki regarding ethical conduct of research involving human subjects.

Results

Since 2002, six patients with classic Pompe disease (three males and three females) were diagnosed, treated and followed up at our department. Their clinical and biochemical phenotypes, genotypes and therapeutic regimens are presented in Table 1.

Table 1:

Basal clinical, biochemical and molecular characteristics and therapeutic approaches in six patients with classic Por

Patient	Characteristics at diagnosis				Genotype		CRIM
	Age	Main signs	CK, UI/L	GAA on dbs, nM/h/mL[a]	Allele 1	Allele 2	
1	5 months	H, HCM	721	0.4	c.2481+ 102_2646+31del	c.2481+ 102_2646+31del	–
2	15 days	H, HCM	900	0.5	c.236_246 del	c.236_246 del	–
3	3 months	H, HCM	873	1.8	c.236_246 del	c.236_246 del	–
4	4 months	H, HCM	752	0.4	c.1927G>A	c.1927G>A	+
5	3 months	H, HCM	458	1.4	c.1571 A>T	c.1935 C>A	+
6	5 days	H, HCM, A	856	0.7	c.1714C>T	c.del exon 1-12	+

[a]Normal activity >6.1 nM/h/mL. H, hypotonia; HCM, hypertrophic cardiomyopathy; A, arrhythmi

Patients 1–5: Standard treatment with alglucosidase alpha (20 mg/kg every other week)

Five patients (patients 1–5) were treated with a standard dose of alglucosidase alpha (20 mg/kg every other week) started at 3.2±1.4 months of age. One patient (patient 2) was treated within the first month of life. In two CRIM-negative patients, the application of the tolerance induction protocol did not prevent the development of immune response against ERT (Table 1). CRIM-positive patients did not

develop any antibody response. In all the patients, the standard dose of ERT warranted substantial improvement or normalization of the LVMI (Figure 1). Three out of five patients (patients 3–5) reached the sitting position while on treatment at 9.6±1.0 months of age. All patients required ventilatory support from 10.1±3.0 months of age. Four patients died (mean age 22.2±11.9 months); one patient has survived until now, but required tracheostomy and gastrostomy support (patient 5, actual age 5 years). Time courses of LVMI, CK concentrations and CHOP-INTEND scores in patients on a standard dose of ERT are depicted in Figure 1.

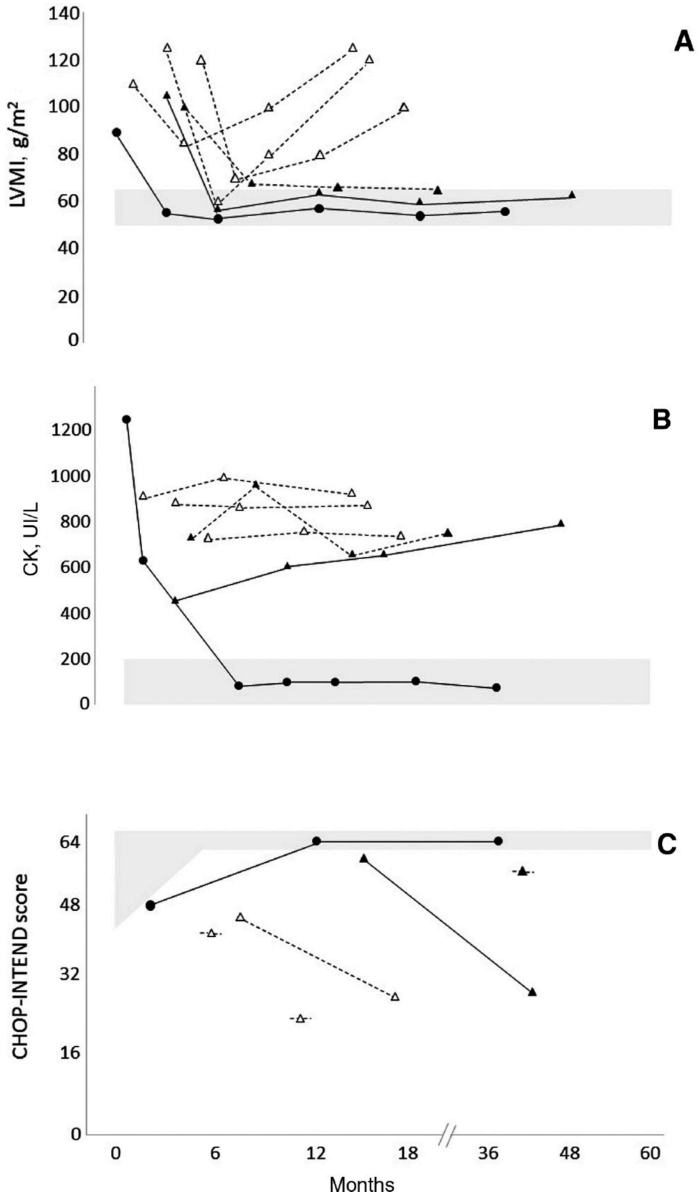

Figure 1:

Time course of LVMI (panel A), creatine kinase (CK, panel B) and CHOP-INTEND (panel C) in six patients with classic Pompe showing positive (filled tags) or negative (empty tags) CRIM during treatment with alglucosidase alpha at a standard dose (20 mg/kg every other week, triangles) or at a boosted dose (40 mg/kg/week, circles). Continuous and dotted lines indicate positive and negative prognosis *quoad vitam*, respectively. Gray areas represent normal ranges.

Patient 6: Early treatment with a higher dosage of alglucosidase alpha (40 mg/kg/week)

In this male patient, the clinical onset occurred prenatally with fetal bradycardia (heart rate 110/min, normal value 120–160/min) from 29 weeks' gestation to birth at 38 weeks' gestation. Fetal echocardiography was repeatedly normal. At birth, the patient suffered from respiratory distress rapidly corrected by oxygen supplementation, accompanied by bradycardia (98 beats/min) and hypotonia. The APGAR scores at 1, 5 and 10 min were 3, 6 and 8, respectively. From the third day of life, multiple episodes of supraventricular tachycardia (heart rate of 280–300/min) were registered, requiring treatment with adenosine, amiodarone and electric cardioversion. The biochemical diagnosis of Pompe disease was established on the fifth day of life by evidencing a near-complete GAA deficiency on dried blood (Table 1). Echocardiography revealed hypertrophic cardiomyopathy (Figure 1). Pre-treatment CK was 1223 U/L (normal values 20–200 U/L).

A high dosage (40 mg/kg/week) of alglucosidase alpha was started from 14 days of age. This treatment warranted early and stable normalization of cardiac morphology, CHOP-INTEND score and CK concentration (Figure 1). Concentrations of lactate dehydrogenase, aldolase and aspartate aminotransferase were steadily normal. A combined antiarrhythmic therapy (amiodarone and propranolol) was maintained for 2 months and then simplified (amiodarone only). During the first year, the patient regularly achieved neuromotor milestones, sitting and walking unaided at 7 and 13 months, respectively. To date, the patient has shown normal neurologic development except for a delay in speech (3 years of age). ERT was administered using recommended infusion rates, with an infusion duration of 6 h and 36 min. No infusion-related reactions or adverse effects from the higher dosage of ERT were registered.

Discussion

The natural history of classic Pompe disease is unavoidably characterized by a rapid progression to death within the first year of life. In the recent years, many successful efforts have been made to modify this outcome, including the development of a targeted ERT and the implementation of newborn screening programs warranting treatment anticipation [5]. These advances significantly ameliorated the prognosis of patients with classic Pompe disease, with cardiologic and neuromotor improvements and prolonged survival [8]. Response to treatment, however, is heterogeneous among patients and is influenced by modifiable and non-modifiable factors [10]. Early initiation of ERT is advantageous [9], [10], [11]. Patients detected at newborn screening and treated early or very early with a standard dosage of ERT reach satisfactory motor capabilities in early childhood and avoid mechanical ventilation but show persistent hyperCKemia and residual muscle weakness while on treatment [11], [18]. Some advantageous clinical effects were reported with the later use of higher doses of ERT in CRIM-positive patients with classic Pompe disease [13], [19], [20]. In particular, a

successful experience with the temporary use of alglucosidase alpha 40 mg/kg twice a week in a late-diagnosed infant with classic Pompe disease was recently reported [21].

In this article, we report our experience using different therapeutic regimens in a cohort of patients with classic Pompe disease. In our series, all CRIM-negative patients treated with the standard dose of alglucosidase alpha died within 15 months of age after a temporary amelioration of their clinical picture, even in the case of early ERT initiation. Unfortunately, the development of an immune response against ERT was not prevented by the application of the immune tolerance protocol. Available results by using this latter approach, indeed, are heterogeneous [22], [23].

CRIM-positive patients treated with ERT at the standard dosage shared a poor prognosis as well, after provisional clinical improvements. Delayed ERT initiation likely had a role in this respect. One patient survived requiring mechanical ventilation and nutritional support, presenting worsening motor skills and persistent hyperCKemia reflecting disease progression.

In light of the previous suggestions on the potential advantages provided by either ERT anticipation or later booster doses, we combined these two strategies in one patient with classic Pompe disease. The observed prenatal onset with persistent fetal bradycardia is a newly described clinical presentation, extending the clinical clues for the antenatal diagnosis of classic Pompe disease to fetal arrhythmias [24]. In this patient, early four-fold dosage of alglucosidase alpha was well tolerated and warranted not only normal motor development, but also long-lasting normalization of markers of muscle damage and disease progression, an unmet target using early ERT at a standard dosage. These findings, indeed, are consistent with an arrest (rather than a slow-down) of disease progression at the muscle level. This approach, however, did not prevent the occurrence of speech delay, an increasingly recognized feature in survivors with classic Pompe disease [25].

In conclusion, we suggest that early higher dosage of alglucosidase alpha (40 mg/kg/week) may further improve clinical prognosis in patients with classic Pompe disease. Studies on more patients treated by this approach are needed. Hopefully, the issues arising from the eventual use of early higher-dose ERT will be resolved by upcoming new treatments for Pompe disease.

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Chapter VI: Lysosomal storage disorders: functional assessment to identify suitable outcome measures for the standard of care in children with Pompe disease

We already explained how ERT changed the natural history and outcome of IOPD patients.

The new phenotype of these 'long survivors' is mainly characterized by myopathic features[1], similar to those observed in some types of congenital muscular dystrophies. Moreover, an involvement of central nervous system (CNS) has been described[2,3].

In the literature there is not a comprehensive and homogeneously collected data about both functional motor and cognitive long-term evolution of this new population of children, so that is impossible to compare data and to establish which is the better approach to these patients.

In this study, we monitored disease progression up to three years in eight young patients with Pompe disease.

Based on the literature data and the long term personal experience, we selected validated functional scales for neuromuscular disorders and compared the results to identify a simple and reliable protocol for the follow-up of children with Pompe disease, which can be used to quantitatively compare patients with each other and the same patient over time. Moreover, we evaluated cognitive functions using developmental/cognitive tests.

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Part of this work has been published as:

Ricci F, Brusa C, Rossi F, Rolle E, Placentino V, Berardinelli A, Pagliardini V, Porta F, Spada M, Mongini T. " Functional assessment tools in children with Pompe disease: A pilot comparative study to identify suitable outcome measures for the standard of care". Eur J Paediatr Neurol. 2018 Nov;22(6):1103-1109. doi: 10.1016/j.ejpn.2018.08.001. Epub 2018 Aug 18.

Background

Pompe disease (PD) is a rare condition caused by mutations in the gene encoding for the enzyme alpha-glucosidase (GAA) or acid maltase, which has a key role in the degradation of intralysosomal glycogen. GAA deficiency results in an abnormal intracellular accumulation of glycogen, with dysfunction of autophagic processes and degeneration of the cell, in particular muscle fibers.[1] The disease has a highly variable clinical spectrum, ranging from very severe infantile forms to adult-onset forms with minor limitations.[2,3] Since 2000, an enzyme replacement therapy (ERT) with recombinant GAA is available and disease natural history has changed, especially in the severe infantile form, usually lethal within the first year of age.[4-6] The new phenotype of these 'long survivors' is mainly characterized by myopathic features,[5] similar to those observed in some types of congenital muscular dystrophies. Moreover, an involvement of central nervous system (CNS) has been described.[7,8] We have not found comprehensive and homogeneously collected data about both functional motor and cognitive long-term evolution of this new population of children.[9]

In this study, we monitored the disease progression up to three years in eight young patients with PD with different age, degree of severity and in various stages of therapy. Based on literature data and long term personal experience in evaluating children with neuromuscular diseases, we selected a series of validated functional scales for neuromuscular dis- orders and compared results to identify a simple and reliable protocol for the long-term follow-up of children with PD. Moreover, we evaluated CNS functions using cognitive/ developmental scales.

Materials and methods

Inclusion criteria and patient selection

This is a prospective observational study on children with Pompe disease followed at the Center for Neuromuscular Diseases (Child Neurology Section) and at the Metabolic Diseases Unit of Regina Margherita Hospital (Turin, Italy). Inclusion criteria were: age at baseline up to 16 y.o.; established diagnosis of Pompe disease (absent or markedly reduced GAA enzyme activity on dried blood spot-DBS, lymphocytes or muscle biopsy, confirmed by the presence of a double DNA mutation); regular attendance to centers for treatment and clinical evaluations.

Patients were defined as classic infantile-onset Pompe Disease (IOPD, onset in the first year of life with cardiomyopathy) and childhood late-onset Pompe Disease (LOPD, early symptoms or diagnosis between 1 y.o. and 12 y.o. without cardiomyopathy), according to the literature.[10]
All parents signed written informed consent prior of the inclusion in the study.

Procedures

At baseline (T0) all patients were clinically evaluated by two physiotherapists with training and experience in neuromuscular diseases evaluation and in motor function scales administration. The assessment protocol included muscular strength test (MRC, Medical Research Council), evaluation of range of motion of principal joints (ROM), timed test (6MWT, 6 Minute Walk test) and functional scales according to age and functional level of each patient.
ROM measurements were taken with goniometer at shoulders, elbows, wrists, hips, knees and ankles. Muscle strength was assessed in 11 groups of upper and lower limbs muscles (shoulder abduction, elbow flexion and extension, hip flexion, extension, abduction and adduction, knee flexion and extension, ankle flexion and extension) by using Medical Research Council scale (MRC). This kind of evaluation requires a very good collaboration of patient, which limits its use in children younger than 5 years.
The 6MWT measures the maximum distance a subject, instructed to walk on a flat hard surface as fast as possible, can walk over a total of 6 min. Normative data collected by healthy children are available since the age of 4.[11-13]
Motor functions were investigated using scales previously validated for neuromuscular diseases according to the age of each patient (Children's Hospital of Philadelphia Infant Test of Neuromuscular Disorders-CHOP Intend),[14,15] Hammersmith Functional Motor Scale-HMFS,[16] Expanded version of the Hammersmith Functional Motor Scale-HMFSE,[17] North Star Ambulatory Assessment-NSAA,[18] Motor Function Measure Scale for Neuromuscular Diseases 20 and 32-MFM20 and MFM32[19,20] and the Gait, Stairs, Gowers, Chair, Arms Functional Test-GSGCA.[21]
The score of each functional scale was assigned by physiotherapists independently, with the support of a video recording.
All patients were re-evaluated during the clinical follow-up visits in a three years' time from T0; the re-assessment protocol included muscular strength test (MRC), evaluation of range of movement of principal joints (ROM), timed test (6MWT) and the functional scales which better assessed motor functional level.
Cognitive functions were assessed using the Bayley Scales of Infant and Toddler Development e Third Edition (Bayley- III22,23) for children younger than 3 years of age, the Wechsler Preschool and Primary Scale of IntelligenceeThird Edition (WPPSI-III24) for children 3e6 years of age and the

Wechsler Intelligence Scale for ChildreneFourth Edition (WISC-IV25) for children older than 6 years of age. The tests were repeated, when possible, after two years during clinical follow-up visits (time-span from 23 months to 30 months).

Data analysis

Considering the small sample size, raw data and descriptive statistical parameters were used.

Results

A total of 8 patients were included: 4 IOPD patients (all treated with ERT, 3 males and 1 female, age range 2 months-2 years and 4 months) and 4 LOPD patients (two treated with ERT, 2 males and 2 females, age range 4 years and 3 months-14 years and 6 months). Population details are reported in Table 1.

Table 1 – Population details.

ID	Form	Age at diagnosis	Age at T0 evaluation	Sex	ERT	CRIM
1	Classical Infantile Pompe	3 m	7 m	M	yes	–
2	Classical Infantile Pompe	4 m	3 y 4 m	F	yes	+
3	Classical Infantile Pompe	3 m	1 y 4 m	M	yes	+
4	Classical Infantile Pompe	5 d	2 m	M	yes	+
5	Non-Classical Infantile Pompe	7 y	11 y 5 m	M	No	+
6	Non-Classical Infantile Pompe	3 m	4 y 3 m	F	No	+
7	Non-Classical Infantile Pompe	9 y	14 y 6 m	M	yes	+
8	Non-Classical Infantile Pompe	2 m	9 y 1 m	F	yes	+

ERT: Enzyme Replacement Therapy; CRIM: Cross-Reactive Immunological Material; d: days; m: months; y: years; M: male; F: female.

Global overview on MRC, ROM, 6MWT and functional scales results at T0 are reported in Table 2.

Table 2 – Motor functional assessment overview at T0.

ID	Age (months-m, years-y)	Form	MRC	ROM limitations	6MWT	Functional scales						
						CI	MFM-20	HMFS	HMFSE	NSAA	GSGCA	MFM-32
1	7 m	Classical Infantile		Upper limbs: no Lower limbs: Hips: yes knees: yes ankles: yes		46/64						
2	3 y 4 m	Classical Infantile		Upper limbs: no Lower limbs: Hips: yes knees: yes ankles: yes		52/64	26/60	8/40	10/66			
3	1 y 4 m	Classical Infantile		Upper limbs: no Lower limbs: Hips: yes knees: yes ankles: yes		60/64						
4	2 m	Classical Infantile		Upper limbs: no Lower limbs: Hips: no knees: no ankles: no		48/64						
5	11 y 5 m	Late Onset Infantile	100%	Upper limbs: no Lower limbs: no	492 mt	64/64	60/60	40/40	66/66	34/34	5/32	96/96
6	4 y 3 m	Late Onset Infantile		Upper limbs: no Lower limbs: no		64/64	60/60	40/40	66/66	34/34		
7	14 y 6 m	Late Onset Infantile	100%	Upper limbs: no Lower limbs: no	520 mt	64/64	59/60	40/40	66/66	33/34	5/32	95/96
8	9 y 1 m	Late Onset Infantile	70%	Upper limbs: no Lower limbs: Hips: yes knees: yes ankles: yes	233 mt	64/64	48/60	38/40	48/66	18/34	19/32	71/96

MRC is expressed as average percentage for all muscular districts according to the formula: [total MRC score/(number of muscles evaluated x 5)] x 100. About ROM limitations, not degree but joints location of limitation is reported because more useful for the present study. For subject 6, GSGCA and MFM-32 could not be used at T0 because validated for older ages (5 years and 6 years respectively); he could not cooperate for the 6MWT neither.
MRC: Medical research Council; ROM: Range of Motion; CI: CHOP Intend; MFM20 and MFM32: Motor Function Measure Scale for Neuromuscular Diseases 20 and 32; HMFSE: Expanded version of the Hammersmith Functional Motor Scale-HMFSE; NSAA: North Star Ambulatory Assessment, GSGCA: Gait, Stairs, Gowers, Chair, Arms Functional Test; y: years; m: months.

Three out of four IOPD patients were tested with the CHOP Intend, being the only test validated for their age. At T0, results showed motor function impairment and the test permitted to functionally stratify patients (ID1 46/64, ID3 60/64, ID4 48/64). One patient (ID2, 3-years and 4-months old at first evaluation, able to sit but not to stand) was tested with multiple tests according to age (CI,

MFM20, HMFS, HMFSE). Results showed, as expected according to age,14e17 too high scores with the CI (52/64), being this scale developed for children with severe neuromuscular compromise, and an underestimation with a floor effect for the HMFS and HMFSE (8/40 and 10/66 respectively). MFM-20, with more items related to upper limb and head function, showed the most useful motor functional evaluation according to the disease features (26/60).

ID1, ID3 and ID4 were re-evaluated in the follow up, whereas ID2 died shortly after T0 for cardiopulmonary arrest. ID1 showed a significant functional deterioration correlated with progressive development of high and sustained titer antibody against rhGAA despite immunosuppression; CI documented the deterioration (46/64 at T0, 29/64 after 1 year). This patient died short after. ID3 also showed a progressive clinical deterioration despite regular ERT infusions and negative antibody titer; CI documented the deterioration (60/ 64 at T0, 30/64 after 2 years) associated with worsening of ROM limitations. ID4 had a good clinical response to ERT and CI documented the clinical improvement (48/64 at T0, 64/64 after 1 year).

The T0 evaluation in LOPD patients non ERT-treated (ID5 and ID6) confirmed maximal scores in all motor functional scales administered according to age. GSGCA and MFM-32 could not be used for ID6 because validated for older ages (5 years and 6 years respectively); he could not cooperate for the 6MWT neither. ID5 after 1 year showed a slight clinical worsening with fatigability in sportive practice, correlating with a slight "decalage" on NSAA (34/34 at T0, 33/34 after 1 year) and a significant reduction at the 6MWT (492 m at T0, 434 after 1 year, 58 m lost). ERT was offered to the patient. ID6 was repeatedly evaluated in three years-time-span and showed stable maximal score at NSAA and good distance at 6MWT, when she could start to perform the test (500 m at 6 years of age). She is still non-ERT treated and in clinical follow-up.

The T0 evaluation in ERT-treated LOPD patients (ID7 and ID8) showed a slight motor function limitation for ID7 (detected by MFM-20, NSAA and MFM-32 but not by other tests) and a significant motor function limitation for ID8 (detected by all functional tests) associated with ROM limita- tions in lower limbs and relevant weakness at MRC. Both pa- tients had low antibody titer.

ID7 was repeatedly evaluated in the following three years, reaching stable maximal score at NSAA and good distance at 6MWT with a gain of 58 m (503 m at T0, 561 after 3 years), correlating with an optimal clinical course. ID8, despite stable low antibody titer and increase in ERT dosage (30 mg/kg every other week), showed progressive loss of motor function detected by NSAA (18/34 at T0, 13/34 after 1 year and 12/34 after 2 years) and 6MWT (233 m at T0, 182 m after 1 year, not performed after 2 years).

The T0 cognitive/developmental evaluation was possible in 6 patients. Four of them obtained scores within the normal range: ID3 (Composite Score of the Cognitive Scale 95, assessed by BayleyeIII),

ID4 (Composite Score of the Cognitive Scale 85, assessed by BayleyeIII), ID7 (Full Scale Intelligent Quotient 89, assessed by WISCeIV) and ID 8 (Full Scale Intelligent Quotient 108, assessed by WISCeIV). Two of them obtained scores above the normal range: ID5 (Full Scale Intelligent Quotient 119, assessed by WISCeIV) and ID6 (Full Scale Intelligent Quotient 121, assessed by the WPPSIeIII). In two patients (ID1 and ID2), the severe functional impairment limited the reliability of cognitive evaluation with the Bayley- III scale.

Re-evaluation was performed in 4 LOPD patients. All of them obtained scores within the normal range, with mild worsening in two cases (ID5 Full Scale Intelligent Quotient 107 and ID6 Full Scale Intelligent Quotient 107) and improvement in two cases (ID7 Full Scale Intelligent Quotient 96 and ID8 Full Scale Intelligent Quotient 110). None of the two IOPD patients was available for the re-evaluation. See Table 3 for cognitive details.

Table 3 – Cognitive/Development evaluation results.

ID	Form	ERT	Age at T0 evaluation (months-m, years-y)	Test	IQ/Composite score of cognitive scale	Re-test (at least after 2 years)
1	Classical Infantile Pompe	yes	7 m	ne	–	–
2	Classical Infantile Pompe	yes	3 y 4 m	ne	–	–
3	Classical Infantile Pompe	yes	1 y 4 m	Bayley-III	95	na
4	Classical Infantile Pompe	yes	2 m	Bayley-III	85	na
5	Non-Classical Infantile Pompe	No	11 y 5 m	WISC-IV	119	107
6	Non-Classical Infantile Pompe	No	4 y 3 m	WPPSI-III	121	107
7	Non-Classical Infantile Pompe	yes	14 y 6 m	WISC-IV	89	96
8	Non-Classical Infantile Pompe	yes	9 y 1 m	WISC-IV	108	110

IQ: intelligence quotient; WPPSI III: Wechsler Preschool and Primary Scale of Intelligence 3rd edition; WISC IV: Wechsler Intelligence Scale for Children 4th edition; CI: Confidence Interval 95%; ne: not evaluable; na: not assessed; m: months; y: years.

Discussion

The aim of this study was to establish which motor functional scales could better assess children with Pompe disease, both infantile-onset form and late-onset form. To our knowledge, there is not a univocal consensus on which instrument is useful to assess motor functions in this population. We chose seven different functional scales commonly used and validated in infantile population affected by neuromuscular diseases, and administered them to our cohort, according to age of validation and functional level.

CHOP Intend was used to assess all the 4 IOPD subjects. This instrument allows to quantify motor repertoire in very young children or in patients with very low functional level. This scale can be used in Pompe children who are less than 2 years old and in older children that cannot seat without support. Only one subject (ID2) could be assessed by MFM-20, HMFS and HMFSE according to age and functional level. Our results suggest that MFM-20 better investigates and differentiates motor functions in children with Pompe disease who can sit without support, starting from the age of 2. In our IOPD cohort MRC was not used due to the young age. ROM evaluation showed limitations in three patients (ID1, ID2, ID3) correlating with low motor functional level. Three patients were evaluated at follow up. Two patients showed significant motor functional deterioration documented

by CI and ROM limitations worsening. One patient died short after. One patient (ID4) had a good clinical response to ERT and CI documented the clinical improvement.

All 4 LOPD children were evaluated by CI, MFM-20, HMFS, HMFSE and NSAA. Chop Intend showed a ceiling effect in all these children, due to their age and their functional status. Two children gained maximum score in all other scales (ID5 and ID6), showing no motor clinical signs of the disease. Comparing results obtained by two children with clinical signs but ambulatory (ID7 and ID8), MFM-20 and HMFS were not appropriate while HMFSE, NSAA, GSGCA and MFM-32 were appropriate according to functional level. Considering that HMFSE investigates a lower functional status and that GSGCA is time-saving but mainly a quantitative scale, these two scales seem not suitable to functional stratify children with Pompe disease. The comparison between NSAA and MFM-32 shows that these two scales have an overlapping of 12 activities but are different for total number of items (17 tested in NSAA and 32 in MFM-32), age of validation (from 4 years in NSAA and from 6 years in MFM-32) and in kind of items (in MFM-32 many items investigate upper limbs function which does not result affected in our population). Considering that our two patients showed some results in both scales, it could be preferred to use NSAA instead of MFM-32 to extend spectrum of age and to reduced time of administration. In consequence of these considerations, we suggest the use of NSAA in children with Pompe disease who age more than 4 years and can walk for ten meters without support. All the four patients were evaluated at follow up with NSAA and 6MWT. Both tests could document motor functional stability, worsening or improvement. To better verify these considerations a wider cohort of children need to be assessed.

The study of the cognitive profile is an element of novelty in metabolic and neuromuscular diseases. Recent studies utilizing CNS neuroimaging in IOPD ERT-treated children evidenced white matter abnormalities.26,27 Two previously published studies in children with IOPD showed QI scores in the lower normal range without any evidence of cognitive decay over time.26,28 In our cohort, we tested 6 patients, all of them with a QIT/Cognitive Composite Score in the normal range (between 85 and 121 at T0). In two of them, a slight decline of IQ was observed after two years, but still in the normal range and without any clinical significance. Further- more, we did not find any significant differences between Wechsler Index Scores in late-onset patients suggesting that there are no specific areas of weakness or strength in the cognitive functioning of these subject. Our results seem to suggest that in PD children the effects of glycogen storage in the central nervous system (CNS) does not influence the global cognitive functionality, differently than in other lysosomal diseases (i.e. mucopolisaccaridoses and sphingolipidoses), where intellectual disabilities are early evident with a severe course. This important observation needs confirmation and deeper investigation on a larger young PD patients cohort, including late-surviving IOPD patients.

Conclusions

Based on study results, we suggest that motor functions in children with Pompe disease, both classic infantile-onset and non-classic late-onset form, could be better assessed by Chop Intend, MFM-20 and NSAA, according to age and functional level. Chop Intend can be used in children who are less than 2 years old, since to our knowledge this is the only one scale validated for newborn and toddler with neuromuscular dis- eases. This instrument can also be used in older children that cannot seat without support. MFM-20 can be used to investigate motor functions in children who are able to sit without any support, starting from the age of 2 years and with no limits of age. NSAA is suitable for children who are more than 4 years old and can walk for ten meters without support. Evaluation should be completed with ROM measurement, MRC evaluation and 6MWT when possible, according to age and functional level (see Table 4).

Table 4 – Proposed motor functional evaluation scales for children with Pompe disease (y: years).

Age	Functional level	Motor Functional Scale
0-2 y	Any	CI
2-18 y	Not able to sit without support	CI
2-18 Y	Able to sit without support	MFM-20
4-18 y	Able to walk	NSAA

CI: CHOP Intend; MFM20: Motor Function Measure Scale for Neuromuscular Diseases 20; NSAA: North Star Ambulatory Assessment; y: years.

The evaluation should be done every six months, because of the progressive natural history of the disease, the rapid changes typical of develop- mental age and the need to document ERT effects. The complete motor functional evaluation should take no more than 45 min to be completed. About cognitive functions, classical intelligence scales (WISC, WPPSI) are not sufficient in children with Pompe disease, and should be complemented with specific tests addressing attention, memory, visual perception, visual-motor integration and executive functions. Swallowing and feeding functions, sensory functions, communication, psychosocial and neuroradiological domains have not directly been addressed in this study but should be included in further standards of care and outcome measures definition.

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Chapter VII: Lysosomal storage disorders: early diagnosis is fundamental because damage starts in the fetal period. A lesson from Fabry disease.

Fabry disease (FD, OMIM 301500) is an X-linked lysosomal disorder (LSD) caused by a deficiency of α-galactosidase A (α-GAL A) activity that results in the progressive accumulation of globotriaosylceramide (Gb3) and related glycosphingolipids, particularly in cellular lysosomes and body fluids[1]. The clinical phenotype includes a broad spectrum of clinical severity ranging from classic phenotype, that may present in childhood with acroparesthesias, angiokeratomas, corneal opacities, gastrointestinal symptoms, neuropathic pain, and hypohidrosis, followed in adulthood by renal failure, cardiac and cerebrovascular disease, and premature death; late onset FD instead, presents with cardiovascular involvement (hypertrophic cardiomyopathy with arrhythmias and conduction abnormalities), with very rare occurrences of renal (albuminuria, proteinuria) and cerebrovascular involvement[2]. In heterozygous females, clinical manifestations vary from asymptomatic to the classic severe phenotype, largely depending on X-chromosome inactivation[3]. Analysis of biomarkers (e.g., Gb3 and its deacylated form, lyso-Gb3) in plasma, urine, and DBS are useful for both diagnosis and follow-up[4]. Recently, pilot newborn screening (NBS) programs for FD, based on enzyme activity assay in DBS have been implemented worldwide using several analytical techniques. Piemonte have been the first region worldwide to perform it between 2003-2005.

It is known that accumulation begins early, sometimes prenatally, as demonstrated by the presence of cellular inclusions of Gb3 in the kidney, myocardiocytes and fetal cornea[5]. Gb3 inclusions were also found in the maternal part of the placenta of a heterozygous mother carrying an unaffected child and in both maternal and fetal sides of a heterozygous mother carrying an affected child[6].

The extent of the metabolic progression before symptoms is unknown. Through the analysis of lyso-Gb3 we demonstrated an early accumulation of the latter, already observable at birth, in a male affected by classic Fabry disease. Such a progressive metabolic trend during the pre-symptomatic period implies the potential definition of a metabolic threshold useful for a preventive therapeutic approach of classic Fabry disease. Additionally, the consistent increase of Lyso-Gb3 in the neonatal period in classic Fabry disease suggests Lyso-Gb3 as a useful marker for improving the specificity of newborn screening for Fabry disease.

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Part of this work has been published as:

Spada M, Kasper D, Pagliardini V, Biamino E, Giachero S, Porta F. "Metabolic progression to clinical phenotype in classic Fabry disease". Ital J Pediatr. BioMed Central, 2017 Jan 3;43(1):1.

Background

Fabry disease (OMIM 301500) is an X-linked lysosomal storage disorder due to α-galactosidase A (α-Gal A) deficiency. The enzymatic defect leads to progressive accumulation of globotriaosylceramide (Gb3) and related glycosphingolipids in the vascular endothelium, particularly in kidney, brain, and heart. Affected males with complete or near-complete α-Gal A deficiency exhibit the classic clinical phenotype of Fabry disease with onset of angiokeratomas, acroparesthesias, hypohidrosis, and corneal opacities in childhood, followed by renal failure, cardiac and cerebrovascular disease, and premature death [1, 2]. A wide spectrum of later-onset variants have been described in patients with residual α-Gal A activity, including the "renal variant" and the "cardiac variant". Since 2001, an effective enzyme replacement therapy (ERT) is available [3].

Recently, globotriaosylsphingosine (LysoGb3), a deacylated form of Gb3, was identified as a new pathogenetic effector and hallmark of Fabry disease, representing a promising non-invasive marker for monitoring the dis- ease [4]. Differently from plasma Gb3, plasma LysoGb3 was shown to be dramatically increased in both males with classic Fabry disease and symptomatic females heterozygous for mutations in the α-galactosidase A gene [5]. Since the clinical phenotype of Fabry disease is invariably preceded by earlier progressive lysosomal storage of glycosphingolipids [6], the analysis of peripheral LysoGb3 may allow investigation of patients' metabolic phenotype even in the pre-symptomatic period.

Here we report LysoGb3 analysis in two brothers with classic Fabry disease with 14 years age difference, giving a picture of metabolic phenotype and natural progression of Fabry disease from the neonatal period to childhood.

Methods

The genealogy of the two brothers with classic Fabry disease is depicted in Fig. 1 (panel a).

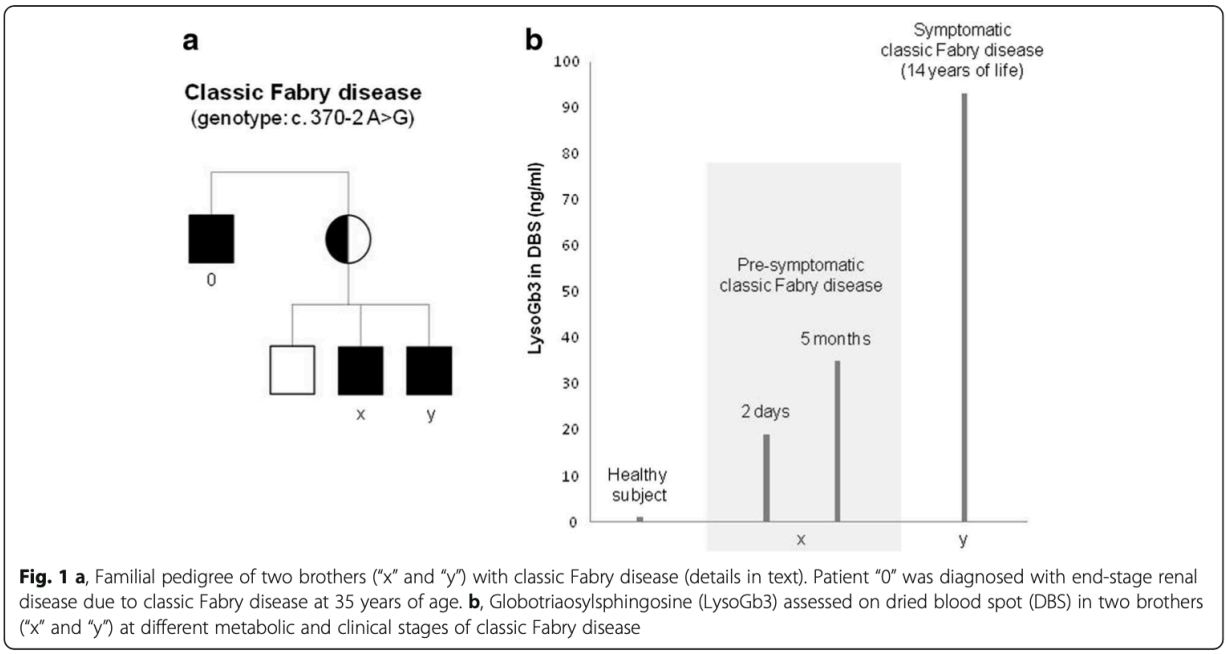

Fig. 1 a, Familial pedigree of two brothers ("x" and "y") with classic Fabry disease (details in text). Patient "0" was diagnosed with end-stage renal disease due to classic Fabry disease at 35 years of age. **b**, Globotriaosylsphingosine (LysoGb3) assessed on dried blood spot (DBS) in two brothers ("x" and "y") at different metabolic and clinical stages of classic Fabry disease

The clinical course of the first-born brother was uneventful until 11 years of age, when persistent acroparesthesias and burning pain were reported. A definite diagnosis of classic Fabry disease was made on the basis of biochemical and molecular data (α-Gal A activity = 0.2 nmol/h/ml, normal value >2 nmol/h/ml; genotype c. 370–2 A > G). A basal LysoGb3 analysis on dried blood spot was obtained just before starting ERT. Based on familiar anamnesis, the second-born brother was diagnosed with classic Fabry disease in the neonatal period (α-Gal A activity = 0.7 nmol/ h/ml; normal value >2 nmol/h/ml; genotype c. 370–2 A > G), undergoing LysoGb3 analysis at 2 days of life. A further LysoGb3 measurement was performed at 5 months.

LysoGb3 was measured in dried blood spot samples by high-sensitive electrospray ionization liquid chromatography tandem mass spectrometry (ESI LC-MS/MS). A 7- point serum calibrator for lysoGb3 quantification (covering the analytic range from 0–120 ng/mL; lower limit of quantification: 1.5 ng/mL) and three calibrator levels (3, 30 and 100 ng/mL) for quality control were used (ARCHIMED Life Science GmbH, Vienna, Austria; www.archimedlife.com).

Results

Blood LysoGb3 concentrations were consistent with patients' age and clinical picture, with lower levels in the asymptomatic neonate and higher levels in the symptomatic child. LysoGb3 in the second-born doubled during the first 5 months of life, reaching ~40% concentration observed in the symptomatic period (Fig. 1, panel b). The comparison of LysoGb3 concentrations in the two brothers with classic Fabry disease revealed its 5-fold increase from the neonatal period to childhood (Fig. 1, panel b). The neonatal LysoGb3 concentration in classic Fabry dis- ease, moreover, exceeds that observed in normal subjects by over 15 times (Fig. 1, panel b).

Discussion

In 2006, we were the first to demonstrate a high incidence of later-onset Fabry disease as opposed to classic Fabry dis- ease [7], subsequently confirmed in other studies [8, 9]. A variable symptom-free interval characterizes all forms of Fabry disease, and clinical phenotype is preceded and sustained by progressive glycosphingolipids storage [3]. In re- cent years, little was known about pre-symptomatic Fabry disease, especially since invasive procedures were invariably required for any pathological assessment. For in- stance, serial renal biopsies showed that Gb3 storage even precedes microalbuminuria in patients with Fabry disease [10]. Recently, podocyturia was identified as an early useful marker of renal damage in Fabry disease [11, 12].

LysoGb3 is a new, easily measurable marker and pathogenic effector of Fabry disease [4]. Smid and colleagues recently described the superior diagnostic utility of lysoGb3 compared to Gb3 to discern non-classical, uncertain or patients having no Fabry disease [13].

LysoGb3 can be measured in plasma or in dried blood spots [14], representing a potential new avenue to the comprehension of metabolic progression of Fabry disease from the pre-symptomatic to the symptomatic period. This potential of LysoGb3, indeed, may be useful for monitoring patients detected through newborn or selective screening for Fabry disease, improving the sensitivity of the clinical approach to prevent irreversible organ damage.

In this study, the comparison of LysoGb3 in two brothers with complete α-Gal A deficiency describes the extent of glycosphingolipids storage during childhood in classic Fabry disease and its relationship with clinical onset. The early and consistent increase of LysoGb3 observed in the neonatal period in classic Fabry disease is consistent with its fetal storage [6], making LysoGb3 a potential useful tool for improving the specificity of newborn screening for Fabry disease. Moreover, a substantial increase of LysoGb3 was documented during infancy in classic Fabry disease, suggesting an early plateau during the pre-symptomatic period. The observation of such a progressive metabolic trend in pre-symptomatic and early-symptomatic patients with classic Fabry disease implies the potential definition of a metabolic threshold (i.e. a LysoGb3 cut-off) useful for addressing an early preventive therapeutic approach to Fabry disease.

Conclusions

These observations suggest a new nosological classification of Fabry disease, based on the metabolic phenotype instead of the clinical phenotype. Early screening for Fabry disease and longitudinal pre-symptomatic non- invasive biochemical monitoring are essential to this definition. Anticipating clinical attention on patients' metabolic phenotype may represent a new frontier for the optimization of medical management of Fabry disease.

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Summary, perspectives and conclusive remarks

In the past decade, advances in liquid chromatography-mass spectrometry (LC-MS) have revolutionized untargeted metabolomics analyses. By mining metabolomes more deeply, researchers are now primed to uncover key metabolites and their associations with diseases. The employment of untargeted metabolomics has led to new biomarker discoveries and a better mechanistic understanding of diseases with applications in precision medicine.

In Italy about five hundred thousand babies are born every year and about 1:2581 child is affected by phenylketonuria or another form of hyperphenylalaninemia, and this is just one of the existing rare metabolic diseases which, if not immediately diagnosed, can lead to very serious disability.

Newborn screenings represent one of the fundamental chapters in the history of preventive and social medicine. In fact, the term "screening" indicates a moment of presumptive identification of diseases which, if not diagnosed and treated early, can have serious consequences and even cause death.

Screening is therefore of fundamental importance for the health of children and their growth prospects.

The expanded newborn screening test consists in taking some drops of blood from the heel of the baby within the first 72 hours of life: a minimally invasive method that allows to identify over 40 early and rare metabolic diseases and avoid the worst consequences, such as very serious disabilities and early deaths.

Neonatal screening exists in Italy since the ’70 –’80, but in 1992 it became mandatory for three pathologies: congenital hypothyroidism, cystic fibrosis and phenylketonuria. Today the obligation has been extended to about 40 rare metabolic diseases thanks to the approval of Law 167/2016.

The progressive development of new low-cost technologies that can be carried out on large numbers of samples, has made possible to diagnose more patients in a pre-clinical and asymptomatic phase of the disease and to promptly treat them.

The early and timely start of a correct therapy has allowed the survival and has improve the quality of life of many children.

The early diagnosis has generated a new phenotype of pathology previously unknown to the clinician for which clear guidelines and evaluating tools are often lacking.

So is important to carry out a correct follow-up of these patients, to avoid hyper-medicalization resulting in the generation of stress and anxiety for patients.

Furthermore, the arrival of new therapies, as in the case of lysosomal diseases, has been a great stimulus for the early identification of patients, before that the organ damage is irreversible.

In the case of Pompe disease, neonatal screening is synonymous of survival. Life-saving therapies are in fact now available for these pathologies and can make the difference between life and death.
For Fabry disease, which usually manifests later, early diagnosis represents the possibility of careful follow-up , because, as demonstrated, the damage is already present in the neonatal period, and for a proper family screening.
The use of DBS, derived from newborn screening, applied to LC-MS, are literally revolutionizing the diagnostic and follow-up approach not only in the field of metabolic diseases, but also in that of genetics.