

# Determination of Acid $\alpha$ -Glucosidase Activity in Blood Spots as a Diagnostic Test for Pompe Disease

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**Background:** Pompe disease is an autosomal recessive disorder of glycogen metabolism that is characterized by a deficiency of the lysosomal acid  $\alpha$ -glucosidase. Enzyme replacement therapy for the infantile and juvenile forms of Pompe disease currently is undergoing clinical trials. Early diagnosis before the onset of irreversible pathology is thought to be critical for maximum efficacy of current and proposed therapies. In the absence of a family history, the presymptomatic detection of these disorders ideally can be achieved through a newborn-screening program. Currently, the clinical diagnosis of Pompe disease is confirmed by the virtual absence, in infantile onset, or a marked reduction, in juvenile and adult onset, of acid  $\alpha$ -glucosidase activity in muscle biopsies and cultured fibroblasts. These assays are invasive and not suited to large-scale screening.

**Methods:** A sensitive immune-capture enzyme activity assay for the measurement of acid  $\alpha$ -glucosidase protein was developed and used to determine the activity of this enzyme in dried-blood spots from newborn and adult controls, Pompe-affected individuals, and obligate heterozygotes.

**Results:** Pompe-affected individuals showed an almost total absence of acid  $\alpha$ -glucosidase activity in blood spots. The assay showed a sensitivity and specificity of 100% for the identification of Pompe-affected individuals.

**Conclusions:** The determination of acid  $\alpha$ -glucosidase activity in dried-blood spots is a useful, noninvasive diagnostic assay for the identification of Pompe disease. With further validation, this procedure could be adapted for use with blood spots collected in newborn-screening programs.

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Pompe disease, or glycogen storage disease type II, is an autosomal recessive disorder of glycogen metabolism that is characterized by a deficiency of lysosomal acid  $\alpha$ -glucosidase. As a consequence, Pompe-affected individuals are unable to degrade glycogen stored in the lysosome, leading to the accumulation of glycogen in lysosomal storage vacuoles. Morphologically, this produces an increase in the size and number of lysosomes in the cell. Clinically, the manifestation of Pompe disease is characterized by a broad and continuous spectrum of clinical severity ranging from severe (infantile) to relatively attenuated (adult). The infantile-onset form is characterized by massive cardiomegaly, macroglossia, progressive muscle weakness (including respiratory muscles), and marked hypotonia, with death occurring within the first 2 years of life. The juvenile- and adult-onset forms manifest as slower progressive muscular disorders that are limited to skeletal muscle, with death usually occurring from respiratory failure (1). The heterogeneous presentation of Pompe disease results, at least in part, from the occurrence of different mutations in the lysosomal acid  $\alpha$ -glucosidase gene, which can lead to variable effects on the functional capacity of the mutant enzyme (2, 3).

Pompe disease has a reported incidence of 1 in 201 000 births in the Australian population (4). However, recent studies based on carrier detection in the general population have indicated that the incidence is much greater, ~1 in 40 000 births, in both the United States (5) and The Netherlands (6). On the basis of their findings, Martiniuk et al. (5) indicated that many adult cases are not recognized as a result of milder mutations.

Although definitive treatment for Pompe disease is not currently available, two main treatment strategies are being developed. Correction of the enzyme deficiency by enzyme replacement therapy is well advanced (7–9). The success of enzyme replacement therapy in mice has led to the start of a phase II clinical trial in Pompe patients (10). Gene therapy using viral vectors is also under development (11–14), and targeted disruption of the gene in different mice strains is being used to modulate disease severity in this model (15). It is thought that early

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diagnosis and treatment will lead to major improvements in the efficacy of therapies for this disorder.

The clinical diagnosis of Pompe disease is confirmed by the virtual absence, in infantile onset, or marked reduction, in juvenile and adult onset, of acid  $\alpha$ -glucosidase activity in leukocytes (16,17), muscle biopsies (18,19), cultured fibroblasts (20), and urine (21). Where a family history is known, prenatal diagnosis can be made by determining the acid  $\alpha$ -glucosidase activity in cultured amniotic cells and/or in chorionic villus biopsies (22) and also by mutation analysis (23,24). The current diagnostic methods for Pompe disease are time-consuming, costly, and invasive; in addition, specific testing for Pompe disease often is not performed until other disease possibilities are exhausted. Diagnosis of the attenuated forms of Pompe disease can take months or even years; consequently, some adult patients may go undiagnosed.

We previously evaluated the concentration of acid  $\alpha$ -glucosidase protein in plasma and dried-blood spots as a screening marker for Pompe disease (25). However, diagnosing Pompe disease on the basis of protein concentration will not identify those Pompe patients who have mutant protein without catalytic activity. Consequently, this study reports the measurement of acid  $\alpha$ -glucosidase activity in dried-blood spots as a diagnostic method for Pompe disease. Accordingly, a sensitive immune-capture enzyme activity assay for the measurement of acid  $\alpha$ -glucosidase was developed and used to determine the enzyme activity of this protein in dried-blood spots from newborn and adult controls, Pompe-affected individuals, and heterozygotes.

### Materials and Methods

#### PATIENT SAMPLES

Control dried-blood spots used in this study were de-identified. Blood spots were either part of the routine samples collected at 48–72 h after birth by the South Australian Newborn Screening Centre at the Women's and Children's Hospital, Adelaide, South Australia or samples collected from adult blood donors by the Australian Red Cross Blood Bank in Adelaide, South Australia. Blood spots were also obtained from previously diagnosed Pompe patients and "obligate" heterozygotes.

#### REAGENTS

Pharming BV (The Netherlands) provided recombinant acid  $\alpha$ -glucosidase protein purified from rabbit milk (10). The purity of this protein is >99%, and the preparation of affinity-purified sheep anti-acid- $\alpha$ -glucosidase polyclonal antibody and the monoclonal antibody 43D1 has been described previously (25).

#### PREPARATION OF BLOOD-SPOT CALIBRATORS AND QUALITY-CONTROL SAMPLES

Blood spots containing recombinant acid  $\alpha$ -glucosidase were used as calibrators for immunoquantification of acid  $\alpha$ -glucosidase protein and the corresponding enzyme

activity. The recombinant acid  $\alpha$ -glucosidase concentrations in the blood-spot calibrators were 200, 100, 50, 25, 12.5, and 0  $\mu\text{g/L}$ ; calibrators were prepared as described previously (25).

#### IMMUNOQUANTIFICATION OF ACID $\alpha$ -GLUCOSIDASE

The acid  $\alpha$ -glucosidase protein concentration in blood spots was determined by a polyclonal/monoclonal (43D1) sandwich immunoassay (25).

#### DETERMINATION OF ACID $\alpha$ -GLUCOSIDASE ACTIVITY

Acid  $\alpha$ -glucosidase activity was determined by capture with affinity-purified anti-acid  $\alpha$ -glucosidase polyclonal antibody. Microtiter plates (Immulon 4; Dynatech Technologies) were coated overnight at 4 °C with affinity-purified anti-acid  $\alpha$ -glucosidase polyclonal antibody (4 mg/L; 100  $\mu\text{L}$ /well) diluted in 0.1 mol/L  $\text{NaHCO}_3$ . Coated plates were washed twice with 20 mmol/L Tris-HCl–250 mmol/L NaCl, pH 7.2. The plates were then frozen, lyophilized, and stored at 4 °C in the presence of desiccant.

Acid  $\alpha$ -glucosidase enzyme was captured by placing single 3.0-mm diameter disks containing 3.6  $\mu\text{L}$  of whole blood from the dried-blood spots into the coated microtiter wells with 100  $\mu\text{L}$  of bovine serum albumin (10 g/L; Sigma-Aldrich Fine Chemicals) in phosphate-buffered saline (10 mmol/L phosphate–150 mmol/L NaCl), pH 7.4. The microtiter plates were shaken (20 °C for 1 h) and incubated at 4 °C for 23 h. In each assay, blood-spot calibrators with 0–200  $\mu\text{g/L}$  recombinant acid  $\alpha$ -glucosidase and a quality-control blood spot were also included. After incubation, the microtiter plates were again shaken at 20 °C for 1 h, the blood-spot filters were removed by aspiration, and the plates were washed twice with 20 mmol/L Tris-HCl–250 mmol/L NaCl, pH 7.2.

Enzyme activity of the immune-captured acid  $\alpha$ -glucosidase was determined by adding to each well 50  $\mu\text{L}$  of 10 g/L bovine serum albumin in phosphate-buffered saline, pH 7.4, and 50  $\mu\text{L}$  of 3.25 mmol/L 4-methylumbelliferyl  $\alpha$ -D-glucoside (Sigma-Aldrich) in 0.1 mol/L sodium acetate buffer, pH 4.0. The microtiter plate was shaken at room temperature for 5 min, sealed in a plastic bag, and then incubated for 24 h at 37 °C. The enzyme reaction was stopped by the addition of 100  $\mu\text{L}$ /well glycine buffer (200 mmol/L glycine–158 mmol/L sodium bicarbonate–146 mmol/L sodium hydroxide, pH 10.7). This mixture (200  $\mu\text{L}$  from each well) was added to 1.5 mL of glycine buffer. The tubes were mixed well, and the relative fluorescence of each tube was measured on the Perkin-Elmer LS-5 spectrophotofluorimeter, using the microflow cell and autosampling system. The instrument settings for excitation and emission wavelengths/slit widths were 366 nm/15 nm and 446 nm/15 nm, respectively. With each analysis, a point calibration of 4-methylumbelliferone (710 pmol; Koch-Light Laboratory, Ltd.) was run in triplicate. The enzyme activity of the blood-spot specimens was calculated by reference to the point calibration

and corrected for incubation time (24 h) and sample volume (3.6  $\mu\text{L}$ ).

#### ASSESSMENT OF BLOOD-SPOT INTEGRITY

To ensure that the absence or the reduction of acid  $\alpha$ -glucosidase activity was not attributable to adverse changes in blood spots during collection, storage, and transport to the laboratory for analysis, another related enzyme activity, chosen for its similar properties, was determined. Neutral  $\alpha$ -glucosidase activity at pH 6.0 was determined in the supernatant following immune-capture of the acid  $\alpha$ -glucosidase protein from blood-spot samples. After the capture step, 25  $\mu\text{L}$  of supernatant was placed into an empty well and mixed with 25  $\mu\text{L}$  of 1 g/L bovine serum albumin in phosphate-buffered saline, pH 7.4, and 50  $\mu\text{L}$  of 3.25 mmol 4-methylumbelliferyl  $\alpha$ -D-glucoside in 0.1 mol/L sodium acetate buffer, pH 6.0. After incubation in a sealed plastic bag for 24 h, the wells were processed in the same way as the enzyme capture wells.

Blood spots from the adult control population ( $n = 82$ ) were used to determine the median and the range for the neutral  $\alpha$ -glucosidase activity at pH 6.0. In addition, the stability of the blood spots was evaluated by analyzing the effects of high temperature (70  $^{\circ}\text{C}$ ) and humidity (95%) for various periods over 24 h on the acidic and neutral  $\alpha$ -glucosidase activities in blood spots

### Results

#### IMMUNE-CAPTURE ASSAY FOR ACID $\alpha$ -GLUCOSIDASE

The proportion of enzyme captured by the antibody-coated plates was measured as  $>95\%$  by immunoquantification of the uncaptured protein. The specific activity of the captured enzyme was determined to be 38.9  $\mu\text{mol} \cdot \text{h}^{-1} \cdot \text{mg}^{-1}$ , which was 29% of the enzyme in free solution (134  $\mu\text{mol} \cdot \text{h}^{-1} \cdot \text{mg}^{-1}$ ; results not shown).

Using blood-spot calibrators containing 0–200  $\mu\text{g/L}$  recombinant acid  $\alpha$ -glucosidase, we observed a linear response over the biological range (Fig. 1). The stability of the enzyme in dry blood spots was investigated by determining the median activity in sets of 16 newborn blood spots stored for 1, 4, 12, 26, and 52 weeks. The activity was determined to be stable for at least 12 weeks; beyond that a decrease in the average activity of 30% was observed in blood spots stored for 26 and 52 weeks. Precision studies were conducted using two blood-spot calibrators (25 and 200  $\mu\text{g/L}$ ) and an adult blood-spot sample (60  $\mu\text{g/L}$ ). Interassay CVs were 16%, 15%, and 17%, respectively, at 25, 200, and 62  $\mu\text{g/L}$ , based on 20 observations over 30 days. Intraassay CVs based on 24 observations were 14%, 12%, and 15%, respectively, for the blood-spot samples used for the precision studies.

#### ACID $\alpha$ -GLUCOSIDASE ACTIVITY IN BLOOD SPOTS FROM CONTROL POPULATIONS, POMPE PATIENTS, AND CARRIERS

The median concentration of acid  $\alpha$ -glucosidase protein in the newborn population ( $n = 96$ ) was 75.9  $\mu\text{g/L}$  with 5th

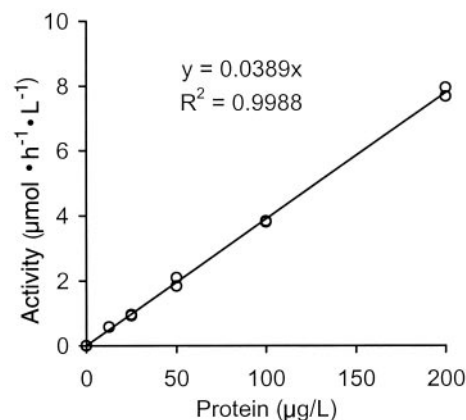


Fig. 1. Relationship between enzyme activity and acid  $\alpha$ -glucosidase protein concentration in blood spots.

The enzyme activity in acid  $\alpha$ -glucosidase blood-spot calibrators was determined by the immune-capture assay as described in *Materials and Methods*. A linear response was observed at acid  $\alpha$ -glucosidase protein concentrations of 0–200  $\mu\text{g/L}$ .

and 95th percentiles of 29.3 and 143.3  $\mu\text{g/L}$ , respectively. The corresponding median enzyme activity of 96 blood spots was 3.5  $\mu\text{mol} \cdot \text{h}^{-1} \cdot \text{L}^{-1}$  with 5th and 95th percentiles of 1.5 and 7.0  $\mu\text{mol} \cdot \text{h}^{-1} \cdot \text{L}^{-1}$ , respectively (Fig. 2). The median concentration of the acid  $\alpha$ -glucosidase protein in the adult control population was 31.7  $\mu\text{g/L}$  with 5th and 95th percentiles of 15.4 and 54.3  $\mu\text{g/L}$ , respectively. The corresponding median enzyme activity from duplicate blood spots was 1.3  $\mu\text{mol} \cdot \text{h}^{-1} \cdot \text{L}^{-1}$  with 5th and 95th percentiles of 0.54 and 2.5  $\mu\text{mol} \cdot \text{h}^{-1} \cdot \text{L}^{-1}$ , respectively (Fig. 2). The median neutral  $\alpha$ -glucosidase activity in adult control blood spots ( $n = 82$ ) was 24  $\mu\text{mol} \cdot \text{h}^{-1} \cdot \text{L}^{-1}$  with 5th and 95th percentiles of 14.3 and 52.9  $\mu\text{mol} \cdot \text{h}^{-1} \cdot \text{L}^{-1}$ , respectively. The acid  $\alpha$ -glucosidase protein concentration and enzyme activity were measured in dry blood spots from 17 individuals with Pompe disease and 3 obligate heterozygotes (Fig. 2 and Table 1). The concentrations of acid  $\alpha$ -glucosidase protein in 2 of the 17 Pompe patients were within the range obtained for adult controls, whereas all Pompe patients had enzyme activities well below those of the adult control population. The Pompe obligate heterozygotes had protein concentrations and enzyme activities in the lower quartile of the adult control population ranges (Table 1).

A significant correlation was observed between acid  $\alpha$ -glucosidase protein concentration and activity in blood spots from the newborn control population ( $r = 0.813$ , Pearson correlation; Fig. 3A). Similar results were evident in the blood spots of the adult control population ( $r = 0.682$ , Pearson correlation; Fig. 3B). However, no significant correlation was evident between acidic and neutral  $\alpha$ -glucosidase activity.

When quality-control blood spots were treated for periods up to 24 h at either 70  $^{\circ}\text{C}$  (dry oven) or 37  $^{\circ}\text{C}$  with 95% humidity (cell culture incubator), we observed a decreasing trend in neutral  $\alpha$ -glucosidase activity to give final activities that were 66% and 48%, respectively, of the

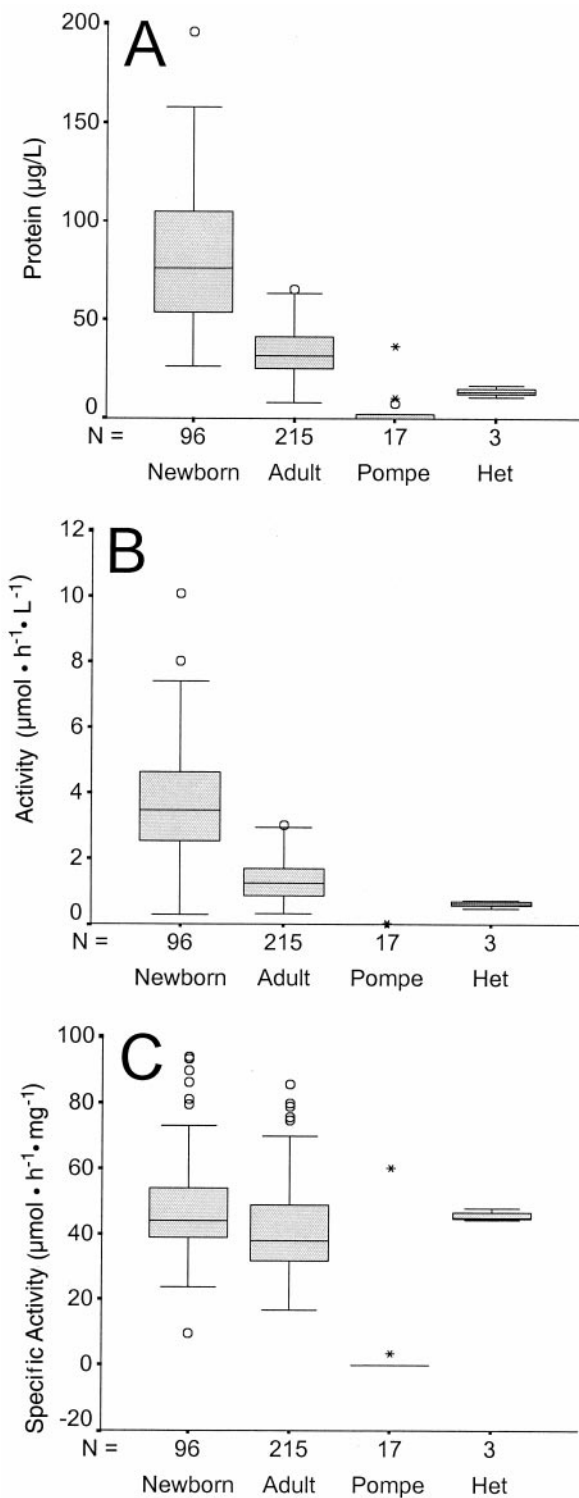


Fig. 2. Acid  $\alpha$ -glucosidase in blood spots from the newborn and adult control populations, Pompe-affected individuals, and heterozygotes.

The concentration of immune-captured acid  $\alpha$ -glucosidase protein (A) in blood spots and enzyme activity from duplicate blood spots (B) were determined as described in *Materials and Methods*. Specific activity was expressed as a ratio of enzyme activity to acid  $\alpha$ -glucosidase protein concentration (C). n = number of samples in each group. Central bars show the median value for each group, shaded areas show the 25th and 75th percentiles, and top and bottom bars show the limits of the range.  $\circ$  and \* represent outliers and extreme outliers, respectively. Het, obligate heterozygote.

**Table 1. Acid  $\alpha$ -glucosidase protein and activity in blood spots from controls, Pompe-affected individuals, and heterozygotes.**

Disorder	Onset	Protein concentration, $\mu\text{g/L}$	Enzyme activity, $\mu\text{mol} \cdot \text{h}^{-1} \cdot \text{L}^{-1}$
Newborn control (n = 96)		75.9 <sup>a</sup> (26–196)	3.5 <sup>a</sup> (0.3–10)
Juvenile control (n = 12)		23.4 <sup>a</sup> (16.6–44.5)	0.8 <sup>a</sup> (0.4–2.5)
Adult control (n = 215)		31.7 <sup>a</sup> (8.1–65)	1.3 <sup>a</sup> (0.3–3.0)
Heterozygote	Adult <sup>b</sup>	13.6	0.65
Heterozygote	Adult <sup>b</sup>	10.5	0.47
Heterozygote	Adult <sup>b</sup>	16.6	0.74
Pompe	Infantile	36.6	0
Pompe	Juvenile	10.1	0
Pompe	Juvenile	0	0
Pompe	Juvenile	0	0
Pompe	Adult	0.6	0
Pompe	Adult	0	0
Pompe	Adult	0	0
Pompe	Adult	0	0
Pompe	Adult	0.8	0
Pompe	Adult	0	0
Pompe	Adult	1.9	0
Pompe	Adult	0	0
Pompe	Adult	0	0
Pompe	Adult	0.6	0.04
Pompe	Adult	0.04	0
Pompe	Adult	2.0	0
Pompe	Adult	7.2	0

<sup>a</sup> Median (range) for newborn, juvenile, and adult populations.

<sup>b</sup> Heterozygotes were adult when blood-spot samples were taken.

original values after 24 h. In comparison, acid  $\alpha$ -glucosidase activity decreased to 79% and 67%, respectively, of the original values, indicating a greater stability of the acid  $\alpha$ -glucosidase activity relative to neutral  $\alpha$ -glucosidase.

**Discussion**

This is the first reported study to investigate acid  $\alpha$ -glucosidase activity in dried-blood spots as a potential screening/diagnostic marker for Pompe disease. In an earlier study we reported that it is feasible to reliably measure acid  $\alpha$ -glucosidase protein concentrations in either dried-blood spots or plasma. In addition, we demonstrated a good correlation between the absence or reduced concentrations of acid  $\alpha$ -glucosidase protein and the incidence of Pompe disease (25). However, some patients (3 of 17), including both infants and adults, showed substantial acid  $\alpha$ -glucosidase protein concentrations in blood spots. This is thought to result from specific mutations that lead to the absence of, or a marked reduction in, the catalytic capacity of the mutant enzyme, although normal processing is not affected (1). Thus, it was thought that the determination of acid  $\alpha$ -glucosidase activity in dried-

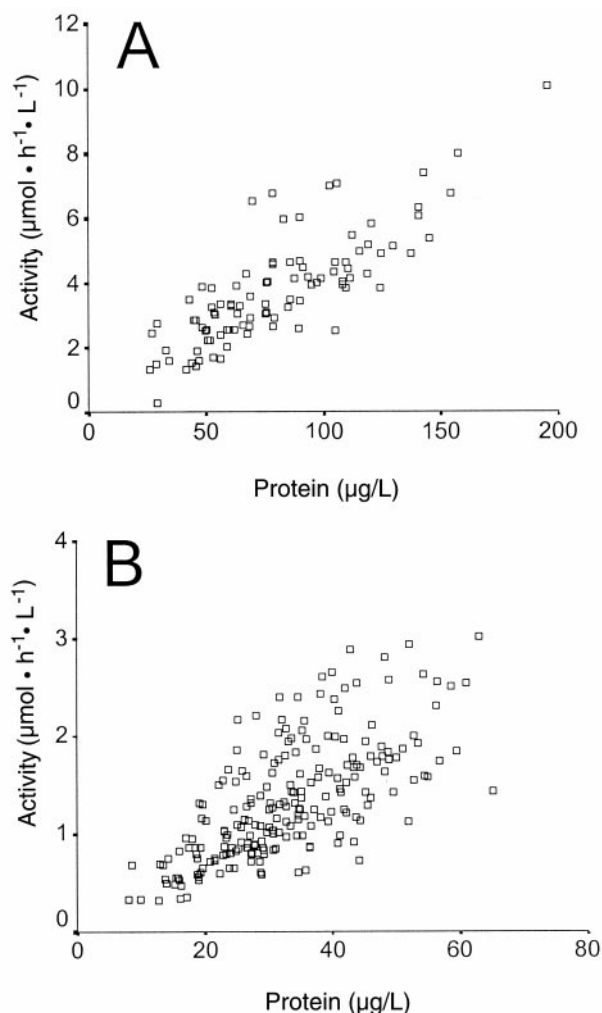


Fig. 3. Relationship between acid  $\alpha$ -glucosidase protein and enzyme activity in blood spots from newborn and adult control populations.

The concentration of immune-captured acid  $\alpha$ -glucosidase protein (A) in blood spots and enzyme activity from duplicate blood spots (B) were determined as described in *Materials and Methods*. There was a significant positive correlation of 0.813 (Pearson correlation) between the protein concentration of acid  $\alpha$ -glucosidase enzyme and its activity in blood spots from the newborn control population (A). Similar results were evident in the blood spots of the adult population, with a significant Pearson correlation of 0.682 (B).

blood spots may provide a more sensitive and specific marker for the identification of Pompe disease.

The immune-capture assay was demonstrated to capture >95% of acid  $\alpha$ -glucosidase protein from blood spots; however, the activity of the captured enzyme was ~29% of the activity of the free or uncaptured protein. This reduction in enzyme activity of the immune-captured protein is thought to be the result of steric restriction produced by interaction between the fixed enzyme and the 4-methylumbelliferyl  $\alpha$ -D-glucoside substrate, or alternatively, the polyclonal antibody may have an inhibitory effect on the enzyme activity.

The ability to validate blood-spot integrity is an important factor in an assay where samples may be collected in different areas and transported by different means. The

use of neutral  $\alpha$ -glucosidase activity to validate blood-spot integrity is justified given that the acid  $\alpha$ -glucosidase activity appears to be slightly more stable to extremes of temperature and humidity than the neutral  $\alpha$ -glucosidase. Consequently, any adverse conditions should be reflected in the activity of neutral  $\alpha$ -glucosidase before acid  $\alpha$ -glucosidase. The lower end of the adult control range ( $n = 82$ ) for neutral  $\alpha$ -glucosidase activity was  $12 \mu\text{mol} \cdot \text{h}^{-1} \cdot \text{L}^{-1}$  and has been set as the minimum acceptable value in our laboratory.

In this study, we observed significant differences in both the protein concentration and enzyme activity between the newborn and adult populations, with the newborn population having protein concentrations and enzyme activities ~2.5-fold higher than the adult population. Both the protein concentration and the enzyme activity in blood spots from juveniles ( $n = 12$ ) were within the adult range. The specific activity of the enzyme was similar in both the newborn and adult populations (median values, 44 and  $38 \mu\text{mol} \cdot \text{h}^{-1} \cdot \text{mg}^{-1}$ , respectively; Fig. 2C), indicating that similar isoforms of the acid  $\alpha$ -glucosidase protein may be involved in both populations. This is further supported by the significant correlations between the acid  $\alpha$ -glucosidase protein concentration and enzyme activity in each population (Fig. 3).

The wider range of enzyme activity observed in the newborn control population compared with the juvenile and adult populations (Fig. 2B and Table 1) is thought to result from the higher concentrations of leukocytes present in whole blood collected from newborns. The different collection methods used for the newborn and adult controls were evaluated and shown to have no effect on the enzyme activity. In contrast to the wider range of enzyme activity observed in the newborn population, the lower range in each population was approximately the same, which would produce similar cutoff values for identification of affected individuals.

The use of acid  $\alpha$ -glucosidase protein as a potential marker for the diagnosis of Pompe patients yielded a sensitivity and specificity of 88% and 100%, respectively, when we used the adult control range. In comparison, acid  $\alpha$ -glucosidase activity yielded 100% sensitivity and 100% specificity. Of the 17 patient samples used in this study, 16 had no detectable enzyme activity in blood spots, with 1 sample having  $0.04 \mu\text{mol} \cdot \text{h}^{-1} \cdot \text{L}^{-1}$ , substantially less than the lower end of the control range ( $0.3 \mu\text{mol} \cdot \text{h}^{-1} \cdot \text{L}^{-1}$ ). However, a larger cohort of newborn controls will be required for validation of this assay as a mass screening procedure.

In conclusion, this study demonstrates that it is possible to identify Pompe disease/patients on the basis of the enzyme activity of acid  $\alpha$ -glucosidase in dried-blood spots. This method is simple, inexpensive, and noninvasive compared with existing diagnostic methods and may also have application in newborn screening for Pompe disease.

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