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ORIGINAL ARTICLE

# Monocytes from male patients with ankylosing spondylitis display decreased osteoclastogenesis and decreased RANKL/OPG ratio

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## Abstract

**Summary** The present study investigates the osteoclastogenic capacity of peripheral blood mononuclear cells (PBMCs) in male patients with ankylosing spondylitis (AS). We demonstrated that monocytes from these patients display a lower capacity to generate osteoclasts compared to cells from healthy controls, and osteoclastogenesis was negatively correlated with disease duration.

**Introduction** Ankylosing spondylitis (AS) is a disease characterized by new bone growth that leads to syndesmophyte formation but AS patients frequently present with low bone mineral density/fractures. Osteoclastogenesis in AS patients is poorly studied and controversial. The aim of this study is to determine if the osteoclastogenic capacity of PBMCs is different in AS patients compared to controls and the relationship between osteoclastogenesis and clinical/laboratory parameters.

**Methods** PBMCs from 85 male AS patients and 59 controls were tested for CD16+ cells and induced to differentiate into osteoclasts over 3 weeks in vitro. Serum levels of RANKL, osteoprotegerin (OPG), C-terminal telopeptide of type I collagen (CTX), and amino-terminal pro-peptide of type I collagen (P1NP) were also evaluated.

**Results** PBMCs from AS patients had fewer CD16+ cells and produced fewer osteoclasts compared to controls. Apoptosis occurred less frequently in osteoclasts obtained from AS patients than in osteoclasts from the controls. A lower RANKL/OPG and CTX/P1NP were observed in AS patients compared to controls. AS patients taking NSAIDs presented no difference regarding the number of OCs produced and the percentage of CD16+ cells compared to controls. However, patients taking TNF inhibitors (TNFi) presented lower OC numbers than controls. A negative correlation was demonstrated between the number of osteoclasts generated from PBMCs of AS patients and disease duration.

**Conclusion** Monocytes from male AS patients display a lower capacity to generate osteoclasts in vitro compared to cells from controls. Osteoclastogenesis was negatively correlated with disease duration. This finding supports the idea that osteoclasts play a role in the physiopathology of bone disease in AS patients.

**Keywords** Ankylosing spondylitis · Apoptosis · CTX · Osteoclastogenesis · Osteoprotegerin · P1NP · RANKL

## Introduction

Bone remodeling is a continuous process throughout life that involves a coordinated and balanced resorption of old bone by osteoclasts and adequate formation of new bone by osteoblasts in a balanced and sequential manner referred to as coupling [1]. Bone diseases may be associated with an uncoupling between the formation and resorption processes [1].

Osteoclasts are cells that are derived from circulating mononuclear precursors; osteoclastogenesis arises from the fusion of monocytes in the presence of the cytokines macrophage-colony stimulating factor (M-CSF) and receptor activator of NF- B ligand (RANKL) modulated by a complex signaling network that involves the receptor activator of nuclear factor NF- B (RANK), osteoprotegerin (OPG), and RANKL, all of which belong to the tumor necrosis factor family [2].

In human peripheral blood, three functionally different subsets of monocytes have been identified and characterized based on their expression of the surface markers CD14 and

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CD16; these are considered monocyte osteoclast precursors [3]. The contribution of monocytes as precursors is well documented during inflammation and, as a result, includes populations of immune cells that are normally not maintained by monocyte recruitment, such as populations of osteoclasts in bone [3]. Inflammation induces an expansion of CD16-expressing monocytes [4], resulting in an increased contribution of immune cells. In this way, the CD16-expressing monocytes contribute to the shaping of these immune cell populations during inflammation. The influence of an affected distribution of circulating monocytes on osteoclast formation during inflammation has also been observed when osteoclastogenesis of monocytes from patients with inflammatory bone loss has been studied [5].

Osteoclastogenesis in patients with ankylosing spondylitis (AS) is poorly studied and controversial [6, 7]. AS is a chronic inflammatory disease characterized by new bone growth that leads to syndesmophyte formation and subsequent vertebral ankylosis [8, 9]. On the other hand, AS patients frequently present with low bone mineral density and fractures that can be associated with systemic inflammation and decreased mobility [10, 11]. Interestingly, inflammation is associated with trabecular bone loss leading to osteoporosis but is also associated with new bone formation, leading to progressive ankylosis of the spine and sacroiliac joints [12].

Acute stages of inflammation in patients with AS are marked by a predominance of mononuclear cell infiltrates, including macrophages and T cells, and also by an increased number of osteoclasts (OCs) [13]. Accordingly, radiographic damage in AS is initially characterized by erosive changes followed by a distinct anabolic skeletal response, which results in excessive bone formation [14].

Studies that evaluated osteoclastogenesis in AS patients presented different results, and some studies have demonstrated a decrease in osteoclast differentiation in these patients [7, 15]. However, there is no data regarding the influence of clinical disease parameters, inflammatory markers, and therapy in AS osteoclastogenesis.

The study of osteoclastogenesis, OC activity and apoptosis of these cells in AS patients, and the association of these parameters with clinical and radiological variables may help to clarify the pathophysiology of disease development and progression.

## Methods

### Patients and controls

Eighty-five male AS patients, aged ≥ 18 to ≤ 55 years, followed by the Spondyloarthritis Outpatient Clinic at the Hospital das Clinicas da Universidade de Sao Paulo, were invited to participate in this study. All patients fulfilled the modified New York Classification criteria [16] and gave written informed consent in accordance with the Declaration of Helsinki. Fifty-nine age-matched healthy individuals who worked at the Universidade de Sao Paulo, School of Medicine, or their family members were included as a control group. Exclusion criteria for the control group were bone-associated metabolic diseases, including osteoporosis, and exclusion criteria for both groups were drugs that interfere with bone metabolism (bisphosphonates, teriparatide, anticonvulsants, anticoagulants), except drugs for AS treatment. This study was approved by the Local Ethics Committee on Human Research at the Sao Paulo University-CAPPesq 0061/11.

Individual characteristics such as age, race, body mass index (BMI), and a questionnaire of risk factors for osteoporosis (personal history of fracture, hours/week of physical activity practice, past and current smoking, alcohol intake) in AS patients and control subjects were recorded. Demographic and clinical characteristics of patients were evaluated. The use of medications such as nonsteroidal anti-inflammatory drugs (NSAIDs), glucocorticoids, methotrexate, and tumor necrosis factor-α inhibitors (TNFi) were collected from the medical records.

### Clinical outcomes of AS

Clinical indexes, including Bath Ankylosing Spondylitis Disease Activity (BASDAI), Bath Ankylosing Spondylitis Functional (BASFI), Bath Ankylosing Spondylitis Metrology (BASMI), and Ankylosing Spondylitis quality of life (ASQoL) were measured as previously described [17].

Ankylosing Spondylitis Disease Activity Score (ASDAS-PCR) was measured as recommended previously [18].

### Radiographic assessment

The Modified Stoke Ankylosing Spondylitis Score (mSASSS) was evaluated using lateral radiographic images of the cervical and lumbar spine in AS patients. The scoring system, developed by Creemers et al. [19], was applied to the lower border of the 12th thoracic vertebrae, all five lumbar vertebrae, and the upper border of the sacrum and the lower border of the 2nd cervical vertebra up to and including the upper border of the 1st thoracic vertebra. The following corresponding nominal scoring system was used, 0=no abnormality; 1 = erosion, sclerosis, or squaring; 2 = syndesmophyte; and 3 = total bony bridging at each site.

### Bone mineral density

Bone mineral density (BMD) at the lumbar spine, femoral neck, and total hip were measured using dual-energy X-ray absorptiometry (DXA) (Hologic Inc., Bedford, MA, USA;

Discovery model). The precision error for BMD measurements was determined by standard ISCD protocols.

## Cell culture

Blood samples (100 ml) were collected, and peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll density and dextran sedimentation. PBMCs were plated at $1.5 \times 10^6$ cells/cm$^2$ in 48-well plates. Cells were maintained in α-MEM from Sigma-Aldrich, Inc. (St Louis, MO, USA) supplemented with 10% FBS from HyClone Laboratories, Inc. (Logan, Utah, USA), 1% penicillin-streptomycin from Sigma-Aldrich, Inc. (St Louis, MO, USA), 50 ng/ml RANKL (recombinant protein produced as described elsewhere) [20], and 10 ng/ml M-CSF from Peprotech, Inc. (Rocky Hill, NJ,USA). Differentiation progressed for 21 days with regular medium changes at 3–4-day intervals [21, 22].

## Quantification of OC precursors

To evaluate whether the capacity to generate osteoclasts in AS patients was related to the percentage of circulating precursors, flow cytometry was used to quantify osteoclast precursors. Mononuclear cells ($1 \times 10^6$) were incubated at room temperature for 30 min with two antibodies, 10 μl of FITC-conjugated anti-CD14 monoclonal antibody and 5 μl of PE-conjugated anti-human CD16 monoclonal antibody (BD Biosciences, Mississauga, Canada). After washing with phosphate-buffered saline (PBS), cells were analyzed by flow cytometry using FACScan and the cell-surface antigen was quantified. Data were analyzed with the CellQuest software (Becton Dickinson, Mississauga, Canada) [21, 22].

## Differentiation assay

PBMCs were differentiated for 21 days in 48-well plates, then washed with PBS and stained for tartrate-resistant acid phosphatase (TRAP) activity (Sigma-Aldrich, Inc., St Louis, USA). TRAP-positive multinucleated (≥ 3 nuclei) cells were counted as osteoclasts, and results are expressed as the number of osteoclasts per well [21, 22].

## Osteoclast apoptosis

Osteoclast apoptosis was determined as described [21, 22]. Briefly, after 21 days of differentiation, cells were maintained for 24 h without M-CSF and RANKL in α-MEM with 5% FBS. Cells were then fixed, permeabilized, labeled using a TACS TdT Blue Label in situ apoptosis detection kit (R&D Systems, Minneapolis, USA), and counterstained with nuclear fast red. Osteoclasts were identified based on multinuclearity. Criteria for apoptosis included the presence of ≥ 3 blue-labeled nuclei and nuclear fragmentation. A total of 100 osteoclasts were examined in each sample.

## OPG and sRANKL serum levels

To study the association between the capacity to generate osteoclasts and the RANKL/RANK/OPG system, serum levels of RANKL and OPG were measured. Fasting blood samples were collected and serum were stored at − 80 °C. Serum OPG was assayed by ELISA (Biomedica, Vienna, Austria), which uses a monoclonal anti-OPG antibody to capture OPG from serum. Serum levels of uncomplexed (free) sRANKL were measured with an enzyme immunoassay (Biomedica, Vienna, Austria) according to the manufacturer's instructions. The inter-assay and intra-assay CVs were 6.5 and 2.6% for OPG, 6.9 and 4.8% for sRANKL, respectively [23]. Samples were consecutively selected for testing, and all tests were performed in duplicate. The detection limit established by the manufacturer was 0.14 pmol/l for OPG and 0.02 pmol/l for sRANKL.

## CTX/P1NP serum levels

Fasting blood samples were collected from all participants and serum were stored at − 80 C for further analysis. A serum bone formation marker, amino-terminal pro-peptide of type I collagen (P1NP), a serum bone resorption marker, C-terminal telopeptide of type I collagen (CTX) were analyzed using an automated Roche electrochemiluminescence system (E411, Roche Diagnostics®, Mannheim, Germany). The following coefficients of variation (CVs) were obtained, 2.2% for P1NP, 2.5% for CTX [24].

## Cytokines and inflammatory markers

The analysis of IL-6, IL1β, and TNFα were performed with the Human Bone Magnetic Bead Panel from the Milliplex® Map Kit (Cat.No. HBNMAG-51 K, Billerica, MA), according to the manufacturer's instructions. Plates were run on a Luminex® 200™-xMAP® Technology machine, and the data were collected using the Luminex xPONENT® software. Analysis of the cytokine/chemokine median fluorescent intensity (MFI) was performed with the Milliplex® Analyst software (v.5.1). The inter-assay coefficient of variation for all cytokines tested was 11.93%. Serum levels of IL-17 were measured with high sensitivity ELISA kit (eBioscience (Viena, Austria)) and coefficient of variation was 3.6%. C-reactive protein (CRP) was evaluated in AS patients.

## Statistical analysis

Results are expressed as the mean ± standard deviation (SD) for continuous variables or percentages for categorical

variables. Data from AS patients were compared to data from healthy controls using *t* test (normal distribution) or Mann Whitney test (non-normal distribution) for continuous variables and chi-squared test for categorical variables. The number of osteoclasts was correlated (Spearman's coefficient) with clinical and laboratory parameters. A generalized linear model (GLM) with gamma distribution and long-link function was constructed to identify the factors associated with number of OCs in the AS group. The analysis included treatment (non-steroidal anti-inflammatory drugs (NSAIDs) and tumor necrosis factor-α inhibitor (TNFi)) and clinical parameters. Statistical significance was accepted at $p < 0.05$. Analyses were performed with the SPSS software version 20.0 for Windows [25].

## Results

### Patients and controls

The demographic characteristics of 85 male AS patients and 59 healthy controls are shown in Table 1. No difference was observed regarding the frequency of personal history of fracture ($p > 0.05$), past and current smoking ($p > 0.05$), or alcohol intake ($p > 0.05$) between AS patients and controls. The frequency of physical activity practice was lower in AS patients compared to healthy controls (40.0 vs. 57.6%. $p = 0.037$) (Table 1 ).

The AS patients presented with a mean of 17.4 ± 9.72 years of disease duration, and 77% were HLA-B27 positive. AS clinical parameters were as follows: BASDAI (2.73 ± 1.87), BASFI (3.68 ± 2.52), BASMI (3.47 ± 2.40), ASQol (5.92 ± 4.97), and ASDAS-ESR (1.74 ± 0.82). Regarding radiographic damage (mSASSS), the AS patients presented with a score of 27.0 ± 17.8. At the moment of the study, the treatments were NSAIDs (76.5%), prednisone (11.8%), methotrexate (4.7%), sulfasalazine (45.9%), and TNF inhibitors (43.5%).

### AS patients vs. healthy controls—in vitro experiments

#### In vitro osteoclastogenesis

The capacity to generate osteoclasts in vitro from PBMCs was significantly lower in AS patients than in controls ($p = 0.014$) (Fig. 1). Monocytes from AS patients with disease duration ≥ 15 years presented a lower capacity in generating osteoclasts compared with monocytes from AS patients with disease duration lower than 15 years (517.9 ± 636.2 vs. 800.7 ± 801.6, $p = 0.036$).

No difference was observed regarding the osteoclast number associated with inflammatory or disease activity index evaluated in AS patients: ASDAS PCR ≥ 2.1 and ASDAS PCR < 2.1 (622.2 ± 723.0 vs. 677.5 ± 624.5, $p = 0.651$); BASDAI ≥ 4 and BASDAI < 4 (752.0 ± 943.4 vs. 606.6 ± 529.2, $p = 0.853$).

No difference was observed in the number of osteoclasts generated from AS patients treated with NSAIDs without TNFi therapy at the time of the study (723.35 ± 792.3 osteoclasts/well, $n = 40$) compared with the number of osteoclasts from healthy controls (764.43 ± 561.9 osteoclasts/well) ($p = 0.10$). However, osteoclastogenesis was lower in AS patients taking TNFi without NSAIDs therapy at the moment of the study (582.51 ± 717.56 osteoclasts/well, $n = 12$) compared to controls (764.43 ± 561.9 osteoclasts/well) ($p = 0.047$). N.

($n = 3$) compared to control group ($p > 0.05$).

#### Circulating osteoclast precursors

The percentage of CD16+ cells in PBMCs was lower in AS patients (25.06 ± 8.59%) than in the control group (28.59 ± 10.20%, $p = 0.026$). No difference was observed concerning CD14+ cells in PBMC preparations between AS patients and healthy controls ($p > 0.05$).

Regarding therapy used at the time of the study, no difference was found concerning the percentage of CD16+ PBMCs in AS patients who used NSAIDs without TNFi (24.63 ± 8.01%) compared to the control group (28.6 ± 10.20%), $p = 0.080$. No difference was observed in CD16+ cells between AS patients who were using TNFi therapy without NSAID and healthy controls ($p > 0.05$).

#### RANKL/OPG serum levels

A lower RANKL/OPG ratio was found in AS patients compared to the control group (0.05 ± 0.03 vs. 0.07 ± 0.07, $p = 0.046$) (Fig. 2), but no difference was observed in RANKL serum levels between the two groups, respectively (0.19 ± 0.11 vs. 0.23 ± 0.13, $p = 0.066$) and OPG serum levels (5.20 ± 3.18 vs. 4.34 ± 1.52, $p = 0.199$).

#### CTX/P1NP serum levels

A lower CTX/P1NP ratio was found in AS patients compared to healthy control (0.008 ± 0.003 vs. 0.010 ± 0.003, $p < 0.001$) but no difference was observed in CTX serum levels between the two groups (0.462 ± 0.277 vs. 0.471 ± 0.197, $p = 0.410$).

#### Apoptosis

The percentage of cells undergoing apoptosis was significantly lower in AS patients than in the control group, respectively (31.8 ± 32.5% vs. 44.5 ± 34.3%, $p = 0.007$) (Fig. 3). No association was found between the percentage of apoptotic osteoclasts in AS patients taking NSAIDs without TNFi therapy and the control group (35.65 ± 31.28% vs. 44.53 ± 34.3%, $p = 0.171$). Osteoclasts from AS patients taking TNFi therapy

**Table 1** Demographics characteristics of ankylosing spondylitis (AS) patients and healthy controls (CT)

| | AS patients $n = 85$ | Healthy controls $n = 59$ | $p$ |
|---|---|---|---|
| Age, years | 42.6 ± 9.0 | 40.7 ± 10.0 | 0.232 |
| White race, % | 85.9 | 72.9 | 0.053 |
| BMI, kg/m$^2$ | 27.3 ± 4.4 | 27.1 ± 2.7 | 0.721 |
| History of fracture, % | 23.5 | 27.1 | 0.697 |
| Physical activity, h/week | 4.03 ± 2.12 | 5.11 ± 1.87 | 0.042 |
| Current smoker, % | 14.1 | 8.5 | 0.302 |
| Alcohol intake, > 3 U/day | 0 | 0 | 1.000 |

Data are expressed as mean (SD) or percentage (%)

*BMI* body mass index

without NSAID exhibited less apoptosis than healthy controls (11.0 ± 8.94% vs. 44.53 ± 34.3%, $p < 0.001$).

### Cytokine serum levels and inflammatory parameter

The IL6 had significantly higher circulating levels in AS patients when compared to healthy controls ($p = 0.002$). No difference was observed regarding IL17 ($p = 0.208$), IL1β ($p = 0.109$), and TNFα ($p = 0.487$) in circulating levels between AS patients and healthy controls. The frequency of patients' samples and healthy controls' samples ≥ minimum limit of detection for IL17 was 85 and 75%, respectively. The mean ± SD of CRP in AS patients was 8.8 ± 11.17 mg/L.

### Bone mineral density

BMD at the lumbar spine was higher in AS patients compared to healthy controls (1.104 ± 0.206 vs. 1.041 ± 0.117 g/cm$^2$, $p = 0.023$), but total femur BMD was lower in AS patients than in the control group (0.951 ± 0.130 vs. 1.003 ± 0.114 g/cm$^2$, $p = 0.017$).

AS patients who were only taking NSAIDs presented no difference related to lumbar spine BMD compared with healthy controls (1.085 ± 0.220 vs. 1.041 ± 0.117 g/cm$^2$, $p = 0.503$). AS patients taking TNFi presented with higher BMD at the lumbar spine compared to healthy controls (1.186 ± 0.206 vs. 1.041 ± 0.117 g/cm$^2$, $p = 0.019$). No difference regarding age was observed between AS patients taking NSAIDs and healthy controls (43.62 ± 8.53 vs. 40.73 ± 10.0 years, $p = 0.137$) and between AS patients taking TNFi and controls (43.83 ± 11.08 vs. 40.73 ± 10.0 years, $p = 0.378$).

### Osteoclastogenesis and disease parameters in AS patients

A negative correlation was found between disease duration and the number of osteoclasts ($r = -0.220$, $p = 0.043$). No other correlation was found regarding other clinical, laboratory, radiographic damage, disease activity (BASDAI), or functional indexes (BASFI) or mobility (BASMI) ($p > 0.05$) (data not shown).

A generalized linear model with gamma distribution analysis (including disease duration, NSAID, and TNFi therapies) demonstrated that disease duration was independently negatively associated with osteoclastogenesis: for each year of disease duration, a decrease of 16.6% in the mean osteoclast number was found ($p < 0.001$) (Table 2).

## Discussion

This study demonstrated that PBMCs from AS patients presented a lower capacity to generate osteoclasts in vitro and a lower percentage of CD16+ cells than did PBMCs from healthy controls. In agreement with these findings, a lower RANKL/OPG and lower CTX/P1NP ratio were found in AS patients compared to controls.

This study was carefully designed by taking into account some important points to avoid confusing factors: an age limit was established since older subjects can present with new bone formation due to spine osteoarthritis [26]. Furthermore, only male subjects were evaluated since the severity and the clinical characteristics in AS patients could be different according to gender [27], and more importantly, sex steroid hormones play an important role in bone resorption [28]. In addition, patients and controls were matched based on race, age, and body mass index, which are essential factors related to bone metabolism.

Our data suggest that bone metabolism in AS patients is clearly unbalanced, although the cellular and molecular mechanisms leading to bone cell uncoupling have not been elucidated. Receptor activator of nuclear factor-κB ligand (RANKL) has been identified as a potent osteoclast-stimulating cytokine, whereas osteoprotegerin (OPG) acts as its decoy receptor, inhibiting the actions of RANKL [29, 30]. AS patients had a low RANKL/OPG ratio compared to healthy controls; this finding was different from other studies in SpA patients that showed an increase in RANKL serum levels [29]. Regarding the serum OPG concentrations in

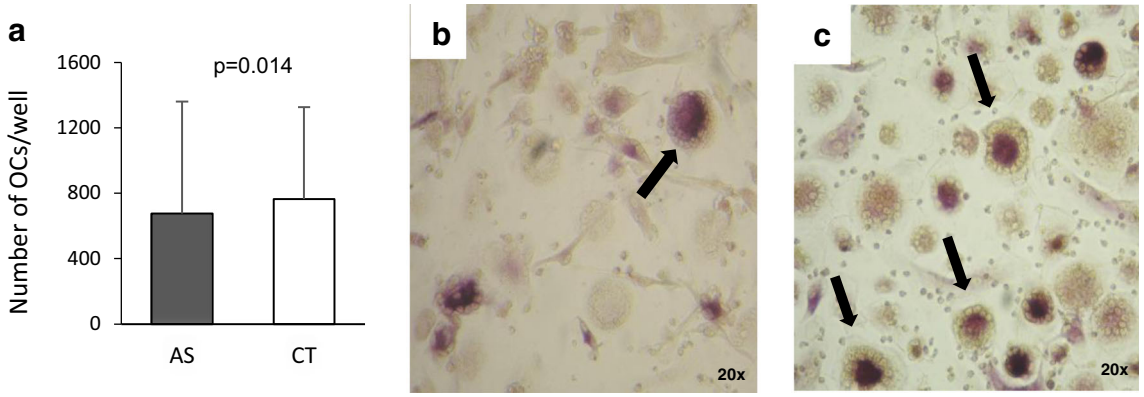


**Fig.1** **a.** Number of osteoclasts from peripheral blood mononuclear cell ($1 \times 10^6$ cell) of Ankylosing Spondylitis (AS) and healthy controls (CT), after 21 days in culture **b.** Photomicrographs of TRAP-positive OC culture from AS patients and. **c.** Photomicrographs of TRAP-positive OC culture healthy controls. Arrows indicate matures osteoclasts

patients with SpA, research groups have reported distinct results compared to healthy controls [31]. In our study, a lower RANKL/OPG and CTX/P1NP ratio were observed in AS patients, suggesting a lower bone resorption. One possible interpretation of this finding is that more than 75% of AS patients in the present study had more than 10 years of disease duration with lower inflammatory activity and lower bone turnover [32]. In clinical practice since bisphosphonate, an antiresorptive drug, is widely used for treatment of bone disease, including AS [33], based on our present data, the question that remains is if bisphosphonates should be used in AS patients with long disease duration. Van Der Weijden et al. [34] showed that patients with AS for less than 10 years are at elevated risk for low BMD and Magrey et al. [35] demonstrated that in AS patients older age was not a significant risk factor for low BMD. Based on the literature and our in vitro findings, bisphosphonates should be considered for early AS disease rather than in patients with a long disease duration.

Based on the literature and our in vitro findings, bisphosphonates could be indicated preferentially at the onset of the disease, rather than a disease with a long disease time.

In fact, our data are in accordance with the scarce data from the literature that show that monocytes from AS patients with a long disease duration had lower osteoclastogenic capacity compared to healthy individuals [7, 16]. These findings suggest that there is an uncoupling between osteoclasts and osteoblast remodeling due to an exhausted bone turnover process, marked by excessive neoformation.

Moreover, a lower percentage of CD16+ subtypes in our study could lead to decreased bone degradation by limiting the homing of precursors to the bone surface. In agreement with our data, Surdarcki et al. [36] demonstrated that there were fewer subtypes of no-classical monocytes in AS patients compared to control individuals. Although the function of monocytes is well characterized in inflammation, their role in AS disease is not fully understood [5].

Contrasting data from studies in AS patients with early disease [37, 38] indicate that an increase in osteoclastogenesis has been identified in bone cells in areas close to bone resorption [37, 38]. Some studies found that proinflammatory cytokines were involved in osteoclastogenesis in the sacroiliac joint, including TNFα, IL6, and IL1β [37]. Im et al. [6], analyzing only 20 AS patients, demonstrated that PBMCs from

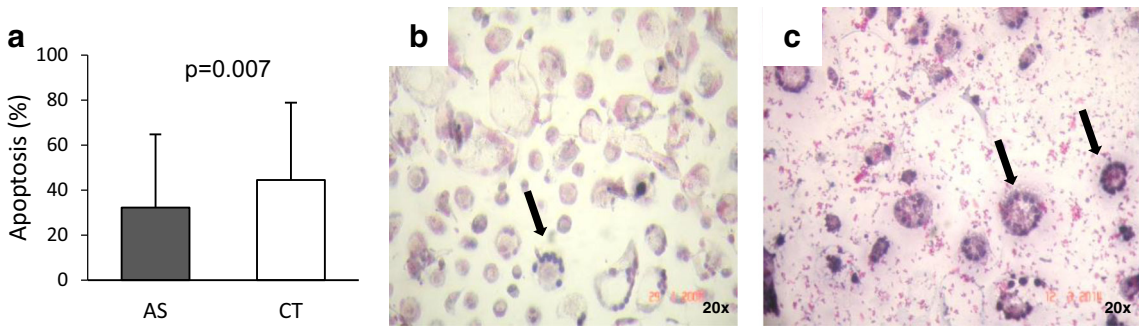


**Fig. 2** **a** Apoptosis in vitro—differentiated osteoclasts from AS patients and healthy controls, **b** photomicrographs of apoptotic cells from AS patients, and **c** photomicrographs of apoptotic cells from healthy controls. Arrows indicate apoptotic osteoclasts

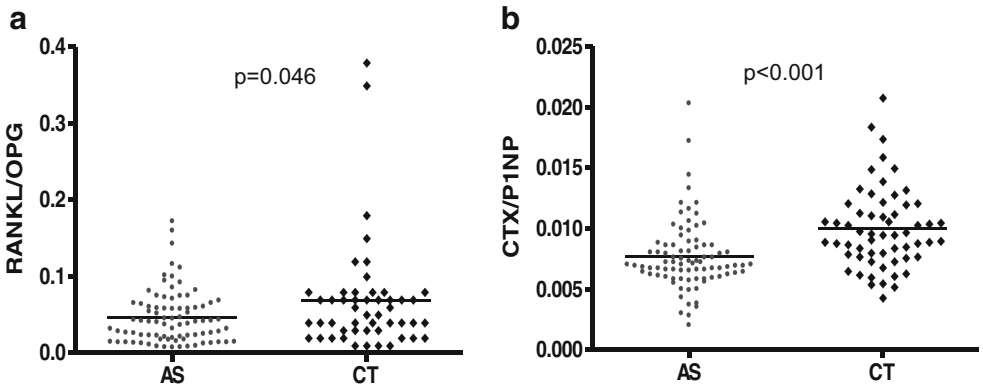


**Fig.3** **a** Ratio serum receptor activator nuclear factor κB (RANKL) and osteoprotegerin (OPG) in patients with ankylosing spondylitis (AS) and healthy controls (CT). **b** Ratio serum C-terminal telopeptide of type I collagen (CTX) and amino-terminal pro-peptide of type I collagen (P1NP) in patients with ankylosing spondylitis (AS) and healthy controls (CT)

AS patients had a higher capacity to generate osteoclasts compared with a control group, although osteoclast activity was not increased. Furthermore, increased osteoclastogenesis was found to be associated with radiographic ankylosis of the sacroiliac joint, but not with disease activity or parameters of systemic inflammation. In our study, the lower capacity to generate osteoclasts in vitro in AS patients was not associated with any disease activity index, inflammatory parameters, or radiographic damage.

Differently than expected, the percentage of cells undergoing apoptosis was significantly lower in AS patients compared to healthy control that might be explained by a compensatory mechanism due to a primary decrease in osteoclastogenesis/osteoclast precursors. Similar findings in Colina et al.' study demonstrated that a lower rate of apoptosis of osteoclasts in AS patients was associated with elevated antiapoptotic factor expression [7].

Interestingly, PBMCs of AS patients taking NSAID therapy did not exhibit a decrease in osteoclastogenesis in vitro, but PBMCs of patients taking only TNFi had lower capacity to generate osteoclasts compared to controls. Previous studies had shown that celecoxib and diclofenac reduce osteoclastogenesis in vitro [39, 40], and another study reported that NSAIDs inhibit murine osteoclastogenesis in vitro and in murine arthritis models in vivo [41]. However, several studies with human osteoclasts did not confirm these effects of cyclooxygenase (COX) inhibitors [42]. There is the possibility that NSAIDs affect bone health by inhibiting COX enzymes, which reduces the synthesis of prostaglandins (PGs), which are multifunctional regulators of bone metabolism [42].

Regarding the finding of low osteoclastogenesis in AS patients who take TNFi, previous studies showed that TNFα stimulates the formation and activity of osteoclasts and that osteoclastogenesis is suppressed by TNFi in TNFalpha- and TNFRI-deficient mice in vitro [43].

As expected, a higher BMD at the lumbar spine but not at the total hip was observed in AS patients in our study compared to controls, suggesting that this increase in bone mass could be secondary to the presence of syndesmophytes in the spine, which is an unreal interpretation of BMD measured by DXA [44]. A decrease in BMD at the total hip indicates that these patients show a lower bone mass secondary to several factors, including less physical activity and less mobility in the lower extremities [45]. However, AS patients taking NSAIDs had similar rates of osteoclastogenesis and lumbar spine BMD compared to healthy controls, suggesting that NSAIDs could counterbalance bone turnover and may have an effect on bone neoformation in these patients [46, 47].

On the other hand, in AS patients taking TNFi without NSAIDs, osteoclastogenesis was significantly lower, and lumbar spine BMD was higher compared to the control group. These findings are in accordance with the literature showing that TNFi therapy could inhibit osteoclastogenesis and have an impact on tissue bone repair in AS patients [48]. Moreover,

**Table 2** Generalized linear model: Number of osteoclasts associated disease duration and treatment (nonsteroidal anti-inflammatory drugs (NSAID) and tumor necrosis factor inhibitor (TNFi)

| Parameter | Coefficient | Std. error | 95% Wald confidence interval | | Wald chi-square | *p* |
|---|---|---|---|---|---|---|
| | | | Lower | Upper | | |
| Intercept | 895.91 | 155.93 | 590.283 | 1201.536 | 33.01 | *< 0.001* |
| NSAID | 77.61 | 109.91 | 137.799 | 293.025 | 0.50 | 0.480 |
| TNFi | − 75.20 | 103.00 | − 277.073 | 126.668 | 0.53 | 0.465 |
| Disease duration (years) | − 15.64 | 4.54 | − 24.546 | − 6.734 | 11.85 | *0.001* |

Italic: *p*-value statistically significant

the influence of TNFi therapy on bone formation and radiographic progression in AS is still controversial.

In conclusion, these findings demonstrated that monocytes from AS patients show a lower capacity to generate osteoclasts compared to control individuals, decreasing the capacity of bone resorption in this disease, reinforcing the idea that osteoclasts may play a role in the physiopathology of bone repair in AS patients.

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## Compliance with ethical standards

This study was approved by the Local Ethics Committee on Human Research at the Sao Paulo University-CAPPesq 0061/11.

**Conflicts of interest** Valeria F. Caparbo, Carla GS Saad, Julio CB Moares, Artur J de Brum-Fernandes, and Rosa MR Pereira declare no conflicts of interest and no competing financial interests.

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