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DOI: https://doi.org/10.1016/j.bbadis.2019.07.005

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Journal Article
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Originally published at:
Heywood, Wendy E; Doykov, Ivan; Spiewak, Justyna; Hallqvist, Jenny; Mills, Kevin; Nowak, Albina (2019). Global glycosphingolipid analysis in urine and plasma of female Fabry disease patients. Biochimica et Biophysica Acta. Molecular Basis of Disease, 1865(10):2726-2735.
DOI: https://doi.org/10.1016/j.bbadis.2019.07.005

Global Glycosphingolipid Analysis in Urine and Plasma of Female Fabry Disease Patients

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Keywords: Fabry disease; glycosphingolipid; biomarker, galabiosylceramide, lyso-Gb_3; ceramide dihexoside

Abstract

Fabry disease (FD) is an X-linked lysosomal storage disorder caused by deficiency of α-galactosidase-A, which results in accumulation of the glycosphingolipid (GSL) globotriaosylceramide (Gb_3). Gb_3 and globotriaosylsphingosine (lyso-Gb_3) levels in plasma and urine are used routinely for diagnosis and treatment monitoring. FD female patients are problematic to diagnose and to predict when to begin treatment. Further biomarkers are needed to detect pre-symptomatic females that will develop the chronic symptoms associated with FD. A LC-MS/MS glycosphingolipidomic assay was developed to measure lyso-Gb_3 and GSLs from the lysosomal GSL degradation pathway, including globoside (Gb_4), Gb_3, ceramide dihexosides (CDH) and ceramide monohexosides (CMH). We analysed plasma and urine from a cohort of Fabry patients, grouped according to clinical symptoms and independent of treatment status (asymptomatic females n=18, symptomatic females n=18, males n= 27 and control urines n=16 and control plasmas n=58).

Multivariate and subsequent univariate analysis showed urine GSLs which had highest significance in identifying asymptomatic females were total levels of CDH, in particular the long chain isoforms C22:1,C22:0,C22:1-OH,C22:0-OH,C24:2,C24:0,C24:2-OH,C24:1-OH,C24:0-OH,C26:0 which likely represent Galabiosylceramide (Ga_2) and not lactosylceramide. These long chain Ga_2 isoforms were found to be 5-fold elevated and more statistically significant ($p<0.0001$) than plasma lyso-Gb_3 ($p< 0.01$) in identifying asymptomatic Fabry female patients. Receiver operating characteristic curve analysis gave an area under the curve of 0.82 ($p=0.001$) for lyso-Gb_3 and 0.88 ($p=0.0006$) for long-chain CDH isoforms indicating the long chain CDH isoforms were as, if not more, a better biomarker for the identification of female FD patients.

Introduction

Fabry disease (FD) is an X-linked, lysosomal storage disorder (LSD) that is caused by mutations of the *GLA*-gene that lead to markedly reduced or absent activity of the lysosomal enzyme alpha-galactosidase (GLA). This results in a progressive accumulation of the glycosphingolipid (GSL) globotriaosylsphingosine (Gb_3) [1]. Traditionally, Gb_3 has been an accepted biomarker for FD but in recent years the deacylated version of Gb_3, lyso-Gb_3, has been observed to be more indicative of disease burden and monitoring the response to treatment [2]. The presence of lyso-Gb_3 in the circulation is not completely understood but is thought to be an inadvertent product of promiscuous acid ceramidase activity on Gb_3 [3]. Female FD patients who are heterozygous for the disease are typically harder to detect and monitor biochemically using Gb_3 [4]. Lyso-Gb_3 so far is proving to be more useful for identifying FD females [4-6]. However, not all FD females can be detected using plasma lyso-Gb_3 levels and usually require a mixture of glycosphingolipid, enzymology and genetics to diagnose. Therefore there is a need for better and additional biomarkers [5].

The GSL degradation pathway involves multiple steps, by highly specific enzymes, that result in the breakdown of the ganglioside GM_1 and globoside (Gb_4), finally resulting in the production of sphingosine which is then recycled (figure 1). This results in various intermediate compounds including Gb_3, lactosylceramide (LacCer), glucosylceramide (GlcCer) and ceramide. Galabiosylceramide (Ga_2 or Gb_2) is also a substrate of alpha-galactosidase and is known to accumulate in FD but not to the extent as Gb_3. To add to the complexity of GSLs, they also consist of multiple isoforms resulting from the different fatty acid chain lengths, double bonds and fatty acid modifications such as hydroxylation [7]. Modifications also can occur on the sphingosine backbone [8] which is pertinent to the discovery of the lyso-Gb_3 analogues that are relevant in FD [9]. The role and the function of the different isoforms for many GSLs are largely unknown [10] and with the effect on the overall GSL degradation pathway in FD patients has not been fully investigated. Recent mouse studies have indicated other GSL species could be affected [11, 12] and therefore, in this study we have looked at the GSL degradation pathway in more detail to see if any changes are also observed in humans. We have developed a multiplex UPLC LC-MS/MS glycosphingolipidomc screen to quantitate the main isoforms of the four GSL species globoside (Gb_4), Gb_3, ceramide dihexoside (CDH) and ceramide monohexoside CMH) and lyso-Gb_3 (including analogues) [13]. Our primary objective was to use this assay to study the effect on the metabolic flux of other glycosphingolipids upstream and downstream of the enzymatic defect found in FD. The secondary aim was to see if any of these other lipids were of use for aiding in the diagnosis of FD, in particular in the

detection of FD females. Finally, we have compared those GSL species to the gold standard plasma lyso-Gb_3, the currently used biomarker, to see if they can aid in the diagnosis of FD.

Methods

Patient samples

FD plasma and urine samples were obtained from University Hospital Zurich, Zurich, Switzerland, all with a *GLA*-mutation confirmed diagnosis, who presented for routine annual examinations at the specialized FD centre. All patients participated in this study were treated in the same hospital. All patients had a comprehensive work-up, including medical history, cardiac, renal, and neurological evaluations. ERT was initiated according to the written local guidelines. Accordingly, ERT was indicated in all males with pathogenic *GLA* mutation, independent from age, phenotype, and symptoms. In females, ERT was indicated if they had proteinuria of more than 300 mg per day, FD-typical kidney biopsy findings, signs of FD cardiomyopathy, such as left ventricular hypertrophy or arrhythmia, if the FD patients had stroke or transient ischemic attack (TIA), persistent FD-related neuropathic pains despite conventional analgetic therapy, and or gastrointestinal symptoms. In all patients, ERT was prescribed at the licensed dose of either 0.2 mg/kg body weight of recombinant agalsidase-a (Replagal) or 1 mg/kg body weight agalsidase-b (Fabrazyme) and given intravenously every 14 days. Each patients ERT status is indicated in supplementary data file 1 which includes all known clinical data and corresponding glycosphingolipid values. This study was conducted in accordance with the principles of the Helsinki Declaration and approved by the Zurich Ethical Committee; the approval number is 2017-00386. All patients signed a written informed consent.

Samples for analysis consisted of 27 males (19-65 yrs median age 47) all on ERT and symptomatic. Thirty-seven female patients were included and divided into 2 experimental groups FD female asymptomatic (age range 21-65 yrs, median 36.5 yrs) where patients were mostly not on ERT and a FD female group who were symptomatic (age range 19-78 yrs, median 51 yrs) where the majority of patients were on ERT. Control samples used in this analysis (plasma n= 64 M: F 32:32, urine n=12 M: F 4:8) were from healthy volunteers (age range 22-61 yrs, median 29 yrs).

Sample preparation for LC-MS/MS analysis

Plasma lyso-Gb_3 analysis: Lyso Gb_3 was extracted from 100 μl of plasma using 1 ml of acetone: methanol (1:1 v/v) containing 2 ng/ml di-methyl psychosine internal standard (Avanti Polar Lipids inc). Samples were shaken for 20 min, sonicated for 15 min in a sonicator bath and shaken again for 20 min at room temperature (RT). After centrifugation at 16000 g, supernatants was transferred and evaporated on a rotational evaporator at RT. Samples were reconstituted in 100 μl of methanol prior to LC-MS/MS analysis.

Urine lyso-Gb_3 analysis: Lyso-Gb_3 was extracted from 500 μl of urine using 600 μl 0.2% formic acid (FA), containing 50 μl of Internal standard solution at 0.1 μg /ml in methanol, followed by 50 μl of methanol. Samples were shaken for 20 min at RT and then cleaned using C18 SPE. 1 ml C18 cartridges were primed with 1 ml of methanol:chloroform (2:1 v/v), then equilibrated 2x 1ml 0.1 % FA. Samples are added and cartridges washed with 0.1 % FA. Lyso-Gb_3 was eluted in 500 μl of methanol:chloroform (2:1 v/v). Samples were dried using a rotational evaporator at RT and reconstituted 100 μl of methanol prior to LC-MS/MS analysis.

Plasma GSL analysis: GSLs were extracted from 50 μl of plasma using a 1 ml solution containing 0.5 μg/ μl C17-CTH (Matreya LLC, USA) and 0.5 μg / μl [D_3]C16:0-Lactosylceramide (Matreya LLC, USA) internal standard in chloroform:methanol (2:1 v/v). Samples were shaken for 20 min at RT, sonicated for 15 min, shaken again for 20 min. 200 μl of ice-cold PBS was added and samples were shaken again for 20 min. Samples were spun at 16000 g for 10min. Using a glass pipette the bottom layer is carefully extracted and placed in a 5 ml glass vial. Samples were dried under nitrogen and reconstituted in 1 ml of chloroform. Samples were cleaned using C18 SPE cartridges (Biotage, UK). Cartridges were primed with 1 ml acetone:methanol (9:1 v/v) then equilibrated twice with chloroform before the addition of sample. Cartridges were washed 2 x 1 ml chloroform and then eluted with 700 μl of acetone:methanol (9:1 v/v). Samples were dried under nitrogen and reconstituted in 200 μl of methanol prior to MS analysis.

Urine GSL analysis: GSLs were extracted from 50 μl of urine using 200 μl solution containing 0.5 μg/μl C17-CTH (Matreya LLC, USA) and 0.5 μg/μl [D_3]C16:0-Lactosylceramide (Matreya LLC, USA) internal standard in methanol:chloroform (2:1 v/v). Samples were shaken for 1 hr at RT. 150 μl of isopropanol was added and samples centrifuged for 10 min at 16000 g. Supernatants were transferred to a new vial and evaporated using a rotational evaporator at RT. Samples were reconstituted in 100 μl of methanol prior to MS analysis.

UPLC-MS/MS analysis

An ACQUITY UPLC system from Waters Corporation (Milford, MA) was used for the separation of GSLs before quantitative tandem mass spectrometry analysis. The reverse-phase UPLC methods used for plasma and urine GSL and lyso-Gb_3 analysis are described in supplementary data table S-1. The UPLC system was coupled to a Xevo TQ-S (Waters Ltd, UK) mass spectrometer operated in the multiple reaction monitoring (MRM) mode. MRM transitions corresponding to isoforms of CMH, CDH, Gb3, Gb4, and lyso-Gb_3 are given in supplementary data Table S-5. Internal standard transitions were included in all MRM files. Transition dwell times were set automatically with minimum of 0.03 sec and minimum of 8 data points per peak. The divert valve of the mass spectrometer was programmed to discard the UPLC effluent before (0 to 1 min) and after (2.8-3.5 GSL 5 min methods) and 8-10 min (10 min methods) to reduce system contamination. Galabiosylceramide (Gb2) is a structural isomer of lactosylceramide (LacCer) a product of alpha galactosidase from ganglioside degradation (figure 1 A). Being structural isomers these two GSLs have the same molecular weight and cannot be separated by conventional reverse phase chromatography. Similarly, it is not possible to separate structural isomers GlcCer and galactosylceramide (GalCer). Therefore, they are referred to as total CDH and total CMH in this study. We demonstrate that there is no requirement to separate these molecules using normal phase chromatography methods such as a HILIC chromatography.

The concentrations of lyso-Gb_3 and its GSLs were evaluated using the TargetLynx 4.1 software (Waters). C17-CTH was used as the internal standard for the quantification of Gb_3 and Gb_4 and [D_3]C16:0-Lactosylceramide for quantitation of CDH and CMH. Dimethylpsychosine was used as IS for lyso-Gb_3. Calibration curves were linear the origin was excluded. Urinary lipids were ratioed to urinary creatinine levels as described previously [14]. Further methods details are available in the supplementary information.

Data analysis: Excel and GraphPad Prism v 6 were used for all composition, comparative and ROC analysis. Both parametric and non-parametric analysis was used for comparisons where appropriate. Multivariate analysis including principal components and OPLS-DA analysis was performed using SIMCA v 14 (Umetrics, Sweden). Where there is no data a 0 value for that molecule is given for analysis.
Spearman Correlation matrix analysis was performed using an in-house Python v 3.6 script (URL https://www.python.org/).

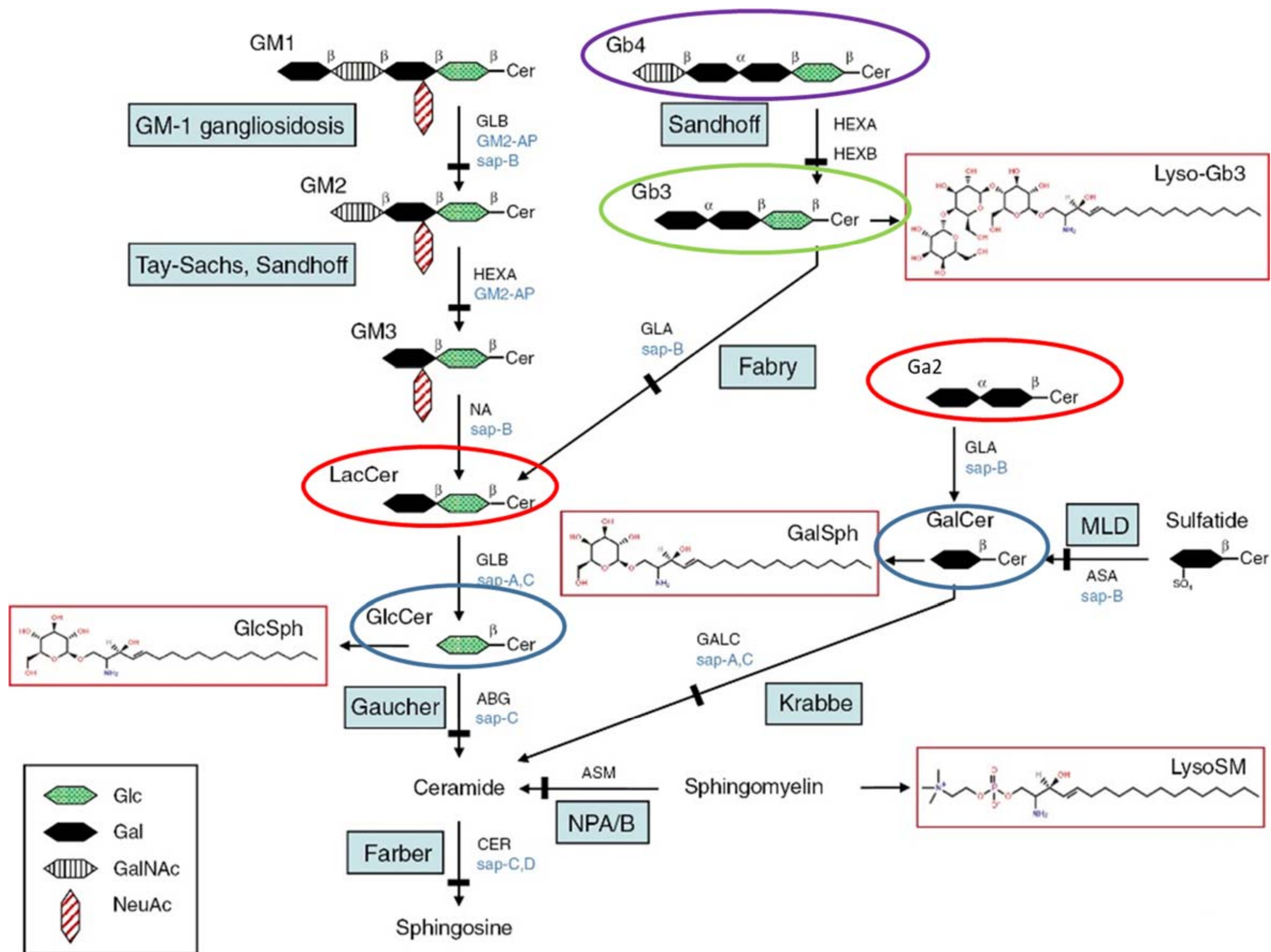


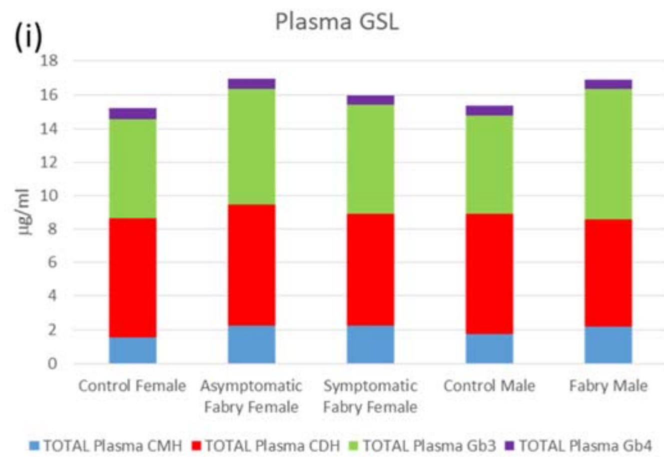
Figure 1. A. Glycosphingolipid degradation pathway (Polo et al 2017[15]) with highlighted GSL species analysed including Lyso-Gb_3. LacCer and Ga2 consist of CDH and GlcCer and GalCer consist of CMH.

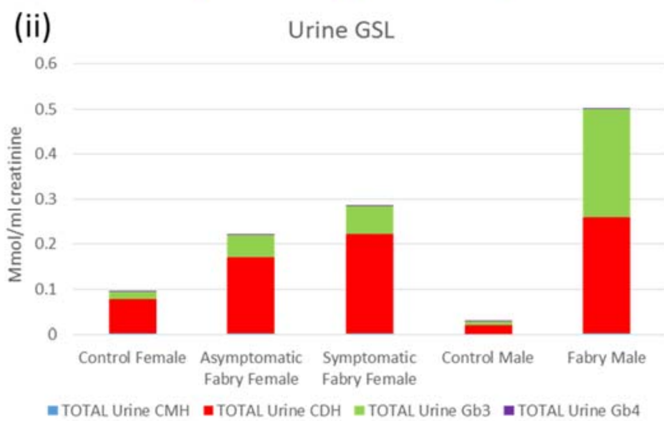
Results

Glycosphingolipid Profile and Composition in Normal and Fabry Disease Patients

Figure 3 shows the total averaged abundance levels of each GSL in (i) plasma and (ii) urine. In control subjects, CDH and Gb_3 are the most abundant GSLs present in both plasma and in urine. Additionally, CDH makes up 47% and Gb_3 38% of total plasma GSL in healthy controls. For total GSLs in urine, CDH consists of approximately 72% in females and 59% in males, with Gb_3 making up 22% of total glycosphingolipids in females and 35% in males, respectively. Plasma Gb_4 and CMH levels are much lower (Gb_4 = ~4%, CMH =~11%) and in urine (Gb_4~0.31%, CMH=~6%) in both males and females. Surprisingly, control females have a much higher level of total GSLs in urine than control males (figure 3 (ii)) of which the majority is comprised of CDH and which confirms previous reports [16]. Furthermore, the proportion of CDH in female urine was also observed to be higher than in men (CDH~72% in females vs ~59% in males, $p<0.03$). However, males were observed to have a greater proportion of Gb_3, 22% in females vs 35% in males ($p<0.02$).

Analysis of patient samples demonstrated the composition of the CDH and Gb3 in FD does not appear to change due to the enzymatic defect observed in FD (figure 2iii), with FD females having on average 74% CDH vs 55% in FD males ($p<0.001$), and 24% Gb_3 vs 41% in males ($p<0.001$). In FD, plasma GSL levels do not alter greatly with only moderate total increases in all FD groups (figure 3 (i)). However, in urine, the increase is much more profound in both males and females which confirms why urine Gb_3 is typically used for diagnosis before lyso-Gb_3 was discovered [14].





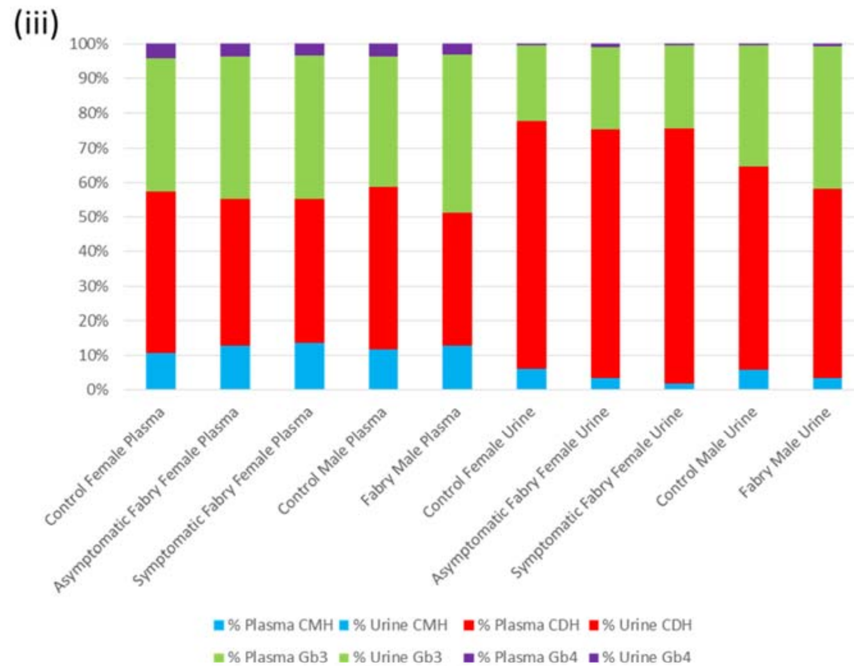


Figure 2. Overview of the approximate average levels and composition of each GSL species in (i) plasma and (ii) urine for Control Female (Ctl), Fabry Female Asymptomatic , Fabry Female Symptomatic, Control Male and Fabry Male who are all symptomatic. (iii) Shows percentage composition comparison of each measured GSL species. CMH and Gb_4 are prevalent in plasma compared to urine. Changes in total levels of CDH and Gb_3 are more evident in the urine in Fabrydisease. Females have both higher urinary CDH and Gb_3 levels which both increase in Fabry disease which also increase in males but Gb_3 levels and composition are higher in males.

Glycosphingolipidomic changes observed in Fabry Disease Patients

Figure 4 shows that when comparing the total levels of each GSL in the FD groups, it was observed that there is a small but significant increase of approximately 1.4 fold of CMH in both FD female groups compared to controls ($p<0.001$). This increase is also observed in the FD male group but to a lesser extent of 1.3 fold ($p<0.05$). The only other significantly altered GSL in plasma was a slight increase of Gb_3 in the FD male group of appx 1.2 fold ($p<0.01$).

However greater changes in other GSLs were observed in urine. The lesser abundant GSLs CMH and Gb_4 were observed to be significantly affected in FD patient urine. CMH is slightly elevated (1.6 fold) in the male FD group ($p<0.05$) but Gb_4 was observed to be 3-fold elevated in all FD groups. Urinary Gb_3 has been previously shown to be significantly elevated by a much larger factor in all FD groups, and was observed in this study to increase by approximately 2.9-fold ($p<0.05$) in females and 25-fold in males ($p<0.01$). CDH was also observed to be elevated in all FD groups but by a greater fold change in the FD female groups by >2.7-fold in the asymptomatic group ($p<0.05$) and 17-fold in the male FD group ($p<0.01$). Statistical analyses of grouping female patients by ERT status as opposed to grouping according to symptomatic status, showed negligible differences (supplementary figure S4). Therefore, grouping by symptomatic status with an asymptomatic female group was used to identify GSLs that could potentially aid in biochemical identification of suspected female FD patients.

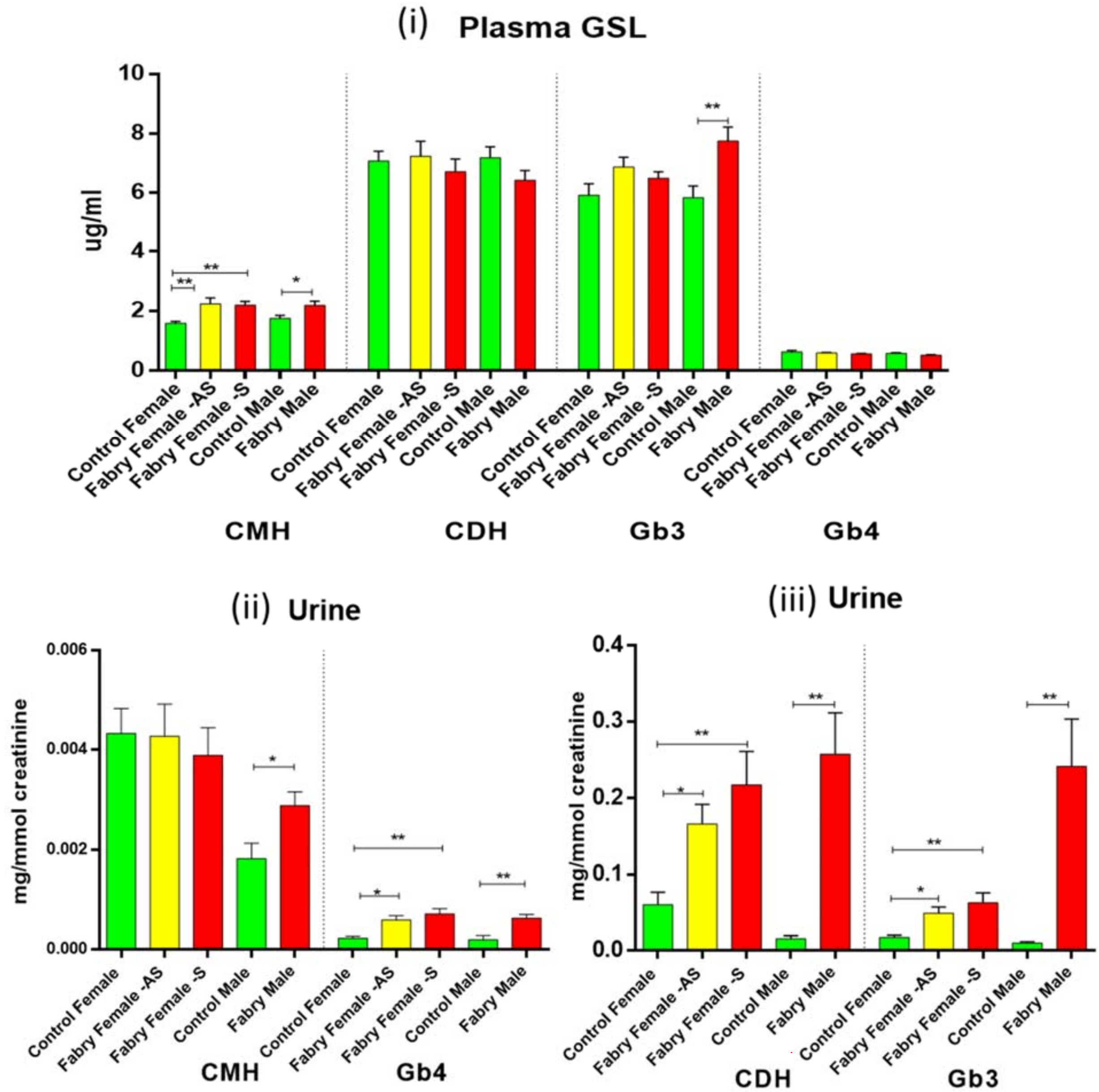


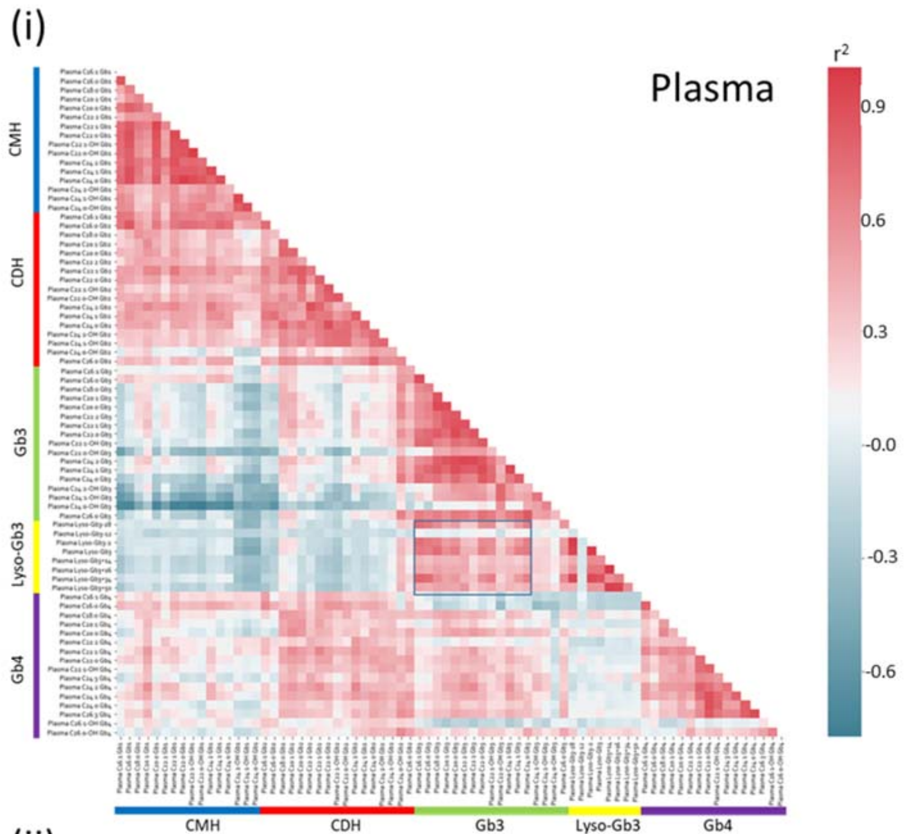
Figure 3. Comparison of each GSL species in Fabry patients. (i) Mean ± SEM plasma values of CMH, CDH, Gb_3, and Gb_4. Significance in the Fabry female group for plasma is determined by one way ANOVA and for Fabry males by unpaired t-test (ii) Mean ± SEM urine values for the lower abundant GSL species CMH and Gb_4 in urine. (iii) Mean ± SEM urine values for the higher abundant GSL species CDH and Gb_3 in urine. Significance of urine GSLs was determined by use of non-parametric Kruskal Wallis for the Fabry female groups and Mann-Witney test for the Fabry male group comparison. * p<0.05, **p<0.01. AS Fabry Females = asymptomatic Fabry females, S Fabry Females =Symptomatic Fabry Females, Ctl Male = Control males, Ctl Female = Control females.

Relationship between GSLs and their isoforms in Plasma and Urine

All various GSLs and their isoforms analysed in both FD patient plasma and urine were subjected to Pearson Correlation analysis in order to highlight the relationships between the various GSL species and isoforms. As control samples have much lower GSLs in urine, this analysis was only performed on the FD patient GSL values. The plasma correlation matrix demonstrated (Figure 2), as expected, a strong relationship of the GSL isoforms within their own GSL species group i.e. Gb_3 isoforms correlate with all other Gb_3 isoforms. However, a poor relationship was observed between the individual GSL group species. CDH appears to show a greater degree of correlation with CMH, Gb_3, and Gb_4. Lyso-Gb_3 and analogues [17] were assayed in plasma in order to see if there may be a particular Gb_3 isoform that is more susceptible to conversion to lyso-Gb_3, therefore giving an indication of the mechanism of generation of lyso-Gb_3. The area of strongest correlation with lyso-Gb_3 is indicated on the plasma correlation matrix and does demonstrate a strong correlation with Gb_3. In particular, the shorter isoform chains of Gb_3 demonstrated the highest correlation with lyso-Gb3 levels and particularly the Gb_3 isoforms of C16 and C18 (supplementary data figure S-1).

In the urine analyses, no significant relationship was observed between the GSL species. However, a weak relationship was observed between CDH and Gb_3 and particularly between the C24:1 and C24:2 hydroxylated isoforms of CDH and Gb_3 (urine correlation matrix plot figure 2ii). This may indicate this modification is independent to the expression of the GSLs.

(i)



(ii)

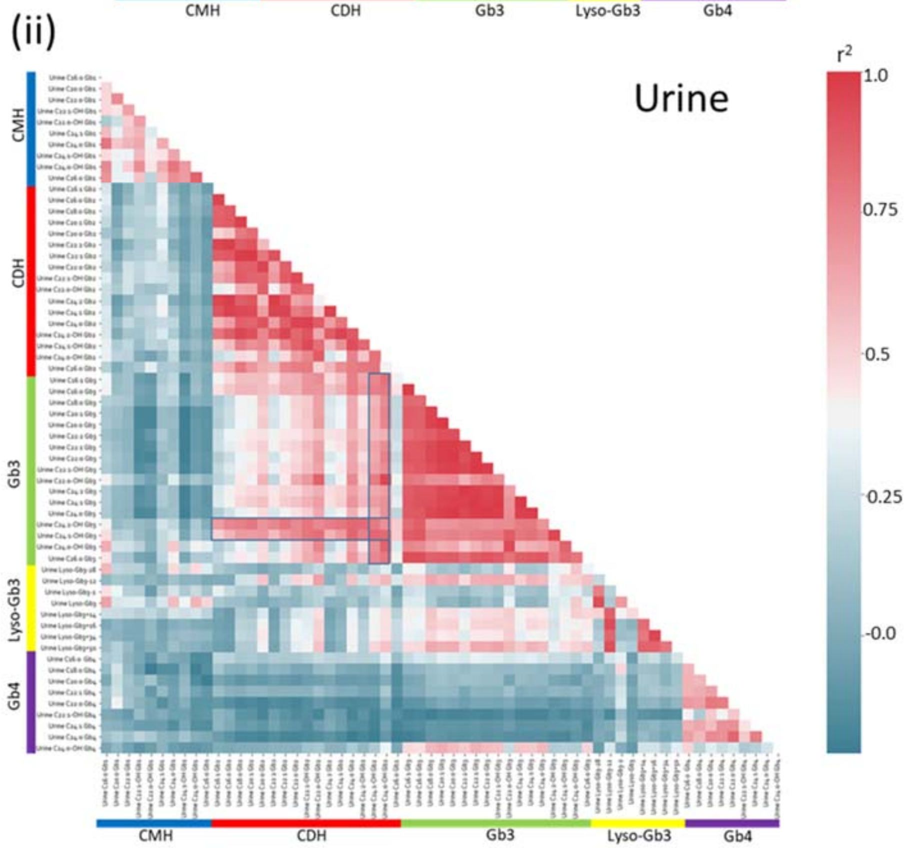


Figure 4. Pearson correlation matrix of all the glycolipids quantitated in plasma and urine of Fabry patients (i). Red indicating a strong correlation and blue a negative correlation between each species. Plasma correlation shows no significant correlation between each of the individual glycolipids with each other. Lyso-Gb_3 is highlighted in the boxed area and demonstrates a stronger correlation with Gb_3 and more so with the short chain isoforms C16, C18 of Gb_3. (ii) Urine analyses also demonstrated no relationship in expression between the GSL species. However, the hydroxylated long-chain species of CDH and Gb_3 appear to show a correlation between each other (highlighted in box area).

Multivariate analysis of Glycosphingoplipidomic screen of Female Patients with Fabry Disease

In order to identify which GSLs, or specific GSL isoforms, could have potential in aiding the diagnosis of FD in females, a multivariate analysis of the data was also performed. Figure 4 shows Principal Component Analysis (PCA) of both urine (A) and plasma (B). All samples demonstrated a separate clustering of the control and FD patients; the first two components (t[1] and t[2]) explaining 55% and 12% of the variation for plasma and the first two components (t[1] and t[2]) explain 41% and 12% of the variation in urine. The FD groups demonstrated a far more varied distribution in both plasma and urine. The male group particularly shows a wide clustering in urine, more so than plasma and which is unsurprising as GSLs were observed to be affected more in the urine FD of FD patients.

In an attempt to identify female specific markers, a further statistical OPLS-DA analysis was performed on the FD female samples. A significant separation in both the urine and plasma GSL profiles was observed (OPLS-DA parameters are given in supplementary data table S-6). Interestingly, there appeared to be no clustering of the GSL profiles based on symptomatic status in FD females in urine or plasma. As shown by corresponding loading plots the GSLs in the plasma analyses that appear to drive the changes were increased levels of C18 CDH and Gb_4 and reduced levels of all CMH isoforms. In urine, there was a clear GSL profile for control and FD females which was driven by increased levels of lyso-Gb_3, lyso-Gb_3-analogues and longer chain CDH isoforms C22:1, C22:0, C22:1-OH, C22:0-OH, C24:2, C24:0 C24:2-OH, C24:1-OH, C24:0-OH, C26:0.

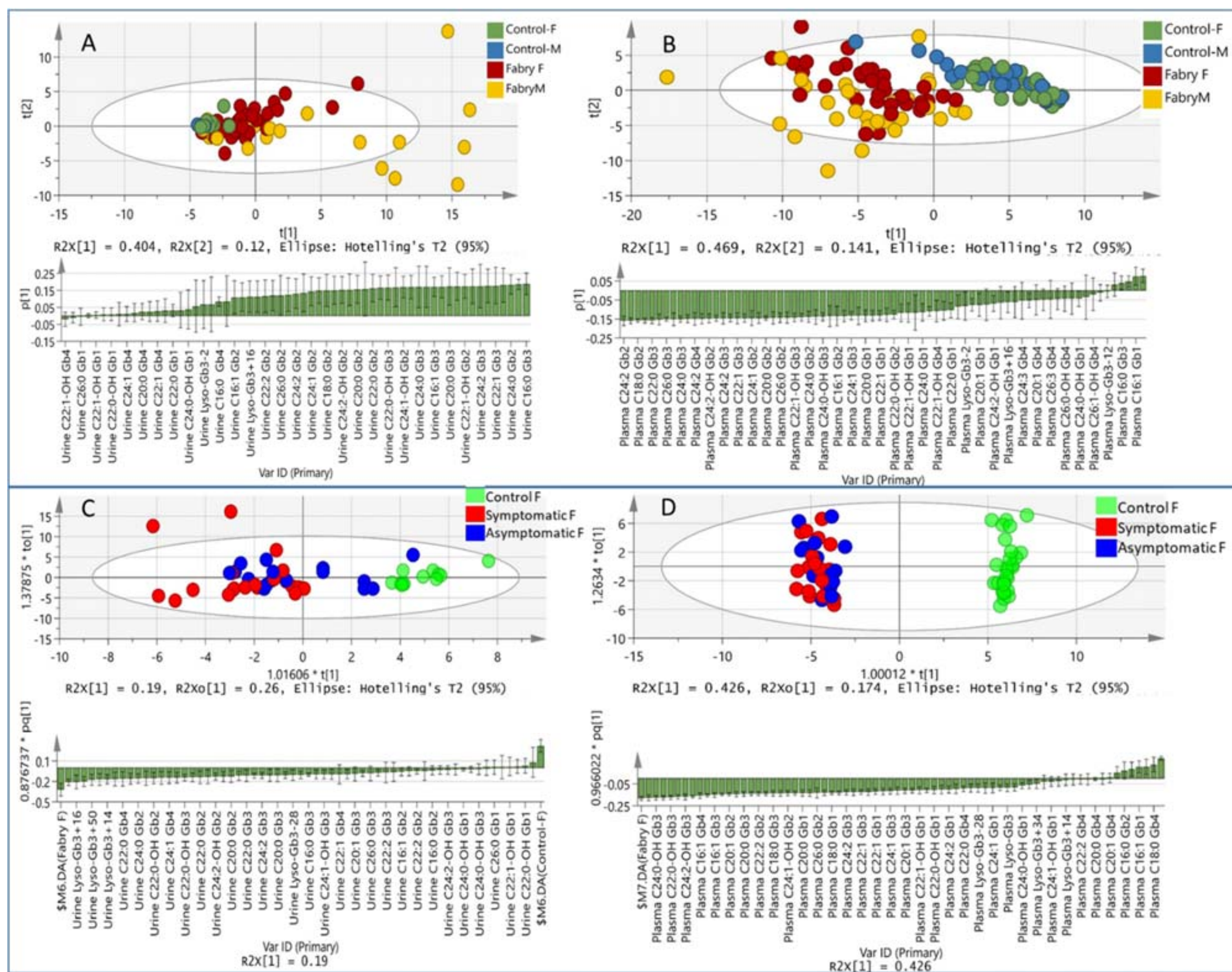
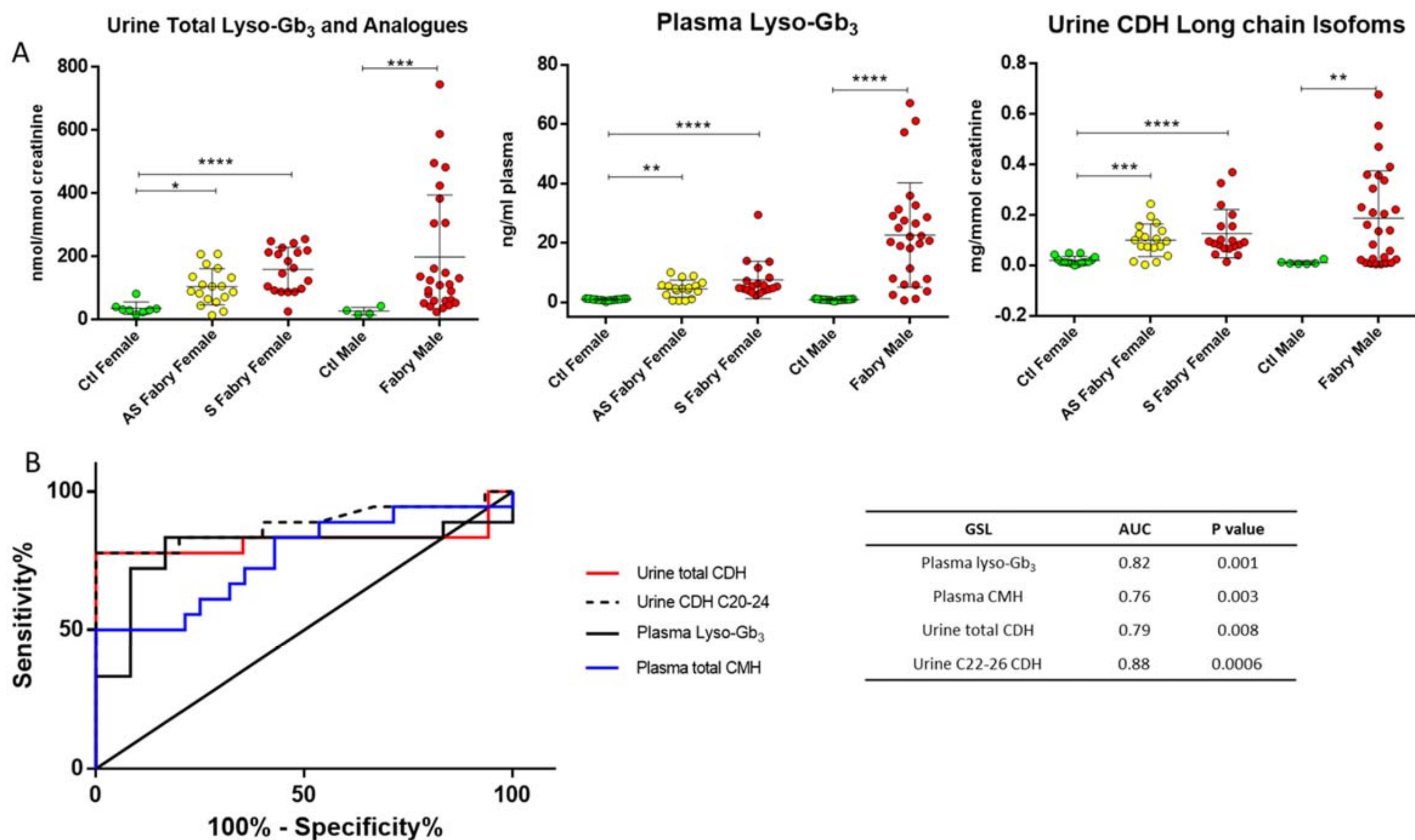


Figure 5. Multivariate analysis of GSLs in Fabry female patients. Scatter (top) and corresponding loading plots (bottom) of GSL PCA analysis of male and female control and Fabry patient urine (A) and plasma (B). Further sub-analysis of Fabry female patient samples using OPLS-DA is shown for urine (C) and plasma (D).

Glycosphingolipids biomarkers that could aid in the identification of female Fabry Disease Patients

Multivariate analysis indicated that the urinary long chain CDH fatty acid isoforms C22:1, C22:0, C22:1-OH, C22:0-OH, C24:2, C24:0 C24:2-OH, C24:1-OH, C24:0-OH and C26:0 contributed the greatest to driving the differences between FD females and controls. In plasma, lyso-Gb_3 analogues and also total CMH levels contributed to the changes in FD female patients.

Analogues of lyso-Gb3 are more predominant in urine than lyso-Gb_3 (supplementary figure S2C) therefore analogue levels were included in the total levels reported. Lyso-Gb_3 showed a significant difference in symptomatic FD females in plasma but less significantly for the asymptomatic female group in urine. In plasma, lyso-Gb_3 demonstrated only a small change in the asymptomatic female group. This indicated that lyso-Gb_3 is a marker of disease severity but offers little information in regards to confirming a diagnosis in asymptomatic heterozygous FD females. However, total urine CDH levels can be used to help identify FD females (figure 5(iii)) and in particular, the use of the 10 long chain isoforms of CDH C22:1, C22:0, C22:1-OH, C22:0-OH, C24:2, C24:0 C24:2-OH, C24:1-OH, C24:0-OH, C26:0 which demonstrate even better specificity ($p<0.001$) than total CDH ($p<0.05$) in its ability to discriminate FD female patients. Importantly, the asymptomatic female group who are the most difficult to diagnose, are more distinguishable from female controls when quantitating the longer chain CDH isoforms compared to using plasma lyso-Gb_3 (figure 6). A ROC analysis (figure 6B) of plasma CMH lyso-Gb_3 and urine total CDH and long chain CDH demonstrated the urine long chain CDH has the highest AUC 0.88 for detecting female FD patients.



GSL	AUC	P value
Plasma lyso-Gb_3	0.82	0.001
Plasma CMH	0.76	0.003
Urine total CDH	0.79	0.008
Urine C22-26 CDH	0.88	0.0006

Figure 6. Comparison of Female Fabry biomarkers (AS Fabry Females = asymptomatic Fabry females, S Fabry Females =Symptomatic Fabry Females, Ctl Male = Control males, Ctl Female = Control females). **A** Currently used biomarkers lyso-Gb_3 in urine and plasma show that plasma lyso-Gb_3 is more specific and has even greater significance in symptomatic patients. However long chain CD also have comparable ability to distinguish asymptomatic female patients from controls. **B** ROC analysis comparing sensitivity and specificity of all GSLs altered in Female Fabry patients. Significance was determined by use of non-parametric Kruskal Wallis for the Fabry female groups and Mann-Witney test for the Fabry male group comparison. * $p<0.05$, **$p<0.01$, ***$p<0.001$,****$p<0.0001$.

Discussion

This glycosphingolipidomic assay has provided significant amounts of useful data regarding GSL catabolism in humans and allowed us to obtain a global overview of GSL composition and abundance in both urine and plasma. To our knowledge, this is the first specific analyses in human samples targeting specifically all those GSLs directly upstream and downstream of the catabolic enzyme defect observed in FD. We did not include the gangliosides GM1, GM2, and GM3 as they are present in very low amounts. Whilst their degradation pathway is not directly related to Gb_3 catabolism (figure 1), their expression would be interesting to observe in FD to see if this branch if the GSL degradation pathway is affected.

The mechanism of generation of lyso-Gb_3 in FD by non-specific action of acid ceramidase has been proposed but the mechanism as yet not fully understood [3]. Multiple correlation analysis in figure 4 shows that lyso-Gb_3 and some of its analogues have the strongest correlation with the Gb_3 isoforms C16:1 and C18:0 (r^2>0.7, p<0.0001). This relationship indicates these Gb_3 isoforms may be more likely to be converted to lyos-Gb_3 and/or its analogues. Interestingly, these isoforms are not the abundant isoforms of Gb_3 in plasma. Supplementary figure S2 shows the composition of the Gb_3 isoforms and lyso-Gb_3 analogues [17] and demonstrates that the C16:1 and C18:0 isoforms of Gb3 are the 5th and 6th most abundant isoforms. In total they comprise approximately 7% and 5% of total Gb_3. This observation needs to verified by other independent cohorts but these isoforms may hint at a mechanism of lyos-Gb_3 formation which may or may not involve acid ceramidase [16, 18].

It was not possible to include lyso-Gb_3 in any GSL composition analyses because the levels are much lower (ng/ml range) compared to GSLs (µg/ml range). Figure 2 showed that there are significant differences in the urine GSL composition between males and females that are unchanged in FD. In addition, Figure 3 demonstrated that there are significant changes in the total levels of CDH and Gb_3 in both males and females. This observation that total levels increase but the composition does not, indicate that CDH is probably an equally an important substrate for alpha-galactosidase and is more relevant in Female FD than Gb_3. This explains previous reports that have observed increased CDH in female FD patients [14, 16]. This study also indicated that Gb_4 and CMH, the upstream and downstream glycolipids to Gb3 respectively, are more abundant in plasma and form a much lower proportion of the GSL profile in urine. Thus, this indicating that the kidney is probably the primary source of CDH and Gb_3. In FD disease, plasma Gb_4 levels were unaffected but the downstream glycolipid, CMH, was increased. This observation was unexpected as the CDH to CMH step is downstream of the defect observed in the GSL degradation pathway caused by FD. Therefore, there may be another mechanism/pathway in FD tissues that causes this unpredicted increase of CMH. As

in urine, the predominant GSLs in plasma were Gb_3 and CDH which do not appear to change significantly in FD and confirms previous findings on the use of plasma Gb_3 as a poor biomarker for FD disease [19].

Ferraz *et al* used various mouse models of GSL degradation disorders and measured the main GSL and lyso compounds. In tissues from the FD model mouse, it was observed there were no other sphingolipid changes in the FD mouse model apart from Gb_3 and lyso-Gb_3. CMH was not affected and indicating no feedback on GSL synthesis to avoid Gb_3 accumulation [12]. However, our data in humans demonstrated that CMH is increased in plasma and therefore indicates that there may be subtle different pathways between mice and human glycosphingolipid homeostasis. Kamani *et al* used a FD mouse model and also measured other products of the GSL degradation pathway including the mono and dihexasylceramides (CDH) [11] and also did not observe an elevation in CMH. However, significant elevation of CDH was observed in the kidney tissue of FD mice and confirming our observations in humans in this study.

GSL species comprise of multiple isoforms based on modification of the fatty acid chain. Little is understood about the biological relevance of each isoform. We took an unbiased analysis approach by using multivariate statistics to see if there were isoforms within the GSL profile with greater specificity for FD females. We confirmed plasma lyso-Gb_3 and also identified urinary long chain CDH isoforms as having greater changes in FD females, indicating they may have use clinically. Subsequent univariate analysis confirmed plasma lyso-Gb_3 as an excellent marker relevant to disease progression. However, the long chain urinary CDH isoforms were highly significant ($p<0.001$) and more so than lyso-Gb_3 ($p<0.01$) for the asymptomatic female group, indicating the long chain CDH isoforms could also be useful for identifying FD females. Gb_3 and CDH are the predominant GSLs in urine and this is demonstrated in figure 3. In control females, the percentage of CDH is ~79% and Gb_3 is ~22% whilst in males, these values are ~ 59% CDH and ~35% Gb_3. In FD, this composition only changes for males with an increase of Gb_3 to 40%. This indicates the involvement of CDH could also be exploited for the diagnosis and as a feature in FD as both GSLs increase in FD to a similar degree in urine.

The analysis of CDH has been performed previously in FD heterozygotes [14] but is confounded by the fact that LacCer and Ga_2 are structural isomers with the same mass. Both LacCer and Ga_2 differ by configuration at a single chiral centre where the LacCer sugar moiety consists of a glucose and a galactose and Ga_2 consists of 2 galactose units (figure 1). These two isomers do not separate by conventional reverse phase chromatography. Boutin *et al* developed a method using normal phase chromatography to separate LacCer and Gb_2 and assessed the two isomers in FD disease urine [16]. They described that Gb_2 was not significantly different between males and females but LacCer was.

Therefore, it is likely LacCer is the driving dihexosylceramide GSL that contributes to our observation of differing CDH levels between females and males in this study. Boutin *et al* did not describe any significant changes in the levels of LacCer in FD but did for Ga_2. The limitation of our study is that LacCer levels, although not altered in FD according to Boutin et al, could affect the sensitivity and specificity of Ga_2 detection. However, the Boutin *et al* study showed that hydroxylated and long chain LacCer could not be detected in urine. Therefore, the long chain CDH isoforms described in our study are likely to be comprised entirely of Ga_2 isoforms and this explains why the long chain isoforms are more specific at detecting the FD patients. The normal phase LC-MS/MS method developed by Boutin *et al* may overall be a more specific method for the analysis of Ga_2, LacCer, and Gb_3 isoforms. However due to issues with complex matrices and throughput the use of normal phase methodology such as HILIC or amide column chromatography is not widespread compared to reverse phase chromatographic methods in clinical laboratories. Longer equilibration is required for HILIC analysis compared to reverse phase thereby increasing LC-MS/MS run times which makes it less attractive for high throughput analysis in many laboratories and exact buffer composition has to be tightly controlled which can be problematic for routine analysis [17, 20-22]. A clinical method to quantitatively measure lyso-compounds has been described previously using reverse phase C18 chromatography. The authors also describe that the use of a normal phase amide chromatography method was found only suitable as a qualitative second-tier method to separate the two hexosylsphingosines, glucosylsphingosine and galactosylsphingosine, that are relevant to Gaucher and Krabbe disease [15]. Our study highlights that by analysing the long chain CDH isoforms the well-established standard reverse phase methodology already employed in most diagnostic laboratories to diagnose Fabry patients could be easily augmented to include this additional biomarker in future FD studies. Our study however was unable to confirm that Gb_3 could discriminate the FD female patients unlike Boutin *et al* who showed that urinary Gb_3 was better than Ga_2. Urinary Gb_3 has been shown previously to be useful as a biomarker in FD disease but less so for the identification of females and has largely been superseded by plasma lyso-Gb3 as the biomarker of choice for diagnosing and monitoring treatment for heterozygotes [19]. To test the specificity of CDH, we compared its levels with plasma and urine lyso-Gb_3 (plus analogues) [23]. Urine lyso-Gb_3 appears to perform poorly compared to plasma lyso-Gb_3 and is likely due to the lower levels of lyso-Gb_3 in urine. In our study, plasma lyso-Gb_3 appears to be more specific for the symptomatic FD female group confirming its association with disease severity/progression [24]. It does, however, have limited use in detecting the asymptomatic heterozygote females. Suspected female FD patients all undergo biochemical confirmation by analysis of urinary Gb_3 and lyso-$Gb_{3,}$ Therefore, we propose that urinary Ga_2 (long chains) could be easily added into existing LC-MS/MS Gb_3 assays. One of the interesting questions

raised from these results is what the potential contribution to the disease pathology could come from raised Ga_2 levels? Does Ga_2 also have a lyso-compound that can also cause disease pathology like lyso-Gb_3 has been shown to do [25, 26]?

Conclusions: Urinary CDH is the more prominent GSL in females which means changes associated with FD are more likely to be detected. We demonstrate that the longer chain CDH isoforms C22:1, C22:0, C22:1-OH, C22:0-OH, C24:2, C24:0 C24:2-OH, C24:1-OH, C24:0-OH, C26:0 of Ga_2 in urine are elevated in FD and can discriminate asymptomatic heterozygotes better than Gb_3 and lyso-Gb_3. We propose it may be more powerful to combine and analyse lyso-Gb_3 and Ga_2 together, particularly for females where there is an uncertainty of diagnosis. Furthermore, this could easily be incorporated into the conventionally used LC-MS analysis being employed in chemical pathology laboratories.

Acknowledgments: This study has been part funded by the UCL Biological Mass Spectrometry Centre. All research at Great Ormond Street Hospital NHS Foundation Trust and UCL Great Ormond Street Institute of Child Health is made possible by the NIHR Great Ormond Street Hospital Biomedical Research Centre. The views expressed are those of the author(s) and not necessarily those of the NHS, the NIHR or the Department of Health. We would also like to thank the Peto foundation for their generous donations.

Supporting information:

Supporting information file S-1: Excel dataset of GSLs and isoforms detected in this study

Supplementary Data:

- Table S1. Table of mass spectral analysis parameters used in all 4 assays.
- Table S2: Gradient parameters 1
- Table S3: Gradient parameters 2
- Table S4: Gradient parameters 3
- Table S5: Mass transitions and conditions for all internal standards, GSLs, lyso-gb3 and analogues used in this study
- Table S6: Coefficient of variation values for 3 QC samples run throughout the assay
- Additional information on determination of GSL concentrations.
- Supplementary figure S1 - Individual Pearson correlation analysis of the Gb3 isoforms C16:1 and C18:0
- Table S6. OPLS-DA models for Fabry female vs female controls
- Supplementary figure S2. Gb3 and lyso-Gb3 analogues composition.
- Supplementary figure S3. Age association of long chain CDH isoforms in the asymptomatic Fabry female group.

- Supplementary figure S4. Comparison of each GSL species in Fabry patients according to ERT status for female samples.
- Supplementary figure S5. Comparative analysis of biomarkers in Fabry patients grouped accrording ot ERT status.

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