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Original Research Article

Identification and Characterization of New Proteins in Podocyte Dysfunction of Membranous Nephropathy by Proteomic Analysis of Renal Biopsy

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Abstract: Interstitial fluid, obtained by gentle centrifugation of the renal biopsy specimen, is highly enriched in elements directly secreted by the kidney tissue and is suitable for proteomic analysis. Here we describe the first clinical application of renal interstitial fluid analysis in a subset of samples obtained from patients affected by idiopathic membranous nephropathy.

We included in the study fifty-one patients with different pathologic diagnoses. We identified the proteomic pattern of idiopathic membranous nephropathy with mass spectrometry analysis by comparing these samples with two controls: normal kidney and IgA nephropathy. Proteomic results were validated by immunofluorescence analysis of renal tissues and Western blot of serum, urines and podocyte cell cultures.

We observed an increased expression of PDZ and LIM domain protein 5 (PDLI5) and LIM domain binding protein 3 (LDB3) providing first evidence of the differential expression of these LIM domain-related proteins in kidney and urines of patients with idiopathic membranous nephropathy.

Interstitial fluid can be considered a valuable biological fluid in the discovery phase of biomarkers. In order to validate its clinical use, it is pivotal to assess the availability of the biomarkers in 'usual' samples: blood and/or urine. PDLI5 and LDB3 share a common LIM domain suggesting a possible role in the cytoskeleton organization and they appear up-regulated in glomeruli of patients affected by idiopathic membranous nephropathy. Furthermore the two proteins become highly abundant in the urine of patients affected by idiopathic membranous nephropathy. In conclusion, our approach may be considered a novel method for identifying candidate biomarkers for patients suffering from membranous nephropathy and other glomerulonephrites.

Keywords: Membranous nephropathy, proteomics, podocyte, interstitial fluid, proteinuria, nephrotic syndrome.

INTRODUCTION

Idiopathic membranous nephropathy (iMN) is the most common glomerulonephritis and the first cause of nephrotic syndrome in the adult population. iMN can have an indolent and benign course, although about 30% of patients progress toward end stage renal failure (ESRF) within ten years [1-4]. The most likely pathogenesis of iMN is autoimmune and this hypothesis is corroborated by the observation of thickened glomerular basal membrane in histological preparations, as a consequence of subepithelial deposits of immune complexes. One of the first antigens reported to be involved in the human iMN in a rare neonatal condition is neutral endopeptidase [5, 6] (NEP), supporting the theory that podocytes act as a source of antigens for the *in situ* formation of subepithelial immune complex deposits. Recently Beck LH *et al.* identified new candidate autoantigens involved in the classic adult form of iMN: M-type phospholipase A2 receptor (PLA2R), a glycoprotein expressed by podocytes, colocalizes with IgG in the subepithelial immune complex deposits [7]. A second research group (Prunotto M *et al.*) found that IgG4 from iMN patients reacted toward aldose reductase (AR) and manganese superoxide dismutase (SOD2), thus adding two more targets to the MN antingen list [8], which has been hypothesized to be further extending [9-11].

Despite progress in the understanding of etiopathogenesis, the diagnosis of iMN is definitively established only by renal biopsy, which usually shows predominant IgG subepithelial deposits [12, 13] and thickening of the glomerular basal membranes. The pathological features and the clinical characteristics at presentation have a poor prognostic value. About 30% of patients may enter a spontaneous remission; other patients may benefit from an immunosuppressive

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regimen [14-19]. Both spontaneous remission and response to the immunosuppressive regimen is unpredictable [20].

This study aims at developing a new approach in the process of discovery of proteins involved in the pathogenesis of iMN by focusing on a biological sample, the renal interstitial fluid (IF), obtained from biopsy specimens [21]. This pilot study confirms that the application of our approach is successful in identifying proteins specifically up-regulated in the disease process and that the method is applicable to standard renal biopsy procedures without interfering with down-stream histological evaluation. The presence of a specific protein or peptide in the IF suggests its importance as a potential renal biomarker. Once novel biomarkers have been identified in IF, which differentiate among the various renal pathologies, their presence in more easily accessible samples such as urine and plasma can be tested. Indeed IF should be considered an invaluable biological fluid in the discovery phase of biomarkers, but in the process of validation for clinical use, it is pivotal to assess the availability of the biomarker in 'usual' samples: renal tissue, blood and/or urine.

In this study we identified the proteomic pattern of iMN with mass spectrometry analysis, comparing iMN IF samples with two control groups, normal kidney and patients with diagnoses other than iMN. The proteomic results were validated with immunofluorescence analysis on renal tissues and Western blot on serum, urines and human podocyte cell lines, providing the first evidence of the expression of LIM domain-related proteins in kidney and urines of iMN patients.

MATERIALS AND METHODS

Patients and Controls

Fifty-one patients were included in the study. We studied 38 patients, from the Nephrology Dialysis and Renal Transplantation Division, three urologic patients from the Urologic Division (Table **1**) and 10 healthy blood donors (HBD). After local Ethics Committee approval, informed consent was obtained from all participants.

Samples from 24 patients, who underwent kidney biopsy from January to June 2010, and 5 biopsies from one cadaveric donor kidney, were also used for proteomic analysis in IF. Six iMN, 6 IgA nephropathy (IgAN) and 3 control kidneys (from cadaveric kidney and from two patients with a normal pathologic diagnosis, who had undergone kidney biopsy for minimal urinary abnormalities) were used for immunofluorescence analysis. Samples from 10 iMN, 10 IgAN, 10 HBD and three urologic patients were tested by Western-blot analysis (Table **1**). Kidney biopsy proven diagnoses were: idiopathic membranous nephropathy (iMN, n=10), IgA nephropathy (IgAN, n=10), mesangio-proliferative nephropathy with IgM depositions (IgMN, n=3), amyloidosis (AMIL, n=1), membranous proliferative glomerulonephritis (MPGN, n=3), intra and extra-capillary glomerulonephritis (IECG, n=1), minimal change disease (MCD, n=1), focal segmental glomerulosclerosis (FSGS, n=2), acute tubular necrosis (ATN, n=1), chronic interstitial nephritis (CIN, n=1), benign nephroangiosclerosis (BNA, n=1), diabetic nephropathy (DN, n=1), normal kidney (NK, n=3). Bladder pathology evaluations were: papillary urothelial carcinoma (PUCa, n=3), one with low grade and two with high grade. Healthy blood donor (HBD, n=10) did not undergo renal biopsy, but provided sera and urines. Cases of secondary MN were excluded from the present study, in particular patients with cancer, connective tissue diseases and active infectious diseases. All the samples of kidney, serum and urine were collected at the time of biopsy and prior to any immunosuppressive treatment (Table **1**).

Kidney Biopsy and Interstitial Fluid Extraction

Biopsy and IF extraction were performed as previously described [21]. In brief, renal biopsy specimens were centrifuged at 3000g for 7 min in a column with glass fiber filter (Roche), rehydrated with physiologic solution and underwent standard histological procedures. Compared to our previous protocol [21], in order to increase the recovery of proteins from the centrifugation filter, it was washed twice with 50ul of ultrapure water and centrifuged 15 minutes at 13,000g. Centrifuged fractions were pooled and stored at -80°C.

Proteomic Analysis

Samples were thawed on ice, spun at 5000r.c.f. and the protein concentration, determined by a standard Bradford assay, were normalized with physiologic solution to a final concentration of 0.25ug/ul (1.2ug tot), treated with 5ul of ammonium bicarbonate (AMBIC) 100mM, reduced with Dithiothreitol (DTT 10mM, 1ul in AMBIC 100mM) at 56°C for 30min and alkylated with 4-vinyl pyridine (55mM, 1ul in AMBIC 100mM) at room temperature in the dark for 1h. The resulting protein mixtures were digested with TPCK-modified sequencing grade trypsin (final ratio of enzyme to substrate 1:50 w/w) at 37°C overnight. Samples were then acidified with 1ul 5% formic acid (FA) solution and dried in a vacuum evaporator. Peptides were resuspended in 30ul of 1% FA/acetonitrile 98:2 solution. Analyses were performed by ESI-Q-TOF Accurate-Mass G6520AA (Agilent Technologies), controlled by MassHunter (v. B.02.00) and interfaced by a CHIP-cube to an Agilent 1200 nano-pump. Chromatographic separation was performed on a high capacity loading chip, with a 150mm, 300Å, C18 column. Each biological replicate was run twice; analytical controls (a mix of baker's yeast enolase and bovine serum albumin tryptic digests) were run daily to monitor chromatographic performances. Resulting mzData.xml raw data were searched against human SwissProt database with Mascot v. 2.3: search results were re-scored with Mascot Percolator [22] percolator score $\geq$ 50, at least two significantly scoring peptides/protein at 1% false discovery rate (detailed MIAPE-compliant methods are provided as Supplemental Methods).

Label-free Relative Quantification

Exported re-scored Mascot search results were cross-related to the corresponding MS1 profiles (mzXML format, converted from the vendor's raw data by trapper, http://sourceforge.net/projects/sashimi/files/trapper (MassHunter converter) by the quantitative software Ideal-Q [23], v. 1.024;

Table 1. Clinical characteristics of patients.

Patient	Pathologic Diagnosis	Gender	Tx Kidney	Age at diagnosis	SCr (mg/dl)	Pr (g/d)	Alb (g/dl)	Micro Hae	Hb (g/dl)	SBP (mmHg)	DBP (mmHg)	Body Weight (kg)	Height (cm)	Race	HbsAg	HIV	HCV	ACE/ARB	Diabetes
1	iMN	F	N	29	0,5	2,3	3,35	Y	12,1	125	75	98	168	Cauc	N	N	N	N	N
2	iMN	M	N	62	2,0	10,0	2,4	Y	13,9	192	97	74	175	Cauc	N	N	N	Y	Y
3	iMN	M	N	59	0,9	4,0	2,7	Y	15.7	130	80	98	182	Cauc	N	N	N	N	N
4	iMN	F	N	39	0,8	4,9	3,29	Y	13,6	118	76	66	162	Cauc	N	N	N	Y	N
5	iMN	F	N	85	1,0	11,5	2,3	Y	10,1	130	63	63	170	Cauc	N	N	N	Y	N
6	iMN	M	N	38	0,7	5,0	3,9	N	13,0	150	80	80	175	Cauc	N	N	N	N	N
7	iMN	M	N	43	1.15	4.6	1.6	N	11	135	80	107	170	Cauc	N	N	N	Y	Y
8	iMN	M	N	35	0.93	5	2.04	N	14.1	130	70	93	188	Cauc	N	N	N	N	N
9	iMN	M	N	34	0.81	1.3	4	Y	12.8	125	80	90	168	Cauc	N	N	N	N	N
10	iMN	F	N	47	0.6	10	2.5	N	11	130	75	83	164	Cauc	N	N	N	N	N
11	IgAN	M	N	41	3,2	9,1	2,9	Y	8,5	140	80	103	180	Cauc	N	Y	Y	Y	N
12	IgAN	M	N	36	1,0	0,1	4,3	Y	15,2	140	80	90	183	Cauc	N	N	N	Y	N
13	IgAN	M	N	41	0,5	0,0	3,9	Y	13,9	108	56	53	163	Cauc	N	N	N	Y	N
14	IgAN	M	N	42	0.6	0.4	4	Y	14.7	120	70	61	168	Cauc	N	N	N	N	N
15	IgAN	F	N	28	1	0.5	3	Y	12.7	110	78	64	160	Cauc	N	N	N	Y	N
16	IgAN	M	N	20	0.94	0.3	4.2	Y	13.9	123	65	62	182	Cauc	N	N	N	N	N
17	IgAN	M	N	65	1	0	4	Y	13.2	120	80	74	174	Cauc	N	N	N	N	N
18	IgAN	M	N	22	0.87	0.6	3.5	Y	15.4	130	70	70	172	Cauc	N	N	N	Y	N
19	IgAN	M	N	43	1.2	0.75	3	Y	17.3	120	70	92	170	Cauc	N	N	N	N	N
20	IgAN	M	N	33	1.4	0	4.1	Y	15.1	126	66	88	183	Cauc	N	N	N	N	N
21	IgMN	M	N	22	0,9	0,5	3,8	N	15,3	137	90	64	170	Cauc	N	N	N	Y	N
22	IgMN	M	N	56	0,7	6,1	3,26	Y	15,4	114	58	65	155	Cauc	N	N	N	N	N
23	IgMN	F	N	36	0,8	1,3	4	N	13,7	139	93	120	160	Cauc	N	N	N	N	N
24	AMIL	F	N	50	0,6	3,0	3,1	Y	12,5	131	86	68	165	Cauc	N	N	N	N	N
25	MPGN	F	N	77	1,9	0,6	NA	N	9,6	140	80	59	152	Cauc	N	N	N	N	N
26	MPGN	F	N	74	1,0	1,9	2,6	Y	11,2	168	83	58	163	Cauc	N	N	Y	N	N
27	MPGN	F	N	42	0,8	1,4	2,4	Y	12,8	106	69	56	160	Cauc	N	N	N	N	N
28	IECG	M	N	85	4,5	6,3	2,4	Y	9,3	150	60	70	160	Cauc	N	N	N	N	Y
29	MCD	M	N	21	0,7	6,8	1,8	N	13,1	129	80	75	170	Cauc	N	N	N	Y	N
30	FSGS	F	N	25	0,7	5,4	2,5	N	14,1	110	70	56	165	Cauc	N	N	N	N	N
31	FSGS	F	Y	44	1,7	8,2	3,72	Y	11,2	142	89	63	173	Cauc	N	N	N	N	N
32	ATN	M	N	61	9,0	13,0	3,3	N	8,8	156	77	88	174	Cauc	N	N	N	N	N
33	CIN	F	N	48	0,7	0,2	4,02	Y	13,9	130	77	92	165	Cauc	N	N	N	Y	N
34	BNA	F	N	60	0,8	0,5	3,75	Y	11,7	149	87	72	160	Cauc	N	N	N	N	N

Table 1. contd….

Patient	Pathologic Diagnosis	Gender	Tx Kidney	Age at diagnosis	SCr (mg/dl)	Pr (g/d)	Alb (g/dl)	Micro Hae	Hb (g/dl)	SBP (mmHg)	DBP (mmHg)	Body Weight (kg)	Height (cm)	Race	HbsAg	HIV	HCV	ACE/ARB	Diabetes
35	DN	M	N	39	0,8	0,3	4,3	N	12,9	120	80	58	173	African	N	N	N	N	Y
36	NK	F	N	84	1.1	0	4.1	N	11.5	130	70	75	160	Cauc	N	N	N	N	N
37	NK	F	N	19	0.6	0	4.2	Y	12	117	63	75	175	Cauc	N	N	N	N	N
38	NK	M	N	44	1	0	4	Y	14.5	117	75	80	185	Cauc	N	N	N	N	N
39	*PUCa low grade	M	N	74	1.04	4	4.02	Y	12.2	140	95	90	170	Cauc	N	N	N	Y	Y
40	*PUCa high grade	M	N	89	1.88	3.2	3.8	Y	10.4	150	80	91	165	Cauc	N	N	N	Y	Y
41	*PUCa high grade	M	N	70	1.12	3	3.03	Y	12.6	140	90	110	180	Cauc	N	N	N	N	Y
42	HBD	M	N	37	1.1	0.02	4	N	14.4	130	80	89	185	Cauc	N	N	N	N	N
43	HBD	M	N	25	0.8	0	4.1	N	15	135	80	90	192	Cauc	N	N	N	N	N
44	HBD	F	N	23	0.9	0	3.8	N	11.8	90	60	58	170	African	N	N	N	N	N
45	HBD	M	N	20	0.8	0	4	N	13.8	110	75	78	180	Cauc	N	N	N	N	N
46	HBD	F	N	42	0.9	0	4.4	N	12.1	95	60	55	170	Cauc	N	N	N	N	N
47	HBD	F	N	40	1	0	4.3	N	11	100	60	52	158	Cauc	N	N	N	N	N
48	HBD	F	N	32	0.9	0.1	4	N	10.5	100	70	60	160	Cauc	N	N	N	N	N
49	HBD	M	N	27	1	0	4	N	14.8	90	60	65	170	Cauc	N	N	N	N	N
50	HBD	M	N	30	1.1	0	4	N	13.5	107	68	63	180	Cauc	N	N	N	N	N
51	HBD	M	N	31	1	0	4.1	N	15	100	80	78	175	Cauc	N	N	N	N	N

Legend. Biopsy pathologic diagnosis (*Bladder pathology evaluation), gender, patients age at diagnosis, serum creatinine (SCr), proteinuria (Pr), albuminemia (Alb), hemoglobin (Hb), systolic blood pressure (SBP), diastolic blood pressure (DBP), body weight, height, race (Caucasian=Cauc). Presence or absence (Yes=Y, No=N) of: kidney transplant (Tx Kidney), microhaematuria (Micro Hae), HbsAg, Anti-HIV, Anti-HCV, ACE/ARB, Diabetes. Biopsy pathologic diagnosis: idiopathic membranous nephropathy (iMN), IgA nephropathy (IgAN), mesangioproliferative nephropathy with IgM depositions (IgMN), amyloidosis (AMIL), membranous proliferative glomerulonephritis (MPGN), intra and extra-capillary glomerulonephritis (IECG), minimal change disease (MCD), focal segmental glomerulosclerosis (FSGS), acute tubular necrosis (ATN), chronic interstitial nephritis (CIN), benign nephroangiosclerosis (BNA), diabetic nephropathy (DN), normal kidney (NK), papillary urothelial carcinoma (PUCa). Healthy blood donor (HBD) did not underwent biopsy.

pooled search iMGN unique hits were manually validated and quantified on the moverz/RT 2D map. Peptide raw XIC data were exported and elaborated through the DanteR tool (http://omic.pnl.gov/software/DanteR.php) [24]; briefly, peak areas were Log2 transformed, normalised (central tendency), then corresponding protein intensities were obtained from the peptides through an analysis of variance (p-value<0.05, $Log_2 0.5$< protein ratio >$Log_2 2$) and the inferred values were evaluated for statistical significance of differentially expressed proteins throughout the groups under study [24].

The approach was validated on a group of 5 biopsy specimens from the same normal kidney; retention time reproducibility was evaluated by linear correlation of IDEAL-Q corrected elution times over the whole dataset (R2= 0.999), with an eXtracted Ion Current (XIC) CV of 20%. False discovery rate was estimated through a concatenated decoy database search and was lower than 1% in all the search results. Percolated hits were visualised by protein family grouping and exported as XML or CSV with MudPIT scoring, reporting only unique peptide hits and the highest ranked proteins of each family.

Antibodies

Primary: Mouse monoclonal anti-human LIM domain binding protein 3 antibody (LDB3); mouse monoclonal anti-human PDZ and LIM domain protein 5 antibody (PDLI5); and mouse monoclonal anti-human Synaptopodin antibody were provided by Abnova (Abnova Corporation, Taipei, Taiwan). Protein A purified rabbit polyclonal anti-human Four and half LIM domains 1 antibody (FHL-1) and a mouse monoclonal IgG1 anti-human glyceraldehyde-3-phosphate dehydrogenase antibody (GAPDH) were provided by Santa Cruz (Santa Cruz Biotechnology Inc., Santa Cruz CA, USA).

Secondary: Goat anti-mouse IgG2a-Alexa 488 conjugate; goat anti-mouse IgG1-Alexa 568 conjugate; goat anti-mouse IgG2b-Alexa 568 conjugate; goat anti-mouse IgG2a-Alexa 633 conjugate; goat anti-mouse IgG2b-Alexa 633 conjugate were all purchased from Invitrogen (Paisley, Scotland, UK). Donkey anti-rabbit Ig-FITC conjugate and sheep anti-mouse HRP conjugate were from GE Healthcare (Little Chalfont, Buckinghamshire, UK). Goat anti-rabbit IgG-Rhodamine conjugate and goat anti-rabbit IgG-HRP conjugated were

provided by Santa Cruz (Santa Cruz Biotechnology Inc., Santa Cruz CA, USA). DAPI Vectashield® Mounting Medium was provided by Vector Laboratories (Vector Laboratories, Inc., Burlingame, CA, USA).

Immunofluorescence

Tissue samples for immunofluorescence staining were: six iMN, six IgAN and three control kidneys. Renal biopsy specimens were stored in a bio-bank at -80°C. Three-micrometer frozen kidney samples were cut by a cryostat, fixed with Acetone, permeabilized with Triton 0.1%, coated with 3% BSA for 1h at 20°C. Primary antibody (1:20) was incubated for 1h at 37°C; the secondary antibody (1:1000) was later incubated for 1h at 20°C. Finally slides were closed with DAPI Vectashield® Mounting Medium. Biopsy samples were examined by immunofluorescence microscopy (Olympus BX41 Microscopy). Laser Confocal images (double-triple and co-localization staining) were obtained with Leica TCS SP2 (Leica Microsystems, Heidelberg, GmbH). Images of the glomerular area and labeled sections were traced digitally and intensity levels were analyzed with Leica Confocal Software v2.61 (Leica Microsystems Heidelberg, GmbH). Leica images were elaborated drawing a region of interest (ROI) around every single glomerulus in each slide; glomerular areas were normalized by background subtraction (using whole frame of image). Finally statistical data were analyzed using one way ANOVA.

Western Blot of Serum, Urine and Podocyte Extracts

Serum separation [21], urine samples preparation [25] and protein concentration assay [25] were performed as previously described. Analysis of protein expression in serum and urine of 10 iMN, 10 IgAN, 10 HBD and three urologic patients was performed by Western blot assay [25] with GAPDH loading control. Western blotting was assessed also on human conditionally immortalized podocyte cell lines [26] under normal and oxidating conditions [8]. Urine protein extracts of patients and controls were normalized to 0.8ug per loaded sample in order to refer to relative abundance instead of the whole proteinuria. In brief, each sample was loaded onto a 7.5% SDS-PAGE and then transferred to nitrocellulose membrane. After blocking for 1h at 20°C in a blocking solution, the membranes were incubated overnight at 4°C with the primary antibody (1:200) in blocking solution. Then the membranes were incubated with secondary antibody conjugated with horseradish peroxidase (1:2000) for 2h at 20°C. The signal was detected by NocexECL HRP Chemiluminescent Substrate Reagent Kit (Invitrogen, Paisley, Scotland, UK). Mini-PROTEAN Tetra Cell was used to perform 3 gel runs at the same time (one for iMN patients, one for IgAN patients and one for controls). Detailed information for serum and urine preparation, cell culture of podocyte and Western blot are described in Supplemental Methods.

RESULTS

Proteomic Analysis

Subjects (24 patients enrolled in the study and 5 biopsies from the cadaveric donor kidney as controls) were divided into three clinical classes for subsequent analyses: A) iMN patients (**1**-**6**, Table **1**); B) normal kidney (**38**, Table **1**) and C) patients with a pathological diagnosis other than iMN (**11**-**13** and **21**-**35**, Table **1**). The renal biopsies from patients were processed to obtain the IF samples according to a procedure already published by our group [21]. Samples were digested and analyzed through liquid chromatography-mass spectrometry to examine IF specific proteins. Two independent runs were performed for each sample. The analysis of variance of the A vs B + C groups showed a set of statistically significant differentially expressed candidate proteins (Supplementary Table **1**). We focused our attention on a group of proteins integrating a LIM domain and functionally related, which were significantly over-expressed in this dataset (Table **2**). The members of this class of proteins which we validated are FHL1 (Four and a half LIM domains protein 1), LDB3 (LIM Domain-Binding protein 3, also known as Cypher or ZASP) and PDLI5 (PDZ and LIM domain 5 protein). LIM-domain proteins have never been correlated to renal diseases until now.

Immunofluorescence Analysis

We performed immunofluorescence analysis of tissue cryosections in order to confirm the results of proteomic analysis. We investigated the expression of FHL1, LDB3 and PDLI5 in renal tissue of three controls, six IgAN and six iMN. We found that LDB3 and PDLI5 are strongly and specifically expressed in glomeruli of all iMN patients, while in IgAN patients both proteins are very weakly and focally expressed and completely absent in renal tissue from controls (Fig. **1**). FHL1 is weakly and focally expressed in iMN patients, while it is not expressed in IgAN and controls.

Semi-quantitative analysis of FHL1, LDB3 and PDLI5 expression was assessed through confocal morphometric evaluation of iMN and IgAN vs control patients. Glomerular expression of PDLI5 and LDB3 among iMN, IgAN patients

Table 2. LIM Domain Proteins Over-expressed in Our Dataset

UniProt AC	PEP	Description	n.peptides	Sequence Coverage (%)	iMN vs. B+C Ratio (Log_2)	P value
PDLI5_HUMAN	92	PDZ and LIM domain protein 5	2	6	2.1	$2 \cdot 10^{-3}$
LDB3_HUMAN	68	LIM domain-binding protein3	2	10	7	$1 \cdot 10^{-6}$
FHL1_HUMAN	52	Four and a half LIM domains protein 1	2	10	0.07	0.04

Legend. iMN differentially expressed LIM -domain proteins, sorted for decreasing identification confidence (percolator post error probabilities, PEP).

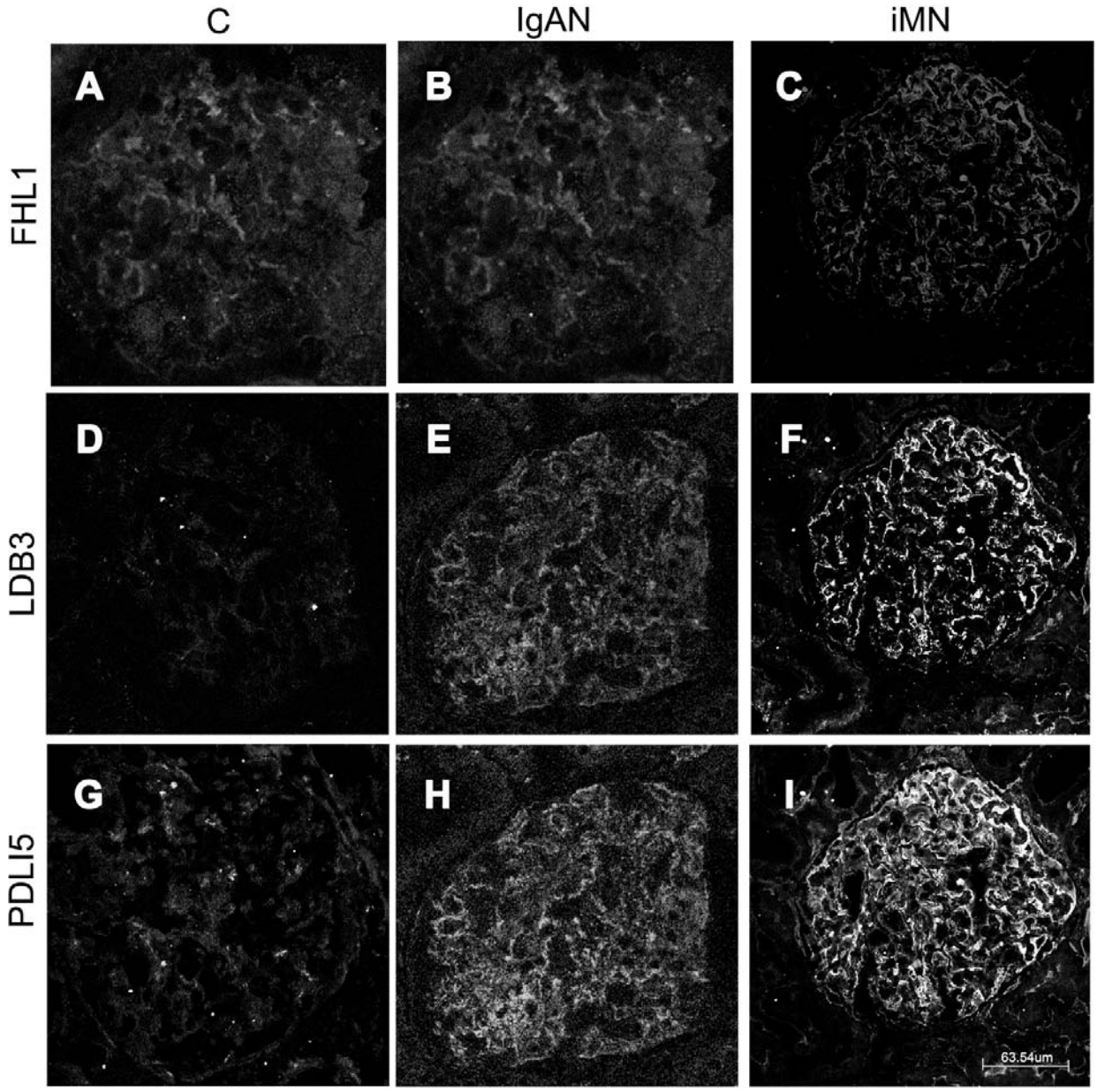

Fig. (1). FHL1, LDB3 and PDLI5 expression by immunofluorescence analysis. LDB3 and PDLI5 are high expressed, while FHL1 is focally expressed in renal biopsies of iMN patients. Images A through C are stained for FHL1 (A from control, B from IgAN and C from iMN). D through F are stained for LDB3 (D control, E from IgAN and F from iMN). G through I stained for PDLI5 (G from control, H from IgAN and I from iMN). FHL1, LDB3 and PDLI5 staining are absent in renal biopsies from control tissue (A, D, G) and are very weakly expressed in IgAN (B, E, H). FHL1 is focally expressed in renal biopsies of iMN patients (C, L), LDB3 and PDLI5 and highly expressed in glomerular region of the same patients (F, I).

and control subjects was significantly different, as judged by Kruskal Wallis analysis (Fig. **2**). PDLI5: χ^2=25.890 with 2 d.f. P <0.001; LDB3: χ^2=24.713 with 2 d.f. P <0.001. PDLI5 is respectively more expressed in iMN than IgAN patients and both groups show higher expression than control patients. LDB3 is more highly expressed in iMN patients and IgAN patients than in control subjects, but there is no significant difference between iMN and IgAN patients for LDB3 expression.

The localization of the two proteins was defined by confocal microscopy: LDB3 and PDLI5 are expressed in podocyte cells, as confirmed in images at high magnification (Fig. **3**: **D, H**). The podocyte localization has been confirmed by Synaptopodin costaining (Fig. **3**).

Western Blot Analysis of Serum, Urine and Podocyte Cell Lines

To evaluate the potential of these candidates as clinical biomarkers, we analyzed the expression of FHL1, LDB3 and PDLI5 in serum and urine of 10 iMN (1-10, Table **1**), 10 IgAN patients (11-20, Table **1**) and 10 HBD (42-51, Table **1**). LDB3 and PDLI5 are expressed in sera of analyzed patients

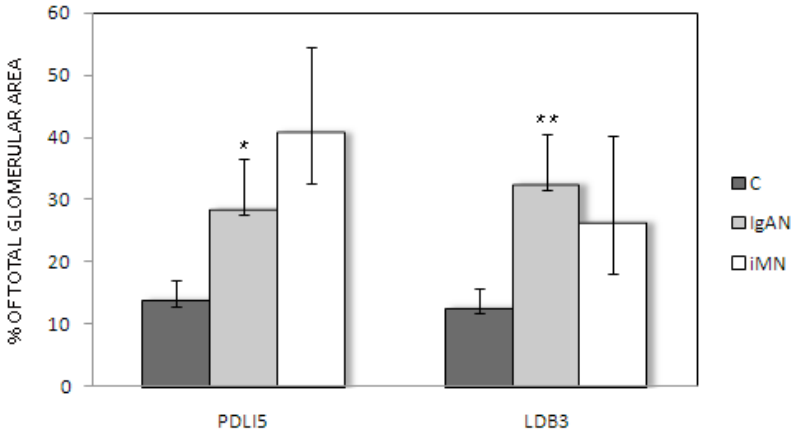

Fig. (2). Morphometric analysis. Morphometric semi-quantitative analysis of PDLI5 and LBD3 expression in glomerular region of iMN and IgAN patients vs controls. Results are expressed as percentage of the positive area for each antibody on total glomerular area. We analized all glomeruli on each slide. Results have high statistical significance for iMN and IgAN vs controls (*PDLI5: P <0.001; **LDB3: P<0.001).

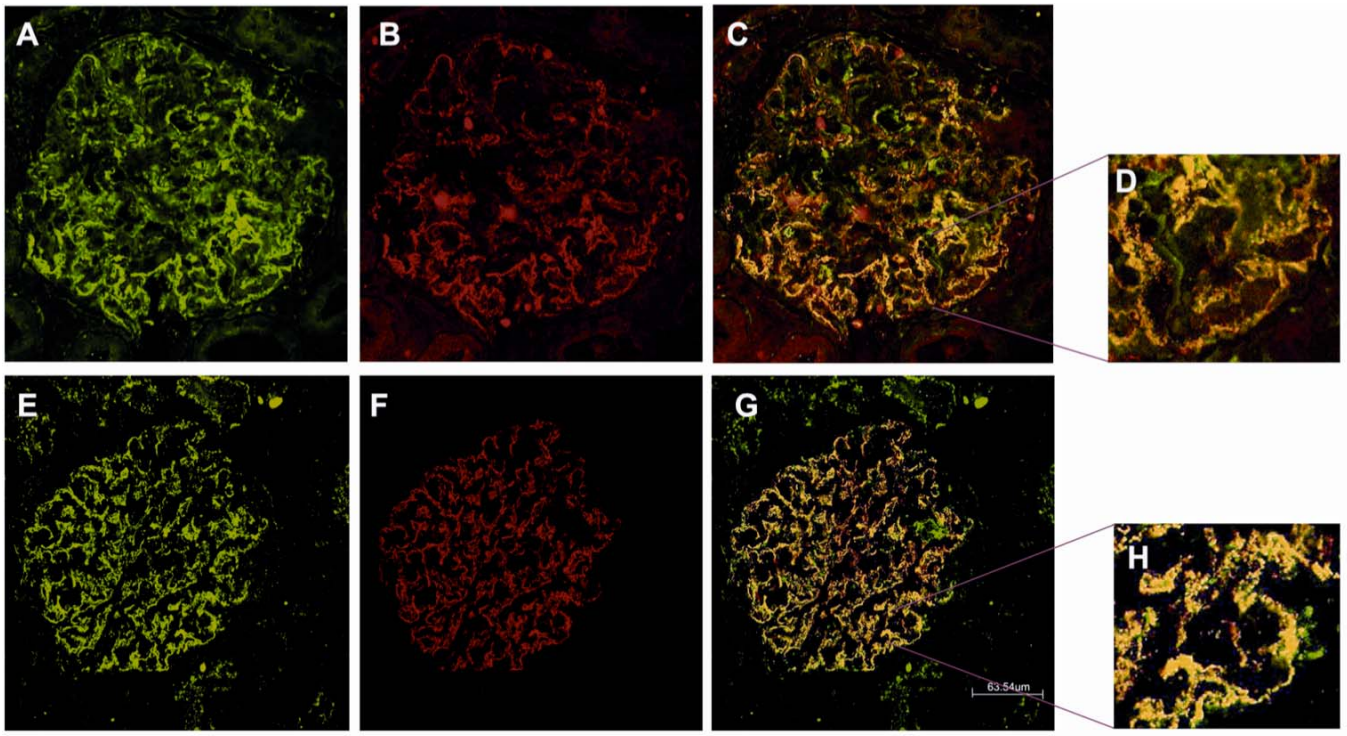

Fig. (3). LDB3 and PDLI5 expression in podocyte cells. LDB3, PDLI5 and Synaptopodin confocal laser scanning image of iMN patients. LDB3 (A) and Synaptopodin (B) do co-localize; merge image is reported (C). Both PDLI5 (E) and Synaptopodin (F) do colocalize and merge image is reported (G). High magnification merge images are reported: LDB3-Synaptopodin (D); PDLI5-Synaptopodin (H).

and controls (Fig. **4A**). They are completely absent in the urine of control subjects, while they are present in that of the IgAN group and highly abundant in the urine of iMN patients (Fig. **4B**). Western blot could not detect the presence of FHL1 in urine or serum (not shown).

Urine from three patients with urinary abnormality not related to glomerular disease were tested by Western blot analysis to confirm the hypothesis of a podocytic origin of PDLI5 and LDB3. All three patients were diagnosed for papillary urothelial carcinoma and their micro-haematuria and proteinuria did not have renal origin (Clinical details is provided in Table **1**). Western blot did not show the existence of any specific bands of LDB3 and PDLI5 in these patients with urinary abnormalities related to urothelial carcinoma (Fig. **4C**).

The podocyte expression of the proteins was further evaluated by Western blot on human podocyte cell lines extracts confirming the expression of LDB3 and PDLI5 in these cells (Fig. **4D**), while the expression of FHL1 was absent. Electrophoresis shows the existence of a band of

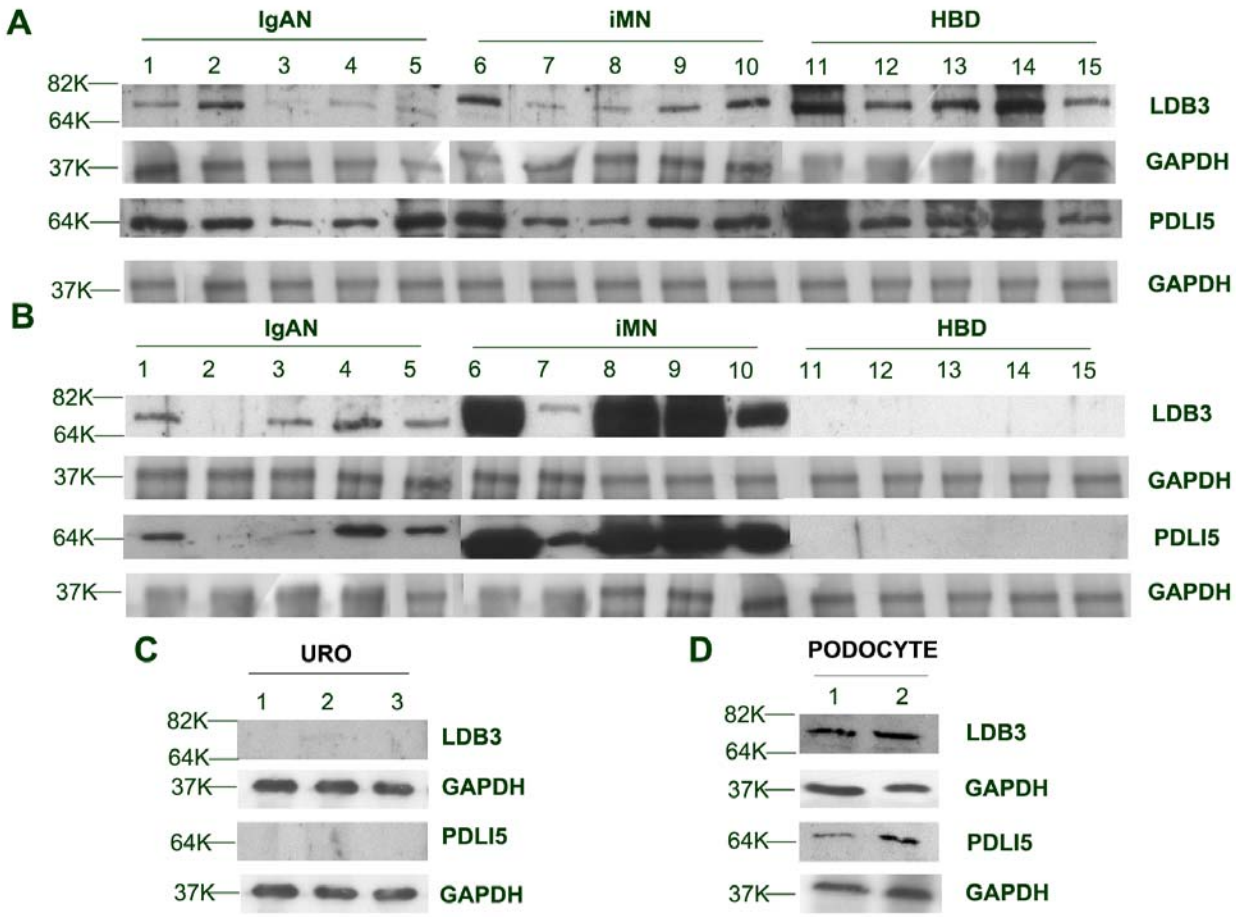

Fig. (4). LDB3 and PDLI5 Western blot of serum, urine and podocyte cell lines. Western Blot with LDB3 (77.1 KDa), PDLI5 (63.9 KDa) and GAPDH loading control antibodies of serum, urine and podocyte cell lines. A) LDB3 and PDLI5 expression in sera. Lane 1-5 are IgAN patients; lane 6-10 are iMN; lane 11-15 are controls, HBD. LDB3 and PDLI5 are present in sera of analyzed patients and controls. B) LDB3 and PDLI5 in urines. Lane 1-5 are IgAN; lane 6-10 are iMN; lane 11-15 are controls, HBD. LDB3 and PDLI5 are completely absent in the urine of control subjects, while they are present in the urine of IgAN patients and they are highly abundant in the urine of iMN patients. C) Western Blot of urine of three urologic patients. LDB3 and PDLI5 are completely absent in the urine of this patients. D) Western Blot of podocyte cell lines extracts in normal (1) and oxidating conditions (2) showing bands of LDB3 and PDLI5. Note that PDLI5 is more expressed in cells under oxydating condition.

about 63.9 kDa for PDLI5 and about 77.1 kDa for LDB3. We did not find any difference in the expression of LDB3 in stressed podocyte cell lines, whereas PDLI5 was strongly overexpressed. This oxidative stress condition resembles the state of podocyte in membranous nephropathy (Fig. **4D**).

DISCUSSION

Based on our comparative proteomic analysis we obtained a list of proteins significantly expressed in iMN patients. In this study, we focused our attention on a group of them showing a common structural feature - the LIM domain - which is well represented among differentially expressed proteins of our dataset, namely FHL1, LDB3 and PDLI5.

Unlike other published studies [27], which required extensive tissue isolation and pre-fractionation procedures, in the present approach we were able to determine some iMN preferentially expressed proteins through a straightforward high-throughput proteomics approach. The key step of our strategy was a new method for obtaining a biological fluid from renal tissue, as previously described [21]. The procedure to obtain the fluid is simple and easily applicable to standard renal biopsy procedures. Although our approach has many advantages compared to other strategies, IF analysis may have some methodological weaknesses: in consideration of the experimental protocol there is the risk of IF contamination by non renal proteins and in particular from plasma proteins. This possibility has been explored in our previous article [21] in which we have effectively described the presence of plasma protein (transthyretin and haptoglobin). However, for the most part, IF overall protein composition is remarkably different from that of serum. Furthermore, serum proteins do not have a large impact on the final results; they are shared between disease and control samples and, therefore, were excluded from the candidate list after the statistical elaboration of data.

FHL1 is the most widely expressed member of the FHL family of proteins, consisting of four and a half highly conserved LIM domains. It is strongly expressed in skeletal muscle and to a lesser extent in heart; expression has never

been reported in the kidney until now [28]. A multifunctional and integral role for FHL1 has been implicated in muscle development, structural maintenance and signaling. It contains four LIM domains and a novel single zinc finger domain in the N-terminal region [29-31].

We could not confirm FHL1 as a specific iMN marker, as proteomic analysis preliminarily suggested: it is weakly expressed in glomeruli of iMN patients. Neither Western blot indicated a specific band in serum, urine or podocytic lines.

LDB3 is a member of the ALP (alpha-actinin-associated LIM protein) and enigma family and contains three LIM domains and one PDZ domain. LDB3 interacts *via* LIM domain with several phosphocreatine kinase (PKC) isoforms and *via* PDZ domain with alpha actinin [32, 33] (ACTN). It is expressed primarily in skeletal muscle and to a lesser extent in heart; it is also detectable in brain and placenta. LDB3 mRNA and protein are present in several cell lines and dog kidney [34], but no expression has been reported in human kidney so far. It may function as an adapter in cytoskeletal assembly, in the targeting and clustering of membrane proteins. It colocalizes with ACTN at sites of actin anchorage, such as the intercalated discs of cardiac muscle cells, since mutations in LDB3 can produce various cardiomyopathy.

PDLI5 is a member of the enigma family. It contains three LIM domains interacting with various PKC isoforms and one PDZ domain interacting with actin and alpha actinin. It is specifically expressed in heart and skeletal muscle [35], but no expression has been previously reported in human kidney. It is involved in heart development, regulation of cardiomyocyte expansion, heart hypertrophy, regulation of dendritic spine morphogenesis in neurons and postsynaptic growth. Its expression is commonly increased in the brain of patients with bipolar disorder, schizophrenia, and major depression [36, 37]. The functional LIM domain is involved in cytoskeleton organization by interaction with actin through the mediation of alpha-actinin. Due to interactions with the cytoskeleton, cytoplasmatic LIM-domain proteins are also involved in many cellular physiological activities, such as cell shape modulation, cell motility and integrin-dependent adhesion and signaling [38].

We found a strong expression of LDB3 and PDLI5 in glomeruli of all iMN patients, also confirmed by semi-quantitative analysis in confocal microscopy. In the glomeruli of IgAN patients LDB3 is expressed in a similar fashion to patients affected by iMN, but PDLI5 is more highly expressed in iMN glomeruli than in IgAN glomeruli suggesting PDLI5 as the most specific marker for iMN. Both of the two proteins are weakly expressed or completely absent in controls. Western blot analysis on urine further confirms these results. These proteins are detectable in sera of analyzed patients and controls without significant differences between them. A noteworthy feature of these markers is their specific increase in the urine of the iMN patients suggesting their release from podocytes in the urinary space. We can exclude that the presence of these proteins in urine could derive from urinary cellular debris of podocytes because our preparative method includes a preliminary centrifugation step of the sample.

By using immunofluorescence technique we found a co-localization of these two proteins with Synaptopodin confirming their podocyte expression, supported also by Western blot analysis of human podocyte cell lines in normal and stressed condition. Notably, PDLI5 expression levels are higher in peroxide stressed cells, a condition miming the oxidative stress on podocytes in iMN patients.

LDB3 and PDLI5 have never been described in the kidney compartment so far and our report is the first description of the presence of these components in the podocyte cytoskeleton. Our preliminary findings could be consistent with the hypothesis, recently reviewed by Mundel P and Reiser J [39], regarding the transition to a motile podocyte foot process state in response to external insults. The typical expression of these LIM domain-containing proteins by cells with a muscular phenotype could be related to the actin cytoskeleton plasticity [40, 38], also described in the proteinuric dysfunctional state of the podocyte.

In conclusion, the main contribution of this study is the identification of a protein family (LIM proteins) specifically expressed in podocyte of glomeruli from iMN patients; these biomarkers are easily detectable in organic fluids, thus encouraging the prospect of a minimally invasive diagnostic procedure.

FUTURE DIRECTIONS

Our data suggest that LDB3 and PDLI5 are overexpressed in iMN. These preliminary data will require confirmation in a larger number of samples. For this reason, in the future, we are considering the need to repeat the morphometric semi-quantification of these proteins in the glomerulus of a large number of patients affected by iMN and other glomerulonephrites other than iMN. At the same time our data suggest that these proteins are present in the urine of the affected patients. For this reason the development of an assay that permits a quick and convenient measurement in the urine is one of our priorities. In particular, the development of an ELISA will improve our understanding of the role of these markers in a larger population of nephrotic patients. If the role of this family of LIM proteins is confirmed in iMN and nephrotic patients, the identification of molecules able to stabilize the cytoskeleton of the podocytes interacting with this system could lead to the development of novel agents with antiproteinuric activity.

CONCLUSIONS

Interstitial fluid can be considered a valuable biological fluid in the discovery phase of biomarkers. We identified the proteomic pattern of idiopathic membranous nephropathy on IF with mass spectrometry analysis and these results were validated by immunofluorescence analysis on renal tissues and Western blot of serum, urines and podocyte cell cultures. We observed an increased expression of PDLI5 and LDB3 providing the first evidence of the differential expression of these LIM domain-related proteins in kidney and urines of patients with idiopathic membranous nephropathy. In conclusion, our approach may be a method for identifying candidate biomarkers for patients suffering from membranous nephropathy and other glomerulonephrites.

CONFLICT OF INTERESTS

None of the authors have potential conflict of interest. None declared/applicable.

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SUPPLEMENTARY MATERIAL

Supplementary material is available on the publisher's web site along with the published article.

ABBREVIATIONS

ACTN	=	alpha actinin
ALP	=	alpha-actinin-associated LIM protein
AMBIC	=	ammonium bicarbonate
AMIL	=	amyloidosis
AR	=	aldose reductase
ATN	=	acute tubular necrosis
BNA	=	benign nephroangiosclerosis
CIN	=	chronic interstitial nephritis
DN	=	diabetic nephropathy
DTT	=	Dithiothreitol
ELISA	=	Enzyme-Linked ImmunoSorbent Assay
ESI-QTOF	=	Elettrospray Quadrupole Time-of-Flight
ESRF	=	end stage renal failure
FA	=	formic acid
FHL1	=	Four and a half LIM domains protein 1
FSGS	=	focal segmental glomerulosclerosis
GAPDH	=	glyceraldehyde-3-phosphate dehydrogenase
HBD	=	healthy blood donor
IECG	=	intra and extra-capillary glomerulonephritis
IF	=	interstitial fluid
IgAN	=	IgA nephropathy
IgMN	=	mesangioproliferative nephropathy with IgM depositions
iMN	=	idiopathic membranous nephropathy
LDB3	=	LIM Domain-Binding protein 3
MCD	=	minimal change disease
MPGN	=	membranous proliferative glomerulonephritis
NEP	=	neutral endopeptidase
NK	=	normal kidney
PDLI5	=	PDZ and LIM domain 5 protein
PKC	=	phosphocreatine kinase
PLA2R	=	M-type phospholipase A2 receptor
PUCa	=	papillary urothelial carcinoma
SDS-PAGE	=	Sodium Dodecyl Sulphate - PolyAcrylamide Gel Electrophoresis
SOD2	=	manganese superoxide dismutase
TPCK	=	Trypsin treated with L-1-tosylamido-2-phenylethyl chloromethyl ketone
XIC	=	eXtracted Ion Current

REFERENCES

[1] Honkanen E, Tornroth T, Gronhagen-Riska C. Natural history, clinical course and morphological evolution of membranous nephropathy. Nephrol Dial Transplant 1992; 7(Suppl 1): 35-41.

[2] Davison AM, Cameron JS, Kerr DN, *et al.* The natural history of renal function in untreated idiopathic membranous glomerulonephritis in adults. Clin Nephrol 1984; 22: 61-7.

[3] Cattran DC. Idiopathic membranous glomerulonephritis. Kidney Int 2001; 59: 1983-94.

[4] Zucchelli P, Ponticelli C, Cagnoli L, *et al.* Long-term outcome of idiopathic membranous nephropathy with nephrotic syndrome. Nephrol Dial Transplant 1987; 2: 73-8.

[5] Debiec H, Guigonis V, Mougenot B, *et al.* Antenatal membranous glomerulonephritis due to anti-neutral endopeptidase antibodies. N Engl J Med 2002; 346: 2053-60.

[6] Debiec H, Nauta J, Coulet F, *et al.* Role of truncating mutations in MME gene in fetomaternal alloimmunisation and antenatal glomerulopathies. Lancet 2004; 364: 1252-9.

[7] Beck LH Jr., Bonegio RG, Lambeau G, *et al.* M-type phospholipase A2 receptor as target antigen in idiopathic membranous nephropathy. N Engl J Med 2009; 361: 11-21.

[8] Prunotto M, Carnevali ML, Candiano G, *et al.* Autoimmunity in membranous nephropathy targets aldose reductase and SOD2. J Am Soc Nephrol 2010; 21: 507-19.

[9] Ronco P, Debiec H. Advances in membranous nephropathy: success stories of a long journey. Clin Exp Pharmacol Physiol 2011; 38: 410-6.

[10] Bruschi M, Carnevali ML, Murtas C, *et al.* Direct characterization of target podocyte antigens and auto-antibodies in human membranous glomerulonephritis: Alfa-enolase and borderline antigens. J Proteomics 2011; 74(10): 2008-17.

[11] Cavazzini F, Magistroni R, Furci L, *et al*. Identification and characterization of a new autoimmune protein in membranous nephropathy by immunoscreening of a renal cDNA library. PLoS One. 2012; 7(11): e48845

[12] Imai H, Hamai K, Komatsuda A, *et al.* IgG subclasses in patients with membranoproliferative glomerulonephritis, membranous nephropathy, and lupus nephritis. Kidney Int 1997; 51: 270-6.

[13] Noel LH, Aucouturier P, Monteiro RC, *et al.* Glomerular and serum immunoglobulin G subclasses in membranous nephropathy and anti-glomerular basement membrane nephritis. Clin Immunol Immunopathol 1988; 46: 186-94.

[14] Ponticelli C, Zucchelli P, Passerini P, *et al.* A 10-year follow-up of a randomized study with methylprednisolone and chlorambucil in membranous nephropathy. Kidney Int 1995; 48: 1600-4.

[15] Ponticelli C, Passerini P, Salvadori M, *et al.* A randomized pilot trial comparing methylprednisolone plus a cytotoxic agent versus synthetic adrenocorticotropic hormone in idiopathic membranous nephropathy. Am J Kidney Dis 2006; 47: 233-40.

[16] Cattran DC, Alexopoulos E, Heering P, *et al.* Cyclosporin in idiopathic glomerular disease associated with the nephrotic syndrome: workshop recommendations. Kidney Int 2007; 72: 1429-47.

[17] Praga M, Barrio V, Juarez GF, *et al.* Tacrolimus monotherapy in membranous nephropathy: a randomized controlled trial. Kidney Int 2007; 71: 924-30.

[18] Fervenza FC, Cosio FG, Erickson SB, *et al.* Rituximab treatment of idiopathic membranous nephropathy. Kidney Int 2008; 73: 117-25.

[19] Beck LH, Jr., Fervenza FC, Beck DM, *et al.* Rituximab-Induced Depletion of Anti-PLA2R Autoantibodies Predicts Response in Membranous Nephropathy. J Am Soc Nephrol 2011; 22: 1543-50.

[20] Ponticelli C, Passerini P. Can prognostic factors assist therapeutic decisions in idiopathic membranous nephropathy? J Nephrol 2010; 23: 156-63.

[21] Magistroni R, Cantu M, Ligabue G, *et al.* Interstitial fluid obtained from kidney biopsy as new source of renal biomarkers. J Nephrol 2011; 24(3): 329-37.

[22] Brosch M, Yu L, Hubbard T, *et al.* Accurate and sensitive peptide identification with Mascot Percolator. J Proteome Res 2009; 8: 3176-81.

[23] Tsou CC, Tsai CF, Tsui YH, *et al.* IDEAL-Q, an automated tool for label-free quantitation analysis using an efficient peptide alignment approach and spectral data validation. Mol Cell Proteomics 2010; 9: 131-44.

[24] Polpitiya AD, Qian WJ, Jaitly N, *et al.* DAnTE: a statistical tool for quantitative analysis of -omics data. Bioinformatics 2008; 24: 1556-8.

[25] Magistroni R, Ligabue G, Lupo V, *et al.* Proteomic analysis of urine from proteinuric patients shows a proteolitic activity directed against albumin. Nephrol Dial Transplant 2009; 24(5): 1672-81.

[26] Saleem MA, O'Hare MJ, Reiser J, *et al.* A conditionally immortalized human podocyte cell line demonstrating nephrin and podocin expression. J Am Soc Nephrol 2002; 13(3): 630-8.

[27] Miyamoto M, Yoshida Y, Taguchi I, *et al.* In-depth proteomic profiling of the normal human kidney glomerulus using two-dimensional protein prefractionation in combination with liquid chromatography-tandem mass spectrometry. J Proteome Res 2007; 6: 3680-90.

[28] Greene WK, Baker E, Rabbitts TH, *et al.* Genomic structure, tissue expression and chromosomal location of the LIM-only gene, SLIM1. Gene 1999; 232: 203-7.

[29] Morgan MJ, Madgwick AJ. Slim defines a novel family of LIM-proteins expressed in skeletal muscle. Biochem Biophys Res Commun 1996; 225: 632-8.

[30] Wilhelmsen KC, Blake DM, Lynch T, *et al.* Chromosome 12-linked autosomal dominant scapuloperoneal muscular dystrophy. Ann Neurol 1996; 39: 507-20.

[31] Quinzii CM, Vu TH, Min KC, *et al.* X-linked dominant scapuloperoneal myopathy is due to a mutation in the gene encoding four-and-a-half-LIM protein 1. Am J Hum Genet 2008; 82: 208-13.

[32] Faulkner G, Pallavicini A, Formentin E, *et al.* ZASP: a new Z-band alternatively spliced PDZ-motif protein. J Cell Biol 1999; 146: 465-75.

[33] Frey N, Olson EN. Calsarcin-3, a novel skeletal muscle-specific member of the calsarcin family, interacts with multiple Z-disc proteins. J Biol Chem 2002; 277: 13998-14004.

[34] Lechuga S, Alarcon L, Solano J, *et al.* Identification of ZASP, a novel protein associated to Zona occludens-2. Exp Cell Res 2010; 316: 3124-39.

[35] Ueki N, Seki N, Yano K, *et al.* Isolation, tissue expression, and chromosomal assignment of a human LIM protein gene, showing homology to rat enigma homologue (ENH). J Hum Genet 1999; 44: 256-60.

[36] Kato T, Iwayama Y, Kakiuchi C, *et al.* Gene expression and association analyses of LIM (PDLIM5) in bipolar disorder and schizophrenia. Mol Psychiatry 2005; 10: 1045-55.

[37] Horiuchi Y, Arai M, Niizato K, *et al.* A polymorphism in the PDLIM5 gene associated with gene expression and schizophrenia. Biol Psychiatry 2006; 59: 434-9.

[38] Kadrmas JL, Beckerle MC. The LIM domain: from the cytoskeleton to the nucleus. Nat Rev Mol Cell Biol 2004; 5: 920-31.

[39] Mundel P, Reiser J. Proteinuria: an enzymatic disease of the podocyte? Kidney Int 2010; 77: 571-80.

[40] Zheng Q, Zhao Y. The diverse biofunctions of LIM domain proteins: determined by subcellular localization and protein-protein interaction. Biol Cell 2007; 99: 489-502.

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