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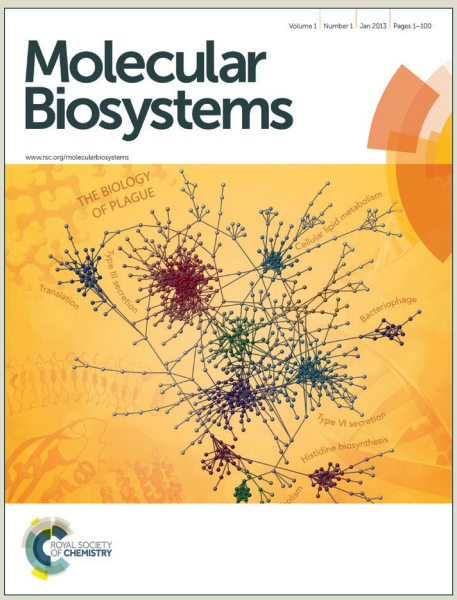

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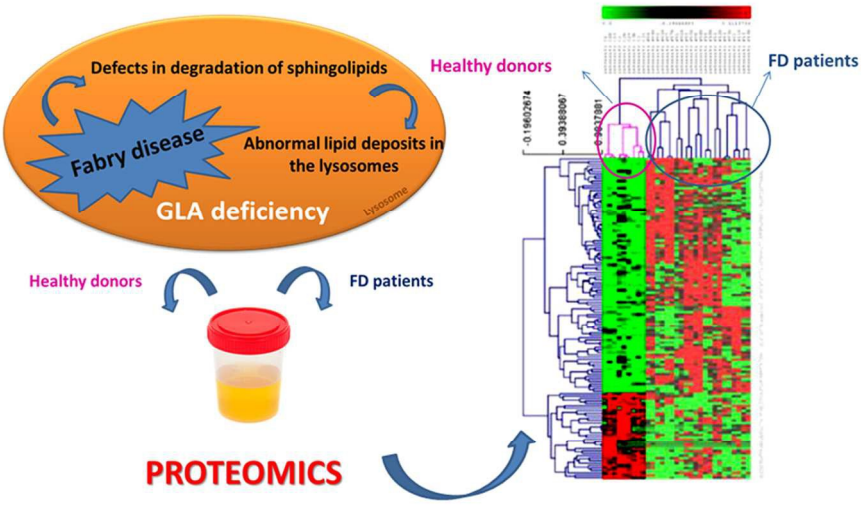
Table of contents

Early markers of Fabry disease revealed by proteomics

V. Matafora *et al.*



Discovery of early urinary biomarkers for Fabry disease in male and in female adult patients revealed by proteomics

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Fabry disease (FD) is an X-linked lysosomal storage disorder due to a deficiency of the lysosomal hydrolase α-galactosidase A (α-GalA) that leads to intra-lysosomal accumulation of globotriaosylceramide (Gb3) in various organ systems. As a consequence, a multisystems disorder develops, culminating in stroke, progressive renal and cardiac dysfunction. Enzyme replacement therapy (ERT) offers a specific treatment for patients affected by FD, though monitoring of treatment is hampered by a lack of surrogate markers of response. Remarkably, due to the high heterogeneity of the Fabry phenotype, both diagnostic testing and treatment decisions are more challenging in female than in men; thus, reliable biomarkers for Fabry disease are needed, particularly for female patients. Here we use a proteomic approach for the identification of disease-associated markers that can be used for early diagnosis of FD as well as for monitoring the effectiveness of ERT. Our data show that the urinary proteome of Fabry naïve patients is different from that of normal subjects. Additionally, biological pathways mainly affected by FD are related to immune response, inflammation, and energetic metabolism. In particular, up-regulation of uromodulin, prostaglandin H2 d-isomerase and prosaposin in the urine of FD patients was demonstrated; these proteins might be involved in kidney damage at tubular level, inflammation and immune response, respectively. Furthermore, comparing the expression of these proteins in Fabry patients before and after ERT treatment, a decrease of their concentration was observed thus demonstrating the correlation between the identified markers and effectiveness of the pharmacological treatment.

Introduction

Fabry disease (FD) is a rare, inherited multisystem disorder that results from a deficiency in the lysosomal hydrolase alpha-galactosidase A (α-GalA) [1]. This causes a progressive deposition of globotriaosylceramide (Gb3) in vascular lysosomes, resulting in systemic accumulation of neutral glycosphingolipids in plasma and tissue [2]. Classic FD form has usually its onset in childhood with mild symptoms [3] while, in middle age, is characterized by cardiovascular and end-stage renal disease [4]. A breakthrough in the treatment of Fabry patients has been accomplished by successful genetic engineering production of α-GalA and development of a direct protein-substitution therapy [5]. So far, the most efficient method for the diagnosis of Fabry disease in males is the measurement of deficient α-GalA enzyme activity [6], but in females this measurement is unreliable, as they usually have normal enzyme activity. Levels of Gb3 in plasma and urine have been measured for the above purposes [7], although recent systemic analysis revealed that Gb3 is not an ideal marker for the diagnosis or for monitoring the response to treatment. Indeed, when measured in random samples of whole urine, Gb3 may be normal in some of the patients and in female heterozygotes [8,9]. Thus, biomarkers predicting disease progression and quickly responding to the therapy may be useful to follow FD patients. Recently, it has been published a preliminary study on the identification of several FD specific biomarkers using a proteomic analysis on peripheral blood mononuclear cells (PBMC) from FD patients and healthy individuals; obviously, further investigations are needed to assess the validity of these markers [10]. Here, we describe a qualitative and quantitative comparison of the urinary proteome of FD patients with respect to healthy subjects. Noteworthy, we also added in the study patients treated with Enzyme Replacement Therapy (ERT) in order to monitor the behaviour of FD deregulated proteins upon treatment. Indeed, our purpose is on one hand to better understand the biological processes hiding behind this pathology and on the other hand to identify potential biomarkers useful as diagnostic and prognostic tools,

especially in women. Preliminary results recently obtained by our group [11-13] suggested the value of this scientific approach for urinary protein profiling. In particular, we analysed urinary proteins using a label free approach coupled with LC-MS/MS analysis. We used a one shot strategy without any pre-fractionation step as we recently demonstrated that this approach allows robust results in terms of sensitivity and reproducibility [13]. These features are essential for a biomarkers discovery study.

Materials and methods

Patients characteristics

A total of 35 Caucasian subjects was recruited: 12 healthy subjects, 11 naïve Fabry patients and 12 permanently ERT-treated patients that were different from the naive ones (Table 1). Healthy donors (age and gender matched with patients) were volunteers; although no biochemical data are available for them, it is useful to note that no evident disease was detected during the course of the study. Control samples were collected and processed exactly as samples from naïve and ERT patients. Gender and age were selected in order to be heterogeneous. The patients cohorts studied exhibited analogous characteristics with respect to age, body mass index (BMI) and urinary creatinine levels (Table 2). The study was carried out in accordance with the Declaration of Helsinki (2000) of the World Medical Association. The Ethical Committee of the Second University of Naples approved the study protocol and informed consent was obtained from all patients. Patients' age, gender, type of mutation and clinical data were reviewed and recorded (Table 1).

Table 1− Patients' clinical data

Pz	age/sex	genotype	exon	Plasmatic enzymatic activity (nmol/h/mg)	Plasmatic creatinine (mg/dl)/GFR (ml/min)	Urine protein/ creatinine	weight (kg)/BMI	AP	ERT	from
P1	30/F	I354K	7	5.9	0.7/123	0.040	62/22	110/65	na	na
P2	22/F	I354K	7	8.2	0.7/170	0.047	64/22	120/60	na	na
P3	26/F	I354K	7	5.2	0.8/175	0.074	68/24	115/70	na	na
P4	38/F	c.1073-1074 ins A	7	22	0.6/124	0.047	59/22	120/70	na	na
P5	15/F	c.1073-1074 ins A	7	26.7	0.5/101	0.014	48/20	115/50	na	na
P7	60/F	na	na	3.9	0.9/60		63/24,3	120/70	na	na
P9	19/F	IVS6-1G>A	7	0.7	0.8/94		70/na	120/75	na	na
P10	19/F	G360D	7	33.7	0.8/94	0.063	48/na	110/60	na	na
P11	30/F	G360D	7	25	0.7/95	0.049	55/na	120/60	na	na
P6	9/M	c.1073-1074 ins A	7	0	0.3/192	0.060	31/16	95/60	na	na
P8	50/M	g.10571 C > A	7	0.3	0.9/na	0.96	70/24	120/80	na	na
E3	59/F	G749A	5	2.1	0.9/90	0.51	67/25	110/70	Fabrazyme	2002
E4	66/F	512A	3	2.9	0.9/80	0.53	68/26	130/70	Fabrazyme	2002
E5	26/F	512a	3	2.4	0.7/106	0.29	65/21	110/60	Fabrazyme	2003
E9	50/F	na	na	4.2	0.9/94	0.11	66/24	120/70	Fabrazyme	2005
E10	55/F	G260	5	8.03	1.2/89	0.66	68/27	130/70	Fabrazyme	2001
E11	50/F	G260	5	12	0.6/112	0.67	58/23	110/70	Fabrazyme	2005
E1	30/M	G749A	5	0.2	0.7/104	0.95	73/25.5	130/70	Fabrazyme	2001
E2	36/M	G749A	5	0.3	0.8/110	0.32	74/25	120/70	Fabrazyme	2001
E6	52/M	T483C	3	0.2	1.2/65	0.37	63/22,3	110/60	Fabrazyme	2001
E7	27/M	na	na	0.16	0.8/110	0.18	59/20,6	120/70	Fabrazyme	2004
E8	28/M	na	na	0.1	0.8/110	0.24	65/21	120/70	Fabrazyme	2005

P, naive patients; E, patients treated by ERT (enzymatic replacement therapy); M, male; F, female; na, not available; GFR, glomerulal filtration rate; BMI, body mass index; AP, arterial pressure.

Table 2 − Population means relative to age, body mass index and urine

Parameter	Näive	Healthy	ERT	p (One-Way Anova)*
age (y)	31.9 (16.2)	32.2 (4.2)	39 (16.0)	0.32675
BMI (Kg/m^2)	21.4 (2.8)		23.1 (2.8)	0.21227
urine creatinine (mmol/L)	7.3 (1.2)	9.8 (4.9)	8.9 (4.6)	0.05760

*Means Comparison using Bonferroni and Scheffe' Test

Sample preparation and label free proteins quantitation

Collection methodology and sample preparation were exactly the same for naïve and ERT patients as well as for healthy subjects. Briefly, 50 ml of second morning urine were added with 250 µl of protease inhibitor cocktail, centrifuged (4000g, 15min, 4°C) to remove intact cells and debris and frozen (-80°C). After thawing, samples were subjected to a further cycle of centrifugation in order to avoid clogging phenomena potentially occurring during supernatant concentration. Concentrated supernatants were washed with centrifugal concentrators (Microcon devices YM-10, Millipore).
Total protein content was assessed by Bradford's method [14] and 100µg of each sample were processed by FASP procedure [15]. Peptide mixtures were purified on StageTips (Proxeon Biosystems, Denmark) [16]. LC-MS/MS (liquid chromatography and tandem mass spectrometry) was performed using a LTQ-Orbitrap mass spectrometer (ThermoScientific, Bremen, Germany). Briefly, purified tryptic digests were injected in a capillary chromatographic system (EasyLC, Proxeon Biosystems); peptide mixtures were separated on a home-made 15 cm long fused silica capillary (75-µm inner diameter, Proxeon Biosystems) filled with Reprosil-Pur C18 3 µm resin (Dr. Maisch GmbH, Ammerbuch-Entringen, Germany). A gradient of eluents A (distilled water with 2% (v/v) acetonitrile, 0.5 % (v/v) formic acid) and B (80% acetonitrile in distilled water with 0.1% (v/v) formic acid) was used to achieve separation from 4% B to 50% B in 95 min (0.2 ml/min flow rate). MS analysis was performed as reported previously [17]. Raw MS files were processed with MaxQuant software (v 1.1.1.25) [18] making use of the Andromeda search engine [19]. MS/MS peak lists were searched against the UniProtKB/Swiss-Prot protein sequence database (release 2010_10 of 07-October-2010) in which trypsin specificity was used with up to two missed cleavages allowed. Searches were performed selecting alkylation of cysteine by carbamidomethylation as fixed modification, and oxidation of methionine and N-terminal acetylation as variable modifications. Mass tolerance was set to 5 ppm and 0.6 Da for parent and fragment ions respectively. The false discovery rate for both peptides and proteins was set at 0.01. For protein quantitation, the "match between run" option was selected; a time window of 3 minutes and a minimum ratio count of 2 were considered. All the raw files of the proteomic data and search parameters were loaded on Peptide Atlas repository (accession number PASS00644).
Biological Networks Gene Ontology (BiNGO) program package (v 2.44) [20] with the Cytoscape plugin (v 2.8.0) were used to find statistically over-represented Gene Ontology (GO) categories [21]. The analysis was done using 'hyper geometric test', and all GO terms that were significant with $p < 0.001$ (after correcting for multiple term testing by Benjamini and Hochberg false discovery rate corrections) were selected as over-represented.

Statistical analysis

MultiExperiment Viewer (MEV) software (v 4.6.2) was used for hierarchical clustering. Hierarchical trees were constructed for proteins quantified (y axis) and samples analysed (x axis). By using MEV, T-tests was applied on each of the intensities of the quantified proteins and p value cut-off was set to 0.01 or 0.05 as reported in the text.

Western Blot analysis

Thirty µg of proteins were diluted in loading buffer (10 g/l SDS, 10% glycerol, 1% 2-mercaptoethanol, 5 mM TrisHCl, pH 6.8), boiled, separated on 8% SDS-PAGE and transferred onto polyvinylidene difluoride membrane by electroblotting. The membrane was washed with phosphate-buffered saline (PBS), blocked with 2 g/l high purified casein in PBS and probed with the antibody. Secondary goat anti-rabbit IgG + IgM alkaline phosphatase conjugate was then added (dilution 1:5,000). CSPD chemiluminescent substrate (Tropix, Bedford, MA) was used for detection. To test for equal loading of proteins, anti-β-actin antibody (Santa Cruz Biotechnology) and 0.5% Ponceau S in acetic acid were used. Uromodulin and β-actin abundances in the urine of control group and naive patients were quantified by densitometric analysis and normalized by creatinine values. WB analysis was performed on randomly selected samples.

Mouse liver lysosome enrichment

As an *in vivo* model of Fabry disease, we used the α-Gal A-deficient mouse described previously [22].
Frozen mouse liver was added with protease inhibitors (Sigma Aldrich, St. Louis, MO,USA) and homogenized in lysis buffer (0.25 M sucrose, 10 mM Tris/HCl pH 7.4) by using a glass dounce homogenizer. Lysosomal fraction was enriched by differential centrifugation at 4°C: briefly, cell debris and nuclei were removed by centrifugation at 1,000g for 10 min. The supernatant was collected and centrifuged at 12,000g for 15 min. The pellet, containing mitochondria, was removed while the supernatant was added with CaCl2 8 mM. Samples were further centrifuged at 25,000g for 15 min and the pellet, containing the enriched lysosomal fraction, was washed twice in KCl 10 mM Tris/HCl pH 7.4. Lysosomal proteins were recovered by adding 0.1% NP40 and separated by 1D gel electrophoresis. A total of 30 µg of proteins was loaded on polyacrylamide gradient gel. Reduction, alkylation and enzymatic hydrolysis of proteins extracted from gel bands were performed as previously described [23]. LC-MS/MS analysis and label-free quantification were performed as described above. Two biological and two technical replicates were analyzed.

Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis

KEGG pathway analysis was performed with the KEGG pathway mapper tool (http://www.genome.jp/kegg/tool/map_pathway2.html) using the Uniprot ID of the modulated proteins.

Results

Label free quantification of differentially expressed proteins in urine from FD patients: identification of FD biomarkers

In order to discover urinary markers of FD, we compared the urinary proteome of FD patients with those of healthy donors. In particular, urinary samples were collected from 11 naïve patients and 12 healthy subjects (Table 1). Males and females samples were analysed in order to find FD biomarkers to be used in a sex-independent manner. Control specimens were grouped four by four and analysed in triplicate (groups C, Cb and Ct) while patients' specimens were analyzed one by one in duplicate (P and Pb). Tryptic peptides obtained from disease and normal urinary proteins were analysed by nanoLC-ESI-MS/MS. 557 protein groups were univocally identified and quantified. All the intensities for each protein quantified were averaged for each group of controls and patients (controls, named C, are the average of the groups C, Cb and Ct while patients, named P, are the average of samples from P1 to P11). Mean ratios were calculated, thus providing an indication of the relative abundance of a given protein in the two groups of samples. All the intensities were tested with Student t-test, resulting in the identification of 133 proteins (out of the 557 identified) undergoing significant changes in na ïve patients versus healthy controls (Fig. 1A and Table S2).
Technical reproducibility of the label free quantification approach was proved to be really excellent ($R^2 > 0.96$). Furthermore, label free quantification of urinary proteins showed a very good homogeneity for the two groups of patients ($R^2 > 0.94$). On the contrary, critical differences were detected when comparing patients *vs* controls ($R^2 < 0.64$) (Fig. S1). For statistical analysis we used Multi Experiment Viewer (MeV) software. Hierarchical trees constructed for proteins quantified (y axis) and samples analyzed (x axis) clearly show the perfect grouping of Fabry patients' specimens with respect to the control ones (Fig. 1B and C). Furthermore, technical replicates of the same sample are always coupled, thus confirming the reproducibility of the label free quantitative analysis.

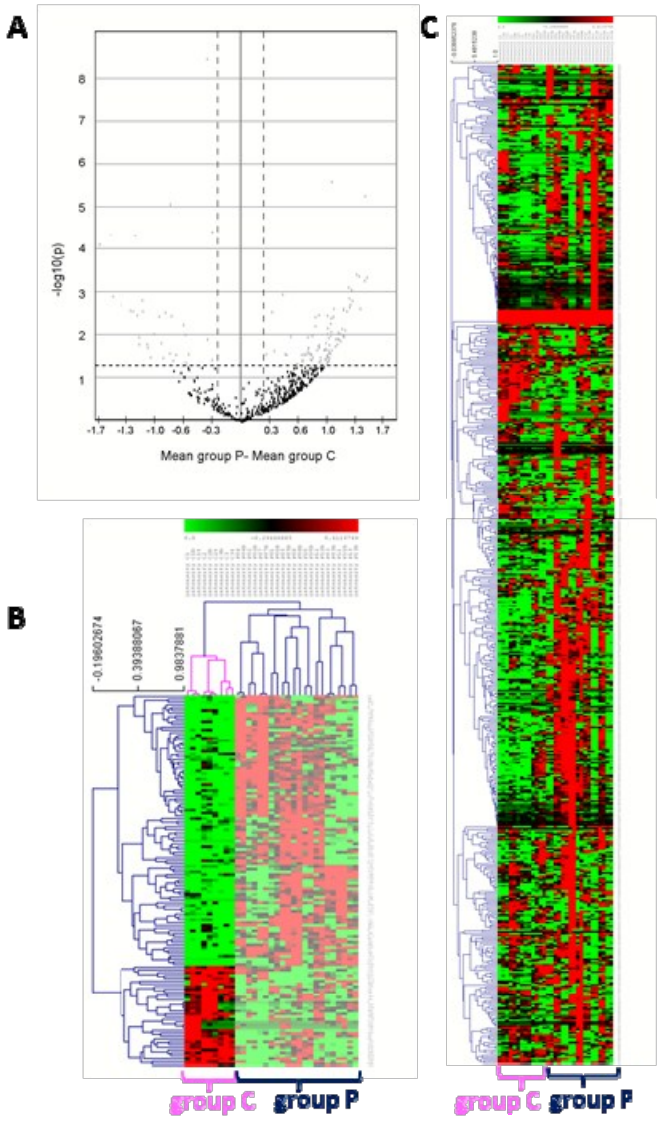

Fig. 1. Label free quantification of proteins. Volcano Plot (A) showing the difference between the means of patients (P) and control (C) (x axis) for each protein quantified plotted against the negative log10 p-value associated with its t-value (y axis). Significant proteins are represented as green spots. Hierarchical clustering (HCL) of significant genes (B). HCL of the proteins differentially expressed (p value < 0.05) in the urine of naïve Fabry patients (upper right) with respect to healthy controls (upper left). Hierarchical tree of 557 quantified proteins (C). Fabry patients' specimens co-cluster (upper right) as well as control ones. Technical replicates of the same sample are always coupled.

Urinary biomarkers of FD are proteins related to inhibition of digalactosylceramide metabolism

Only proteins with a fold change greater than 2 (91 entries corresponding to 16.5% of the total) were considered for bioinformatic analyses (Table S2). These proteins were functionally categorized based on universal Gene Ontology

(GO) annotation terms [21] using the Biological Networks Gene Ontology (BiNGO) program package [20]: 89, 82, and 87 proteins were linked to at least one annotation term within the GO cellular component, biological process, and molecular function categories respectively, while 18, 29 and 12 terms exhibited significance ($P < 0.001$) as over-represented terms compared with the entire list of UniProtKB/Swiss-Prot entries (Table S1). In cellular component category, GO terms related to extracellular space were over-represented together with GO terms related to plasma membrane and lysosomal proteins (Table S1). In biological process category (Table S1 and Fig. 2A), 55% of GO terms enriched are related to immune response and/or inflammation. Notably, this percentage is 5 times higher with respect to that found in human urine by Adachi and colleagues in 2006 [24]. Finally, in the molecular function category, 12 GO terms are significantly enriched (Table S1 and Fig. 2B). All of them are related to the enzyme inhibitor activity, in particular to phospholipase and endopeptidase like uteroglobin and annexins (Table S2), which are enzymes supposed to be involved in digalactosylceramide metabolism usually affected in Fabry disease.

Fig. 2. Enrichment analysis. Enrichment of Biological process GO terms (A): significantly over-represented GO biological process terms for the set of urinary proteins deregulated in a significant way (pvalue < 0.05) in the group of naïve Fabry patients with respect to controls. This set of proteins was compared with the entire list of UniProtKB/Swiss-Prot entries, and significantly over-represented GO terms ($P < 0.001$) are shown. The ratio shown is the number of urinary and entire UniProtKB/Swiss-Prot proteins annotated to each GO term divided by the number of urinary and entire UniProtKB/Swiss-Prot proteins linked to at least one annotation term within the indicated GO biological process categories.. Enrichment of Molecular function GO terms (B):

significantly over-represented GO molecular function terms for the set of significant proteins as visualized in Cytoscape. Dark grey categories are most significantly over-represented. White nodes are not significantly over-represented, they are included to show the grey nodes in the context of the GO hierarchy. The area of a node is proportional to the number of genes in the test set annotated to the corresponding GO category.

In order to understand the biological implications of proteins found differentially expressed in Fabry patients' urine, we also performed a KEGG Pathways analysis. Pathways mostly affected are related to glycolysis, pyruvate metabolism, lysosome and sphingolipid metabolism (Fig. 3 and Table S2). Notably, the up-regulation of Beta-galactosidase (Table S2) in Fabry naïve patients versus healthy subjects indicates that this enzyme might have a role in the intra-lysosomal accumulation of glycosphingolipids, which is a major symptom of the disease. Moreover, in this context a key role might be played also by prosaposin, a highly conserved glycoprotein involved in the hydrolysis of sphingolipids mediated by specific lysosomal hydrolases. Together, these findings confirm that the deregulated urinary proteins reflect Fabry pathology.

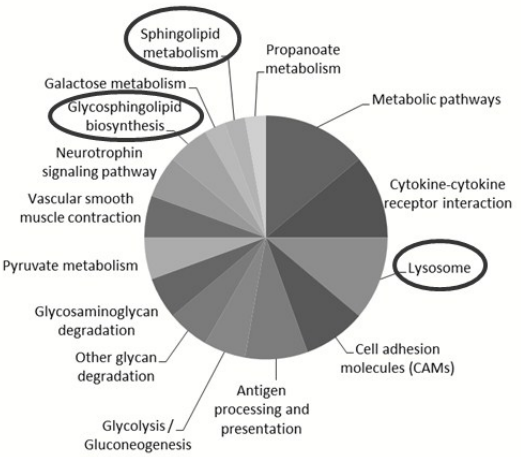

Fig. 3. KEGG pathway analysis of proteomic data. In the panel, the pie charts of the modulated pathways are reported.

Validation of uromodulin as biomarker of Fabry disease

Interestingly, some of the proteins found de-regulated by label free quantification have already been observed in the past, like prostaglandin H2 d-isomerase and the Ig kappa chain V-III (Table S2) [25], confirming the consistency of our data. On the contrary, while in a previous report by Vylet and colleagues, uromodulin was shown to be down-regulated in the urine of Fabry patients [26], our results clearly showed the opposite (Table S2). In order to validate our findings, western blot analyses were performed and uromodulin expression in Fabry patients was found significantly up regulated with respect to healthy subjects (Fig. 4).

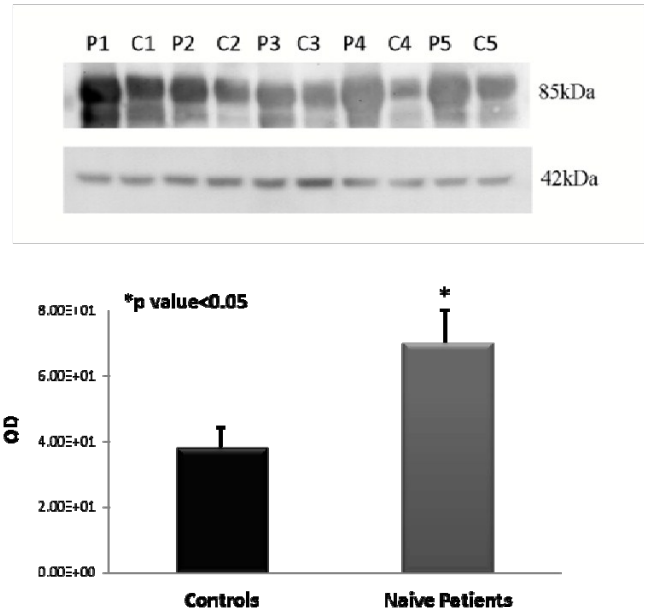

Fig. 4. Uromodulin biomarker validation. Western Blot analysis: uromodulin (85kDa) was quantified by densitometric analysis in the urine of Fabry naïve patients with respect to healthy subjects. To test for equal loading of proteins, anti-β-actin (42 kDa) antibody was used. Protein abundances, normalized by patients' creatinine values, are expressed as optical density in the bar graph below each western blot.

Cross-validation of prosaposin as marker of Fabry disease in mouse liver tissue

Prosaposin is the precursor protein for four glycoproteins (SAPs) A, B, C and D, which are sphingolipid activator. Prosaposin has been shown to be secreted into body fluids, whereas the mature SAPs are located within the lysosome. Recently, prosaposin has been claimed as potential marker of Fabry disease in urine samples from pediatric Fabry disease patients, these patients were all males [27]. Therefore, we decided to further validate the behaviour of this protein in naïve patients. In particular, we aimed at investigating at what level prosaposin maturation is impaired. Therefore, we investigated this protein in the tissue using a Fabry mouse model. Exactly, selective lysosome enrichment using liver tissues from control and Fabry mice was used to monitor prosaposin differential expression (Fig. S2). Lysosomal protein extracts were separated on SDS-PAGE. The entire gel lanes, both from control and Fabry mice were cut and digested as described before (Fig. S3). LC-MS/MS analysis led to the identification and quantitation of 1130 unique proteins. Five different peptides of PSAP were found in the low molecular weight region (20-10kDa) of the gel. They were sequenced and quantified as described above, proving that the mature form of PSAP are significantly up-regulated in liver lysosomes of Fabry mice (Fig. 5 and Fig. S4). Therefore, the abundance of urinary prosaposin might reflect

what happens at the lysosomal level in Fabry disease affected patients.

Fig. 5. Proteomic analysis of the intensity levels for prosaposin (PSAP) in Fabry liver tissue with respect to controls.

Levels of FD biomarkers are restored at basal values in ERT patients

Once validated as putative FD biomarkers, we analysed the abundance of these proteins in ERT patients in order to test their behaviour upon enzyme treatment.

In particular, we analysed the urinary proteome of ERT patients with the same label free approach used for Fabry patients and healthy controls. We compared the proteins intensities distributions from Fabry patients (P) with respect to healthy donors (C) and ERT-treated Fabry patients (E). In particular, we reported the box plot analysis of quantified expression levels for uromodulin (UMOD) (Fig. 6A), prostaglandin-H2 d-isomerase (PTGDS) (Fig. 6B) and prosaposin (SAP1) (Fig. 6C) showing their up-regulation in Fabry patients with respect to controls. Interestingly, upon ERT the concentration of UMOD, PTGDS and SAP1 is similar to the value observed in healthy control, leading to the speculation that it might reflect the effectiveness of the pharmacological treatment.

Moreover, for uromodulin, we also validated the MS quantitative data by western Blot analysis (Fig 4 and 6D). To test for equal loading of proteins, β-actin staining was used. Protein abundances were normalized by patients' creatinine values. These results might be clinical relevant as, if confirmed on a larger cohort of patients, they open up the possibility to use these proteins as markers to monitor the response to treatment.

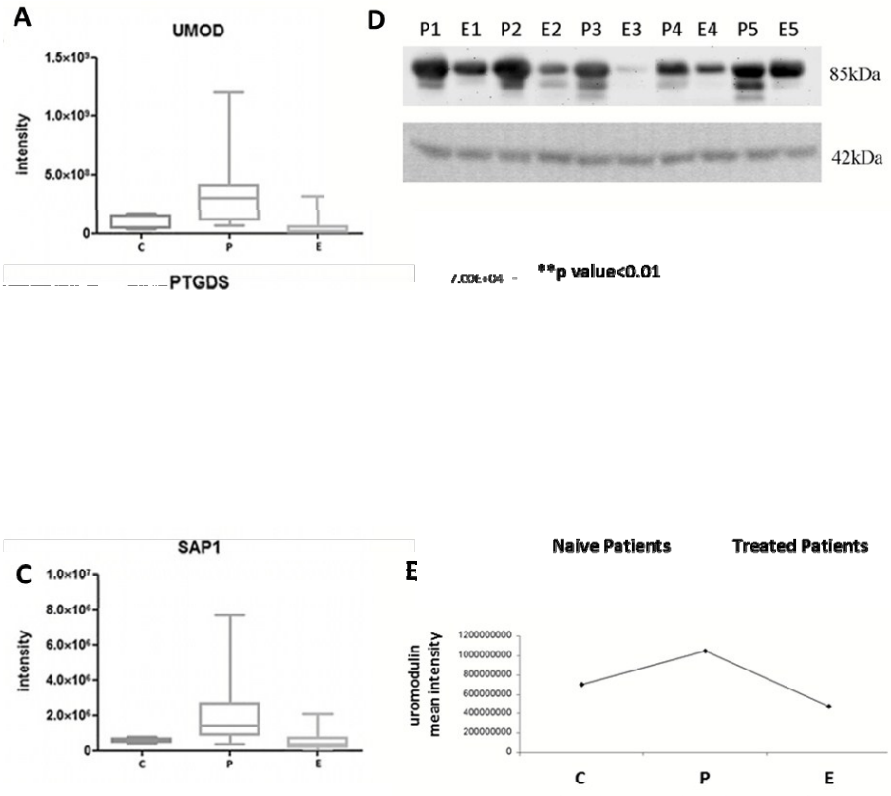

Fig. 6. FD biomarkers in ERT patients. Comparison of the label free intensities distributions from Fabry patients (P) with respect to healthy donors (C) and ERT-treated Fabry (E) for the proteins as indicated. Box plot analysis of quantified expression levels for uromodulin (UMOD) (panel A), prostaglandin-H2 d-isomerase (PTGDS) (panel B) and saposin (SAP1) (panel C) showing their up-regulation in Fabry patients with respect to controls as well as to patients under ERT. Western Blot analysis: uromodulin (85kDa) was quantified by densitometric analysis in the urine of Fabry naïve patients with respect to patients after enzymatic replacement therapy (panel D). To test for equal loading of proteins, anti-β-actin (42 kDa) antibody was used. Protein abundances,

normalized by patients' creatinine values, are expressed as optical density in the bar graph below. Panel E shows mean intensity values for uromodulin as detected by MS.

Discussion

Despite the great potential of –omic sciences in elucidating biological processes and monitoring disease progression, a comprehensive proteomic and analysis on FD patients has not been performed so far, with the exception of some targeted analyses [26,28] and very preliminary studies [11,12,25]. Only recently, using a proteomic approach on PBMC, several specific FD markers were identified, namely calnexin, g-enolase and galectin-1 [10]. Here, we describe the use of MS–based label-free protein quantification for the characterization of the urinary proteome of FD patients. Notably, we have not limited the analysis to adult male Fabry patients as biomarkers to guide diagnosis and treatment decisions are particularly needed for female Fabry patients.

We identified several proteins able to discriminate the urinary proteomics profile of all naïve patients affected by FD from that of healthy subjects. Our results point at proteins involved in biological pathways specifically modified by the pathology. We attempted to validate putative FD markers by alternative technique and also to follow them upon ERT.

In particular, up-regulation of potential markers of both kidney function and glycosphingolipid metabolism impairment was successfully confirmed by western blot analysis and lysosome isolation, respectively. In order to better understand the biological mechanisms induced by FD, proteins undergoing significant changes were functionally categorized. Surprisingly, the GO enrichment and KEGG pathway analysis highlighted an impairment of the immune system as well as an inflammatory status induced in the host by the disease. The analysis also revealed that, in patients, proteins with phospholipid inhibitor and serine type endopeptidase inhibitor activities are particularly enriched with respect to healthy subjects. The latter are involved in modulation of serine proteases, which in mammals serve functions in blood clotting, immune system, and inflammation. On the contrary, altered regulation of proteins with phospholipids inhibitor activity, like uteroglobin and annexins (Table S2), is supposed to affect also glycosphingolipid metabolism via ceramide production. Taking into account that the cohort of naïve patients analyzed is quite heterogeneous, the finding that specific groups of proteins were consistently deregulated in all patients, despite age and gender, highlights specific pathways modified by the disease. The activation of proteins involved in the inflammation is important since Fabry disease has been associated with many inflammatory conditions including fever of unknown origin [29]. Among the potential markers identified, particularly interesting is prosaposin, a protein precursor of 4 small peptides serving as sphingolipid hydrolase activators. As mentioned before, this proteins was also found more aboundant in urine from pediatric male FD patients [27], we confirmed this marker in adults and, more remarkable, in femal patients. Moreover, up-regulation of prosaposin was also investigated in the liver of a FD mouse model: indeed saposin B is a lysosomal activator of α-GalA, and the liver is the tissue with the highest concentration of lysosomes. In FD mice we detected an up-regulation of the mature form of PSAP, compatible with a compensatory mechanism induced by α-GalA deficiency in glycosphingolipid metabolism, as suggested by the up-regulation of beta galactosidase. A Few proteins found deregulated in this investigation have already been observed in previous studies (prostaglandin H2 d-isomerase (Fig. 4B) and the Ig kappa chain V-III (Table S2) [25]. On the contrary, differently from what reported in literature [26], we found up-regulation of uromodulin in the urine of Fabry patients. Usually, uromodulin mutations associated with kidney disease (UMAK) result in a marked decrease in the synthesis of uromodulin, as well as in the accumulation of abnormal uromodulin in tubular cells, leading to tubular cell death [30]. Notably, all patients showed almost normal values for both creatinine and GFR and therefore uromodulin up-regulation could be considered as a very early marker of kidney damage at tubular level, particularly useful for female patients for whose the measurement of α-GalA is not a reliable diagnostic marker [8]. Uromodulin was also measured in ERT patients where a kind of "normalization" was observed, thus demonstrating the effectiveness of the pharmacological treatment. These last results may be clinical relevant since uromodulin has been implicated in the pathogenesis of renal interstitial fibrosis, a pathology that is also typical in patients affected by Fabry disease. In addition, recent studies have identified uromodulin as a risk factor for kidney disease and have proposed that the urinary concentration of uromodulin may be a valuable biomarker for the development of chronic kidney disease [31]. This hypothesis is further strengthened by data of our group [13] indicating that uromodulin, although it is not a specific biomarker for Fabry patients, is significantly increased in the urine of hypertensive patients, a disease often associated with progressive renal failure.

Indeed, this study indicates that Fabry naïve patients can be classified and separated from healthy subject simply on the basis of their urinary proteomic patterns. Although we have analyzed groups of Fabry and ERT patients carrying mutations in different exons, it is worth to note that the positive effect of ERT on the urinary abnormalities found by proteomics has also been recently claimed in other studies performed on the urines of heterozygous adult female Fabry patients. In this study, several peptides specific for Fabry patients were found differently excreted respect to control subjects and, upon ERT, were restored to normal levels [32].

Lastly, we identified several pathways specifically modulated in Fabry patients. Essentially, these pathways are related to glycolysis, lysosome and sphingolipid metabolism. Interestingly, in this last pathway we found up-regulated Beta-galactosidase in Fabry naïve patients versus healthy subjects indicating that this molecule might have a role in the accumulation of glycosphingolipids, which is a major symptom of Fabry pathology.

Conclusions

Using a proteomic approach, we found several proteins as potential urinary biomarkers for Fabry disease in male and in female patients. Interestingly, we showed that some of these proteins are restored to normal levels during ERT and could therefore be useful markers to monitor ERT response. Our results might have important clinical implications since they might be used, instead of measurements of α-GalA enzyme activity, for an earlier detection of FD and for a better follow-up of ERT.

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Notes

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