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Urinary Lipid Profiling for the Identification of Fabry Hemizygotes and Heterozygotes

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Background: **Fabry disease is an X-linked lysosomal storage disorder resulting from a deficiency of the lysosomal hydrolase, α-galactosidase, for which enzyme replacement therapy is now available. In this study, we aimed to identify Fabry heterozygotes not only for genetic counseling of families but because it is becoming increasingly obvious that many heterozygous (carrier) females are symptomatic and should be considered for treatment.**

Methods: **We measured 29 individual lipid species, including ceramide, glucosylceramide, lactosylceramide, and ceramide trihexoside, in urine samples from Fabry hemizygotes and heterozygotes and from control individuals by electrospray ionization tandem mass spectrometry. Individual analyte species and analyte ratios were analyzed for their ability to differentiate the control and patient groups.**

Results: **The Fabry hemizygotes had increased concentrations of the substrate for the deficient enzyme, ceramide trihexoside, as well as lactosylceramide and ceramide, along with decreased concentrations of both glucosylceramide and sphingomyelin. Ratios of these analytes improved differentiation between the control and Fabry groups, with the Fabry heterozygotes generally falling between the Fabry hemizygotes and the control group.**

Conclusions: **These lipid profiles hold particular promise for the identification of Fabry individuals, may aid in the prediction of phenotype, and are potentially useful for the monitoring of therapy in patients receiving enzyme replacement.**

Fabry disease is an X-linked inborn error of glycosphingolipid metabolism that is attributable to a deficiency of the lysosomal hydrolase, α-galactosidase *(1)*. This deficiency leads to the accumulation of ceramide trihexoside (CTH)[4] and related glycosphingolipids with terminal α-galactosyl residues in the tissues and body fluids, including heart, liver, kidney, vascular endothelial cells, and plasma *(2)*. Classically affected hemizygous males suffer from acroparesthesias, angiokeratoma, and occlusive vascular disease of the kidney, heart, or brain. "Cardiac" and "renal" variants have been described, which present with late-onset manifestations primarily limited to the heart *(3)* or kidney *(4)*, respectively. Heterozygous females have a more variable expression because of random X chromosome inactivation, with some patients experiencing few or mild symptoms and others developing skin, ocular, and renal manifestations, premature strokes, and myocardial infarctions, although these usually occur later in life than in affected males *(5)*.

In the absence of a family history of Fabry disease, diagnosis is typically made on the basis of clinical suspicion and by the demonstration of reduced or no α-galactosidase activity in blood, leukocytes, or cultured skin fibroblasts *(6)*. To date, more than 200 Fabry disease-causing mutations have been described. Most mutations are private (unique to each family) with the exception of a few common ones, thus precluding genotype/phenotype correlations in most cases. Although clinical onset usually occurs in childhood, disease presentation can be subtle and often mistakenly attributed to other disorders *(2)*. As a result, diagnosis of a patient may not occur until

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[4] Nonstandard abbreviations: CTH, ceramide trihexoside; LC, lactosylceramide; GC, glucosylceramide; Cer, ceramide; PC, phosphatidylcholine; ESI-MS/MS, electrospray ionization tandem mass spectrometry; and SM, sphingomyelin.

the third decade of life, when the pathology of the disorder is often quite advanced *(7)*. Identifying female heterozygotes with this X-linked condition is more complicated. The range of enzyme activity in Fabry carriers is broad, and differentiation from unaffected individuals is not always clear. Furthermore, mutation detection can be time-consuming and may not be informative. The identification of heterozygotes has profound implications for genetic counseling of affected families, particularly because female carriers can display severe symptoms of the disease *(8)*.

Until recently, treatment for patients with Fabry disease was limited to symptomatic management of pain and the end-stage complications of renal failure, cardiac disease, and stroke. However, the use of recombinant α-galactosidase has been demonstrated to be an effective means of therapy to treat Fabry patients and is now well established within the clinical environment *(9)*. The recent development of enzyme replacement therapy for Fabry disease has led to the need to identify biomarkers of this disorder for monitoring response to therapy and for prediction of disease progression, which could lead to earlier commencement of therapy. Heterozygotes should also be scrutinized for clinical manifestations of Fabry disease because consideration should be given to offering enzyme replacement therapy to females before, or early in, the disease process.

The emerging postgenomic science of metabolomics is concerned with detecting global changes in the distributions and concentrations of metabolites and offers the possibility of characterizing surrogate profiles of disease. In this study, we established a profile of sphingolipid species in the urine of Fabry hemizygotes and heterozygotes and in unaffected controls. This approach has enabled Fabry hemizygotes and heterozygotes to be accurately identified and shows particular promise for improved clinical assessment of Fabry disease and related inherited metabolic disorders.

Materials and Methods

PATIENT SAMPLES AND REAGENTS

Urine samples from Fabry hemizygotes and heterozygotes were provided through the Australian Fabry Support Group and stored at −20 °C. Samples from healthy control individuals were obtained with informed consent. All solvents were of HPLC grade and were used without further purification. *N*-Palmitoyl-*d3*-lactosylceramide [LC C16:0 (*d3*)] and *N*-palmitoyl-*d3*-galactosylceramide [GC C16:0 (*d3*)] were from Matreya Inc., *N*-heptadecanoyl-D-erythro-sphingosine (Cer C17:0) was from Avanti Polar Lipids, phospatidylcholine (PC C28:0) was from Sigma Chemical Co., and CTH C17:0 was synthesized as described previously *(10)*.

EXTRACTION OF LIPIDS

Urinary lipids were extracted according to the method of Bligh and Dyer *(11)*. Each 1.5-mL aliquot of urine was extracted with 5.6 mL of chloroform–methanol (1:2 by volume) containing 400 pmol of each of the following internal standards: LC C16:0 (*d3*), GC C16:0 (*d3*), Cer C17:0, CTH C17:0, and PC C28:0. The mixture was shaken for 10 min and then allowed to stand at room temperature for an additional 50 min. The samples were partitioned with the addition of 1.9 mL each of chloroform and water and shaken for 10 min. To facilitate phase separation, the mixture was centrifuged (3000*g* for 2 min), and the upper phase was removed by aspiration. The lower phase was washed with 1.0 mL of Bligh and Dyer synthetic upper phase, mixed, and then centrifuged (3000*g* for 2 min). The upper phase was removed by aspiration, and the lower chloroform layer containing the glycolipids was dried under a gentle stream of nitrogen at 50 °C. The glycolipid extract was reconstituted in 150 μL of methanol containing 10 mmol/L ammonium formate.

MASS SPECTROMETRY

Sphingolipids were analyzed by electrospray ionization tandem mass spectrometry (ESI-MS/MS) on a PE Sciex API 3000 triple-quadrupole mass spectrometer with a turbo-ionspray source (200 °C) and Analyst 1.1 data system. Samples (20 μL) were injected into the electrospray source with a Gilson 233 autosampler, with methanol as the carrying solvent at a flow rate of 80 μL/min. N_2 was used as the collision gas at a pressure of 2×10^{-5} Torr. Molecular species of GC, LC, Cer, and CTH were identified by precursor ion scanning for mass-to-charge ratio (*m/z*) 264, and molecular species of sphingomyelin (SM) and PC were identified by precursor ion scanning for *m/z* 184 in positive-ion mode. Semiquantification of individual GC, LC, Cer, CTH, SM, and PC species was performed in multiple-reaction monitoring mode with the ion pairs shown in Table 1. Each ion pair was monitored for 100 ms with a resolution of 0.7 atomic mass units at half-peak height and averaged from continuous scans over the injection period. Lipid concentrations were calculated by relating the peak height of each species to the peak height of the corresponding internal standard, with the SM species being related to the PC C28:0.

STATISTICAL ANALYSIS

The Mann–Whitney *U*-test was used to determine the difference between control and patient groups for each analyte.

Results

ASSAY PERFORMANCE

We assessed the recovery of urinary lipids by adding representatives of each lipid type to control urine. The recovery of each of the lipids from the urine was between 79% and 87%. We determined the limit of detection for each lipid type by adding to a control urine a deuterated or nonphysiologic species of lipid and determining the concentration that gave a signal three times higher than the signal for the urine alone. The calculated limits of

detection in urine were 2.7 nmol/L for Cer, CTH, and PC; 5.4 nmol/L for GC; and 10.8 nmol/L for LC. We determined the linearity of the response for each lipid type over the assay range by adding increasing concentrations of each lipid type to a control urine sample. The ratios of the signals for the lipid species to the signals for the internal standards were then plotted against the concentration of added lipid. All lipid types (Cer, GC, LC, CTH, SM, and PC) gave linear responses (R^2 >0.99) over the range 2.7–2700 nmol/L.

To assess the reproducibility of the mass spectrometric analysis of the sphingolipids, we determined intraassay and interassay CVs, using a Fabry urine sample. The intraassay CVs (n = 10) were 9%, 8%, 5%, and 7% for CTH C24:1, LC C24:1, SM C22:0, and GC C24:0, respectively, and the interassay CVs (12 measurements by three operators over 5 days) were 12%, 13%, 13%, and 20% for CTH C24:1, LC C24:1, SM C22:0, and GC C24:0, respectively. The concentrations of the individual lipid species in the urine sample were 2.4 μmol/L CTH C24:1, 131 nmol/L LC C24:1, 65 nmol/L SM C22:0, and 11 nmol/L GC C24:0.

GLYCOLIPID ANALYSIS

We measured a total of 29 lipid species, including CTH, GC, LC, Cer, SM, and PC. The concentration of each of the lipid species was related to the total PC concentration. We determined the total PC concentration by summing eight individual molecular species of PC. As shown in Table 1, the concentrations of several lipid species differed significantly between the control and patient groups, with the

Table 1. Relative concentrations and Mann–Whitney *U* values for lipid analytes in urine from controls and Fabry heterozygotes and hemizygotes.

		Mean (SD) relative concentrations[b]			Mann–Whitney *U* values[c]	
Analytes	MRM,[a] *m/z* values	Controls (n = 24)	Heterozygotes (n = 13)	Hemizygotes (n = 15)	Controls vs heterozygotes	Controls vs Fabry patients
Cer C16:0	538.7/264.4	30 (34)	32 (15)	43 (24)	95	73[d]
Cer C24:0	650.7/264.4	22 (16)	14 (5)	26 (14)	90	107
Cer C24:1	648.7/264.4	10 (7)	8 (3)	21 (14)	141	60[d]
GC C16:0	700.6/264.4	50 (56)	35 (13)	44 (27)	133	152
GC C22:0	784.7/264.4	48 (31)	26 (18)	36 (15)	62[d]	109
GC C24:0	812.7/264.4	41 (17)	27 (16)	33 (14)	63[d]	119
GC C24:1	810.8/264.4	18 (10)	13 (6)	18 (8)	89	148
LC C16:0	862.4/264.4	160 (84)	540 (419)	458 (424)	37[e]	63[d]
LC C20:0	918.7/264.4	133 (104)	128 (103)	274 (144)	128	69[d]
LC C22:0	946.7/264.4	371 (342)	328 (318)	563 (288)	107	80[e]
LC C22:0-OH	962.7/264.4	145 (54)	168 (115)	533 (362)	125	71[g]
LC C24:0	974.8/264.4	100 (39)	204 (121)	618 (478)	62[d]	2[d]
LC C24:1	972.8/264.4	87 (37)	285 (206)	398 (220)	34[d]	2[d]
CTH C16:0	1024.8/264.4	131 (99)	296 (260)	2097 (1616)	87	35[d]
CTH C18:0	1052.7/264.4	98 (47)	102 (81)	888 (639)	126	33[d]
CTH C20:0	1080.9/264.4	130 (102)	143 (120)	1401 (1066)	128	35[d]
CTH C22:0	1108.9/264.4	99 (40)	281 (261)	3619 (2804)	68[d]	26[d]
CTH C24:0	1136.9/264.4	121 (47)	289 (256)	4484 (3623)	78[e]	11[d]
CTH C24:1	1134.9/264.4	86 (48)	338 (296)	3992 (3073)	43[d]	4[d]
SM C16:0	703.9/184.1	454 (184)	271 (112)	337 (80)	42[d]	70[d]
SM C22:0	787.8/184.1	386 (105)	224 (100)	226 (48)	47[d]	0[d]
SM C24:0	815.8/184.1	336 (111)	161 (104)	181 (53)	43[d]	4[d]
Cer (total)		61 (54)	53 (21)	90 (49)	119	82[e]
GC (total)		158 (110)	101 (50)	131 (61)	83[e]	129
LC (total)		996 (577)	1654 (952)	2844 (1510)	77[e]	26[d]
CTH (total)		665 (345)	1450 (1227)	16 481 (12 606)	97	19[d]
SM (total)		1175 (375)	656 (307)	744 (165)	42[d]	12[d]
CTH C24:1/SM C22:0		0.2 (0.1)	1.7 (1.8)	17.2 (12.7)	22[d]	0[d]
LC C24:1/GC C24:0		2.1 (0.8)	12.4 (9.5)	115.4 (81.6)	3[d]	0[d]
PC (total)		178 (96)	814 (834)	175 (104)		

[a] MRM, multiple-reaction monitoring. *m/z* values for internal standards and PC species were as follows: Cer C17:0, 552.7/264.4; GC (*d3*) C18:0 (703.8/264.4); LC (*d3*) C16:0, 865.6/264.4; CTH (*d3*) C16:0, 1027.6/264.4; PC C14:0, 678.5/184.1; PC C32:0, 734.7/184.1; PC C32:1, 732.7/184.1; PC C34:2, 758.5/184.1; PC C36:2, 786.6/184.1; PC C36:4, 782.6/184.1; PC C38:4, 810.8/184.1.

[b] All lipid analytes except PC (total) are reported as nmol/μmol PC; PC (total) is in nmol/L.

[c] Mann–Whitney *U* values were calculated for the control group compared with the heterozygous group and the control group compared with the Fabry group.

[d] P <0.01.

[e] P <0.05.

SM C22:0 species providing total discrimination between the Fabry hemizygotes and unaffected controls. The Fabry patients had increased median values of the total amount of CTH, LC, and Cer, whereas only LC was significantly increased in the heterozygotes ($P < 0.05$). SM concentrations were lower in the Fabry patients compared with the control group, whereas both SM and GC were lower in the heterozygotes than in the control group.

A selection of specific glycolipids that provide the best differentiation between the control and patient groups are shown in Fig. 1. The increases in Cer, LC, and CTH were greatest in the C24:1 species, and the decreases in GC and SM were greatest in the C22:0 species.

LIPID PROFILES

The finding that LC and CTH are increased and that GC and SM are decreased in the Fabry patients and/or heterozygotes enables relationships between these analytes to be assessed. A scatter plot of CTH C24:1 plotted as a function of SM C22:0 is shown in Fig. 2A, and LC C24:1 is plotted against GC C24:0 in Fig. 2B. Such plots enable better differentiation between the control and Fabry patient groups than do the plots of single analytes (Fig. 1). The GC C24:0 species was used in this plot as opposed to GC C22:0 simply because it was more abundant. Although not distinct, the heterozygous group generally fell between the Fabry patient and control group. A scatter plot of the ratio CTH C24:1/SM C22:0 against the ratio LC C24:1/GC C24:0 is shown in Fig. 2C. This profile enabled the complete differentiation of all three groups with the exception of one symptomatic heterozygous patient. Additionally, two Fabry individuals who had undergone renal transplants appeared at the lower end of the Fabry patient group. We then examined this permutation of sphingolipids in the Fabry hemizygotes according to their genotypes (Fig. 3). Three patients with the genotype delAA717-718 are clustered together, whereas three patients sharing the genotype M284T appear dispersed in this plot.

Discussion

The CTH concentration in plasma or urine has been used to follow disease progression and to monitor the efficacy of enzyme replacement in Fabry disease *(9, 12, 13)*. Nonetheless, the intralysosomal accumulation of one lipid (CTH) is likely to disrupt the flow of lipids in the endosome/lysosome pathway, leading to secondary disturbances of other glycolipids *(14)*. In Fabry patients, we noted not only an increase in the substrate for the deficient enzyme but also increases in LC and Cer as well as decreases in GC and SM. It has been reported that a fluorescent analog of LC accumulates in the lysosomes of cells from patients with Fabry disease as well as some other lipid storage disorders, but not in other lysosomal storage disorders or in controls *(15)*. This analog accumulated in the Golgi complex in healthy fibroblasts. No mechanism has been proposed for this, but the different trafficking and storage of this lipid analog might reflect disturbed functions of the lipid flow in the degradative endocytic pathway. We would suggest that the increases in LC and Cer and decreases in GC and SM observed in Fabry urine most likely reflect perturbed intracellular transport of lipids in renal epithelial cells.

In this study we used ESI-MS/MS to semiquantify several lipid species in urine. The absolute quantification of individual lipid species by MS requires either deuterated internal standards for each lipid species under investigation or the preparation of calibration curves for each species to calculate response ratios for the different acyl chain lengths. With the current lack of availability of deuterated and single-acyl-chain species for these lipid types, these are not practical approaches to this problem. To overcome this limitation, we first demonstrated the linearity of response for the different lipid types over the concentration range found in urine and then used these assays to semiquantify the lipid species. This enabled reproducible lipid determinations for a large number of individual species. The precision and accuracy of the mass spectrometric analysis were further validated by the intraassay and interassay CVs, which were <9% and 20%, respectively.

An initial statistical analysis was performed on the 19 lipid analytes, expressed as nmol/L of urine and nmol/μmol of creatinine (data not shown). For all analytes except CTH, the values for the heterozygous group were substantially increased above both the Fabry and control groups, whereas there was relatively little difference between the two latter groups. As shown in Table 1, the concentration of the total PC was higher in the heterozygotes than in either the control group or the hemizygotes, thus indicating greater urinary sediment in the former. PC is one of the most abundant cell membrane lipids; therefore, normalizing the urinary samples for total PC will effectively correct for the amount of urinary sediment. We have used PC to correct for the amount of urinary sediment in earlier studies *(16)*, and SM has also been proposed for this purpose *(17)*. However, because SM concentrations are potentially altered through the aberrations of lipid trafficking observed in lysosomal storage disorders *(14)*, we have continued to use PC as a representative cellular lipid to correct for the amounts of urinary sediment. In contrast to other low-molecular-weight analytes in urine, such as creatinine, sugars, and amino acids, which are the result of glomerular filtration, urinary lipids are derived from the urinary sediment. This cellular material results from the shedding of the internal lining of the renal tubuli and the urinary tract.

Whereas CTH reflects primary storage in Fabry disease, the measurement of other lipids in urine has enabled a lipid profile for Fabry disease to be constructed. The measurement of CTH on its own was not sufficient to identify all Fabry patients, although the SM C22:0 species did provide complete differentiation between the hemizygotes and the control group (Table 1). Furthermore, the

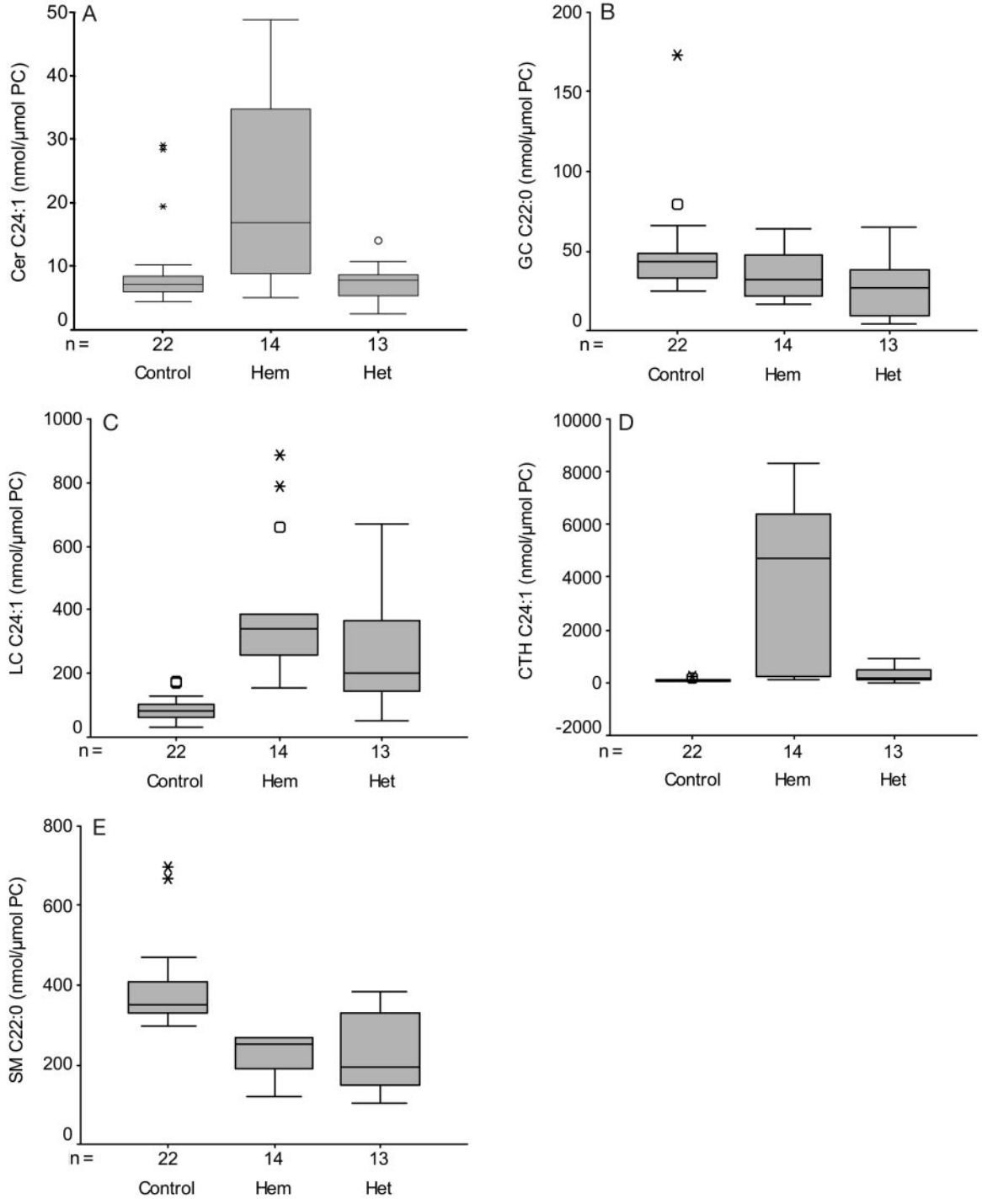

Fig. 1. Relative concentrations of individual lipid species in urine from Fabry hemizygotes (*Hem*), heterozygotes (*Het*), and control individuals.

Lipids were extracted by the method of Bligh and Dyer *(11)* and analyzed by ESI-MS/MS as described in *Materials and Methods*. The *line inside* each *box* indicates the median value for each group, *shaded areas* indicate the 25th and 75th percentiles, and the *top* and *bottom error bars* indicate the limits of the range. ○ and ∗ represent statistical outliers and extreme outliers, respectively. n = number of samples in each group.

use of lipid ratios (Fig. 2), in which analytes that are increased in the disease state are expressed as a ratio to analytes that are decreased, improved differentiation between controls and affected patients (Table 1). This approach enabled the complete differentiation of Fabry hemizygotes, heterozygotes, and controls. The best demarcation of the three groups was achieved by plotting the ratio CTH C24:1/SM C22:0 against LC C24:1/GC C24:0 (Fig. 2C). One anomaly was a Fabry heterozygote who had been diagnosed with clinical manifestations of

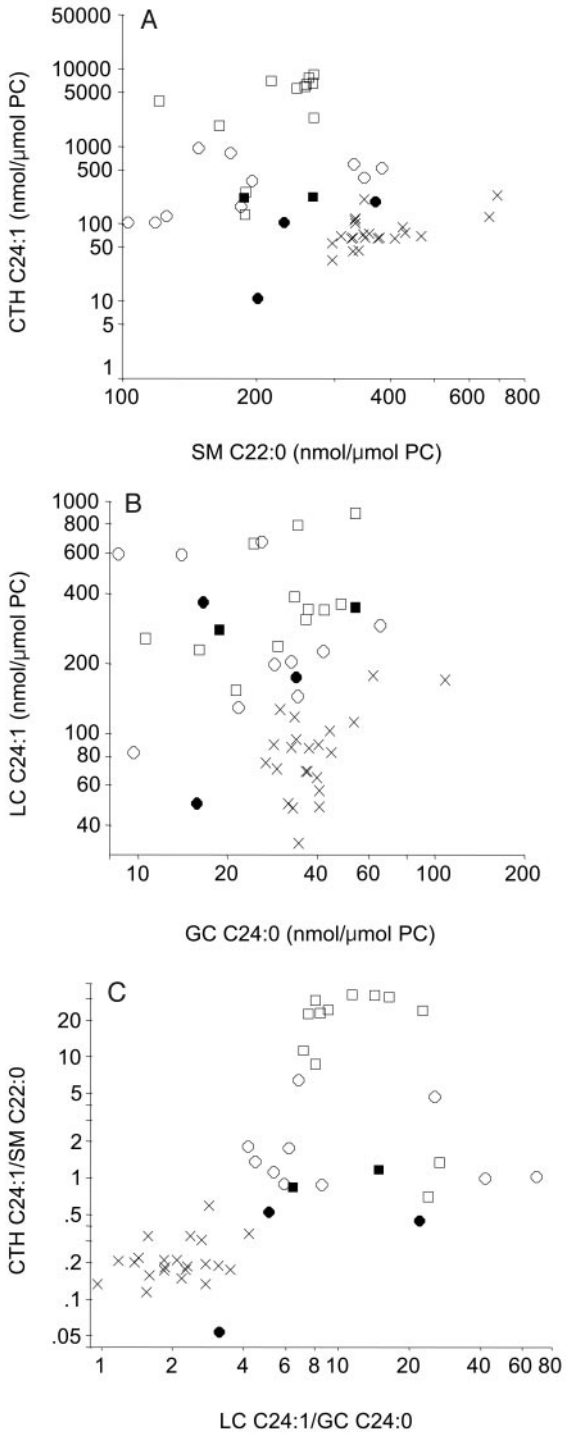

Fig. 2. Lipid ratios in urine from Fabry hemizygotes, heterozygotes, and controls.

Urine samples from controls (×), Fabry heterozygotes who had been diagnosed with clinical symptoms (●), Fabry heterozygotes for whom clinical details were unavailable (○), Fabry hemizygotes (□), and Fabry hemizygotes who had undergone renal transplant (■) were analyzed for individual lipid species by ESI-MS/MS. (*A*), CTH C24:1 plotted as a function of SM C22:0; (*B*), LC C24:1 plotted as a function of GC C24:0; (*C*), CTH C24:1/SM C22:0 ratio plotted as a function of LC C24:1/GC C24:0 ratio.

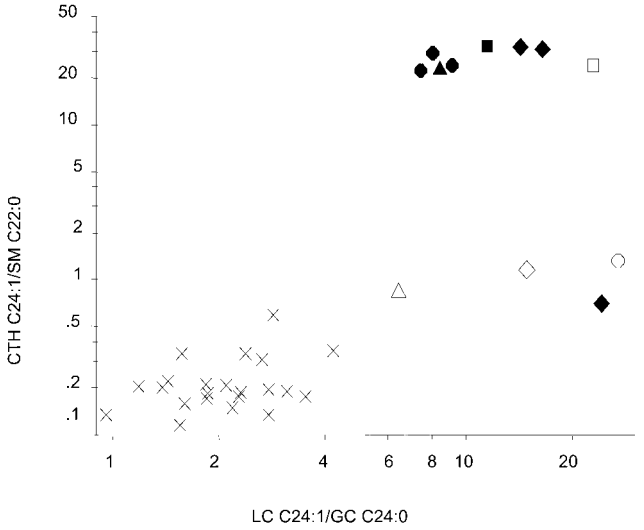

Fig. 3. Lipid ratios in Fabry hemizygotes according to genotypes.

Urine samples from controls (×) and Fabry hemizygotes with genotypes of T141I (□), R227Q, (○), del E358 with renal transplant (△), M284T with renal transplant (◇), M284T (♦), delC895 (■), delAA717-718 (●), and C52R (▲) were analyzed for individual lipid species by ESI-MS/MS. The ratio CTH C24:1/SM C22:0 was plotted as a function of the LC C24:1/GC C24:0 ratio.

the disease. This patient was separated from all other individuals but warrants further investigation. Clearly, more samples from heterozygous patients need to be tested to fully validate this approach. Despite the variation (CV up to 20%), it is still likely that this lipid-profiling approach will be more sensitive than current methods of heterozygote detection. Decreased plasma α-galactosidase activities will correctly identify 60–70% of heterozygotes *(18)*, and experience in the National Referral Laboratory for the Diagnosis of Lysosomal, Peroxisomal and other Genetic Disease (Women's and Children's Hospital, Adelaide, Australia) has shown a similar value, with 16 of 28 known heterozygotes having leukocyte α-galactosidase activities below the reference interval.

Although some mutations are associated with the classic phenotype, genotype–phenotype correlations, as with all lysosomal storage disorders, have been problematic *(19, 20)*. The genotype R227Q is known to be associated with the classic phenotype *(21)*; although we showed here that two of the patients with this genotype were at the higher end of the group of Fabry hemizygotes, one patient was clearly at the lower end (Fig. 3). This patient, who was 60 years of age at the time of the study, presumably does not have the classic form of the disease and also warrants further investigation. A more extensive study of Fabry hemizygotes covering many more phenotypes/genotypes is needed if this lipid-profiling approach is to be evaluated for predicting disease severity in Fabry patients. In addition, we studied two patients who had undergone renal transplants, and both of these individuals fell below the range for Fabry hemizygotes (Fig. 2C). This exemplifies the opportunity for these lipid profiles to monitor therapy for Fabry disease.

The use of lipid profiles to diagnose Fabry hemizygotes and identify heterozygotes has positive implications, not only for genetic counseling purposes, but so that consideration can be given for prophylactic treatment for these individuals. It has been suggested that patients entering renal dialysis clinics should be screened for Fabry disease so that organ failure can be detected at an earlier stage, enabling appropriate intervention *(22)*. The lipid profiles produced from the different lipid analytes hold promise for monitoring of therapy for patients receiving enzyme replacement. This is an intriguing possibility that markers reflecting primary storage, lysosomal hypertrophy, and secondary changes may be appropriate for monitoring the components of pathology for Fabry disease as well as for charting individual responses to enzyme replacement therapy. Such characterization for this disorder could pave the way for similar profiles, based on several different analytes, to be generated for other lysosomal storage disorders. The concept of metabolomics and the use of metabolites as surrogate markers of disease activity will potentially be very useful for the characterization of many complex genetic disorders.

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