Urinary CD80 Discriminates Among Glomerular Disease Types and Reflects Disease Activity

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Introduction: Heterogeneity of nephrotic diseases and a lack of validated biomarkers limits interventions and reduces the ability to examine outcomes. Urinary CD80 is a potential biomarker for minimal change disease (MCD) steroid-sensitive nephrotic syndrome (NS). We investigated and validated a CD80 enzyme-linked immunosorbent assay (ELISA) in urine in a large cohort with a variety of nephrotic diseases.

Methods: A commercial CD80 ELISA was enhanced and analytically validated for urine. Patients were from Mayo Clinic (307) and Nephrotic Syndrome Study Network Consortium (NEPTUNE; 104) as follows: minimal change disease (MCD, 56), focal segmental glomerulosclerosis (FSGS, 92), lupus nephritis (LN, 25), IgA nephropathy (IgAN, 20), membranous nephropathy (MN, 49), autosomal dominant polycystic kidney disease (ADPKD, 10), diabetic nephropathy (DN; 106), pyuria (19), and controls (34). Analysis was by Kruskal−Wallis test, generalized estimating equation (GEE) models, and receiver operating characteristic (AUC) curve.

Results: Urinary CD80/creatinine values were highest in MCD compared to other glomerular diseases and were increased in DN with proteinuria >2 compared to controls (control = 36 ng/g; MCD = 139 ng/g, $P <$ 0.01; LN = 90 ng/g, $P < 0.12$; FSGS = 66 ng/g, $P = 0.18$; DN = 63, $P = 0.03$; MN = 69 ng/g, $P = 0.33$; ng/g, $P =$ 0.07; IgA = 19 ng/g, $P = 0.09$; ADPKD = 42, $P = 0.36$; and pyuria 31, $P = 0.20$; GEE, median, P vs. control). In proteinuric patients, CD80 concentration appears to be independent of proteinuria levels, suggesting that it is unrelated to nonspecific passage across the glomeruli. CD80/creatinine values were higher in paired relapse versus remission cases of MCD and FSGS ($P < 0.0001$, GEE).

Conclusion: Using a validated ELISA, urinary CD80 levels discriminate MCD from other forms of NS (FSGS, DN, IgA, MN) and primary from secondary FSGS.

Aproximately 30 million Americans have chronic kidney disease, with diabetes and high blood pressure being the leading cause of end-stage kidney disease.[1] Nephrotic syndrome (NS) is the second most frequent cause of chronic kidney disease in the first 3 decades of life, requiring dialysis or kidney

transplantation for survival. Idiopathic nephrotic syndrome is relatively rare in adults, with an annual incidence of 2 per 100,000. The most common causes among adults are focal segmental glomeruloclersis (FSGS) and membranous nephropathy (MN).[2,3]

CD80 plays a role in innate and adaptive immunity activation. It has been implicated in the pathogenesis of nephrotic syndrome along with a possible role of B- and T-cell−mediated immunity in minimal change disease (MCD, FSGS) and lupus nephritis (LN).[4−8] Inducible podocyte CD80 expression *in vitro* and *in vivo* has been described, and circulating CD80 levels in serum in MCD have been quantitated.[9−11] Overexpression of CD80 in podocytes leads to slit

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diaphragm protein rearrangement *in vitro* and proteinuria in animal models.[9,10] In addition, inducible podocyte CD80 expression *in vitro* and *in vivo* has been initially described in induced models of proteinuria, such as puromycin-treated cultured podocytes, mice and cultured podocytes treated with lipopolysaccharide (LPS), α3 integrin and nephrin knockdown mice, and a murine model of LN.[10] These initial observations have not been reproduced in some subsequent studies.[12–14]

Using immunohistochemistry, CD80 was detected in podocytes in FSGS and other nephropathies.[11,15–17] Recent studies in animal models and clinical samples, which included positive and negative controls, have shown that glomerular CD80 was detectable only in tubular and activated infiltrating immune cells but not in podocytes.[13,18–20] However, using combined immunostatining, immunogold labeling *in situ* hybridization, and cell-specific RNA sequencing analysis, a possible renal source of urinary CD80 has been recently proposed to be glomerular endothelial cells and podocytes.[21]

CD80-deficient mice treated with LPS did not show proteinuria, suggesting that CD80 induction by LPS could be causative of proteinuria.[10] However, it has also been discussed that the effect could be immune mediated, as this knockout mouse model was not podocyte specific.[22] Studies using CD80 and T-cell receptor knockdown and wild-type irradiated mice supplemented with bone marrow suggest that albuminuria induced in mice by toll-like receptor ligands is dependent on the expression of toll-like receptors exclusively in hematopoietic cells and on CD80 expression exclusively in nonhematopoietic cells.[23] T-cell receptor–induced tumor necrosis factor–α secretion by hematopoietic cells is a prominent mediator of renal CD80 induction and resultant albuminuria in this model.[23]

CTLA4 (CD154) is expressed in activated T cells and functions as an immune checkpoint. CTLA4 binds with high avidity to CD80, blocks the binding of CD80 and CD86 to CD28, and suppresses T-cell activation.[24] Clinical trials of biological agents that block the CD80–CD28 pathway are in progress for LN (NCT0242993; NCT017148174), FSGS, and MCD (NCT02592798), and transplantation (belatacept NCT01729494; NCT02327403).[25] Thus, validated assays are needed to assess and to monitor this pathway before and after treatment.

Several studies report high uCD80 excretion in subjects with active MCD compared to remission and other glomerular diseases in relapse.[11,15,26–28] A recent report did not show differences in the urinary CD80/creatinine ratio in MCD with respect to other nephrotic diseases.[29] Of note, this study included only 4 FSGS patients (with an outlier), and one-third of MCD samples appeared to have high uCD80 levels, suggesting that subgroups of patients can be stratified according to their uCD80 levels.[29]

Glomerular CD80 expression and uCD80 have also been suggested as predictors of abatacept response.[15,17,30] Garin *et al* found strong glomerular CD80 staining in an MCD patient and weak staining in 2 FSGS samples. Urinary CD80 excretion in the MCD patient with positive CD80 glomerular staining dramatically dropped following abatacept treatment, with temporary improvement in urinary protein/creatinine ratio (uPCR).[15] The FSGS patients with CD80 detected in kidney biopsy samples by immunohistochemistry experienced partial or complete remission of proteinuria following abatacept treatment. However, there was no uCD80 analysis, and there were no negative control FSGS cases without podocyte CD80 expression, which are both weaknesses of the study.[17]

The uCD80/creatinine ratio may be a good indicator of steroid response and a predictor of long-term renal function in pediatric MCD.[31] The CD80 assays used in these and other studies have not been validated for urine samples, and urinary concentrations are low and challenging to accurately quantitate. To fill this gap, we enhanced and validated a commercial CD80 enzyme-linked immunosorbent assay (ELISA) to use specifically in urine. We hypothesized that urinary CD80 excretion could discriminate among glomerular diseases and could be used to assess disease activity.

MATERIALS AND METHODS

Urine Collection
A strict urine storage protocol was followed to reduce variability.[32] This included addition of protease inhibitors (protease cocktail for conditioned medium; Sigma Aldrich, St. Louis, MO) before storage at −80 °C until testing. With the exception of a few samples kept at 4 °C up to 7 days, all samples were processed and frozen within 4 hours of collection. One freeze–thaw cycle led to precipitation in some samples; thus, at the time of testing, samples were thawed, and precipitates, if present, were solubilized by incubating at 37 °C for up to 10 minutes, with occasional mixing. Samples were centrifuged at 1000 *g* at room temperature for 10 minutes and the supernatant used for testing.

Urinary CD80 Measurement
A commercial CD80 sandwich ELISA (catalog number BMS291INST, Fisher Scientific, Pittsburg, PA) was enhanced with biotinyl tyramide amplification (ELAST ELISA amplification system, Perkin Elmer Health

Sciences, Boston, MA) for this study. Full analytical validation data are contained in the Supplementary Methods and Supplementary Results.

Human Studies

This study was approved by the Mayo Clinic Institutional Review Board, and subjects provided informed consent for this study. After approval of the Nephrotic Syndrome Study Network Consortium (NEPTUNE) Ancillary Studies Committee, biospecimens of NEPTUNE subjects with biopsy-proven glomerular diseases (MCD and FSGS) were obtained from the Neptune Consortium Biobank (n = 94). The samples were supplemented with additional cases (n = 210) from prevalent glomerular or renal disease cases at Mayo Clinic; MCD (n = 53), FSGS (n = 43), diabetic nephropathy (n = 106), IgA (n = 28), MN (n = 54), LN (n = 31), ADPKD (n = 9), pyuria (n = 19), and subjects without kidney disease (controls n = 34); 65 MCD and 43 FSGS samples were from the same subjects at different time points with active versus remission disease status. Fresh or waste urine obtained from a clinical sample was used (24-hour or random urine collections).

The FSGS subjects were classified as having primary, secondary, or genetic forms after reviewing the medical records and using published criteria.[33] Genetic FSGS cases were defined by having a family history of FSGS or a positive genetic test result. Subjects with a pathology favoring secondary FSGS (presence of segmental foot process effacement, and without a genetic cause of FSGS) or a known maladaptive, viral, or drug-induced FSGS, or with subnephrotic proteinuria, were classified as secondary FSGS. Subjects with diffuse and extensive foot process effacement and/or presented with clinical nephrotic syndrome (high proteinuria uPCR ≥3.5, hypercholesterolemia, and low serum albumin) were considered primary FSGS. There were 48 urine samples from primary FSGS subjects with uPCR ≥2 g/g and 27 with uPCR <2 g/g; and 7 urine samples from secondary FSGS subjects and uPCR ≥2 g/g and 5 with uPCR <2 g/g.

Clinical laboratory data used in the analyses included serum and urine creatinine by enzymatic isotope dilution mass spectrometry–traceable assay, estimated glomerular filtration rate (eGFR) by the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) (adult) or Schwartz (children) equations, urine albumin by an immunoturbidometric assay, and total urine protein by pyllogallo red.

Statistical Methods

CD80 values were normalized to urine creatinine concentration (nanograms per gram [ng/g]). For values in

which CD80 fell below the detectable range, a random value was imputed between 0 and the limit of quantification of the assay (0.04 ng/ml) using a uniform probability distribution. Urinary CD80 levels in unpaired samples with different levels of proteinuria were compared using Kruskal–Wallis test. CD80 levels in samples with uPCR ≥2 were compared across disease types using generalized estimating equation (GEE) models with interaction terms to test for differences by renal diagnosis. uCD80 distributions were not normally distributed, and so they were log transformed for GEE analysis. Urine CD80/creatinine levels across diseases were adjusted for proteinuria using uPCR as a continuous variable in the GEE model. We did not consider familial cases of FSGS in the GEE analysis of CD80 levels between FSGS subtypes because of the low number of cases in our cohort (n = 3). The clinical utility of the CD80 ELISA for separating MCD and FSGS subjects with relapse (uPCR >3) versus remission (uPCR <1) disease, subjects with uPCR ≥2 with MCD versus FSGS/MN/IgA, subjects with uPCR ≥2 with MCD versus FSGS, subjects with uPCR≥2 with primary and genetic versus secondary FSGS, or subjects with uPCR ≥2 with primary versus secondary FSGS was performed using receiver operating characteristic curve analysis (SAS Institute, Cary, NC).

RESULTS

Analytical Validation of ELISA to Measure CD80 in Urine

A commercial CD80 ELISA (e-Bioscience, Thermofisher, Waltham, MA) was modified using an enhancer (ELAST ELISA amplification system, PerkinElmer, Waltham, MA) to increase sensitivity and allow the low levels found in urine to be measured. The enhanced assay was analytically validated before subject samples were assayed. The enhanced assay showed high sensitivity (to 0.04 ng/ml), reproducibility (interassay CV = 12% at 0.118 ng/ml, CV = 11.5% at 0.396 ng/ml), and good spiked-recovery and linearity after dilution (Supplementary Figure S1 and Supplementary Table S1). CD80 was stable in urine after freeze–thaw and when stored for more than 12 months at −80 °C (see Supplementary Results). We also tested the Abcam (Cambridge, UK) and R&D Systems (Minneapolis, MN) ELISA kits for measuring CD80 levels in urine. The detection range for the Abcam CD80 ELISA kit in cell culture supernatant, serum, or plasma is 78 to 5000 pg/ml and the detection limit is 31 pg/ml. The detection range in cell culture supernatant for R&D Systems CD80 ELISA kit is 62.5 to 4000 pg/ml. However, we found them unsatisfactory at detecting low levels of urinary CD80.

Table 1. Characteristics of urine samples by disease and control groups

Variable	ADPKD	Control	Diabetic nephropathy	FSGS	IgAN	Lupus	MCD	MN	Pyuria
Subjects, n	10	34	106	92	20	25	56	49	19
Samples, n	10	34	108	129	29	34	109	58	19
Data source									
Mayo Clinic, n (%)	10 (100)	34 (100)	108 (100)	75 (58)	29 (100)	34 (100)	59 (54)	58 (100)	19 (100)
NEPTUNE, n (%)	0 (0)	0 (0)	0 (0)	54 (42)	0 (0)	0 (0)	50 (46)	0 (0)	0 (0)
Age, yr, median (IQR)	55 (48, 66)	55 (41, 72)	67 (59, 75)	36 (17, 52)	44 (39, 50)	35 (23, 48)	17 (7, 49)	59 (51, 68)	72 (55, 80)
Female (%)	9 (90)	11 (32)	40 (37)	43 (33)	8 (28)	28 (82)	60 (55)	9 (16)	13 (68)
uPCR, median (IQR)	0.1 (0.1, 0.1)	0.1 (0.0, 0.1)	0.3 (0.1, 1.8)	3.2 (1.1, 5.4)	1.5 (0.7, 2.4)	1.2 (0.3, 3.3)	1.3 (0.1, 6.4)	2.4 (0.8, 4.6)	0.3 (0.1, 0.4)
≥ 2, n (%)	0 (0)	0 (0)	26 (24)	82 (64)	10 (34)	12 (35)	51 (47)	33 (57)	0 (0)
<2, n (%)	10 (100)	34 (100)	82 (76)	47 (36)	19 (66)	22 (65)	58 (53)	25 (43)	19 (100)
eGFR, median (IQR)	61 (55, 67)	—	52 (35, 71)	45 (25, 75)	43 (25, 58)	79 (62, 100)	101 (75, 128)	53 (39, 73)	—
Serum albumin, median (IQR)	4.2 (4.0, 4.2)	—	3.3 (2.6, 4.4)	3.6 (2.8, 4.0)	4.0 (3.9, 4.1)	3.7 (2.8, 4.4)	3.8 (2.7, 4.4)	3.5 (3.0, 4.1)	—
CD80/creatinine, median (IQR) [ng/g creatinine]	42 (33, 90)	36 (22, 57)	36 (20, 68)	48 (24, 86)	28 (8, 46)	53 (19, 83)	75 (38, 158)	48 (26, 82)	31 (14, 44)
<2, Median (IQR)	42 (33, 90)	36 (22, 57)	30 (18, 54)	33 (12, 63)	28 (10, 44)	36 (12, 71)	46 (23, 70)	31 (13, 39)	31 (14, 44)
≥ 2, Median (IQR)	—	—	63 (37, 124)	66 (29, 98)	19 (7, 52)	90 (19, 395)	139 (84, 221)	69 (45, 96)	—

ADPKD, autosomal dominant polycystic kidney disease; eGFR, estimated glomerular filtration rate; IQR, interquartile range; MCD, minimal change disease; MN, membranous nephropathy; uPCR, urinary protein/creatinine ratio.

This was likely due to antigenic sites masked by protein−protein interaction or problems with antibody−antigen recognition and binding in urine, as these kits were not developed for use with urine samples.

Subject Demographics and Laboratory Test Results

Urine samples were obtained from patients with FSGS (n = 92), MCD (n = 56), IgAN (n = 20), LN (n = 25), MN (n = 49), autosomal dominant polycystic kidney disease (ADPKD; n = 10), diabetic nephropathy (DN; n = 106), pyuria (n = 19), and no known renal disease (controls; n = 34) (Table 1). Most subjects were European American, with a median age of 44 years, and 43% were female. Median eGFR (ml/min per 1.73 m^2) differed by disease type (45 for FSGS, 101 for MCD, 43 for IgAN, 79 for LN, 53 for MN, 52 for DN, and 61 for ADPKD cases $P < 0.01$). The MCD and FSGS cohorts included pediatric and adult patients (MCD 53% pediatric, FSGS 28% pediatric). The median uPCR was 3.2 g/g (interquartile range [IQR] = 1.1, 5.4) for FSGS and 1.3 g/g (IQR = 0.1, 6.4) for MCD. The percentage of patients receiving immunosuppressive treatment at the time of urine collection was 69% for MCD, 46% for FSGS, 59% for MN, and 52% for LN. Controls were healthy donors or subjects with normal urinalysis results and eGFR >60, median age 55 years, and 32% were female. Urinary CD80 concentrations were higher among 24 MCD and 16 FSGS subjects with high degrees of proteinuria (uPCR >3 g/g) as compared to low levels (uPCR <1 g/g) ($P < 0.0001$) (Figure 1a). The uCD80

values were higher in MCD compared to FSGS subjects ($P = 0.01$). Receiver operating characteristic curve analysis showed that the uCD80 test performed well to differentiate relapse (uPCR >3 g/g) versus remission (uPCR <1 g/g) in both MCD and FSGS cases (AUC = 0.83 and 0.81 respectively) (Figure 1b and 1c). Urinary CD80 was under the limit of quantification (<0.04 ng/ml = <40 pg/ml) in 30% of MCD and FSGS samples of patients considered in relapse, 73% FSGS samples from individuals considered in remission, and 46% MCD samples considered in remission (Figure 2). In addition, uCD80 values were under the limit of quantification (<0.04 ng/ml = <40 pg/ml) in 53% of all samples analyzed, in 64% of 129 FSGS urine samples, and in 42% of 109 MCD urine samples.

Urinary CD80 Discrimination of LN and MCD Proteinuric Diseases From Other Kidney Diseases

We next evaluated the influence of glomerular disease type and protein excretion on uCD80 excretion (Figure 2 and Table 2). The GEE analyses were performed on a natural log scale. The uCD80 excretion increased with uPCR among MCD, FSGS, LN, and MN subjects but not among IgA subjects (Figure 2). MCD, and DN subjects with a uPCR value ≥ 2 g/g, had higher uCD80 levels than controls (Table 2). Subjects with FSGS, pyuria, ADPKD, IgAN, and MN who had a uPCR value ≥ 2 g/g did not have elevated uCD80 excretion compared to controls (Table 2). We observed high uCD80 excretion in some individuals with LN; however, the mean uCD80 was not significantly different
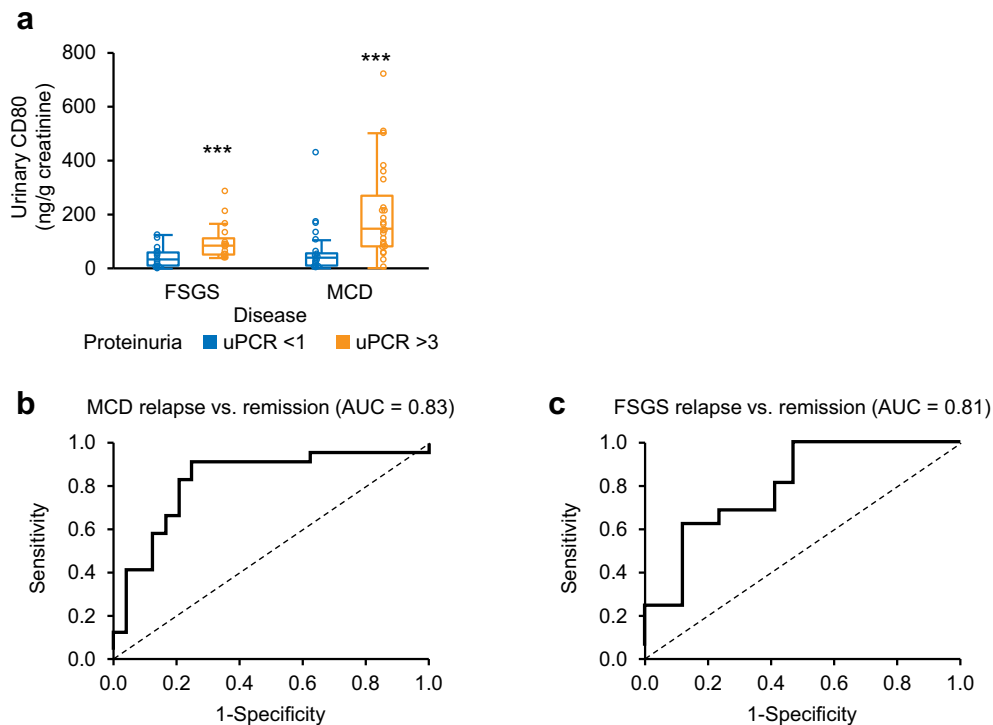
Figure 1. CD80/creatinine ratio is a good marker of disease activity for minimal change disease (MCD) and focal segmental glomerulosclerosis (FSGS) cases. (a) Scatter plot of urinary CD80/creatinine shows values measured in 15 FSGS subjects (15 active visits and 16 remission visits) and 25 MCD subjects (25 active visits and 27 remission visits). *P* values were analyzed with generalized estimating equation analysis of relapse versus remission log transformed CD80/creatinine (MCD vs. FSGS, *P* < 0.0001. ***Relapse versus remission, *P* ≤ 0.0001). (b,c) Receiver operating characteristic curve of baseline urinary CD80/creatinine ratio to predict remission for (b) MCD and (c) FSGS. AUC, area under the curve; uPCR, urinary protein/creatinine ratio.

from that of controls (Table 2). The low number of LN samples likely affected the ability to detect differences.

Elevated uCD80/creatinine values (expressed in nanograms per gram [ng/g]) were observed when proteinuria was ≥2 g/g among patients with MCD (139) compared to patients with FSGS (66), MN (69), or IgA (19) (Table 2). Importantly, uCD80 excretion was not significantly different in MCD compared to LN before or after adjusting for urine protein (*P* = 0.86 and 0.71, respectively), whereas significant differences persisted between MCD and IgA (*P* < 0.01) after adjusting for proteinuria, indicating that the differences were not simply a reflection of a nonspecific protein leak.

Finally, we investigated whether uCD80 excretion could differentiate among forms of FSGS when the uPCR was ≥2g/g. Urinary CD80/ creatinine ratios were significantly lower in secondary compared to primary FSGS (Figure 3a). Receiver operating characteristics analysis also confirmed that uCD80 could differentiate primary and genetic from secondary FSGS with an AUC of 0.78 (Figure 3b). A cut-off for CD80/creatinine of 42.1 ng/g differentiated primary from secondary FSGS with a sensitivity of 75% and a specificity of 75%. When genetic cases of FSGS were excluded from the analysis, the AUC of primary versus secondary FSGS was 0.74 (Figure 3c), and the strongest uCD80/creatinine threshold was 39.4

ng/g, with a sensitivity of 77% and specificity of 69%. There were no significant differences between uCD80 levels in primary and genetic FSGS cases (AUC = 0.52).

Sensitivity Analysis

We performed receiver operating characteristic analysis to investigate whether the uCD80/creatinine ratio could differentiate MCD from other glomerular diseases when the uPCR was ≥2 g/g. The area under the curve (AUC) was 0.77 for MCD versus FSGS/MN/IgAN (Figure 4a). A cut-off for CD80/creatinine of 108.9 ng/g resulted in sensitivity of 65% and specificity of 83% for the diagnosis of MCD. CD80/creatinine values also discriminated MCD versus FSGS (AUC = 0.76) (Figure 4b) and MCD versus primary FSGS (AUC = 0.73) (Figure 4c); a cut-off of 123.5 ng/g differentiated MCD from primary FSGS with a sensitivity of 59% and a specificity of 81%.

DISCUSSION

Data from this study demonstrate that the uCD80/creatinine ratio helps to discriminate MCD from other forms of NS and primary from secondary FSGS. Accumulated data by several independent groups using 4 different immunoassays now demonstrates that uCD80 is consistently elevated in relapsed MCD with
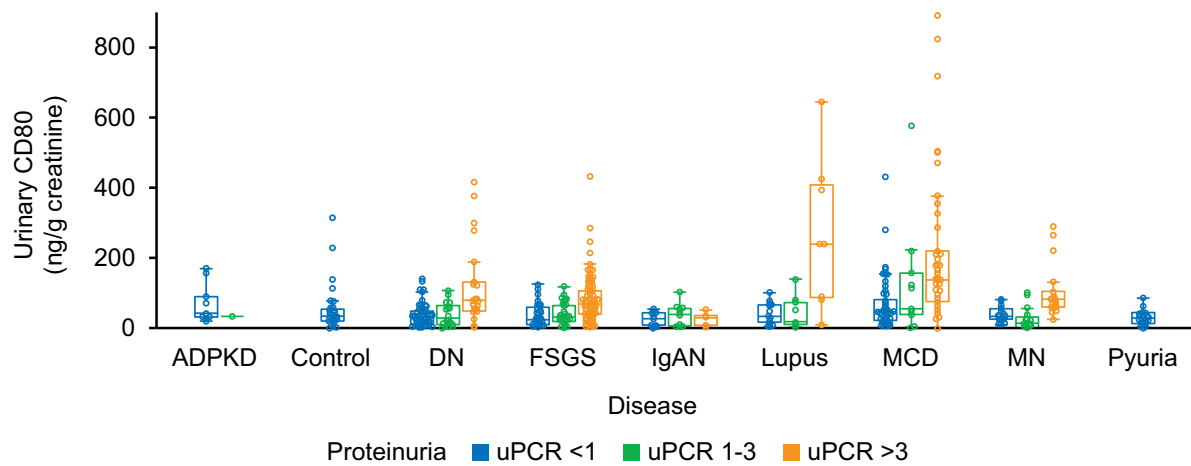
	ADPKD	Control	Diabetic nephropathy	FSGS	IgAN	Lupus	MCD	MN	Pyuria
uPCR 1–3 vs. uPCR <1			0.92	0.09	<0.01	0.49	0.17	0.92	
uPCR >3 vs. uPCR 1–3			<0.01	<0.01	0.11	<0.01	0.10	<0.01	
uPCR >3 vs. uPCR <1			<0.01	<0.01	0.49	<0.01	<0.01	0. 53	
ULQ (%)	80	65	54	64	69	41	42	43	47

FSGS patients: ULQ	n	%
Relapse	8/27	30
Remission	74/102	73
Total	82/129	64

MCD patients: ULQ	n	%
Relapse	6/22	27
Remission	40/87	46
Total	46/109	42

Figure 2. Urine CD80/creatinine differs by disease type and status. Urinary CD80/creatinine levels were evaluated in unpaired and paired samples of nephrotic diseases with, autosomal dominant polycystic kidney disease (ADPKD), pyuria, and controls. Urinary CD80/creatinine values were elevated in proteinuric samples, with the exception of IgA nephropathy (IgAN), but not in healthy volunteers, patients with pyuria, or those with ADPKD. Patients in remission with CD80 under the limit of quantification (ULQ; <0.04 ng/ml = <40 pg/dl) were 73% for focal segmental glomerulosclerosis (FSGS) and 46% for minimal change disease (MCD). DN, diabetic nephropathy; Lupus, lupus nephritis; MN, membranous nephropathy; uPCR, urinary protein/creatinine ratio.

respect to other nephrotic diseases (our current results and previous reports summarized in Supplementary Table S2).[11,15,26–28] The source of CD80 may be from

Table 2. Urinary CD80/creatinine ratio discriminates MCD from controls and other proteinuric kidney diseases

| Disease type	No. of samples	CD80 (ng/g creatinine), median (IQR)	P vs. control	P vs. MCD
Control	34	36 (22, 57)	REF	<0.01
MCD	51	**139 (79, 221)**	<0.01	REF
FSGS	82	66 (29, 98)	0.18	<0.01
IgAN	10	19 (7, 52)	0.09	<0.01
Lupus	12	**90 (19, 395)**	0.33	0.29
MN	33	69 (45, 96)	0.12	<0.01
Diabetic nephropathy	26	63 (37, 124)	0.03	0.01
Pyuria	19	31 (14, 44)	0.20	<0.01
ADPKD	10	42 (33, 90)	0.36	<0.01

ADPKD, autosomal dominant polycystic kidney disease; FSGS, focal segmental glomerulosclerosis; IgAN, IgA nephropathy; MCD, minimal change disease; MN, membranous nephropathy; REF, referent (normal nonproteinuric control cases without glomerular disease).
Generalized estimating equation (GEE) analysis of log-CD80 across controls, pyuria, ADPKD, and samples with proteinuria ≥2 for FSGS, IgAN, lupus, MCD, MN, and diabetic nephropathy.
Data in boldface type denotes that CD80 levels (ng/g creatinine) in the disease type are statistically different respect to CD80 levels in normal nonproteinuric control cases (REF).

the kidney itself, as recently reported by Cara-Fuentes *et al.*, who examined CD80 expression by glomerular endothelial cells and podocytes in nephrotic patients and LPS-injected mice.[21]

Others have published CD80 immunostaining of murine and human podocytes with nephrotic syndrome.[10,11,15–17] Subsequent studies using positive/negative controls and more specific antibodies suggest that positive staining of podocytes observed in early studies was nonspecific binding of secondary antibodies (Supplementary Table S3).[3,15,17–20] Thus, the source of uCD80 may be systemic (filtered from plasma, nonrenal), tubular, from interstitial inflammatory cells, or produced locally at the actual podocyte at levels below the limit of quantification by immunostaining. CD80 antigenic sites may be masked by interaction with other proteins. Recent studies combining immunostaining, immunogold labeling, and *in situ* hybridization techniques suggest that the source of CD80 may be from the kidney itself.[21] In addition, several reports describe a lack of correlation between serum and urine CD80 supporting a renal source.[11,29] Immunodetection
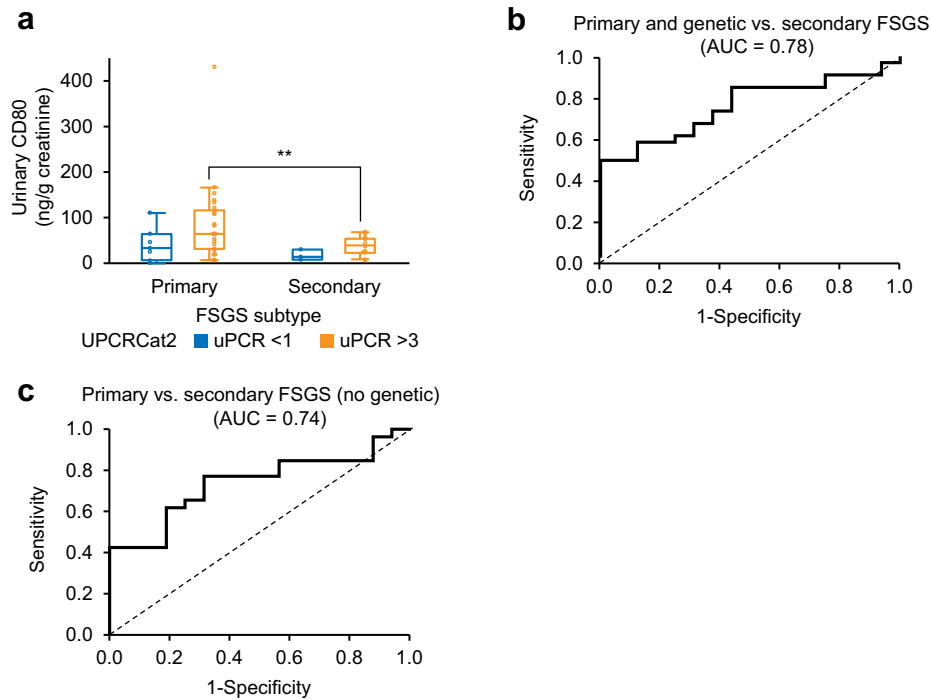
Figure 3. Urinary CD80/creatinine (ng/g creatinine) is higher in primary and genetic versus secondary focal segmental glomerulosclerosis (FSGS). CD80/creatinine ratio was measured in FSGS subjects with relapse (uPCR>3) versus remission (uPCR<1) disease. (a) Area under the curve (AUC) analysis of CD80/creatinine in FSGS subclasses (**$P < 0.01$ primary versus secondary, generalized estimating equation analysis). (b) AUC analysis of CD80/creatinine to predict primary and genetic FSGS cases versus secondary FSGS (AUC = 0.78). (c) AUC analysis of CD80/creatinine to predict primary versus secondary FSGS (AUC = 0.74). uPCR, urinary protein/creatinine ratio.
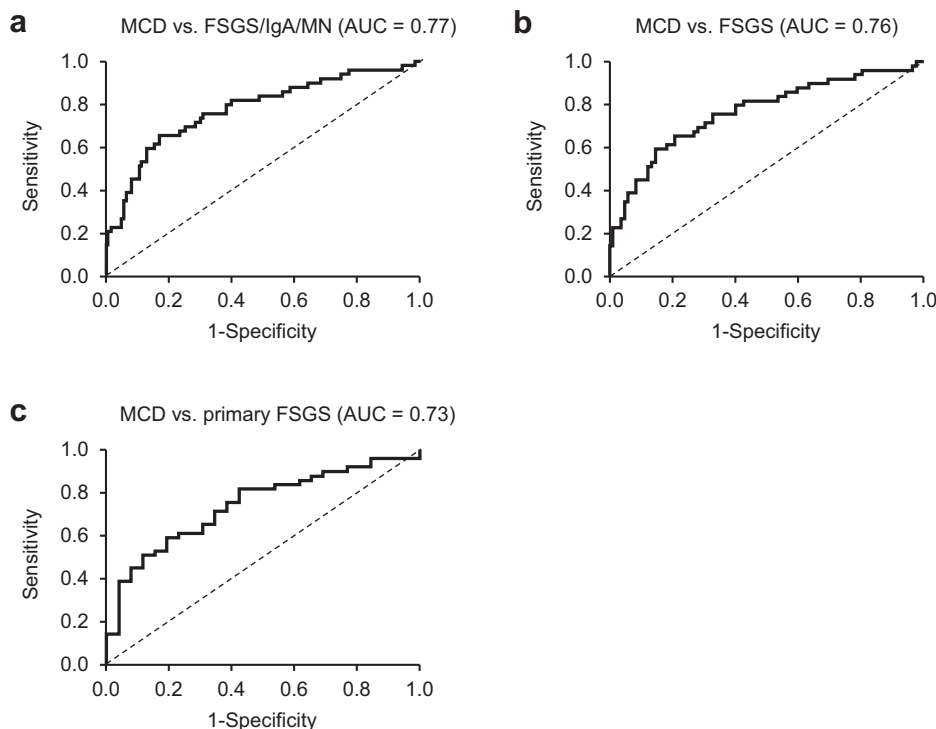
Figure 4. Urinary CD80/creatinine (ng/g creatinine) could differentiate minimal change disease (MCD) from other glomerular diseases when the urinary protein/creatinine ratio (uPCR) was ≥2 g/g. (a) Area under the curve (AUC) analysis value was 0.77 for MCD versus generalized estimating equation focal segmental glomerulosclerosis (FSGS)/membranous nephropathy (MN)/IgA nephropathy (IgAN). (b) The AUC was 0.76 for MCD versus FSGS. (c) The AUC was 0.73 for MCD versus primary FSGS.

using other primary antibodies with higher specificity and affinity (higher K_A) and adequate controls needs to be done to provide definitive evidence.

The CTLA4 inhibitor abatacept modulates its B-cell ligand receptors directly through the CD80/CD86 interaction, thereby modulating T-cell (or podocytes) activation and priming. In 1 study, CD80 was detected on renal biopsy samples from 5 FSGS patients (1 patient with rituximab-resistant recurrent FSGS after transplantation, and 1 patient with steroid-resistant primary FSGS); all experienced partial or complete remission of proteinuria after abatacept treatment. However, uCD80 was not used as an indicator to asses response to abatacept.[17] In clinical trials, a subgroup of abatacept-treated LN patients experienced a 20% to 30% greater reduction in mean uPCR compared with placebo.[34] A recent study supported the efficacy of abatacept for the steroid-resistant MCD.[30] Abatacept induced transient resolution of proteinuria in MCD patients with high uCD80.[22] In addition, a case report of a young adult with severe relapsing MCD with elevated uCD80 has shown sustained disease remission in response to abatacept therapy.[35] Another trial is evaluating abatacept in resistant MCD and FSGS.[36] However, other studies failed to demonstrate benefit from abatacept and belatacept (another CD80 inhibitor) in FSGS, further supporting the concept that only a subgroup of patients are responsive, and that biomarker(s) to select potential responders is needed to avoid exposing patients to (expensive) drugs and their side effects.[13,15,37–40]

A threshold urinary CD80/creatinine ratio ≥325 ng/g has been proposed as a marker for steroid responsiveness, and as a predictior of favorable outcome in pediatric MCD.[31] In addition, FSGS subjects with a uCD80/creatinine ratio ≥325 ng/g creatinine achieved remission and did not experience a renal function decline.[31] However, a previous report suggested that the proposed uCD80/creatinine cutoff of 325 ng/g creatinine may not be a good indicator of steroid sensitivity in idiopathic nephrotic syndrome.[28] Unfortunatly, we were unable to evaluate the predictive value of CD80 for steroid responsiveness in our current cohort. This will be a subject of future investigation.

Urinary CD80 distinguished MCD from FSGS and other glomerular diseases (excluding LN) with an AUC of 0.925.[27] Our data support the ability of uCD80 to differentiate MCD from other nephrotic diseases (FSGS, MN, and IgA, AUC = 0.74) and from primary FSGS (AUC = 0.69) but with a lower discriminatory power than Ling et al found.[31] Therefore, uCD80 could be useful for distinguishing MCD from FSGS in children, who usually do not undergo renal biopsy. High uCD80 levels would favor MCD, potentially avoiding a second biopsy in nonresponders to therapy.

The present study extends prior findings. The increased assay sensitivity and the larger number of FSGS samples tested allowed us to see differences in urinary CD80/creatinine between FSGS in relapse and remission that were missed in most of previous reports with low sample sizes and that did not use an enhanced assay.[26,27,41] In addition, previous studies were done mainly in children, in whom biopsies are usually not performed and among whom some steroid-sensitive FSGS cases could have been miscategorized as MCD. Our study included only biopsy-proven FSGS and MCD cases; CD80 was undetectable in most proteinuric secondary FSGS cases and a subset of primary FSGS cases (∼40%), indicating that not all relapsed primary FSGS subjects excrete uCD80. This was not surprising, because of the heterogeneity of FSGS pathogenesis and immunotherapy response.

High uCD80 has been reported in a case report of genetic FSGS.[42] We did not observe differences between genetic and primary FSGS uCD80 levels; nonetheless, few genetic FSGS cases were represented in our cohort (n = 3). Larger numbers of individuals should be included in future studies to make any deductions about the significance of urinary CD80 in genetic forms of FSGS.

Urinary CD80/creatinine was elevated in LN cases, as was reported by Ling et al. but contrary to the results reported by Garin et al.[11,27,43] Others have reported that soluble CD80 is elevated in plasma of systemic lupus erythematosus (SLE) patients, with high expression of CD80 in CD4+ T cells associated with LN.[27,43] In our study uCD80 excretion was higher in the SLE cases but was not statistically different from MCD.

Diabetes type I is an autoimmune disease, and immunotherapies with distinct immunologic targets, including anti CD80 therapy with abatacept, have resulted in temporary improvement (decreased proteinuria) in some individuals with new onset of diabetes type I.[44] Diabetes type II has also been linked to desregulation of innate and adaptive immunity.[45] In the current study, we found that urinary CD80 excretion was higher in subjects with diabetic nephropathy with uPCR >3 with respect to cases with lower proteinuria. Thus, further studies regarding the direct role of renal CD80 expression in the progression of diabetic nephropathy, and the role of other immune coactivators, remains a promising area for future investigation.

IgA nephropathy was an interesting outlier in the current study; uCD80 excretion was not elevated in this patient group, with or without proteinuria. These data suggest that the pathways of immunoactivation and its role in disease progression differ in IgA nephropathy, and that therapies targed at CD80 may not be effective.

Limitations of the current study were its retrospective nature (some patients were already on immunosuppressive treatment), few secondary FSGS cases, and lack of follow-up samples in treated cases, which would have permitted us to evaluate whether CD80 outperforms proteinuria to monitor therapy. Another limitation is our definition of relapse (uPCR > 3) versus remission (uPCR < 1). A uPCR of 3 or slightly higher may represent early remission and rising uPCR of 1 or less can represent an early relapse. Although it is difficult to have such information in collaborative studies, it would be important to acknowledge that as a minor limitation. The enhanced commercial CD80 assay detected the cytokine in the vast majority of our samples and our analytical validation confirmed that it is a robust assay in urine; however, we were unable to fully validate the specificity of the ELISA-coupled CD80 antibodies using liquid chromatography with tandem mass spectrometry/mass spectroscopy (LC-MS/MS) because of the low concentration of CD80 in urine and the high cost of the antibody concentrations necessary for immuno-enrichment. In addition, we did not address whether the source of CD80 was renal or systemic.

We confirmed previous findings concerning the potential elevation of uCD80 in the urine of subjects recruited from multiple institutions (Nephrotic Syndrome Study Network Consortium [NEPTUNE] and Mayo Clinic). The uCD80/creatinine ratios were lower in the MCD subjects than in previous reports. These differences could be a consequence of using different ELISAs and standards, the use of urine concentrators in previous studies, our current assay modifications (including enhanced ELISA), and the fact that previous publications did not report rigorous assay validation. It is also common to observe racial differences in glomerular disease manifestations and biomarker expression. Subjects studied by Ling *et al*. were Asian Chinese, and those of Mishra *et al*. were Asian Indian, whereas our population was predominantly European American and African American.

In conclusion, uCD80 excretion differentiated among several specific nephrotic diseases (MCD, LN, DN, and some cases of FSGS). Higher uCD80 levels were observed in patients with proteinuria with these pathologies compared to other nephrotic diseases (MN, IgA, secondary FSGS) . It is possible that uCD80 could serve as a predictive marker for responsiveness to specific immunosuppressant agents. Future prospective studies of immunosuppressive therapy using larger numbers of responder and nonresponder subjects (with urines obtained prior to immunosuppression exposure) could address this question. Further investigation to determine whether there is a correlation between urine CD80 and immune phenotypes in patients who have relapsed could be helpful to better understand the role of CD80 in disease pathogenesis, and to identify patients who may benefit from immunotherapies.

DISCLOSURE

ACKNOWLEDGMENTS

SUPPLEMENTARY MATERIAL

Supplementary File (PDF)
Supplementary Methods.
Supplementary Results.
Table S1. Analytical performance of enhanced CD80 ELISA assay.
Table S2. Urine CD80 measured in nephrotic syndrome.
Table S3. Podocyte staining in human and animal models.
Figure S1. Reproducibility of dose response standard curve and controls for modified sandwich ELISA assays for CD80. Standard curves and controls were run using two different lots of CD80 ELISA kit.

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