

# Immunohistochemical Analysis of Osteoblasts in Zygapophyseal Joints of Patients with Ankylosing Spondylitis Reveal Repair Mechanisms Similar to Osteoarthritis

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**ABSTRACT. Objective.** New bone formation of the spine is a typical feature of ankylosing spondylitis (AS). It is unknown whether new bone formation is part of a physiological repair process or a unique pathological entity of the disease.

**Methods.** We analyzed zygapophyseal joints from patients with AS and osteoarthritis (OA) undergoing spinal surgery for rigid hyperkyphosis (AS) or radiculopathy caused by severe OA. In 17 patients with AS, 11 with OA, and 5 controls we performed immunohistochemical analysis of osteoprotegerin (OPG), nuclear factor- $\kappa$ B ligand (RANKL), and osteocalcin (OC) expression in osteoblasts and determined the trabecular thickness in AS and OA patients and controls. Osteoclasts were detected by tartrate-resistant alkaline phosphatase (TRAP) staining.

**Results.** Trabecular thickness was significantly lower in patients with AS compared to OA ( $p = 0.01$ ). The absolute number of CD56+ osteoblasts ( $p < 0.001$ ) and OC+ ( $p = 0.002$ ), OPG+ ( $p = 0.003$ ), and RANKL+ osteoblasts ( $p = 0.03$ ) in AS patients was also significantly lower than in OA patients. The percentages of OC+, OPG+, and RANKL+ osteoblasts did not differ between AS and OA ( $p > 0.05$  in all cases). In controls, the percentages of OPG+ ( $p = 0.013$ ) and OC+ ( $p = 0.034$ ) but not RANKL+ ( $p > 0.05$ ) osteoblasts were significantly lower compared to AS patients. The frequency of TRAP+ osteoclasts in AS patients was significantly lower compared to OA ( $p < 0.001$ ), but higher compared to controls.

**Conclusion.** Immunohistochemical analysis of zygapophyseal joints suggested that osteoblast activity is similar in AS and OA, indicating that new bone formation is possibly a physiological function of repair in both diseases. (J Rheumatol First Release Feb 15 2010; doi:10.3899/jrheum.090986)

## Key Indexing Terms:

ZYGAPOPHYSEAL JOINT      ANKYLOSING SPONDYLITIS      OSTEOARTHRITIS  
IMMUNOHISTOCHEMICAL ANALYSIS           OSTEOBLASTS

Ankylosing spondylitis (AS) is a chronic inflammatory disease predominantly affecting the axial skeleton that can lead to bone erosions, new bone formation, and ankylosis of the

spine. The burden of disease is determined by the grade of acute inflammation causing inflammatory back pain and stiffness and by new bone formation causing a reduction of spinal mobility<sup>1,2</sup>. The mechanisms leading to new bone formation in AS and the link between inflammation and new bone formation is poorly understood, but is a matter of intensive discussion<sup>3,4</sup>.

Histopathological features of acute inflammation are well described: studies from intervertebral discs<sup>5</sup>, femoral heads<sup>6,7</sup>, sacroiliac joints<sup>8,9</sup>, manubriosternal junction<sup>10</sup>, and zygapophyseal joints<sup>11,12</sup> suggest that subchondral inflammation at the interface between bone and cartilage — a subchondral osteitis — is the primary site of the AS immunopathology. In recent immunohistochemical studies of femoral heads and zygapophyseal joints from patients with AS we have reported mononuclear cell aggregates, cartilage destruction, high microvessel density, and increased osteoclastic activity at sites of acute inflammation<sup>7,11</sup>.

This is the first study analyzing the mechanisms of new

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bone formation in AS in human bone samples derived from the spine by immunohistochemistry. We investigated whether there is a difference in the amount of osteoblasts, osteoblast function, and new bone formation in the spine of patients with AS and with OA. We chose zygapophyseal joints for analysis because they are available after surgical procedures in patients with AS and OA, and it is well described that the presence of syndesmophytes often goes together with an ankylosis of the zygapophyseal joints<sup>13,14</sup>. We concentrated our histopathological analysis on osteoblasts and indicators of osteoblast activation such as osteoprotegerin (OPG), osteocalcin (OC), and the receptor activator of nuclear factor- $\kappa$ B ligand (RANKL)<sup>15-18</sup>.

## MATERIALS AND METHODS

**Patients.** Zygapophyseal joints were obtained from 17 AS patients who had severe kyphosis with advanced ankylosis in the lumbar spine and who underwent surgery for polysegmental correction of rigid hyperkyphosis. Fourteen AS patients were male, the mean age was  $47.3 \pm 9.2$  years, mean disease duration  $21.4 \pm 8.9$  years; 76% of patients were HLA-B27+; 11 AS patients were taking nonsteroidal antiinflammatory drugs and 3 were taking disease modifying drugs; no AS patient was receiving tumor necrosis factor blockers. Spinal osteoporotic fractures and signs of diffuse idiopathic skeletal hyperostosis could be excluded radiographically<sup>19</sup>. Zygapophyseal joints were obtained from patients with OA ( $n = 11$ ) who underwent surgery of the lumbar spine because of neurological deficits in the lower limbs caused by compression of nerve roots. During this process either the superior or inferior articular processes or the entire zygapophyseal joint were removed. Eight of 11 patients were female, mean age 67 years (range 53–78 yrs).

All patients gave consent to participate in this study.

Control samples were taken from autopsies of 5 non-AS patients who died from cardiovascular diseases and had no history of spinal diseases.

The study was approved by the local ethical committee of the Charité Universitätsmedizin Berlin, Campus Benjamin Franklin.

**Tissue assessment.** The zygapophyseal joints were cut into slices and fixed in 4% buffered formalin. After decalcification with ethylene diaminetetraacetic acid, sections 4–6  $\mu$ m thick were prepared and stained by hematoxylin and eosin (H&E). Sections were examined using a microscope allowing light and immune fluorescence microscopy (BX60, Olympus, Germany).

**Immunohistochemistry.** Immunohistochemistry of the paraffin-embedded zygapophyseal joints was performed as described<sup>11</sup>, to detect CD56+ osteoblasts and tartrate-resistant acid phosphatase-positive (TRAP+; Sigma-Aldrich Co, Taufkirchen, Germany) osteoclasts.

RANKL was detected by a polyclonal rabbit antibody (dilution 1:20; 12.5  $\mu$ g/ml; Peprotech, Rocky Hill, NJ, USA) as validated by Pettit, *et al*<sup>20</sup> and as recently used in an immunohistochemical analysis of synovial membranes from patients with spondyloarthritis (SpA)<sup>21</sup>. In the latter report the antibody was further validated by Western blot analysis. OPG was detected by a monoclonal antibody (clone 98A1071, dilution 1:50, 10  $\mu$ g/ml; Acris, Hiddenhausen, Germany), also used in an analysis of synovial membranes from SpA patients<sup>21</sup>. OC was detected by a monoclonal antibody (clone 190125, dilution 1:50, 4  $\mu$ g/ml; R&D Systems). CD56+ osteoblasts were analyzed with a CD56 antibody (clone 123C3, dilution 1:200; Monosan, Uden, Netherlands). For control experiments, we omitted the primary antibodies and used isotype controls. Quantitative analysis was performed as described<sup>7,11</sup>. For quantification of CD56+ osteoblasts, OPG+ osteoblasts, OC+ osteoblasts, and RANKL+ osteoblasts, and TRAP+ osteoclasts in the bone marrow, 10 high-power fields (HPF) of one section per patient were analyzed and the absolute number was divided by 10 to obtain the average number of cells per one HPF. For this analysis, tissue sections were chosen with a detectable joint space, which were either partially (Figure 1A) or completely (Figure 1B and 1C) ankylosed, as well as

zygapophyseal joints with open joint spaces (Figure 1D). Areas in close vicinity to these joint spaces with predominant new bone formation, mostly in the absence of inflammatory cells, were analyzed.

Thickness of trabecular bone in zygapophyseal joints was analyzed in H&E stained tissue samples. A quantification was performed by measuring the mean relative amount of trabecular bone surface area as a percentage of the total bone marrow area in 10 HPF from each patient.

**Statistical analysis.** Mann-Whitney test was used for analysis and a  $p$  value  $< 0.05$  was considered significant.

## RESULTS

**Thickness of trabecular bone in zygapophyseal joints of AS patients.** We first addressed the question if there are general differences in the formation of trabecular bone in the zygapophyseal joints of AS and OA patients compared to controls without spinal diseases. In AS patients the thickness of trabecular bone was significantly lower ( $24.91\% \pm 13.4\%$  of total surface area) compared to OA patients ( $37.37\% \pm 12.5\%$ ;  $p = 0.01$ ), but higher compared to controls ( $17.0\% \pm 6.3\%$ ;  $p = 0.047$ ; Figure 2). The thickness of trabecular bone in OA patients compared to controls was also significantly higher ( $p < 0.001$ ). These data give evidence that new bone formation is increased in both spinal diseases, but clearly more in OA.

**Frequency of osteoblasts.** During bone formation under physiological conditions osteoblasts are the main cellular component, mostly under the control of Wnt signaling<sup>16</sup>. Therefore, we first analyzed the density of CD56+ osteoblasts in AS and OA patients and controls. The density of CD56+ osteoblasts in AS patients ( $13.49 \pm 3.72$  per HPF) was significantly lower than in OA patients ( $23.35 \pm 6.58$  per HPF;  $p < 0.001$ ) and did not differ from controls ( $15.64 \pm 1.76$ ; Figure 3).

**RANKL, OPG, and OC expression in osteoblasts in zygapophyseal joints.** Similarly, the absolute number of OC+ and OPG+ osteoblasts (AS: upper left panel of Figure 4A-4C, OA: lower left panel of Figure 4A-4C) in zygapophyseal joints was significantly lower in AS compared to OA ( $p = 0.02$ ,  $p = 0.001$ , respectively; upper right panels of Figure 4A, 4B), but significantly higher compared to controls ( $p = 0.001$ ,  $p = 0.003$ ; upper right panels of Figure 4A, 4B). In contrast, comparing the relative amount of osteoblasts for the whole group of osteoblasts attached to trabecular bone that was OC+ or OPG+, we found no significant differences between AS and OA ( $p > 0.05$  in all cases; lower right panels, Figure 4A, 4B); however, in controls the percentage of OC+ (vs AS:  $p = 0.003$ ) and OPG+ (vs AS:  $p = 0.013$ ) osteoblasts was significantly lower compared to AS patients (lower right panels, Figure 4A, 4B).

Similar to the analysis of CD56+ osteoblasts, the frequency of RANKL+ osteoblasts was also significantly higher in OA than in AS ( $p = 0.03$ ; upper right panel, Figure 4C) and did not differ significantly between AS and controls. Interestingly, there was no difference in the percentage of RANKL+ osteoblasts out of the whole group of osteoblasts between the groups ( $p > 0.05$  in all cases; lower right panel, Figure 4C).

**Frequency of osteoclasts in zygapophyseal joints.** The fre-

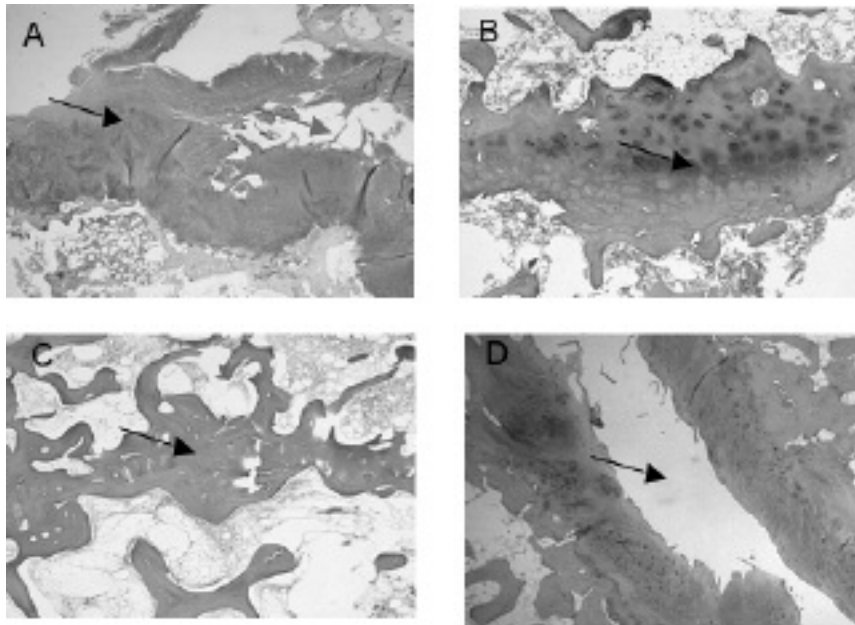


Figure 1. Tissue sections were chosen from zygapophyseal joints that were either partly (A) or completely (B, C) ankylosed as well as joints with open joint spaces (D).

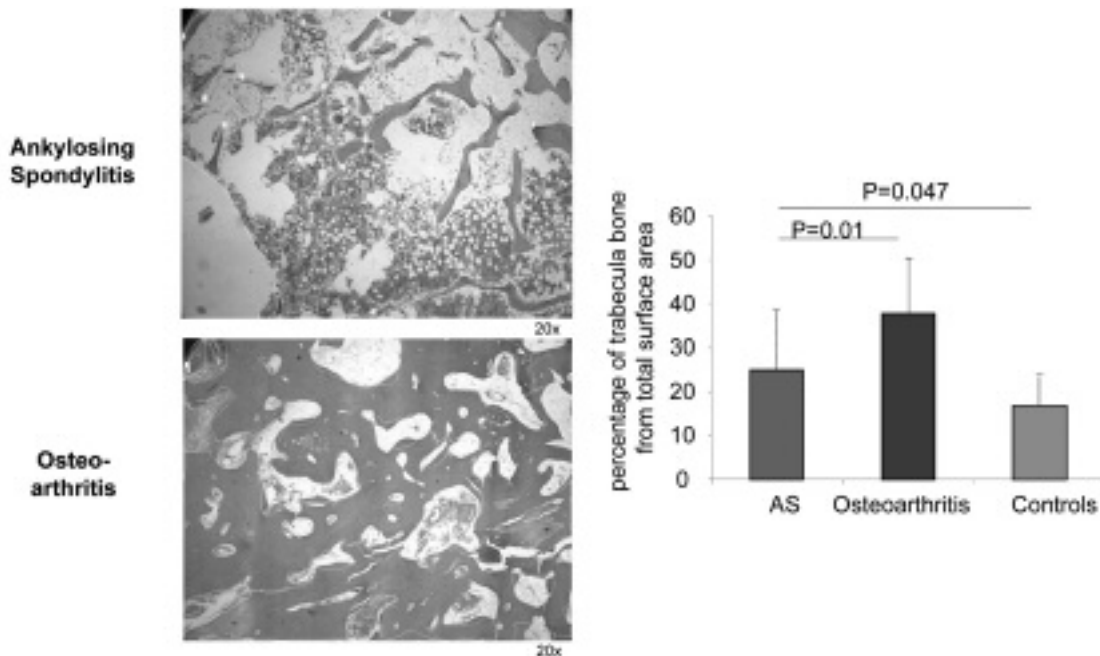


Figure 2. Thickness of trabecular bone in zygapophyseal joints of patients with AS and OA. The mean relative amount of trabecular bone surface area (indicated by thin white line in both panels, left) as a percentage of the total bone marrow area was significantly higher in OA compared to AS patients and autopsy samples, and higher in AS patients compared to controls without spinal diseases. H&E stain; original magnification 20 $\times$ , both photographs.

frequency of TRAP+ osteoclasts in zygapophyseal joints of AS patients was significantly lower compared to OA patients ( $p < 0.001$ ) but higher in comparison to autopsy samples ( $p < 0.001$ ; Figure 5).

## DISCUSSION

Longterm outcome of AS is predominantly determined by

the severity of ankylosis in the spine. Although there is agreement that the disease starts with inflammation in the sacroiliac joints and the spine, there has been continuing debate whether new bone formation in AS can be seen as a kind of physiological response to local damage caused by inflammation, or instead as abnormal bone remodeling with excessive new bone formation being a distinct part of AS<sup>4</sup>.

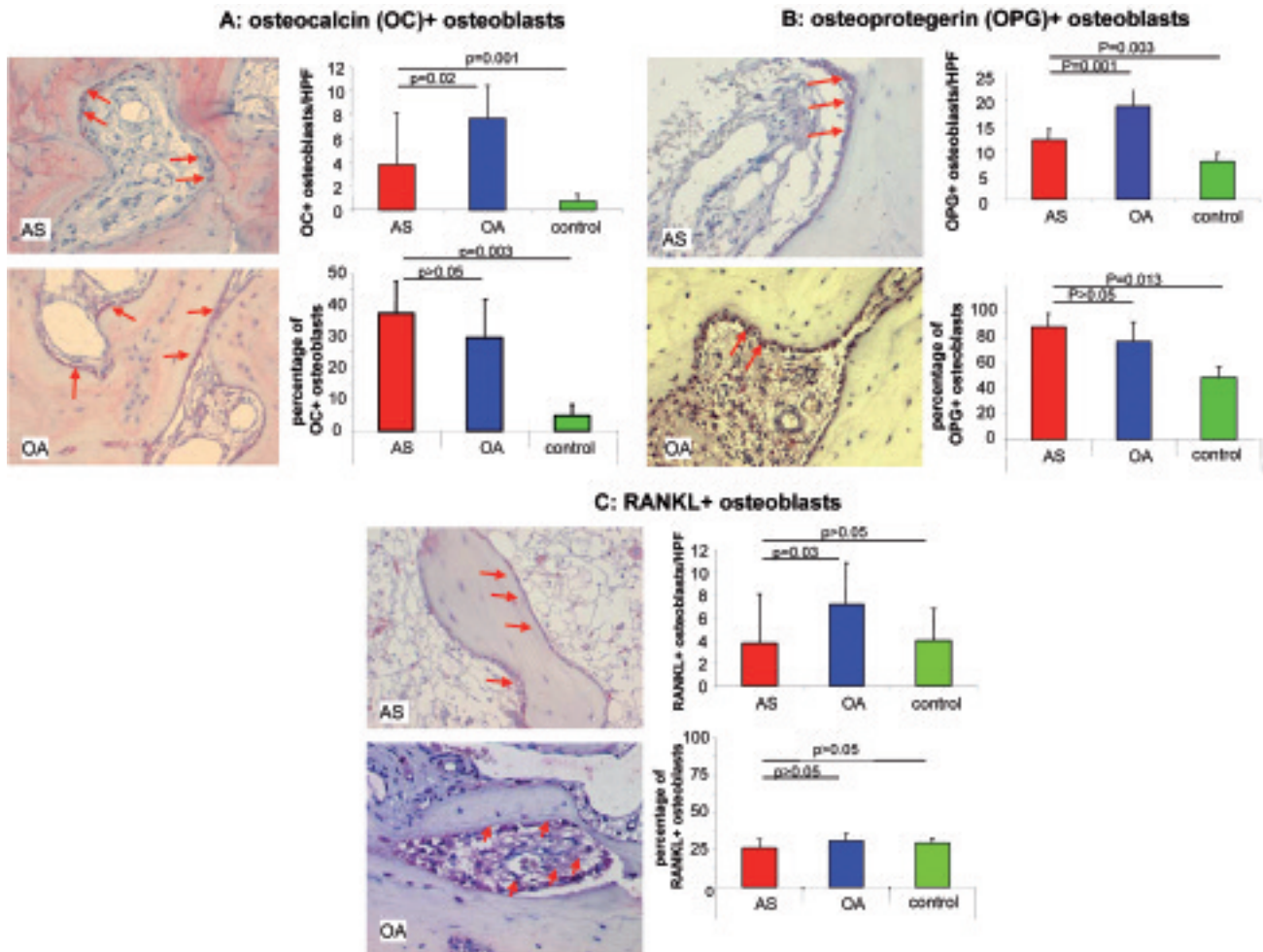


Figure 4. RANKL, OPG, and OC expression in osteoblasts in zygapophyseal joints of AS (A–C upper left panels), OA (A–C lower left panels), and controls. The absolute numbers of OPG+, OC+, and RANKL+ osteoblasts in zygapophyseal joints were significantly higher in OA than in AS (upper right panels, A–C). Percentages of OPG+ and OC+ osteoblasts in AS and OA did not reveal any significant differences and were significantly higher compared to controls (lower right panels, A–C). The percentages of RANKL+ osteoblasts did not differ among AS patients, OA patients, and controls (C).

We analyzed osteoblasts in immunohistological sections from zygapophyseal joints from AS patients in comparison to joints from patients with a degenerative spine disease (OA) in which new bone formation is also frequently observed<sup>22,23</sup>. As the most prominent result of our study, osteoblast activity was not clearly different between patients with AS and those with OA, but was clearly higher compared to zygapophyseal joints from autopsy samples without spine disease.

Osteoblasts are the key cellular component for bone formation, and they are counterbalanced by the bone-resorbing activity of osteoclasts. OA patients showed even higher numbers of osteoblasts and, accordingly, we observed greater thickness of trabecular bone in OA patients compared to AS. We further characterized the osteoblasts by analyzing the expression of OC, OPG, and RANKL in these cells. The percentage of OC- and OPG-expressing osteoblasts was similar in AS and OA patients, and was significantly higher than in controls. Interestingly, we observed no differences in the per-

centage of RANKL-expressing osteoblasts in AS, OA, and controls, indicating that the bone-degrading pathway was not downregulated in AS patients.

Zygapophyseal joints were chosen for investigation because they are available after surgeries of the spine in both diseases. Radiographic analysis and historical studies have shown that zygapophyseal joints are frequently involved during the process of ankylosis in AS<sup>13,14</sup>. Therefore, new bone formation in zygapophyseal joints of AS patients is believed to reflect new bone formation at other sites such as vertebral bodies<sup>13</sup>. The fact that we studied osteoblasts and new bone formation at the trabecular bone of zygapophyseal joints might be a limitation of the study. However, we performed our analysis at sites in close vicinity to remaining joint spaces or partially or completely ankylosed zygapophyseal joints, which are the most likely sites of newly formed bone. Additionally, detailed histomorphological studies by Ball<sup>23</sup> and Cruickshank<sup>24</sup> show that syndesmophyte growth takes its

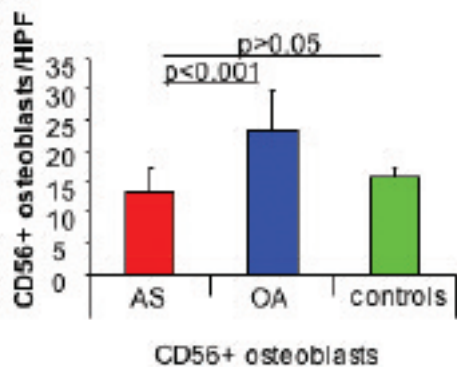
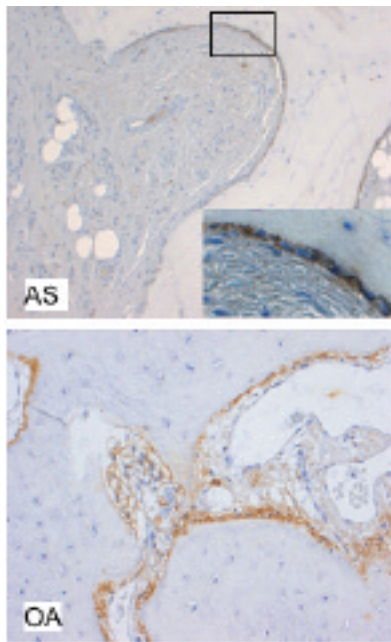


Figure 3. The absolute number of CD56+ osteoblasts in AS patients was significantly lower compared to OA patients and did not differ from autopsy samples.

origin from trabecular bone within the same dense formations of fibrous tissue that we could observe in our samples<sup>4,25</sup>. We believe our results likely reflect new bone formation in general for both diseases, and they can therefore be used for comparison of the 2 spinal diseases. Age and sex matched samples from AS and OA patients were not assessable because surgery in OA patients is performed at an older age and more often in women. The approach used here is most probably as close as we can get to address this question in humans.

The upregulation of osteoblast activity in AS and OA might be at least partly communicated through Wnt signaling in both diseases — recent *in vitro* and *in vivo* analyses showed that Wnt signaling regulates the expression of OPG, which we found was elevated in comparison to controls<sup>16,17,26-28</sup>. The regulation of the Wnt pathway is directly linked to the presence or absence of inflammation. DKK-1 has recently been shown to be a crucial inflammatory molecule that downregulates new bone formation, and that was

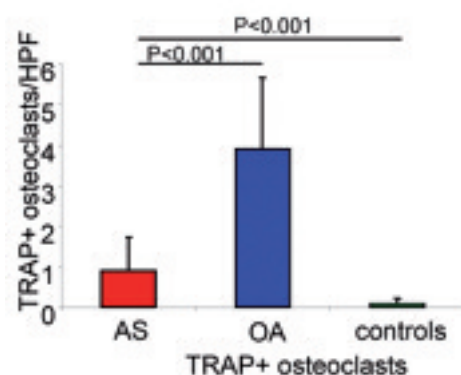
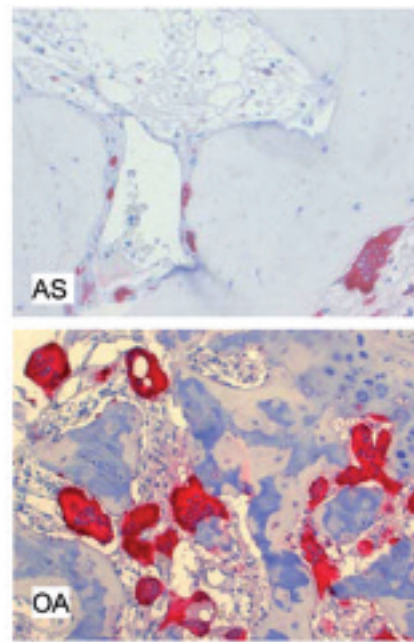


Figure 5. The frequency of TRAP+ osteoclasts in zygapophyseal joints of AS patients was significantly lower compared to OA but higher compared to controls.

found to be at low levels in the serum of AS patients with long disease duration<sup>3</sup>. Similarly, Wnt signaling has also been identified as an important regulator of bone mass and bone cell function in OA<sup>29</sup>. Mechanical loading causes a decrease of negative regulators of the Wnt signaling pathway such as DKK-1<sup>3,30,31</sup>.

Our data show that increased new bone formation caused by osteoblasts is not specific for AS and does occur in different diseases that damage the spine, through inflammation or mechanical loading or other causes, as in diffuse idiopathic skeletal hyperostosis<sup>19</sup>. Interestingly, syndesmo-phytes of the spine, as a manifestation of new bone formation in AS, are present in only about 25% of AS patients with a disease duration less than 10 years<sup>32</sup>, and in only about 50% of AS patients with a mean disease duration of 20 years<sup>33</sup>. Thus, not all AS patients do develop new bone formation and ankylosis of the spine. This resembles OA of the spine, where similarly only a subgroup of patients develops severe new bone formation, mostly as a consequence of

degenerative disc disease<sup>34-36</sup>. Thus, new bone formation might not be an inherent part of diseases such as AS or OA, but could occur, possibly on a similar genetic background, independently of the cause of damage. It might be of interest in future research to look for similarities, in genetics for example, in AS and OA patients with a similar amount of new bone formation.

The increased number of osteoclasts in OA but also in AS compared to controls indicates that an ongoing process of bone remodeling is present in both diseases.

In summary, we present evidence that the amount of new bone formation in OA is at least as high as that in AS. Our data indicate that similar mechanisms might be involved in AS, probably induced by inflammation, and in OA, induced by mechanical stress. Nonetheless, even if new bone formation in AS were part of a repair process it might still be a relevant target for treatment in patients with rapidly progressing disease<sup>37</sup>.

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