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Article

Light and Shadows in Newborn Screening for Lysosomal Storage Disorders: Eight Years of Experience in Northeast Italy

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Abstract: In the last two decades, the development of high-throughput diagnostic methods and availability of effective treatments has increased interest in newborn screening for lysosomal storage disorders. However, long-term follow-up experience is needed to clearly identify risks, benefits and challenges. We report our 8-year experience of screening and follow up on about 250,000 neonates screened for four lysosomal storage diseases (Pompe disease, mucopolysaccharidosis type I, Fabry disease, Gaucher disease), using enzyme activity assay by tandem mass spectrometry, and biomarker quantification as second tier test. Among the 126 positive newborns (0.051%), 51 infants were confirmed as affected (positive predictive value 40%), with an overall incidence of 1:4,874. Of these, 3 infantile-onset Pompe disease, 2 neonatal-onset Gaucher disease and 4 mucopolysaccharidosis type I patients were immediately treated. Furthermore, another four Gaucher disease patients needed treatment in the first years of life. Our study demonstrated the feasibility and effectiveness of newborn screening for lysosomal storage diseases. Early diagnosis and treatment allow the achievement of better patient outcomes. Challenges such as false positive rates, the diagnosis of variants of uncertain significance or late-onset forms, and the lack of treatment for neuronopathic forms, should be addressed.

Keywords: newborn screening; lysosomal storage disease; Pompe disease; Mucopolysaccharidosis type I; Gaucher disease; Fabry disease; second tier test; tandem mass spectrometry; glycosaminoglycans; lysosphingosine

1. Introduction

Lysosomal storage disorders (LSDs) are inborn errors of metabolism that include about 60 different inherited diseases [1]. They are caused by the deficiency or absence of specific lysosomal enzyme or transporter activities, resulting in the accumulation of uncatabolized macromolecular substrates within lysosomes [2–4]. LSDs can present at any age with multisystemic involvement and progressive courses. Enzyme analysis in leukocytes/lymphocytes, biochemical assays demonstrating

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accumulation, and gene analysis can confirm the diagnosis [5,6]. The treatment (enzyme replacement therapy ERT, hematopoietic stem cell transplantation HSCT, chaperones, and gene therapy) can significantly improve outcomes, but the progressive nature of these diseases makes early diagnosis essential [7].

Unfortunately, diagnosis is often delayed because of non-specific symptoms that frequently arise when organ damage is already irreversibly severe [8–13]. This has motivated the development of newborn screening (NBS) programs in the last two decades that use multiplex analytical techniques [5,14,15], such as fluorometry coupled with digital microfluidics (DMF) and electrospray ionization tandem mass spectrometry (MS/MS), for the simultaneous quantification of several lysosomal enzyme activities [16].

In Europe, the first pilot NBS project for LSD was performed in the Piemonte Region in Northwest Italy. Between 2003 and 2005, 37,104 male newborns were screened for Fabry disease (FD) using a fluorometric assay. Twelve positive neonates were identified and confirmed with a specific *GLA* variant (1:3,092 males). Only one patient carried a variant associated with the classic phenotype, demonstrating a high incidence of later-onset forms [17]. A similar study, using a fluorometric assay for FD, was performed in 2008 in Galicia (Spain) on 14,600 newborns. Of these, 106 resulted positive and 37 carried a *GLA* variant, indicating a very high overall incidence (1:395). However, only one male patient carried a variant associated with the classic phenotype, 11 (including a female neonate) carried variants of uncertain significance (VUS) and 25 carried polymorphisms [18].

After the development of multiplex high-throughput assays (DMF and MS/MS), these methods were used to screen for multiple LSDs simultaneously on deidentified neonatal dried blood spot (DBS). Pilot studies using MS/MS to assay 4 LSDs (Pompe disease PD, Fabry disease FD, Gaucher disease GD and Niemann Pick A/B disease NPD) were performed in Austria and Hungary on 34,736 and 40,024 anonymous neonatal DBS, respectively. Both found an overall incidence of about 1:2,300 [19,20]. Subsequently, a similar study performed in Belgium analyzed around 20,000 newborn samples for PD, FD and MPSI using an MS/MS assay to establish reference ranges. Reference intervals were found to depend on sex and gestational age (higher enzyme activities in female and premature newborns) [21]. A limitation of these studies is the lack of follow-up data.

A retrospective study conducted in Italy had identified a high incidence of LSDs, which constituted the most frequent class of inborn errors of metabolism (overall incidence 1:8,275) [22]. In 2012, a small pilot program conducted in Umbria region of Italy assessed four lysosomal disorders (PD, GD, FD and MPSI) using a fluorometric assay. One patient with GD was found among 3,403 newborns screened [23].

In 2015, the Regional Health Administration of Northeast Italy expanded its NBS program to include four LSDs: GD, PD, FD, MPSI, using a multiplex high-throughput MS/MS assay. A second-tier test (2TT) of positive DBS (GD, FD, MPSI) was used to reduce the recall rate. Here, we report our 8-year experience with about 250,000 neonates screened, discuss the role of 2TT and briefly describe the patients' outcomes summarizing the "lights and shadows" of our experience.

2. Materials and Methods

2.1. Study population

DBS from 248,616 newborns were consecutively collected from September 2015 to August 2023 at the Regional Center for Expanded Newborn Screening, Padua University Hospital. Informed consent was obtained from parents.

2.2. Determination of enzyme activities

The DBSs were assayed for the enzyme activities deficient in PD, GD, FD and MPSI (α-galactosidase A [α-GalA] for FD, acid α-glucosidase [GAA] for PD, acid β-glucocerebrosidase [GCase] for GD and acid α-L-iduronidase [IDUA] for MPS I) using a flow-injection-MS/MS analysis, as previously reported [24]. The cut-offs (0.2 MoM) were recalculated monthly to avoid an increase

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in false-positives in winter and false-negatives in summer, due to seasonal changes in temperature and humidity during transport.

2.3. Second tier tests

Second tier tests were introduced in 2016 for GD and FD and in 2019 for MPSI. Newborns who had initial screening results below the cutoff for GCase, α-GalA or IDUA activity, were tested for LysoGb1, LysoGb3 and glycosaminoglycans (GAGs), respectively.

DBS lysosphingolipids (LysoGb1, LysoGb3) were measured by LC-MS/MS as previously described [25,26]. GAGs levels were measured by methanolysis followed by LC-MS/MS with a method optimized for DBSs based on that developed by Zhang et al [27].

2.4. Confirmatory testing

Screen-positive newborns were referred to our Clinical Unit for confirmatory testing, including clinical evaluation, enzyme activities in leukocytes/lymphocytes, substrate quantification (plasma LysoGb1 for GD, LysoGb3 for FD, urinary GAGs for MPSI and urinary Glc4 for PD) and mutation analysis.

For PD and MPSI, which can require immediate intervention, results were obtained within 24 hours for urinary Glc4 and GAGs, respectively. Treatment was started immediately in patients with infantile-onset PD (ERT) and Hurler disease (ERT+HSCT). In patients with the neonatal form of GD, treatment was started after ethics discussion. The other patients were followed and treated only when symptoms appeared. Family members were counseled and offered testing and medical evaluations.

3. Results

3.1. Screening outcomes

Of the 248,616 screened newborns (119,512 males, 129,104 females), 126 positive cases were referred to our Clinical Unit for confirmatory testing (0.05%). Screening results and patients with a confirmed diagnoses are reported in Table 2.

Table 2. Newborn screening results and confirmed diagnosis.

LSD	Total screened	Positive NBS	Patients with confirmed disorder	Pseudodeficit	VUS	Carrier Status	Benign variant	No variant	Lost before confirmatory testing
GD	248,616	18	13	0	3	0	0	0	2
PD		48	16	17	7	4	0	0	4
FD		31	18	0	10	/	1	2*	0
MPSI		29	4	21	2	2	0	0	0
TOT		126	51	38	22	6	1	2	6

* Both were females.

3.1.1. Mucopolysaccharidosis type I

For MPSI, 29/248,616 newborns (0.012%) had low IDUA activity in DBS (range 0.1 to 2.2 µmol/L/h). Of these, 4/29 were confirmed as affected due to low IDUA activity in lymphocytes, elevated GAGs in urine, as well as two pathogenic alleles at genetic analysis. The overall MPSI incidence was 1:62,154 births.

During the first 3 years of screening (2015-2018, 112,446 newborns), when we only tested IDUA activity in DBS, the recall rate was 0.024% (27 newborns). 25/27 newborns were false positive on confirmatory testing, presenting normal urinary GAGs and genetic variants known to be pseudodeficency alleles (21 newborns, 18 of African origin), variants of unknown significance VUS (n = 2) or were carriers (n = 2) (false positive rate 0.022%, PPV 7.4%). The residual enzyme activities

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in these newborns (0.10-2.21 nmol/L/h) overlapped that of two true positive MPSI patients (0.12-0.22 µmol/l/h).

Since 2019 (136,170 newborns), GAG analysis in DBS was developed as a 2TT. The recall rate during this period was 0.015% (2 patients) and no false positives were referred to the clinic (predictive value 100%).

The four affected newborns were immediately investigated and treated (Table 3).

Table 3. Clinical and biochemical characteristic of MPSI patients.

	Pt.1 (female)	Pt.2 (female)	Pt.3 (male)	Pt.4 (female)
Diagnosis				
IDUA activity on DBS	0.22 µmol/l/h	0.17 µmol/l/h	0.17 µmol/l/h	0.12 µmol/l/h
GAGs on DBS HS nv <3.2 µg/ml; DS nv <2.7 µg/ml	NA	NA	HS 6.3 µg/ml, DS 33 µg/ml	HS 7.8 µg/ml, DS 41 µg/ml
uGAGs HS nv <4.6 mg/mmol crea; DS nv <38.1 mg/mmol crea	HS 148.9 mg/mmol crea, DS 172 mg/mmol crea	HS 121.9 mg/mmol crea and DS 80 mg/mmol crea	HS 60.9 mg/mmol crea and DS 58 mg/mmol crea	HS 274 mg/mmol crea and DS 287.7 mg/mmol crea
Molecular analysis (DNA)	homozygous c.1598C>G	homozygous p.Tyr201*	c.603C>G + c.-14_10del	homozygous c.46_57del12
Protein	homozygous p.Pro533Arg	homozygous p.Tyr201*	pTyr201* + intronic variant	homozygous p.Tyr201*
Phenotype	Hurler/Scheie	Hurler	Hurler	Hurler
Treatment				
ERT (laronidase 100 U/kg)	30 days	45 days	19 days	9 days
HSCT	no	6 months	6 months	6 months
Follow-up				
Age at last follow up	6.5 years old	6 years old	1.5 years	1 year
Clinical features	diffuse corneal clouding	mild coarse facial features, deformity of lumbar vertebral bodies, diffuse corneal clouding.	Mild deformity of vertebral bodies, diffuse corneal clouding.	deformity of thoracic and lumbar vertebral bodies, diffuse corneal clouding.
Cognitive function	WPPSI III: 93	WPPSI III: 121	Bayley 3: 75	Bayley 3: 100
Chimerism	/	94% donor	86% donor	100% donor
IDUA nv 1.9-15 µmol/l/h	/	6.49 µmol/l/h	3.49 µmol/l/h	12.60 µmol/l/h
uGAGs	HS 9.2 mg/mmol crea, DS 15.4 mg/mmol crea	HS 2.6 mg/mmol crea, DS 5.5 mg/mmol crea	HS 3.9 mg/mol crea, DS 12 mg/mmol crea	HS 8.4 mg/mmol crea, DS 13.2 mg/mmol crea

NA: not available, HS: heparan sulfate, DS: dermatan sulfate, crea: creatinine.

The first patient, a female of African origin (Morocco), had the intermediate Hurler/Scheie phenotype. She began ERT with laronidase (100 U/kg/weekly) one month after birth with good clinical and biochemical responses.

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The other 3 patients (of which pt.2 and pt.4 were siblings), were of Italian origin, and affected by Hurler syndrome. ERT with laronidase (100 U/kg/weekly) was started early. GAGs normalized, although patients developed mild features of MPSI, such as coarse facial features (n = 3), conductive and sensorineural hearing loss (n = 2), corneal clouding (n = 3), mild dilatation of the periventricular spaces on brain MRI (n = 3). Due to severe phenotype, at 6 months of age, they received an allogenic HSCT. All patients achieved high donor chimerism and normal IDUA levels. GAGs remained normal after discontinuation of ERT, 6 months after HSCT. Currently, they have no neurologic involvement.

3.1.2. Pompe disease

For PD, 48/248,616 newborns (0.019%) needed to be clinically evaluated. Two pathogenic variants of the GAA gene were found in 16/48 newborns, of which 3 were infantile-onset (IOPD) and 13 late-onset (LOPD) patients. Findings in 28 other newborns included a VUS (n = 7), a known pseudodeficiency allele or predicted non-pathogenic variant (n = 17), or to be carriers (n = 4). Four newborns were lost to follow-up prior to confirmatory testing due to relocation out of the region. The incidence of PD was 1:15,539 (IOPD 1:82,872, LOPD 1:19,124) and the PPV was 52%.

DBS GAA values in affected and pseudodeficiency/carrier newborns overlapped (0.45-1.94 µmol/l/h and 1.14-3.22 µmol/l/h, respectively). Only the urinary assay of Glc4 and cardiologic assessment allowed rapid identification of IOPD patients. Molecular analysis helped to confirm IOPD and distinguish LOPD patients from pseudodeficiency and carrier newborns.

The 3 IOPD patients were referred between day 3 and day 14 of life. All had increased levels of muscle necrosis markers (CPK 653-1063U/L) and uGlc4 (26.5-71.2 mmol/mol crea, nv < 7.4), hypertrophic cardiomyopathy (LVMI 128-232 g/m^2). In our laboratory, we assayed the CRIM status on PBMC, then confirmed by molecular analysis (Pt.1 CRIM pos c.1933G>A, c.2237G>A – p.Asp645Asn, p.Trp746*; Pt.2 CRIM neg c.2560C>T, del exons 4-8 – p.Arg854*, del exon 4-8; Pt.3 CRIM neg homozygous c.236_246del – p.Pro79Argfs*13). They started ERT (alglucosidase alfa, Genzyme Corp.) between day 5 and day 19 of life, with good responses. To date, all are alive (mean age 4.5 years). Two patients (Pt.1, 5.5 years old, and pt.3, 3.0 years old) have age-appropriate motor development with no signs of cardiomyopathy and normal biochemical test results, including CPK and uGlc4. Pt.2, CRIM negative, developed anti-rhGAA antibodies after 6 months of ERT (max titre 1:102,400), associated with delayed psychomotor development and persistence of cardiomyopathy [28].

Among patients with LOPD, the typically Caucasian IVS1 variant was the most common pathogenic variant, present in 12/13 LOPD cases, 6 of which were homozygous. Over 8 years (mean follow-up 3.6 yrs, range 0.5-7), none of the patients carrying a LOPD or VUS variant developed symptoms and none was receiving ERT.

Among patients with pseudodeficiency alleles, we found a high incidence of the Asiatic variant c.2065 G>A (p.Glu689Lys), alone (n = 1) or in the complex allele c.(1726G>A, 2065G>A) p.(Gly576Ser; Glu689Lys) (n = 10). Moreover, 5 European newborns carried the predicted non-pathogenic variant p.Val222Met. Patients carrying a single variant or pseudodeficiency alleles were dismissed after communication of molecular assay result.

3.1.3. Fabry disease

For FD, of the 248,616 newborns screened (119,512 males), 31 newborns (29 males) were referred for confirmatory testing due to low enzyme activity. The α-GalA enzyme activities in DBS of positive patients ranged from 0.45 to 3.45 µmol/L/h (n = 29, mean 1.45 µmol/L/h, SD 0.82). LysoGb3 testing was conducted on 24 DBS with low α-GalA activity. LysoGb3 values ranged from 0.22 to 8.06 nmol/L (mean 1.38 nmol/L, SD 1.74, nv <1.45 nmol/l). Values were abnormal in 7 newborns.

All 29 males were confirmed to have low α-GalA activity in lymphocytes and a genetic variant in the *GLA* gene, with an incidence of 1:4,121 males. The two female newborns with low DBS α-GalA activity were negative at molecular testing.

Molecular analysis identified 18 newborns carrying known pathogenic variants, associated with the later-onset form of FD, 6 carrying previously unclassified *GLA* variants, 4 the debated

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p.Ala143Thr variant and 1 carrying a haplotype considered benign (-10C>T, IVS2-77_81del15, IVS4-16A>G, IVS6-22C>T). The most common variant was p.Asn215Ser (n = 5). Other variants were associated with ethnic origin. Three unrelated newborns of Asiatic origin carried the splicing variant IVS4+919G>A [12], 3 unrelated patients of African descent carried the pathogenic variant p.Arg363His, 3 African patients had arginine substitutions at position 356 (p.Arg356Gln, n = 2; and p.Arg356Gly, n = 1). Ten patients carried a VUS. One patient carrying an unclassified variant (p.Ile91Thr) had abnormal DBS lysoGb3 at birth (8.06 nmol/L), that increased during follow-up (13.62 nmol/L at 1 yr), suggesting that this variant may be pathogenic. All mothers were positive for the same mutation as their children.

All patients participated in regular follow-up, except 4 whose parents refused additional medical examinations. Because all our patients carried variants associated with the later-onset form or unclassified variants, we decided to follow them every 12 months to avoid overmedicalization. After 8 years of NBS and follow-up, none of our patients (mean age at last visit 4.5 years, range 0.7-7.8 years) have symptoms or signs of disease and none is treated. All presented a progressive increase of LysoGb3, which was abnormal at last follow-up in 21/25 patients (mean 1.88 nmol/l, SD 2.2, range 0.35-13.62).

3.1.4. Gaucher disease

For GD, positive NBS results were found in 18 neonatal DBS; 16 were referred to our Clinic for confirmatory testing, while 2 infants were lost due to relocation. GD was confirmed in all newborns, with an overall incidence of 1:15,539 births. Two newborns affected by neonatal form of GD were already symptomatic at recall. The other newborns were predicted to have GD1.

Gcase activity on DBS in confirmed patients ranged from 0.38 to 2.3 uM/h and the value did not correlate with disease subtype or severity. Conversely, LysoGb1 in DBS from patients with neonatal onset GD were 7,262 nmol/L and 1,698 nmol/l (nv < 33.31), respectively, while the mean in GD1 patients was 130.81 nmol/L (n = 14, range 45.55-323). Thus, DBS LysoGb1 at birth can clearly discriminate the disease phenotype. Molecular analysis was available for all 16 patients. Of the two patients with neonatal onset GD, both of Balkan origin, one was homozygous for p.(His294Gln+Asp448His) and the other compound heterozygous p.Asp448His, p.(His294Gln+Asp448His). Of 11 genotypes associated with GD1, p.Asn409Ser (n = 17) was the most common allele in our cohort, as expected in the Italian population. Three patients carried at least one VUS (p.Thr408Met or p.Glu365Lys).

Except for the 2 patients who relocated after diagnosis of GD, most patients (n = 14) received follow-up evaluations (mean age at last visit 3.8 years, range 0.2-7.0 years). Thus far, 6/16 patients exhibited clinical manifestations and required therapeutical intervention. The neonatal onset GD patients presented at birth with anemia and low platelet counts, petechiae, hepatosplenomegaly, and cholestatic hepatitis. They were treated with ERT beginning at 13 and 4 days of life and obtained hematological and visceral improvement, Unfortunately, ERT does not cross the blood-brain barrier, and the first patient presented gradual neurological involvement with delayed psychomotor development [29], while the second patient showed neurological involvement in the first month of life with hypertonus, strabismus, dysphagia, stridor and central apnea. Four GD1 patients developed signs of disease at a mean age of 3 yrs (range 2-4 years). Three patients developed hepatosplenomegaly, one patient developed bilateral femur osteonecrosis [30]. All started ERT, with good clinical and biochemical outcomes.

4. Discussion

We report our experience after 8 years of LSDs newborn screening on about 250,000 newborns, which is the largest study to date reported in Europe.

Among the 126 newborns who underwent confirmatory tests, we found 51 affected patients (16 with PD, 13 with GD, 18 males with FD, 4 with MPSI). Of them, 45/51 are currently in follow-up (15 with PD, 11 with GD, 15 males with FD, 4 with MPSI).

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For MPSI, we found an incidence of 1:62,154, which is higher that clinically reported (1:100,000) [31–34]. In Europe, there are only a few other experiences with NBS for MPSI, such as the small experience in the Umbria Region of Italy (3,403 newborns) [23], and a pilot project on 64,907 newborns conducted in the Italian Regions of Tuscany and Umbria [35]. No true positives were identified in either project, while a total of 15 recalled newborns were false positives at confirmatory testing. In the USA, NBS for MPSI is included in the Recommended Uniform Screening Panel (RUSP). The incidence ranged between 1:35,509 (Washington State) to 1:219,793 (Illinois) [36] and a high number of pseudodeficiencies is found in programs that do not use 2TT (GAGs quantification or DNA sequencing). Conversely, in Taiwan, where the incidence was about 1:73,000, the rate of false positives was very low without 2TT (0.0013% on 294,196 newborns) [37,38]. This was attributed to population homogeneity in Taiwan and a very low frequency of pseudodeficiency.

Our experience confirmed a high false positive rate due to pseudodeficiency, more prevalent in African and African-American populations [35,39]. Since February 2019, when we added GAGs quantification on DBS as a 2TT [27], our two-test algorithm has reduced false-positives and recall rates, with a PPV of 100% [40]. The outcome of our patients confirms that early HSCT can change the natural history of the severe disease form, preventing neurological involvement and allowing a normal psychomotor development.

During the study period, one patient was clinically diagnosed at the age of 10 months with Hurler syndrome. The patient was born in a neighboring country and did not undergo neonatal screening for lysosomal diseases. At diagnosis he presented with severe organ involvement (coarse facies, lumbar hump, joint stiffness, heart valve disease, visceromegaly, umbilical hernia, corneal opacity and severe deafness). This confirms the importance of neonatal screening for MPSI in avoiding diagnostic delay. No screen-negative patients were clinically diagnosed, with a negative predictive value of 100%.

The incidence of PD from our NBS data was 1:15,539 (IOPD 1:82,872; LOPD 1:19,124). The reported clinical prevalence of PD worldwide is 1:40,000 [41], whilst a previous Italian study estimated an incidence of 1:120,743 [22]. The difference with our results could be explained by recent immigration from Africa, where there is a higher incidence of consanguinity, but also by the identification of a high number of LOPD patients, as reported from other screening programs. Among 48 newborns with a positive NBS result, 3 patients had IOPD, 13 were predicted with LOPD and 7 carried a VUS (PPV 52%).

In IOPD, treatment should be started as soon as possible; delay of a few days can influence outcomes [42,43]. Our IOPD patients (n = 3) started ERT very early, between 5 and 19 days of life. Two had good outcomes, one patient, CRIM negative, developed a non-compactum myocardium and psychomotor delay [28], due to the formation of anti-ERT antibodies [44].

In patients with LOPD, ERT is associated with better outcomes when started before irreversible muscle damage occurs [45,46]. The outcome of LOPD is difficult to predict. Most of our patients carried the c.-32-12T>G variant (i.e., IVS1), which is the most frequent variant in the Caucasian population (90% of patients) [47], with phenotypic variation in the age of symptom onset from early childhood to late adulthood [48,49]. Until now, our LOPD patients (mean age 3.4 years, range 0.3-6.9 years) have no clinical signs or symptoms, all have age-appropriate development, and none is receiving ERT.

Our data demonstrate a high incidence of pseudodeficiencies, especially of the Asiatic variant p.Glu689Lys, alone (n = 1) or in the complex allele p.(Gly576Ser; Glu689Lys) (n = 7). Moreover, 5 European newborns carried the predicted non-pathogenic variant p.Val222Met.

Despite these reservations, NBS for PD is widespread worldwide. In the USA, it was the first LSD to be included in the RUSP, added in 2015. All reported programs confirmed a higher-than-expected incidence of disease and in particular a high number of LOPD. In Taiwan, the first NBS pilot study for PD was conducted in 2005. The Taiwanese cohort is unique, because almost all the IOPD patients are CRIM positive (due to high frequency of the p.Asp645Glu mutation), LOPD cases lack the IVS1 variant, common in Caucasian population, and there is a high frequency of the pseudodeficiency allele p.(Gly576Ser; Glu689Lys).

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For GD, among 18 newborns with positive NBS, we diagnosed 13 patients with GD1, 2 patients with neonatal onset GD, while 3 patients carried a VUS (PPV 100%). The overall incidence was 1:16,574, three times higher than clinically estimated (1:40,000-1:60,000) [50]. The most frequent allele was p.Asn409Ser (n = 17, associated with phenotype GD1), interestingly followed by the p.(His294Gln+Asp448His) allele (n = 4), of Balkan origin, associated with a GD2/3 phenotype [51]. This is probably due to recent immigration from the Balkans.

NBS for GD is still controversial, and in the USA it is not included in the RUSP. Only a few States have reported their experience, such as Missouri and Illinois (43,701 and 219,973 newborns, respectively), which found an incidence of about 1:43,000 [52,53]. In New York State (65,605 newborns), a very high incidence was found (1:4374), and this likely reflects New York City's large Jewish population, in which the p.Asn409Ser allele is frequent [54]. Several NBS programs for GD have been conducted in East Asia where, due to different ethnic backgrounds, a lower incidence of disease was reported (1:73,743 to 1:103,134), and most patients suffered from neuronopathic forms (GD2/3), for which few therapeutic options are available [37,55–58].

There are few data on the measurement of LysoGb1 in the neonatal period. Its use as a 2TT appears promising. In our population, all the neonates with increase LysoGb1 were confirmed as true positive, with a PPV of 100%. LysoGb1 at birth correlated with disease burden, in agreement with genotype, as patients harboring neuropathic alleles returned significantly higher plasma concentrations than GD patients with non-neuropathic disease. Interestingly, 6/16 patients, including 4 GD1 patients, started ERT in the first years of life due to clinical manifestations (hepatosplenomegaly, osteonecrosis). This underlines the importance of NBS for GD to prevent irreversible complications and avoid diagnostic delays.

In our study, FD was identified and confirmed in 28 males, while one newborn carried a benign variant. The 2 female newborns were found to be false positives (overall PPV 90%). The overall incidence of α-GalA deficiency was 1:8,573 (1:4,121 males), which is similar to previous reports from NBS programs [59], but higher than the clinically estimated incidence (1:40,000) [60], likely due to the recognition of previously undiagnosed later-onset forms of FD. Ten patients carried unclassified variants. It is difficult to predict their pathogenicity because FD may occur later in life. Clinically, our patients presented no symptoms or signs at the last visit (0.7-7.8 years old), although they show a progressive increase of LysoGb3. All mothers carried the same mutation as their children.

In the USA, FD is not included in the RUSP, but local laws promoted by NBS advocates and parents allowed its implementation in NBS in several States. These programs confirmed the high incidence of the disease (1:1,790 to 1:15,558), but also the high number of false positives [59]. Conversely, in Taiwan, NBS for FD was started in 2006. The disease prevalence was very high, due the diffusion of the IV4+919G>A variant (82%, 1:1,600 males), associated with cardiac involvement [61].

We tested the utility of lysoGb3 as a 2TT. Only 7/29 patients had abnormal DBS LysoGb3 at birth, indicating that a normal result cannot exclude FD. A recent report of two brothers with classic FD (genotype c. 370–2 A > G) showed that LysoGb3 was elevated in the first days of life and that it increased significantly during infancy in these patients [62]; therefore, LysoGb3 values may contribute to the complete identification of the phenotype.

We did not detect any heterozygotes among 129,104 females screened, which confirms that the current enzyme based NBS approach misses most female carriers due to X-chromosome inactivation [63]. Some authors suggest first-tier screening with *GLA* gene sequencing in female newborns. This method has been applied in Taiwan, where 21 variants account for approximately 98% of variants [64]. In Italy and throughout Europe, mutational heterogeneity hampers the use of molecular analysis for high-throughput screening, which could also increase the number of VUS.

5. Shadows

NBS for LSDs (MPSI, PD, GD and FD) has several limitations:

- A high number of false positives and pseudodeficiencies, which impact families and health care systems. Our results demonstrate that biochemical 2TTs are effective to reduce the recall rate for

MPSI (GAGs quantification) and GD (LysoGb1). In FD the use of LysoGb3 in the neonatal period is debated [59]. The development of a 2TT is important for all the screened diseases. Proposal to reduce false positive rate for PD includes both biochemical (creatine/creatinine over GAA ratio by MS/MS) [65], and/or molecular 2TTs [66–68] and postanalytical tools, e.g., CLIR [69,70].

- Identification of an increasing number of VUS, especially for PD and FD. Some of these patients may never develop symptoms; however, they require ongoing monitoring, which causes anxiety to families and costs for healthcare systems. Future improvement in the management of these patients is likely to come from a better understanding of the genotype/phenotype correlation and natural history of the disease.

- NBS detects newborns across the full phenotypic spectrum of severity, although not all subtypes benefit equally from early detection. Central nervous system damage for neuropathic LSDs such as GD2 cannot be treated currently [71]. Although having the benefit of informing genetic counseling for parents, an untreatable disease may not fit the criteria for NBS. Potential new therapies for the central nervous system are under development.

- Ethical issues due to the high incidence of late-onset forms. For these disorders, onset is often unpredictable and some "patients in waiting" may never develop symptoms [72], resulting in unnecessary anxiety and medical intervention, but also the potential that they will be treated as "vulnerable children" [73]. Challenges remain in identifying better biomarkers for phenotype prediction and the best tailored strategy for the follow-up, management and treatment of these patients.

In conclusion, our experience showed that NBS for these four LSDs is feasible and extendable to larger populations. The incidence of screened LSDs appears to be higher than clinically estimated in our country, but compatible with other screening studies. The possibility of including 2TTs for three of these diseases has changed the false positive rate.

Our experience also outlines the importance of early diagnosis in improving disease outcomes when treatments are available.

Remaining challenges include determining the best management of infants with late-onset phenotypes and those carrying VUS. Long-term follow-up of these patients is an emerging problem. Sharing data in international databases will provide valuable information on genotype-phenotype correlations, natural history, the role of biomarkers, and the impact of early treatment, all of which will pave the way to optimized management of these patients.

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