

# Osteopontin might be involved in bone remodelling rather than in inflammation in ankylosing spondylitis

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**Objectives.** To determine whether osteopontin (OPN) is increased in patients with AS and to investigate its relationship to inflammatory disease activity and bone remodelling process.

**Methods.** This cross-sectional study included 30 patients with AS and 23 age- and sex-matched healthy controls. We assessed clinical characteristics and laboratory parameters including the ESR, CRP, lipid profiles, the Bath AS disease activity index (BASDAI) and the Bath AS radiographic index (BASRI). To evaluate bone metabolism, we tested ALP, OCN and C-telopeptide of type I collagen (CTX-I). Plasma levels of OPN, TNF- $\alpha$  and IL-6 were measured by ELISA, and mRNA expression in peripheral blood mononuclear cells (PBMCs) was performed by RT-PCR. Changes in OPN level were also evaluated in eight patients after the treatment with a TNF- $\alpha$  blocker.

**Results.** Patients with AS had significantly higher plasma OPN, TNF- $\alpha$  and IL-6 levels and more mRNA expression than healthy controls. Plasma OPN levels were correlated with serum ALP, OCN and CTX-I levels, but not with ESR, CRP, lipid profiles, BASDAI or BASRI. Treatment with a TNF- $\alpha$  blocker did not alter OPN levels, although it reduced the disease activity.

**Conclusions.** Patients with AS had higher levels of OPN compared with controls. The plasma OPN level was correlated with serum ALP, OCN and CTX-I levels, but not with disease activity in AS. OPN might be involved in bone remodelling rather than in inflammation in AS.

**KEY WORDS:** Osteopontin, Ankylosing spondylitis, Bone remodelling, Disease activity.

## Introduction

The inflammatory process of AS usually involves the sacroiliac joints, and the vertebral column is typically affected as the disease progresses. The production of several pro-inflammatory cytokines, such as TNF- $\alpha$  and IL-6, is increased in patients with AS [1–3]. The findings that TNF- $\alpha$  is overexpressed in sacroiliac joints and IL-6 levels are correlated with ESR and CRP in the patients with AS suggest that these cytokines are associated with the process of inflammation [1–4]. In addition to spinal inflammation, a characteristic feature of AS is new bone growth that leads to syndesmophyte formation and ankylosis of the vertebrae [5]. Moreover, it has been reported that the majority of patients with AS had reduced bone density, and patients with syndesmophytes have greatly reduced bone density than do those without [6]. This suggests that bone growth and bone loss occur in parallel in AS patients. However, the pathogenesis of this process remains obscure.

Osteopontin (OPN) is a secreted phosphoglycoprotein weighing ~34 kDa that was identified from the bone's extracellular matrix [7]. OPN is expressed by various cell types, including osteoclasts, osteoblasts, chondrocytes, macrophages, activated T cells, smooth muscle cells and epithelial cells, and is present in several tissues including bone, kidney, placenta, smooth muscle and secretory epithelia [8–10]. There is evidence suggesting that OPN acts as a pro-inflammatory cytokine and plays an important role in regulating inflammation [11]. It can be induced by inflammatory mediators such as IL-1, TNF- $\alpha$  and platelet-derived growth factor [8]. At sites of inflammation, it promotes macrophage infiltration and dendritic cell migration to lymph nodes [12, 13]. OPN is concerned in human atherosclerosis formation, especially in advanced atherosclerotic plaque, may act as positive regulators of vascular calcification [14, 15]. OPN is also involved in the bone remodelling process [16–19]. Bone cells secrete OPN

physiologically during the process of bone remodelling. Osteoclasts may be the source of OPN in the cement lines of bone during remodelling [16, 19]. The function of OPN in osteoclasts may be to promote cell adhesion and chemotaxis during bone resorption [16, 20]. In addition, OPN exists *in situ* in osteoblasts and accumulates in mineralized bone matrix during endochondral and intramembranous ossification [16, 18]. OPN enhances osteoblastic differentiation and proliferation, and increases ALP activity [21–23]. OPN is thought to be a candidate molecule for the bone remodelling process in AS, in that it can induce both bone formation and resorption.

In the murine CIA model, it has been reported that plasma OPN levels and expression of OPN mRNA in peripheral blood mononuclear cells (PBMCs) are markedly elevated at points corresponding to arthritis flares [24, 25]. Moreover, the use of specific antibodies reacting to OPN could inhibit inflammatory cell infiltration and bony erosion in arthritic joints [25]. Overexpression of plasma OPN protein and OPN mRNA in PBMC have also been reported in human RA [26, 27].

These observations led us to hypothesize that OPN may be involved in the pathogenesis of AS through inflammation and/or the bone remodelling process. Therefore, we examined whether OPN was increased in patients with AS and assessed the association between OPN expression and disease activity parameters, bone remodelling markers and the expression of pro-inflammatory cytokines such as TNF- $\alpha$  and IL-6. We found that OPN might be involved in bone remodelling rather than in inflammation in AS patients.

## Methods

### Study design and patient population

This cross-sectional study included 30 patients with AS and 23 healthy controls. Consecutive patients with AS were selected from an outpatient rheumatology clinic at Severance Hospital, Seoul, Korea. The study was approved by the institutional ethics committee and we obtained informed patient consent from all of the patients and healthy volunteers. Patients diagnosed with AS according to the 1966 New York criteria were included in the study. All patients were selected independently of disease activity status. Exclusion criteria were systemic diseases such as diabetes mellitus, congestive heart failure, pregnancy, infectious process,

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Submitted 23 May 2008; revised version accepted 4 September 2008.

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impaired renal function (creatinine clearance <60 ml/min), elevated hepatic enzyme levels (twice the upper limit of normal level) and history of anti-TNF- $\alpha$  treatment (infliximab, etanercept or adalimumab) prior to this study. Controls were healthy individuals matched for age and sex. We followed OPN expression in eight patients who were treated with a TNF- $\alpha$  blocker after enrolment in this study to compare OPN levels before and after anti-TNF- $\alpha$  treatment.

#### Clinical and laboratory assessment

Age, sex, medication history, BMI, duration of disease and extra-articular manifestations of AS were recorded. Complete blood count and routine biochemical analysis, including lipid profiles and HLA-B27, were studied. We evaluated the following disease activity markers: ESR, CRP and Bath AS disease activity index (BASDAI). Bath AS radiology index (BASRI) was evaluated for grading radiographic changes. ALP and OCN were measured to assess bone formation, and serum C-telopeptide of type I collagen (CTX-I) was measured to assess bone resorption (ALP; Eiken Chemical Co., Tokyo, Japan; OCN, CTX-I; Roche Inc., Mannheim, Germany). OCN and CTX-I were measured by electrochemiluminescence immunoassays (ECLIAS) according to the manufacturer's recommendations. The intra- and interassay coefficients of variation (CVs) of OCN were 1.4–4.0% and 1.8–6.5%, respectively. Intra- and interassay CVs were 1.0–4.6% and 1.6–4.7% for CTX-I, respectively.

#### Plasma protein and cytokine analysis

Plasma concentrations of OPN, TNF- $\alpha$  and IL-6 were measured by sandwich ELISAs (R&D Systems Inc., Minneapolis, MN, USA) according to the manufacturer's recommendations. The intra- and interassay CVs of OPN were 2.9–4.0% and 5.4–6.6%, respectively. Intra- and interassay CVs were 4.6–5.2% and 5.4–7.4% for TNF- $\alpha$ , and 3.0–5.8 and 6.3–9.6% for IL-6. All assays represented duplicate measurements. A standard curve was drawn by plotting OD vs the log of recombinant OPN, TNF- $\alpha$  and IL-6 concentrations, and the coefficient of determination ( $R^2$ ) of these were 0.9970, 0.9943 and 0.9993, respectively.

#### RNA extraction and RT-PCR analysis of gene expression of OPN and cytokines

Expressions of OPN and cytokine mRNA were measured by RT-PCR using specific primers and probes in all AS patients and healthy controls (OPN: forward 5'-GGAACAAGGATAGG TAGGCT-3', reverse 3'-AGTTTAGCTTTGTGATTTTCG-5'; TNF- $\alpha$ : forward 5'-GTGGAGATCTCTTCTTGAC-3', reverse 3'-AGTGCCCTTAACATTCTAA-5'; IL-6: forward 5'-ACTCA CCTCTTCAGAACGAA-3', Reverse 3'-GTCTCCTCATTGAA TCCAGA-5'). PBMCs were obtained by Ficoll-Hypaque density gradient centrifugation. Total RNA was extracted from PBMCs using TRIzol reagent (Life Technologies, Mississauga, ON, Canada). One microgram of RNA was combined with superscript II (Invitrogen, Burlington, ON, Canada) in a 20- $\mu$ l reaction volume for 30 min at 42°C and then heated to 95°C for 4 min to inactivate the enzyme and denature RNA-cDNA hybrids. cDNA amplification was performed at a final concentration of 1  $\times$  PCR buffer, 1.5 mmol/l MgCl<sub>2</sub>, 200  $\mu$ mol/l deoxynucleoside triphosphate (dNTP), 10 pmol/l of TNF- $\alpha$ , IL-6, OPN primers or forward 5'-GAAGGTGAAGGTGGAGT-3', reverse 3'-GAAGATGGTGGATTTTC-5' (GAPDH) primers and 1.25 U of AmpliTaq DNA polymerase (Promega Corp., Madison, WI, USA) in a total volume of 50  $\mu$ l. Amplification condition were as follows: an initial denaturation step at 94°C for 2 min, followed by denaturation at 94°C for 30 s, primer annealing at 50°C for 30 s, and extension at 72°C for 1 min. PCR samples were performed in 50  $\mu$ l volumes, and each PCR sample underwent a 30-cycle amplification (28-cycle amplification for

GAPDH), which ensured that the reactions did not reach the plateau phase. Final PCR products were electrophoresed on 1.5% agarose gel (ImageMaster VDS; Amersham Biosciences, Uppsala, Sweden) containing 0.2  $\mu$ g/ml ethidium bromide. mRNA bands were quantitated by densitometry using the TINA 2.0 Image program and normalized to GAPDH.

#### Statistical analysis

The Student's *t*-test and chi-square test were used to compare baseline demographic and clinical data and differences in means in OPN, TNF- $\alpha$  and IL-6 plasma levels and mRNA expression between AS patients and controls. The Mann-Whitney test was used to compare plasma levels and mRNA expression of OPN, TNF- $\alpha$  and IL-6 between patients with AS and healthy controls. We used the Wilcoxon signed-rank test to compare OPN expression before and after TNF- $\alpha$  blocker treatment. The partial correlation coefficient adjusted for age and sex was used to evaluate the strength of association ( $\rho$ ) between OPN, TNF- $\alpha$ , IL-6 and clinical variables in patients with AS. Clinical variables in the correlation analysis were ESR, CRP, ALP, OCN, CTX-I, BASDAI and BASRI. Statistical significance was set at the level of  $P=0.05$ . All analyses were performed using SPSS version 12.0 (SPSS Inc., Chicago, Illinois, USA).

## Results

#### Patient characteristics

Thirty patients with AS were compared with twenty-three healthy controls. There was no significant difference in the mean age or sex ratio between the two groups. The clinical features and laboratory values of the AS patients are given in Table 1.

#### Plasma OPN and cytokine levels and serum bone remodelling marker levels

Plasma levels of OPN, TNF- $\alpha$  and IL-6 are shown in Table 2. Plasma levels of OPN were higher in patients with AS than in controls ( $P<0.001$ ) as were plasma levels of TNF- $\alpha$  and IL-6 ( $P<0.001$  and  $<0.001$ , respectively). Serum levels of ALP and OCN in patients with AS were more elevated compared with controls ( $94.17 \pm 31.78$  vs  $47.09 \pm 11.41$  IU/l,  $P<0.001$ ;

TABLE 1. Basic characteristics of AS patients ( $n=30$ )

	Min.	Max.	Mean $\pm$ s.d.
Age, yrs	18	63	33.4 $\pm$ 12.7
Disease duration, yrs	0.6	30	7.0 $\pm$ 7.7
BMI, kg/m <sup>2</sup>	17.5	26.7	21.2 $\pm$ 3.5
BASDAI	1.0	9.1	5.1 $\pm$ 2.2
BASRI	2	12	6.0 $\pm$ 2.7
ESR, mm/h	2	120	40.1 $\pm$ 31.2
CRP, mg/dl	0.100	8.300	1.487 $\pm$ 1.591
Total cholesterol, mg/dl	92	240	160.8 $\pm$ 36.2
HDL cholesterol, mg/dl	19	68	48.2 $\pm$ 11.6
LDL cholesterol, mg/dl	35	159	92.1 $\pm$ 34.3
Triglyceride, mg/dl	35	328	104.4 $\pm$ 68.8
ALP, IU/l	59	208	90.1 $\pm$ 31.8
OCN, ng/ml	7.74	58.54	20.38 $\pm$ 11.54
CTX-I, ng/ml	0.064	0.900	0.333 $\pm$ 0.216
Sex, male, % <sup>a</sup>		25 (83.3)	
HLA-B27 positivity, %		28 (93.3)	
Peripheral joint arthritis, %		11 (36.7)	
Uveitis, %		5 (16.7)	
Enthesitis, %		4 (13.3)	
Current use of NSAIDs, %		27 (90.0)	
Current use of corticosteroid, %		3 (10.0)	
Current use of SSZ, %		18 (60.0)	

<sup>a</sup>One postmenopausal woman was included. HDL: high density lipoprotein; LDL: low density lipoprotein.

TABLE 2. Comparison of OPN, TNF- $\alpha$  and IL-6 plasma levels and mRNA expression in AS patients and healthy controls

	AS (n=30)	Control (n=23)	P
OPN, ng/ml	80.49 $\pm$ 16.98	26.99 $\pm$ 7.25	<0.001
TNF- $\alpha$ , pg/ml	62.99 $\pm$ 11.95	19.01 $\pm$ 4.79	<0.001
IL-6, pg/ml	40.67 $\pm$ 4.79	11.22 $\pm$ 1.47	<0.001
OPN mRNA (-fold)	123.90 $\pm$ 19.50	81.65 $\pm$ 19.51	<0.001
TNF- $\alpha$ mRNA (-fold)	118.78 $\pm$ 14.40	77.11 $\pm$ 24.51	<0.001
IL-6 mRNA (-fold)	123.64 $\pm$ 19.53	57.87 $\pm$ 19.79	<0.001

mRNA expression is represented as fold-induction compared with GAPDH. Results are shown as mean  $\pm$  s.d.

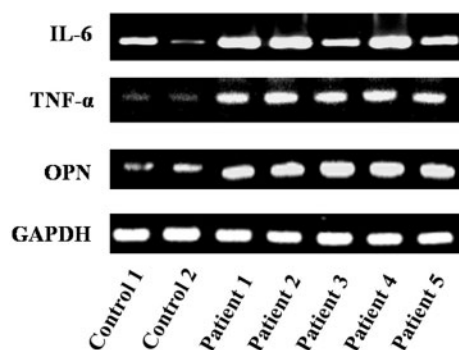


FIG. 1. OPN mRNA expression as well as TNF- $\alpha$  and IL-6 mRNA expressions from PBMCs are higher in patients with AS than in controls.

TABLE 3. The relationship between ESR, CRP, ALP, BASDAI and BASRI and the plasma levels of TNF- $\alpha$ , IL-6 and OPN

	OPN		TNF- $\alpha$		IL-6	
	r	P	r	P	r	P
ESR	0.091	NS	0.420	0.033	0.176	NS
CRP	0.275	NS	0.368	0.028	0.515	0.007
ALP	0.282	0.033	0.133	NS	0.394	NS
OCN	0.542	0.004	-0.062	NS	0.016	NS
CTX-I	0.600	<0.001	0.279	NS	0.285	NS
BASDAI	0.020	NS	0.006	NS	0.278	NS
BASRI	-0.344	NS	0.215	NS	-0.012	NS

r = partial correlation coefficient adjusted for age and sex.

20.38  $\pm$  11.75 vs 15.53  $\pm$  3.81 ng/ml,  $P=0.046$ , respectively). The serum CTX-I level was also elevated in patients with AS compared with controls (0.33  $\pm$  0.22 vs 0.21  $\pm$  0.10,  $P=0.009$ ).

#### OPN and cytokines mRNA expression in PBMC

OPN, TNF- $\alpha$  and IL-6 mRNA expression in PBMCs are shown in Fig. 1. OPN mRNA expression was higher in patients with AS than in controls ( $P < 0.001$ ). TNF- $\alpha$  and IL-6 mRNA expressions were also higher in patients with AS than in controls ( $P < 0.001$  and  $< 0.001$ , respectively; Table 2).

#### Correlation between plasma OPN, TNF- $\alpha$ and IL-6 levels and clinical parameters

Plasma OPN level did not show any correlation with either plasma TNF- $\alpha$  or IL-6 levels ( $r = -0.143$ ,  $P = 0.450$ ;  $r = 0.293$ ,  $P = 0.116$ , respectively). The relationships between clinical parameters and plasma OPN, TNF- $\alpha$  and IL-6 levels are shown in Table 3. When the OPN level was compared with ESR, CRP, BASDAI and BASRI, no correlations were found, but positive correlations were found between plasma OPN level and serum ALP, OCN and CTX-I levels ( $r = 0.416$ ,  $P = 0.028$ ;  $r = 0.566$ ,  $P = 0.001$ ;  $r = 0.619$ ,  $P < 0.001$ , respectively). Plasma OPN level had no correlation

with any of lipid profiles and BMI. Plasma TNF- $\alpha$  level had a positive correlation with ESR and CRP, and plasma IL-6 level had a positive correlation with CRP. BASDAI and BASRI did not correlate with plasma TNF- $\alpha$  or IL-6 levels.

#### Subgroup analysis of plasma OPN levels and OPN mRNA expression in AS

Subgroup analysis of plasma OPN levels and OPN mRNA expression were performed in patients with AS. Age, sex, disease duration, presence of peripheral arthritis, uveitis, enthesitis and spinal syndesmophyte and medication history had no correlation with plasma OPN levels or mRNA expression.

#### Comparison before and after treatment with TNF- $\alpha$ blocker

Eight of the thirty AS patients received treatment with a TNF- $\alpha$  blocker after enrolment in this study. Six were treated with etanercept (25 mg twice a week) and two with infliximab (3 mg/kg at 0, 2 and 6 weeks). Blood samples were taken after 2 months of etanercept treatment or before the third infusion of infliximab. ESR, CRP and BASDAI levels were significantly decreased after treatment with a TNF- $\alpha$  blocker. However, there was no change in plasma OPN or mRNA expression and in serum levels of APL, OCN and CTX-I (Fig. 2).

#### Discussion

In this study, we found that patients with AS had higher plasma levels of OPN compared with controls and that OPN mRNA expression was also higher in AS patients. This suggests that OPN might be related to AS in some way. Although there have been several reports showing that plasma OPN levels and mRNA expression in PBMCs are elevated in other disease states and animal models [24–27], there has been no study reporting elevated OPN levels and increased gene expression in patients with AS; this is the first study showing that OPN levels are elevated in patients with AS compared with healthy controls and suggests that OPN is involved in the pathogenesis of AS.

We first considered the possibility that OPN is related to the inflammatory process in AS. It has been reported that OPN is related to the arthritis flares in murine CIA [24, 25], and has a correlation with disease activity in patients with RA [26, 27]. In our study, however, OPN had no correlation with inflammation or disease activity in AS patients. Plasma OPN levels and mRNA expression in PBMCs had no correlation with ESR or CRP levels or with BASDAI, the validated parameters reflecting disease activity. On the other hand, the plasma levels of TNF- $\alpha$  and IL-6, well-known inflammatory cytokines in AS, were significantly elevated in patients with AS compared with healthy controls and correlated with ESR and CRP levels, which were similar to the previous reports that TNF- $\alpha$  and IL-6 levels are elevated and correlated with acute-phase reactants [1–3]. This result shows that, unlike TNF- $\alpha$  and IL-6, OPN is not directly involved in the inflammatory process in AS. To further investigate this dissociation, we followed plasma OPN levels, OPN mRNA expression and disease activity parameters in eight patients who were treated with a TNF- $\alpha$  blocker after enrolment in this study. Plasma OPN levels and mRNA expression in PBMCs were unchanged, although disease activity indices such as ESR and CRP levels and BASDAI were significantly decreased after treatment. This also supports the notion that OPN plays little or no role in inflammation or disease activity in AS. Interestingly, in RA, another classic example of chronic inflammatory arthritis, increased plasma OPN is well-correlated with disease activity [26, 27]. This observation led us to hypothesize that the role of OPN depends on the underlying disease, even though the diseases belong to the same category. Recently reported findings regarding



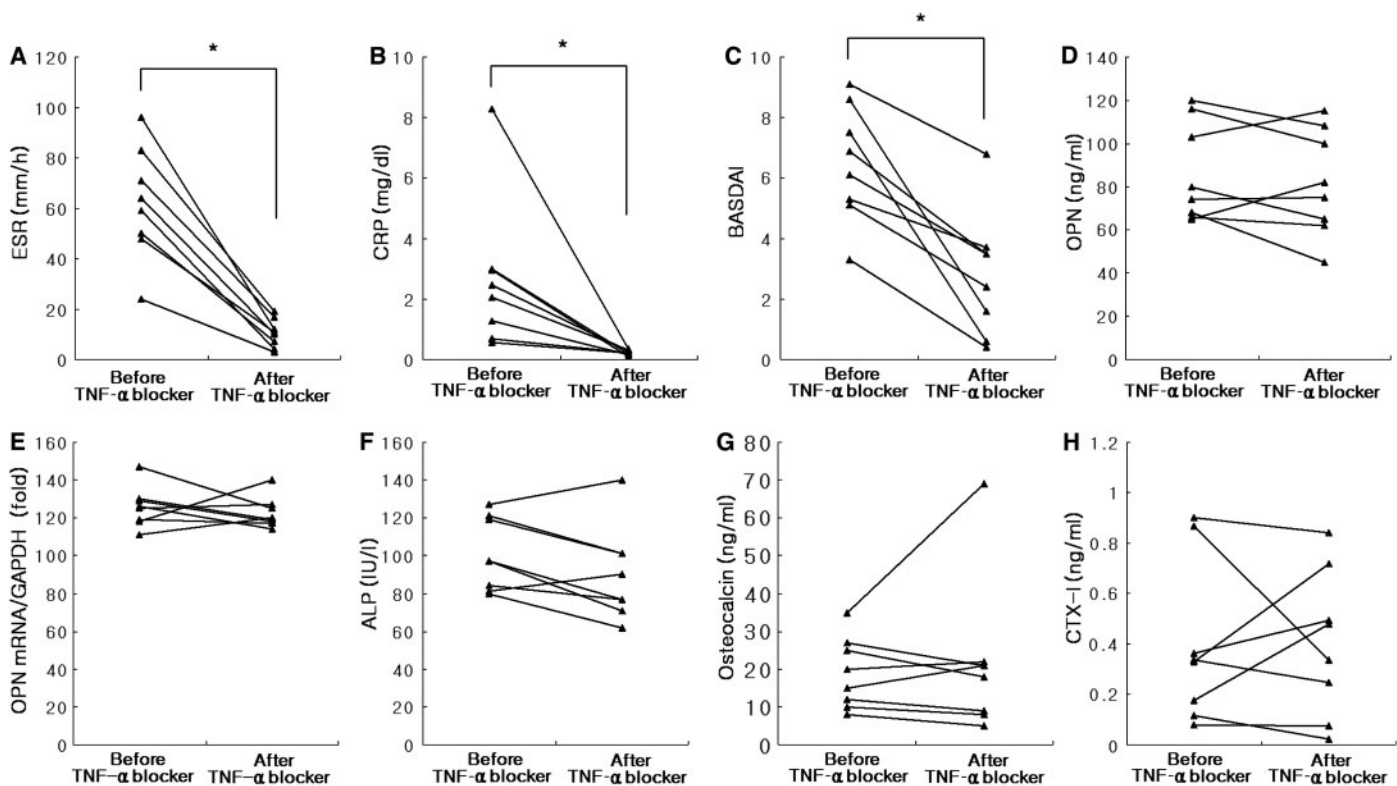


FIG. 2. ESR and CRP levels and BASDAI were checked along with plasma OPN, OPN mRNA in PBMCs and bone remodelling markers of eight patients with AS before and after treatment with a TNF- $\alpha$  blocker. Etanercept was used in six patients and infliximab was used in two patients. ESR, CRP and BASDAI decreased dramatically after treatment; however, there was no change in plasma OPN level or OPN mRNA expression, and in serum levels of bone remodelling markers. (A) ESR level; (B) CRP level; (C) BASDAI; (D) plasma OPN level; (E) OPN mRNA expression; (F) ALP level; (G) OCN level; (H) CTX-I level; \* $P < 0.05$ .

OPN in inflammatory bowel disease is one of the examples that the role of OPN differs depending on the underlying disease. Although the plasma OPN levels were elevated in patients with ulcerative colitis and Crohn's disease, representative inflammatory bowel diseases, a significant correlation between plasma OPN level and disease activity was observed only in the patients with ulcerative colitis, but not in the patients with Crohn's disease [28].

Another possible explanation for elevated OPN in AS patients is that OPN is an epiphenomenon of inflammation. That is, OPN is not involved in the inflammatory process even though it is stimulated by pro-inflammatory cytokines such as TNF- $\alpha$  or IL-6. However, because OPN had no correlation with TNF- $\alpha$  or IL-6 levels in AS patients, this is unlikely.

One possible role of OPN in AS is in the bone remodelling process. Although a characteristic feature of AS is new bone formation, it has also been reported that patients with syndesmophytes have a greater reduction in bone density than those without [5, 6]. Bone remodelling markers, such as serum levels of ALP, OCN and CTX-I, were elevated in the patients with AS compared with controls. We found a positive correlation between plasma OPN levels and two bone formation markers: serum ALP ( $r = 0.416$ ,  $P = 0.028$ ) and serum OCN ( $r = 0.566$ ,  $P = 0.001$ ). These results suggest that OPN in AS may be related to the formation of new bone such as syndesmophytes. Similarly, we found that plasma OPN levels were well correlated with serum CTX-I levels, a bone resorption marker ( $r = 0.619$ ,  $P < 0.001$ ). This suggests that OPN plays a role in bone loss as well as in bone formation in AS patients, which is supported by previous reports showing that OPN can promote osteoclastic activity [16, 19, 20], and can also enhance osteoblastic differentiation and proliferation to increase ALP activity [21–23].

The relationship between inflammation and bone damage in AS is not fully defined [29]. The reports that disease activity in AS

did not correlate with structural progression and that radiographic progression of AS was independent of the treatment with etanercept, along with our study, suggest that bone damage in AS can be progressive even if the inflammation is well controlled, and OPN is of concern in the structural progression in AS, independent of inflammation [30, 31]. However, we did not observe any correlation between plasma OPN levels and BASRI. We cannot explain this discrepancy, but believe that this may be because BASRI is a parameter representing graded radiographic changes due to chronic persistent stimuli rather than a single insult, and because new bone formation is not always correlated with the degree of inflammation. Further evaluation regarding the correlation between BASRI and the area under the curve of OPN will be useful in clarifying the role of OPN in the formation of syndesmophytes in AS.

OPN has been documented in atherosclerosis, especially in advanced atherosclerotic plaque formation [14, 15]. It is possible that the increased plasma OPN levels in the patients with AS were due to atherosclerosis, unrelated to the pathogenesis of AS. To rule out this possibility, we evaluated the lipid profiles and BMI of the patients with AS and compared them with the plasma OPN levels. The mean age and BMI of the patients were 33.4 yrs and 21.5 kg/m<sup>2</sup>, and the mean LDL cholesterol level was 92.1 mg/dl. There were only two patients taking a kind of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor for the control of hyperlipidaemia. Moreover, plasma OPN levels were not correlated with any of the lipid profiles and BMI. All these findings considered, we believe that the elevated plasma OPN levels were not related to the atherosclerosis formation in this study.

Our study had several limitations. The cohort number was not large enough for strong statistical analyses and BASDAI did not correlate with cytokine levels. However, the lack of correlation

between TNF- $\alpha$  or IL-6 levels and BASDAI have been reported in other, similar studies [1, 3]; BASDAI may be more affected by ongoing medical treatment than ESR and CRP levels.

In conclusion, we found that OPN was elevated in AS patients compared with healthy controls and that elevated OPN might play a role in the process of bone remodelling, rather than in inflammation, in AS. We believe that these findings will be helpful in understanding the pathogenesis of AS and in developing new drugs to modify bone remodelling in AS.

### Rheumatology key messages

- Patients with AS had higher levels of OPN compared with controls.
- OPN might be involved in bone remodelling rather than in inflammation in AS.

*Disclosure statement:* The authors have declared no conflicts of interest.

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