The role of osteopontin as a candidate biomarker of renal involvement in systemic lupus erythematosus

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Abstract

Objective

Kidney biopsy is the gold standard for the diagnosis of lupus nephritis (LN). Conventional biomarkers of disease activity or renal function, such as complement levels, anti-dsDNA, serum creatinine, urinary sediment and proteinuria, do not have a sensitive diagnostic and prognostic value, therefore new biomarkers are needed to help predict or monitor LN. Osteopontin (OPN) is a pro-inflammatory molecule detectable in serum and renal tissue. The aim of this study was to evaluate OPN as a biomarker of renal involvement in patients with systemic lupus erythematosus (SLE) and correlate its levels with disease activity and laboratory features.

Methods

OPN was measured in the serum and urine of SLE patients with active LN (n=14), LN in remission (n=20), SLE without kidney involvement (n=22) and age- and sex-matched healthy controls (HC, n=20).

Results

OPN levels were significantly higher in urine than in serum in both groups of patients and controls ($p<0.001$). Serum OPN levels were higher in the LN patients than in HC and in SLE patients without renal involvement ($p<0.0001$ and 0.0032, respectively), regardless of the phase of renal activity. SLE patients without renal involvement and controls showed similar serum levels. We detected a direct correlation between low complement levels and OPN serum levels in patients with LN ($p=0.014$; $R=0.438$). Moreover, a higher percentage of patients with LN, compared to SLE without LN and HC, showed abnormal serum OPN.

Conclusion

Our data suggest that serum OPN could be considered a biomarker of renal involvement, without differentiating between active and remission LN.

Key words

osteopontin, lupus nephritis, systemic lupus erythematosus, biomarkers, glomerulonephritis

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Introduction

Systemic lupus erythematosus (SLE) is a chronic, multi-factorial autoimmune disease with several clinical manifestations ranging from mild-moderate to severe, life-threatening. Above all, lupus nephritis (LN) is one of the most severe complications in terms of quality of life and life expectation. Since 2004, LN has been histologically classified into 6 classes, and kidney biopsy is critical to guide the therapeutic management (1). Biomarkers of renal function and SLE disease activity currently used in clinical practice – serum creatinine, glomerular filtration rate, proteinuria, anti-dsDNA and C3/C4 complement fractions levels – are not able to predict renal flare, to discriminate the different histological classes of nephritis, and so they are not useful in providing accurate prognostic information. Several new potential biomarkers belonging to innate and adaptive immunity, including chemokines, cytokines and adhesion molecules, were studied both on murine models of lupus and LN patients but their clinical application has not been established yet (2-6).

Osteopontin (OPN), previously called early T-lymphocyte activation protein, is a 60-kd phosphorylated glycoprotein expressed in inflamed tissue by immune cells, such as natural killer cells, activated T cells, macrophages, and fibroblasts (7, 8). OPN can boost pro-inflammatory Th1 cell response and restrain Th2 response; it stimulates T-cell and B-cell proliferation, interferon-α production, CD40 ligand expression and production of antibodies. Based on these considerations, the OPN gene, located at 4q22, has been studied in several studies as candidate gene for SLE (7, 9-12). A very recent meta-analysis evaluated the relationship between circulating OPN level and disease activity, and association between OPN polymorphisms and SLE susceptibility on 9 studies including overall 1938 SLE patients and 3009 controls: the results of the meta-analysis suggest that circulating OPN levels were significantly higher in the SLE group than in the control group, OPN had a positive correlation with SLE activity measured by SLEDAI and its expression in SLE patients was associated with renal disease (13).

Urine, perhaps more than serum, may be used as an additional source for potential biomarkers in LN due to the easy accessibility and for being an anatomical and functional product of the status of the kidney (2, 3). To date, there are limited data on OPN levels in the urine of SLE patients.

The aim of this cross-sectional study was to evaluate serum and urine OPN in SLE patients with and without kidney involvement.

Materials and methods

We enrolled consecutive patients with SLE diagnosed according to the 1997 revised ACR Classification Criteria (14). The whole cohort was divided according to kidney involvement: patients with active nephritis (A-LN, n=14), patients with nephritis in remission (R-LN, n=20), patients affected by SLE without renal involvement (NR-SLE, n=22). We included sex and aged healthy adults as control group (HC, n=20). Active nephritis was defined according to the 2012 American College of Rheumatology Guidelines for management of LN (15).

All the patients we underwent a complete physical examination; serological data were collected and clinical indices – Disease and Renal Activity by SLEDAI 2-K and RENAL-SLEDAI – were calculated (16).

All the participants gave their written informed consent before enrolment in the study. The protocol was approved by the local Ethics Committee and the study was conducted according to the Helsinki Declaration.

Specimen collection

Serum and urine samples from patients and controls were collected to test OPN levels. Serum samples were obtained from peripheral whole blood and frozen at -20°C until tested. Urine samples were also frozen at -20°C until analysed.

ELISA for OPN

Serum and urine samples were tested in duplicate using a sandwich enzyme-linked immunosorbent assay (Quan-

Table I. Demographic and clinical features of SLE patients and healthy controls.

Cohort	A-LN n=14	R-LN n=20	NR-SLE n=22	HC n=20	*p*-value
Male:Female	2:12	4:16	0:22	2:18	0.070
Age, years (mean±SD)	34.58 ± 12.04	38.63 ± 11.45	42.82 ± 11.21	31.52 ± 10.31	0.137
Disease duration, months (mean±SD)	157.75 ± 109.19	140.58 ± 98.21	125.90 ± 123.68	n.e.	0.731
Hypocomplementaemia (n/tot; %)	11/14, 79%	11/20, 55%	10/22, 46%	n.e.	0.030
SLEDAI-2K (mean±SD)	10.33 ± 5.85	1.84 ± 2.98	0.91 ± 2.28	n.e.	0.008
R-SLEDAI (mean±SD)	7.50 ± 4.18	0.68 ± 2.00	0	n.e.	0.000
Proteinuria mg/24h (mean±SD)	2636.18 ± 3050.69	314.38 ± 284.38	0	0	0.012
Serum Creatinine mg/dL (mean±SD)	1.01 ± 0.59	0.92 ± 0.33	0.67 ± 0.13	n.e.	0.028

A-LN: active lupus nephritis; HC: healthy controls; n.e.: not evaluated; NR-SLE: non-renal SLE; R-LN: remission lupus nephritis; R-SLEDAI: renal SLEDAI; SLE: systemic lupus erythematosus; SLEDAI: Systemic Lupus Erythematosus Disease Activity Index.
For non-parametric variables Kruskal-Wallis test was performed to analyse all the four groups together; for parametric variables one-way ANOVA was performed to compare the four groups together.

Table II. Serum and urinary osteopontin levels in patients and controls.

	A-LN n=14	R-LN n=20	NR-SLE n=22	HC n=20	*p*-value
Serum osteopontin ng/ml (median - interquartile range)	154.6 97.9 – 231.7	121.1 105.6 – 156.8	97.96 85.2 – 113.2	85.6 68.2 – 106.3	0.0004
Urinary osteopontin ng/ml (median - interquartile range)	837.9 386.5 – 1193	826.4 343.3 – 1026	901.2 309.7 – 1108	730.3 462.6 – 1107	0.9866

A-LN: active lupus nephritis; HC: healthy controls; NR-SLE: non-renal SLE; R-LN: remission lupus nephritis; SLE: systemic lupus erythematosus. Kruskal-Wallis test was performed to analyse all four groups together.

tikine kit for Human Osteopontin Immunoassay; R&D Systems Inc., Minneapolis, MN, USA) according to the manufacturer's instructions.
Anti-dsDNA antibodies were measured by immunofluorescence (IF) on *Crithidia luciliae*.

Statistical analysis
Parameters were compared against each other using the Student's *t*-test where the data were normally distributed. Non-parametric data instead were compared with Mann-Whitney U-test. Statistical tests including correlation coefficients (R) were calculated using Graph Pad Prism. A *p*-value of <0.05 was considered significant.

Results

Patients and controls characteristics
We enrolled 56 SLE patients recruited from the Lupus Clinic of the Sapienza University of Rome between January 2015 and June 2017; patients were divided into 3 groups:
Fouteen subjects with active nephritis (A-LN) (M:F=2:12, mean age 34±12, mean disease duration 157.75 months); 20 subjects with remission nephritis (R-LN) (M:F=4:16, mean age 38±11, mean disease duration 140.58 months); 22 subjects affected by SLE without renal involvement (NL-SLE) (M:F=0:22, mean age 42±11, mean disease duration 125.90 months). The control group was composed of 20 sex- and age-matched healthy controls (M:F=2:18, mean age 31±10). Table I shows the demographic and clinical characteristics of the four groups. Higher rates of hypocomplementaemia, R-SLEDAI, SLEDAI-2K, proteinuria and serum creatinine were found in the active nephritis group compared to the remission and non-renal SLE groups ($p<0.005$).
When comparing the three subsets of patients according to the presence or absence of clinical and serological manifestation included in the SLEDAI score, we did not find any difference except for the nephritis items of the SLEDAI (proteinuria and urinary sediment), which drove the attribution to the three groups.

OPN serum and urinary levels
Table II shows the median serum OPN level of the four groups [A-LN: 154.6 (133.8), R-LN: 121.1 (51.2), NR-SLE: 97.96 (28), HC: 85.6 (38.1) ng/ml]. Serum OPN was significantly higher in A-LN compared to the other 3 ($p<0.0004$); moreover, patients with kidney involvement (active and in remission) showed significantly higher OPN levels compared to NR-SLE and HC ($p=0.0032$ and $p=0.0001$, respectively) (Fig. 1).
Urinary OPN levels were significantly higher compared to serum levels in the whole cohort ($p<0.0001$); however, median urinary OPN levels did not significantly differ between the 4 groups [A-LN: 837.9 (806.5), R-LN: 826.4 (682.7), NR-SLE: 901.2 (798.3), HC: 730.3 (644.4) ng/ml] (Table II).
A significantly higher percentage of A-LN had abnormal serum OPN levels – a serum level higher than the 97th percentile – compared to SLE patients without renal involvement and to the healthy controls ($p=0.005$ and $p=0.0002$, respectively); similarly, a higher percentage of R-LN had abnormal serum OPN levels compared to SLE patients without renal involvement and of healthy controls ($p=0.029$ and $p=0.003$, respectively) (Fig. 2).

Correlation between OPN and laboratory clinical manifestation, pathological classification and treatment

We found an inverse correlation between C3 and serum OPN levels in patients with LN (r=-0.34, p=0.02), and a direct correlation between serum OPN and SLEDAI-2K/R-SLEDAI (r=0.35, p=0.01 and r=0.32 and p=0.02, respectively).

No correlation was found between urinary or serum OPN and other clinical manifestations, disease duration, proteinuria, serum creatinine and anti-dsDNA.

No significant difference was found in the serum and urinary OPN levels regarding the different treatment patterns, concerning the use of glucocorticoids, immunosuppressant drugs and hydroxychloroquine.

Data about histologic class of kidney biopsy were available for 30 out of 34 patients with LN: 17 (58%) patients had class IV nephritis, 4 (14%) patients had class III nephritis, 2 (7%) patients had class II nephritis and 2 (7%) patients had class V nephritis. Evaluating the serum concentration of OPN in the different classes, class III nephritis showed higher median levels [146.50 (15.52) ng/ml] compared to class V LN [100.20 (21.59) ng/ml] (p=0.01). No other differences were detected.

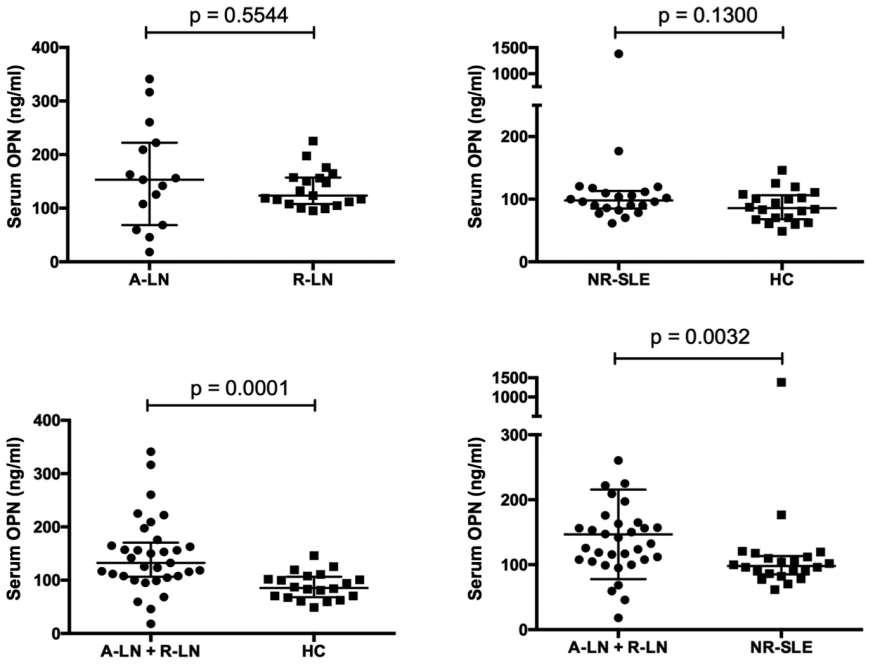

Fig. 1. Serum OPN levels in SLE patients with and without kidney involvement compared to healthy controls.
A-LN: active lupus nephritis; HC: healthy controls; NR-SLE: non-renal SLE; OPN: osteopontin; R-LN: remission lupus nephritis; SLE: systemic lupus erythematosus.

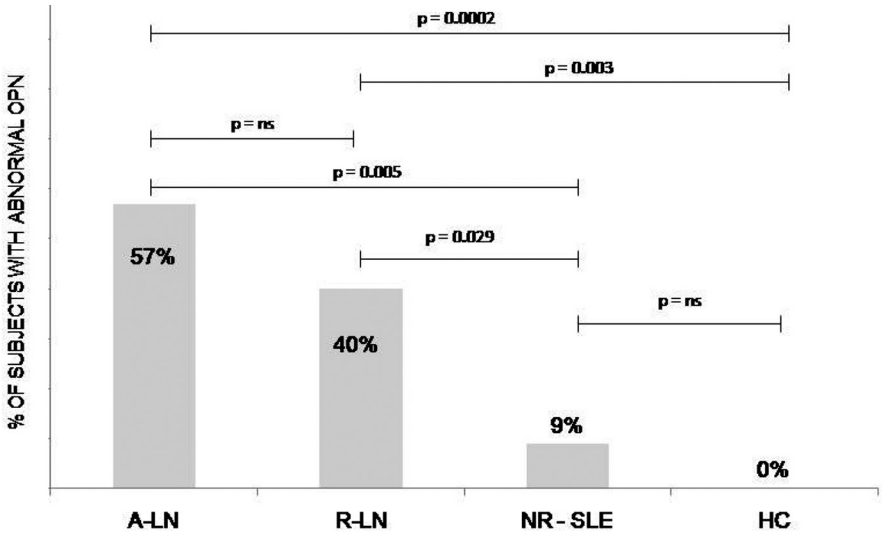

Fig. 2. Abnormal serum OPN in SLE patients with and without kidney involvement compared to healthy controls.
A-LN: active lupus nephritis; HC: healthy controls; NR-SLE: non-renal SLE; OPN: osteopontin; R-LN: remission lupus nephritis; SLE: systemic lupus erythematosus.

Discussion

The results of our study confirm a role for serum, but not urinary, OPN as a marker of kidney involvement in patients with SLE.

OPN is a pivotal molecule in driving inflammation and tissue damage; its concentration in serum seems to be significantly correlated with disease activity in SLE patients. Our study confirmed that patients with lupus nephritis have higher serum levels of OPN compared to lupus patients without renal involvement and healthy controls; however, urinary OPN seems not to differentiate patients with LN. Table III summarises the results of previous studies investigating serum OPN levels in patients with SLE. The first report of OPN in the serum of a SLE patient dates back to 1995 when the protein was found by Western blot analysis in a murine model of lupus and in the sera of one SLE patient and one rheumatoid arthritis patient but not in a healthy subject (17). In 2005, Wong *et al*. evaluated for the first time the serum levels of OPN in 54 SLE patients, half of whom had renal involvement; the authors showed that OPN levels were significantly higher in the whole SLE population compared to controls, regardless the renal involvement and no difference between patients with and without NL was found (7). Similar results were also presented by Afify *et al*. who studied serum OPN in 40 patients with SLE – 20 with and 20 without LN – compared to healthy subjects (18). Unlike our study, neither Wong *et al*. nor Afify *et al*. divided the patients ac-

Table III. Summary of previous studies on OPN in adult SLE patients.

Type of study	number of SLE patients	SLE sub-groups R-SLE	NR-SLE	Healthy controls	Blood	Urine	Correlations	Effect of immuno-suppressant on OPN	Reference
Cross-sectional	54	27	27	40	yes	no	IL-18, SLEDAI	n.r.	(7)
Cross-sectional	40 (paediatric)	20	20	30	yes	no	IL-18, SLEDAI, proteinuria, creatinine	n.r.	(19)
Prospective (12 months follow-up)	64 (23 adults)		22	62 (40 adults)	yes	no	anti-dsDNA, AMS, SDI	n.r.	(24)
Cross-sectional	101			115	yes	no	renal involvement, SLEDAI, SDI, proteinuria, use of RAA antagonists Urine: IL-18, albumin, thrombin activity	No effect	(25)
Cross-sectional	56	29	27	50	yes	yes	Plasma and urine: anti-dsDNA	Discordant effects	(24)
Cross-sectional	163	36	127	180	yes	no	Renal and joint involvement	n.r.	(28)
Cross-sectional	240			240			ESR, creatinine, SLEDAI-2K, SDI, renal involvement, APS syndrome.	n.r.	(23)

AMS: adjusted mean SLEDAI; APS: anti-phospholipid syndrome; A-LN: active lupus nephritis; ESR: erythrocyte sedimentation rate; HC: healthy controls; n.r.: not reported; NR-SLE: non-renal SLE; OPN: osteopontin; RAA: renin-angiotensin antagonists; R-LN: remission lupus nephritis; SDI: Systemic Lupus International Collaborting Clinics (SLICC) Damage Index; SLE: systemic lupus erythematosus.

cording to the LN activity at the study entry. Our study was designed as the first one to explore the possible role of OPN as a marker of renal activity, enlisting 3 different SLE populations: patients with active nephritis, patients with nephritis in remission and patients with no history of nephritis. The results of our study confirmed that the cytokine levels were significantly higher in the whole population of patients with LN compared with those without renal involvement; however, when we compared active LN and nephritis in remission, the serum levels of OPN did not differ significantly. Quaglia *et al*. (19) investigated the possible associations between serum OPN and various clinical and serological manifestations of SLE in 101 patients and 115 healthy controls. The authors divided the study subjects into tertiles, based on the OPN values – low, medium and high – and showed that OPN was significantly higher in patients than in controls. In addition, the highest concentrations of OPN were associated with SLE renal involvement, defined according to the histological diagnosis of NL or clinical confirmation of proteinuria, active urinary sediment or chronic renal failure (20).

In inflammatory conditions, metalloproteinases and thrombin cleave the OPN, increasing its pro-inflammatory effect (20). Therefore, the cleaved OPN could represent the "active" form detectable in the inflamed tissue. In this light, Kitagori *et al*. evaluated the utility of the two forms of osteopontin (OPN), the full one and its cleaved form (OPN N-half), in plasma and urine as markers of disease activity in lupus nephritis (LN) (21). Samples were collected from patients with SLE with and without renal involvement, IgA nephropathy, minimal change nephrotic syndrome (MCNS) diabetic nephropathy and healthy volunteers (21). Similarly to our results, plasma OPN full concentration was significantly higher in LN, non-LN, DN and MCNS than in HC ($p<0.01$, $p<0.001$) without significant difference among the different kidney diseases; considering the whole population, plasma OPN N-half concentration was below the detection sensitivity (21).

The consideration that OPN is a molecule richly expressed both by resident kidney cells (mesangial and tubular epithelium) and the monocytic cell renal infiltrate, was the premise of our study: to explore the role of this cytokine, as possible urinary biomarker of lupus nephritis we investigated the presence and concentration in urine. In our population we found significantly higher levels of OPN in the urine compared to the serum; however, analysing the four groups we found no significant differences either between patients with and without NL, nor between patients and controls. Kitagori *et al*. found that urine OPN N-half concentration was significantly higher in patients with LN than HC (21). However, similarly to us, the authors did not find any difference in urinary full OPN suggesting that the N-half form of the cytokine could reflect renal inflammation.

Besides regulating inflammation, OPN is also involved in tissue fibrosis and could be responsible for collagen deposition, fibrosis and end stage renal disease as demonstrated in different murine models of kidney disease (22). Quaglia *et al*. found an association between abnormal OPN levels and clinical features of chronic renal involvement – chronic kidney disease > class

II or creatinine levels >1.2 mg/dl (19). Similarly, Wirestam *et al*. also found a correlation between OPN and creatinine levels in 240 consecutive SLE patients; however, the authors aimed at evaluating OPN as a biomarker of disease activity and chronic damage and did not stratify the population according to the renal involvement (23). On the contrary, we did not find a positive correlation between serum nor urinary OPN and creatinine. Moreover, none of our patients with available kidney section had a class VI LN, the more severe class of glomerulonephritis associated with diffuse histological signs of scaring and to end-stage renal disease.

A further aim of this study was to evaluate possible correlations of serum and urinary OPN with disease activity and serum biomarkers of active disease – complement levels and anti-dsDNA – and markers of kidney involvement (proteinuria, creatinine and urinary sediment). In our population OPN serum levels correlated with SLEDAI and renal SLEDAI scores. Similarly, Wong and Quaglia detected a positive correlation between the serum levels of the cytokine and the SLEDAI score (7, 19). Rullo *et al*. in the cross-sectional phase of their study found no baseline association between serum levels of OPN and any single manifestations of the disease nor with the disease activity; in the longitudinal phase, conducted on a subset of SLE patients, the authors observed a direct correlation between increased baseline levels of OPN and mean increase in SLEDAI score over the 6 months of follow-up (24). Differently, Kitagori *et al*. did not show any correlation between serum and urine OPN and disease activity (21).

As for the SLE serology, we did not find an association with dsDNA positivity; similarly, Rullo *et al*. showed only a tendency of anti-dsDNA positive patients to have higher OPN levels (24). Interestingly, we observed an inverse correlation between C3 levels and levels of OPN; and higher rate of abnormal OPN in patients with active urinary sediment (41% *vs*. 15%). Similarly to all the previous reports, in our study we did not find any correlation between serum and urinary levels of OPN and glucocorticoids, immunosuppressants or antimalarials.

In 2006, Sasaki *et al*. studied the role of OPN in a mouse model of lupus glomerulonephritis demonstrating a hyperexpression of OPN – both protein and mRNA – in tubular epithelium, independently from the histological class (25). Furthermore, in a different murine study, Ka *et al*. demonstrated that also mesangial cells, stimulated with LPS, were capable of producing OPN (26). Data on kidney expression of OPN in SLE patients are scant. Okada *et al*. studied the renal tissue of 46 biopsy of various immune-mediated glomerulonephritis, including 12 patients affected by SLE, confirming in human that OPN participates to in the interstitial inflammatory infiltrate (27). In our study, higher serum levels of cytokines were detected in patients with class III LN compared to class V; however, we should consider that a half of our patients had a class IV and the comparison between the latter and the other classes was limited by the number discrepancy. Kitagori *et al*. also studied the correlation between urinary OPN N-half and histopathological classes of kidney biopsies, showing a lower OPN N-half in class II LN and no difference among class III/IV/V of lupus nephritis (21).

Our study has some limitations: the number of patients included and the lack of a follow-up observation. Moreover, the definition of "abnormal" levels of OPN was arbitrary in the present study as well as in the previous ones. We defined altered the OPN values greater than 97 percentile of healthy subjects; Rullo *et al*. have considered abnormal values above the third quartile, calculated on serum of healthy subjects while Quaglia *et al*. divided the patients according to the OPN values into 3 groups: low, medium, and high, according to tertiles of the serum concentration (19, 24). The different definition of the normal cut-off makes it difficult to compare the different studies.

A further consideration relates to the therapy practiced by patients at the time of collection; in fact, the majority of patients with active NL were already treated with immunosuppressive drugs: it cannot be excluded that glucocorticoids and immunosuppressants affect the levels of the cytokine.

Finally, to further determine the role of OPN in the pathogenesis of LN, the cytokine expression should be investigated locally, in the kidney sections of patients with renal involvement.

In conclusion, the results of this study allow confirming an increase in OPN levels in SLE patients with renal commitment. This is the first study enrolling SLE patients according to their renal involvement. We investigated the levels of OPN also in the urine and found values significantly higher than in serum, but unable to differentiate patients from controls. Based on data available up to now, serum, but not urinary OPN could be proposed as a marker of organ involvement more than a biomarker of renal activity. Further investigation on a larger cohort followed up prospectively is needed to validate our results.

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