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Fabry disease urinary globotriaosylceramide/creatinine biomarker evaluation by liquid chromatography–tandem mass spectrometry in healthy infants from birth to 6 months

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ABSTRACT

Fabry disease is an X-linked lysosomal storage disorder caused by deficiency of alpha-galactosidase A, resulting in accumulation of the principal substrate, globotriaosylceramide (Gb_3), in various physiological fluids and tissues in affected patients. The recognition that accumulation of Gb_3 begins *in utero*, combined with the fact that the diagnosis of the disease is often delayed until after the development of irreversible tissue damage, has generated pressure to develop techniques for the early, pre-symptomatic diagnosis of the disease. Measurements of urinary Gb_3 have been shown to be useful for the diagnosis of Fabry disease in adults. The objective of this work was to measure the Gb_3/creatinine biomarker in urine of healthy infants from birth to 6 months, including the establishment of reference ranges for urinary Gb_3 excretion at various postnatal ages, in male and female infants. We employed liquid chromatography–tandem mass spectrometry (LC–MS/MS) to determine Gb_3/creatinine ratios in urine specimens dried on filter paper and mailed to the laboratory by participating parents. A total of 728 urine specimens were obtained at intervals from birth to 6 months of age from 68 healthy infants (35 male and 33 female). Parental participation was good, with 90% of the expected specimens received by the laboratory. The results of the analyses were grouped by the age of the infants into four periods. We have determined that both postnatal age and sex have an effect on urinary Gb_3 excretion levels which vary considerably in newborns. We conclude that screening for Fabry disease by measurement of urinary Gb_3 excretion is unlikely to be reliable before 30 days of age.

Introduction

Fabry disease (OMIM 301500) is an X-linked inherited lysosomal storage disorder caused by deficiency of alpha-galactosidase A (α-Gal A; EC 3.2.1.22) activity, resulting in glycosphingolipid accumulation, mainly globotriaosylceramide (Gb_3, or GL-3 or CTH), in various tissues and physiological fluids [1,2]. Accumulation of Gb_3 has been shown to begin *in utero* [3,4], but clinical manifestations of the disease generally do not develop until later in childhood, with acroparesthesias, abdominal pain and diarrhea; proteinuria, an indicator of significant renal damage, may develop as early as 10 years of age [5–7], underscoring the importance of early diagnosis and initiation of treatment [8–12]. Fabry disease is probably underdiagnosed because of the great variability in clinical symptoms and its phenotypic heterogeneity. The long delay from onset of symptoms to confirmation of a diagnosis averages 14 years in men and 16 years in women [8].

The establishment of enzyme replacement therapy (ERT) for Fabry disease in 2001 [2,13,14] has had a significant impact on the otherwise relentlessly progressive nature of the disease [15,16]. However, the earlier development of irreversible end organ damage, before the initiation of ERT, has led to the belief that optimum results are only achievable if treatment is started earlier, before the development of overt clinical symptoms of the disease.

A methodology for high-risk screening based on the measurement of urinary Gb_3 in adults and children older than 3 years with Fabry disease was previously shown by our team using liquid chromatography–tandem mass spectrometry (LC–MS/MS) [17–20] to be an efficient and rapid method for the detection of most Fabry hemizygotes and heterozygotes. The advantages of this methodology are numerous: the use of filter paper for the collection of urine specimens, which can be transported to laboratories by regular post, eliminates time-consuming and labor-intensive extraction, centrifugation and evaporation steps, facilitates urine collection by parents or patients, and reduces costs.

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The non-invasive and innovative methodology developed for high-risk screening for Fabry disease enabled us to measure urinary Gb_3/creatinine ratios in urine specimens collected and dried onto filter paper and establish normal values for adults and children 3–17 years of age [17,18]. However, no such reference values existed for infants less than 12 months of age, making the use of the technique for the identification of infants affected with pre-symptomatic Fabry disease uncertain. Our past experience with a young child (unpublished results) who was studied at 21 days of age was found to have urinary Gb_3/creatinine levels close to five times normal, based on reference values established for older children. The child was subsequently found to have a pseudo-deficiency mutation (D313Y) for Fabry disease [21], raising important questions about the significance of the finding. Was the increased urinary Gb_3 excretion due to the effects of the pseudo-deficiency mutation, or to the fact that all newborns excrete increased quantities of Gb_3 in the first few weeks of life? It became particularly important to answer this question in order to evaluate the feasibility of neonatal urine screening for Fabry disease. Moreover, high-risk screening for Fabry disease in a family affected by the disease often leads to cascade screening and analysis of urinary Gb_3 in infants and siblings for the purposes of genetic counseling, which raises ethical issues for later-onset diseases and carrier detection.

In this paper, we have measured the Gb_3/creatinine excretion in the urine of healthy infants from birth to 6 months of life and thereby, examined the effect of postnatal age and sex on urinary Gb_3 excretion, as determined by LC–MS/MS.

Materials and methods

This project was approved by the Research Ethics Board of the Faculty of Medicine and Health Sciences and the Centre Hospitalier Universitaire de Sherbrooke (CHUS).

Study subjects

Recruitment of participants started at CHUS in February 2008 ending in April 2008. Couples whose newborns experienced an uneventful delivery and post-partum evolution were chosen at random with the help of the nursing staff to participate in this project. Great care was taken during the recruitment process to explain the role of parents in collecting urine samples from their babies, with a demonstration of the collection procedure, over the next 6 months with emphasis on the voluntary participation of parents. A follow-up reminder telephone call was made to parents each sampling day in order to maximize compliance.

Inclusion criteria

In order to be included in the study, babies had to be full-term and normal at birth, without any anomalies or health problems related to the delivery process; participation of parents was voluntary, after receiving information and response to their questions; parents had to be able to perform 13 urine collections over a 6 month period; parents had to be able to read and sign the consent form.

Exclusion criteria

Babies were excluded from the study if: they had any type of anomaly or confirmed inborn error of metabolism, malformation, other health problems or birth weight less than 2500 g; babies experienced problems related to the delivery process; parents were unable to give an informed consent; parents failed to collaborate with the urine collection schedule.

Study protocol

Urine samples were obtained at postnatal ages 2, 3, 4, 6, 10, 14, 21 and 28 days, then at 2, 3, 4, 5 and 6 months. Urine was collected on Whatman 903 filter paper by parents according to previously published methodologies [17–19,22]. The urine was left to dry overnight in the open, placed in ordinary paper envelopes, and returned by regular postal service to the mass spectrometry laboratory.

Parents' compliance in sending the urine sample of their babies from birth to 6 months of age was an important issue for the success of this research project. The protocol had to be simple and worry-free for them. Coded (alphanumeric codes) kits for each baby were prepared before recruitment started, comprising: 13 pre-stamped and pre-addressed, coded envelopes, each containing two filter papers, two absorbent pads with a plastic shield (sponsored by private companies, such as Cascades Inc., to assist them in collecting the urine using ultra-absorbent diapers), urine collection schedule and instructions, a copy of the consent form and our coordinates to reach us. A total of 988 kits were prepared for the entire research project.

Processing and analysis of urinary Gb_3 and creatinine

Processing of samples, liquid chromatography and mass spectrometry parameters, and resulting quantification method were performed according to previously published methods [17–19]. In summary, for each urine filter paper to be analyzed, a 5-cm diameter filter paper disc was punched out. Internal standards were added to the filter papers: $C_{17:0}$ for Gb_3 and d3-creatinine for creatinine. Elution was performed by rotary shaking filter papers with 4 ml of methanol in glass vials for 60 min, followed by homogenization of eluates for 30 s (to break up cells and extract Gb_3). Ten microliters were injected in the LC–MS/MS using a Quattro micro tandem quadrupole instrument (Waters Micromass, Manchester, UK) with electrospray ionization operated in positive-ion-mode (ES+). Waters QuanLynx software was used to quantify Gb_3/creatinine data.

Statistical analysis

The sample size large enough to compare different parameters and obtain statistically significant data over different time periods with a power of at least 80% was estimated to require 25 participants for each sex, for each postnatal period studied. In order to ensure an adequate sample size in the event of withdrawals, we recruited a total of 76 participants, 39 boys and 37 girls.

We divided the 6-month period of the study into four time periods according to homogeneity within each period: Period 1 = less than 6 days of age; Period 2 = from 6 to 29 days; Period 3 = from 30 to 90 days; and Period 4 = more than 90 days. We pooled the data for each period according to sex, using the proc univariate procedure of SAS (Statistical Analysis System, version 9.1.3, SAS Institute Inc., Cary, NC, USA). The variable of Gb_3/creatinine was transformed into logarithms ($1 + Gb_3$/creat.) because the distributions of the raw data were skewed; the distribution of the transformed data was normal. We performed the ANOVA with repeated measures on the transformed data using the proc mixed procedure of the Statistical Analysis System (SAS) for comparison analysis for means of log ($1 + Gb_3$/creatinine) values for each cohort and for each period studied. The independent variables were the sex of the babies and time periods. For description, it is order Statistics (median, minimum and maximum) of raw data of Gb_3/creatinine which are presented. We used the SPSS software (version 16.0 for Windows) to produce the cumulative sum plots for Gb_3/creatinine levels for each child.