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Moonlighting newborn screening markers: the incidental discovery of a second-tier test for Pompe disease

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Purpose: To describe a novel biochemical marker in dried blood spots suitable to improve the specificity of newborn screening for Pompe disease.

Methods: The new marker is a ratio calculated between the creatine/creatinine (Cre/Crn) ratio as the numerator and the activity of acid α-glucosidase (GAA) as the denominator. Using Collaborative Laboratory Integrated Reports (CLIR), the new marker was incorporated in a dual scatter plot that can achieve almost complete segregation between Pompe disease and false-positive cases.

Results: The (Cre/Crn)/GAA ratio was measured in residual dried blood spots of five Pompe cases and was found to be elevated (range 4.41–13.26; 99%ile of neonatal controls: 1.10). Verification was by analysis of 39 blinded specimens that included 10 controls, 24

samples with a definitive classification (16 Pompe, 8 false positives), and 5 with genotypes of uncertain significance. The CLIR tool showed 100% concordance of classification for the 24 known cases. Of the remaining five cases, three p.V222M homozygotes, a benign variant, were classified by CLIR as false positives; two with genotypes of unknown significance, one likely informative, were categorized as Pompe disease.

Conclusion: The CLIR tool inclusive of the new ratio could have prevented at least 12 of 13 (92%) false-positive outcomes.

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Key Words: creatine; Collaborative Laboratory Integrated Reports; newborn screening; Pompe disease; second-tier test

INTRODUCTION

Acid α-glucosidase (GAA) deficiency (Pompe disease; glycogen storage disease type II; OMIM 232300) results in different clinical phenotypes depending on age at onset, degree of organ involvement, and severity of muscle disease.[1] Traditionally, Pompe disease is classified in an infantile-onset and a late-onset variant. Patients with infantile-onset Pompe disease suffer from cardiomyopathy, progressive cardiorespiratory decline, and death, usually by the end of the first year of life. Late-onset Pompe disease has been classified based on age at onset as childhood, juvenile, and adult-onset disease, but more recently classifications have been attempted that are based on symptomatology such as limb girdle and diaphragmatic weakness pattern, rigid spine syndrome, scoliosis, and cardiocerebrovascular pattern.[2] Severity of this emerging multisystemic and progressive disease correlates best with length of time since onset of symptoms.[3] The nonspecific and variable presentations that overlap with other, more common etiologies explain why Pompe disease is often undiagnosed in older patients. But efforts to increase understanding and awareness of this previously untreatable condition have been under way since 2006, when recombinant human GAA was

approved by the US Food and Drug Administration as enzyme replacement therapy.[4]

The incidence of Pompe disease of any phenotype varies greatly among different ethnicities, from an estimated 1:14,000 in African Americans[1] to 1:600,000 in a study from Portugal.[5] In the United States the overall incidence was initially estimated to be approximately 1:40,000,[6] but newborn screening now reveals a higher prevalence of Pompe disease ranging from 1 in 5,500 in Missouri to 1 in 28,000 in Washington with most infants identified being affected with late-onset Pompe disease.[7,8] At the genotype level, at least 563 variants are listed in the online database curated by Erasmus University,[9] almost 100 of which are classified as benign pseudodeficiency alleles that cause low enzyme activity in vitro with no associated clinical phenotype.[10] As a direct consequence, early newborn screening pilot programs for Pompe disease showed a high frequency of the c.[1726A;2065A] pseudodeficiency allele in the Asian population, generating a large number of false-positive results.[11,12] Reports of screening performance have varied widely among different pilot programs. For example the positive predictive value may range from 80% to as low as 0.37%.[8]

Table 1 Percentiles of reference ranges (not adjusted for covariates)

Marker	Count	1%ile	10%ile	50%ile	90%ile	99%ile
Acid α-glucosidase (GAA)[a] (nmol/ml/h)	55,161	5.61	7.62	12.14	19.33	28.40
Creatine (Cre) (nmol/ml)	1,896	241	321	425	567	729
Creatinine (Crn) (nmol/ml)	1,896	47.4	60.1	76.6	96.7	118
Cre/Crn ratio	1,896	3.5	4.2	5.1	6.1	7.1
(Cre/Crn)/GAA ratio	1,896	0.2	0.3	0.5	0.8	1.1

[a]Same values as reported in ref. 21.

Although response to enzyme replacement therapy is highly variable and complicated by the risk of an immune response against the recombinant enzyme, clinical trials have shown prolonged survival and better quality of life in early-onset patients especially when treatment is initiated very early in life.[13] Although enzyme replacement therapy appears to be well tolerated by patients with late-onset Pompe disease,[14] the benefits of treatment are maximized only when initiated before irreversible symptoms develop.[15] The availability of a newborn screening test, a treatment option, and evidence that early treatment improves outcomes led to the addition of Pompe disease to the Recommended Uniform Screening Panel in March 2015.[16]

To minimize the number of false-positive results requiring unnecessary patient contact, genotyping of borderline cases has been adopted as for other lysosomal disorders.[17] At the biochemical level, the Taiwan newborn screening program proposed to calculate the neutral α-glucosidase to GAA ratio.[18] Second-tier tests have shown clinical utility for other lysosomal disorders,[19,20] but no other known marker of Pompe disease measurable in blood spots has been described yet. We report here the incidental discovery and preliminary validation of a new marker applicable to the differential diagnosis between Pompe disease and false-positive cases.

MATERIALS AND METHODS

Reference population
Residual samples of routine specimens ($N = 1,896$) collected at <250 hours of age and screened for three lysosomal disorders were also analyzed by flow infusion tandem mass spectrometry to determine a preliminary reference range of the creatine/creatinine (Cre/Crn)/GAA ratio. Percentiles of the reference ranges, not adjusted for covariates, are shown in **Table 1**.

True-positive and false-positive cases
The initial validation of the new ratio was based on the analysis of known cases with Pompe disease collected at age <250 hours and tested for six lysosomal enzymes, four lysophosphatidylcholines, creatine, and creatinine (12-plex assay). Three cases were detected prospectively as part of newborn screening in the Commonwealth of Kentucky since February 2016.[21] Two additional true-positive samples, also collected within 10 days of age, were follow-up specimens for abnormal newborn screening results from other state programs (IRB 15-0055393). As no residual samples of genotype-confirmed false-positive cases were available to measure creatine and creatinine, an exchange of specimens with the New York State newborn screening program under an approved New York State quality improvement project was arranged. Thirty-nine samples were sent blindly and later their case resolution was provided after return of the results and interpretation shown in **Table 2**. In this group, a diagnosis of early infantile-onset Pompe disease was made based on the presence of cardiac abnormalities. Nonclassical infantile cases had no cardiac involvement, but did present prior to 1 year of age with deficiencies in muscle tone. None of the predicted late-onset cases have developed symptoms.

To evaluate the potential utility of the Cre/Crn ratio in blood spots for Duchenne/Becker muscular dystrophy (DMD), 36 blinded samples of clinically genotyped unaffected cases, female carriers, and DMD patients were provided by EGL Genetics (Atlanta, GA) (IRB 00090208).

Analytical methods
The activity of GAA, five other lysosomal enzymes, and the concentrations of four lysophosphatidylcholines were measured by flow infusion tandem mass spectrometry.[22] Unrelated to lysosomal disorders, other conditions are under consideration for inclusion in the Recommended Uniform Screening Panel. One of them is guanidinoacetate methyltransferase deficiency (OMIM 601240), which is detectable in neonatal blood spots by measuring guanidinoacetic acid (Guac).[23–26] Rather than relying on a single compound, testing a profile of Guac, Cre, and Crn is preferable as it provides an opportunity to calculate ratios.[27] To begin covering guanidinoacetate methyltransferase deficiency as part of a comprehensive supplemental newborn screening panel, these markers have been incorporated in our standard procedure for the analysis of amino acids, acylcarnitine, and succinylacetone in dried blood spots by tandem mass spectrometry.[28] Briefly, stable isotope labeled internal standards d_3-Crn, d_2-Guac, and d_3-Cre were purchased from CDN Isotopes (Point-Claire, Quebec, Canada). Methanol solutions of each internal standard were added to the amino acid and acylcarnitine internal standard solution with no other modifications of the existing method being necessary. The triple quadrupole instrument was optimized for multiple reaction monitoring of mass transitions m/z 114.1 to 44.1, 117.1 to 47.1, 188.2 to 90.0, 191.2 to 93.1, 174.2 to 101.1, and 176.2 to 103.1 for Crn, d_3-Crn, Cr, d_3-Cr, Guac, and d_2-Guac, respectively, as previously described.[23] These experiments

Table 2 Demographic information, biochemical results, and genotypes of blinded specimens exchanged for verification of the new marker.

Case ID	Age at collection (h)	Birth weight (g)	Sex	GAA activity (NY)	Genotype (unresolved cases only)	Resolution By NY	GAA activity (Mayo)	Cre	Crn	(Cre/Crn)/GAA ratio	Resolution By CLIR
01	12	2,193	M	n/a		Pompe	0.8	333.2	93.3	4.6	Pompe
03	395	2,193	M	n/a		Pompe	0.4	140.6	50.4	6.3	Pompe
04	695	2,193	M	n/a		Pompe	0.2	66.5	31.7	9.1	Pompe
06	36	2,880	F	1.8		FP	1.9	361.0	74.1	2.5	FP
07	43	3,270	F	0.9		Pompe	0.7	326.6	64.5	7.5	Pompe
09	625	1,150	F	2.0		FP	2.0	240.3	41.5	2.9	FP
10	56	4,200	M	2.2		FP	1.2	435.6	75.5	4.7	FP
12	693	2,008	F	1.2		FP	0.9	159.0	38.8	4.6	FP
13	25	3,652	F	1.6		Pompe	1.0	584.8	99.2	5.7	Pompe
14	46	3,695	F	1.7		FP	1.3	363.7	68.5	4.1	FP
16	47	3,280	M	2.0	p.V222M/p.V222M	*Unknown significance*	2.1	501.9	78.5	3.1	*FP*
17	38	3,110	M	1.7		Pompe	1.4	393.1	83.6	3.4	Pompe
18	117	4,080	F	1.7		FP	1.0	351.0	61.0	5.7	FP
21	51	2,830	F	1.0		FP	1.0	270.2	65.5	4.3	FP
22	31	3,720	M	0.6		Pompe	0.6	321.0	63.4	8.9	Pompe
23	24	3,868	M	0.9		Pompe	0.6	307.4	67.0	7.1	Pompe
24	63	2,830	F	0.6		Pompe	0.7	473.4	93.7	7.0	Pompe
26	44	3,090	M	0.9	p.V222M/p.V222M	*Unknown significance*	0.9	367.8	78.3	5.2	*FP*
27	25	3,117	M	1.4		FP	1.5	324.8	69.6	3.1	FP
28	28	3,748	M	1.0		Pompe	0.7	494.4	97.2	7.2	Pompe
29	44	2,580	M	0.6		Pompe	0.7	417.0	83.0	7.1	Pompe
30	39	3,236	F	0.7		Pompe	1.0	387.2	73.4	5.5	Pompe
31	41	2,970	M	1.4	p.V222M/p.V222M	*Unknown significance*	1.7	517.8	87.0	3.6	*FP*
33	2306	2,902	M	0.5	p.W746C_p.N815_P.E689K/p.G576S_P.E689K	*FP*	0.5	209.1	32.6	11.9	*Pompe*
34	33	3,225	M	1.1	p.P690L_p.G576S_p.E689K/p.P690L_p.G576S_p.E689K	*Unknown significance*	1.2	598.7	90.1	5.6	*Pompe*
35	4	880	F	0.8		Pompe	0.9	527.4	84.5	7.3	Pompe
36	46	3,665	M	1.0	p.E721Rfs/p.D91N_p.W746L	*Unknown significance*	1.3	716.2	102.9	5.5	*Pompe*
38	41	2,900	F	0.5		Pompe	0.5	379.6	71.3	10.1	Pompe
39	1	2,480	F	0.2		Pompe	0.1	615.2	92.4	49.2	Pompe

CLIR, Collaborative Laboratory Integrated Reports; Cre, creatine; Crn, creatinine; F, female; FP, false positive; GAA, acid α-glucosidase; M, male; Mayo, Biochemical Genetics Laboratory, Mayo Clinic; n/a, not available (three samples of a patient born before screening for Pompe disease was initiated by New York State); NY, New York State Department of Health. Gaps in case ID reflect embedded normal controls (not shown). GAA activity expressed as nmol/ml/hour. Creatine and creatinine concentrations expressed as nmol/ml. Bold italic type indicates discrepancies of case resolution.

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were then added to the panel of precursor ion, neutral loss, and multiple reaction monitoring scans for acylcarnitine, amino acid, and succinylacetone determination. Genotyping was performed by Sanger sequence analysis of all coding exons and 20 bp at each intron/exon boundary of the GAA gene.

Postanalytical interpretation

A workflow designed to implement prospective screening for lysosomal disorders has been described.[21] Briefly, a 6-plex lysosomal panel is analyzed and the results are matched against covariate-adjusted reference intervals.[27] This analysis is accomplished using postanalytical interpretive tools created by Collaborative Laboratory Integrated Reports (CLIR; https://clir-mayo.edu), a multivariate pattern recognition software originally developed for the interpretation of newborn screening for metabolic disorders by analysis of amino acids and acylcarnitines.[29,30] If the score is greater than zero, another tool, called the dual scatter plot, is used to establish a differential diagnosis between Pompe disease and false-positive cases.[21] If the case is classified as either Pompe disease (score coordinates in the lower right quadrant) or indeterminate (score coordinates in the upper right quadrant where true- and false-positive cases cosegregate) a repeat analysis is performed including C20–C26 lysophosphatidylcholines (10-plex). Cases with a score again > 0 are tested with the 10-plex version of the dual scatter plot. A still informative score constitutes an abnormal result that before the recognition of the new marker described here would have triggered a referral to initiate follow up.

RESULTS

During the postanalytical validation of an expanded newborn screening panel (Recommended Uniform Screening Panel plus other candidate conditions, including three disorders of creatine metabolism) a Pompe case was noted to have a Cre/Crn ratio of 9.0, substantially higher than the 99%ile of the reference population (7.1). A biological rationale for the elevated ratio among Pompe patients can be postulated following the observation of serum creatine and creatinine levels at the higher and lower ends of normal reference ranges, respectively, in adult-onset patients with Pompe disease.[31] Therefore, we hypothesized that the Cre/Crn ratio could be a marker of early muscle involvement already present in the newborn period and potentially an opportunity for introducing a multitier approach for Pompe disease newborn screening. Although testing of additional cases failed to confirm the initial finding (range 4.71–5.47, $N = 4$; **Figure 1a**), an attempt was made to further integrate the Cre/Crn ratio with the residual GAA activity (**Figure 1b**), which resulted in a clear separation from the reference population (**Figure 1c**). At this stage, however, it was still unknown to what extent the potential marker would behave in false-positive cases with similar and overlapping residual GAA activity (**Figure 1b**).
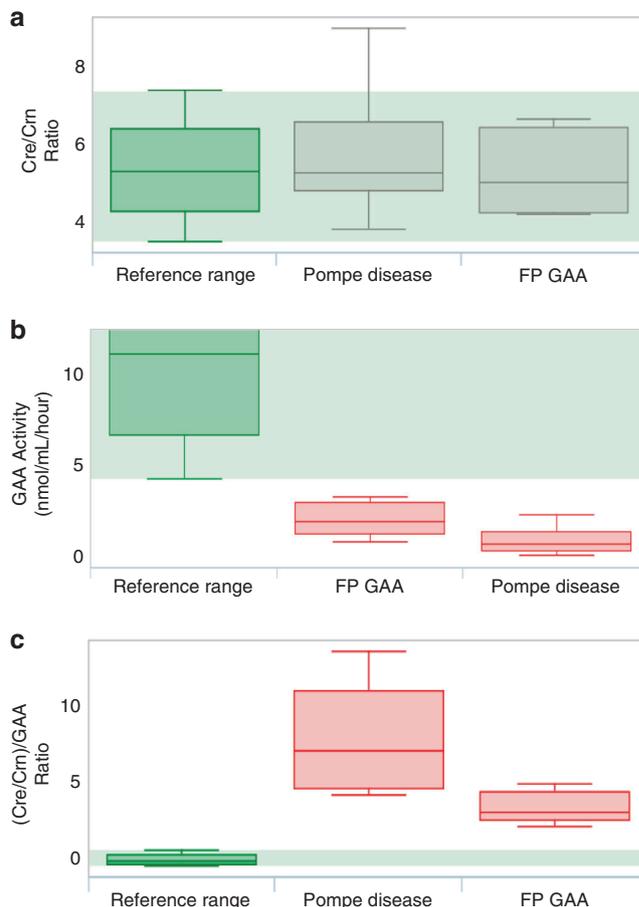
Figure 1 Box plot comparison of reference and disease ranges (1%, 10%, 50%, 90%, and 99%iles, respectively). Color coding as follows: green, reference population; gray, not informative; red, informative. Informative status is assigned when the median of a disease range is outside of the peripheral percentiles. (**a**) Cre/Crn ratio. (**b**) Residual GAA activity. (**c**) (Cre/Crn)/GAA ratio. Cre, creatine; Crn, creatinine; FP, false positives; GAA, acid α-glucosidase.

The analysis of 39 blinded samples with the 10-plex assay resulted in a distribution of cases as follows: 10 normal cases, based on GAA activity; 16 likely Pompe disease; 8 likely false-positives; and 5 indeterminate outcomes. These results were disclosed to the New York program, which then shared the genotypes and outcomes for these cases. All results matched, particularly the five indeterminate cases, which were classified as harboring variants of uncertain significance. With this information, it was possible to establish disease ranges for the Cre/Crn and (Cre/Crn)/GAA ratios in false-positive cases (**Figure 1a,c**), and create a 12-plex dual scatter plot (**Figure 2a**) that achieved complete separation between the two groups of confirmed cases. **Figure 2b** shows the combined scores and quadrant localization of the 24 concordant cases, 8 false positives, and 16 true positives. **Figure 2c** shows the scores of the remaining cases. The three cases that were resolved as false positives harbor the same genotype, homozygosity, for the p.V222M variant. This variant has been
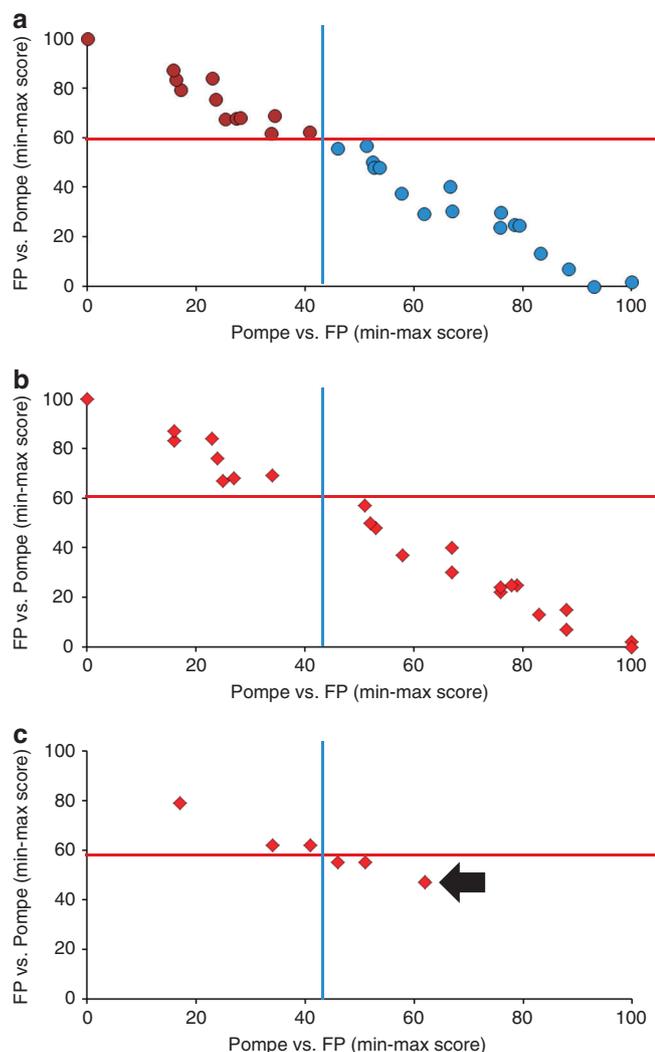
Figure 2 Collaborative Laboratory Integrated Reports dual scatter plot (12-plex) applied to the differential diagnosis between Pompe disease and false-positive cases. Each plot is divided in four quadrants. Lower right: consistent with Pompe disease (light blue circles). Upper right: indeterminate (both conditions are possible). Upper left: consistent with false-positive cases (purple circles). Lower left: neither condition. (**a**) Distribution of scores of cases confirmed by genotyping. (**b**) Distribution of scores of blinded cases (red diamonds) with concordance of resolution. (**c**) Distribution of scores of blinded cases resolved by the New York program as genotype of unknown significance (arrow indicates case 34). See **Table 2** for details. FP, false positives.

reported as likely benign or pseudodeficient,[9] and these cases were not included in a summary of Pompe disease in a recent report of post–newborn screening evaluation of Pompe disease in New York State.[32] For these reasons, we are considering these cases to be false positives properly recognized by the CLIR tools. The genotypes of the final two cases resolved by CLIR as Pompe disease were p.E721Rfs//p. D91N_p.W746L (case 34) and homozygosity for p.P690L_p. G576S_p.E689K (case 36), respectively. Both cases were listed in the publication mentioned above;[32] case 34 as "Pompe

disease/variant of unknown significance 2" and case 36 as "Pompe disease/Pathogenic-10." The latter case is consistent with the CLIR classification and therefore considered a correct outcome. On the other hand, variant p.P690L is a recurrent variant of unknown significance found in unrelated families.[33] Pending further characterization of this genotype, for the purpose of the current study, case 34 was considered to represent a false-positive outcome, incorrectly classified by CLIR.

DISCUSSION

This report describes preliminary but promising evidence of a biochemical second-tier test to improve the specificity of newborn screening for Pompe disease. Additional validation studies are necessary and are undergoing in several ways: (i) continuing prospective screening of lysosomal disorders;[21] (ii) routine clinical offering of the 12-plex assay as a standalone orderable second-tier test; (iii) future potential demand for the new expanded panel; and (iv) sharing of specimens and results of newly confirmed cases as part of an ongoing collaborative effort centered around the CLIR database. If confirmed, a biochemical second-tier test for Pompe disease not only is more cost effective than molecular genetic analysis but also allows a faster turnaround time of results. To achieve best outcomes,[13] early-onset Pompe disease should be considered a time-critical condition for which newborn screening results should be made available to care providers by the 5th day of life, a goal not readily achievable when using molecular genetic analysis as a second-tier test. Another benefit of a reliable biochemical second-tier test is the avoidance of anxiety and costs associated with the frequent discovery of genotypes of uncertain significance, which eventually turn out to be unaffected individuals with pseudodeficient GAA activities.[8,34,35]

It is also possible to begin exploring whether the Cre/Crn ratio alone, the (Cre/Crn)/GAA ratio, or additional permutations of ratios could be relevant to the expansion of the biochemical phenotype of other conditions with prominent skeletal and cardiac myopathy and consequent elevation of creatinine phosphokinase, for example, very long chain fatty acid oxidation disorders[36] and particularly DMD and related disorders.[37] For proof of concept, preliminary testing of blood spotted on filter paper from residual clinical samples of genotyped DMD patients showed consistent elevations of the Cre/Crn ratio (**Figure 3**; age 3–11 years, $N = 10$, range 6.42–8.99; age 25–39 years, $N = 10$, range 4.71–10.44; controls of age 21–60 years, $N = 11$, range 2.55–4.96; see **Table 1** for neonatal reference percentiles). As a next step we are seeking neonatal/infantile blood spot specimens of DMD patients to evaluate whether one or more permutations of ratios integrating creatine and creatinine could be used as a second-tier test or even as an alternative primary test for newborn screening of DMD and related disorders.

The traditional concept of a second-tier test is to increase specificity of the primary screening without requiring additional patient contact and specimen collection.[38] In the case of
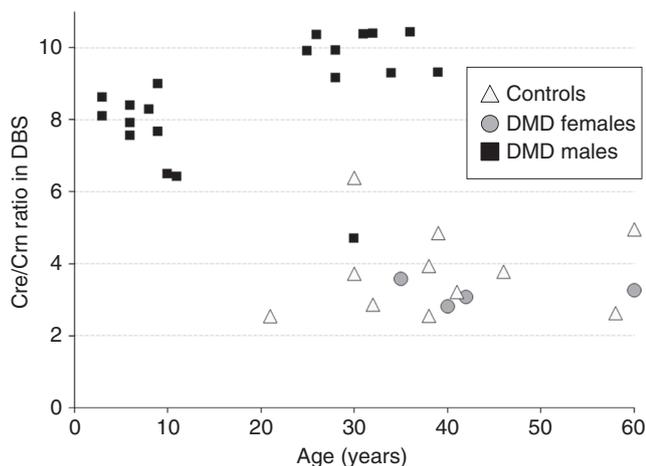
Figure 3 Scatter plot of creatine/creatinine ratio in blood spots of DMD patients, DMD carriers, and controls. Cre, creatine; Crn, creatinine; DBS, dried blood spots; DMD, Duchenne muscular dystrophy.

Pompe disease, this evaluation is achieved by calculating a ratio between the already measured enzyme activity (GAA), other lysosomal and peroxisomal markers, and two unrelated markers added to the primary screening panel to target unrelated conditions, namely the disorder of creatine metabolism. As all the markers are measured as part of an expanded test, in essence the second-tier test for Pompe disease differs from those previously developed because of the potential to become "built in" and therefore could be performed in every sample and become part of the primary screening. Similar to the concept of protein moonlighting, a phenomenon by which an enzyme can perform more than one function,[39] this observation provides credence to the possibility of also uncovering potentially many more moonlighting biochemical markers, and new impetus to the systematic review of markers and calculated ratios in all target conditions, not limited to those biologically linked to a given condition. Using the CLIR software it has become readily possible to rapidly screen the whole spectrum of calculated ratios across all types of markers, and to seek evidence of clinical utility especially when targeting the differential diagnosis between true- and false-positive cases. This effort to discover secondary biomarkers, in parallel to the calculation of covariate-adjusted reference percentiles, could pave the way to a global implementation of precision newborn screening,[21] defined as the sustainable achievement of performance metrics substantially better than historical standards.[40]

DISCLOSURE
The authors declare no conflict of interest.

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