



Characterization of the plasma proteomic profile of Fabry disease: Potential sex- and clinical phenotype-specific biomarkers

Laura López-Valverde^{a,b,1}, María E. Vázquez-Mosquera^{a,b,1}, Cristóbal Colón-Mejeras^{a,b}, Susana B. Bravo^{b,c}, Sofía Barbosa-Gouveia^{a,b}, J. Víctor Álvarez^{a,b}, Rosario Sánchez-Martínez^d, Manuel López-Mendoza^e, Mónica López-Rodríguez^{f,g}, Eduardo Villacorta-Argüelles^h, María A. Goicoechea-Diezhandinoⁱ, Francisco J. Guerrero-Márquez^j, Saida Ortolano^k, Elisa Leao-Teles^l, Álvaro Hermida-Ameijeiras^{a,b,2,*}, María L. Couce^{a,b,3,*}

^a Unit of Diagnosis and Treatment of Congenital Metabolic Diseases. RICORS-SAMID, CIBERER. University Clinical Hospital of Santiago de Compostela, Choupana s/n, Santiago de Compostela, A Coruña 15706, Spain

^b Health Research Institute of Santiago de Compostela (IDIS), University Clinical Hospital of Santiago de Compostela, Choupana s/n, Santiago de Compostela, A Coruña 15706, Spain

^c Proteomic Platform, University Clinical Hospital of Santiago de Compostela, Choupana s/n, Santiago de Compostela, A Coruña 15706, Spain

^d Internal Medicine Department, Alicante General University Hospital-Alicante Institute of Health and Biomedical Research (ISABIAL), Pintor Baeza 12, Alicante 03010, Spain

^e Department of Nephrology, Hospital Universitario Virgen del Rocío, Manuel Siurot s/n, Sevilla 41013, Spain

^f Internal Medicine Department, Hospital Universitario Ramón y Cajal, IRYCIS, Colmenar Viejo, Madrid 28034, Spain

^g Faculty of Medicine and Health Sciences, Universidad de Alcalá (UAH), Av. de Madrid, Alcalá de Henares 28871, Spain

^h Department of Cardiology, Complejo Asistencial Universitario de Salamanca, Pº. de San Vicente 58, Salamanca 37007, Spain

ⁱ Nephrology Department, Hospital Gregorio Marañón, Dr. Esquerdo 46, Madrid 28007, Spain

^j Department of Cardiology, Internal Medicine Service, Hospital de la Serranía, San Pedro, Ronda, Málaga 29400, Spain

^k Rare Diseases and Pediatric Medicine Research Group, Galicia Sur Health Research Institute-SERGAS-UVIGO, Clara Campoamor 341, Vigo 36213, Spain

^l Centro de Referência de Doenças Hereditárias do Metabolismo, Centro Hospitalar Universitário de São João, Prof. Hernâni Monteiro, Porto 4200-319, Portugal

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ABSTRACT

Fabry disease (FD) is a X-linked rare lysosomal storage disorder caused by deficient α -galactosidase A (α -GalA) activity. Early diagnosis and the prediction of disease course are complicated by the clinical heterogeneity of FD, as well as by the frequently inconclusive biochemical and genetic test results that do not correlate with clinical course. We sought to identify potential biomarkers of FD to better understand the underlying pathophysiology and clinical phenotypes. We compared the plasma proteomes of 50 FD patients and 50 matched healthy controls using DDA and SWATH-MS. The >30 proteins that were differentially expressed between the 2 groups included proteins implicated in processes such as inflammation, heme and haemoglobin metabolism, oxidative stress, coagulation, complement cascade, glucose and lipid metabolism, and glycocalyx formation. Stratification by sex revealed that certain proteins were differentially expressed in a sex-dependent manner. Apolipoprotein A-IV was upregulated in FD patients with complications, especially those with chronic kidney disease, and apolipoprotein C-III and fetuin-A were identified as possible markers of FD with left ventricular hypertrophy. All these proteins

Abbreviations: α -GalA, α -galactosidase A; AUC, area under the curve; CKD, chronic kidney disease; Clin FD, FD with clinical complication; Clin FemFD, female FD with some clinical complication; CTL, control; DDA, data-dependent acquisition; DIA, data-independent acquisition; ERT, enzyme replacement therapy; FD, Fabry disease; GFR, glomerular filtration rate; GB3, globotriaosylceramide; LC-MS/MS, liquid chromatography-tandem mass spectrometry; LSD, lysosomal storage disease; Lyso-GB2, lyso-lactosylceramide; Lyso-GB3, globotriaosylsphingosine; LV, left ventricle; LVH, left ventricular hypertrophy; MRI, magnetic resonance imaging; MLR, multiple linear regression; No Clin FD, FD without some clinical complication; No Clin FemFD, female FD without clinical complication; NT-proBNP, N-terminal pro-brain natriuretic peptide; SWATH, sequential window acquisition of all theoretical fragment ion spectra; Tpl, troponin I; VUS, variants of unknown significance.

* Corresponding authors.

E-mail addresses: alvaro.hermida.ameijeiras@sergas.es (Á. Hermida-Ameijeiras), maria.luz.couce.pico@sergas.es (M.L. Couce).

¹ These authors contributed equally

² (+34 981951263)

³ (+34 981950162)

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had a greater capacity to identify the presence of complications in FD patients than lyso-GB3, with apolipoprotein A-IV standing out as being more sensitive and effective in differentiating the presence and absence of chronic kidney disease in FD patients than renal markers such as creatinine, glomerular filtration rate and microalbuminuria. Identification of these potential biomarkers can help further our understanding of the pathophysiological processes that underlie the heterogeneous clinical manifestations associated with FD.

Introduction

Fabry Disease (FD; OMIM #301500) is an inherited X-linked lysosomal storage disease (LSD) caused by pathogenic variants in the *GLA* gene (OMIM *300644) that induce a deficiency in the activity of the lysosomal enzyme α -galactosidase A (α -GalA, EC 3.2.1.22).^{1,2} This deficiency leads to the accumulation of globotriaosylceramide (GB3) and other naturally occurring glycosphingolipids such as globotriaosylsphingosine (lyso-GB3) in plasma, urine, and multiple cell types, primarily endothelial, renal, cardiac, and neuronal cells.^{3,4} FD is a rare disease with an estimated overall incidence of 1 per 40,000 live births. The most recent newborn screening studies estimate the prevalence of FD between 1 in 1500 and 1 in 8000, although many of those diagnosed do not develop clinical symptoms.^{5–7}

Clinically, the disease is characterized by broad phenotypic variability, ranging from a severe multiorgan involvement (the so-called classic phenotype) to atypical late-onset forms.⁸ The classic phenotype, which features little to no α -GalA activity, emerges during childhood and is characterized by angiokeratomas, neuropathic pain, gastrointestinal disturbances, hypohidrosis, intolerance to heat and exercise, and cornea verticillata, progressing in adulthood to severe organ dysfunction, including kidney and heart disease and cerebrovascular events. The late-onset phenotype, in which onset typically occurs in adulthood, involves significant residual α -GalA activity and is associated with single organ damage, usually renal or cardiac involvement.^{9,10} Moreover, affected women can have very mild or severe forms due to random X-chromosome inactivation.^{11,12} Kidney and heart dysfunction are the main complications in FD. Kidney disorder in FD is characterized by proteinuria and a decrease in glomerular filtration rate (GFR) which, over the years, can lead to renal failure,^{11,13} while FD heart disease can manifest as infiltrative hypertrophic cardiomyopathy, arrhythmias, coronary artery disease, valve disorders or heart failure.^{13,14}

The prognosis includes progressive damage to vital organs with age, which can lead to organ failure. End-stage renal disease and cardiovascular or cerebrovascular complications significantly worsen life expectancy.¹⁵ Current treatments are based on enzyme replacement therapy (ERT) and/or the use of pharmacological chaperones. Treatment initiation depends on the degree of organ involvement and can improve quality of life as well as disease course.¹⁶ For these reasons, early diagnosis is essential to improve management. However, this is difficult in FD patients, particularly in women, due to the highly variable clinical presentation, the frequent inconclusive results of biochemical and genetic tests, and the lack of correlation between genotype, α -GalA activity, and clinical severity of the disease.^{1,13,17}

Biomarker identification in FD research is a growing field that has significant potential to overcome current challenges associated with delayed diagnosis and to enhance the effectiveness of care. Proteomics technologies applied to biological fluids from FD patients can potentially help identify disease-related protein markers. Indeed, recent studies have sought to identify urinary^{18–22} and plasma^{23–28} biomarkers using proteomics approaches. Here, we describe the plasma proteome profile of a Fabry patient cohort, and identify proteins linked to factors such as sex and main associated complications, in order to further our knowledge of biomarkers for potential use in clinical practice. This study is the deepest untargeted plasma proteomics study of FD patients performed to date, and reveals previously unknown plasma proteome patterns linked to the most prevalent clinical manifestations, notably chronic kidney disease (CKD) and left ventricular hypertrophy (LVH).

Material and methods

Experimental design

This study was approved by the Galician (Spain) Research Ethics Committee of Santiago-Lugo (Xunta de Galicia/Servizo Galego de Saúde, registration code: 2020/438 [Ana Estany Gestal; committee technical secretariat]) and was conducted in accordance with the legal and regulatory provisions for the use of human biological samples, in compliance with the Declaration of Helsinki. Informed consent was obtained from each study participant or guardian/legal representative if available.

The overall purpose of the study was to identify plasma biomarkers in FD to improve early detection and diagnosis, define clinical phenotypes, and facilitate the monitoring of the disease. This clinical study had an international, multicentre, prospective, open design, with a control group protocol, and collected biological samples from 50 patients diagnosed with FD and 50 sex- and age-matched healthy subjects. All participants were recruited over a 24-month period, from October 2020 until September 2022, and all procedures took place in a single visit. The overall study workflow is depicted in Fig. 1.

Study population

Biological samples from patients with a diagnosis of FD, both treated and naïve, who were recruited through clinicians from FD reference centres in Spain and Portugal. The age- and sex-matched group of healthy controls were recruited from volunteers and nonmedical staff at the University Clinical Hospital of Santiago.

The following inclusion criteria were applied: (i) male or female, aged 6–75 years; (ii) informed consent provided by participants or their legal representatives; (iii) FD patients: diagnosis biochemically confirmed by a decrease in the enzymatic activity of α -GalA and/or by the presence of pathogenic variants in *GLA*; (iv) healthy controls: with no family history of lysosomal storage disorders and no clinical signs of FD, considered healthy with no previous history of diabetes mellitus, atherosclerotic vasculopathy, or other inflammatory diseases. The exclusion criteria were as follows: (i) inconclusive genetic diagnosis (i.e. carriers of variants of unknown significance [VUS] or variants with conflicting interpretations of pathogenicity); (ii) any medical or psychological disorder that could interfere with the patient's ability to provide informed consent; (iii) inability or unwillingness to provide informed consent; (iv) participation in a study with an investigational drug within 3 months before providing informed consent.

Sample collection

Two blood samples (3.5 ml each) per study participant were collected directly into purple K2-EDTA tubes and stored at 4°C for up to 24 h. Subsequently, these samples were centrifuged at 10,000 g for 10 min at 4°C, and plasma and leukocytes were collected separately and frozen at -20°C for further analysis.

Clinical variables

Information related to the following variables was collected from each FD patient: demographic variables (sex, age, age at diagnosis, and time of evolution); genotype/phenotype (*GLA* variant, type of

phenotype: [classic or late-onset]); associated CKD, defined as abnormalities of kidney structure or function, present for >3 months, with implications for health and classified based on cause; GFR category (GFR is the volume of fluid filtered from the renal glomerular capillaries into the Bowman's capsule per unit of time; decreased GFR corresponds to <60 mL/min/1.73m²); albuminuria category (microalbuminuria corresponds to >30 mg/L);²⁹ associated LVH, defined as the thickening of the walls (>9 mm in women and >10 mm in men, determined by echocardiography) of the left ventricle and increase in its mass (linear method: >95 g/m² in women and >115 g/m² in men; 2D method: >88 g/m² in women and >102 g/m² in men), which can interfere with the heart's ability to pump blood into the aorta;³⁰ stroke, defined as an episode of acute neurological dysfunction caused by ischemia or haemorrhage that persists >24 h or until death;³¹ therapy (no therapy, ERT or chaperone therapy, age at therapy initiation, and therapy duration); biochemical variables (lyso-GB3 levels, troponin I, N-terminal pro-brain natriuretic peptide [NT-proBNP], creatinine, GFR, and microalbuminuria); maximal left ventricular (LV) wall-thickness and LV mass determined by echocardiography and/or cardiac magnetic resonance imaging (MRI); and α -GalA activity.

Information related to the following variables was collected from each healthy control: age, sex, lyso-GB3 levels, and α -GalA activity.

Biochemical analysis

Lyso-GB3 determination was based on measuring the amount of accumulated substrate present in plasma. It needed a minimum of 100 μ L plasma volume (for determination in duplicate). As a standard solution, 500 μ g/ml Lyso-Lactosylceramide (Lyso-Gb2 – Internal standard) dissolved in DMSO/methanol was used. After incubation for 2 h at 37°C and filtering of the supernatant, readings were taken using liquid chromatography-tandem mass spectrometry (LC-MS/MS) (HPLC 1200 Series, Agilent Technologies, and AB Sciex API 4000) in positive

polarity.

For measurement of α -GalA enzymatic activity in leukocytes, samples were sonicated and centrifuged for 10 min at 10,000 rpm at 4°C, and the supernatant collected. The Bradford technique was used to quantify total protein. For measurement of α -GalA activity, a solution of 4-methylumbelliferyl- α -D-galactoside in citrate-phosphate buffer (substrate) was added to the solution, as well as N-acetyl-D-galactosamine, an inhibitor of α -galactosidase B but not α -galactosidase A. After 20 h incubation at 37°C, the reaction was stopped with an ethylenediamine solution. The resulting fluorescence, caused by liberation of 4-methyl-umbelliferone from the substrate, is considered proportional to the enzymatic activity of the sample (excitation, 355 nm; emission, 460 nm; SPECTRAmax M2 Molecular Devices).

Plasma proteomic analysis

To ensure global and quantitative protein identification and quantification, an equal amount of protein was first depleted to eliminate high abundance proteins, as previously described.^{32,33}

Next, samples were concentrated on a 10 % SDS-PAGE gel. The protein band was detected by Sypro-Ruby fluorescent staining (Lonza, Basel, Switzerland), excised, and processed for in-gel and manual tryptic digestion, as previously described.³⁴ Briefly, the band was washed with 50 mM ammonium bicarbonate in 50 % methanol (HPLC grade) and dehydrated in acetonitrile (HPLC grade). The gel was reduced in 10 mM dithiothreitol (Sigma-Aldrich) in 50 mM ammonium bicarbonate (Sigma-Aldrich) at 56°C for 30 min and alkylated with 55 mM iodoacetamide (Sigma-Aldrich) in 50 mM ammonium bicarbonate for 20 min at room temperature in darkness. Subsequently, the gel sections were rinsed with 50 mM ammonium bicarbonate in 50 % methanol and dehydrated with acetonitrile. Tryptic digestion was performed by adding modified porcine trypsin (Promega) at a final concentration of 20 ng/ μ L in 20 mM ammonium bicarbonate for 16 h at 37°C. To extract the

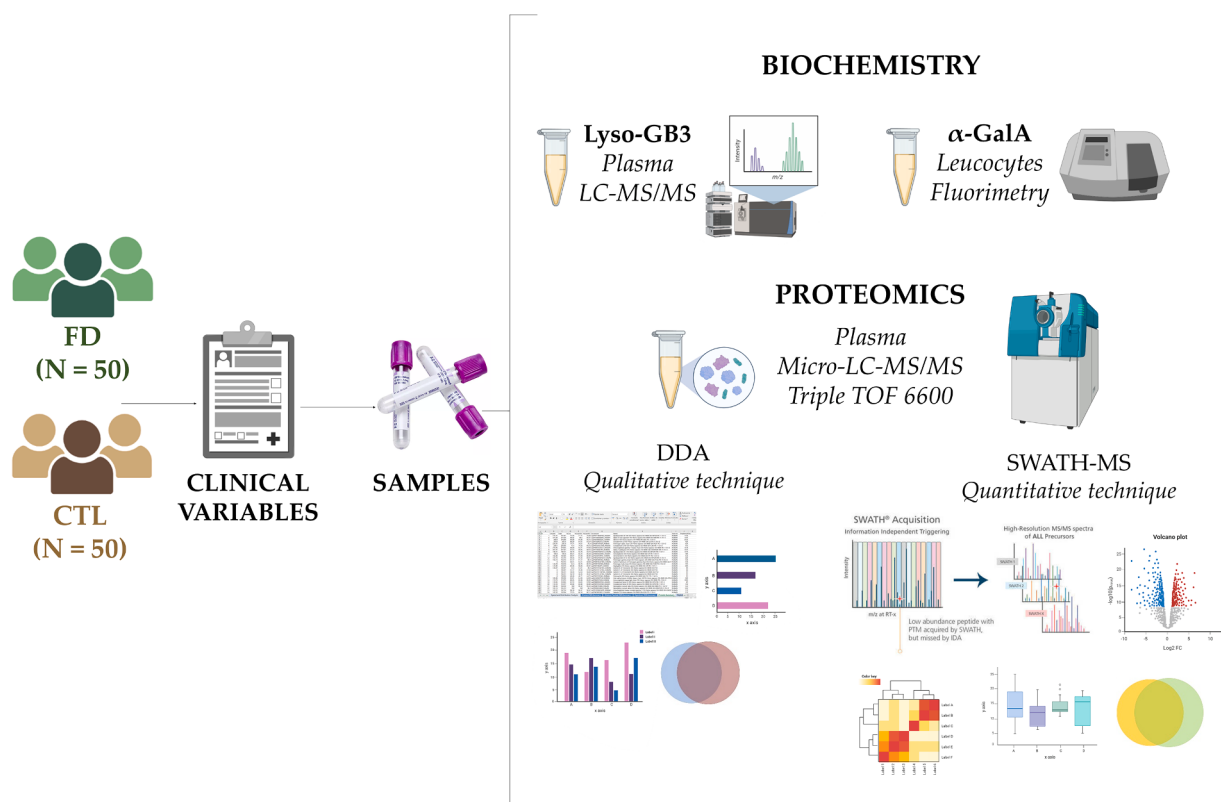


Fig. 1. Workflow of the overall clinical study. α -GalA, α -galactosidase A; CTL, control; DDA, data-dependent acquisition; FD, Fabry disease; LC-MS/MS, liquid chromatography-tandem mass spectrometry; lyso-GB3, globotriaosylsphingosine; SWATH, sequential window acquisition of all theoretical fragment ion spectra.

tryptic peptides generated, three 20-min incubations were performed in 40 μ L 60 % acetonitrile and 0.5 % formic acid, and the samples pooled and vacuum dried in a SpeedVac for storage at -20°C for further analyses.

Digested peptides ($> 4 \mu\text{g}$ of each sample) were separated using reverse phase chromatography. The gradient was created using a micro liquid chromatography system (Eksigent Technologies nanoLC 400, Sciex) coupled to a high-speed Triple TOF 6600 mass spectrometer (Sciex, Foster City, C) with a microflow source, as described previously.³² Qualitative analysis was performed using a data-dependent workflow (DDA) as described previously.³² Further details can be found in the Supplementary Material and Methods.

Quantitative analysis was performed by sequential window acquisition of all theoretical fragment ion spectra (SWATH-MS). To build the mass spectrometry (MS/MS) spectral libraries, pools of peptide solutions per condition were analysed using a shotgun data-dependent acquisition (DDA) approach by micro-LC-MS/MS (Sciex, USA), as described above and previously.^{32,35,36} After creation of the library, protein quantification was performed for individual samples by SWATH-MS (a data-independent acquisition [DIA] method for sample acquisition) using a TripleTOF® 6600 LC-MS/MS system (Sciex, USA) (see Supplementary Material and Methods for details).

Functional analysis

Analysis of the qualitative plasma proteome was performed using the open-access software FunRich (Functional Enrichment Analysis Tool version 3.1.3; <http://funrich.org/index.html>) for functional enrichment (Gene Ontology (GO)-Biological processes).

Statistical analysis

Epidemiological and statistical software was used (EPIDAT 4.0) to estimate the number of biological samples needed to achieve statistical power to identify potential significant differences between two groups. For identification of diagnostic biomarkers, candidate biomarkers were required to be present in 90 % of FD patients and ≤ 10 % of controls; assuming a 1:1 patient/control ratio, a confidence level of 95 %, and a statistical power of 100 %, 50 blood samples were required from patients and controls, respectively.

Statistical analysis was performed using GraphPad Prism 8 (GraphPad Inc) and SPSS Statistics (version 26.; IBM Corp). All statistical tests were two sided, and a p-value < 0.05 was considered statistically significant. The details of experiments can be found in the Fig. legends and tables.

Clinical and biochemical characteristics were compared between groups using the Mann-Whitney U-test for quantitative variables and Pearson's chi-squared and Fisher's exact tests for qualitative variables. The hypergeometric test was used to select the enriched biological processes and Fisher's exact test to compare between groups.

To analyse the quantitative proteomic results, normality tests (Shapiro-Wilk test) were performed if possible, and the log₂FC was calculated with the mean or median. Significant differences between groups were assessed using the Mann-Whitney U-test or Student's t-test. Proteins with p-values < 0.05 and log₂FC $\geq +0.6$ and ≤ -0.6 were selected. In comparisons in which groups had a $n \geq 15$, the p-value of those proteins influenced by confounding factors (sex, age) was adjusted by binary logistic regression. For regression, the variables were rescaled using Min-Max scaling due to the magnitude of the values. Spearman and Pearson rank analyses were used to analyse the correlation between proteins and lyso-GB3, or between proteins and other biochemical variables. The areas under the curve (AUC) of the ROC curves were calculated using GraphPad Prism 8.

Results

Clinical, biochemical, and genetic characteristics of the FD cohort

Fifty FD patients and 50 control (CTL) subjects, matched by sex and age, were enrolled in this study. FD patients were 44 % female, and the median age was 47.50 (10–75) years. The median follow-up time since diagnosis was 6.5 (0–34) years. Twenty patients had the classical phenotype and 30 had the late-onset phenotype. Thirty-one patients were receiving specific treatment for FD with ERT ($n = 21$, 67.7 %) or chaperone therapy ($n = 10$, 32.3 %), with a median age at therapy initiation of 47 (8–62) years. The treated FD patients in our cohort were predominantly male and older, with a later diagnosis than untreated patients. Moreover, these treated patients had more complications, mainly involving the kidney and heart, and showed greater alterations in lyso-GB3 levels and enzymatic activity. Interestingly, 31.6 % of untreated patients had organ damage and were therefore susceptible to specific treatment for FD. Three of the most common manifestations in FD were assessed: stroke (12 % of patients); CKD (44 %); and LVH (58 %). Biochemical variables related to these conditions were altered in FD patients, as detailed in Table 1. Plasma lyso-GB3 levels were increased in FD patients, with a median of 74.20 (3.2–1767) ng/mL (normal range, 0.3–8.25 ng/mL), while α -GalA activity in leucocytes was reduced, at 1.05 (0–23.9) nmol/h/mg (normal range: 4.8–23 nmol/h/mg). Male FD patients were more likely to be receiving treatment, had a higher incidence of CKD and LVH, and showed greater alterations in lyso-GB3 levels and reduced enzymatic activity, as well as greater alterations in other variables, in particular NT-proBNP, LV wall thickness, and microalbuminuria (Table 1). As detailed in Fig. 2, the two most frequent genetic variants of GLA in the study cohort were c.713G>A ($n = 14$, 25 %) and c.902G>A ($n = 7$, 12.5 %), both missense variants.

Plasma proteome of FD patients vs healthy controls

An initial qualitative analysis of the FD and CTL plasma proteome using MS/MS identified 335 and 313 proteins, respectively (Supplementary Table 1 and Supplementary Table 2) (Fig. 3a). Of these, 271 were common to both groups, 64 were exclusive to the FD group, and 42 were exclusive to the CTL group. Analysis of the processes associated with the proteins unique to each group showed a greater proportion of proteins associated with cell communication/signal transduction and metabolism in the FD group, and of those associated with protein metabolism and cell growth and/or maintenance in the CTL group, with statistical significance reached for the latter effect (Fig. 3b).

SWATH-MS analysis detected 8 downregulated ($\text{FC} \leq 0.65$; $p < 0.05$) and 28 upregulated ($\text{FC} \geq +1.5$; $p < 0.05$) proteins in the FD vs CTL groups (Fig. 3c) (Supplementary Table 3). By studying the correlation between these differentially expressed proteins and lyso-GB3, we detected significant correlations between lyso-GB3 and the upregulated proteins HBA ($r = 0.361$, $p = 0.010$), HBB ($r = 0.390$, $p = 0.005$), PRDX2 ($r = 0.323$, $p = 0.022$), CAH1 ($r = 0.390$, $p = 0.005$), DIAC ($r = -0.358$, $p = 0.011$), IGHG3 ($r = -0.302$, $p = 0.033$), FA9 ($r = -0.320$, $p = 0.023$), and GPX3 ($r = -0.299$, $p = 0.035$).

Analysis stratified by sex (Supplementary Table 4 and Supplementary Table 5) revealed 14 and 11 proteins upregulated exclusively in female and male FD patients, respectively. In females, expression of ANT3, HRG, FINC, G3P, 6PGD, FA9, and SODC differed most clearly from the profile observed in males, while in males, 1433Z, S10A8, CD99, HBG2, A2GL, FHR2 differed most clearly from the profile observed in females. Proteins downregulated in the FD versus CTL groups included C1QB and PLMN in women and CO5 and vitronectin (VTNC) in men (Fig. 4a). Moreover, irrespective of sex, 12 proteins were upregulated ($\text{FC} \geq 1.5$; $p < 0.05$) and 5 downregulated ($\text{FC} \leq 0.65$; $p < 0.05$) in the FD versus CTL groups (Fig. 4b). Analysis of the ROC curves allowed us to filter out markers for which AUC values exceeded 0.75, and revealed upregulation of HBD, HBB, HBA, DIAC, DEF3, FCN2, APOH, and S10A9,

Table 1
Clinical and biochemical characteristics of the cohort.

Characteristic	FD n = 50*	CTL n = 50*	p-value ^a	Female FD n = 22*	Male FD n = 28*	p-value ^a
Sex	22/50 (44)	25/50 (50)	0.548			
Female	28/50 (56)	25/50 (50)				
Male						
Age (Y)	47.50 (17)	45.50 (19)	0.692	44.5 (17)	48 (16)	0.604
Age at diagnosis (Y)	40 (30)			37.50 (26)	42.50 (32)	0.597
Time of evolution (Y)	6.50 (12)			5.50 (16)	7 (13)	0.883
Phenotype	20/50 (40)			8/22 (36.4)	12/28 (42.9)	0.642
Classic	30/50 (60)			14/22 (63.6)	16/28 (57.1)	
Late-onset						
Treatment	31/50 (62)			6/22 (27.3)	25/28 (89.3)	<0.0001
Treated	19/50 (38)			16/22 (72.7)	3/28 (10.7)	
Untreated						
Type of therapy	21/31 (67.7)			4/6 (66.7)	17/25 (68)	>0.9
ERT	10/31 (32.3)			2/6 (33.3)	8/25 (32)	
Chaperone						
Age at therapy initiation (Y)	47 (12)			44 (12)	48 (20)	0.841
Therapy duration (Y)	3 (5)			5.50 (6)	3 (5)	0.596
CKD^b	22/50 (44)			5/22 (22.7)	17/28 (60.7)	0.007
LVH^c	29/50 (58)			7/22 (31.8)	22/28 (78.6)	0.001
Stroke	6/50 (12)			4/22 (18.2)	2/28 (7.1)	0.385
Lyso-GB3 (ng/mL)	74.20 (105.6)	3.45 (1.1)	<0.0001	29.2 (65.6)	97.25 (210.7)	<0.0001
α-GalA (nmol/h/mg)	1.05 (6.9)	17.45 (7.4)	<0.0001	7.1 (6.2)	0.0 (0.6)	<0.0001
Elevated Troponin I (>40 pg/mL)	12/40 (30)			4/17 (23.5)	8/23 (34.8)	0.443
Elevated NT-proBNP (>125 pg/mL)	23/44 (52.3)			6/19 (31.6)	17/25 (68)	0.017
Elevated LV wall thickness^d (mm)	35/50 (70)			12/22 (54.5)	23/28 (82.1)	0.035
Elevated LV mass^e (g/m²)	15/39 (38.5)			4/17 (23.5)	11/22 (50)	0.092
Decreased GFR^f (<60 mL/min/1.73m²)	8/50 (16)			1/22 (4.5)	7/28 (25)	0.064
GFR stages						0.560
G1 (normal or high: ≥90)	22/50 (44)			12/22 (54.5)	10/28 (35.7)	
G2 (mildly decreased: 60-89)	20/50 (40)			9/22 (40.9)	11/28 (39.3)	
G3a (mildly to moderately decreased: 45-59)	3/50 (6)			1/22 (4.5)	2/28 (7.1)	
G3b (moderately to severely decreased: 30-44)	2/50 (4)			0/22 (0)	2/28 (7.1)	
G4 (severely decreased: 15-29)	2/50 (4)			0/22 (0)	2/28 (7.1)	
G5 (kidney failure: <15)	1/50 (2)			0/22 (0)	1/28 (3.6)	
Microalbuminuria (>30 mg/L)	23/49 (46.9)			6/21 (28.6)	17/28 (60.7)	0.042
Microalbuminuria stages						0.053
A1 (normal to mildly increased: <30)	26/49 (53.1)			15/21 (71.4)	11/28 (39.3)	
A2 (moderately increased: 30-300)	15/49 (30.6)			5/21 (23.8)	10/28 (35.7)	
A3 (severely increased: >300)	8/49 (16.3)			1/21 (4.8)	7/28 (25)	

Abbreviations: CTL, Control; CKD, Chronic Kidney Disease; ERT, Enzyme Replacement Therapy; FD, Fabry Disease; GFR, Glomerular Filtration Rate; LV, Left Ventricle; LVH, Left Ventricular Hypertrophy; (Y), Years. Bold font denotes significant values (p<0.05).

* n/N (%); median (RQ).

^a Mann-Whitney U test (quantitative variables); Pearson's chi-squared and Fisher's exact tests (qualitative variables).

^b CKD is defined as abnormalities of kidney structure or function, present for >3 months, with implications for health, and CKD is classified based on cause, GFR category, and albuminuria category.

^c LVH is defined as thickening of the walls of the left ventricle and an increase in is mass, which can interfere with the heart's ability to pump blood into the aorta.

^d >9 mm in women and >10 mm in men (echocardiography).

^e Linear method: >95 g/m² in women and >115 g/m² in men; 2D method: >88 g/m² in women and >102 g/m² in men.

^f GFR is the volume of fluid filtered from the renal glomerular capillaries into Bowman's capsule per unit of time.

and downregulation of ITIH, CETP, and CO3 (Fig. 4c). The same approach was applied to proteins that were differentially expressed in the FD group for only one sex, revealing upregulation of ANT3, HRG, and FINC in women and 1433Z in men, and downregulation of C1QB and PLMN in women and CO5 and VTNC in men (Fig.s 4d-e).

Clinical phenotypes

Comparison of FD patients with clinical complications (CKD and/or LVH and/or Stroke; Clin FD) (n = 35) versus those without (No Clin FD) (n = 15) revealed no significant differences in lyso-GB3 or α-GalA levels (Fig. 5a). Analysis of plasma protein expression in FD patients with an associated complication showed upregulation of APOA4, APOC3, and QSOX1 and downregulation of IGHG3 and FETUA (Fig. 5b) (Supplementary Table 6). ROC curves were generated to determine which markers best distinguished between these 2 FD subgroups. Lyso-GB3 was unable to distinguish between the 2 groups (AUC=0.6610). AUC values for APOC3, QSOX1, IGHG3, FETUA and APOA4 were higher than those obtained for lyso-GB3, and APOA4 was the only marker that

surpassed the AUC threshold of 0.75 (Fig. 5c). While APO4 expression differed according to sex, statistical significance was maintained in both groups after adjusting the p-value, with significant upregulation observed in the Clin FD subgroup relative to both the No Clin FD and the CTL group (Fig. 5d).

Due to the heterogeneity of clinical manifestations in women, we ensured an appropriate distribution of samples into 2 groups according to the absence or presence of complications, and compared the plasma proteome in female patients with (Clin FemFD; n = 11) versus without (No Clin FemFD; n = 11) clinical complications. There were no significant differences between groups in either lyso-GB3 levels or α-GalA enzymatic activity (Fig. 6a). Three proteins were downregulated in the Clin FemFD group (IGHG3, VTNC, and FCN2; Fig. 6b; Supplementary Table 7): for all 3 proteins AUC values were significantly higher than those obtained for lyso-GB3 (Fig. 6c). IGHG3 was the protein that best distinguished between the Clin FemFD and No Clin FemFD subgroups (AUC= 0.8347), but VTNC was the only of the three proteins that marked the differences with both No Clin FemFD and CTL groups with a similar trend in the latter (Fig. 6d).

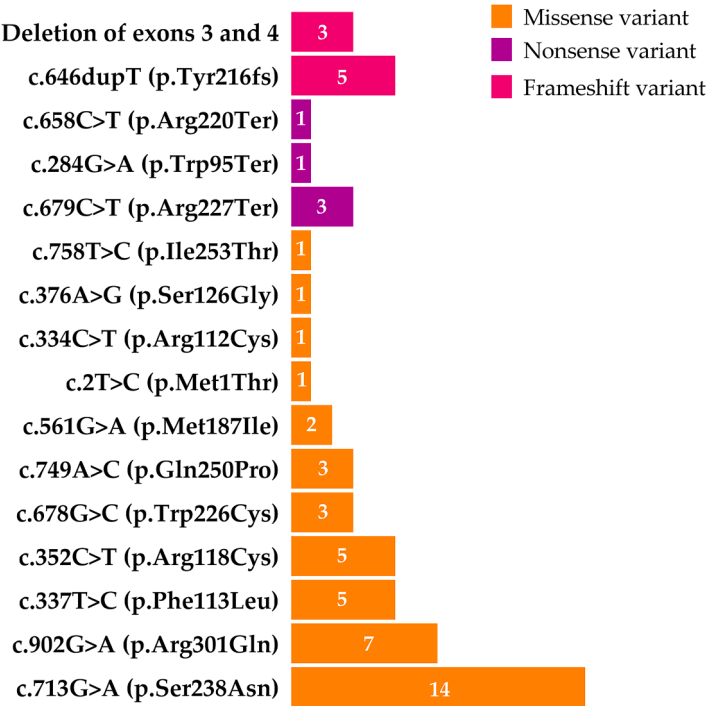


Fig. 2. *GLA* variant distribution in the FD cohort. The frequency of the variant types (missense, nonsense, and frameshift variant) is represented. In the 50 FD patients, 16 variants were repeated 56 times: 11 missense (n= 43, 76.8 %), 3 nonsense (n= 5, 8.9 %), and 2 frameshift (n= 8, 14.3 %). *FD*, *Fabry Disease*.

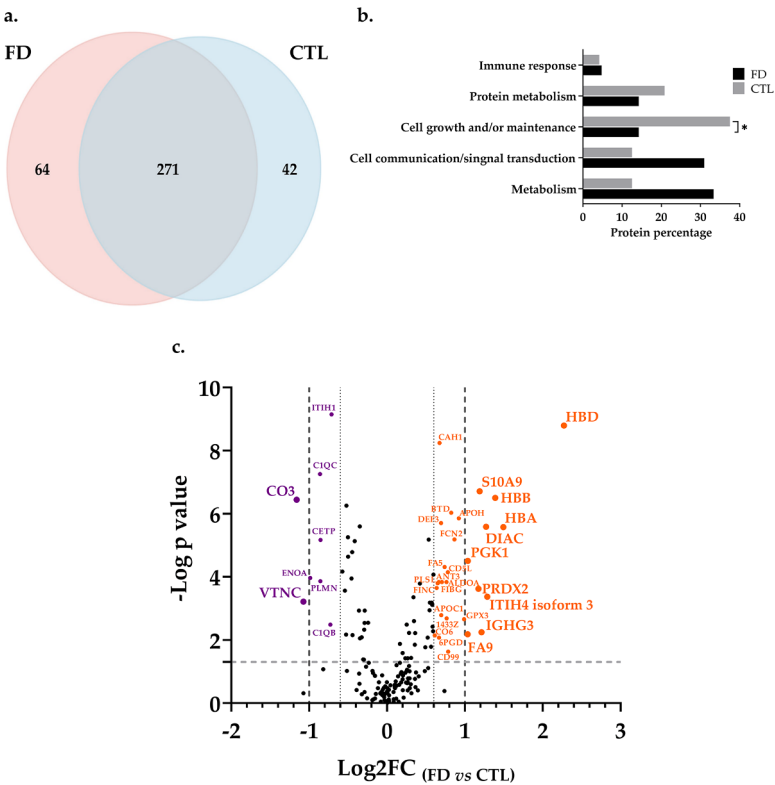


Fig. 3. Comparison of the plasma proteome of FD patients vs healthy controls. a) Venn diagram showing the proteins identified in FD (pink) and CTL plasma (blue). b) Biological processes associated with proteins identified only in FD (black bars) and proteins identified only in CTL (grey bars) plasma. c) Volcano plot depicting significantly ($p<0.05$) downregulated ($\text{Log2FC} \leq -0.6$, purple) and upregulated ($\text{Log2FC} \geq +0.6$, orange) proteins in FD vs CTL. *CTL*, *control*; *FD*, *Fabry disease*.

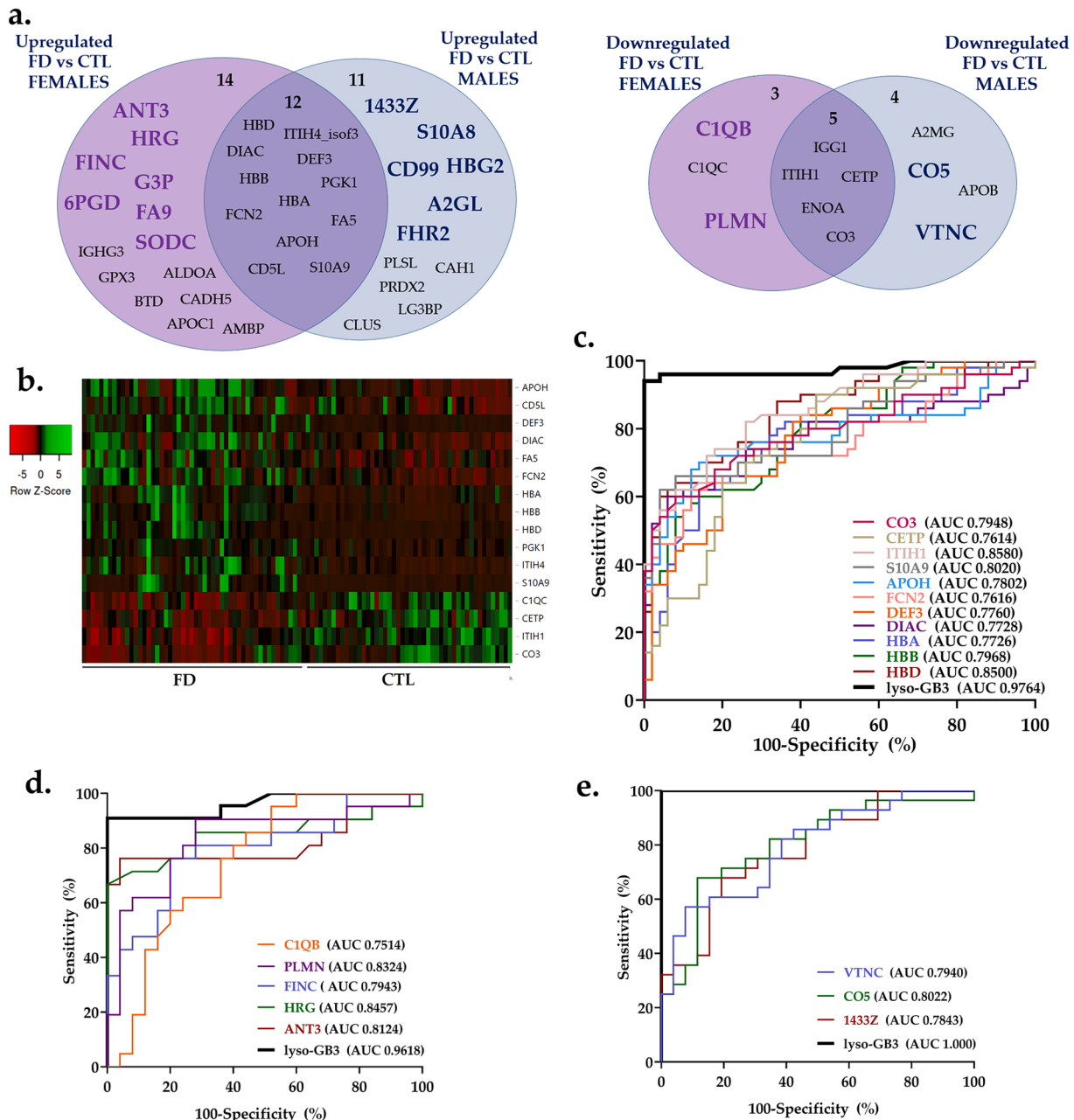


Fig. 4. Comparison of the plasma proteome of FD patients vs healthy controls, stratified by sex. a) Venn diagram showing plasma proteins that were differentially expressed in female (purple) between male (blue) FD patients relative to their respective controls. b) Heatmap depicting differences in protein expression (z-score) between FD and CTL groups, regardless of sex. ROC curves of proteins for which AUC values were ≥ 0.75 in FD vs corresponding controls: male and female (c); female only (d); and male only (e). AUC, area under the curve; CTL, control; FD, Fabry disease; lyso-GB3, globotriaosylsphingosine.

CKD-FD

Significantly lower α -GalA activity and higher lyso-GB3 levels were observed in the CKD FD ($n = 22$) versus No Clin FD ($n = 15$) groups (Fig. 7a). Expression levels of 4 proteins were significantly altered in the CKD FD versus the No Clin FD group: APOA4 and HPTR (upregulated), and A2MG and IGHG3 (downregulated), although A2MG was discarded from further analyses as significance was lost after adjusting for confounding factors (Fig. 7b) (Supplementary Table 8). Analysis of the ROC curves for these proteins, together with those of lyso-GB3 and the diagnostic markers commonly used in CKD (GFR, creatinine, and microalbuminuria), revealed highest AUC values for APOA4 and HPTR (0.8833 and 0.7697, respectively), even surpassing that of lyso-GB3 (Fig. 7c), and showed significantly greater differences in the CKD FD

group relative to both the No Clin FD and CTL groups (Fig. 7d). In addition to showing a significant correlation with GFR ($r = -0.399$, $p = 0.004$), creatinine ($r = 0.499$, $p = 0.00022$), and microalbuminuria ($r = 0.347$, $p = 0.0135$), APOA4 had a higher AUC than these markers (0.8833, $p < 0.0001$). Comparison of FD patients with CKD as the sole complication (Only CKD FD, $n = 4$) against the No Clin FD subgroup revealed that while differences in α -GalA activity persisted, differences in lyso-GB3 levels were attenuated (Fig. 8a). APOA4 remained significantly upregulated, APOH, CBPB2, and DIAC were upregulated, and ENOA was downregulated (Fig. 8b) (Supplementary Table 9). Analysis of ROC curves for these proteins showed higher AUC values than markers such as lyso-GB3, creatinine, and microalbuminuria whose AUC values failed to reach significance ($p > 0.05$) (Fig. 8c). In comparisons of the Only CKD FD group with both the No Clin FD and CTL groups,

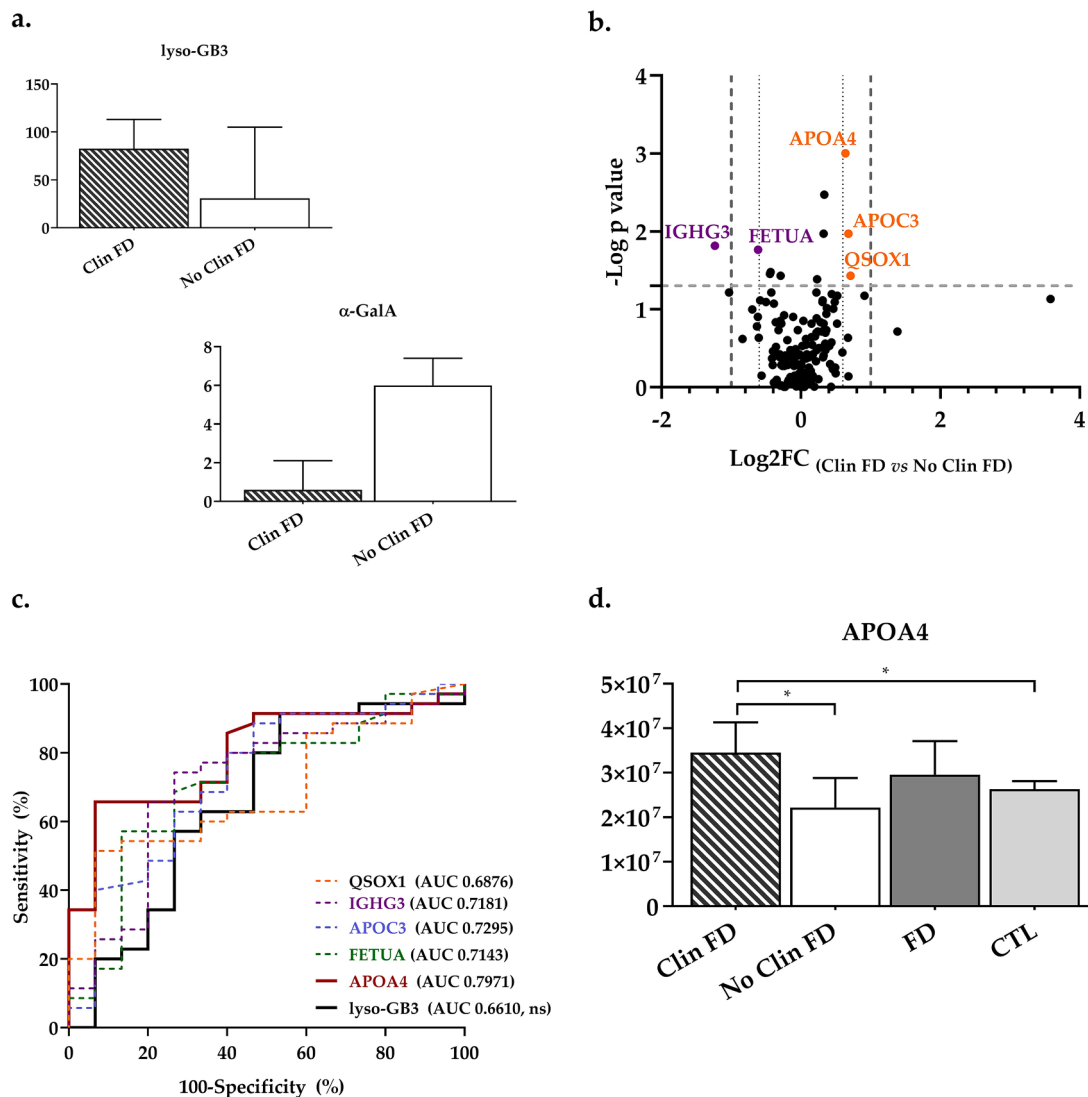


Fig. 5. Comparison of the plasma proteome of FD patients with clinical complication vs those without. a) Plasma levels of lyso-GB3 and enzymatic activity of α -GalA in Clin FD and No Clin FD groups. b) Volcano plot depicting significantly ($p < 0.05$) downregulated (Log2FC ≤ -0.6 , purple) and upregulated (Log2FC $\geq +0.6$, orange) proteins. c) ROC curves of proteins that were differentially expressed in Clin FD vs No Clin FD patients. d) Normalized MLR area of APOA4, α -GalA, α -galactosidase A; AUC, area under the curve; Clin FD, FD with clinical complication; CTL, control; FD, Fabry disease; lyso-GB3, globotriaosylsphingosine; MLR, multiple linear regression; No Clin FD, FD without clinical complication. * $p < 0.05$.

expression levels of the 5 aforementioned proteins were also significantly altered (Fig. 8d).

LVH-FD

Comparison of patients with LVH (LVH FD, $n = 29$) versus the No Clin FD ($n = 15$) group revealed significantly decreased α -GalA activity and increased lyso-GB3 levels (Fig. 9a), upregulation of APOC3, QSOX1, and APOC2, and downregulation of FETUA, IGHG3, SODC, and TSP4 with Log2FC limits set (Fig. 9b) (Supplementary Table 10). In subsequent analyses of the corresponding ROC curves, only AUC values for FETUA and APOC3 exceeded those of lyso-GB3, although only the APOC3 AUC value exceeded the 0.75 threshold (Fig. 9c). None of the AUC values for potential markers surpassed that of NT-proBNP (0.9477) or troponin I (0.8583). FETUA was the only protein for which expression levels differed significantly in the LVH FD group versus the No Clin FD and CTL groups (Fig. 9d).

Comparison of FD patients with LVH as the sole complication (Only LVH FD, $n = 9$) with the No Clin FD group showed no significant differences between groups in either lyso-GB3 levels or α -GalA activity

(Fig. 10a). In terms of differentially expressed proteins, 6 were upregulated (TTHY, QSOX1, APOC2, APOC3, HEMO, and CO3) and 8 downregulated (IGHG3, FCN2, CATA, CRACD, IGD, FETUA, FHR1 and HRG) (Fig. 10b) (Supplementary Table 11): in all cases AUC values exceeded those of lyso-GB3, which failed to reach significance (0.7333). Only for TTHY (0.8395) and IGHG3 (0.8296) did AUC values exceed those of the biochemical markers NT-proBNP (0.8295) and troponin I (0.8295) (Fig. 10c), and with FETUA, CATA, and FCN2 are the ones with the best ROC curve (≥ 0.8) (Fig. 10d).

CKD+LVH-FD

Comparison of FD patients with both LVH and CKD (CKD+LVH FD, $n = 16$) against those without clinical complications (No Clin FD, $n = 15$) revealed that the former group had higher levels of lyso-GB3 (Fig. 11a) and markedly reduced α -GalA activity, which approached negligible levels. Of the proteins that were differentially expressed between groups (Fig. 11b) (Supplementary Table 12), APOA4, HPTR and APOC3 had AUC values that exceeded both the 0.75 threshold and the AUC of lyso-GB3. APOA4 even outperformed troponin I, a marker of cardiac disease,

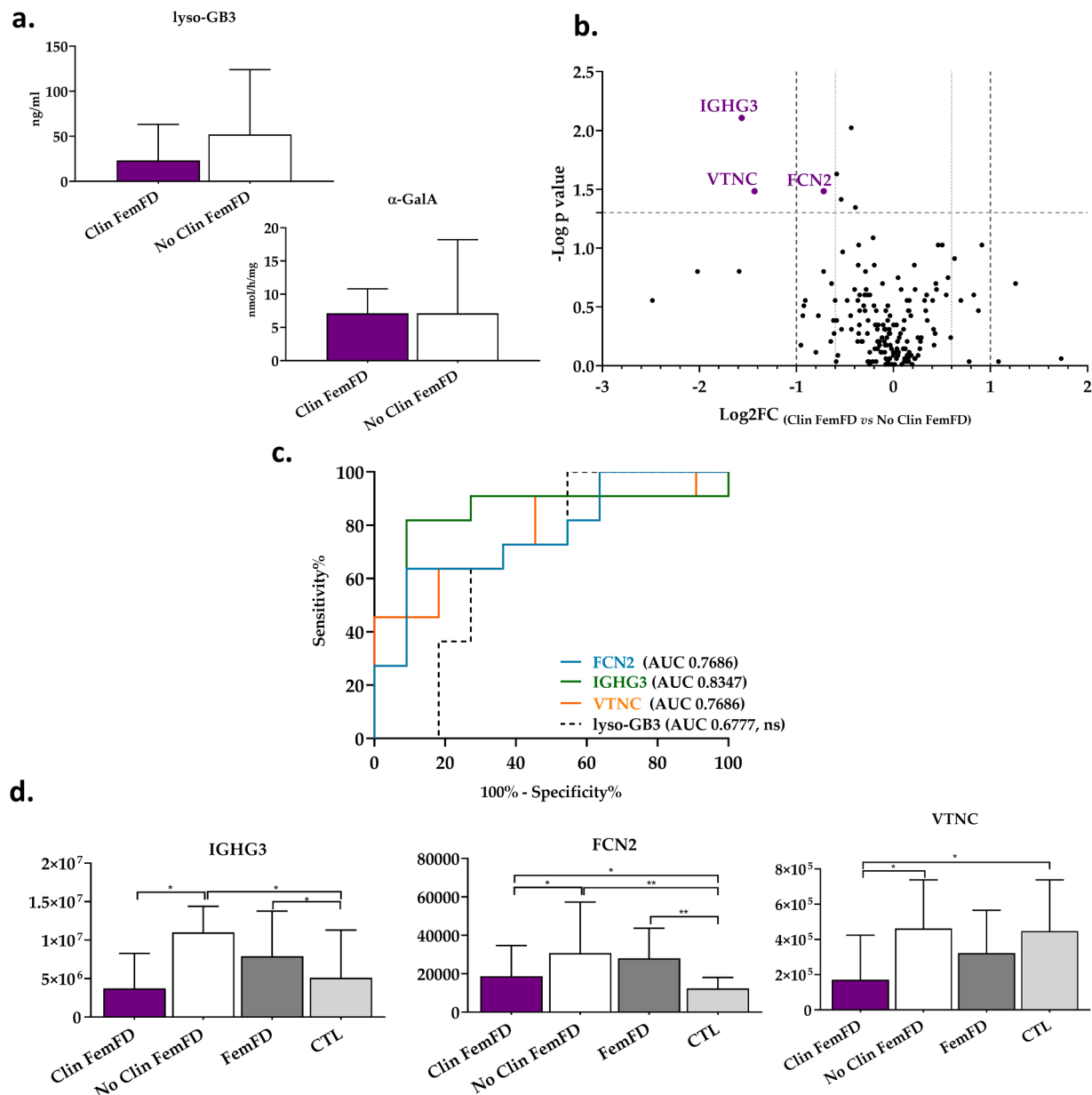


Fig. 6. Comparison of the plasma proteome of female FD patients with clinical complication vs those without. A) Plasma levels of lyso-GB3 and enzymatic activity of α -GalA in Clin FemFD and No Clin FemFD groups. b) Volcano plot depicting significantly ($p < 0.05$) downregulated ($\text{Log2FC} \leq -0.6$, purple) and upregulated ($\text{Log2FC} \geq +0.6$, orange) proteins. c) ROC curves of proteins that were differentially expressed in Clin FemFD vs No Clin FemFD groups. d) Normalized MLR area of IGHG3, VTNC, and FCN2. α -GalA, α -galactosidase A; AUC, area under the curve; Clin FemFD, Female FD with some clinical complication; CTL, control; FD, Fabry disease; FemFD, female FD; lyso-GB3, globotriaosylsphingosine; MLR, multiple linear regression; No Clin FemFD, Female FD without clinical complication. * $p < 0.05$, ** $p < 0.0001$.

and markers of renal disease as shown in the previous sections (creatinine and glomerular filtration rate) (Fig. 11c). APOA4 and HPTR were the only proteins capable of differentiating the CKD+LVH from both the No Clin FD and CTL groups (Fig. 11d).

Discussion

We performed a plasma proteomic analysis to identify differences in protein expression in FD patients versus healthy controls, stratified or not by sex, in order to better understand the pathophysiology of the disease. Furthermore, we characterized the plasma proteome of subgroups of FD patients with the most common associated clinical complications to identify possible biomarkers of clinical evolution.

We identified 17 proteins that were differentially expressed in FD patients versus healthy subjects, regardless of sex. These proteins are

implicated in functions related to endothelial dysfunction and vasculopathy characteristic of FD, including inflammation, heme and haemoglobin metabolism, oxidative stress, coagulation, fibrinolysis, complement system activity, glycolysis, lipid and glycolipid metabolism and glycocalyx formation.^{37–39}

Analysis of ROC curves revealed the highest AUC values for HBA, HBB, HBD, DIAC, S10A9, DEF3, FCN2, CO3, APOH, ITIH1 and CETP, and significant correlations between HBA, HBB, and DIAC expression and lyso-GB3 levels. Increased expression of haemoglobin chains (HBA, HBB, and HBD) has been reported in various inflammatory conditions,^{40–44} and can exert pro-oxidant effects and lead to endothelial dysfunction.^{41,45} A previous study reported a similar trend in FD females and the opposite trend in FD males,²⁶ a discrepancy that may be due to differences in the recruitment of study participants and the sensitivity of the technique used.^{46,47} Altered expression in FD patients of S10A9,

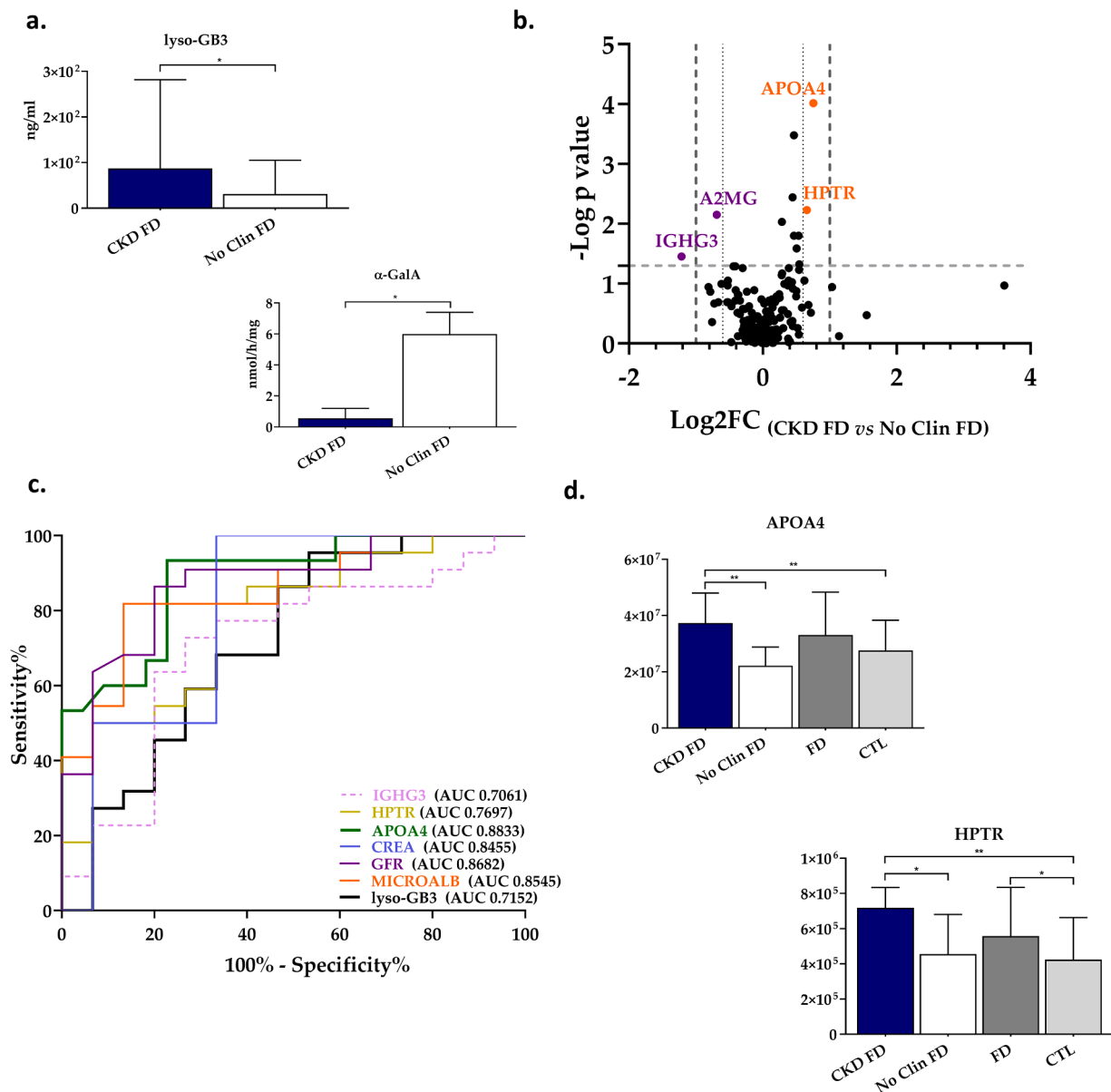


Fig. 7. Comparison of the plasma proteome of FD patients with CKD vs those without clinical complication. a) Plasma levels of lyso-GB3 and enzymatic activity of α -GalA. b) Volcano plot depicting significantly ($p < 0.05$) downregulated ($\text{Log2FC} \leq -0.6$, purple) and upregulated ($\text{Log2FC} \geq +0.6$, orange) proteins. c) ROC curves of proteins that were differentially expressed in CKD FD vs No Clin FD patients. d) Normalized MLR area of differentially expressed proteins. α -GalA, α -galactosidase A; AUC, area under the curve; CKD, chronic kidney disease; CREA, creatinine; CTL, control; FD, Fabry disease; GFR, glomerular filtration rate; lyso-GB3, globotriaosylsphingosine; MICROALB, microalbuminuria; MLR, multiple linear regression; No Clin FD, FD without clinical complication. * $p < 0.05$, ** $p < 0.0001$.

DEF3, APOH, and ITIH1, which are implicated in inflammatory and/or thrombotic processes, suggests that these proteins may also participate in endothelial dysfunction.^{48–55} Increased gene expression of S10A9 has been described in a mouse model of FD.⁵⁶ Activation of the complement cascade in FD has also been reported: expression of iC3b, the product of C3b cleavage, is increased in FD patients.²⁴ In our FD patient cohort, we observed downregulation of CO3 and upregulation of FCN2, a pattern-recognition molecule of the lectin pathway.⁵⁷ Another protein of particular interest is DIAC, a β -N-acetylglucosaminidase involved in GB3 metabolism^{58,59}; increases in DIAC expression in FD may be explained by the increased demand to metabolize excess lyso-GB3. Moreover, DIAC has been proposed as a possible clinical marker of kidney damage in FD patients.⁶⁰

Random X chromosome inactivation in female FD patients can give rise to significant clinical variability.⁶¹ Therefore, we sought to identify differentially expressed proteins in a FD cohort stratified by sex. We

identified differentially expressed proteins implicated in pathophysiological processes such as inflammation and coagulation/fibrinolysis, including ANT3, HRG,^{62–65} FINC,^{66,67} and PLMN^{68,69} in women, and, in line with a previous study in FD patients,⁷⁰ 1433Z in men. In addition, activation of the complement system was detected in both women and men with FD by finding a downregulation of C1QB and CO5 relative to controls, respectively.⁷¹ Epigenetic studies may help to elucidate the mechanisms underlying differential protein expression between sexes.⁷² Interestingly, VTNC expression was downregulated only in male FD patients, with no alterations detected in female counterparts relative to healthy controls. However, VTNC expression was downregulated in the plasma proteome of female FD patients with complications versus those without. Low levels of VTNC are implicated in the pathogenesis of atherosclerotic cardiovascular disease. Chronically high cholesterol suppresses the expression of VTNC, leading to unresolved inflammation due to overexpression of NF- κ B and pro-inflammatory cytokines, and an

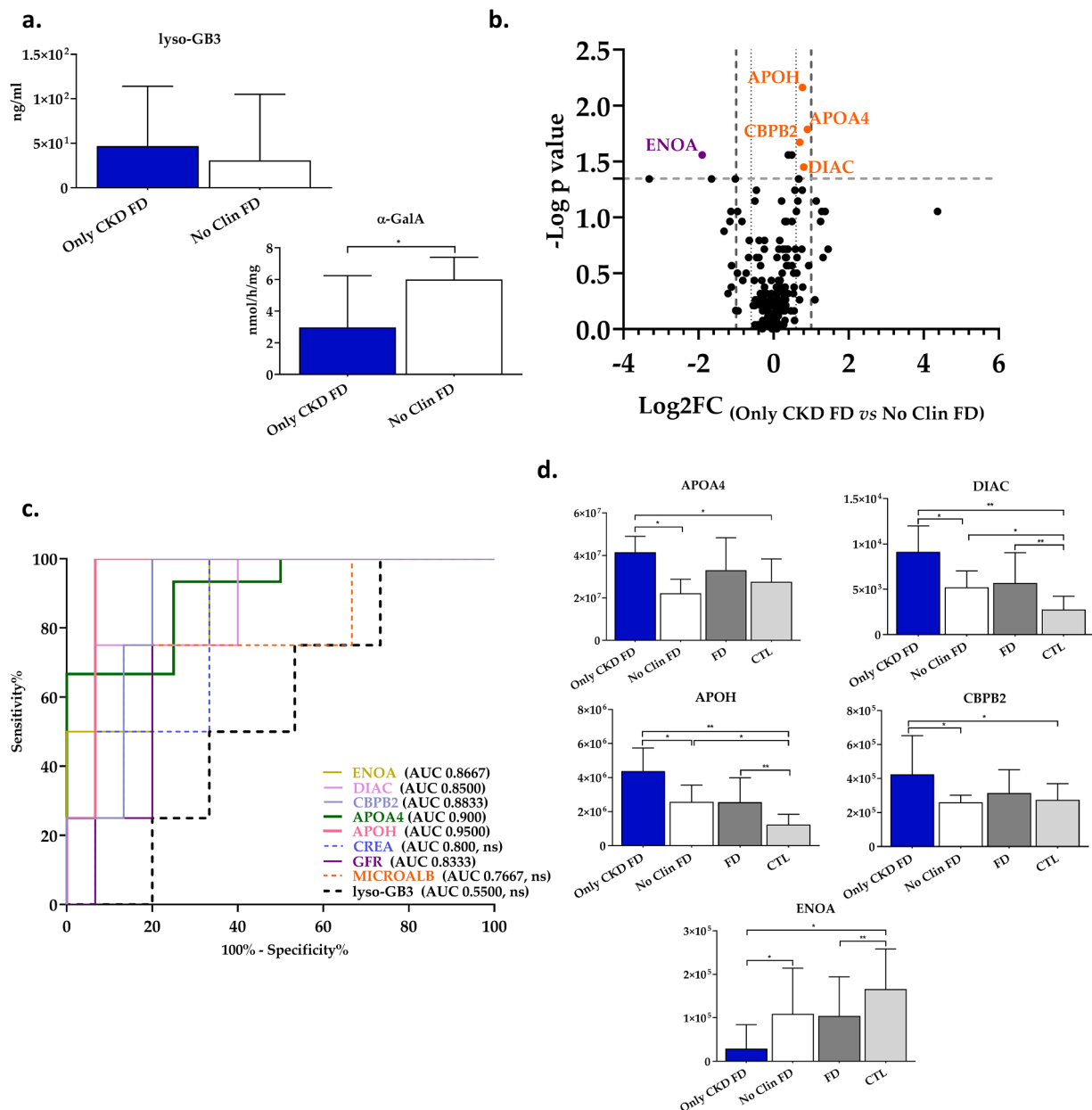


Fig. 8. Only CKD-FD. Comparison of the plasma proteome of Only CKD FD vs No Clin FD. a) Plasma levels of lyso-GB3 and enzymatic activity of α-GalA. b) Volcano plot depicting significantly ($p < 0.05$) downregulated ($\text{Log}_2\text{FC} \leq -0.6$, purple) and upregulated ($\text{Log}_2\text{FC} \geq 0.6$, orange) proteins. c) ROC curves of proteins that were differentially expressed in Only CKD FD vs No Clin FD. d) Normalized MLR area of differentially expressed proteins. α-GalA, α-galactosidase A; AUC, area under the curve; CKD, chronic kidney disease; CREA, creatinine; CTL, control; FD, Fabry disease; GFR, glomerular filtration rate; lyso-GB3, globotriaosylsphingosine; MICROALB, microalbuminuria; MLR, multiple linear regression; No Clin FD, FD without clinical complication. * $p < 0.05$, ** $p < 0.0001$.

unstable plaque caused by the accumulation of immune cells and overexpression of adhesion molecules and metalloproteinases.⁷³ Given that male FD patients predominantly present with complications typical of FD, and vitronectin expression can distinguish between female FD patients with versus without complications, this protein may constitute a valid candidate biomarker of FD clinical course.

The present study is the first to explore potential plasma biomarkers associated with specific clinical phenotypes of FD, with a view to better understanding the variability in clinical manifestations among FD patients. We identified APOA4 as the protein with the greatest capacity to distinguish between FD patients with versus without clinical complications. The upregulation of APOA4 in patients with complications may be due to the presence of CKD, especially in those patients for whom CKD is the only clinical manifestation. APOA4 is a glycoprotein involved in reverse cholesterol transport that modulates both lipid metabolism and

glucose homeostasis.^{74,75} Elevated levels of this apoprotein are associated with impaired renal function independently of classic CKD risk factors in patients with primary CKD^{74,76,77} and in the general population.⁷⁸ Another prospective study found that patients with nondiabetic primary CKD who experienced CKD progression during the observation period had significantly higher baseline APOA4 concentrations than patients who did not progress.⁷⁹ Interestingly, we observed an association between high levels of this protein and the presence of kidney disease in our FD cohort, suggesting that APOA4 may be a valid candidate biomarker for early identification of CKD onset in patients with FD. The most commonly used clinical parameters for identifying renal dysfunction, such as reduced GFR, elevated creatinine, and microalbuminuria, are not sensitive biomarkers for early kidney damage in Fabry disease. Significant lesions have been observed in renal biopsies of Fabry patients without these typical clinical alterations.^{80,81} Since renal

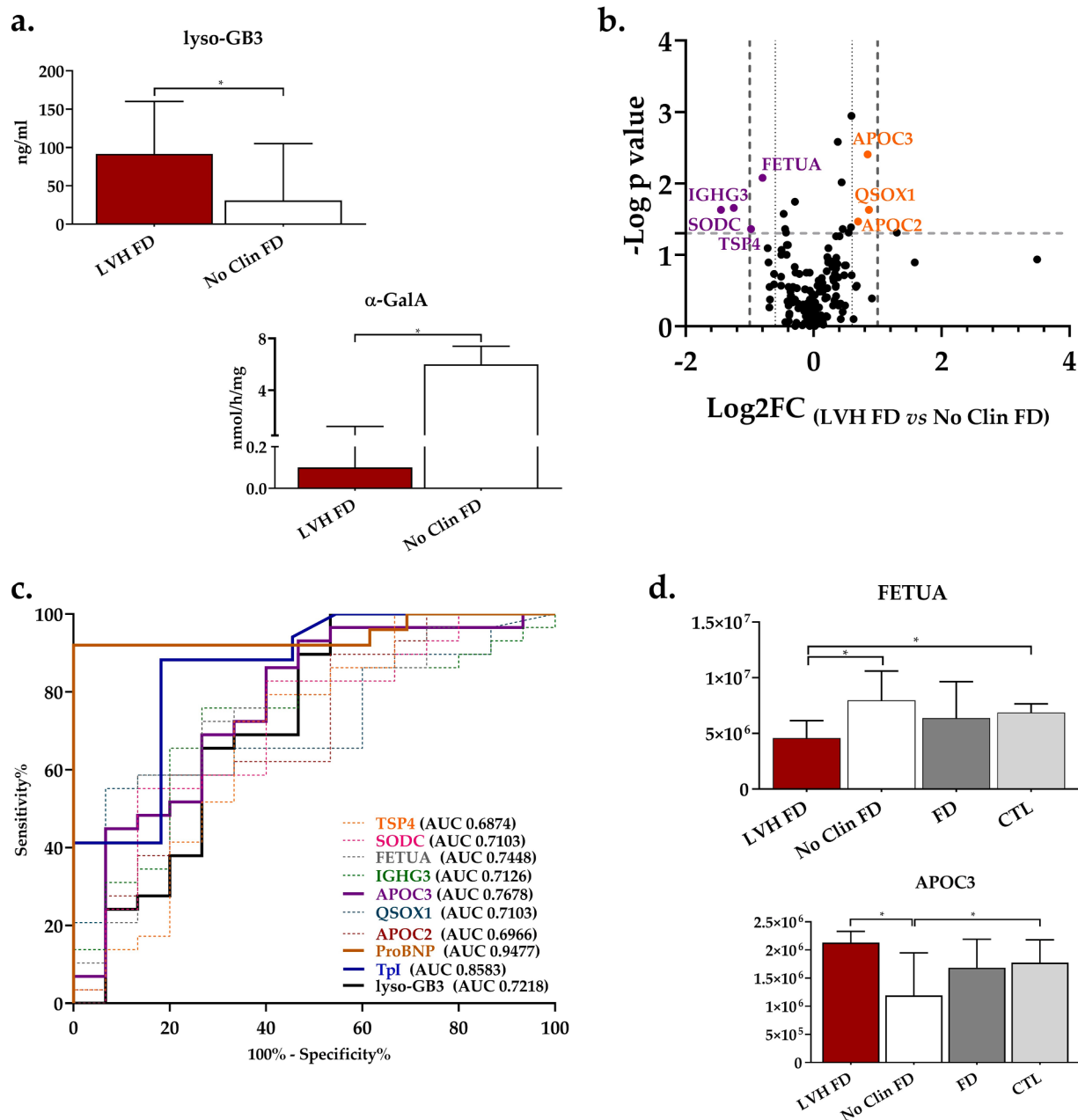


Fig. 9. Comparison of the plasma proteome of FD patients with LVH vs those without clinical complication. a) Plasma levels of lyso-GB3 and enzymatic activity of α -GalA. b) Volcano plot depicting significantly ($p < 0.05$) downregulated ($\text{Log}_2\text{FC} \leq -0.6$, purple) and upregulated ($\text{Log}_2\text{FC} \geq +0.6$, orange) proteins. c) ROC curves of proteins that were differentially expressed in LVH FD vs No Clin FD patients. d) Normalized MLR area of differentially expressed proteins. α -GalA, α -galactosidase A; AUC, area under the curve; CTL, control; FD, Fabry disease; LVH, left ventricular hypertrophy; lyso-GB3, globotriaosylsphingosine; MLR, multiple linear regression; No Clin FD, FD without clinical complication; proBNP, pro-brain natriuretic peptide; Tpl, troponin I. * $p < 0.05$.

biopsy is seldom performed without clinical evidence and ERT does not prevent the deterioration of renal function once clinical symptoms appear⁶ there is a clear need for new biomarkers. In our cohort, APOA4 demonstrated a significant capability to identify Fabry patients with CKD, surpassing GFR, creatinine, and microalbuminuria, and thus potentially serving as a pivotal factor to aid the decision whether to proceed with a renal biopsy or not.

In agreement with our findings in FD patients with CKD, downregulation of ENOA is associated with signalling pathways implicated in tubulointerstitial fibrosis and autophagy in a FD podocyte cell model.⁸² In line with the renal involvement frequently seen in FD, DIAC has been proposed as a marker of kidney damage in FD, which is likely due to diffuse deposition of glycosphingolipids in the glomeruli, tubular

system, and vasculature.⁶⁰ Upregulation of HPTR, haptoglobin-related protein, has already been associated with end-stage renal disease⁸³ and with neutralisation of the oxidative capacity of haemoglobin by specialised high-density lipoprotein particles (HDL3) upon initiation of haemodialysis.⁸⁴

In addition to renal damage, cardiac involvement is a major complication of FD, predominantly manifesting as LVH and constituting the main cause of quality-of-life impairment and death in FD patients.⁸⁵ The proteins APOC3 and FETUA showed the greatest ability to differentiate between the FD patients with LVH and those patients without disease-related clinical complications, and although they do not outperform the usual cardiac markers troponin I and NT-pro-BNP, we consider them to be of relevance in FD patients with the complications.

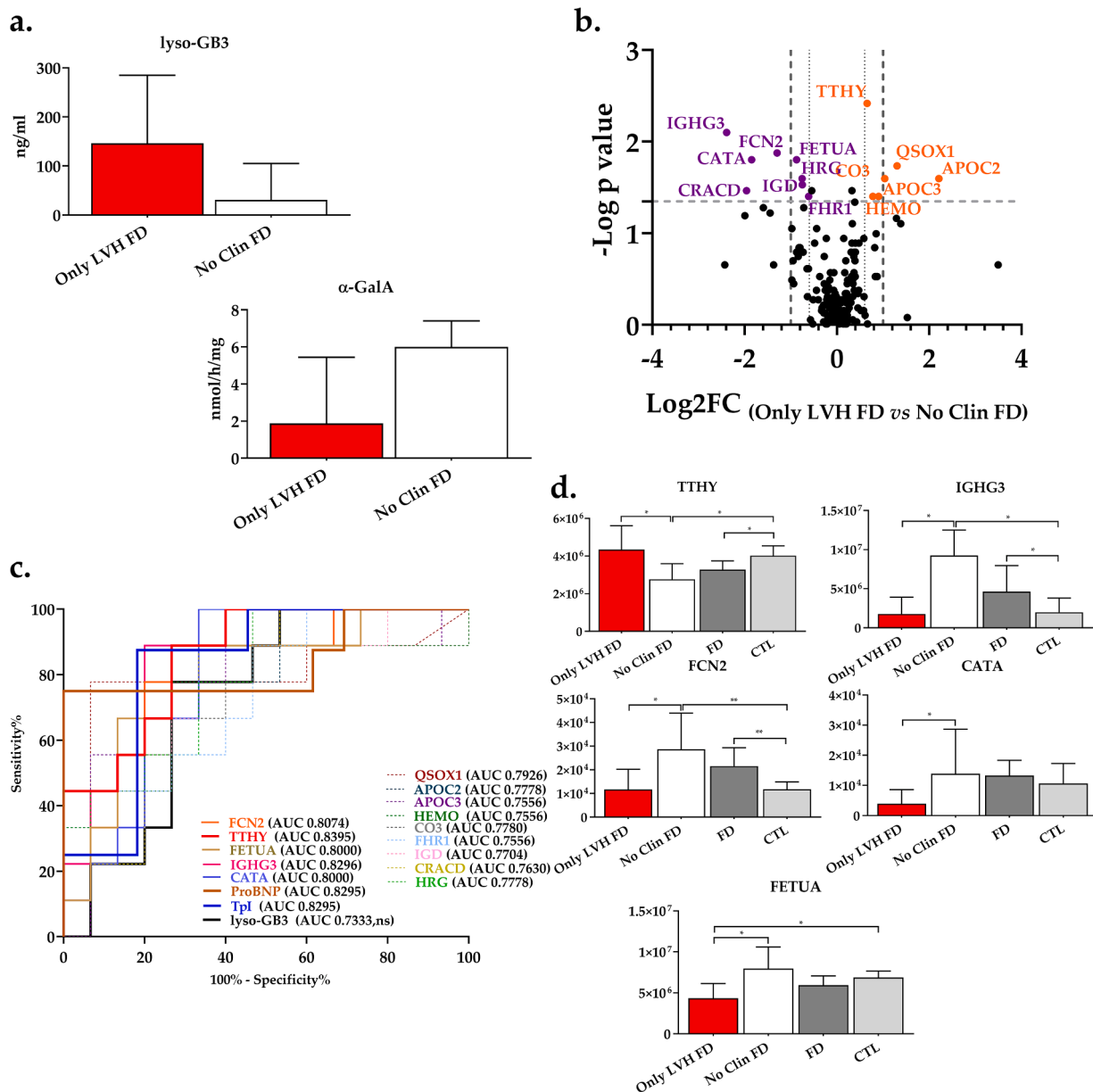


Fig. 10. Comparison of the plasma proteome of Only LVH FD vs No Clin FD. a) Plasma levels of lyso-GB3 and enzymatic activity of α -GalA. b) Volcano plot depicting significantly ($p < 0.05$) downregulated ($\text{Log}_2\text{FC} \leq -0.6$, purple) and upregulated ($\text{Log}_2\text{FC} \geq +0.6$, orange) proteins. c) ROC curves of proteins that were differentially expressed in Only LVH FD vs No Clin FD. d) Normalized MLR area of differentially expressed proteins. α -GalA, α -galactosidase A; AUC, area under the curve; CTL, control; FD, Fabry disease; LVH, left ventricular hypertrophy; lyso-GB3, globotriaosylsphingosine; MLR, multiple linear regression; No Clin FD, FD without clinical complication; proBNP, pro-brain natriuretic peptide; Tpl, troponin I. * $p < 0.05$, ** $p < 0.0001$.

APOC3, which was upregulated in the LVH FD cohort, is an apolipoprotein that inhibits lipoprotein lipase, causing lipid accumulation, and stimulates inflammation via $\text{TNF-}\alpha$.⁸⁶ APOC3 expression is elevated in the plasma of patients with atherosclerotic cardiovascular disease.^{86–88} FETUA, which was downregulated in the LVH FD cohort, is a calcification inhibitor, reduced levels of which have been associated with vascular calcification, a typical feature of atherosclerotic cardiovascular disease.^{89,90} Decreased FETUA expression has been reported in FD patients after ERT treatment, suggesting that its increase in untreated FD patients may constitute a protective response.²⁵ Based on the findings in our FD cohort, we propose the inverse hypothesis, i.e. that reduced FETUA levels are associated with an increased cardiovascular risk.

Our results also indicate alterations in the LVH FD group of plasma levels of proteins that impair endothelial integrity either directly (CRACD⁹¹ and HRG⁶⁵) or indirectly, via their roles in oxidative stress (e.

g. TTHY,^{92,93} QSOX1,⁹⁴ HEMO⁹⁵ and CATA⁹⁶) and complement regulation (IGHG3,⁹⁷ CO3,⁷¹ FCN2,^{52,57} and FHR1^{98,99}).

Limitations and strengths

A limitation of our study is that despite the large number of patients in the FD cohort, there is considerable heterogeneity in terms of time since the onset of the disease, which may have masked the presence of specific earlier biomarkers. Moreover, although our analysis indicated that certain markers associated with clinical complications of FD outperformed those already used routinely, their sensitivity as early markers of clinical progression will need to be assessed prospectively following genetic and biochemical diagnosis of FD. Regarding the technical limitation, the number of proteins quantified in a SWATH-MS analysis is largely constrained by the composition of the spectral library.

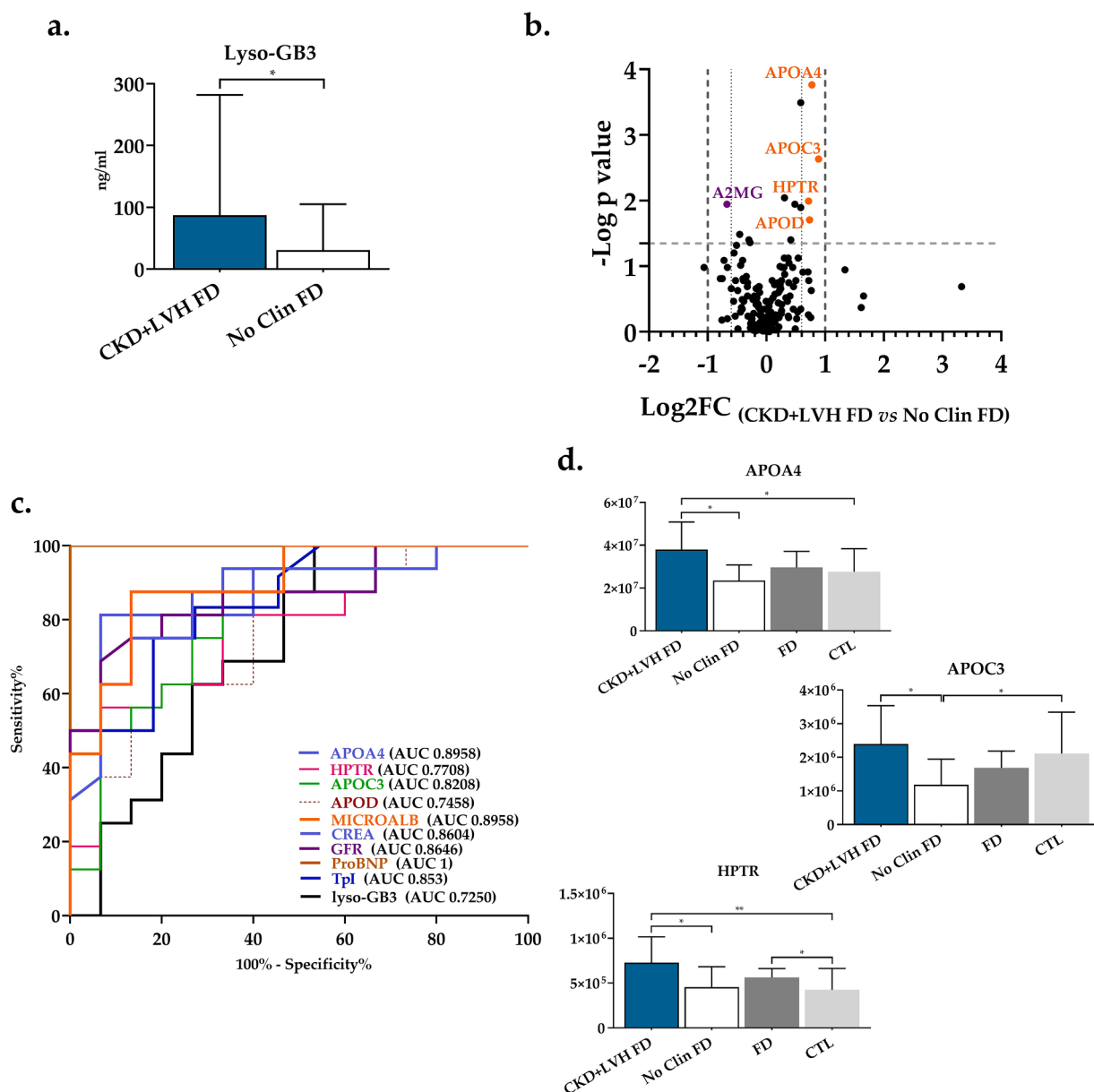


Fig. 11. CKD+LVH-FD. Comparison of the plasma proteome of FD patients with CKD + LVH vs those without clinical complication. A) Plasma levels of lyso-GB3. B) Volcano plot depicting significantly ($p < 0.05$) downregulated ($\text{Log}_2\text{FC} \leq -0.6$, purple) and upregulated ($\text{Log}_2\text{FC} \geq +0.6$, orange) proteins. C) ROC curves of proteins that were differentially expressed in CKD+LVH FD vs No Clin FD patients. D) Normalized MLR area of differentially expressed proteins. *α -Gala*, *α -galactosidase A*; AUC, area under the curve; CKD, chronic kidney disease; CREA, creatinine; CTL, control; FD, Fabry disease; GFR, glomerular filtration rate; LVH, left ventricular hypertrophy; lyso-GB3, globotriaosylsphingosine; MICROALB, microalbuminuria; MLR, multiple linear regression; No Clin FD, FD without clinical complication; proBNP, pro-brain natriuretic peptide; Tpl, troponin I. * $p < 0.05$, ** $p < 0.0001$.

Additionally, SWATH has a tendency to be biased towards the most abundant proteins, and is therefore particularly affected by high sample complexity and/or the protein dynamic range.¹⁰⁰ However, the use of non-targeted analysis by SWATH-MS, a specific independent data acquisition method, allows combination of deep proteomic coverage capabilities with quantitative consistency and precision, and robust reproducibility between experiments.^{101–104} Other key strengths of our study include the large sample size, which exceeds that of any previous untargeted plasma proteomics study in FD; and the inclusion of age- and sex-matched healthy controls, which enabled identification of sex-specific FD biomarkers. Crucially, this is first study to identify potential biomarkers associated with clinical phenotype in FD.

Conclusion

In summary, our plasma proteomic study of FD patients identifies potential novel markers associated with the disease, in both mixed and sex-stratified populations, all of which are implicated in processes typically linked to the endothelial dysfunction characteristic of FD, including inflammation, regulation of coagulation/fibrinolysis, and the complement system. Furthermore, we identify for the first time plasma proteins associated with the predominant clinical phenotypes of FD: APOA4 was associated with the presence of complications in FD, in particular CKD, while FETUA and APOC3 were identified as potential markers of LVH in FD patients. As discussed above, further studies will be needed to confirm these proposed associations, and to explore the role of these proteins in the pathophysiology of FD and their potential as early markers of disease course.

Brief Commentary

Background

Fabry Disease (FD) is a X-linked rare lysosomal storage disorder due to a deficiency in the α -galactosidase A (α -GalA) activity. Patients with FD exhibit a great clinical variability and often experience late diagnosis. This work aims to identify potential biomarkers to better understand the disease's pathophysiology, define clinical phenotypes, and improve both the diagnosis and monitoring of FD.

Translational Significance

Our study represents the deepest untargeted plasma proteomics investigation conducted on FD patients to date. It reveals previously unknown plasma proteome patterns linked to sex and the most prevalent clinical manifestations of the disease.

Data availability

Mass spectrometry proteomics data have been deposited in the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD045528.

CRedit authorship contribution statement

Laura López-Valverde: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **María E. Vázquez-Mosquera:** Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Conceptualization. **Cristóbal Colón-Mejeras:** Writing – review & editing, Methodology. **Susana B. Bravo:** Writing – review & editing, Methodology, Investigation, Formal analysis. **Sofía Barbosa-Gouveia:** Writing – review & editing, Methodology, Formal analysis. **J. Víctor Álvarez:** Writing – review & editing, Methodology. **Rosario Sánchez-Martínez:** Writing – review & editing. **Manuel López-Mendoza:** Writing – review & editing. **Mónica López-Rodríguez:** Writing – review & editing. **Eduardo Villacorta-Argüelles:** Writing – review & editing. **María A. Goicoechea-Diezhandino:** Writing – review & editing. **Francisco J. Guerrero-Márquez:** Writing – review & editing. **Saida Ortolano:** Writing – review & editing. **Elisa Leao-Teles:** Writing – review & editing. **Álvaro Hermida-Ameijeiras:** Writing – review & editing, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **María L. Couce:** Writing – review & editing, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.trsl.2024.02.006](https://doi.org/10.1016/j.trsl.2024.02.006).

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