Hypertension

ORIGINAL ARTICLE

Increased Collagen Turnover Is a Feature of Fibromuscular Dysplasia and Associated With Hypertrophic Radial Remodeling: A Pilot, Urine Proteomic Study

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ABSTRACT: Fibromuscular dysplasia (FMD), a nonatherosclerotic, noninflammatory disease of medium-sized arteries, is an underdiagnosed disease. We investigated the urinary proteome and developed a classifier for discrimination of FMD from healthy controls and other diseases. We further hypothesized that urinary proteomics biomarkers may be associated with alterations in medium-sized, but not large artery geometry and mechanics. The study included 33 patients with mostly multifocal, renal FMD who underwent in depth arterial exploration using ultra-high frequency ultrasound. The cohort was separated in a training set of 23 patients with FMD from Belgium and an independent test set of 10 patients with FMD from Italy. For each set, controls matched 2:1 were selected from the Human Urinary Proteome Database. The specificity of the classifier was tested in 700 additional controls from general population studies, patients with chronic kidney disease (n=66) and coronary artery disease (n=31). Three hundred thirty-five urinary peptides, mostly related to collagen turnover, were identified in the training cohort and combined into a classifier. When applying in the test cohort, the area under the receiver operating characteristic curve was 1.00, 100% specificity at 100% sensitivity. The classifier maintained a high specificity in additional controls (98.3%), patients with chronic kidney (90.9%) and coronary artery (96.8%) diseases. Furthermore, in patients with FMD, the proteomic score was positively associated with radial wall thickness and wall cross-sectional area. In conclusion, a proteomic score has the potential to discriminate between patients with FMD and controls. If confirmed in a wider and more diverse cohort, these findings may pave the way for a noninvasive diagnostic test of FMD. **(*Hypertension*. 2022;79:93–103. DOI: 10.1161/HYPERTENSIONAHA.121.18146.)** • **Supplemental Material**

Key Words: collagen ■ fibromuscular dysplasia ■ hypertension ■ kidney

Fibromuscular dysplasia (FMD) has been defined as an idiopathic, systemic, nonatherosclerotic, noninflammatory vascular disease leading to stenosis, aneurysms, dissections, and occlusion of small- and medium-sized arteries.[1] Although it has been considered a rare disease for many years, its actual prevalence is currently estimated around 4% in the general population.[2] FMD occurs typically in young and middle-aged, otherwise healthy, women; since it may lead to severe, life-threatening complications, such as resistant hypertension, renal failure, stroke, myocardial infarction, its socioeconomic impact is presumably large and mostly overlooked.[1] While FMD was initially considered as a vascular disease limited to a single arterial bed, in view of frequent multivessel involvement (50%–60% of cases), it is nowadays increasingly viewed as a truly systemic

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ORIGINAL ARTICLE

Novelty and Significance

What Is New?

- A proteomic signature including mostly collagen-derived peptides has the potential to discriminate between patients with fibromuscular dysplasia (FMD) and controls with high accuracy.
- In patients with FMD, this molecular signature is associated with hypertrophy of radial arteries.

What Is Relevant?

- Urinary proteomics is a promising tool to unravel the pathophysiology of FMD.
- Urinary proteomics may allow developing a noninvasive diagnostic test of FMD.

Summary

A proteomic score, comprised mostly of peptides related to increased collagen turnover, has the potential to discriminate with high accuracy between patients with FMD and healthy controls, as well as patients with other renal and cardiovascular diseases, and is associated with vascular hypertrophy of medium-sized arteries.

Nonstandard Abbreviations and Acronyms

CAD	coronary artery disease
CKD	chronic kidney disease
eGFR	estimated glomerular filtration rate
FMD	fibromuscular dysplasia

arterial disease.[3,4] Since confirmation of the diagnosis requires computed tomography-, magnetic resonance-, or catheter-based angiography,[1] FMD is greatly underdiagnosed. Furthermore, in the absence of the pathognomonic string of beads, that is, in case of focal stenosis and possibly of unexplained dissections and/or aneurysms in young, middle-aged women (atypical FMD or FMD-like presentation), it remains a diagnosis of exclusion. Therefore, there is a strong need of reliable tools for the diagnosis of FMD.

Urinary peptides have been demonstrated to be highly significantly linked to (patho)physiology.[5–7] Their presence in urine is the result of (1) glomerular filtration, (2) tubular reabsorption, and (3) generation of peptides in the kidney and urinary tract. It is generally not possible to assess with confidence the origin of these peptides, about 70% are thought to come from kidney and urinary tract, and about 30% from blood.[8] According to reports currently available, urinary peptides are especially well suited to depict pathologies (including systemic diseases) that involve fibrosis, vascular and endothelial dysfunction, and of course kidney disease. Based on these observations, the hypothesis was generated that distinct urinary peptides could specifically depict the molecular changes in FMD. As a result, in this study, we aimed toward identifying urinary peptides significantly associated with FMD and combining these into a classifier for discrimination of patients with FMD from healthy controls as well as from patients with other cardiovascular and renal diseases. In addition, we examined the association of the classifier with medium-sized artery geometry and mechanics.

METHODS

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Study Population

The current analysis included 23 patients with FMD from the Belgian multicentre cohort of patients with Fibromuscular Dysplasia study (BEL-FMD) in Brussels and 10 patients from the Italian FMD Initiative study (FMD-ITA) in Pisa, for whom both urinary samples and vascular structure and function measures were available. The samples were separated in a training set, comprised of all 23 patients with FMD from Brussels, and in an independent test set of the 10 patients from Pisa. To each set of patients healthy controls were matched, as described below. BEL-FMD and FMD-ITA include biological samples collection, were approved by local ethics committees, and are part of the European International FMD Registry and Initiative study (FEIRI), including >1000 patients with a diagnosis of FMD.[9] Clinical information and biological samples were collected according to a strictly identical protocol. Vascular function and structure measures were performed as part of an ancillary study of FEIRI, the "very high-frequency ultrasonography for arterial phenotyping in patients with cervico-cerebral artery dissection, hypertension, spontaneous coronary artery dissection and FMD" study (FUCHSIA). All participants with FMD whose datasets and biological samples were used for the present analysis gave explicit consent for use in future studies.

Urinary proteomics profiles for all non-FMD control subjects were extracted from Human Urinary Proteome Database,[10] containing information on >80 000 samples and >20 000 peptides and low molecular weight proteins. All datasets retrieved were fully anonymized and previously published, with relevant references provided below. The included studies were conducted in line with the regulations on the protection of individuals participating in medical research and the Declaration of Helsinki (2013) and received an approval from the responsible review boards.

To each set of patients, healthy controls were matched for age, sex, systolic blood pressure, diastolic blood pressure and estimated glomerular filtration rate (eGFR) at a ratio 2:1. This included 46 and 20 controls in the training and test cohort, respectively. Additional patient-derived datasets for 700

unselected controls were included to further evaluate biomarker specificity in the general population. Datasets for the above mentioned control subjects were derived from population-based studies.[11–13]

Moreover, to ensure that changes in proteomic profile observed in FMD were specific of the disease rather than reflecting more general pathophysiologic mechanisms shared with other vascular and renal conditions, we included 2 additional control groups retrieved from the same database,[10] namely patients with coronary artery disease (CAD) and patients with chronic kidney disease (CKD). For those patients, diagnoses were established based on clinical assessment, in line with the guidelines at the patients' recruitment, and were provided by participating clinical centers. For patients with CKD, diagnosis was based on clinical assessment and/or histopathology. CAD was confirmed by coronary angiography. Specifically, data from 31 patients with CAD[14] with estimated glomerular filtration rate >60 mL/minute per 1.73 m^2, as well as 66 patients with CKD of known etiology[15,16] and without CAD, matched to the whole FMD cohort (n=33) for age and sex, were included. The CKD group included patients with diabetic nephropathy (n=39, 59%), IgA nephropathy (n=14, 21%), vasculitis (n=5, 8%), and others (n=8, 12%). Case-control matching was performed using the R package "MatchIt"[17] based on a "nearest neighbor" matching-method and estimation of the distance-measure using logistic regression.

Urinary Proteomics

Spot morning void urine samples were collected during routine follow-up visits, prepared, and analyzed with capillary electrophoresis-mass spectrometry as described.[18] Sample collection, preparation, and analysis follow established standard operating procedure and are monitored by quality control as described in study by Mischak et al.[18] Quality control is ensured using standard urine samples for the assessment of the analytical performance of capillary electrophoresis-mass spectrometry platform.[10,18] All protocols have been in place for 12 years, conferring very good comparability of datasets. Urine samples (0.7 mL) were mixed with an aqueous solution of 2 M urea, 10 mmol/L NH4OH, and 0.02% sodium dodecyl sulfate (0.7 mL), followed by filtration with Centrisart ultrafiltration devices (20 kDa molecular mass cutoff, Sartorius, Germany). Subsequently, PD-10 desalting columns (GE Healthcare, Germany) were used to remove salts, and samples were lyophilized. Before capillary electrophoresis-mass spectrometry analysis, samples were redissolved in high performance liquid chromatography-grade water, and introduced into a P/ACE MDQ CE (Beckman Coulter) coupled to a micro-TOF-MS (Bruker Daltonic, Germany). Subsequent evaluation of the MS data was conducted using MosaFinder,[10] followed by normalization based on 29 collagen fragments, serving as internal standards.[20] Peptide sequencing was performed by tandem mass spectrometry (MS/MS) using an Orbitrap Velos FTMS (Thermo Finnigan, Bremen, Germany), as described previously.[21]

Statistical Analysis and Classifier Development

Identification of biomarkers discriminating between patients with FMD and controls was performed in the training cohort. Only urinary peptides that were found at a 30% frequency or higher in either case or control group were considered. Statistical analysis was performed using nonparametric Wilcoxon test,[22] followed by adjustment for multiple testing by assessing the false discovery rate according to Benjamini & Hochberg.[23] An adjusted $P<0.05$ was considered statistically significant. Only sequenced peptides found significantly altered were included in subsequent analysis.

A classifier differentiating between FMD cases and controls was developed in a training cohort, by combining significantly altered peptides using the support vector machine integrated in the MosaCluster software,[24] followed by optimization of the support vector machine classifier parameters (C and γ). The performance of the classifier was validated in the test cohort. The receiver operating characteristic plot was obtained and the area under the receiver operating characteristic curve was evaluated (MedCalc 12.7.5.0, Mariakerke, Belgium). The optimal cutoff level for the classification score was determined based on the criterion value corresponding with the Youden index J, providing an equal weight to sensitivity and specificity. The specificity of the classifier was further assessed in unselected group of controls (n=700), patients with CAD (n=31) and CKD (n=66). Of the patients with CKD, 39 were patients with diabetic nephropathy, diagnosis established based on the presence of type II diabetes and macroalbuminuria and/or estimated glomerular filtration rate <60 mL/minute per 1.73 m^2. Diagnosis in the other 27 patients (14 IgA nephropathy, 5 vasculitis, 3 lupus nephritis, 2 focal segmental glomerulosclerosis, 2 membranous glomerulonephritis, 1 minimal change disease) was based on histopathology.

Bioinformatics Analysis

Bioinformatic analysis was conducted to place the urinary proteomics findings in the context of biology. Since changes in peptide abundance are expected to be the result of and reflect changes in protease activity,[5] proteases involved in the generation of peptides associated with FMD were predicted. Protease prediction was performed using a web-based tool, Proteasix,[25] which allows retrieving protease/cleavage sites associations. Only proteases observed to match cleavage site associations retrieved from the literature were considered (observed mode). Predicted proteases as well as parental proteins from which urinary peptides originated were used as an input for pathway enrichment analysis using CluGO v2.3.3[26] (a Cytoscape[27] plugin) and kyoto encyclopedia of genes and genomes pathway database.[28] Kyoto encyclopedia of genes and genomes pathway contains pathway maps representative of the current knowledge on different molecular interaction/ reaction network. Analysis was carried out using default settings, and pathways predicted with $P<0.05$ (Bonferroni step down correction) were considered as significant. Pathways specific for other diseases, not related to FMD, were excluded.

Large and Medium-Sized Artery Structure and Function

Vascular variables were available only in the patients with FMD. Carotid-to-femoral pulse wave velocity was obtained by applanation tonometry (SphygmoCor CVMS; AtCor Medical, Sydney, Australia) to assess large artery (aortic) stiffness. Carotid and femoral waveforms were recorded sequentially at the carotid and femoral sites. Carotido-femoral pulse wave velocity was calculated as the ratio of the surface distance between the 2 recording sites, multiplied for 0.8, and pulse transit time,

according to international guidelines.[29] Two consecutive measurements were recorded and averaged if their difference was <1 m/s, otherwise the median value between 3 measurements was taken.

Carotid ultrasound coupled with automated software detection was used to measure carotid function and structure. Common carotid artery scans (25 frames/seconds) were obtained by high-resolution ultrasound with a 10 MHz linear array transducer (MyLab25; ESAOTE, Florence, Italy) by a trained operator. Longitudinal scans were acquired from each common carotid artery (1 cm proximal to the carotid bulb in a region 1-cm wide and free of plaques) and automatically analyzed by a software based on contour tracking algorithm.[30] The following variables were obtained: Carotid distensibility coefficient=$\Delta A/(A\times\Delta P)$, where A represents the diastolic lumen area, evaluated from the diameter values (assuming the cross-section of the artery to be circular), ΔA represents the stroke change in lumen area, ΔP the local pulse pressure obtained by tonometry.[29] Common carotid intima-media thickness was simultaneously measured,[30] and carotid cross-sectional area (wall cross-sectional area) was also calculated. All parameters were then calculated as the average of the right and left common carotid values. We visually quantified the presence of supernumerary acoustic interfaces within the common carotid wall by the triple signal score.[4,31] Triple signal was identified by visual scoring of B-mode right and left common carotid clips by a single reader. B-mode scans were scored as follows: 1 for the normal pattern, 2 for mild alterations in intima-media thickness echogenicity, 3 for moderate alterations in intima-media thickness echogenicity, and 4 for the triple signal.

Ultrahigh-frequency ultrasound (VevoMD, Visualsonics-Fujifilm; Toronto, Canada) was used to evaluate radial wall thickness, geometry and distensibility. 5 seconds-clips were acquired with the 70 MHz probe on the right and left radial artery at the wrist level. Three end-diastolic frames were selected for each clip: in each frame, global wall thickness, as well as inner and outer layer thickness (leading edge to leading edge), were measured manually on 3 different points, by using a graphic user interface build in Matlab.[32] Systolo-diastolic stroke change in radial lumen (radial distension) was measured by the same automated software used for carotid (Carotid Studio, Cardiovascular Suite 3.0; Quipu srl, Pisa, Italy) and radial distensibility coefficient obtained by the aforementioned formula. Oscillometric brachial pulse pressure was used for radial distensibility coefficient calculation.

RESULTS

Study Population

In the training cohort, mean age at diagnosis of FMD was 47.0±14.1 years, most patients were women (82.6%) and had renal FMD (91.3%) of the multifocal subtype (83%). Characteristics of patients with FMD at diagnosis from the test cohort were similar (age: 48.6±13.5 years, 100% women with renal, multifocal FMD) (Table 1). Since urine samples from patients with FMD were collected during the follow-up, controls were matched considering the variables at sampling. There were no significant differences ($P>0.05$) between patients with FMD and controls for the key matching variables (Table S1 in the Supplemental Material). Clinical characteristics at the time of sampling for the complete FMD cohort, matched controls, and additional patients with CKD and CAD are presented in Table 2. An overview of the study design is presented in Figure 1.

Table 1. Clinical Characteristics of Patients With FMD at Baseline

Variables	Training cohort (n=23)	Test cohort (n=10)
Women (n, %)	19 (82.6)	10 (100)
Age at urine sampling, y	50.5±12.1	58.9±8.3
Age at diagnosis, y	47.0±14.1	48.6±13.5
BMI, kg/m^2	24.1±3.2	24.6±4.6
Systolic BP, mm Hg	142.4±21.1	130.3±13.8
Diastolic BP, mm Hg	87.0±12.0	76.0±8.7
eGFR, mL/min per 1.73 m^2	88.3±18.8	85.3±8.9
Hypertension (n, %)	18 (78.2)	9 (90.0)
Diabetes (n, %)	0 (0.0)	0 (0.0)
Multifocal FMD (n, %)	19/23 (83.0)	10/10 (100.0)
Renal FMD (n, %)	21/23 (91.3)	10/10 (100.0)
Multivessel FMD (n, %)	14 (60.9)	1 (10.0)
Previous interventions for FMD (n, %)	9 (39.1)	4 (40.0)

Values are given as mean±SD, unless otherwise stated. BMI indicates body mass index; BP, blood pressure; eGFR, estimated glomerular filtration rate; and FMD, fibromuscular dysplasia.

Urinary Peptides Associated With FMD

Three hundred thirty-five sequenced peptides were identified as significantly different between urine samples from patients with FMD and matched controls in the training cohort (Table S2). The distribution of these peptides is graphically depicted in Figure 2. Among these 335 peptides, the most prominent (based on the number of peptides) were fragments derived from collagen alpha-1(I), collagen alpha-1(III), collagen alpha-2(I), indicating an increase in collagen degradation. In addition, several urinary peptides derived from CD99, from polymeric immunoglobulin receptor, from sodium/potassium-transporting ATPase subunit gamma, and from fibrinogen alpha were found to be increased in patients with FMD (Table S2).

To obtain insight into the potential mechanisms underlying the changes displayed by the urinary proteome, bioinformatic analysis was conducted. Twenty-three proteases were predicted to be involved in the generation of peptides significantly associated with FMD (Table S3). This includes among others MMPs (matrix metalloproteinases) and cathepsins, with MMP13, MMP9, and MMP25 having the highest number of cleavage events predicted. Those proteases were combined with the parental proteins that gave rise to the 335 peptides associated with FMD. The combined data were used as basis for the bioinformatic pathway analysis. A total of 14 pathways were predicted to be significantly enriched.

Table 2. Characteristics of Controls and Patients With Other Vascular Diseases (CKD, CAD) Matched to Patients With FMD

Variable	FMD cohort (n=33)	Controls cohort (n=66)	CKD cohort (n=66)	CAD cohort (n=31)
Women (n, %)	29 (87.9)	60 (90.9)	58 (87.9)	28 (90.3)
Age, y	53.0±11.6	53.58±19.3	52.9±11.5	54.9±10.3
Systolic BP, mm Hg	130.6±16.4	132.9±20.9	137.4±17.3	134.3±14.0
Diastolic BP, mm Hg	74.7±9.9	78.2±8.1	78.6±11.5	77.7±8.8
eGFR, mL/min per 1.73 m^2	88.0±19.0	87.6±14.1	60.6±59.2*	83.3±14.8

Characteristics are provided at urine sampling. Values are given as mean±SD, unless otherwise stated. FMD patients were compared with other groups using *T*-test for continuous variables and χ^2 test for categorical variables. BP indicates blood pressure; CAD, coronary artery disease; CKD, chronic kidney disease; eGFR, estimated glomerular filtration rate; and FMD, fibromuscular dysplasia.

*P value <0.01.

Upon exclusion of pathways specific for other diseases, 8 pathways were shortlisted (Table S4, Figure 3). The 3 most significantly enriched pathways included protein digestion and absorption, extracellular matrix-receptor interaction, and focal adhesion, with different collagens involved, indicating changes in the extracellular matrix organization. Among other significant pathways, PI3K-Akt signaling, complement and coagulation cascades, IL-17 signaling, antigen processing and presentation, and PPAR signaling were predicted.

Proteomic Classifier Development and Validation

The 335 peptides were combined into a support vector machine-based classifier (C=5120 and γ=0.000032).

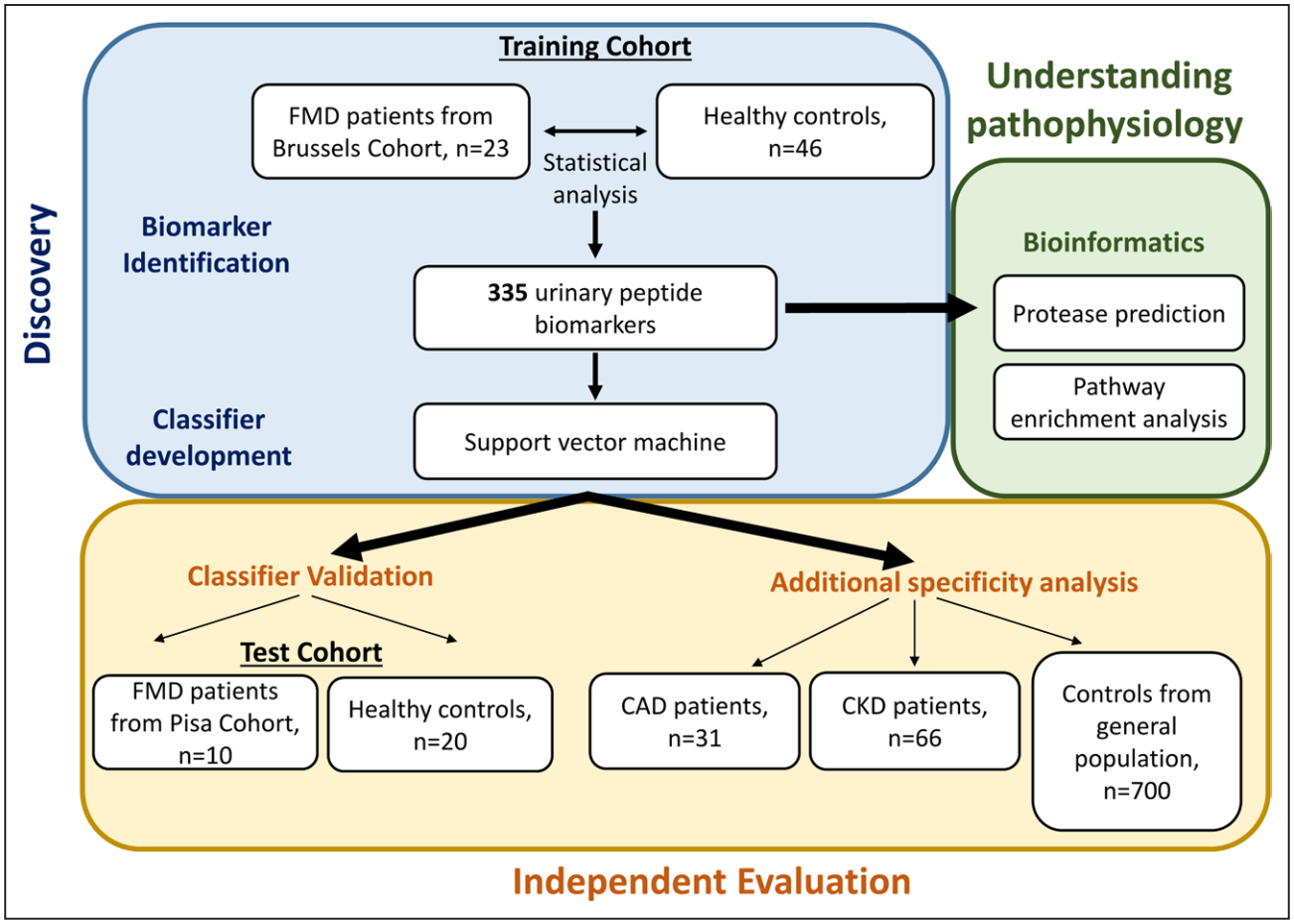

Figure 1. Overview of the study workflow. Information on the main steps and investigated cohorts is given.
Urinary proteomic profiles from patients with fibromuscular dysplasia (FMD) and healthy controls matched for age, sex, blood pressure, and kidney function were compared. Three hundred thirty-five sequenced peptide biomarkers discriminating between cases and controls were identified and subsequently combined using machine learning approach. Performance of the classifier was further assessed in the independent cohorts. In parallel, identified biomarkers were used to investigate biological pathways associated with FMD. CAD indicates coronary artery disease; and CKD, chronic kidney disease.

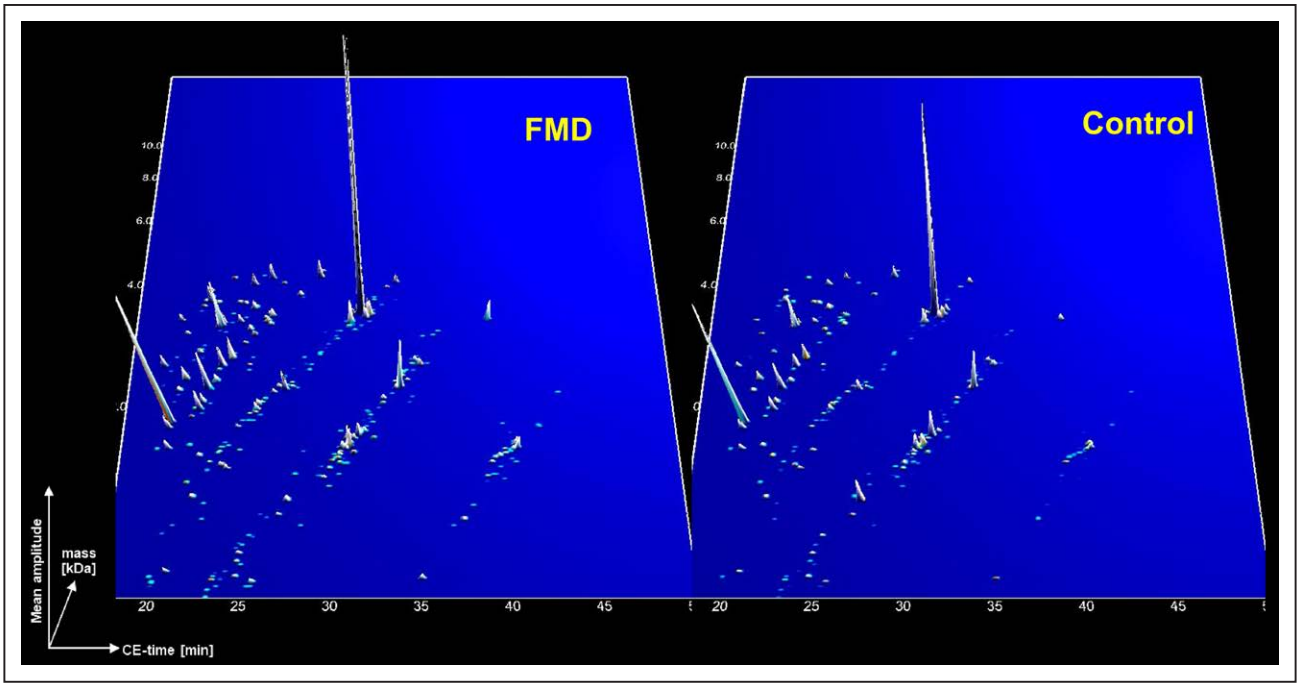

Figure 2. Capillary electrophoresis-mass spectrometry (CE-MS) plot showing the 335 peptides differentially excreted in patients with fibromuscular dysplasia (FMD) vs matched controls in training cohort.
Migration time (minutes) is plotted against molecular mass (kDa, on a logarithmic scale). The average signal intensity is encoded by the peak height.

When using this classifier in the independent test cohort (n=10 patients with FMD and 20 controls), the area under the receiver operating characteristic curve was 1.0 ([95% CI, 0.88–1.00], $P<0.0001$) at the cutoff value of 0.029. The classifier was further evaluated in the cohort of 700 additional controls. Applying the cutoff of 0.029, only 12 out of 700 subjects (1.7% of the entire cohort) scored positive, indicating a specificity of 98.3% in this additional independent cohort, representing the general population. We further examined the performance of the classifier in 2 independent sets of patients, respectively with CKD (n=66) and CAD (n=31). Based on the predefined cutoff of 0.029, the biomarker classifier correctly classified as non-FMD 60/66 patients with CKD (specificity of 90.9%) and 30/31 patients with CAD (specificity of 96.8%). Distribution of the classifier scores is presented in Figure 4A and 4B.

Association Between Proteomic Biomarkers and Arterial Geometry and Stiffness

In the 31 patients with FMD for which imaging data were available, the proteomic score was positively associated with radial wall thickness (r=0.43, P=0.015, Figure 4C), and wall cross-sectional area (r=0.36, P=0.049; Figure 4D) but not with radial lumen, indicating a positive association between proteomic score and hypertrophic remodeling in medium-sized arteries. When association with the 10 most significant peptides was tested, patients having urinary concentrations above the median of a peptide derived from collagen alpha-1(I) chain (sequence: DDGEAGKPG) showed increased radial wall thickness (0.30 [0.26–0.33] versus 0.25 [0.24–0.28] mm, P=0.011). Conversely, proteomic score was not correlated either with aortic or carotid variables. Furthermore, no association of the proteomic score with general characteristics (age, sex, classical cardiovascular risk factors) or additional disease-related characteristics (multifocal type, need for revascularization, age at diagnosis, multivessel involvement) was found.

DISCUSSION

We identified for the first time a urinary proteomic signature strongly associated with FMD. Interestingly, it includes mostly collagen peptides, likely reflecting increased collagen turnover. These findings are consistent with replacement of arterial muscle from the arterial media by a loose collagen network, which is the hallmark of medial FMD, with multifocal thickened fibromuscular ridges alternating with segments of thinning of the vascular wall, leading to the pathognomonic string-of-beads lesion.[33,34] Medial FMD is the pathological counterpart of multifocal FMD, both the most frequent FMD subtype[1] and the most represented in this series. Notably, intimal fibroplasia, another pathological subtype of FMD inducing focal stenosis, is also characterized by circumferential accumulation of fibrous tissue, in this case in the intima.[33,34] The involvement of collagen subtypes in the pathogenesis of FMD is further supported by the recent identification of mutations in *COL5A1* at the origin of rare cases of multifocal FMD and dissections.[35]

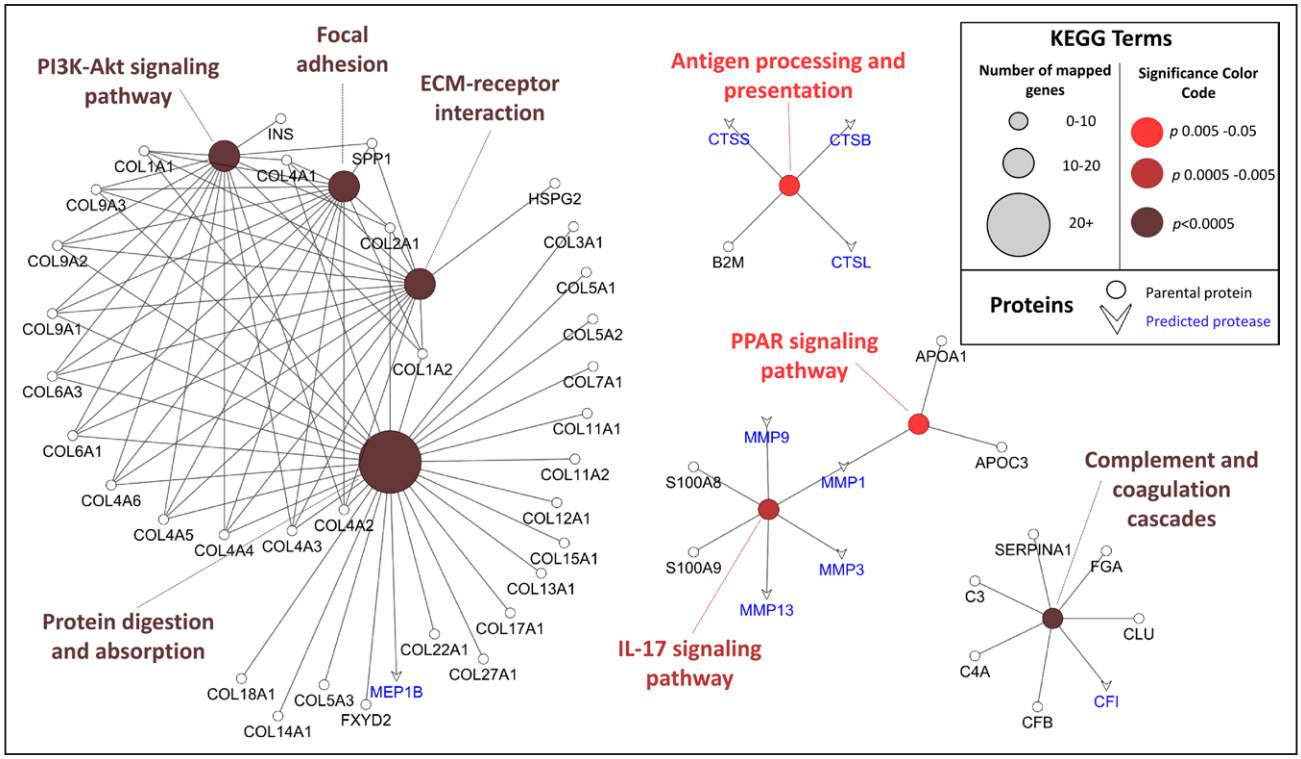

Figure 3. Pathway enrichment analysis. Graphical representation of the shortlisted pathways and associated proteins (parental proteins and predicted proteases) represented by the urinary proteomics data.

Size of the node representing the pathway increases along with the number of proteins associated with the pathway. *P*-values are color coded. APOA1 indicates apolipoprotein A-I; APOC3, apolipoprotein C-III; B2M, Beta-2-microglobulin; C3, complement C3; C4A, complement C4-A; CFB, complement factor B; CFI, complement factor I; CLU, clusterin; COL11A1, collagen alpha-1(XI) chain; COL11A2, collagen alpha-2(XI) chain; COL12A1, collagen alpha-1(XII) chain; COL13A1, collagen alpha-1(XIII) chain; COL14A1, collagen alpha-1(XIV) chain; COL15A1, collagen alpha-1(XV) chain; COL17A1, collagen alpha-1(XVII) chain; COL18A1, collagen alpha-1(XVIII) chain; COL1A1, collagen alpha-1(I) chain; COL1A2, collagen alpha-2(I) chain; COL22A1, collagen alpha-1(XXII) chain; COL27A1, collagen alpha-1(XXVII) chain; COL2A1, collagen alpha-1(II) chain; COL3A1, collagen alpha-1(III) chain; COL4A1, collagen alpha-1(IV) chain; COL4A2, collagen alpha-2(IV) chain; COL4A3, collagen alpha-3(IV) chain; COL4A4, collagen alpha-4(IV) chain; COL4A5, collagen alpha-5(IV) chain; COL4A6, collagen alpha-6(IV) chain; COL5A1, collagen alpha-1(V) chain; COL5A2, collagen alpha-2(V) chain; COL5A3, collagen alpha-3(V) chain; COL6A1, collagen alpha-1(VI) chain; COL6A3, collagen alpha-3(VI) chain; COL7A1, collagen alpha-1(VII) chain; COL9A1, collagen alpha-1(IX) chain; COL9A2, collagen alpha-2(IX) chain; COL9A3, collagen alpha-3(IX) chain; CTSB, cathepsin B; CTSL, procathepsin L; CTSS, Cathepsin S; ECM, extracellular matrix; FGA, fibrinogen alpha chain; FXYD2, sodium/potassium-transporting ATPase subunit gamma; HSPG2, basement membrane-specific heparan sulfate proteoglycan core protein; IL-17, interleukin 17; INS, insulin; KEGG, kyoto encyclopedia of genes and genomes; MEP1B, meprin A subunit beta; MMP1, interstitial collagenase; MMP13, collagenase 3; MMP3, stromelysin-1; MMP9, matrix metalloproteinase-9; PI3K-Akt, phosphatidylinositol 3-kinase/protein kinase B; PPAR, peroxisome proliferator-activated receptor; S100A8, protein S100-A8; S100A9, Protein S100-A9; SERPINA1, alpha-1-antitrypsin; and SPP1, osteopontin.

Also, the increased excretion of collagen degradation peptides—particularly collagen alpha-1(I)—was correlated with the extent of vascular hypertrophy in radial arteries (Figure S1). This is not surprising as the latter have been shown to harbor subclinical hallmarks of FMD using both high-resolution echotracking[31] and more recently ultra-high frequency ultrasound.[36] Two collagen alpha-1(III) chain (COL3A1) fragments were found decreased in FMD. We cannot present a convincing hypothesis explaining the simultaneous increase and decrease of specific COL3A1 fragments. However, it must be based on a distinct biological mechanism, considering the very consistent changes and the low *P*. One potential explanation may be that these peptides overlap with other peptides that are increased in abundance (evident from the data for both COL3A1 fragments reduced in FMD). The increase in the overlapping, essentially competing peptide (eg, $_{807}$GPAGFpGApGQNGEpGGKGERGApG$_{831}$), may consequently result in a decrease in the corresponding overlapping peptide ($_{796}$SpGERGETGPpGP$_{808}$), as observed.

Finally, bioinformatic analysis based on the parental proteins of the differentially excreted peptides, and the proteases predicted to be involved in their generation suggests the implication of a number of pathways such as protein digestion and absorption, extracellular matrix-receptor interaction, and focal adhesion, with different collagens involved, indicating changes in the extracellular matrix organization. Among other significant pathways, involvement of PI3K-Akt signaling, complement and coagulation cascades, and PPAR signaling were predicted. Overexpression of a number of proteins involved in inflammation and immunity is of particular interest.

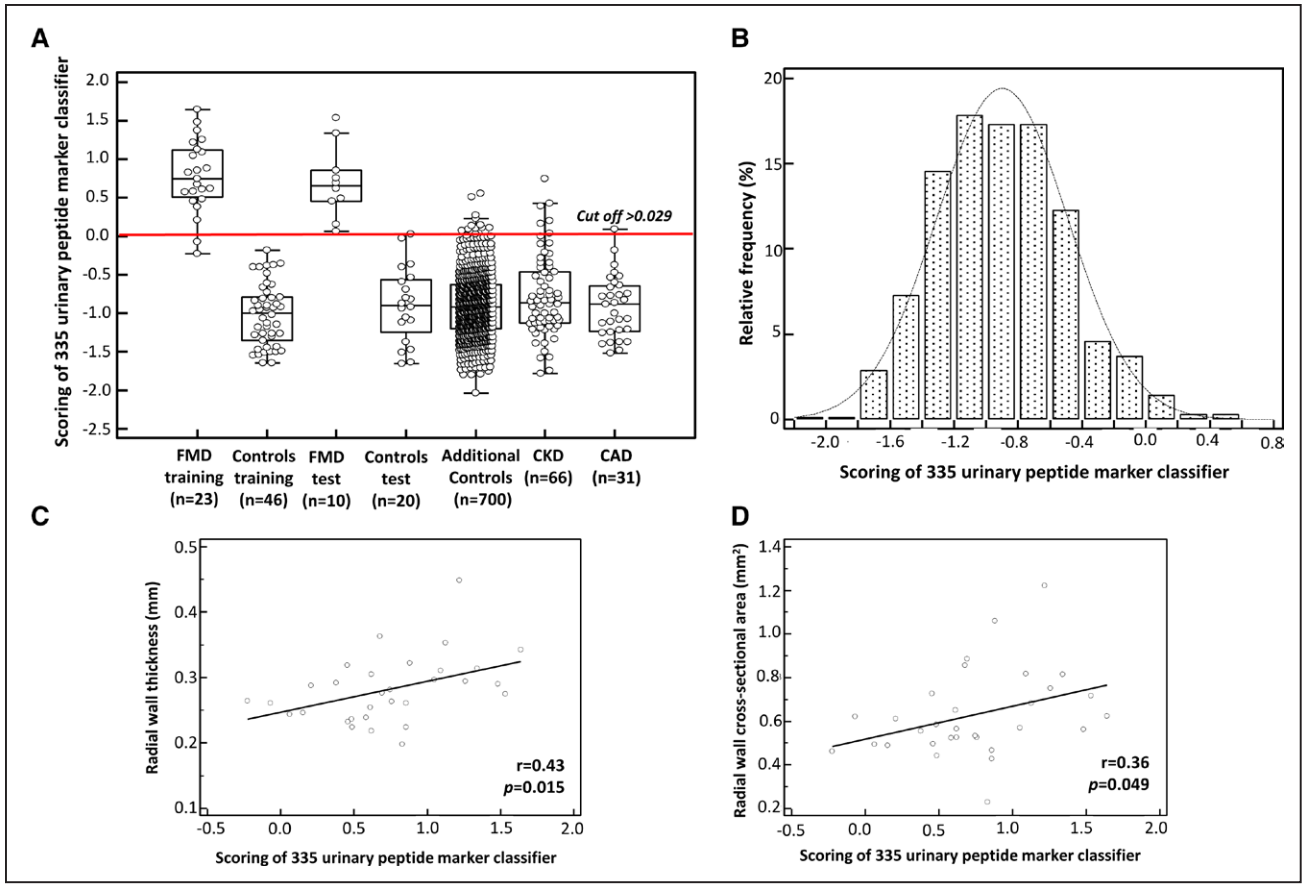

Figure 4. Distribution of the classification scores and correlation of the scoring with arterial characteristics.
A, Box-whisker plot showing the scoring of the 335 urinary peptide marker classifier in the training cohort, the test cohort, an additional set of controls (n=700) and patients with chronic kidney disease (CKD; n=66) and coronary artery disease (CAD; n=31). **B**, Histogram representing the detailed distribution of scores for 700 controls. **C**, Linear correlation between the proteomic score and radial wall thickness. **D**, Linear correlation between the proteomic score and radial wall cross-sectional area. FMD indicates fibromuscular dysplasia.

Indeed, though based on the relative paucity of inflammatory cells in historical samples with advanced FMD lesions,[33,34] FMD has been considered as a noninflammatory disease, the identification of increased biomarkers of inflammation and cytokines, known to trigger arterial fibrosis, in patients with FMD has led to the hypothesis that inflammation may play a role in the incipient phase of the disease.[19] Nevertheless, such interpretations have to be taken with some caution, as prediction of pathways related to inflammation (ie, IL-17 signaling) was based to a large degree on the predicted proteases (see Table S4) rather than on detected peptides themselves.

As a whole, the proteomic signature identified in this work appears highly specific for FMD whether compared with controls from general population, patients with CKD or CAD. Using the classifier, only 12 out of 700 of healthy controls were misdiagnosed for FMD. As systematic screening for FMD was not performed in the controls, we cannot exclude that some of these apparent false positives had in fact undiagnosed FMD. Indeed, based on kidney donor studies, it has been suggested that about 4% of supposedly healthy subjects harbor silent renal FMD lesions.[2] This proportion may be even higher in our control groups of which demographic characteristics were similar to the FMD group, that is, including predominantly middle-aged women.

Notably also, this proteomic score was associated with FMD rather than to one or another clinical aspect of the disease. When investigating the distribution of the FMD specific peptides in subgroups of patients from the entire FMD cohort, of the 335 peptides significantly changed in FMD versus controls, 326 also changed in the same direction when comparing the same controls to 15 patients with multifocal single-vessel renal FMD, only 9 peptides showing changes in the opposite direction. Comparison of the same controls to 12 patients with multifocal renal FMD and lesions of FMD in other arterial beds confirmed the same directional changes in 327 of the 335 peptides, 8 peptides showing a change in the opposite direction. Only 2 patients in the study had no renal involvement, too few to investigate peptide changes in comparison to controls with confidence.

In a recent publication from the Defining the basis of FMD study (DEFINE-FMD), Olin et al[37] reported a distinct

plasma proteomic profile in patients with FMD, with 105 differentially abundant proteins, 37 of which, including CD2AP and PODXL2, were subsequently validated. Direct comparison with our study is hampered by the differences in body fluid analyzed (blood versus urine), analytical platform and compounds assessed. While we performed a global analysis of naturally occurring peptides and small proteins in the urine, in the DEFINE study,[37] samples were processed by Olink protein array, in which panels of selected proteins are measured (in plasma) using corresponding oligonucleotide-labeled antibody probe pairs (proximity extension assay technology).[38] Furthermore, most of the parental proteins from our differentially excreted peptides were not measured by the Olink method. While the performance of our classifier would appear better in terms of replication and receiver operating characteristic curves, our samples size was substantially smaller. Therefore, our results need further confirmation. To draw definitive conclusions, a side-by-side comparison in a statistically well-powered cohort would be needed, where both urine and plasma samples are collected from the same patients.

The current study has several strengths. These include a rigorous, well-validated method of proteomic analysis, replication of our findings in an independent cohort, evaluation of performance of the proteomic classifier both in healthy controls and patients with cardiovascular and renal disease, as well as correlation of components of this classifier with anatomic characteristics of arterial wall.

Limitations of our study includes small sample size and as a result adjustment for potential confounders not being possible. In addition, patients enrolled had predominantly renal, multifocal FMD. While the proportion of patients with multivessel FMD in the training cohort was in line with that reported in contemporary registries (61%), patients from the test group had mostly single-vessel renal FMD. Therefore, it remains uncertain whether the proteomic signature also applies to patients with predominant involvement of nonrenal, particularly cerebrovascular arterial beds and/or patients with focal FMD. Notably, however, urine samples were collected at 2 different sites (Brussels and Pisa) and detected changes in urinary peptides were independent of the sample collection site and clinical presentation.

In conclusion, a proteomic score comprised mostly of peptides related to increased collagen turnover has the potential to discriminate with high accuracy between patients with FMD and controls. The direct association between increased radial wall hypertrophy and the urinary proteomic score in patients with FMD suggests vascular fibrosis in medium-sized arteries as a potential mechanism of disease.

Perspectives

Overall, our findings are a strong incentive to revisit the histology and pathophysiology of FMD to dissect connective tissue abnormalities associated with the pathognomonic string-of-beads lesion. They highlight the critical need to collect arterial fragments of those few patients with FMD still undergoing surgery within a tissue biobank to analyze the components of arterial wall using contemporary methods. Along the same lines, urinary proteomics appears as a promising tool to unravel the pathways involved in the pathophysiology of FMD and related diseases, either alone or in combination with genetic characterization and imaging biomarkers.

Future studies should aim to replicate our findings in a wider and more diverse dataset, also including patients with cerebrovascular presentation, focal and so-called atypical FMD, that is, young to middle-aged women with dissections or aneurysms but without the typical string of beads, in the absence of inflammatory of genetic arteriopathy. It would also be of interest to see if our proteomic classifier is associated with spontaneous coronary artery dissection, with or without extracoronary FMD lesions.[39] Finally, it would be worthwhile to compare in a head-to-head study the relative performance of imaging biomarkers, the urinary proteomic biomarker identified in this study, and the proteogenomic signature reported by Olin et al[37] in the DEFINE-FMD study.

ARTICLE INFORMATION

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Disclosures

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