Increased expression of receptor activator of NF-κB ligand (RANKL) in stimulated T cells from patient with ankylosing spondylitis

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**Abstract**

**Objectives.** Based on the hypothesis, that activated T cells are the major source of RANKL in inflammatory bone-resorptive diseases, we investigate the presence of intracellular (ic) and membrane bound (mb) RANKL on activated T cells from patients with ankylosing spondylitis (AS). Further we determined their relationship to bone mineral density (BMD), disease activity and markers of bone metabolism.

**Methods.** T cell surface and intracellular expression of RANKL was analyzed by flow cytometry. BMD at the lumbar spine and total hip was measured by dual-energy x-ray absorptiometry (DXA) and quantitative computed tomography (QCT).

**Results.** Expression of icRANKL was significantly increased in patients with AS compared to the healthy volunteers, whereas expression of mbRANKL was significantly lower in the group of patients compared to the controls. The cytokine expression was not associated with bone loss that was found with DXA at the total hip in 45% of all cases, and with QCT at the lumbar spine in 48% of all cases.

**Conclusion.** Intracellular RANKL in T cells is overexpressed in patients with AS, that indicates that these cells may act as a storage for soluble RANKL which rapidly can be mobilized and secreted into the circulatory system after an inflammatory stimulus. In contrast, the expression of mbRANKL is significantly decreased in the AS patients, that might be due to a different activity or prevalence of the enzymes, responsible for the ectodomain shedding of RANKL.

**Key words:** ankylosing spondylitis, T cells, receptor activator of nuclear factor kappaB (NF-κB) ligand, osteoprotegerin, interleukin 17, bone mineral density
**Introduction**

Ankylosing spondylitis (AS) is a chronic, disabling rheumatic disease, which initially mainly involves the sacroiliac joints and in later stages affects the vertebral column. Other sites of involvement include the peripheral joints, entheses and extraarticular manifestations. Alongside the inflammation, new bone formation leads to ossification, syndesmophytes and progressive ankylosis. However, besides to new bone formation, bone loss leading to osteoporosis as well as regional osteopenia and thereby to an increased fracture risk is a well reported feature of AS (1,2).

Low bone density in patients with AS is associated with increased biochemical markers of bone resorption such as C-terminal telopeptide of type I collagen (CTx) or deoxypyridinoline (DPD), and correlated in most cases with inflammation assessed by erythrocyte sedimentation rate (ESR) or C-reactive protein (CRP) (3, 4, 5).

Since the identification of the factors involved in the control of osteoclasts, and hence the development of osteoporosis, significant progress in the understanding of bone remodeling and major advances in bone biology have been made. These factors are the tumor necrosis factor α (TNFα) family member receptor activator of NF-κB ligand (RANKL) (6) and the soluble decoy receptor for RANKL osteoprotegerin (OPG) (7, 8), that competes with RANK for binding RANKL and thereby preventing its osteoclastogenic effect. The major role of RANKL in bone is the differentiation of osteoclast precursor cells into mature osteoclasts and the inhibition of osteoclast apoptosis (9), where the osteoblasts represent the prevailing source of RANKL. Furthermore, RANKL regulates lymphocyte maturation and lymph node organogenesis and is also expressed on activated T cells and dendritic cells, showing cell-interactions in the immune system (10). Kong et al. (11) underlined the relationship between bone and immune system by demonstrating that activated T cells regulate bone loss in pathological conditions, such as rheumatoid arthritis.

In contrast to RANKL, OPG is the so called protector of the bone that was identified by the high bone mass phenotyp of OPG transgenic mice, whereas OPG-knockout mice developed severe osteoporosis (12). To summarize, the RANKL/RANK/OPG system plays an outstanding role in bone homeostasis and inflammatory bone resorption.
IL-17 is a proinflammatory cytokine that is secreted as a dimer predominantly by activated human memory T cells. IL-17 was found at high levels in synovial fluids and synovial biopsies in patients with RA. In addition IL-17 promotes osteoclastogenesis and bone destruction through interaction with RANKL in RA synoviocytes and as well cells of the osteoblast lineage (13-15).

Because RANKL is not only expressed by cells of the stromal-osteoblast lineage, but also by activated T cells, the aim of our study was to investigate the osteoclastogenic potential of peripheral blood (PB) T lymphocytes from patients with AS by examining the intracellular and surface expression of RANKL as well as the intracellular production of IL-17 in these cells. Furthermore we studied bone mineral density (BMD), performed by two different methods, namely dual-energy x-ray absorptiometry (DXA) and quantitative computed tomography (QCT) and its correlation to the cytokine expression, disease duration, disease activity, bone metabolism markers and inflammatory markers.

**Patients and Methods**

**Patients**

Twenty-one patients with diagnosed AS (mean age 51±3; range 25-68 yrs) were enrolled in this study. Patients with a history of other bone diseases or cancer, and those who where taking drugs, that are capable of affecting BMD (like bisphosphonates or TNFα blockers, with the exception of calcium and vitamin D supplements) were excluded from the study. The same exclusion criteria were applied to fifteen sex- and age- matched healthy controls (mean age 49 ±4; range 25-69 yrs). All the patients provided informed and written consent to participate. The study protocol was approved by the ethical committee of the Krankenhaus der Barmherzigen Schwestern. Routine laboratory measures were routinely carried out in the laboratory of the clinic according to standard procedures. Additionally aliquots of serum samples were stored at -70 °C until they were analyzed by ELISA. Clinical indices of disease activity (BASDAI) (16) and function (BASFI) (17) were evaluated by disease-specific questionnaires. Global pain of the patients was determined with a visual analog scale (VAS).
Disease activity was determined by erythrocyte sedimentation rate (ESR) and the C-reactive protein (CRP). Disease duration was assessed from the moment of the first symptoms.

**Bone mineral densitometry**

Bone density was measured using DXA (GE healthcare Lunar iDXA ME +200066) and QCT (QCTPRO™, Mindways Software Inc., on Philips MX 8000) at the lumbar spine (L1-L4) and the total hip. According to the World Health Organization (WHO) (18) osteopenia was defined as a T score < -1 and osteoporosis as a T score < -2.5 measured by the DXA technique. Values measured at the lumbar spine by qCT (mg/cm³) below the method-respective fracture threshold of 120 mg/cm³ calcium phosphate are considered to be osteopenic and osteoporotic as values below the method-respective fracture threshold of < 80 mg/cm³ calcium phosphate.

**ELISA**

The serum concentrations of tartrate-resistant acid phosphatase isoform 5b (SB-TR201, Immunodiagnostic Systems, Turku, Finland, standard range: 1 – 10 U/l), free soluble(s)RANKL (BI-20522, Biomedica, Vienna, Austria; standard range: 0-100 pmol/l), osteoprotegerin (BI-20602, Biomedica, Vienna, Austria; standard range: 0-100 pmol/l), cathepsin K (BI-20432, Biomedica, Vienna, Austria; standard range: 0-300 pmol/l), β-CTX (Roche Diagnostics, Vienna, Austria; standard range: 10-6000 pg/ml), total P1NP (Roche Diagnostics, Vienna, Austria; standard range: 5-12000ng/ml), PTH (Roche Diagnostics, Vienna, Austria; standard range: 1.20-5000pg/ml), CRP (Roche Diagnostics, Vienna, Austria; standard range: 1.00-250mg/l) were determined by commercially available ELISA kits according to the manufacturer’s protocol.

**Flow cytometry**

We performed flow cytometric analysis of T cell cytokine production essentially as previously described (19). Freshly isolated peripheral blood mononuclear cells (PBMC) by density centrifugation from heparinized blood samples were stimulated for cytokine production with 10 ng/ml phorbol myristate acetate (PMA), 1.25 µg/ml ionomycin for 4 h at 37 % in a 5 % CO₂ atmosphere and in the presence of 10 µg/ml
Brefeldin A (BFA) (all three are from Sigma Chemical Co, USA). Cells were fixed with a final concentration of 2% formaldehyde, permeabilized by 0.1% saponin (Sigma Chemical Co.) and stained for the intracellular cytokines using (PE) anti-human RANKL and (PE) anti-human IL-17 (both from Bioscience, San Diego, California, USA) in combination with staining for T cell surface markers, using PeCy-7 conjugated anti-CD4 and APC conjugated anti-CD8 (both from Becton Dickinson, San Jose, CA, USA). For staining of membrane bound (mb) RANKL the cells were neither fixed nor permeabilized, because we observed that nonpermeabilized but fixed cells used for surface staining showed a false high expression of membrane bound RANKL similar to the results of the intracellular staining that supposes that formaldehyde makes the cells permeable (data not shown). Additionally we found, that the presence of the secretion inhibitor Brefeldin A during stimulation of the cells did not influence or minimize RANKL expression on the surface of T lymphocytes (data not shown). Stained cells were analyzed by flow cytometry (FACSCalibur; Becton Dickinson, San Jose, CA) and using a Cell Quest Pro software.

**Statistical analysis**

All statistical analyses were performed by SPSS 14.0 software for Windows. All results are presented as means and standard error of the mean (SEM). Differences between the two groups were tested using the Mann-Whitney U test. Correlation analysis was performed using the Spearman test. In all cases, the minimal requirement for establishing statistical significance was < 0.05.

**Results**

**Patient characteristics**

The clinical data are given in Table 1. Disease activity and pain, determined by using BASDAI, BASFI and VAS pain did not show a correlation between inflammatory markers (ESR, CRP) and disease duration. Laboratory parameters of patients (PTH, 25(OH) vitamin D₃, P1NP, β-CTx, Trap 5b, cathepsin K, sRANKL and OPG) are summarized in Table 2. They all were within the normal range.
Frequency of osteopenia and osteoporosis and the relationship of bone density measured by two different methods

Figure 1 shows the prevalence of osteopenia and osteoporosis in patients with AS. Bone loss was detected most frequently with DXA of the total hip with 45% of all cases, and there was a significant correlation between T scores of the total hip and the lumbar spine (LS, Spearman correlation coefficient $r = 0.54; p = 0.01$). In contrast to measurements at the lumbar spine with DXA, QCT showed with 48% of all cases to be the more sensitive method for measuring low bone density in patients with AS than spinal BMD measures with DXA, especially in men. QCT at the lumbar spine classified more male patients as osteopenic than DXA at the lumbar spine ($p \leq 0.05$). The results of the measurements (mg/cm$^3$) of the lumbar spine and total hip by QCT correlated also very well ($r = 0.53; p = 0.01$). Measurements of the total hip by QCT and DXA showed a significant correlation ($r = 0.87; p = 0.001$) but in contrast values of the measurements of the lumbar spine with did not ($r = 0.195; p = 0.42$).

Relationship of BMD, circulatory OPG and RANKL, age, bone and inflammatory markers

Disease activity measures, inflammatory markers, disease duration and measurements of bone metabolism were not found to be significantly correlated with the bone mineral density measured by DXA or qCT. There was a significant relationship between spinal BMD measured by QCT and age ($r = -0.630; p = 0.003$). In regard to the relationship between BMD and the circulating OPG-RANKL system, serum OPG levels were negatively correlated with lumbar spine BMD values, measured by QCT ($r = -0.571; p = 0.025$) and although statistically not significant - they also tended to be negatively correlated with all other BMD measurements. Furthermore serum levels of OPG where positively related with the age of the AS-patients ($r = 0.513; p = 0.025$). However serum RANKL, OPG as well as RANKL/OPG ratio did not show any associations with laboratory parameters, inflammatory markers or disease activity. Only serum RANKL and serum concentrations of TRAP 5b showed to be positively correlated ($r = 0.54; p = 0.17$).

Expression of intracellular and surface RANKL in T cells

Cell surface expression and intracellular expression of RANKL were determined by three colour flow cytometry. Permeabilized, fixed and stimulated freshly isolated T
High intracellular RANKL expression in T-cells in AS

lymphocytes from peripheral blood of patients with AS showed a significantly higher expression of intracellular RANKL within the CD8+ cell population compared to the healthy controls. Similar results were detected within the CD4+ cell population, but did not reach statistical significance. The mean percentages of intracellular RANKL expression was similar on CD4+ and CD8+ cells from patients with AS (mean ± SEM %; 83.9 ± 4.3, median 90.9 and 85.2 ± 4.0, median 91.5) compared with the healthy group (69.6 ± 6.5, median 73.8 and 65.3 ± 6.4, median 69.0) (Figure 2).

In contrast, the expression of surface RANKL on activated PB T cells from patients with AS was with about 2% RANKL+ cells within the CD4+ cell population and 0.8% RANKL+ cells within the CD8+ cell population significantly lower compared to healthy controls (8% RANKL+ cells within the CD4+ cell population, 3% RANKL+ cells within the CD8+ cell population) (Figure 3). Statistically significant differences were detected between the two cell populations in each group, indicating that CD4+ cells have a higher capacity to express membrane bound RANKL. No significant correlations were found between intracellular and surface RANKL on T cells with disease activity, bone density, inflammatory markers and measurements of bone metabolism. Although not statistically significant, TRAP 5b, as a marker of bone resorption tended to be positively correlated with the percentages of RANKL+ CD4 and CD8 cells in patients with AS, examined by intracellular cytoplasmic staining (r=0.15; r=0.11).

Expression of intracellular interleukin 17 in T cells

Because IL-17 is a potent stimulator of RANKL, the essential molecule for osteoclast development, we further performed flow cytometric analysis to examine the expression of intracellular IL-17 in freshly isolated PB T lymphocytes. Patients with AS showed a higher expression of IL-17 in both cell populations, CD4+ and CD8+ cells (mean ± SEM %; 1.7 ± 0.6, median 1.2 and 0.4 ± 0.2, median 0.2) compared to the healthy controls (0.9 ± 0.1, median 0.8 and 0.1 ± 0.04, median 0.1), but did not reach statistical significance. In the patients with AS the expression of IL-17 within the CD4+ cell population was with the minimal level of 1.7±0.6% comparable to the results, revealed by membrane staining for RANKL in CD4+ cells. Furthermore our study was not able to find significant correlations between the proinflammatory cytokine IL-17 and other measurements.
Discussion

Besides formation of new bone in form of syndesmophytes, bone loss is a common complication in AS that has been well documented in several studies (1, 2, 20-23). In this study we compared two different methods in order to determine bone density, namely DXA and QCT. Our own observations showed that osteopenia was apparent in 45% of all patients with AS by using DXA technique at the total hip, whereas five patients showed osteoporotic BMD values. Furthermore, T scores of the total hip and lumbar spine evaluated by DXA correlated nicely as did the results of the total hip and lumbar spine measured by QCT. Consistent with previous research (24-26) we found that BMD values of the lumbar spine were higher measured by DXA technology and lower using QCT, indicating that QCT is the more sensitive method for measuring low bone density at the lumbar spine in patient with AS, even in the presence of syndesmophytes, an abnormal bone outgrowth at various spinal sites, that are decreasing the sensitivity of bone densitometry. These discrepancies are supported by our observations of a negative correlation between BMD values at the lumbar spine measured by QCT and disease duration and age, in contrast to a positive correlation between BMD values at lumbar spine revealed by DXA and disease duration. Further, we observed this phenomenon mainly in men which might be due to the different gender specific clinical manifestations in AS. In men, the pelvis and the spine are most commonly affected, whereas the involvement of the spine is generally less severe in women. However, several studies have been conducted evaluating bone loss in AS with different frequencies of osteoporosis and osteopenia as well as correlations between BMD and several markers of bone metabolism. Diverse to most other studies (2-5), we could neither find a relationship between BMD and biochemical markers of bone turnover, like C-terminal telopeptide crosslinks nor between BMD and markers of disease activity (ESR, CRP, BASDAI). As already outlined by Wendling (27), the development of osteopenia or osteoporosis is influenced by several factors over a period of time, whereas serum concentrations of biological markers are known to be reflective only at a given time point. Additionally, our measurements of bone metabolism in the serum of patients with AS (Table 2), like PTH, P1 collagen, crosslinks, 25(OH) vitamin D₃ did not show any deviations from the normal range.
The discovery of RANKL and its receptors RANK and OPG shows a new cytokine network that is essential for osteoclast development and plays a pivotal role in inflammatory bone resorption. In our correlation study between BMD and circulating levels of OPG and RANKL in patients with AS we found that serum OPG levels were significantly negatively correlated with LS BMD values, determined by QCT, and also showed a non-significant negative relationship with all other BMD measurement techniques. Additionally, serum levels of OPG showed a significant positive correlation with age. These observations can be explained by the compensatory reaction of OPG to encounter age-related bone loss, a phenomenon that also was seen in previous studies (28-32). With regard to serum OPG concentrations in AS, various research groups reported contrasting results, showing decreased (5), increased (3) or nearly normal OPG levels (33) compared to healthy controls. Recently, a study described that serum levels of sRANKL and the RANKL/OPG ratio were significantly higher in patients with AS than in healthy controls. Furthermore, these measurements tended to be related with BMD, suggesting their involvement in the pathogenesis of osteoporosis in AS (33). In contrast to these interesting results, our study neither showed such high serum sRANKL levels and sRANKL/OPG ratios, nor a correlation to BMD. Such discrepancies may have several causes. Analytical variability of the different assays used for the detection of sRANKL serum levels may be one of the main factors accounting for these discordant results. Next to the assay performance, preanalytical factors, like age, sex, menopause or circadian rhythm that may influence serum sRANKL, are not well studied yet. Furthermore it is necessary to mention that some assays detect only free sRANKL while others detect total sRANKL. A further attributable factor may be regional differences and of course the number of subjects enrolled into the study. In contrast to the study of Kim et al. (33) we investigated fewer patients, consequently our statistical power was lower. However, consistently to our results, Hofbauer et al. (34) and further studies (35, 36) showed similar low concentrations of free serum sRANKL levels in healthy people by using the same assay from Biomedica (Vienna, Austria).

The finding of the involvement of immune cells in the regulation of osteoclastogenesis has been first shown by the demonstration, that RANKL is upregulated on T cells after activation (11, 37). Further intense research demonstrated, that both activated T cells and synovial fibroblast like cells in the joints of patients with rheumatoid arthritis (RA) express membrane bound RANKL and
soluble RANKL, thereby promoting focal bone erosions and periarticular bone loss. In addition, a recently published study (38) showed that the expression of RANKL by T cells in chronic periodontal disease also has an important impact on the bone resorption process. Due to the accumulating evidence, that activated T cells are the major source of RANKL in inflammatory bone-resorptive diseases, we sought to investigate the presence of intracellular and membrane bound RANKL on activated T cells from patients with AS analyzed by flow cytometry. This is the first study to show that peripheral blood T cells respond to an in vitro stimulation with ionomycin and PMA with a higher intracellular production of RANKL (~85 %) in patients with AS compared to the healthy controls (~65 %) (Figure 2). This high extent of RANKL expression was observed in both T cell populations, but the increase was significant only for the CD8+ subpopulation. To confirm our results, a different RANKL antibody (MAB6261; R&D) was applied and validated the high intracellular expression of RANKL (data not shown). Our observation might be explained by the strong association between the MHC-class I molecule HLA-B27 and AS (39) for also a previous study has demonstrated a strong antigen specific T cell cytokine response with a significant reduction after infliximab treatment only in CD8+ cells from patients with AS (40). This arthritogenic peptide hypothesis proposes that AS is a consequence of the antigen presentation function of HLA-B27 to CD8+ cells, resulting in an immune mediated pathogenesis. Furthermore, the well established intimate interactions between bone cells and immune cells (11, 41) show an important role for CD8+ cytotoxic T cells and CD4+ T helper cells in AS. Additionally, an immunohistologic study of sacroiliitis in AS patients showed cellular infiltrates of T cells (CD4+ and CD8+) and macrophages in the sacroiliacal joints (42). Our novel results of high amounts of intracellular RANKL in T lymphocytes indicates that these cells may act as a storage for soluble RANKL which rapidly can be mobilized and secreted into the circulatory system after an inflammatory stimulus, where it exerts its systemic effects. In light of this interpretation, our results would be in agreement with the up-regulated serum levels of RANKL and sRANKL/OPG ratios in patients with AS that were published previously (33).

RANKL is synthesized as a membrane bound protein and is converted into a soluble form by ectodomain shedding that has been shown to be mediated by numerous proteinases. At the moment no definite RANKL sheddase has been identified. However, a disintegrin and metalloproteinase domain family (ADAM) members 10, 17
High intracellular RANKL expression in T cells in AS

and 19 have been shown to exhibit RANKL shedding properties in vitro as well as different matrix metalloproteinases (MMP), like MMP3, MMP7 or MMP14 (43-45). Somewhat unexpectedly, we found that the cell surface expression of RANKL on activated PB T cells was significantly lower in the group of patients with AS compared to the healthy controls. We can only hypothesize, that this difference may be caused by a different activity or prevalence of the enzymes, responsible for the ectodomain shedding of RANKL. In this case, the shedding of surface RANKL into the soluble form would be accelerated and thereby exhibit strong systemic effects. However, the minimal extent of mbRANKL expression was not comparable to the high amount of RANKL revealed by intracellular staining. Although some studies have been performed investigating the functional difference between membrane-bound and soluble RANKL, it is not exactly clear whether membrane bound RANKL or soluble RANKL is more efficient in supporting osteoclastogenesis. On the one hand, soluble forms of RANKL derived from human T cells were demonstrated to have potent function in bone and immune homeostasis (47) and on the other hand membrane-bound RANKL, derived from murine bone marrow stromal cells, was shown to have more potent osteoclastogenic activity than sRANKL (48). Further studies are needed that focus on the biological and pathological importance of ectodomain shedding of RANKL as well as on the contribution of mbRANKL and sRANKL in osteoclast development.

Since the discovery, that the T cell cytokine IL-17 does not only play a role in inflammatory responses, but also contributes to joint destructions, several studies were performed that focused its role in the pathogenesis of RA. IL-17 was shown to be present in synovial fluids and synovial biopsies in patients with RA, as well to be an important inducer of RANKL and thus a potent regulator in osteoclastogenesis and bone erosions (13-15).

Finally we showed that the expression of IL-17 in PB T cells was higher in patients with ankylosing spondylitis compared to the healthy volunteers. The major source of IL-17 was with 1.7 ± 0.6% in the AS-patients and 0.9 ± 0.1% in the controls the activated CD4+ T cell subset. Additionally, a recent study demonstrated the existence of a new, distinct Th cell lineage, termed Th17, next to the classical CD4+ T cell subpopulations Th1 and Th2 (45). The result of elevated intracellular IL-17 expression in T lymphocytes which we found in patients with AS is in concordance with the study of Kim et al. (33) that found up-regulated serum levels of IL-17 in
patients with AS. However, we did not observe any correlations with sRANKL or intracellular RANKL, as was demonstrated in the forementioned study. Taken together, the results presented here underline the involvement of immune cells in the regulation of osteoclastogenesis and indicate that activated T cells may act as the major source of soluble RANKL in inflammatory bone-resorptive diseases, such as ankylosing spondylitis. Moreover, this is the first study to show that intracellular RANKL is more abundantly expressed than the membrane-bound form opening interesting questions in regard of the form in which RANKL is primarily produced, possible processing steps leading to the maturation of the molecule, its localization within the cell, the shutteling of RANKL to the membrane and the release of RANKL into the serum which could happen directly by exocytosis or in form of membrane shedding.

Acknowledgements

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**Table 1. Clinical characteristics of 21 patients with ankylosing spondylitis**

<table>
<thead>
<tr>
<th></th>
<th>Mean ± SEM (range)</th>
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<tbody>
<tr>
<td><strong>Age</strong></td>
<td>51.3 ± 3.3 (range 25-68 yrs)</td>
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<tr>
<td><strong>Sex</strong></td>
<td></td>
</tr>
<tr>
<td>male</td>
<td>10 (47.6%)</td>
</tr>
<tr>
<td>female</td>
<td>11 (52.4%)</td>
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<tr>
<td><strong>BASDAI</strong></td>
<td>4.7 ± 0.5 (range 1.3 – 8.3)</td>
</tr>
<tr>
<td><strong>BASFI</strong></td>
<td>3.8 ± 0.4 (range 1.2 – 8.1)</td>
</tr>
<tr>
<td><strong>VAS pain</strong></td>
<td>5.0 ± 0.6 (range 1.0 – 9.0)</td>
</tr>
<tr>
<td><strong>Disease duration (yr)</strong></td>
<td>25.4 ± 3.4 (range 4.0 – 53.0 yrs)</td>
</tr>
<tr>
<td><strong>ESR 1h (mm/h)</strong></td>
<td>21.2 ± 4.2 (range 4.0 – 84.0mm/h)</td>
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<tr>
<td><strong>CRP (mg/dl)</strong></td>
<td>9.1 ± 3.0 (range 0.41 – 49.5mg/dl)</td>
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</table>

Data are expressed by mean ± SEM. Abbreviations: BASDAI, Bath Ankylosing Spondylitis Disease Activity Index; BASFI, Bath Ankylosing Spondylitis Functional Index; VAS, Visual Analogue Scale; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein.
### Table 2. Laboratory parameters in patients with ankylosing spondylitis

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Mean ± SEM</th>
<th>Normal Range</th>
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<tbody>
<tr>
<td>PTH (pg/ml)</td>
<td>59.4 ± 5.6</td>
<td>15.0 – 65.0</td>
</tr>
<tr>
<td>total P1NP (ng/ml)</td>
<td>52.1 ± 6.1</td>
<td>16.0 – 74.0</td>
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<tr>
<td>CTx (pg/ml)</td>
<td>0.4 ± 0.0</td>
<td>0.0 – 1.01</td>
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<tr>
<td>Cathepsin K (pmol/l)</td>
<td>11.8 ± 2.6</td>
<td>not established</td>
</tr>
<tr>
<td>Trap5b (U/l)</td>
<td>2.8 ± 0.1</td>
<td>2.59 – 3.31</td>
</tr>
<tr>
<td>sRANKL (pmol/l)</td>
<td>0.1 ± 0.0</td>
<td>0.0-2.7 (see ref. 36)</td>
</tr>
<tr>
<td>OPG (pmol/l)</td>
<td>3.3 ± 0.4</td>
<td>1.2-6.6 (see ref. 50)</td>
</tr>
<tr>
<td>25 (OH) vitamin D$_3$ (ng/ml)</td>
<td>48.9 ± 7.5</td>
<td>7.5 – 120</td>
</tr>
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</table>

Data are expressed by mean ± SEM. Abbreviations: PTH, parathyroid hormone; total P1NP, total procollagen type 1 amino-terminal pro-peptide; CTx, C-terminal telopeptide crosslinks; Trap 5b, tartrate-resistant acid phosphatase isoform 5b; RANKL, receptor activator of NF-κB ligand; OPG, osteoprotegerin.
Figure 1. Comparison of the relative frequencies of osteopenia and osteoporosis measured at different sites and with two different methods in male and female patients with AS. *Significant different from QCT LS in men (p ≤ 0.05). Abbreviations: TH, total hip; LS, lumbar spine; DXA, dual-energy x-ray absorptiometry; QCT, quantitative computed tomography.
Figure 2. Intracellular expression of receptor activator of NF-κB ligand (RANKL) in activated peripheral blood (PB) T lymphocytes in ankylosing spondylitis (AS) and healthy controls (HC) as determined by flow cytometry. Black bars indicate the expression of patients with AS, and white bars show the expression of healthy controls. Each bar represents the mean percentage of RANKL+ cells ± SEM per group. Expression of RANKL in CD8+ cells was significantly higher in AS-patients than in HC. *Significant different from RANKL expression in CD8+ cells of HC (p < 0.05).
Figure 3. Cell surface expression of RANKL in activated T cells analyzed by flow cytometry. **Significant different from membrane bound RANKL expression on CD4+ cells of HC (p < 0.01). ++Significant different from membrane-bound RANKL expression on CD8+ cells of HC (p < 0.01) For definitions, see Figure 2 legend.


33. Kim HR, Kim HY, Lee SH. Elevated serum levels of soluble receptor activator of nuclear factors-KappaB ligand (sRANKL) and reduced bone mineral density in patients with ankylosing spondylitis (AS). J Rheumatol 2006; 45:1197-200.


