Osteopontin and disease activity in patients with recent-onset systemic lupus erythematosus

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Title: Osteopontin and Disease Activity in Patients with recent-onset Systemic Lupus Erythematosus: Results from the SLICC Inception Cohort

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Abstract

Objective. In cross-sectional studies, elevated osteopontin (OPN) levels have been proposed to reflect, and/or precede, progressive organ damage and disease severity in systemic lupus erythematosus (SLE). We aimed, in a cohort of recent-onset SLE, to determine whether raised serum OPN levels precede damage and/or associate with disease activity or certain disease phenotypes.

Methods. We included 344 patients from the Systemic Lupus International Collaborating Clinics (SLICC) Inception Cohort who had 5-years of follow-up data available. All patients fulfilled the 1997 American College of Rheumatology (ACR) criteria. Baseline sera from patients and from age- and sex-matched population-based controls were analysed for OPN using ELISA. Disease activity and damage were assessed at each annual follow-up visit using the SLE Disease Activity Index 2000 (SLEDAI-2K) and the SLICC/ACR damage index (SDI), respectively.

Results. Compared to controls, baseline OPN was raised fourfold in SLE cases ($p<0.0001$). After relevant adjustments in a binary logistic regression model, OPN levels failed to significantly predict global damage accrual defined as SDI≥1 at 5 years. However, baseline OPN correlated with SLEDAI-2K at enrolment into the cohort ($r=0.27$, $p<0.0001$), and patients with high disease activity (SLEDAI-2K≥5) had raised serum OPN ($p<0.0001$). In addition, higher OPN levels were found in patients with persistent disease activity ($p=0.0006$), in cases with renal involvement ($p<0.0001$) and impaired estimated glomerular filtration rate ($p=0.01$).

Conclusion. The performance of OPN to predict development of organ damage was not impressive. However, OPN associated significantly with lupus nephritis and with raised disease activity at enrolment, as well as over time.

Systemic lupus erythematosus (SLE) is a multi-systemic inflammatory rheumatic disease that often shows periods of flares followed by remissions. Distinguishing ongoing inflammation attributed to SLE from established organ damage caused by the disease, medication or co-morbidities remains a challenge for the clinician. The spectrum of phenotypes complicates the search for biomarkers that adequately reflect active disease and/or increasing organ damage.

Osteopontin (OPN), an extracellular matrix protein with multiple functions, has been reported to be involved in inflammation (1). Local production and elevated circulating levels of OPN have been observed in several autoimmune diseases, such as multiple sclerosis (2), rheumatoid arthritis (3) and SLE (4, 5). Overexpression of OPN in lupus-prone mice induces B-cell activation and subsequent production of anti-dsDNA antibodies (6, 7), which is a hallmark of SLE. Intracellular OPN has been implicated in numerous cellular processes and its expression is required for toll-like receptor (TLR)-9-dependent production of interferon (IFN)-α (8), a central cytokine in the SLE pathogenesis (9).

Elevated OPN levels have been found to distinguish SLE from healthy individuals (4, 5, 10). Furthermore, associations between OPN and SLE disease activity (11) as well as with organ damage accrual (12) have been reported. In addition, elevated OPN levels have been suggested to precede the development of organ damage in a study including predominantly paediatric SLE cases (13). We have previously investigated serum OPN in a cross-sectional Swedish SLE cohort where OPN appeared to reflect current global organ damage (4). OPN was also found to associate with lupus nephritis, antiphospholipid syndrome (APS), as well as with individual clinical and laboratory criteria of APS. In addition, OPN levels showed significant correlations with SLE disease activity, particularly in newly diagnosed cases.

The aims of this study were to determine whether OPN (i) predicts future organ damage, (ii) reflects current and/or persistent disease activity, and (iii) associates with certain disease phenotypes using a longitudinal international inception cohort of recent-onset SLE.

Materials and Methods

The SLICC Inception Cohort

The Systemic Lupus International Collaborating Clinics (SLICC) Inception Cohort was recruited from 31 centres from 11 countries in North America, Europe and Asia from 2000-2011 as previously described (14, 15). Briefly, all clinical data were submitted to the coordinating centre at the University of Toronto and patients were reviewed annually. Laboratory tests necessary to evaluate disease activity, including complement proteins and autoantibodies, and parameters related to organ damage were performed at the recruiting centers. Exceptions for this were OPN and estimated glomerular filtration rate (eGFR) based on serum creatinine.

Patients and controls

SLE cases were enrolled within 15 months (mean 6 months, range 0-15) of SLE diagnosis, which was based on the fulfilment of at least 4 of the American College of Rheumatology 1997 (ACR-97) criteria (16). We selected patients from the inception cohort who had baseline serum available and for which there were 5 years of annual follow-up data completed. In addition, absence of organ damage at baseline was a requirement. Systemic Lupus Erythematosus Disease Activity Index 2000 (SLEDAI-2K) (17), clinical SLEDAI (scores for complement consumption and increased DNA binding subtracted from SLEDAI-2K), serological activity (scores for complement consumption and increased DNA binding only) and SLICC/ACR damage index (SDI) (18) were assessed at each visit. Patients with 'persistent disease activity' were defined as having SLEDAI-2K scores of ≥5 at ≥3 separate occasions

during the 5-year follow-up. At baseline, peripheral venous blood was drawn from each individual. Sera were prepared and stored at -70°C until analysed.

Sera from population-based controls matched 1:1 according to sex and age included in the EIRA cohort (acronym for the Swedish *Epidemiological Investigation of Rheumatoid Arthritis*) served as controls for the OPN analyses (19).

This study was approved by the SLICC data coordinating centre's institutional research ethics board at the University Health Network (File#: 00-0279). Each of the 33 participating centre's institutional research ethics boards approved the SLICC inception cohort study.

OPN immunoassay

A serum- and plasma-validated enzyme-linked immunosorbent assay (ELISA) kit (Quantikine®, R&D Systems, MN, USA) was used to analyse OPN levels in SLE and control sera. All OPN assays were performed in Linköping (Sweden), and the analyses were in accordance with the manufacturers' instruction. Briefly, serum (diluted 1:25) was added to microwells pre-coated with monoclonal antibodies directed against human OPN. After incubation and washing, a horseradish-peroxide conjugated polyclonal anti-OPN antibody was added and the plate incubated, followed by washing and addition of tetramethylbenzidine substrate. The enzymatic reaction was stopped by adding 2 N sulfuric acid and read at 450 nm (plate reader Sunrise, Tecan, Männedorf, Switzerland; software Magellan version 7.1, Tecan).

Creatinine and eGFR

Serum creatinine was determined using an enzymatic colorimetric method at the Clinical chemistry laboratory (Linköping University Hospital, Sweden). The 4-variable modification of diet in renal disease (MDRD) equation was used to calculate eGFR (20).

Statistics

Sample-size calculation (for comparing two groups) revealed that sera from 208 individuals were needed to detect a significant difference in OPN levels between SLE patients with versus without any organ damage at follow-up. This calculation was based on: (i) a power of 80%; (ii) a standard deviation (SD) of 36.8 ng/mL, which was the OPN level (SD) in patients with permanent organ damage using data from our pilot-study (4); and (iii) the approximation that at least 25% of the SLE patients would develop any kind of organ damage during the 5-year follow-up.

Independent samples t-tests were used to evaluate differences in OPN levels between SLE patients and controls, and between patients meeting and not meeting specific ACR criteria.

Pearson correlation analyses between OPN and disease activity measures (erythrocyte sedimentation rate, SLEDAI-2K, clinical SLEDAI and serological activity) as well as between OPN and the total number of fulfilled ACR criteria were performed. Significant associations were further analysed in a univariate general linear model (GLM) with adjustment for age, sex, race/ethnicity and daily glucocorticoid dose at baseline. In addition, the association between OPN and nephritis was adjusted for eGFR.

ANOVA was used to evaluate differences in OPN levels between patients with ‘no damage’, ‘moderate damage’ and ‘extensive damage’.

Binary logistic regression was used to predict damage accrual (global SDI, as well as organ domains of SDI) with adjustments for baseline data on age, sex, race/ethnicity, SLEDAI-2K and glucocorticoid therapy. Binary logistic regression was used to predict persistent disease activity with adjustments for baseline data on age, sex, race/ethnicity and glucocorticoid therapy.

Statistical significance was set at $p<0.05$, along with 95% confidence interval (CI). Statistical analyses were performed with SPSS Statistics 22 (IBM, Armonk, NY, USA) or GraphPad Prism, version 5.04 (GraphPad Software, La Jolla, CA, USA).

Results

There were a total of 344 SLE cases (315 women and 29 men; mean age 34.0 years, range 12-73) included in the study. The majority of patients (n=200, 58%) were of Caucasian ethnicity. Of the 344 controls (315 women and 29 men; mean age 34.4 years, range 15-73), 327 (95%) were of Caucasian race/ethnicity. Detailed characteristics of the study populations are found in Table 1.

Baseline OPN levels are increased in SLE

Circulating levels of OPN were markedly higher in SLE patients (mean 45.4 ng/ml, 95% CI 41.4-49.4) than in the controls (mean 11.8 ng/ml, 95% CI 10.4-13.3, p<0.0001; Figure 1A). OPN levels correlated inversely with age, both among the patients (r=-0.17, p=0.002) and the controls (r=-0.27, p<0.0001). No differences were observed between men and women among the controls regarding OPN levels. However, among SLE patients, men displayed higher OPN levels (mean 79.5 ng/ml, 95% CI 47.0-111.9) compared to women (mean 42.3 ng/ml, 95% CI 39.1-45.4, p<0.0001). There were no significant differences in baseline disease activity (SLEDAI-2K) between men (mean 4.3, 95% CI 2.77-5.9) and women (mean 5.0, 95% CI 4.5-5.6, p=0.45). However, clear differences in OPN levels were identified between patients of Caucasian race/ethnicity (mean 38.2 ng/ml, 95% CI 34.7-41.7) compared to non-Caucasians (mean 55.4 ng/ml, 95% CI 47.4-63.5, p<0.0001). Such difference was not found among the controls (Caucasians mean 11.8 ng/ml, 95% CI 10.3-13.3; non-Caucasians mean 12.3 ng/ml, 95% CI 5.5-19.1; p=0.87). Patients of non-Caucasian race/ethnicity had higher disease activity (mean 6.1, 95% CI 5.2-7.0) compared to Caucasians (mean 4.1, 95% CI 3.5-4.7, p=0.0002).

OPN failed to predict damage accrual in adjusted analyses

At the 3-year follow-up visit, 63 (18%) SLE patients had developed any damage (i.e. SDI≥1), and 98 (29%) showed damage after 5 years. Since only 18% had an SDI score of ≥1 3 years post inclusion, we focused mainly on the 5-year data. A weak correlation was found between baseline OPN and damage accrual after 5 years (r=0.15, p=0.006). However, in a binary logistic regression analysis with adjustments OPN levels failed to predict future global damage when defined as SDI≥1 with a ROC area under curve (AUC) of 0.67 (p=0.061; Table 2). Examining each domain of SDI separately rendered no statistically significant association with OPN levels. However, age and SLEDAI-2K at baseline significantly predicted organ damage development at 5 years (Table 2).

We did not identify any significant differences in baseline OPN levels when separating patients' SDI after 5 years into 'no damage' (i.e. SDI=0, n=246, mean 41.9 ng/ml, 95% CI 38.6-45.3), 'moderate damage' (SDI 1-2, n=84, mean 52.5, 95% CI 40.0-65.1) and 'extensive damage' (SDI≥3, n=14, mean 63.4 ng/ml, 95% CI 38.6-88.2).

OPN reflects disease activity and renal involvement

Baseline OPN correlated with SLEDAI-2K (r=0.27, p<0.0001), clinical SLEDAI (r=0.22, p<0.0001) and serological activity (r=0.24, p<0.0001) at enrolment into the cohort. Using a binary variable for anti-dsDNA (positive/negative) showed that patients positive for anti-dsDNA had significantly higher OPN levels (mean 55.7 ± 3.8 ng/ml, n=150) compared to those that were negative (mean 37.4 ± 1.9, n=194), p<0.0001. Patients with low

complement (C3 and/or C4) had higher levels of OPN (mean 54.7 ± 4.1, n=130) compared to those with normal complement (mean 39.7 ± 2.1, n=214), p=0.0003. Patients with a SLEDAI-2K score of ≥5 had higher levels of OPN (mean 56.6 ng/ml, 95% CI 48.3-64.9) than patients with SLEDAI-2K<5 (mean 38.5 ng/ml, 95% CI 34.7-42.2; p<0.0001; Figure 1B). The erythrocyte sedimentation rate correlated with OPN (r=0.38, p<0.0001). The above-mentioned associations remained significant after adjustments for age, sex, race/ethnicity and glucocorticoid therapy in a univariate GLM analysis.

We further evaluated associations with different disease phenotypes (i.e. fulfilled ACR criteria). Only the renal disorder criterion (ACR-7) reached statistical significance with higher levels of OPN (mean 63.7 ng/ml, 95% CI 49.9-77.5, n=75) compared to those without renal involvement (mean 40.3 ng/ml, 95% CI 37.1-43.5, n=269; p<0.0001; Figure 2A). The association with nephritis remained significant after adjustments for age, sex, race/ethnicity, glucocorticoid therapy and eGFR in a univariate GLM analysis. 83 patients had an impaired eGFR (≤90 mL/mean/1.73 m^2), but only 12 patients had an eGFR below 60. Higher levels of OPN were found in patients with an impaired eGFR (mean 54.6 ng/ml, 95% CI 42.3-66.9, n=83) compared to those with eGFR above 90 (mean 42.5, 95% CI 38.9-46.0, n=261; p=0.01; Figure 2B). Of the 75 patients meeting the renal ACR criterion, patients with an impaired eGFR had higher OPN levels (mean 96.8 ± 24.5 ng/ml, n=18) compared to those with normal eGFR (mean 53.3 ± 4.2, n=57), p=0.006. A weak correlation between OPN levels and the total number of fulfilled ACR criteria (r=0.17, p=0.001) was identified.

OPN predicts persistent disease activity

To further examine the association between OPN and disease activity, we separated patients based on persistent disease activity (defined as SLEDAI-2K scores of ≥5 at ≥3 separate occasions during the 5-year follow-up). Higher levels of OPN were found among the 51 patients (15%) with persistent disease activity (mean 62.0 ng/ml, 95% CI 43.8-80.5) compared to those without (mean 42.5 ng/ml, 95% CI 39.08-45.9; p=0.0006; Figure 3A). To evaluate the possible impact of organ damage on OPN levels in cases with persistent disease activity (n= 51), those patients who had developed any damage (i.e. SDI≥1) after 5 years (n= 18) were compared to those without any damage (n=33). No statistically significant difference in OPN levels was observed (Figure 3B).

Using a binary logistic regression model with adjustments, OPN levels were associated with persistent disease activity (p=0.011, AUC=0.66; Table 3). Further adjustment for damage (SDI) at 5 years did not change this association (p=0.012, AUC=0.66).

Discussion

In SLE, OPN has been proposed as a useful biomarker of disease activity (4, 11), as well as of organ damage (4, 12, 13). Most previous studies had a cross-sectional design, but in the present study we aimed to dissect whether baseline OPN levels could be predictive of future organ damage in a longitudinal cohort. Our results confirm some of the previous reports indicating that OPN is associated with disease activity and lupus nephritis, rather than being a marker of future damage progression.

In line with previous findings by our group and others (4, 5, 10), OPN levels were elevated in SLE patients compared with population-based healthy controls. According to Rullo *et al.*, increased circulating OPN levels have been reported to precede increased 'cumulative' disease activity and organ damage in SLE patients especially in paediatric SLE (13). In a cross-sectional pilot study, we evaluated OPN in a cohort of Swedish SLE patients and found that circulating OPN levels were associated with global organ damage (4). In the present study, OPN levels at entry into the SLICC cohort were not significantly associated with damage accrual after 5 years, using SDI≥1 as cut-off. However, a larger group of patients with more extensive damage accrual observed during a longer time period is required to further resolve this issue.

Since OPN showed an inverse correlation with age, and because differences were observed between men and women, as well as between Caucasians and non-Caucasians, these factors were adjusted for in the statistical analyses. Rullo *et al.* (13) reported that high circulating OPN levels preceded increased 'cumulative' SLE disease activity and organ damage over 12 months. In contrast to their study, the SLICC inception cohort consists mainly of adult SLE cases, and there may also be differences between the studies regarding

race or ethnicities which could have affected the divergent conclusions of OPN levels as a potential biomarker of future organ damage.

In line with earlier reports, we observed an association between OPN and disease activity, using the SLEDAI-2K (4, 5). In our previous pilot study, we noted a robust correlation between SLEDAI-2K and OPN ($r=0.67$, $p=0.028$) when we restricted the analysis to patients with recent-onset disease (4). In the present study, patients with active disease (i.e. SLEDAI-2K≥5) had higher OPN levels compared to those with no/low disease activity (i.e. SLEDAI-2K<5), and higher OPN levels were also found in patients with persistent disease activity.

We further investigated associations of baseline OPN with different clinical manifestations. Patients meeting the lupus nephritis criterion displayed higher levels of OPN, which corroborates the finding in our pilot study (4). Patients with impaired renal function had higher OPN levels, but we did not find an association between OPN and the renal domain of SDI. However, such relation has previously been reported (4, 11, 12), and lupus-prone mice with nephritis have been shown to express OPN associated with macrophage infiltration (21). Furthermore, anti-OPN therapy in nephritic rats reduces albuminuria and invasion of macrophages (22), and OPN knock-out mice have less recruitment of macrophages as well as reduced renal fibrosis (23).

The reason for elevated OPN in SLE remains unclear, but it could be of relevance to the SLE pathogenesis that the intracellular expression of OPN in plasmacytoid dendritic cells (pDCs) is required for TLR-9-dependent production of IFN-α (8). In addition, mutations in tartrate-resistant acid phosphatase (TRAP) cause spondyloenchondrodysplasia, an unusual recessive disease associated with short stature, brain calcifications and lupus-like autoimmunity (24). OPN is a substrate for TRAP, and TRAP has been shown to co-localize and physically interact

with OPN in pDCs and macrophages (25). Lack of TRAP leads to hyperphosphorylation of OPN and enhanced TLR-9 signalling in pDCs with subsequent IFN-α production, which can cause the lupus-like autoimmunity seen in spondyloenchondrodysplasia patients. Thus, future studies focusing on potential associations between IFN-α and OPN in SLE are highly warranted.

The present study has several strengths, especially the extremely well-characterised SLE population and the prospective study design using a large international inception cohort of SLE patients with 5 years of follow-up data. Some limitations should also be mentioned. Although all cases were incident and enrolled up to 15 months from diagnosis (mean time 6 months), it cannot be excluded that the baseline sample may have been taken at a time-point when the patient already received immunosuppressive therapy or antimalarials. Even though the control subjects were matched according to sex and age, the great majority (95%) were Caucasians which did not reflect the race/ethnicity distribution of the SLE cases (58% Caucasians). Thus, it cannot be excluded that this difference, as well as the potential impact of environmental factors, may have influenced the disparity of OPN levels between patients and controls. The relatively small number of damage events over 5 years probably reflects well-controlled patients, but generates uncertainties in predicting damage accrual. Finally, OPN was analysed at baseline only and we acknowledge that the predictive value of OPN for different outcome measures (such as SLE flares or damage accrual) may vary over time in established disease.

To summarize, in early SLE, OPN is elevated and appears to be associated with renal involvement and higher disease activity at sampling, as well as over time. We found no distinct association with accumulation of organ damage. Based on this, we suggest that

raised OPN at SLE onset identify cases with risk of high and persistent disease activity but may not necessarily lead to accrual of damage within 5 years of follow-up.

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Figure legends

Figure 1. Serum osteopontin (OPN) levels. (A) Baseline levels of OPN were significantly higher among patients with SLE (mean 45.4 ng/ml, n=344) compared to controls (mean 11.8 ng/ml, n=344). **(B)** Patients with raised disease activity (SLEDAI-2K≥5) had higher baseline levels of OPN (mean 56.6 ng/ml, n=131) than patients with low/no disease activity (SLEDAI-2K<5; mean 38.5 ng/ml, n=213).

Figure 2. Serum osteopontin (OPN) levels in SLE cases with or without renal involvement. (A) Patients meeting the renal disorder criterion (ACR-7) had significantly higher baseline levels of OPN (mean 63.7 ng/ml, n=75) compared to those without renal involvement (mean 40.3 ng/ml, n=269). **(B)** Higher OPN levels were found in patients with impaired eGFR (mean 54.6, n=83) compared to those with normal eGFR (mean 42.5, n=261).

Figure 3. Baseline osteopontin (OPN) levels in patients with persistent disease activity. (A) Higher levels of OPN were found in the 51 patients with persistent disease activity (mean 62.0 ng/ml) compared to those without (mean 42.5 ng/ml, n=293). **(B)** To investigate the possible impact of damage on OPN levels in cases with persistent disease activity, we compared patients who had developed any damage (i.e. SDI≥1) after 5 years to those without any damage. No significant difference in OPN levels was observed between patients with any damage (mean 81.5 ng/ml, n=18) compared with those without (mean 51.4 ng/ml, n=33).