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LysoGb3 quantification facilitates phenotypic categorization of Fabry disease patients: Insights gained by a novel MS/MS method

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ABSTRACT

Background: Fabry disease (FD) is an X-linked lysosomal storage disease resulting from pathogenic variants in the *GLA* gene coding α-galactosidase A (AGAL) and cleaving terminal alpha-linked galactose. Globotriaosylceramide (Gb3) is the predominantly accumulated sphingolipid. Gb3, deacylated-Gb3 (lysoGb3), and methylated-Gb3 (metGb3) have been suggested as FD biomarkers.
Materials and Methods: We developed a novel LC-MS/MS method for assessing lysoGb3 levels in plasma and Gb3 and metGb3 in urine and tested 62 FD patients, 34 patients with *GLA* variants of unknown significance (VUS) and 59 healthy controls. AGAL activity in white blood cells (WBCs) and plasma was evaluated in parallel.
Results: In males, lysoGb3 concentrations in plasma separated classic and late-onset FD patients from each other and from individuals carrying *GLA* VUS and healthy controls. Calculating AGAL activity/plasmatic lysoGb3 ratio allowed to correctly categorize all females with classic and majority of patients with late-onset FD phenotypes. Correlation of AGAL activity in WBCS with lipid biomarkers identified threshold activity values under which the biomarkers' concentrations increase.
Conclusion: We developed a novel simplified LC-MS/MS method for quantitation of plasma lysoGb3. AGAL activity/plasma lysoGb3 ratio was identified as the best predictor for FD. AGAL activity correlated with plasma lysoGb3 and corresponded to individual FD phenotypes.

1. Introduction

Fabry disease (FD, OMIM 301500) is an X-linked lysosomal storage disorder (LSD) caused by the deficient activity of the lysosomal alpha-galactosidase A (AGAL, E.C. 3.2.1.22). AGAL is a hydrolytic enzyme cleaving terminal alpha-linked galactose in saccharide structure(s) of glycosphingolipids. AGAL deficiency results from pathogenic variants (mutations) in the *GLA* gene (*Xq22.1*). Globotriaosyl ceramide (Gb3) and digalactosylceramide are the predominant accumulated non-degraded substrates [1].

Majority of X-hemizygous male FD patients present with a clinical phenotype hallmarked by cardiac, neurological and renal involvement. Additional symptoms include peripheral neuropathy, angiokeratomas, abnormal sweating (hypohidrosis and/or hyperhidrosis) and lymphedema [2]. A subset of FD patients develop a late-onset disease that is often limited to the heart (cardiac variant, late-onset variants). Additionally, specific *GLA* variants have been associated with relatively high residual AGAL activity and mild to moderate FD [1]. Females heterozygous for *GLA* mutations are also affected, but their phenotype is (in most cases) variable and mitigated. As a result, many FD female patients are diagnosed late or retrospectively by screening in the family [1,3].

FD diagnostics integrate clinical assessment, residual AGAL activity testing in plasma and/or peripheral white blood cells (WBCs) and molecular genetic identification of *GLA* mutations. Residual AGAL activity

Abbreviations: NP-LC-ESI-MS/MS, normal phase liquid chromatography electrospray ionization tandem mass spectrometry; LO, late onset (phenotype); FD, Fabry disease; LSD, lysosomal storage disease; WBCs, white blood cells.

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may, however, be difficult to interpret in healthy individuals carrying pseudo-deficiency allele(s) or in heterozygous female FD patients, who often present with values comparable to healthy controls. Correct pathogenicity categorization of *GLA* variants of unknown significance (*GLA* VUS) is a further current pitfall hampering efficient identification of FD patients [1,3,4].

Gb3 is the first-generation FD biomarker [5–7]. However, its concentration is relatively low in body fluids of male patients with late-onset FD. There is a significant overlap of the values in FD female patients with non-FD controls [5,8–10]. As a result, Gb3 utility is largely restricted to detection of male patients with classic FD. Different types of deacylated (lyso) sphingolipids have recently emerged as abnormally increased in tissues and body fluids of patients with specific LSDs [9,11–13]. As shown by us [11] and other authors [14–16], some of these molecules may serve as sensitive second-generation biomarkers facilitating differential diagnostics [11,12]. Abnormally increased levels of lysoGb3 were identified in FD patients [17,18].

In FD, lysoGb3 is produced by the action of acid ceramidase [13], which mediates the alternative degradation of the stored Gb3. Vascular endothelial cells are among the cell types most affected by accumulation of Gb3 [17] and one of the major sources of lysoGb3. They are, however, also an important target of the pathological action of lysoGb3 [19]. Necroptosis of these cells was suggested to be triggered by the action of extracellular lysoGb3 consequently resulting in further release of lysoGb3. Nascent lysoGb3 is a competitive inhibitor of AGAL activity at acidic pH. At neutral pH, AGAL does not exhibit any activity toward lysoGb3 [17].

Methylated Gb3 (metGb3) was suggested to serve as a biomarker associated with late-onset cardiac FD resulting from the p.N215S *GLA* mutation [20]. Parallel measurements of AGAL activity and lysoGb3 to increase the diagnostic yields and facilitate patient categorization have been suggested by some authors [21,22].

In this report, we present a novel, sensitive and substantially simplified normal phase liquid chromatography electrospray ionization tandem mass spectrometry assay (NP-LC-ESI-MS/MS) for quantitative analysis of plasma lysoGb3 and urinary Gb3 and metGb3. To validate the diagnostic utility we tested its performance in a well-defined cohort of FD patients.

We further aimed to assess whether the levels of these biomarkers correlate with the residual AGAL activity and can facilitate categorization of different *GLA* variants.

2. Material and Methods

2.1. Patients

The cohort was set in accordance with SAGER guidelines based on biological sex criteria. It consisted of an anonymized group of 12 untreated male FD patients (6 classic FD and 6 late-onset cardiac FD), and 50 untreated heterozygous female FD patients (25 classic FD and 25 late-onset cardiac FD). The study also included 12 males and 22 females with *GLA* variants classified as VUS (*GLA* VUS). *Supplementary Materials and Methods* contain a list of all *GLA* variants identified in the cohort. The diagnosis and phenotypic FD categorization of patients was based on clinical assessment, residual AGAL activity levels and molecular genetic setup of the *GLA* gene. Control (FD unaffected) group included 29 males and 30 females.

This study was approved by ethics committee of General University Hospital in Prague (No. 23/20Grant AZV VES 2021 1.LF UK) and performed in accordance with Helsinki declaration. All material collection and analyses were performed based on a written informed consent obtained from the patients or their legal guardians.

2.2. AGAL activity in peripheral WBCs and plasma

AGAL activity in peripheral WBCs and plasma was measured by fluorometric method using 4-methylumbelliferyl-α-D-galactopyranoside as substrate (final concentration 2.5 mM). N-acetylgalactosamine (final concentration 0.1 M) was used as an inhibitor of α-D-galactosidase B in the WBCs assay [23,24]. For measurements of AGAL activity in plasma, method published by Merta et al. was used [25].

2.3. Plasma lysoGb3 extraction

Plasma samples were processed according to a modified method by Aerts et al. [17].

Briefly, plasma samples were thoroughly homogenized by sonication, 50 µl aliquots placed into Eppendorf tubes (Sigma-Aldrich, No. Z336769) and mixed with 5 µl of internal lysoGb3 standard (D_7-d18:1) (Avanti Polar Lipids, No.860682) in chloroform:methanol (2:1, v/v) (Merck, LC-MS grade) at 5 ng/µl concentration. Twenty-five µl of MilliQ water and 450 µl of chloroform:methanol (1:2, v/v) were added. Samples were vortexed (Biosan, TS-100) for 15 min at 1400 RPM and centrifuged at 10 000 x *g* for 10 min. Supernatant was transferred to another Eppendorf tube. One hundred and fifty µl of chloroform and 225 µl of MilliQ water were added. Samples were vortexed for 10 min at 1400 RPM and centrifuged at 10 000 x *g* for 5 min to separate water and the organic phase. The upper water phase was transferred to a new Eppendorf tube whereas the lower organic phase was re-extracted by 600 µl of methanol:water (1:1, v/v) by vortex mixing at 1400 RPM for 10 min and centrifugation at 10 000 x *g* for 5 min. The upper water phases from both extraction steps were combined and evaporated under a gentle nitrogen stream. The extract was reconstituted by adding 500 µl of water and 500 µl of water-saturated 1-butanol (Sigma-Aldrich, No.537993) and sonicated for 5 min in a sonic bath. Afterwards, the samples were vortexed at 1400 RPM for 10 min and centrifuged at 10 000 x *g* for 5 min. The upper phase was transferred to a glass vial and the lower phase was re-extracted by adding 500 µl of water saturated 1-butanol. The mixture was subsequently vortexed at 1400 RPM for 10 min and centrifuged at 10 000 x *g* for 5 min. Both butanol upper phases were combined in a glass vial and evaporated under a gentle nitrogen stream. Samples were stored at −20 °C prior further analyses.

2.4. Urinary Gb3 and metGb3 extraction

Urinary samples were processed according to a modified method by Abaoui et al. [20].

Gb3 and metGb3 were extracted from 90 µl aliquote of thoroughly homogenized urine by *tert*-butyl methyl ether (Sigma-Aldrich, No.650560). Ten µl of Gb3 (d18:1, D3-18:0) (Matreya, No.1537) internal standard dissolved in chloroform:methanol (2:1, v/v) at 5 ng/µl concentration were used and added to the urine samples. Freezing of the lower water phase was done by placing the samples into a freezer at −80 °C for 40 min. After separation, the upper organic phase was transferred to a glass vial and evaporated under a gentle nitrogen stream. Samples were stored at −20 °C prior to further analyses.

2.5. Isocratic NP-LC-ESI-MS/MS analysis of Fabry biomarkers

Samples were resuspended in 200 µl of methanol with 5 mM NH_4Ac (Sigma-Aldrich, No.73594), sonicated for 5 min in sonic bath and filtered through the hydrophilic PTFE syringe filter (Sigma-Aldrich/Millipore, No. SLLHR04NL). Liquid chromatography was performed on Agilent Infinity 1290 UPLC system (Agilent Technologies). Twenty µl of each sample were introduced by autosampler and separated in isocratic regime on Microsolv Sillica-C column (Microsolv, No.40000–7.5P-2, 75mm x 2.1ID − 4 µm 100A) by normal phase liquid chromatography (NP-LC) **(Fig S1)**. Methanol with 5 mM NH_4Ac was used as a mobile phase at 180 µl/min flow rate. Mass spectrometric analyses were performed on AB/MDS SCIEX API4000 triple quadrupole tandem mass spectrometer (AB/MDS SCIEX) equipped with electrospray ionization. Mass spectrometer operated in positive ion mode, $[M + H^+]^+$ ions were

generated and analyzed in SRM mode. Transition pairs and scan times were as follows: 786.6 → 282.3 (1500 ms) for d18:1 lysoGb3, 793.6 → 289.3 (1500 ms) for D_7-d18:1 lysoGb3 (IST). ESI source and ion optics parameters for lysoGb3 were set as follows: collision gas (7psi), curtain gas (20psi), ion source gas 1 (30psi), ion source gas 2 (50psi), electrospray voltage (5500 V), temperature (200 °C), interface heater (ON), declustering potential (120 V), entrance potential (10 V), collision energy (50 V), collision cell exit potential (12 V). Measured data were processed using the Analyst software (v. 1.6.2). Further details on ESI source of ion optics parameters for Gb3 and metGb3 are provided in the *Supplementary Materials and Methods*.

2.6. Preparation of calibrants and quantification of plasma lysoGb3, urinary Gb3 and metGb3

For quantification of lysoGb3, 25 ng of lysoGb3 (D_7-d18:1) internal standard was mixed with 50 ng of lysoGb3 (d18:1) (Matreya, No.1520). The external calibration sample for Gb3 quantification was prepared by mixing 50 ng of Gb3 (D_3-18:0, d18:1) internal standard with 300 ng of Gb3 (isoform mixture) (Matreya, No.1067). These calibrants were analyzed simultaneously with plasma and urine samples. Concentrations of the monitored biomarkers were calculated using single point calibration with a standard lipid concentration (external calibration standard) corrected by the signal ratio toward internal standard as previously described [7].

2.7. Analytical parameters of lysoGb3: linearity, CV, LOD, LOQ and matrix effect

Linearity of the lysoGb3 assessment was tested by analyzing doublet samples with increasing lysoGb3 standard concentration and constant amount of the internal standard (25 ng/ml). Repetitive analyses of the calibration samples allowed us to calculate the coefficient of variation (CV) in a wide concentration range. The linear range of lysoGb3 (d18:1) standard response was from 0.1 ng/ml to 1000 ng/ml. Limit of detection (LOD) and limit of quantification (LOQ) were calculated as the concentration of lysoGb3 with set cut off value of S/N ratio. The S/N ratio was set for LOD and LOQ to 3 and 10, respectively. Matrix effect was measured using the post extraction method [26] by mixing the extract of pooled plasma from six different donors with 25 ng of internal standard. The same amount of internal standard without plasma extract was analyzed in parallel. The difference of the internal standard signals between these two preparations was used to calculate the impact(s) of matrix from extracted plasma on the lysoGb3 signal.

2.8. Statistical evaluation of data by ROC curves

ROC curves were calculated using Origin 2018 PRO software. The dataset was separated by biological sex (males or females). State (ID) for individual values was set as classic FD, late-onset cardiac FD, *GLA* VUS or control(s). Positive and negative groups were sorted according to ID for each sex category. The entire dataset was divided into three separate subsets differing in the setup of the positive and negative groups: (i) classic FD and late-onset cardiac FD (positive) vs. *GLA* VUS and controls (negative), (ii) *GLA* VUS (positive) vs. controls (negative), and (iii) classic FD (positive) vs. late-onset cardiac FD (negative). Confidence level was set as 95 % for all calculations. Calculations (low vs. high or high vs. low) were selected according to evaluated biomarkers.

3. Results

3.1. Analytical parameters of the method

The linearity, LOD, LOQ, CV and matrix effect of co-extracted compounds on lysoGb3 signal were evaluated and were found satisfactory and comparable to other recently presented methods for lysoGb3 assessment [27,28]. For additional details see *Supplementary Materials and Methods*.

3.2. Plasma lysoGb3 and urinary Gb3 biomarkers

The study was conducted in a cohort of patients with Fabry disease with known phenotype and genotype (for details see *Supplementary Materials and Methods*).

Plasma lysoGb3 concentrations **(**Fig. 1**A and 1C)** were compared to urinary Gb3 levels **(**Fig. 1**B and 1D)**. We also assessed urinary metGb3 **(Fig S2)** as an alternative biomarker to plasma lysoGb3 in patients with cardiac FD.

Plasma lysoGb3 concentrations allowed us to distinguish male patients with classic FD (29.1–179 pmol/ml, 110 ± 66.3) from patients with late-onset cardiac FD (4.9–9.4 pmol/ml, 7.5 ± 1.9). Patients with *GLA* VUS (0.4–1.84 pmol/ml, 1.2 ± 0.45) were well separated from FD patients but had lysoGb3 concentrations indistinguishable from controls (0.2–2.47 pmol/ml, 0.84 ± 0.45).

Findings in heterozygous female FD patients were different from male patients. Female patients with classic FD (3.6–24.7 pmol/ml, 10.8 ± 5.3) were the only group significantly differing from all other female groups. LysoGb3 concentrations did not significantly differ among female patients with late-onset cardiac FD (0.95–4.31 pmol/ml, 1.97 ± 0.96), females with VUS in *GLA* gene (0.42–3.65 pmol/ml, 1.09 ± 0.66) and controls (0.2–1.73 pmol/ml, 0.8 ± 0.39).

Urinary Gb3 levels differentiated male patients with classic FD phenotype (2054–6174 pmol/ml, 4064 ± 1428) from other male groups. Values in late-onset cardiac FD (38–1058 pmol/ml, 256 ± 396) did not significantly differ (except of one outlier) from males with VUS in the *GLA* gene (5.98–111 pmol/ml, 55.6 ± 35.1) and controls (8–225 pmol/ml, 48 ± 43). Males with *GLA* VUS did not differ significantly from control males.

Approximately half (n = 11) of heterozygous classic FD female patients (64–1891 pmol/ml, 570 ± 548) had values clearly elevated in comparison to females with late-onset cardiac FD (8.5–467 pmol/ml, 68 ± 89), females with *GLA* VUS gene (6.2–106 pmol/ml, 29 ± 23) and controls (6.6–117 pmol/ml, 34 ± 30).

Urinary metGb3 measurements showed similar results as Gb3 for both males and females **(Fig S2)**.

3.3. Ratio of AGAL activity and plasma lysoGb3

Ratio of AGAL activity in WBCs or plasma/lysoGb3 concentrations in plasma were calculated in male and female cohorts.

In males, ratio of AGAL activity in WBCs to plasma lysoGb3 **(**Fig. 2**A and 2C)** differentiated classic FD ($8.1{\cdot}10^{-4}$-$8.9{\cdot}10^{-2}$ nmol·mg^{-1}·h^{-1}/pmol·ml^{-1}, 0.02 ± 0.04) and late-onset cardiac FD males (0.32–0.7 nmol·mg^{-1}·h^{-1}/pmol·ml^{-1}, 0.57 ± 0.14) from males with *GLA* VUS (3.8–68.8 nmol·mg^{-1}·h^{-1}/pmol·ml^{-1}, 21 ± 20.1) and healthy controls (20–126 nmol·mg^{-1}·h^{-1}/pmol·ml^{-1}, 81.6 ± 27.6). The ratio of AGAL activity in plasma to plasma lysoGb3 **(**Fig. 2**B and D)** gave comparable results: classic FD males ($7.8{\cdot}10^{-4}$-$7.6{\cdot}10^{-3}$ nmol·ml^{-1}·h^{-1}/pmol·ml^{-1}, $3{\cdot}10^{-3}$ ± $2.6{\cdot}10^{-3}$), late-onset cardiac FD (0.02–0.09 nmol·ml^{-1}·h^{-1}/pmol·ml^{-1}, 0.05 ± 0.02), *GLA* VUS (0.5–12.1 nmol·ml^{-1}·h^{-1}/pmol·ml^{-1}, 2.2 ± 3.5) and controls (3.4–12.7 nmol·ml^{-1}·h^{-1}/pmol·ml^{-1}, 6.6 ± 2.5).

The ratios of AGAL activity in WBCs to plasma lysoGb3 of FD females **(**Fig. 2**A and C)** with classic (0.33–11.7 nmol·mg^{-1}·h^{-1}/pmol·ml^{-1}, 3.41 ± 2.61) and late-onset cardiac FD (1.74–45 nmol·mg^{-1}·h^{-1}/pmol·ml^{-1}, 13.9 ± 10.4) were significantly different from females with *GLA* VUS (12.1–131 nmol·mg^{-1}·h^{-1}/pmol·ml^{-1}, 51.1 ± 30) and controls (26.6–158 nmol·mg^{-1}·h^{-1}/pmol·ml^{-1}, 78.6 ± 30.8). Females with *GLA* VUS were also significantly different from female controls. Similar results were achieved using AGAL activity in plasma for females **(**Fig. 2**B and D)** with classic FD (0.03–0.89 nmol·ml^{-1}·h^{-1}/pmol·ml^{-1}, 0.23 ± 0.2) and late-onset cardiac FD (0.32–3.8 nmol·ml^{-1}·h^{-1}/pmol·ml^{-1},

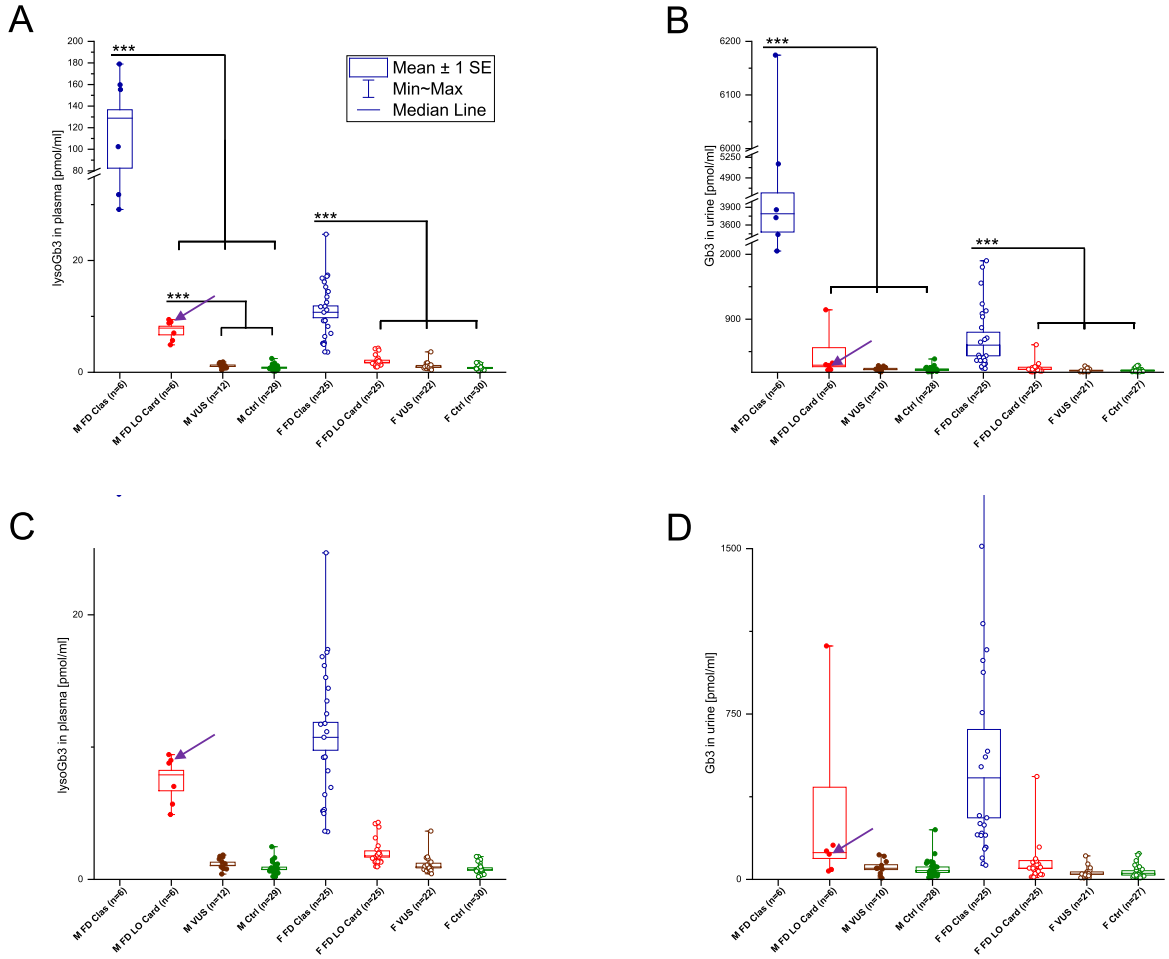

Fig. 1. Plasma lysoGb3 and urinary Gb3 levels in FD patients. A – concentration of lysoGb3 measured by NP-LC-ESI-MS/MS in blood plasma samples; **B** – concentration of Gb3 measured by NP-LC-ESI-MS/MS in urinary samples; **C** – Detail of A; **D** – detail of B. Statistical analysis in **A** and **B** is based on pairwise Student t-tests after ANOVA analysis of the presented dataset where: *** – $p < 0{,}001$ (JMP v. 11.0.0). M FD Clas – males with classic FD; M FD LO Card – males with late-onset cardiac FD; M VUS – males with VUS in *GLA* gene; M Ctrl – male control group; F FD Clas – females with classic FD; F FD LO Card – females with late-onset cardiac FD; F VUS – females with Variants of Unknown Significance in *GLA* gene; F Ctrl – female control group; Arrow – male based on biological sex but the patienťs gender self-identification is female.

1.39 ± 0.89) and differed significantly from females with *GLA* VUS (0.97–6.32 nmol·ml^{-1}·h^{-1}/pmol·ml^{-1}, 3.25 ± 1.45) and controls (1.86–8.87 nmol·ml^{-1}·h^{-1}/pmol·ml^{-1}, 5.75 ± 2.03).

3.4. Statistical ROC evaluation of the biomarkers

Diagnostic performance of individual biomarkers and ratios of AGAL activity to plasma lysoGb3 was evaluated using ROC curves.

In males **(**Fig. 3**A),** lysoGb3 and ratio of AGAL activity in WBCs and plasma to plasma lysoGb3 were confirmed as the best biomarkers with the same score 1.0. Urinary Gb3 and metGb3 had scores 0.91 and 0.87, respectively. In females, plasma lysoGbCer score was 0.95 followed by urinary Gb3 (0.85) and metGb3 (0.76) **(**Fig. 3**B).** This confirmed plasma lysoGb3 as the best biomarker. It was, however, surpassed by both AGAL activity in WBCs/lysoGb3 (0.98) and AGAL activity in plasma/lysoGb3 (0.97) ratios.

We further evaluated the efficacy of the biomarkers to differentiate *GLA* VUS carriers of both sexes from healthy controls. ROC curves in males **(**Fig. 3**C)** revealed the following (categorized from the best performing to the worst performing): AGAL in WBCS/lysoGb3 (0.96), AGAL in plasma/lysoGb3 (0.9), lysoGb3 (0.76), Gb3 (0.59) and metGb3 (0.54). In females, the scores were lower **(**Fig. 3**D):** AGAL in plasma/lysoGb3 (0.83), AGAL in WBCs/lysoGb3 (0.76), lysoGb3 (0.69), Gb3 (0.51) and metGb3 (0.46).

Finally we evaluated the ability of the biomarkers and the ratios to separate patients with the classic variant from those with late-onset cardiac FD. Scores 1.0 were calculated in males for all variables **(**Fig. 3**E).** In females, lysoGb3 scored 0.99 and was followed by AGAL in plasma/lysoGb3 (0.97), Gb3 (0.95), metGb3 (0.91) and AGAL in WBCs/lysoGb3 (0.9) **(**Fig. 3**F).**

3.5. Correlation of plasma lysoGb3 and urinary Gb3 concentrations with AGAL activity in peripheral WBCs

AGAL activity was assessed in plasma and peripheral WBCs. Comparison of activity measured in both materials (plasma and WBCs) in individual subjects is presented in **Fig. S3**. The control range of AGAL activity in WBCs (24.8–103 nmol·mg^{-1}·h^{-1}, 59.7 ± 14.6) was determined for diagnostic purposes in 477 control samples previously. In plasma, the activity in healthy population (2.4–19.4 nmol·ml^{-1}·h^{-1}, 6.1 ± 2.8) was determined for diagnostic purposes by analysis in 322 control samples. Correlations of AGAL activity and plasma lysoGb3 and urinary Gb3 in all analyzed groups of FD patients are presented in Fig. 4.

Plasma lysoGb3 concentration progressively increased in male FD patients with AGAL WBCs activity < 10 % of average in the controls (<6 nmol·mg^{-1}·h^{-1}) **(**Fig. 4**A, C).** As a likely effect of X-linked inheritance

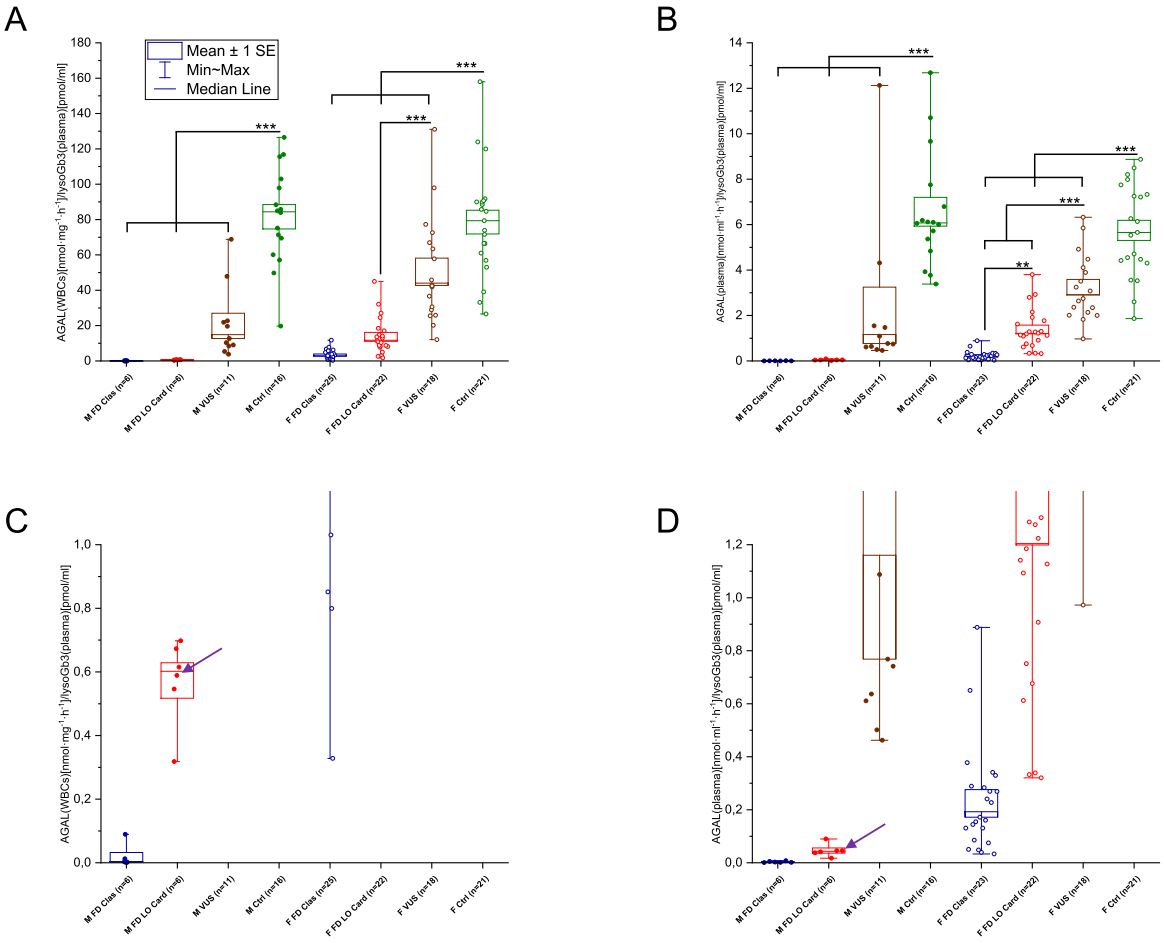

Fig. 2. Ratio of AGAL activity in plasma and peripheral WBCs to plasma lysoGb3 in untreated FD patients and controls. Quantitative analysis performed by NP-LC-ESI-MS/MS. A – Ratio of AGAL activity in WBCs to plasma lysoGb3; B – Ratio of plasma AGAL activity to lysoGb3. C and D – details of A and B to show separation of males with classic and late-onset cardiac FD phenotype. Statistical analysis in **A** and **B** is based on pairwise Student t-tests after ANOVA analysis of the presented dataset where: *** – $p < 0{,}001$ ** – $p < 0{,}01$ (JMP v. 11.0.0). M FD Clas – males with classic FD; M FD LO Card – males with late-onset cardiac FD; M VUS – males with VUS in *GLA* gene; M Ctrl – male control group; F FD Clas – females with classic FD; F FD LO Card – females with late-onset cardiac FD; F VUS – females with Variants of Unknown Significance in *GLA* gene; F Ctrl – female control group; Arrow – male based on biological sex but the patients gender self-identification is female.

such a correlation and threshold effects were not observed among heterozygous female FD patients (Fig. 4**B**). Similar pattern was observed using plasma lysoGb3 correlated to plasma AGAL activity in FD females (**Fig S4**).

Interestingly, urinary Gb3 (Fig. 4**D**) and metGb3 (**Fig. S5**) increased in male classic FD patients with AGAL WBCs activity less than 5 % (<3 $nmol \cdot mg^{-1} \cdot h^{-1}$).

4. Discussion

Herein, we present a robust and easy-to-execute NP-LC-ESI-MS/MS method for analyses of lysoGb3 in plasma and Gb3 and metGb3 in urine. This assay allows isocratic HPLC separation of lysoGb3 from Gb3. The analytical time is short (<3 min). Cheaper and less powerful mass spectrometers can be used. Overall, our protocol should be feasible even in laboratories with limited experience in liquid chromatography techniques therefore facilitating Fabry disease diagnostics and screening.

Use of reverse-phase columns and gradient elution [28–31] are the key technical disadvantages of the current methods for lysoGb3 assessment in the standard laboratory practice. Omitting liquid chromatography (i.e. use of direct infusion of the sample or flow injection mass spectrometry), [32] however, increases the risk of artificial conversion of Gb3 to lysoGb3 during the electrospray ionization and other unwanted (e.g. matrix) effects. Therefore, we utilized isocratic elution chromatography on normal phase (column with Silica-C silicagel) coupled with one-component mobile phase of methanol with ammonium acetate. The use of normal stationary phase brings greater capacity, smaller memory effect(s), and ability to separate lipids according to their classes rather than by individual molecular species based on their fatty acid composition [32,33]. Our new protocol enables separation of Gb3 from lysoGb3 (**Fig S1**) with sufficient separation speed, reproducibility and sensitivity comparable to the latest published methods [27,28]. LysoGb3 measurements are possible from concentrations as low as 140 fmol/ml. The simplicity of the new procedure is comparable to flow injection mass spectrometry (FIA-MS). Importantly, lower power mass spectrometers (e.g. AB/MDS SCIEX API4000) can be used.

The assay was validated by determining concentrations of lysoGb3 in plasma and Gb3 and metGb3 in urine of FD patients and control subjects. Individual biomarkers were evaluated based on their ability to distinguish the groups of patients with different FD phenotypes from controls and phenotypic groups from each other. The assessment was performed both in male and female patients.

Plasma lysoGb3 proved to be the best biomarker for all FD types. LysoGb3 values reliably discriminated FD male patients with both classic and late-onset cardiac phenotypes as well as FD females with the

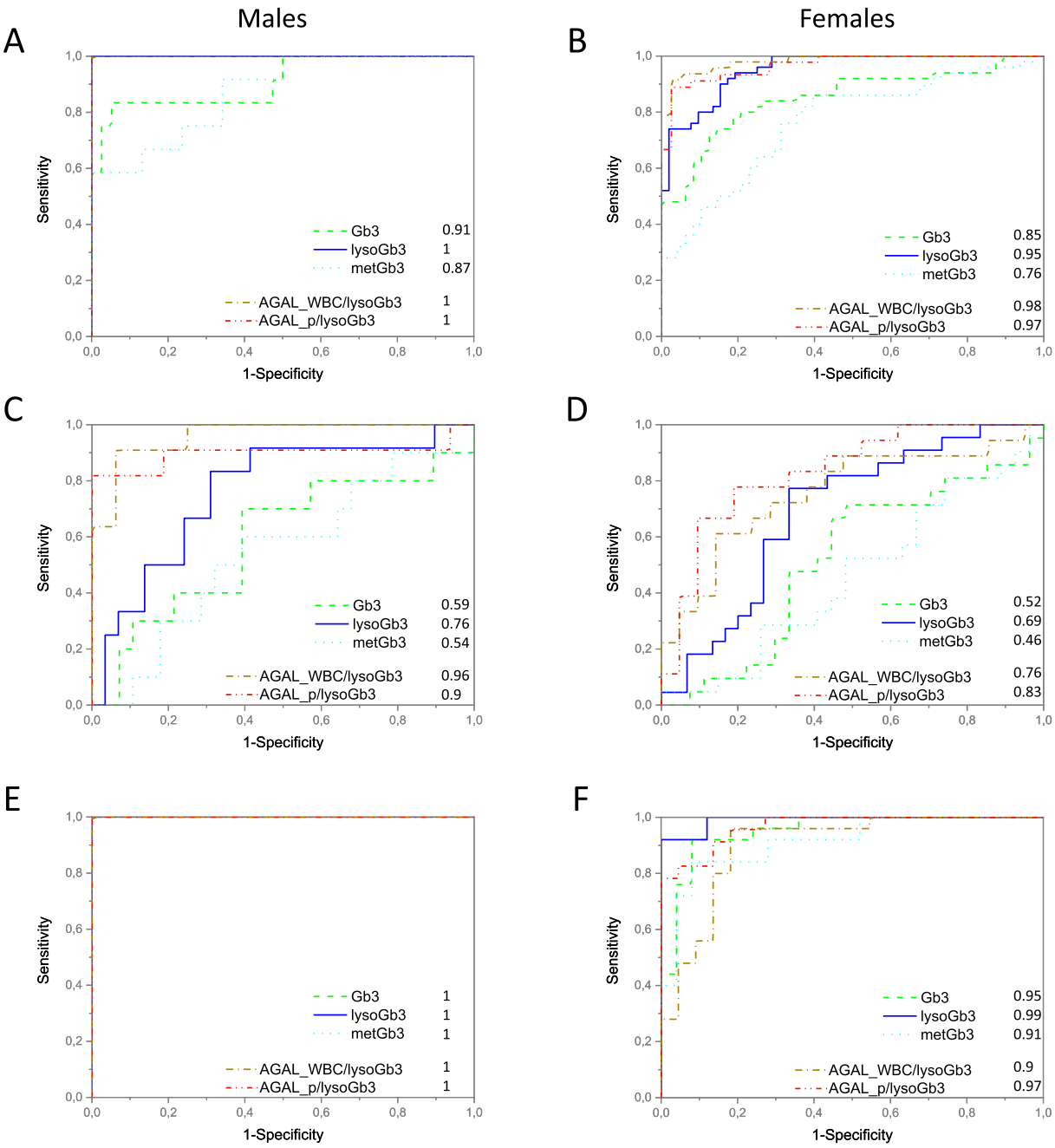

Fig. 3. Statistical ROC evaluation of FD markers. A – Males: classic FD and late-onset FD (positive group) vs *GLA* VUS and controls (negative group); B – Females: classic FD and late-onset FD (positive group) vs *GLA* VUS and controls (negative group); C – Males: *GLA* VUS (positive group) vs controls (negative group); D – Females: *GLA* VUS (positive group) vs controls (negative group); E – Males: classic FD (positive group) vs late-onset cardiac FD (negative group); F – Females: classic FD (positive group) vs late-onset cardiac FD (negative group). AGAL_p/lysoGb3 – ratio of plasma AGAL activity to lysoGb3; AGAL_WBC/lysoGb3 – ratio of AGAL activity in WBCs to plasma lysoGb3.

classic phenotype from controls. However, lysoGb3 (similar to other authors [10,12,18,30]) failed to differentiate females with late-onset cardiac FD from controls in our cohort. We therefore recommend preferential use of lysoGb3 for diagnostics/screening in FD males. Of note, values in males and females with *GLA* VUS did not differ from controls. These measurements may thus provide additional helpful cue to discriminate these *GLA* variants from those causing significant storage [4,9,34].

Urinary Gb3 and metGb3, on the contrary, did not give the expected predictive response **(**Fig. 1**B and S4-6)**. This was particularly surprising for metGb3, which was previously suggested as an alternative biomarker to lysoGb3 in male patients with late-onset cardiac FD carrying *GLA* variant p.N215S [20]. Overall, diagnostic efficacies of Gb3 and metGb3 were substantially lower than the sole use of lysoGb3. We therefore believe that the practical utility of Gb3 and metGb3 in routine FD diagnostics/screening is very limited.

AGAL deficiency is currently considered the key pathogenic contributor to FD. Understanding the relationship between the residual enzymatic activity and the concentration of the biomarker is therefore a critical parameter determining the informative value of the latter. Parallel testing of AGAL activity together with lysoGb3 was previously used to improve the diagnostic efficiency of FD [22]. Correlations of these parameters have been presented in dried blood spots (DBS) [21] and plasma [14]. Baydakova et al. [21] demonstrated improved diagnostic

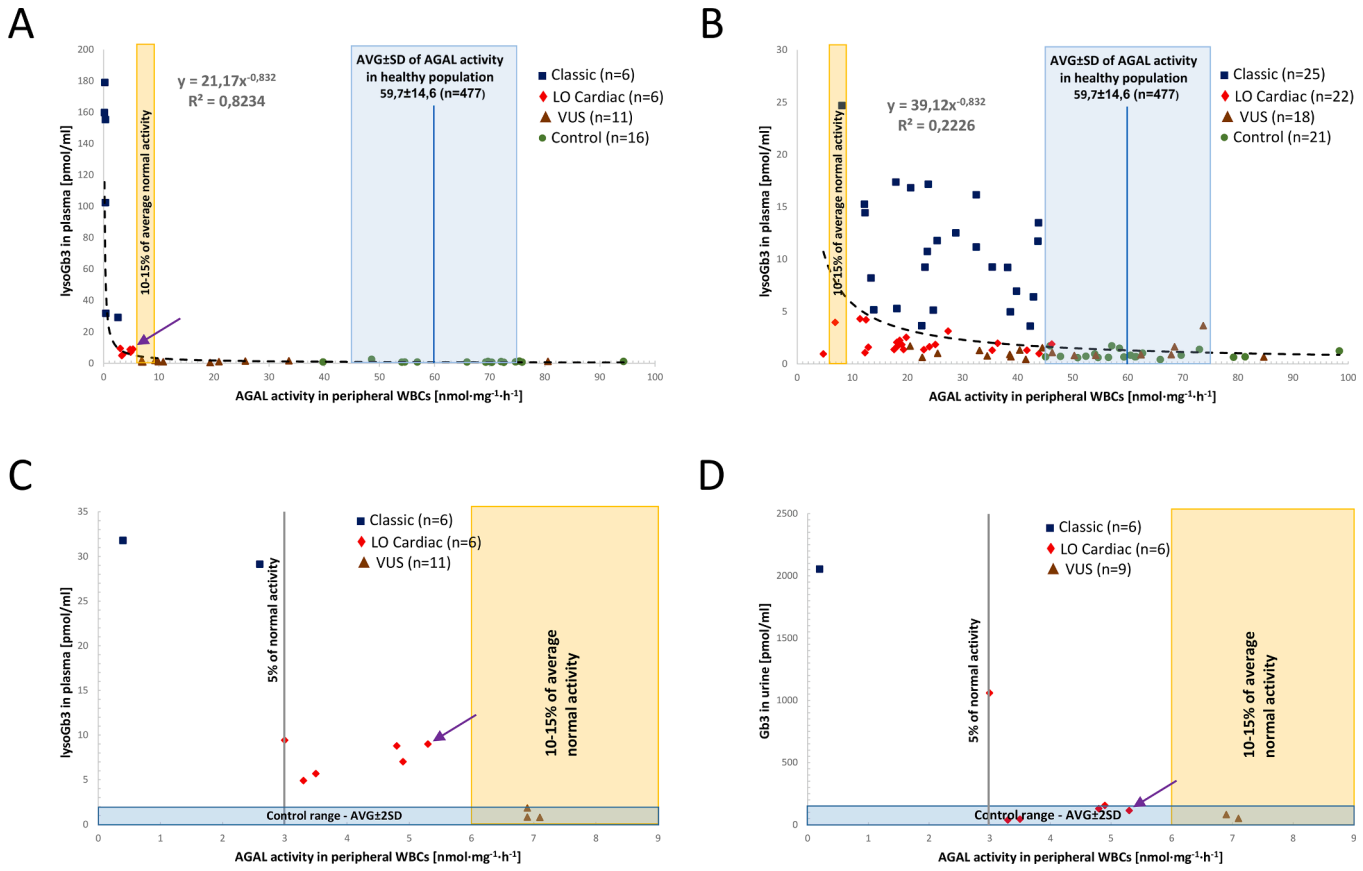

Fig. 4. Correlation of lysoGb3 in plasma with AGAL activity in peripheral WBCs. A – correlation of plasma lysoGb3 to AGAL activity in peripheral WBCs in males; **B** – correlation of plasma lysoGb3 to AGAL activity in peripheral WBCs in females; **C** – detail showing correlation of plasma lysoGb3 to AGAL activity in peripheral WBCs below the minimal activity threshold required for maintaining Gb3 biochemical turnover in males; **D** – detail showing correlation of urinary Gb3 to AGAL activity in peripheral WBCs below the minimal AGAL activity threshold required for maintaining Gb3 biochemical turnover in males. Regression curves were created on the basis of a power mathematical model of dependence. Classic – patients with classic FD; LO Cardiac – patients with late-onset cardiac FD; VUS – subjects with Variants of Unknown Significance in *GLA* gene; Control – control subjects. Arrow – male based on biological sex but the patient́s gender self-identification is female.

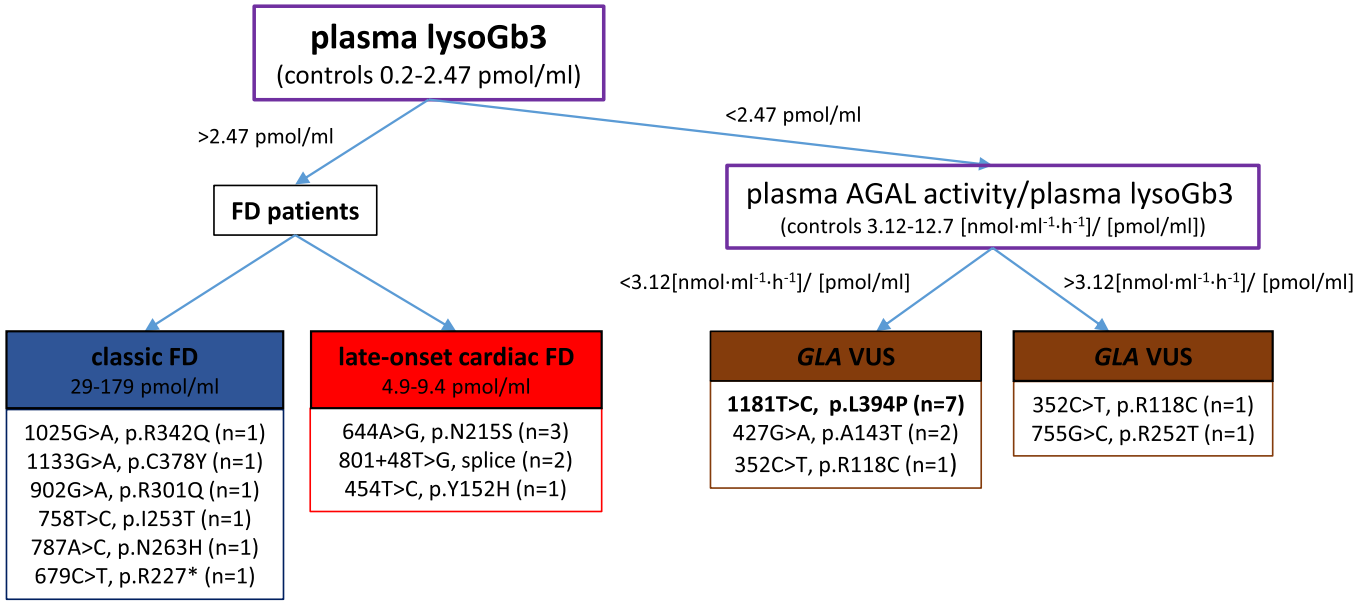

Fig. 5. Summary of biochemical results in the tested male cohort. Correlation of biochemical findings to FD phenotypes and *GLA* genotypes is provided. LysoGb3 plasma concentration discriminates FD (classic and late-onset cardiac) patients. Some *GLA* VUS (e.g. 1181 T > C, p.L394P in 7 patients in our cohort) displayed lower values of plasma AGAL activity/plasma lysoGb3 ratio than controls. Fig S7 provides similar overview in the female part of the cohort.

performance of AGAL activity to lysoGb3 ratios. Nonetheless, limited information on the ability to discriminate classic or late-onset FD was provided.

Our studies (including ROC-based statistics) show that the values of AGAL activity in plasma or peripheral WBCs to plasma lysoGb3 ratio(s) further improve the phenotypic predictive capacity **(**Fig. 3**)** [21]. In FD males, there was a significant difference of both ratios between all phenotypic groups. In females, there was no overlap between the range of values of classic FD and controls. A significant improvement to testing solely lysoGb3 was shown in females with late-onset cardiac FD for both ratios using plasma or WBCs AGAL activity **(**Fig. 3**)**. The calculated values reached almost similar diagnostic efficacy in late-onset cardiac FD females as did plasma lysoGb3 in classic FD females. Based on these results, we suggest to screen male patients by lysoGb3 testing **(**Fig. 5**).** In females, parallel AGAL activity assessment in plasma and ratio calculations improve discrimination/categorization **(Fig S7)**. Last, calculated ratios of AGAL activity to lysoGb3 may (in X-hemizygous male genetic setup) represent an additional biochemical mean to evaluate impacts of *GLA* variants of unknown significance **(**Fig. 5**).**

Earlier work by Leinekugel, P., et al. [35] presumes the existence of a threshold value of enzyme activity that ensures sufficient degradation of sphingolipids to prevent their lysosomal accumulation. The expected critical threshold ranges from 10-15 % of the average catabolic activity in a healthy population. Average control AGAL activity in peripheral WBCs in our study is 60 nmol/mg·h. The minimal value of AGAL activity to maintain sufficient lysosomal catabolism can thus be estimated as 6–9 nmol/mg·h. Males with classic and late-onset cardiac FD presented with residual AGAL activity values below the threshold and plasma lysoGb3 concentrations were above the control range. On the contrary and in agreement with literature-based predictions, we observed that the concentration of lysoGb3 in plasma was normal in all males with WBCs AGAL activity above 6 nmol/mg·h. This group consisted not only of healthy control subjects but also of males with *GLA* VUS. Importantly, correlation with AGAL activity measured in WBCs discriminated a group of male patients with late-onset cardiac FD (unlike to correlations solely using the plasma AGAL activity [14]).

An increase in urinary Gb3 and metGb3 concentrations above the control range was detected only in males with classic FD and one patient with late-onset cardiac FD. This patient had the lowest AGAL activity (3 nmol/mg·h) that corresponded to 5 % of the average activity in the control group (Fig. 4**D**). All other patients with cardiac FD had normal values of urinary Gb3 and metGb3. This result confirms that lysoGb3 is the only biomarker that correlates and reliably reflects the enzyme deficiency in FD.

Distribution of lysoGb3 values in X-heterozygous female FD patients differs from patterns observed in male patients (Fig. 4**B**). The graph of the dependence of lysoGb3 concentration on residual AGAL activity separates the phenotypic groups imperfectly. LysoGb3 concentrations are substantially less increased and residual AGAL activities are higher than in males. As a result, the scatter plot is substantially wider even in patients with the classic FD. We hypothesize that this dispersion is most likely an effect of mosaicism due to X-chromosome inactivation [3,24].

Lastly, we would like to comment on caveats of biological/chromosomal sex and gender identity incongruity in diagnostics of X-linked genetic conditions. Sample processing in our cohort was anonymous. The only data available to us were gender categorization (provided by the patients) and FD histories provided by the clinical collaborators. Surprisingly, lysoGb3 values and the AGAL/lysoGb3 ratio values in one of our female gender samples (Figs. 1, 2, 4) corresponded to values in the male cohort. As a result, we substantially struggled with the correct biochemical/clinical interpretation of the patient's condition and thoroughly re-investigated the biological sex history of the donor (including molecular genetic reassessment). Finally, we found out that although the primary gender information from the collaborating clinician was female (based on the patient's wish to communicate solely the gender and not sex information), the subject's biological sex was male. We highlight this aspect to demonstrate the critical need to communicate the gender information in the context of the biological/chromosomal sex to allow correct clinical, diagnostic, and therapeutic practice.

5. Conclusions

We present a new simple, fast and reliable NP-LC-ESI-MS/MS method for analysis of plasma lysoGb3. In parallel, we measured concentrations of lysoGb3 in blood plasma and Gb3 and metGb3 in urine. Our results clearly show that plasma lysoGb3 is a biomarker of the first choice for Fabry disease. In male FD patients, increased lysoGb3 concentration reliably reflects the extent of the enzymatic defect. In females, the differences are not as distinct as a likely effect of X-chromosome inactivation, but still lysoGb3 surpasses urinary Gb3 and metGb3. To increase the predictive efficiency of plasma lysoGb3, we calculated the ratio of plasma AGAL activity to lysoGb3 and showed its benefits for diagnostics of female patients with late-onset cardiac FD. Correlations of lipid biomarker levels and residual AGAL activity in WBCs and plasma also help to categorize *GLA* VUS variants from those causing FD as defined by clinically relevant storage.

Ethic statement and patients' consent

This study was approved by ethics committee of General University Hospital in Prague No. 23/20Grant AZV VES 2021 1.LF UK and performed in accordance with Helsinki declaration. We confirm that all material collection and analyses were done after written informed consents from patients were obtained. This is a part of study conditions approved by ethics committee.

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CRediT authorship contribution statement

Ladislav Kuchar: Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Linda Berna:** Writing – review & editing, Writing –

original draft, Validation, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Helena Poupetova:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Jana Ledvinova:** Writing – review & editing, Writing – original draft, Validation, Supervision, Methodology, Investigation, Conceptualization. **Petr Ruzicka:** Writing – review & editing, Writing – original draft, Data curation. **Gabriela Dostalova:** Writing – review & editing, Writing – original draft, Supervision, Resources. **Stella Reichmannova:** Writing – review & editing, Writing – original draft, Supervision, Resources. **Befekadu Asfaw:** Writing – review & editing, Writing – original draft, Data curation. **Ales Linhart:** Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Jakub Sikora:** Writing – review & editing, Writing – original draft, Validation, Supervision, Formal analysis, Data curation, Conceptualization.

Data availability

Data will be made available on request.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cca.2024.119824.

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