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Newborn screening for Pompe disease in Italy: Long-term results and future challenges

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ABSTRACT

Pompe disease (PD) is a progressive neuromuscular disorder caused by a lysosomal acid α-glucosidase (GAA) deficiency. Enzymatic replacement therapy is available, but early diagnosis by newborn screening (NBS) is essential for early treatment and better outcomes, especially with more severe forms. We present results from 7 years of NBS for PD and the management of infantile-onset (IOPD) and late-onset (LOPD) patients, during which we sought candidate predictive parameters of phenotype severity at baseline and during follow-up. We used a tandem mass spectrometry assay for α-glucosidase activity to screen 206,741 newborns and identified 39 positive neonates (0.019%). Eleven had two pathogenic variants of the *GAA* gene (3 IOPD, 8 LOPD); six carried variants of uncertain significance (VUS). IOPD patients were treated promptly and had good outcomes. LOPD and infants with VUS were followed; all were asymptomatic at the last visit (mean age 3.4 years, range 0.5–5.5). Urinary glucose tetrasaccharide was a useful and biomarker for rapidly differentiating IOPD from LOPD and monitoring response to therapy during follow-up. Our study, the largest reported to date in Europe, presents data from longstanding NBS for PD, revealing an incidence in North East Italy of 1/18,795 (IOPD 1/68,914; LOPD 1/25,843), and the absence of mortality in IOPD treated from birth. In LOPD, rigorous long-term follow-up is needed to evaluate the best time to start therapy. The high pseudodeficiency frequency, ethical issues with early LOPD diagnosis, and difficulty predicting phenotypes based on biochemical parameters and genotypes, especially in LOPD, need further study.

1. Introduction

Pompe disease (PD) or glycogenosis II (OMIM #232300), is an autosomal recessive lysosomal storage disorder caused by deficiency of the enzyme acid α-glucosidase (GAA), resulting in progressive glycogen accumulation primarily in cardiac, skeletal, and smooth muscles [1–3].

Clinical manifestations are broad, from patients with classic infantile-onset PD (IOPD), presenting in the first months of life with

Abbreviations: CLIR, Collaborative Laboratory Integrated Reports; CRIM, cross-reactive immunological material; DBS, dried blood spot; DMF, digital microfluidics; ECG, electrocardiogram; EF, ejection fraction; EMG, electromyography; ERT, enzyme replacement therapy; GAA, acid α-glucosidase; Glc4, glucose tetrasaccharide; GMFM-88, Gross Motor Function Measure; IOPD, infantile-onset Pompe disease; ITI, immunotolerance induction; LOPD, late-onset Pompe disease; LVMI, left ventricular max index; MFM-20, motor function measurement; MRC, Medical Research Council Scale; MRI, magnetic resonance imaging; MS/MS, tandem mass spectrometry; NBS, newborn screening; nv, normal values; PBMC, peripheral blood mononuclear cells; PD, Pompe disease; PPV, positive predictive value; rhGAA, recombinant human GAA; RUSP, Recommended Uniform Screening Panel; VUS, variants of uncertain significance..

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hypertrophic cardiomyopathy, muscular hypotonia and death due to cardiorespiratory failure within the first 1–2 years of life, to patients with the late-onset form (LOPD), which may manifest at any age with progressive muscle weakness [4].

Diagnosis is established by low GAA activity in dried blood spot (DBS), lymphocytes or fibroblasts, and is confirmed by gene analysis [5,6]. Urinary glucose tetrasaccharide (Glc4), derived from glycogen breakdown, is a specific biomarker in the context of reduced GAA activity or other signs and symptoms of PD. [7,8]

Enzyme replacement therapy (ERT) with recombinant human GAA (rhGAA) has significantly changed the natural history of the disorder [9–11]. Some patients, especially those with IOPD lacking GAA cross-reactive immunological material (CRIM-negative), develop anti-rhGAA antibodies with subsequent adverse events or loss of efficacy, and may require preventive or therapeutic immunotolerance induction (ITI) [12,13]. Recently, an Italian Expert Group formulated consensus recommendations for managing immune response to rhGAA, based on a review of the evidence and their clinical experience [14].

Favorable outcomes with ERT are strongly associated with very early initiation of treatment. In IOPD, treatment should be initiated as soon as possible; delays of even days can influence outcomes [15–20]. In patients with LOPD, ERT is associated with better outcome when started before irreversible muscle damage occurs [21–23]. In the absence of a family history, early (presymptomatic) diagnosis can only be achieved through newborn screening (NBS).

The first NBS pilot study was launched in Taiwan in 2005 using a fluorometric assay [24]. About 1 million newborns were screened between 2005 and 2018 (using tandem mass spectrometry (MS/MS) since 2010), and a higher-than-expected PD incidence was found (1:18,090) [22]. Similar data were obtained by the Japanese NBS program that started in 2013 [25,26].

Subsequent improvement in screening technologies for lysosomal storage diseases led to the development of multiplexed enzyme assays that use fluorescence-based digital microfluidics (DMF) or MS/MS. [27] Using these two methods, several pilot programs around the world have evaluated the feasibility of PD NBS. A summary of the known NBS programs for PD is presented in Supplementary Table 1.

In the United States, pilot projects were started between 2013 and 2014 in Missouri (DMF), New York (MS/MS) and Illinois (MS/MS). All found an unexpectedly high PD incidence (1:23,596–1:10,152), partially due to the identification of a high number of suspected LOPD patients [9,28,29]. The findings of high disease incidence, the technical feasibility of NBS and the benefits of early diagnosis led to the inclusion of PD in the Recommended Uniform Screening Panel (RUSP) in February 2015. To date, PD NBS is routinely performed in 28 USA states and Washington DC (https://nbstrn.org/tools/nbs-vr). Most have reported their experience, in terms of screening methods, prevalence of disease and positive predictive value. All confirmed a higher-than-expected incidence of disease, and in particular a high number of suspected LOPD infants. Little data are available on the follow-up of screen-positive newborns to establish the effect of NBS on IOPD patient outcomes [9,29–31]. Very recently, Huggins et al. reported a systematic evaluation of 20 LOPD patients detected by NBS and followed through the first 2 years of life [23].

In Brazil, a NBS program screened 10,527 newborns using DMF but did not identify any PD patients [32,33]. In Mexico, a PD NBS program was started in 2012 using MS/MS and included 20,018 newborns, identifying only 1 suspected PD patient (carrying a pathogenic variant and a VUS), but the follow-up is not reported [34].

PD NBS is less widespread in Europe. Pilot studies using MS/MS were performed in Austria on 34,736 deidentified neonatal DBS (PD incidence 1:8684) [35], and in Hungary on 40,024 deidentified neonatal DBS (PD incidence 1:4447) [36], but no follow-up data were reported. In Italy, a small study on 3403 newborns was performed in Umbria region between 2010 and 2012 using a fluorometric assay but no affected newborn was identified [37].

In 2015, we established a NBS program using MS/MS assays for 4 lysosomal storage disorders, including Pompe disease. A 17-month initial phase (44,411 newborns) confirmed the feasibility of the test and the high incidence of the disease (1:22,205, all LOPD) [38]. Here, we report 7 years of experience with PD NBS, including genetic and clinical features, epidemiology, and outcomes after long-term follow-up. We present our algorithm for managing IOPD and LOPD patients, and evaluate the advantages and challenges of PD NBS in Italy.

2. Methods

2.1. Study population

DBS from 206,741 newborns were collected consecutively from September 2015 to April 2022 at the North-East Italy Regional Center for Expanded NBS, Veneto Region. Written informed consent was obtained from a parent. Proof of informed consent is available upon request.

2.2. Methods

GAA activity was measured by multiplex MS/MS using the NeoLSD® assay system from Perkin Elmer (Turku, Finland), as previously reported [38]. The kit contained the buffer, mobile phase, substrates, and internal standards for assaying 6 lysosomal enzyme diseases: Pompe disease, Fabry disease, Mucopolysaccharidosis type I, Gaucher disease, Niemann pick type A/B and Krabbe disease [38].

Confirmatory testing included clinical evaluation, cardiologic assessment (electrocardiogram ECG and echocardiogram), biochemical tests (blood CK, AST, ALT, LDH; urine Glc4; GAA enzyme activity in peripheral blood mononuclear cells [PBMC] by MS/MS) and molecular analysis. All test results (except molecular analysis) are ready the day after hospital admission.

2.3. Management and follow-up of positive newborns

Diagnosis and follow-up of positive patients differ by disease form.

IOPD patients were rapidly diagnosed based on increased Glc4 and the cardiologic assessment. Before starting ERT, CRIM status was determined either by genotype prediction or western blot analysis on PBMC, and an ITI protocol was performed accordingly. Patients were monitored closely with clinical, biochemical and instrumental assessments that included plasma and urine biomarkers (CPK, AST, ALT, Glc4), cardiac testing (ECG and Echocardiogram), and pulmonary and feeding status were periodically evaluated; psychomotor development was monitored using age-appropriate scales every month for 6 months, and every 3–6 months thereafter. Anti-rhGAA antibodies were monitored by ELISA (Genzyme Corp.). Complete details and timing of each evaluation are reported in Table 1.

In non-IOPD infants (i.e., LOPD, pseudodeficiency and carriers), clinical decisions were made after molecular analysis was available. Although asymptomatic at birth, for classification purposes we considered NBS-positive newborns with a confirmed PD diagnosis to have LOPD if they had predicted "late-onset" *GAA* variants in homozygosity or compound heterozygosity and lacked cardiac involvement. Predicted pathogenicity for each variant was based on information from the Pompe variant database at the Erasmus Medical Center (http://pompevariantdatabase.nl).

Follow-up for LOPD cases, also if asymptomatic, was conducted every 3 months during the first year and every 6–12 months thereafter, and included evaluation of biomarkers (CPK, AST, ALT, Glc4), cardiac assessment (especially for rhythm disturbances), pulmonary and feeding status, and psychomotor development, with age-appropriate scales (see Table 1 for details and timing). Treatment was only initiated when abnormalities appeared. The modifier variant c.510C > T was investigated to improve phenotype prediction in patients carrying the c-32-13 T > G

Table 1
Proposed follow up of positive newborns.

	IOPD	LOPD/VUS
Clinical evaluation	Every month for 6 months, then every 3–6 months	Every 3 months in the first year, then every 6 months to a year.*
Biochemical evaluation CPK, AST, ALT, LDH	Every month for 6 months, then every 3–6 months	Every 3 months in the first year, then every 6 months to a year.*
Glc4**	Every month for 6 months, then every 3–6 months	Every 3 months in the first year, then every 6 months to a year.*
Cardiac assessment ECG; Echocardiogram (LVMI, EF)	Every month for 3 months, then every 3 to 6 months.	Every 3–6 months through the first year, then ECG every 12 months and Echocardiogram as clinically warranted (every 1–2 years in adult)
Pulmonary assessment and ventilation required (invasive or non-invasive)	Every visit	Every visit
Spirometry	>5 years: every 6–12 months	>5 years: every 6–12 months
Polysomnography	Every 6–12 months	Every 6–12 months
6 min walking test	>5 years: every 6–12 months	>5 years: every 6–12 months
Developmental assessment with age-appropriate scale (Alberta, MFM-20, GMFM-88), MRC score***	Every 3–6 months	Every 3 months in the first year, then every 6 months to a year.*
Feeding evaluation (oral feeding or use of any feeding support such as nasogastric tube or gastrostomy tube), growth and nutritional assessment	Every visit	Every visit
Swallow study	If indicated	If indicated
Anti-rhGAA IgG antibody titers	Every month for 6 months, then every 3–6 months	If ERT is started, every month for 6 months, then every 3–6 months
Other system (speech, hearing vision and cognitive functions)	Every 12–24 months	Every 12–24 months
Brain magnetic resonance imaging (MRI) scans	Every 12–24 months****	Every 12–24 months (with MR angiography)****

While it is a general protocol, the procedure for each patient is highly dependent on the specific clinical manifestation.

* If ERT is started, monthly for 3 months and then every 3–6 months.

** Normal Glc4 values (97.5 percentile) change with the age (16.3 mmol/mol creatinine until 5 months, 7.7 until 2 years, 3.7 until 10 years, then 1.1).

*** All assessments were administered and scored in accordance with standardized test procedures specific for each assessment by an experienced child neurologist.

**** Sedation related risks should be considered.

(IVS1) pathogenic variant. Moreover, to better characterize some LOPD patients, GAA protein was assessed in PBMC using western blot analysis, which allows different forms of GAA to be detected (see supplementary material). In particular, in PMBC GAA is synthesized as a 110 kDa precursor that undergoes a series of complex proteolytic and N-glycan processing events in the lysosome (intermediate forms of 100 and 95 kDa), leading to the mature lysosomal species of 76 and 70 kDa [39–41]. These forms can be quantified versus a reference protein (in our study GAPDH) for interpatient comparisons, and ratios between different forms can be evaluated.

Newborns with pseudodeficiency (changes in the *GAA* gene sequence that result in reduced activity in vitro, but normal activity *in vivo* [9,42,43]) were discharged with no further follow-up. When we find a single pathogenic variant (carrier), there is the rare risk of an undiscovered second variant [6,44]. In these cases, we avoided most invasive tests in asymptomatic infants, but pediatricians and parents were informed to be alert for abnormal clinical symptoms suggestive of the disease.

2.4. Statistical analysis

Data are presented as mean with standard deviation (for continuous variables) and frequency and percentage (for categorical variables). Student's *t*-test was used to compare GAA activity, CPK, left ventricular max index (LVMI) and Glc4 between IOPD, LOPD, pseudodeficiency and carrier newborns. Correlation between variables was performed with regression test (Pearson). Statistical analyses were performed using GraphPad Prism Version 5.00 (GraphPad Software, San Diego, California). *P*-values <0.05 were considered statistically significant differences.

3. Results

3.1. Demographic and genotype

From September 2015 to April 2022, 206,741 newborns were screened for PD.

Confirmatory testing was performed on 39 (0.019%) neonates (16 females, 23 males, 2 twins). Twenty-seven were Europeans, 5 were of Asian descent, 4 were African; for 3 newborns this data was not available. Table 2 presents the biochemical and molecular genetic analysis for NBS-positive newborns.

Two pathogenic variants of the *GAA* gene were present in 11/39 newborns (7 Europeans, 4 of African origin), of which 3 had IOPD (2 CRIM negative, 1 CRIM positive), and 8 had LOPD. The incidence of PD was 1:18,432 (IOPD 1:67,583, LOPD 1:25,344) and the positive predictive value (PPV) was 28%. Twenty-eight newborns had ≥1 VUS ($n = 6$), a known pseudodeficiency allele or predicted non-pathogenic variant ($n = 15$), or were carriers ($n = 4$). Three newborns were lost to follow up prior to confirmatory tests due to the family's relocation out of the region.

The typically Caucasian IVS1 variant was the most common pathogenic variant (23/41, 56% of all pathogenic mutations) and was present in all 8 LOPD cases, 5 of which were homozygous. None of them carried the genetic modifier c.510C > T. Of note, this variant was also found in non-Caucasian newborns (1 Asiatic, 2 neonates from North Africa).

Among pseudodeficiencies, we found a high incidence of the Asiatic pseudodeficiency variant c.2065G > A (p.Glu689Lys), alone ($n = 2$, European) or in the complex allele c.[1726G > A;2065G > A] (p.[Gly576Ser; Glu689Lys]) ($n = 7$, 3 newborns of Asiatic origin, of which 2 were homozygous, and 4 Europeans). Moreover, 5 European newborns carried the predicted non-pathogenic variant p.Val222Met (3 of which were homozygous). Of note, 5 newborns with the same phenotype have been reported in the Hungarian NBS program [36], so that a founder effect is possible.

In silico analysis of novel *GAA* variants identified in this study are reported in Table 3.

3.2. Assessment at diagnosis

1) DBS GAA activity: Although there were statistically significant differences ($p < 0.0001$) between IOPD/LOPD and pseudodeficiency/carrier newborns, it is not possible to utilize DBS GAA levels to discriminate these groups, even by modifying the cut-off, because of overlapping values (Fig. 1a). Of note, we observed seasonal variations of the median of the GAA activity values, probably due to the different stability of the enzyme under different temperature and humidity conditions during transport [45]. Therefore, the cut-off needs to be periodically adjusted to avoid an increase in false positives in winter and false negatives in summer (Fig. 2).

2) Lymphocyte GAA activity: Although there was statistically significant difference ($p = 0.02$) between the values in IOPD/LOPD and pseudodeficiency/carrier newborns, also on this matrix there is an overlap of the values between the groups (Fig. 1b), so that molecular

Table 2
Patient demographics, baseline evaluation and predicted phenotype.

Year	Pt	Sex	Ethnic origin	NBS enzyme activity μM/h	PBMC enzyme activity nmol/h/mg (nv >6.98)	Glc4 mmol/mol crea (nv <16.3)*	CPK U/L (nv 0–295)	ECG: PR interval	Echocardiogram: LVMI g/m2 (nv <65)	Disease-associated variants (coding nomenclature)**	Disease-associated variants (protein nomenclature)	ACMG	Predicted phenotype (http://pompevariantdatabase.nl)
2015	1	F	South Asia	**2.71**	N/A	3.4	69	0.12	32.7	c. [−32-13 T > G]; [1726G > A; 2065G > A]	p.[=,0]; [Gly576Ser; Glu689Lys]	5/1	Unaffected (Pseudodeficiency)
2015	2	F	European	**3.22**	N/A	N/A	142	0.10	49.1	c.-32-13 T > G	p.[=,0]	5	Unaffected (Carrier)
2015	3	F	European	**2.26**	N/A	N/A	73	**0.06**	32.7	c.[533G > A]; [533G > A]	p.[Arg178His]; [Arg178His]	3/3	VUS (found only in NBS)
2016	4	F	North Africa	**1.41**	**1.11**	11	**682**	0.08	28	c.[−32-13 T > G]; [236_246del]	p.[=,0]; [Pro79Argfs*13]	5/5	Affected (LOPD)
2016	5	M	North Africa	**0.61**	**0.41**	**20**	**448**	**0.06**	54.6	c.[−32-13 T > G]; [236_246del]	p.[=,0]; [Pro79Argfs*13]	5/5	Affected (LOPD)
2016	6	M	European	**1.97**	N/A	N/A	N/A	N/A	N/A	c.[−32-13 T > G]; [1726G > A;2065G > A]	p.[=,0]; [Gly576Ser;Glu689Lys]	5/1	Unaffected (Pseudodeficiency)
2016	7	M	N/A	**1.25**	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
2017	8	M	European	**1.43**	N/A	N/A	198	N/A	N/A	c.[664G > A]; [664G > A]	p.[Val222Met]; [Val222Met]	3/3	Unaffected (Predicted non-pathogenic)
2017	9	M	East Asia	**1.92**	N/A	N/A	76	0.08	37.2	c.[1726G > A;2065G > A]; [1726G > A;2065G > A]	p.[Gly576Ser; Glu689Lys]; [Gly576Ser; Glu689Lys]	1/1	Unaffected (Pseudodeficiency)
2017	10	M	European	**2,7**	N/A	N/A	57	0.11	48.4	c.[−32-13 T > G]; [1726G > A;2065G > A]	p.[=,0]; [Gly576Ser;Glu689Lys]	5/1	Unaffected (Pseudodeficiency)
2017	11	M	European	**1.11**	N/A	N/A	N/A	0.10	41.4	c.[−32-13 T > G]; [−32-13 T > G]	p.[=,0]; [=,0]	5/5	Affected (LOPD)
2017	12	F	European	**0.45**	N/A	**27**	**990**	0.10	**187**	c.[1933G > A]; [2237G > A]	p.[Asp645Asn]; [Trp746*]	5/5	Affected (IOPD CRIM positive)
2017	13	M	European	**1.84**	N/A	N/A	N/A	N/A	N/A	c.2238G > C(;) 1154G > A(;) 2110G > A(;) 2065G > A(;)	p.Trp746Cys(;) Arg385His(;) Ala704The (;) Glu689Lys(;)	4/3/3/1	VUS
2017	14	M	East Asia	**1.89**	N/A	N/A	114	N/A	N/A	c.1935C > A	p.Asp645Glu	4	Unaffected (Carrier)
2018	15	M	European	**0.59**	**0.45**	2.5	153	N/A	Normal	c.[−32-13 T > G]; [−32-13 T > G]	p.[=,0]; [=,0]	5/5	Affected (LOPD)
2018	16	M	European	**0.88**	**0.75**	1.6	142	N/A	Normal	c.[−32-13 T > G]; [−32-13 T > G]	p.[=,0]	5/5	Affected (LOPD)
2018	17	M	West Africa	**0.49**	N/A	**71**	**1063**	**0.07**	**232**	c.[2560C > T]; [(692 + 1_693–1)_(1194 + 1_1195–1)del]	p.[Arg854*]; [Leu232Thrfs*41]	5/5	Affected (IOPD CRIM negative)
2018	18	M	European	**1.94**	**0.63**	6.3	290	**0.07**	40.4	c.[−32-13 T > G]; [−32-13 T > G]	p.[=,0]; [=,0]	5/5	Affected (LOPD)
2018	19	M	East Asia	**2.11**	N/A	1.27	82	Normal	N/A	c.2238G > C	p.Trp746Cys	4	Unaffected (Carrier)
2018	20	M	European	**2.52**	N/A	1.9	131	0.08	N/A	c.[664G > A]; [664G > A]	p.[Val222Met]; [Val222Met]	3/3	Unaffected (predicted non-pathogenic)
2018	21	M	European	**1.39**	**1.21**	16	144	0.08	45	c.[−32-13 T > G]; [−32-13 T > G]	p.[=,0]; [=,0]	5/5	Affected (LOPD)
2019	22	F	European	**1.08**	**0.65**	2.6	**500**	0.08	46.3	c.[−32-13 T > G]; [1933G > A]	p.[=,0];[Asp645Asn]	5/5	Affected (LOPD)
2019	23	F	European	**2.07**	**0.99**	1.7	77	N/A	N/A	c.[−32-13 T > G];[701C > T]	p.[=,0]; [Thr234Met]	5/3	VUS
2020	24	M	European	**1.75**	N/A	1.6	120	0.12	48.5	c.[2461G > A]; [664G > A]	p.[Glu821Arg]; [Val222Met]	3/3	Unaffected (predicted non-pathogenic)
2020	25	M	North Africa	**0.73**	**0.21**	**30**	**653**	0.08	**128**	c.[236_246del]; [236_246del]	p.[Pro79Argfs*13]; [Pro79Argfs*13]	5/5	Affected (IOPD CRIM negative)
2020	26	F	European	**2.72**	**3.07**	12	119	0.12	38.5	c.[−32-13 T > G]; [1726G > A; 2065G > A]	p.[=,0]; [Gly576Ser; Glu689Lys]	5/1	Unaffected (Pseudodeficiency)

(*continued on next page*)

Table 2 (*continued*)

Year	Pt	Sex	Ethnic origin	NBS enzyme activity μM/h	PBMC enzyme activity nmol/h/mg (nv >6.98)	Glc4 mmol/mol crea (nv <16.3)*	CPK U/L (nv 0–295)	ECG: PR interval	Echocardiogram: LVMI g/m2 (nv <65)	Disease-associated variants (coding nomenclature)**	Disease-associated variants (protein nomenclature)	ACMG	Predicted phenotype (http://pompevariantdatabase.nl)
2020	27	F	European/ afroamerican	**2.55**	N/A	10	119	0.10	40	c.[−32-13 T > G]; [726G > A]	p.[=,0]; [Ala242=]	5/1	VUS
2020	28	F	European	**1.39**	**1.49**	7.1	107	0.09	Normal	C.[1465G > A]; [664G > A]	p.[Asp489Asn]; [Val222Met]	4/3	Unaffected (predicted non-pathogenic)
2020	29	F	Asia	**1.55**	**3.14**	6.7	77	N/A	N/A	c.[1726G > A;2065G > A]; [1726G > A;2065G > A]	p.[Gly576Ser; Glu689Lys]; [Gly576Ser; Glu689Lys]	1/1	Unaffected (Pseudodeficiency)
2021	30	F	NA	**2**	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
2021	31	F	European	**1.43**	**2**	5.7	133	0.10	Normal	c.886C > A	p.Pro296Thr	3	Unaffected (Carrier)
2021	32	M	NA	**1.19**	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
2021	33	F	European	**0.85**	**0.9**	6.8	141	0.12	28	c.[1048G > A]; [2051C > T]	p.[Val350Met]; [Pro684Leu]	¾	VUS
2021	34	M	European	**1.16**	**4.32**	3.3	100	0.10	38.1	c.[−32-13 T > G]; [726G > A]	p.[=,0]; [Ala242=]	5/1	VUS
2021	35	F	European	**1.14**	**0.69**	2.3	94	0.10	32.4	c.[2238G > C]; [2065G > A]	p.[Trp746Cys]; [Glu689Lys]	4/1	Unaffected (Pseudodeficiency)
2021	36	M	European	**2.33**	N/A	5.5	65	0.09	Normal	c.[664G > A]; [664G > A]	p.[Val222Met]; [Val222Met]	3/3	Unaffected (predicted non-pathogenic)
2021	37	M	European	**1.71**	**6.9**	N/A	135	0.12	Normal	c.[−32-13 T > G]; [271G > A]	p.[=,0]; [Asp91Asn]	5/2	Unaffected (Pseudodeficiency)
2021	38	F	European	**1.89**	N/A	3.4	128	0.12	37.5	c.[−32-13 T > G]; [271G > A]	p.[=,0]; [Asp91Asn]	5/2	Unaffected (Pseudodeficiency)
2021	39	M	European	**1.23**	N/A	7.6	174	0.09	N/A	[1903A > G]; c.[1726G > A;2065G > A]	p.[Asn635Asp]; [Gly576Ser;Glu689Lys]	2/1	Unaffected (Pseudodeficiency)

* Glc4 reference ranges are age dependent: 0–5 months <16.3 mMol/Mol Creatinine, 6–23 months <7.7 mMol/Mol Creatinine, 2–10 years <3.7 mMol/Mol Creatinine, >10 years <1.1 mMol/Mol Creatinine.

** All pathogenic variants were in trans, as confirmed by family studies.

Table 3
Description and in silico analysis of novel GAA variants identified in this study.

Location	Variant (coding nomenclature)	Variant (protein nomenclature)	Type of variant (protein)	Predicted severity	CRIM status	Predictions of pathogenicity	Missense prediction (Align GVGD)	Missense prediction (SIFT)	Missense prediction (Mutation Taster)
Intron 3	c.(692 + 1_693–1)_(1194 + 1_1195–1)del	p. Leu232Thrfs*41	Deletion (frameshift)	pathogenic	Negative	no effect on splicing - causes an out of frame product			
Exon 5	c.701C > T	p.Thr234Met	Substitution (missense)	VUS	Positive	no effect on splicing	Class C0 (GV: 114.06 - GD: 38.84)	Deleterious (score: 0.02)	Deleterious (prob: 82|18 (del | benign))
Exon 5	c.726G > A	p.=	Substitution (silent)	VUS	Unknown	Loss of cryptic splice acceptor and gain of new cryptic splice acceptor site			
Exon 6	c.886C > A	p.Pro296Thr	Substitution (missense)	VUS	Positive	no effect on splicing	Class C35 (GV: 0.00 - GD: 37.56)	Deleterious (score: 0)	Deleterious (prob: 64|36 (del | benign))
Exon 8	c.1154G > A	p.Arg385His	Substitution (missense)	VUS	Positive	no effect on splicing	Class C0 (GV: 101.88 - GD: 15.20)	Tolerated (score: 0.07)	Deleterious (prob: 61|39 (del | benign))
Exon 9	c.2110G > A	p.Ala704Thr	Substitution (missense)	VUS	Positive	no effect on splicing	Class C0 (GV: 165.34 - GD: 0.00)	Tolerated (score: 1)	Benign (prob: 15|85 (del | benign))
Exon 10	c.2461G > A	p.Gly821Arg	Substitution (missense)	VUS	Unknown	Gain of new cryptic splice acceptor site and strengthens a cryptic splice donor site	Class C0 (GV: 242.52 - GD: 0.00)	Tolerated (score: 0.17)	Benign (prob: 36|64 (del | benign))

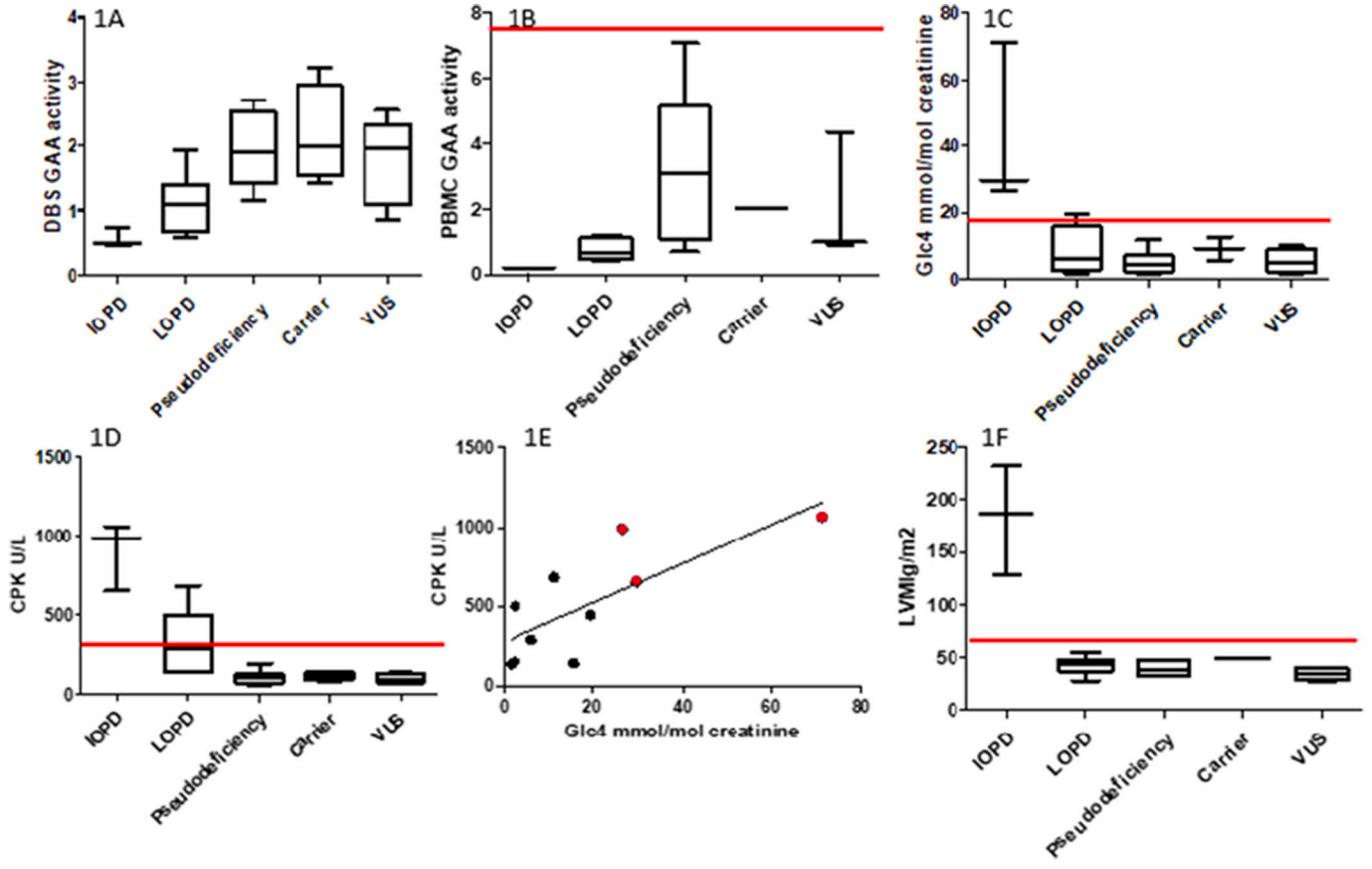

Fig. 1. Baseline distribution of GAA activity in DBS (uM/h) (**1a**), GAA activity in PBMC (nv >6.8 nmol/L/mg protein) (**1b**), Glc4 (nv < 16.3 mmol/mol creatinine) (**1c**), CPK (U/L) (**1d**), LVMI (nv < 65 g/m^2) (**1f**) in IOPD, LOPD, pseudodeficiency, carrier and VUS carrying neonates. Correlation among Glc4 and CPK at first visit in IOPD (red) and LOPD patients (black) (**1e**). Horizontal red lines indicate the cutoffs (not possible in Fig. 1a, because the cutoff of DBS GAA activity had seasonal variations).

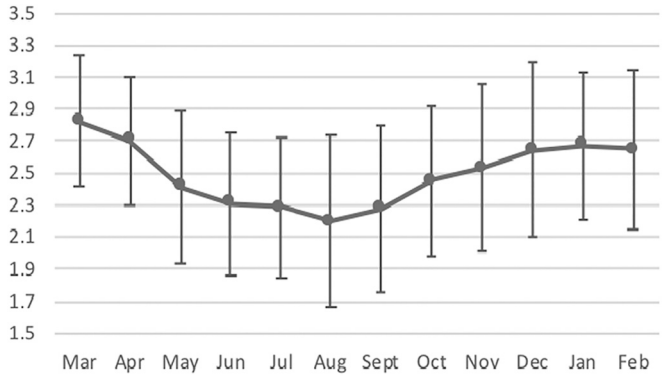

Fig. 2. Seasonal variations of 0.2 multiple of median DBS GAA activity (mean ± SD).

analysis is required to identify pseudodeficiency and carrier newborns.

3) Glc4 values: there was a statistically significant difference between IOPD and LOPD newborns ($p = 0.008$), without overlap of the values, so it effectively and rapidly identifies IOPD patients. No IOPD patients had normal values. All LOPD cases had normal values, except for one newborn who had a borderline value (19.5 mmol/mol creatinine, nv <16.3) (Fig. 1c).
4) CPK values: there was a statistically significant difference between IOPD and LOPD newborns ($p = 0.005$), although with overlapping of values between the groups. No IOPD patients had normal CPK at birth. Interestingly, among LOPD cases, IVS1 homozygous individuals had normal CPK values, while compound heterozygotes had elevated CPK at birth (Fig. 1d). Other markers of muscle necrosis (AST, ALT, LDH) had a similar trend. There was a direct correlation between urine Glc4 and CPK values (r = +0.59) (Fig. 1e).
5) LVMI: hypertrophic cardiomyopathy was present only in IOPD patients and effectively identifies them (Fig. 1f).

3.3. Follow-up of IOPD patients

Three IOPD patients were referred to the Clinical Unit between day 3 and day 14 of life. Two males, CRIM-negative (pt.17 and pt.25, of African origin), had a prenatal diagnosis of hypertrophic cardiomyopathy, while a female, CRIM-positive (pt.12, European), was apparently asymptomatic. At confirmatory tests, all showed increased levels of muscle necrosis markers (CPK, AST, ALT, LDH) and Glc4, short PR interval at ECG, increased LVMI and a molecular analysis suggestive of IOPD. Pt.17 also presented with heart failure (ejection fraction 20%) at birth and needed invasive ventilation and circulatory support. Follow-up data of IOPD patients are summarized in Table 4a and Fig. 3(a-c).

3.3.1. Treatment evaluation

All patients started ERT (alglucosidase alfa, Genzyme Corp., Cambridge, MA) between day 5 and day 19 of life. CRIM-negative patients received a dosage of 40 mg/kg weekly, simultaneously with an ITI protocol (methotrexate, rituximab, IV immunoglobulins [46]), the CRIM-positive patient was treated with an initial dosage of 40 mg/kg every other week without ITI, based on the best evidence at that time. The dosage was then increased to 40 mg/kg weekly when she was 3-year-old, based on recent evidence [11,47].

All patients showed an early response to ERT. Pt.17 developed anti-rhGAA antibodies after 6 months (max. Titer 1:102,400), associated with clinical and biochemical worsening. He was immunomodulated multiple times, with methotrexate, rituximab, IV immunoglobulins, bortezomib and sirolimus. The antibody titer decreased to 1:6400, but clinical benefit remained partial; therefore, at age 2.5 years he started therapy with cipaglucosidase alfa/miglustat (Amicus Therapeutics). Cipaglucosidase alfa is a novel rhGAA, enriched with cellularly derived bis-phosphorylated N-glycans to improve cellular uptake that was administered with miglustat, a pharmacological chaperone that stabilizes the GAA enzyme [48]. A clinical trial in adult patients was ongoing, but the drug had not been approved yet; therefore, it was provided under compassionate use.

- Cardiac status: LVMI of pt.12 and pt.25 normalized after 9 and 2.5 months of ERT, respectively. LVMI of patient 17 also decreased, despite slower compared with other cases (until a minimum of 128 g/m^2 at 3 months of life). Unfortunately, after 6 months of life, pt.17 progressively worsened (LVMI up to 236 g/m2) due to the development of anti-rhGAA antibody. He improved partially after ITI cycles (119 g/m2, with an EF of 48%) and further after the change of ERT (after 1 month: LVMI 72 g/m2, EF 67%); he was stable at the last visit (age 3.5 years), with LVMI 98 g/m2 and EF 66%.
- Motor status: Pt.12 and pt.25 presented with hypotonia and psychomotor delay that progressively improved to an age-appropriate motor development starting at 1 year of life. They never needed feeding or respiratory assistance. Pt.17 initially presented with severe hypotonia and needed feeding (nasogastric tube) and respiratory assistance. In the first month of ERT he improved and was able to eat and breathe independently. His course was complicated by anti-rhGAA antibodies, but after ITI cycles and a change of ERT, his motor status improved; he has been able to walk independently from the age of 2.5 years, although a developmental delay persists.
- Biomarkers: In all our patients, Glc4 normalized after 1 month of therapy, and CPK after 2.5–4 months. In pt.17, biomarkers had progressively increased since the age of 6 months due to the development of anti-rhGAA antibodies (CPK up to 6795 U/L, Glc4 up to 50 mmol/mol creatinine). He improved somewhat after several cycles of ITI (CPK 4728 U/L, Glc4 36 mmol/mol creatinine) and further after switching ERT (after 1 month CPK 2989 U/L, Glc4 32.6 mmol/mol creatinine), and was stable at the last visit (age 3.5 years), with CPK 3115 U/L and Glc4 30.4 mmol/mol creatinine.

Overall outcomes at the last visit: To date, all IOPD patients are alive and in active follow-up (mean age 2.8 years). Pt.12 and pt.25 (3.5 and 1.5-years-old, respectively) have age-appropriate motor development with no signs of cardiomyopathy and normal biochemical testing, including CPK and Glc4. They continue ERT and have not developed anti-rhGAA antibodies or experienced adverse events. Pt.17, at age 3.5 years, presents delayed psychomotor development, but he walks unsupported, and has stable biomarkers and cardiac parameters. He has normal hearing, and does not need respiratory or feeding assistance. Of note, he has cognitive impairment with absence of language and relational difficulties. Brain MRI shows widespread bilateral and symmetrical hyperintensities of centra semiovale white matter. Of note, a previous brain MRI, performed at 1.5 years, showed normal myelinization for age.

3.4. Follow-up of LOPD patients

3.4.1. Demographic and genotype:

We diagnosed 8 newborns with LOPD, 2 of which of Moroccan origin and 6 Europeans (2 were twins). None had a known family history of PD. Five (all European) were homozygous for the common splicing mutation IVS1, 3 were compound heterozygotes for an IVS1 variant and a second variant. An Italian female (pt.22) carried c.1933G > A (p.Asp645Asn) as a second variant, while 2 unrelated newborns (pt.4 and pt.5), both of Moroccan origin, carried the severe mutation c.236_246del (p.Pro79Argfs*13). None presented the modifier variant c.510C > T. All, except 1 (pt.11), are in follow-up after a mean of 3.4 years (range 2–5.5 years). Their detailed follow-up data are reported in Table 4b and in Fig. 3(d-e).

Table 4
Follow up data of infants with IOPD (**4a**), LOPD (**4b**) and VUS (**4c**).

4a: IOPD patients

Patient	Sex	Disease-associated variants (coding nomenclature)	Disease-associated variants (protein nomenclature)	CRIM status	Current age (years)	LVMI g/m2	EF %	CPK U/L (0–228)	Glc4 mmol/mol creatinine	GMFM-88%	MFM-20%	walking	ventilator status	Hearing	feeding status	Brain MRI	ERT dosage	ITI protocol	antibody titer (max/latest)	Cognitive function
12	F	c.[1933G > A]; [2237G > A] c.[2560C > T];	p.[Asp645Asn]; [Trp746*]	+	3.5	57	62	179	3.1 (nv < 3.7)	85.59	96.6	indipendently	no	normal	normal	N/A	alglucosidase 40 mg/kg/week	No	negative	normal
17	M	[(692 + 1_693–1)_ (1194 + 1_1195–1)del]	p.[Arg854*]; [Leu232Thrfs*41]	–	3.5	98	66	3115	30.4 (nv < 3.7)	69	63	wait based gait	no	normal	normal	widespread demyelinization	cipaglucosidase 30 mg/kg/ weekmiglustat	Yes (prophylactic and therapeutic)	1:102,400/ 1:6400	developmental delay
25	M	c.[236_246del]; [236_246del]	p. [Pro79Argfs*13]; [Pro79Argfs*13]	–	1.5	65	66	117	7.4 (nv < 7.7)	80.6	81.6	wait based gait	no	normal	normal	normal	alglucosidase 40 mg/kg/week	Yes (prophylactic)	negative	normal

4b: LOPD patients

Patient	Sex	Disease-associated variants (coding nomenclature)	Disease-associated variants (protein nomenclature)	Current age (years)	ECG; Echocardiogram	CPK U/L (0–228)	Glc4 mmol/mol creatinine	GMFM-88%	MRC scale	walking	ventilator status	feeding status	Psycomotor development	ERT
4	F	c.[−32-13 T > G]; [236_246del]	p.[=,0]; [Pro79Argfs*13]	5.5	normal	273	3,3 (nv < 3,7)	99.44	5/5 for all muscles	independently	no	normal	regular	no
5	M	c.[−32-13 T > G]; [236_246del]	p.[=,0]; [Pro79Argfs*13]	5	normal	704	7,4 (nv < 3,7)	98.61	5/5 for all muscles	independently	no	normal	regular	no
15	M	c.[−32-13 T > G]; [−32-13 T > G]	p.[=,0]; [=,0]	3	normal	251	N/A	76.21	/	independently	no	normal	delay	no
16	M	c.[−32-13 T > G]; [−32-13 T > G]	p.[=,0]; [=,0]	3	normal	197	N/A	81.42	/	independently	no	normal	delay	no
18	M	c.[−32-13 T > G]; [−32-13 T > G]	p.[=,0]; [=,0]	3	normal	208	1 (nv < 3,7)	92.51	/	independently	no	normal	regular	no
21	M	c.[−32-13 T > G]; [−32-13 T > G]	p.[=,0]; [=,0]	2.5	N/A	107	4.7 (nv < 3,7)	78.75	/	independently	no	normal	regular	no
22	F	c.[−32-13 T > G]; [1933G > A]	p.[=,0]; [Asp645Asn]	2	normal	138	2.1 (nv < 7,7)	82.95	/	independently	no	normal	regular	no

4C: Newborns carrying VUS

Patient	Sex	Disease-associated variants (coding nomenclature)	Disease-associated variants (protein nomenclature)	Current age (years)	ECG; Echocardiogram	CPK U/L (0–228)	Glc4 mmol/mol creatinine	GMFM-88%	walking	ventilator status	feeding status	Psycomotor development	ERT
23	F	c.[−32-13 T > G]; [701C > T]	p.[=,0]; [Thr234Met]	2	normal	106	1.8 (<3.7)	77.42	independently	no	normal	regular	no
27	F	c.[−32-13 T > G]; [726G > A]	p.[=,0]; [Ala242=]	1.5	normal	114	3.1 (<7.7)	88	wait based gait	no	normal	regular	no
33	F	c.[1048G > A]; [2051C > T]	p.[Val350Met]; [Pro684Leu]	0.5	normal	141	3.7 (<7.7)	31.32	no	no	normal	regular	no
34	M	c.[−32-13 T > G]; [726G > A]	p.[=,0]; [Ala242=]	0.5	normal	91	1.9 (<7.7)	20.4	no	no	normal	regular	no

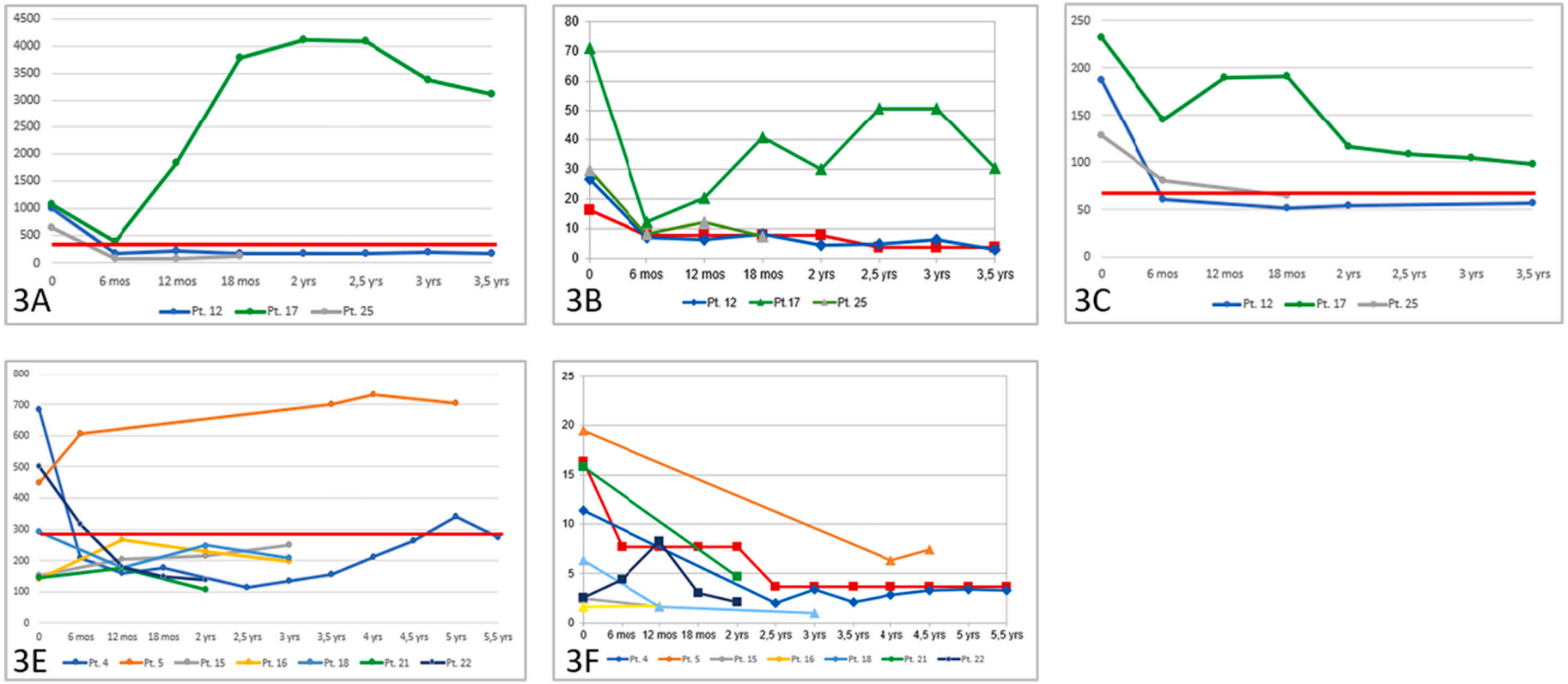

Fig. 3. Trends in serum CPK (U/L) (**3a**), Glc4 (mmol/mol creatinine) **(3b**) and LVM I(g/m^2) (**3c**) during follow up of 3 IOPD patients. Trend in serum CPK (U/L) (**3d**) and Glc4 (mmol/mol creatinine) (**3e**) in LOPD patients. Red lines: normal values.

3.4.2. Clinical course

- Cardiac status: At birth, all IVS1 homozygous newborn and pt.22 (IVS1 + c.1933G > A) had normal cardiologic assessments. Of note, pt.4 and pt.5 (IVS1+ c.236_246del) had a short PR interval at ECG (0.08 s and 0.06 s, respectively), but had good heart function and no hypertrophy. The PR interval normalized at subsequent visits; however, this demonstrates that a short PR interval cannot be used as a parameter for identifying the infantile form at birth. At the last visit, all cases presented normal cardiologic assessments (ECG + echocardiogram).
- Skeletal muscle assessment: All were asymptomatic from birth to the last visit. All presented normal psychomotor development with normal for age motor scale results and, where applicable, muscle strength (MRC scale), 6 min walking test and spirometry. None presented with speech or swallowing disorders. Of note, a muscle biopsy was taken from 2 patients compound heterozygous for the IVS1 variant and the severe c.236_246del variant, at the age of 6 and 18 months respectively, to better characterize their disease status, and showed lysosomal activation and glycogen storage, although the patients were asymptomatic.
- Biomarkers: At birth, all had normal Glc4 (except for a borderline value in pt.5, 19.5 mmol/mol creatinine, nv <6.3). Interestingly, CPK at birth was normal in all IVS homozygous, and elevated in all other LOPD newborns ($p = 0.043$). IVS1 homozygous individuals and pt.22 (IVS1 + c.1933G > A) had normal biomarkers (CPK, Glc4) at the last visit. Pt.4 and pt.5 (IVS1 + c.236_246del), presented elevated muscle necrosis enzymes (CPK 273 U/L and 704 U/L, respectively) at the ages of 5.5 and 5 years respectively; at that time, pt.5 also presented elevated Glc4 (7.4 mmol/mol creatinine, nv <3.7).

Overall, during a follow-up period of up to 5.5 years (mean 3.4, range 2–5.5 years), none of the suspected LOPD patients has developed symptoms, and none are receiving ERT.

3.5. Family studies

We found that the mother and two sisters of pt.4 were affected by PD

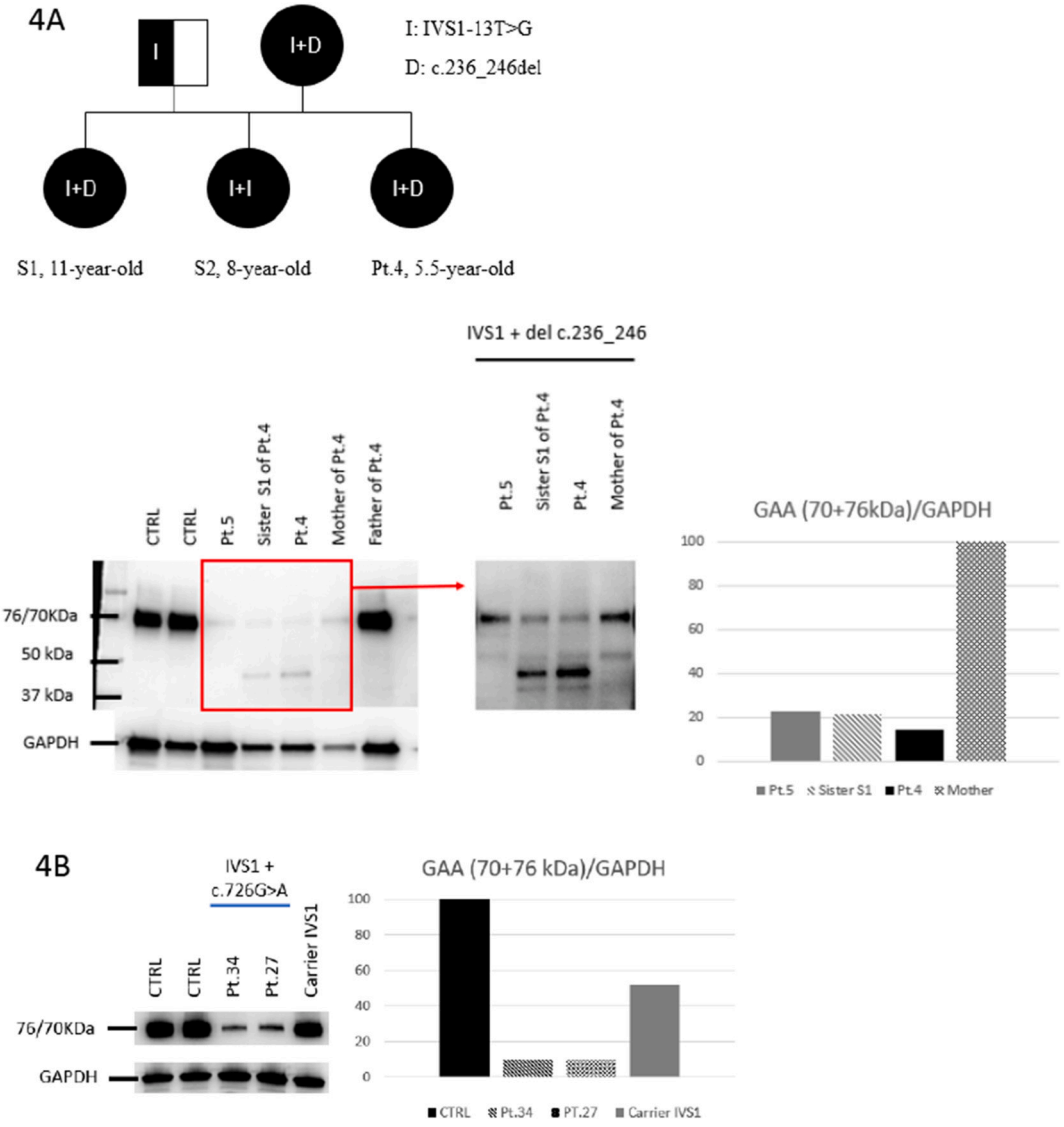

Fig. 4. Western blot analysis of GAA in peripheral mononuclear cells (PBMC) of LOPD patients. **4a:** Family tree of Pt.4 and western blot analysis of GAA in family members. Genotype: Pt.4, Pt.5, Sister S1 of Pt.4, Mother of Pt.4: IVS + del c.236_246; Father of pt.4: heterozygous IVS1; CTRL: wild type. Fig. 4b**:** two patients compound heterozygous for IVS1 + synonymous variant c.726G > A (p.Ala242=). GAPDH (glyceraldehyde-3-phosphate dehydrogenase) is used as a loading control; 76 and 70 KDa bands represent the mature lysosomal forms of the GAA. Quantitative analysis of band intensity was performed using ImageLab (see supplementary materials).

(family tree, Fig. 4A).

- The 33-year-old mother (IVS1 + c.236_246del) was asymptomatic and had normal muscle necrosis enzymes (CPK 242 U/L) and borderline Glc4 (1.8, nv < 1.1).
- The father carried a single IVS1 variant.
- An older sister (S1) (IVS1 + c.236_246del) had pathological muscle biopsy results at age 6 years (myopathic signs, prevalence of type 1 fibers and marked lysosomal activation). At age 11 years, she presented with limb girdle weakness, difficulty in running and climbing stairs, easy fatigability with dyspnea, mild scoliosis, accentuation of the physiological curves of the spine and decreased strength in shoulder girdle muscles with scapular winging. She had elevated biomarkers (CPK 551 U/L, Glc4 3.1-nv <1.1) and myopathic girdle involvement by electromyography (EMG). 6 min walking test was borderline for distance (420 m), with subjective dyspnea. Because of the clinical symptoms, she started treatment with alglucosidase (20 mg/kg every other week).
- Another older sister (S2) age 8 years was homozygous for the IVS1 mutation. At the last visit she had normal biomarkers (CPK, Glc4), but developmental delay due to comorbid Cornelia de Lange syndrome.

The different clinical and biochemical pictures between the mother and two daughters (pt.4 and 11-year-old sister S1) despite them having the same genotype (IVS1 + c.236_246del) and lacking the known modifier gene c.510C > T, prompted Western Blot analysis of GAA. We noted that the mother had a higher amount of mature 76/70 kDa protein than the daughters. Moreover, the two daughters had a protein of about 45 kDa, which might be explained by an erroneous splicing and/or protein degradation (Fig. 4a). However, further studies are needed.

3.6. Variants of uncertain significance

Six newborns, all Caucasian, were found to be carriers of at least one VUS. All were asymptomatic with normal biochemical and cardiologic tests at birth, as well as at the last follow-up visit (mean age 1.13 years, $n = 4$, two lost to follow-up) (Table 4c). Two newborns (pt.27 and pt.34) were compound heterozygous for the IVS1 mutation and the synonymous mutation c.726G > A (p.Ala242=). In silico studies suggests that the latter variant creates an abnormal splice site in exon 5 that could lead to an abnormal transcript. Western blot analysis of GAA protein revealed the amount of mature protein (76/70 kDa) in these infants to be reduced compared to carriers, and similar to PD patients (Fig. 4b). However, further studies are needed.

4. Discussion

4.1. Epidemiology

In the last 7 years, we have screened 206,741 newborns for PD, which is the largest study reported to date in Europe. The overall incidence of PD from our NBS data was 1 in 18,795 (IOPD 1 in 68,914; LOPD 1 in 25,843). The reported clinical prevalence of PD worldwide is 1 in 40,000 [49], whilst a previous Italian study estimated an incidence of 1:120,743 [50]. The difference with our results could be explained by the recent immigration from Africa (4/11 patients), where there is a higher incidence of consanguinity (2/4 our patients, pt.4 and pt.25), but also by the identification of a high number of LOPD patients. The frequency identified in our study is similar to those detected in other screening programs worldwide (Supplementary Table 1), e.g., Taiwan 1:18,436 [51], Illinois 1:23,596 [9,52], Pennsylvania 1:16,095 [30], New York 1:20,190 [53]. Differences appear among different programs and can be explained by the predominant ethnic background, differences in screening assays and the chosen cut-off value, even using the same technologies, the presence of pseudodeficiencies and even the rarity of the disorder itself. For example, the Taiwanese cohort is unique, in that almost all the IOPD patients are CRIM positive (due to the high frequency of the p.Asp645Glu mutation), LOPD cases lack the c-32-13 T > G (IVS1) variant, common in Caucasian population, and there is a high frequency of the pseudodeficiency allele [c.1726G > A; 2065G > A] [54,55]. Thus, the broad Taiwanese experience, although helpful, does not fully address the issue in other parts of the world.

4.2. Phenotype identification

Timely determination of the phenotype is important because prognosis and treatment options are different for IOPD and LOPD. To date, no GAA enzyme assay has been described that can differentiate IOPD vs LOPD using blood samples [56,57]. Of note, the analytical range of the mass spectrometry method is higher than that of the fluorometric assay, allowing more accurate enzyme activity measurements at very low values. Several studies report that this allows for better differentiation between patients with pathogenic mutations, pseudodeficiency alleles and/or benign variants, but our study demonstrated that a high false positive rate persists, and it cannot be eliminated by adjusting the cut-off, because of overlapping values. Studies on fibroblasts or molecular analysis can be useful [58], but they require time and molecular analysis may be difficult to interpret when new variants are identified.

Clinical manifestations (increased LVMI) and Glc4 can help to rapidly and effectively distinguish between patients with IOPD, who should start ERT early, from other patients with low GAA activity (LOPD, pseudodeficiency, carriers) [8]. In our experience all IOPD patients and none of the LOPD patients had abnormal Glc4 and LVMI. It is reported that CPK may be increased at diagnosis also in LOPD patients [23]. In our experience CPK values, although significantly different between IOPD and LOPD newborns, present overlap that does not allow discrimination between phenotypes. Interestingly, CPK was normal at birth only in LOPD patients homozygous for the IVS1 variant.

4.3. IOPD patients

Despite significant cardiac involvement in all our IOPD patients, none were diagnosed clinically. IOPD patients are known to benefit most from early diagnosis and early treatment [59]. The Taiwanese NBS program demonstrated that patients identified through NBS who started ERT early (mean age 11.92 days; range 6–23) had better biological, physical, and developmental outcomes and lower anti-rhGAA antibodies after 2 years of treatment, compared with a group that began ERT just 10 days later [60]. Moreover, Li et al. reported that, among 20 CRIM negative patients, early treated infants (< 4 weeks) showed significant improvements in overall clinical outcomes and biomarkers, compared to those treated later [61]. ERT, possibly associated with an ITI protocol, should be started as soon as possible after the diagnosis is confirmed and CRIM status is established by western blot assay on PBMCs or predicted from genetic analysis [14]. Therefore, to avoid diagnostic and therapeutic delays, NBS programs should establish a follow-up guideline that includes contact information for clinical experts and laboratories providing the necessary services in an accessible and timely fashion.

Of note, it seems that treatment outcomes are influenced more by initial health status and condition rather than chronological age alone [62]. Our experience confirmed this: pt.17 started ERT at 5 days of life, but had a poor clinical condition. His outcome was worse than the other 2 IOPD patients (pt.12 and pt.25), who had started ERT at 12 and 19 days of life, respectively, and have achieved normal cardiac and motor function since 1 year of age. Pt.25, CRIM negative and treated early with ITI + ERT, had optimal cardiac and motor outcomes, similar to that of pt.12, CRIM positive, as reported by Li et al. [61]

The experience from Japan also suggests that the early initiation of ERT, before immune system maturation, reduces the likelihood of anti-rhGAA antibody production [60,63]. In our experience, pt.17 was treated at 5 days of life and developed high titer anti-ERT antibodies

with clinical and biochemical worsening, while the other 2 patients who started ERT later have persistently negative antibody titers at 1.5 and 3.5 years of age. Many factors could explain this difference. Among them, we should also consider that pt.17 had a higher disease burden at baseline which, in the context of Matzinger's "danger model", could produce alarm signals from injured tissues that promote affinity-maturation of IgG antibodies to the ERT [64]. Therefore, it is important to monitor anti-ERT IgG titers routinely in all patients [14], and all specialized centers should have guidelines with information about laboratories that can perform this assay.

Our patients are all treated with a dosage of 40 mg/kg/week, following the most recent literature [11,65–67], and their outcomes overall are good. At the most recent evaluation, at a mean age of 2.7 years (range 1.5–3.5 years), all were alive, ambulatory, and had normal respiratory and feeding functions. Of note, pt.17 presented cognitive impairment and white matter abnormalities at brain magnetic resonance. Before the availability of ERT, cognitive problems were not apparent because most affected infants died early. Our experience also confirms reports of a new phenotype (neurological symptoms and arrhythmias) that has emerged among patients with IOPD as a result of increased long-term survival with ERT [4,55,68–72].

4.4. LOPD patients

Recently, several NBS studies reported a previously unrecognized early biochemical and clinical phenotype of LOPD patients and suggested the need for early treatment in some of these infants (prevalence up to 20% after a follow-up of 15 years) [22,23]. In particular, Huggins et al. revealed early biochemical and kinematic abnormalities during the first 2 years of life among 20 LOPD patients identified through NBS [23]. Lee et al. reported treating pediatric LOPD patients identified through NBS when persistent elevation of CK suggested myocyte injury [22]. Data from long-term follow-up are needed.

The poor genotype-phenotype correlation, even within families, presents a major challenge for predicting phenotypes. All our patients carried the c-32-13 T > G variant (i.e., IVS1), which is the most frequent in the Caucasian population (90% of patients of European descent) [73]. The IVS1 variant causes aberrant splicing of *GAA* exon 2, resulting in at least 8 distinct aberrant splice products [74,75], and functional GAA protein expression that is 10–15% that of healthy controls; therefore, the expected 20%–30% residual enzyme activity in homozygous patients should predict that the majority of patients will remain asymptomatic [73]. However, both IVS1 homozygotes and compound heterozygotes display phenotypic variation in the age of symptom onset from early childhood to late adulthood [3,73], also within families [76]. We found 3 members of a family (pt.4, her sister S1 and mother) with the same genotype (IVS1 + del 236_246), but very different clinical and biochemical outcomes and different mature GAA amount on western blot. This finding has led to the hypothesis that genetic, epigenetic, or environmental modifying factors, may be involved.

None of our patients carried the genetic modifier c.510C > T (p.=), a silent variant that reduces levels of leaky wild-type splicing and leads to early disease onset if present in cis to IVS1 [3,73], suggesting the existence of additional modifying factors, including other putative genetic modifiers.

Personalizing the management of LOPD patients comprises the most complex aspect of PD NBS. Guidelines published in 2017 by the Pompe disease NBS Working Group provide a framework for management of confirmed IOPD patients and "symptomatic" LOPD patients identified through NBS, yet there is no consensus on the definition of "symptomatic" LOPD [55]. However, these guidelines should be revised based on new data available on LOPD cases identified through NBS programs. In particular, the best management of presymptomatic LOPD patients is not clear.

A multidisciplinary approach that includes a pediatric neurological assessment is recommended, to allow for early identification of typical and subtle features [77]. Because muscle weakness may not be evident on routine physical exams, developmental progress should be monitored using a variety of tools [55]. However, there are no specific recommendations for utilization of these tests to determine whether a patient is symptomatic from a musculoskeletal perspective. Although not routinely recommended, muscle biopsy in two of our patients revealed glycogen storage already at age 6 and 18 months, but no symptoms after >5 years of follow-up. It can be hypothesized that either glycogen storage must exceed a threshold before causing muscle weakness or the accumulation alone does not cause muscle weakness. Pulmonary and feeding status should be monitored because they may be affected early [23].

Biomarkers (CPK, Glc4) should be monitored because an increase may precede the onset of PD symptoms [23,78], but the results should be interpreted carefully within the clinical context of each patient. CPK may be elevated for unrelated reasons, (e.g., illness, physical activity), while Glc4 is a more sensitive and specific PD biomarker. In our case series, Glc4 was not elevated at birth in suspected LOPD patients, except marginally in pt.5, who had a progressive increase of Glc4 levels over time. He also had hyperCPKemia and skeletal muscle involvement confirmed by muscle biopsy. These findings might be explained by the severity of the p.Pro79Argfs13* pathogenic variant (in compound heterozygosity with IVS1 variant) or by the presence of unknown modifier variants. Treatment decision for ERT should be based on biomarker value trends, rather than single data points, and on clinical evaluation. Current guidelines recommend that biomarkers be measured every 3 months in the first year of life and every 3–12 months thereafter, to monitor trends.

Cardiac evaluations are recommended every 3 months through the first year of life and then every 3–12 months as clinically warranted [55]. Although there are isolated reports of patients with LOPD and hypertrophic cardiomyopathy [79–81], in our experience, in agreement with recent studies [23], LOPD patients, especially carrying the IVS1 variant [2], do not present cardiac involvement in early infancy. We suggest an electrocardiogram (ECG) and echocardiogram in LOPD patients every 3–6 months in the first year, then a follow-up ECG every 6–12 months because of the risk of arrhythmias. We recommend echocardiogram in infancy only if clinically warranted, while, in adults, it is recommended every 1–2 years due to the risk of aortic dilatation (the earliest reported aortic dilatation occurred at age 28 years [2,82]).

Although there is no consensus yet on whether to initiate ERT, and when, it has been associated with better outcomes when started in the youngest, non-ventilated patients [21,83]; therefore, early diagnosis from NBS and careful follow-up may be important.

Among our LOPD patients diagnosed by NBS, at last visit (mean age 3.4 years, range 2–5.5 years) none had clinical signs or symptoms (except for an increase of biomarkers in pt.5), all had age-appropriate development and none was receiving ERT. We have also diagnosed several families with multiple cases, especially in siblings born before the start of the NBS program. The 11-year-old sister of pt.4 developed clinical and biochemical abnormalities and needed to start ERT. Her symptoms were very subtle and could have been misdiagnosed if she had not been identified because of her sibling and carefully followed-up. This case reinforces the finding that patients with LOPD may develop symptoms and require ERT in childhood. In this family, the mother had the same genotype as her daughters and was asymptomatic, further demonstrating the clinical variability and confirming the poor correlation between genotype-phenotype, especially in LOPD forms [84,85].

4.5. Limitations

Our study presents several limitations and challenges:

- a high number of false positives, especially due to known pseudodeficiency or predicted nonpathogenic variants (15/39 newborns), which impact families and the health care system. This is a common limitation in PD NBS programs, and in most of them pseudodeficiency is

detected more often than true deficiency, especially in Asian populations [26,54,86–89], but also in USA (e.g., in Illinois [52]). Proposals to reduce false positive rates have included biochemical assays (neutral α-glucosidase NAG/GAA ratio and percentage of acarbose inhibition by fluorometry [24,54,90]; creatine/creatinine over GAA ratio by MS/MS [45]), molecular second-tier tests [30,53,91], and postanalytical tools (e.g. CLIR, https://clir.mayo.edu) [31,53,92]. In the future, their wider diffusion could improve false positive rates.

- ethical issues due to the high incidence of suspected LOPD. For every IOPD case, we also identified about 3 suspected LOPD cases. The advantages of early LOPD diagnosis have already been discussed. However, disease onset is unpredictable and some patients may never develop PD symptoms ("patients in waiting") [93], resulting in unnecessary anxiety and medical intervention, but also the possibility that these children will be treated as "vulnerable children" [94]. Challenges remain particularly in identification of better biomarkers for phenotype prediction and the best tailored strategy for follow-up, management and treatment of these patients.

- VUS: NBS for PD resulted in the identification of an increasing number of VUS [25,33,38,87,95,96]. As discussed above, some of these patients may never develop symptoms of PD; however, they require ongoing monitoring [30], which causes anxiety to families and costs for health care systems [97]. Future improvement in the management of these patients is likely to come from a better understanding of the genotype/phenotype correlation and pathogenicity of VUS based on long-term follow-up, the development of a global registry and variant database [98,99], as well as from the identification of new biomarkers.

Notwithstanding these concerns, studies conducted in patients with PD, their parents, or healthcare providers show high support for PD NBS [97,100–105].

As highlighted by Bodamer et al., despite these challenges and potential drawbacks, in diseases with early-onset forms like PD, for which the earliest possible diagnosis and treatment can make a difference between survival with positive outcomes and severe disability or death, the lives of these children outweigh any negative aspects [27].

5. Conclusions

Our study, the largest reported to date in Europe, demonstrates that PD NBS is feasible and readily extendable to the larger Italian newborn populations. The primary goal of NBS is identifying patients who can benefit from early treatment. Early detection and ERT are certainly beneficial for IOPD patients, but also for less severe LOPD patients.

Secondary goals for NBS include shortening the diagnostic odyssey, avoiding misdiagnosis or unwarranted invasive diagnostic tests such as muscle biopsy, identifying carriers or affected relatives, providing information on reproductive options and, for LOPD patients, allowing informed choices later in life. Moreover, NBS provides important information on the prevalence of PD and the prevalent genotypes. It also increases our knowledge about the natural history of the disease.

Remaining challenges include reducing the number of false positives and determining the best management of infants with suspected LOPD and VUS. Long-term follow-up of these patients and sharing data in international database (e.g., Pompe Registry, sponsored by Sanofi Genzyme, http://www.registrynxt.com) are providing valuable information on genotype-phenotype correlations, the natural history of LOPD, its early clinical and biochemical manifestations, and the impact of early treatment, all of which are paving the way to optimized management of these individuals.

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Data availability statement

Data available on request due to privacy/ethical restrictions.

Declaration of Competing Interest

The authors declare no conflict of interest.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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