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# The increased *in vitro* osteoclastogenesis in patients with rheumatoid arthritis is due to increased percentage of precursors and decreased apoptosis — The *In Vitro* Osteoclast Differentiation in Arthritis (IODA) study

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ABSTRACT

Increases in local and systemic bone resorption are hallmarks of rheumatoid arthritis (RA). Osteoclasts are implicated in these processes and their enhanced differentiation may contribute to bone destruction. We observed that *in vitro* osteoclastogenesis varies among healthy individuals and hypothesized that increased osteoclastogenesis could be a marker for the presence of RA. Our objective in the present study was to determine if *in vitro* osteoclastogenesis from peripheral blood mononuclear cells (PBMCs) was different in patients with RA compared to healthy controls and osteoarthritis (OA) patients. Expression of CD14 in PBMCs was quantified and PBMCs were incubated for 21 days in the presence of the osteoclastogenic cytokines M-CSF and RANKL. Differentiation on cortical bone slices permitted the analysis of bone resorption while apoptotic potential was assessed by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling. *In vitro* osteoclastogenesis was higher in PBMCs from RA patients compared to controls, and a similar increase was observed in the percentage of osteoclast precursors in RA patients. Osteoclasts from RA patients showed lower apoptotic rates than osteoclasts from healthy controls. No difference was observed in bone resorption activity between RA patients and controls. Interestingly, the difference in osteoclast number and apoptosis rate allowed the implementation of an algorithm capable of distinguishing patients with RA from controls. In conclusion, our study shows that osteoclast differentiation from PBMCs is enhanced in patients with RA, and this difference can be explained by both a higher percentage of osteoclast precursors in the blood and by the reduced apoptotic potential of mature osteoclasts.



## Introduction

Rheumatoid arthritis (RA) is an inflammatory disease characterized by joint destruction and cartilage loss. Periarticular bone erosions and generalized bone loss are hallmarks of RA and indicate that osteoclasts (OCs), cells specialized in bone resorption, are important for the pathogenesis of joint destruction. In RA joints, the presence of OCs and proinflammatory cytokines lead to pathological bone destruction, irreversible joint damage, pain and loss of function [1]. The participation of OCs in the genesis of joint destruction has been clearly demonstrated by both clinical and experimental data. In RA patients, treatment with Denosumab — a monoclonal antibody that binds RANKL and inhibits osteoclastogenesis and OC activity [2] — decreases the progression of bone erosions without affecting inflammation [3]. RANKL knock-out mice with inflammatory experimental arthritis are protected against periarticular bone erosion, confirming the importance of OCs in this process [4,5].

The intensity of either local or generalized bone resorption by OCs depends on the number of OCs formed, on the intrinsic activity of these cells as well as on their survival. Thus, factors inducing or facilitating osteoclastogenesis, or affecting activity or apoptosis may be important in the pathophysiology of bone destruction. OCs are derived from CD34-positive hematopoietic stem cells, which give rise

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to a monocytic lineage expressing CD14 surface protein [6]. OCs arise by fusion of these precursors in the presence of M-CSF and RANKL, two cytokines indispensable for osteoclastogenesis [7–12]. OC differentiation, survival and resorptive activity are highly influenced by inflammatory cytokines present in RA joints [13–15].

In previous studies, we used human OCs differentiated *in vitro* from peripheral blood mononuclear cells (PBMCs) of self-reported healthy donors to study the role of prostaglandin receptors and synthetic enzymes in the control of these cells [16,17]. We noticed that the number of OCs generated by this technique varies widely among individuals, and this led us to the hypothesis that osteoclastogenesis could be an individual characteristic and a predictor or a biomarker of disease. The present study had two main objectives: 1) to investigate the possible relationships among demographic characteristics, the percentage of CD14+ cells and osteoclastogenic capacity, and to determine if osteoclastogenic capacity is stable over time in a population of self-reported healthy blood donors, and 2) to investigate the possible relationship between the presence of RA and osteoclastogenic capacity using the model of *in vitro* osteoclastogenesis from PBMCs. We also compared the cohort of RA patients to controls and to osteoarthritis (OA) patients in several secondary outcomes, including percentage of OC precursors, bone resorptive activity and apoptotic potential.

## Patients and methods

### *Patients and controls*

Patients satisfying the 1987 American College of Rheumatology (ACR) Classification Criteria for RA [18] and for knee OA [19] and willing to give their informed consent were recruited from the outpatient rheumatology clinics at the *Centre hospitalier universitaire de Sherbrooke* (CHUS). A cohort of control individuals for the RA cohort was recruited from the local population by public advertising in a local newspaper: exclusion criteria for this group included a known diagnostic of Rheumatoid arthritis, Osteoarthritis, Psoriatic arthritis, Ankylosing spondylitis or any other inflammatory or autoimmune disease, Osteoporosis or any form of cancer, and absence of present or past articular symptoms lasting more than one week in the absence of trauma. Twenty of these individuals who agreed to a prospective evaluation of osteoclastogenesis were studied weekly for three consecutive weeks. The following individual characteristics were recorded for this cohort: age, sex, body mass index (BMI), physical activity in hours/week, past and present smoking and alcohol intake. Alcohol intake was recorded as the number of drinks/week, one drink being defined as either one beer, one glass of wine, one ounce of strong alcohol or equivalent. Individuals taking any sort of prescription medication were not included in the cohort of self-reported healthy persons. All participants gave written informed consent to participate in this study, which was approved by the Human Ethics Review Board of the CHUS.

### *Materials*

Fetal bovine serum (FBS) was purchased from Gibco (distributed by Invitrogen Canada, Inc., Burlington, ON, Canada). Macrophage-colony stimulating factor (M-CSF) was from PeproTech, Inc. (Rocky Hill, NJ, USA) and the FITC-coupled anti-human CD14 antibody was from BD Biosciences (Mississauga, ON, Canada). TACS Blue Label kit was from R&D Systems (Minneapolis, MN, USA). Human RANKL-GST fusion protein was produced as described elsewhere [20]. Bovine cortical bone was purchased from a local slaughterhouse, cut in thin slices (200 μm) with a diamond saw, devitalized and used as resorption substrate. Antibodies against calcitonin receptor (CTR) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Caspase-3 fluorogenic substrate was purchased from Calbiochem (Merk, Germany). RNeasy Mini kit and Quantitect Reverse Transcription kit were supplied from Qiagen (Mississauga, ON, Canada) and TaqMan Universal PCR Master Mix was from Life Technologies (Burlington, ON, Canada). All other reagents were purchased from Sigma-Aldrich Canada, Ltd. (Oakville, ON, Canada).

### *Cell culture*

Blood samples (100 ml) were collected and PBMCs were isolated by Ficoll density gradient and dextran sedimentation. Cells were plated at $1.5\times10^6$ cells/cm$^2$ in 48-well plates (either empty or containing a bone slice) or on 12-mm round coverslips in 24-well plates coated with poly-L-lysine. Cells were maintained in α-MEM supplemented with 10% FBS, 1% penicillin–streptomycin, 50 ng/ml RANKL, and 10 ng/ml M-CSF. Cells were allowed to differentiate for 21 days in a 37 °C, 5% $CO_2$, humidified atmosphere with regular (3–4 days) medium changes.

### *Quantification of OC precursors*

Flow cytometry was used to determine the percentage of CD14+ cells among the PBMC samples. After cells were counted, $1\times10^6$ PBMCs were centrifuged and incubated in PBS with a dilution 1:10 of FITC-conjugated anti-human CD14 antibody for 30 min. Then, cells were washed, suspended in PBS and analyzed by flow cytometry using FACScan from Becton Dickinson (Mississauga, ON, Canada) (10,000 events analyzed).

### *Calcitonin receptor staining*

Cells seeded on 12-mm round coverslips in 24-well plates were fixed and incubated in 1% paraformaldehyde for 10 min, then permeabilized with PBS 1% Triton X-100. Cells were stained for TRAP for 30 min. Nonspecific binding was blocked by incubating the cells with 5% skimmed milk in phosphate-buffered saline for 1 h and then the first antibody (goat anti-human CTR) diluted 1:250 in blocking buffer was added. Cells were incubated overnight at 4 °C, then washed and incubated with the secondary antibody coupled to Alexa Fluor 546 (dilution 1:100) for 1 h. Absence of the first antibody was used as negative control. Pictures were taken with a Nikon Eclipse microscope and the Simple PCI software.

### *Differentiation assay*

PBMCs were differentiated for 21 days in 48-well plates. Cells were then washed with PBS and stained for TRAP for 30 min. TRAP-positive multinucleated cells containing three and more nuclei were counted as OCs and the results were expressed as the number of OCs per well. For 41 randomly selected controls, 46 RA and 22 OA patients, the number of nuclei in TRAP+ cells were also recorded by counting 15 fields for each patient's slide with a 20× objective; the cells were classified as small, medium or large OCs if they contained from 3 to 5, 6 to 9, or 10 or more nuclei, respectively; results are expressed as the total number of OCs in each category per 15 fields examined.

### *RT-PCR*

After 21 days of culture, total RNA was extracted from osteoclasts using RNeasy Mini kit. Reverse transcription was done using Quantitect Reverse Transcription kit. Real time PCR was performed using 7500 Applied Biosystems instrument (Life Technologies), with TaqMan Universal PCR Master Mix, and the cathepsin K (Hs00166156) and ß-actin (Hs99999903) TaqMan gene expression assays.

*Resorption assay*

PBMCs were differentiated for 21 days under the same conditions described above, but on devitalized bovine cortical bone slices, two slices per individual studied. Differentiated cells were then incubated for 10 days at 37 °C in 10% $CO_2$. Bone slices were then stained with 0.2% toluidine blue for 3 min to reveal the formation of lacunar resorption pits, washed in water, and air dried before being examined by brightfield microscopy. Photographs of 10 randomly selected fields covering a 2.26 $mm^2$ surface each were taken and the resorption area was quantified using the image analysis program SimplePCI from Compix Inc., Imaging Systems (Cranberry Township, PA, USA) and extrapolated for the whole slice surface. Results are expressed as the average of resorption area in $\mu m^2$ per disk (2 disks per individual); total area of each disk is 27.34 $mm^2$.

*OC apoptosis*

OC apoptosis was determined after 21 days of differentiation *in vitro* using the TACS Blue Label kit following the manufacturer's instructions. Cells were maintained for 24 h without M-CSF or RANKL in 5% FBS, and then were fixed and permeabilized with cytonin. Biotinylated nucleotides were incorporated by terminal deoxynucleotidyl transferase and then, streptavidin-horseradish peroxidase conjugate was added. Upon addition of the substrate, TACS Blue Label, the enzymatic activity on the substrate generated an insoluble blue precipitate in nuclei where DNA fragmentation had occurred. A total of 100 multinucleated (three nuclei or more) cells were examined and OCs with two or more blue nuclei were considered positive for apoptosis.

*Caspase activity assay*

Caspase-3 activity was determined in cultures from 5 RA patients and 4 controls using a caspase-3 fluorogenic substrate to determine if there was a correlation between the levels of apoptosis using the TACS kit and caspase-3 activity. In these assays, staurosporine (1 μM for 3 h) was used as a positive control of apoptosis. The activity of caspase-3 was measured using a fluorogenic peptide substrate, CPP32/Apopain. Differentiated osteoclasts were cultured in the media containing 5% FBS for 24 h without M-CSF and RANKL, then cells were lysed using RIPA buffer (50 mM Tris–HCl pH 8, 150 mM NaCl, 1 mM DTT, 0.5 mM EDTA, 1% Igepal, 0.5% Na deoxycholate, 0.1% SDS) with a protease inhibitor cocktail to extract proteins. Cell lysate proteins (30 μg) were incubated with 100 μM CPP32/Apopain in a reaction buffer (100 mM Hepes pH 7.5, 20% glycerol, 5 mM DTT) for 2 h at 37 °C. Cleavage of CPP32/Apopain was monitored by a fluorescence spectrophotometer at an excitation/emission wavelength pair of 380 nm/405–500 nm.

*Statistical analyses*

The following parameters were compared between the cohort of RA and OA patients and the group of controls: number of OCs, percentage of CD14+ cells, OC resorption, and apoptosis. One-way analysis of variance (ANOVA) was used to determine differences among groups. Categorical variables were presented as frequency and percentage, and statistical significance was determined with the Chi-square test. Continuous variables were presented as mean ± standard error (SEM) and data were compared between groups using the Student's t-test, if the distribution was normal, or with the Mann–Whitney test, if not. Methotrexate, prednisone and bisphosphonate use was not found in the control group. To evaluate potential contributions of medications, rheumatoid factor, age, sex, and smoking to differences in the number of OCs, percentage of CD14+ cells, OC resorption, or apoptosis in the RA group, multivariate linear regression was performed. Univariate and multivariate logistic regressions were utilized to build diagnostic models based on disease markers that were found to be significant. Statistical analyses were performed with SPSS software version 17.0 and logistic regressions were computed using WEKA software version 3.6 [21]. Differences were considered significant when $p<0.05$.

## Results

*Patient cohort and controls*

140 patients with RA, 60 with OA and 105 self-reported healthy donors were evaluated. Demographic and clinical characteristics are shown in Table 1. The only statistically significant difference between the group with RA and the self-reported healthy control group was the fact of having ever smoked, which was higher in the group with RA. The group with OA differed significantly from the group with RA on the following characteristics: age and percentage of women in menopause, which were higher in the OA group, and present and past smoking, lower in the OA group. As anticipated by the definition of healthy control, no use of bisphosphonates, methotrexate or prednisone was found in this group, which was not tested for bone mineral density or the presence of rheumatoid factor. Osteoporosis, defined by a bone mineral density with a t score lower than −2.5 was found in 11.4% of patients with RA and in 5.0% of patients with OA.

The number of osteoclasts after 21 days in culture for controls can be found in Fig. 1A. It varied between a minimum of 19 and a maximum of 1814 with a median and interquartile range for the total population of 267 (57–487) OCs/well. No correlation was found between age and the number of osteoclasts. Multivariate analysis indicated no significant correlation between the demographic characteristics shown in Table 1 and OC number. Fig. 1B shows that no difference was observed between the percentage of OC precursors (CD14+ cells) in men and women of the whole cohort. The median of the whole cohort was 14.8%, with interquartile range from 9.7% to 18.3%. No significant correlation was established when comparing percentage of OC precursors with BMI, smoking, menopause or age.

*Prospective cohort*

Twenty healthy donors were followed for three consecutive weeks. Demographic characteristics of this subgroup are shown in Table 2. There were more women than men (60% *vs* 40%, respectively) and the average age was 33.5 ± 10.7 years. Fig. 1C shows the results from the three visits for the number of OCs. We used the median of the number of OCs/well of the whole self-reported healthy cohort to divide the whole cohort in two groups: low differentiators, with a number of OCs/well of <348, and high differentiators, with ≥348 OCs/

**Table 1**
Demographic and clinical characteristics of the cohorts of rheumatoid arthritis and osteoarthritis patients and controls.

| | Controls (n = 105) | RA (n = 140) | OA (n = 60) |
|---|---|---|---|
| Age, mean ± SD years (min–max) | 58.9 ± 8.1 (27–77) | 60.5 ± 11.5 (23–85) | 68.0 ± 9.4 (45–87)§ |
| Gender, male/female | 45/60 | 45/95 | 20/40 |
| Menopause (% of women) | 83.3 | 84.2 | 97.5* |
| Presently smoking (%) | 16.2 | 18.6 | 5.0* |
| Ever smoked (%) | 37.1 | 62.1§ | 53.3* |
| BMI (mean ± SD) | 26.4 ± 4.6 | 26.6 ± 5.1 | 30.5 ± 6.4*** |
| Use of bisphosphonates (%) | N/A | 31.3 | 10 |
| Use of methotrexate (%) | N/A | 96.4 | N/A |
| Use of prednisone (%) | N/A | 18.6 | N/A |
| Positive RF (%) | N/A | 65.7 | N/A |
| Osteoporosis (%) | N/A | 11.4 | 5.0 |

Mann–Whitney test was used. *p<0.05, ***p<0.001 when compared to RA and §p<0.0001 when compared to controls.

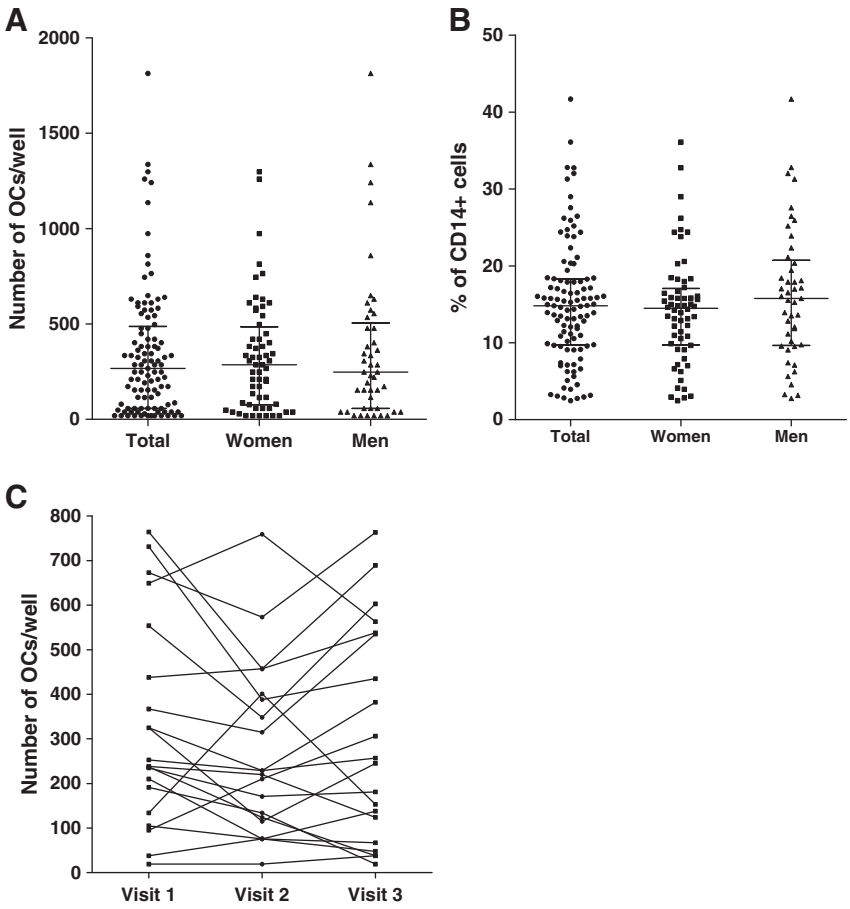


**Fig. 1.** Osteoclastogenesis in a cohort of self-reported healthy individuals. A, Number of OCs/well per individual studied. PBMCs were differentiated for 21 days and multinucleated (three or more nuclei) TRAP-positive cells were counted. Median with interquartile range is shown; Mann–Whitney test was used to compare men and women, no statistically significant difference was detected. B, Number of OC precursors (CD14+ cells) determined by flow cytometry from PBMCs. Median with interquartile range is shown; Mann–Whitney test was used to compare men and women, no statistically difference was detected. C, Number of OCs/well from the prospective cohort was quantified weekly for three consecutive weeks; McNemar's test showed no significant difference in the classification of individuals as high or low differentiators in the three weeks tested.

well. Seventeen donors (85%) stayed in their initial group from visits 1 to 3. One donor went from the low differentiator group in visit 1 to the high differentiator group in visit 2, but returned to low in the third visit. Two others were below the median for visits 1 and 2, but not for visit 3. We used the McNemar analytic test to determine if there were differences in the classification of high and low differentiators between visits 1 and 2, 1 and 3 and 2 and 3, but no significant differences were found. Similar results were found with the percentage of CD14+ precursors (data not shown).

*In vitro osteoclastogenesis*

The capacity to generate OCs from PBMCs was significantly higher in the group of patients with RA (447 ± 38 OCs/well) than in controls (342 ± 33 OCs/well, $p<0.05$) (Fig. 2A). No significant difference was observed for OA patients when compared to RA patients or controls. Multivariate analysis of the group with RA showed no impact of methotrexate, bisphosphonates, prednisone or the presence of rheumatoid factor on the number of OCs. As shown in Fig. 2B, expression of cathepsin K was weakly but statistically significantly correlated with the number of OCs/well ($p=0.0339$, $r=0.2504$) confirming that TRAP-positive cells with 3 nuclei and more are representative of osteoclasts. In Figs. 2C and D, multinucleated TRAP-positive cells were stained for the calcitonin receptor. Omission of the first antibody (negative control) is shown in Figs. 2E and F. As shown in Fig. 2G, the difference in OC numbers between RA patients and controls was most pronounced in the large OC category. Large OCs (10 nuclei and more) were more frequent in RA patients (17 ± 3 OCs) than in controls (7 ± 1 OCs, $p<0.05$). In contrast, the number of small (3–5 nuclei) or medium (6–9 nuclei) TRAP+ cells was not statistically different among the three groups (49 ± 5 in RA *vs* 37 ± 5 in controls and 39 ± 6 in OA, and 22 ± 3 in RA *vs* 14 ± 2 in controls and 16 ± 3 in OA, respectively).

**Table 2**
Demographic characteristics of the prospective cohort.

| | Total | Women | Men |
|---|---|---|---|
| Total | 20 | 12 | 8 |
| Age (years) (min–max) | 33.5 ± 10.7 (23–59) | 31.9 ± 9.5 (24–59) | 35.8 ± 12.5 (23–57) |
| Menopause (% of women) | 8.3 | 8.3 | – |
| BMI (kg/m$^2$) | 23.4 ± 2.4 | 22.8 ± 2.1 | 24.2 ± 2.8 |
| Presently smoking (%) | 15.0 | 8.3 | 25 |
| Ever smoked (%) | 5.0 | 0 | 12.5 |
| Physical activity/week (hours) | 3.7 ± 2.6 | 3.4 ± 3.1 | 4.1 ± 1.7 |
| Alcohol intake drinks/week | 3.9 ± 3.3 | 3.0 ± 3.4 | 5.4 ± 2.5 |

Values for age, BMI, physical activity/week and alcohol intake/week are the mean ± SD.

*Circulating OC precursors*

Using flow cytometry and an anti-human CD14 antibody, we demonstrated that RA patients had relatively more CD14+ cells in their PBMC preparations (18.5 ± 0.8%) than controls (15.1 ± 0.8% CD14+ cells, $p<0.01$) (Fig. 3). Percentage of CD14+ cells in OA patients (16.8 ± 1.1%) was not different than in RA patients or controls.

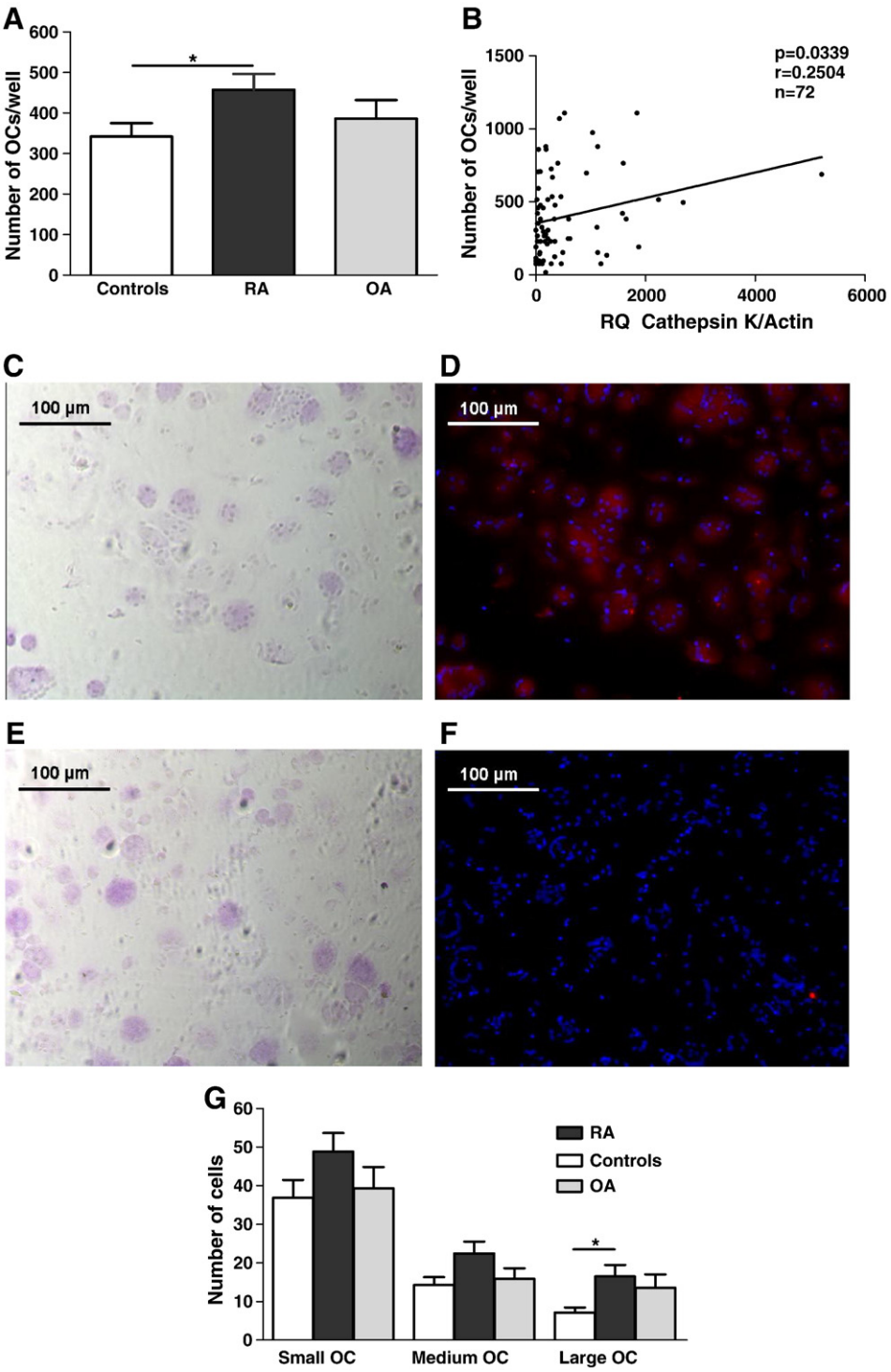


**Fig. 2.** *In vitro* osteoclastogenesis is increased in RA patients when compared to controls. A, PBMCs were differentiated for 21 days and multinucleated (three or more nuclei) TRAP-positive cells were counted. Means ± standard error of the mean (SEM) are shown. n = 105 for controls, 140 for RA and 60 for OA patients. B, Scatter plot presenting the correlation of RQ Cathepsin K/Actin and number of OCs/well. Linear regression shows a direct, linear statistically significant correlation between the two parameters. C and D, TRAP-positive OC culture from a control (C) and staining for calcitonin receptor (D) in red; nuclei are in blue. E and F, TRAP-positive OC culture from a healthy control (E) and negative control for staining of calcitonin receptor (F). *$p<0.05$, Mann–Whitney test was used. G, Distribution of small (3 to 5 nuclei), medium (4 to 9 nuclei) and large (10 and more nuclei) osteoclasts in cultures from randomly selected 41 controls, 46 RA and 22 OA patients. Results are means ± S.E.M. of all studied individuals and represent the total number of OCs in each category per 15 fields examined with a 20× objective.

*Resorptive activity is comparable in OCs from RA patients, OA patients and controls*

OCs differentiated on bovine cortical bone disks, in the presence of M-CSF and RANKL, were used to study the consequence of RA disease on resorptive activity. As shown in Fig. 4A, no statistical difference was found in the areas resorbed by OCs from RA patients (667 000 ± 104 000 µm$^2$) and OA patients (880 597 ± 201 406 µm$^2$) compared to OCs from the control group (585 000 ± 122 000 µm$^2$). Fig. 4B is representative of the resorption paths formed on cortical devitalized bovine bone.

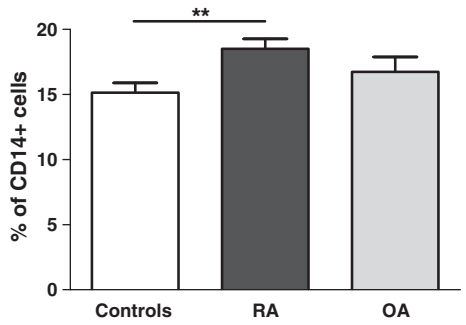


**Fig. 3.** Percentage of monocytes expressing surface antigen CD14 in controls, RA and OA patients. PBMCs were incubated with FITC-conjugated anti-CD14 antibody and analyzed by flow cytometry (results expressed as means ± SEM of the percentages of positive events out of the total 10,000 events). $^{**}p<0.01$, two-tailed Student's t-test.

*Apoptotic potential of in vitro-differentiated OCs*

Fig. 5A shows the percentage of OCs positive for apoptosis by the TUNEL assay in the control, RA and OA groups 24 h after cytokine withdrawal. The percentage of cells undergoing apoptosis was significantly lower in the group of RA patients (12 ± 1%) than in controls (23 ± 1%) and OA patients (20 ± 2%), $p<0.001$ for both comparisons. In nine specimens (5 from RA patients and 4 from healthy controls) caspase-3 activity was quantified and the results correlated to the percentage of apoptosis obtained with the TACS method. A linear correlation was found between both methods ($p=0.0001$, $R^2=0.9442$) as shown in Fig. 5B. Fig. 5C shows TUNEL assay on cells in 5% FBS starved of M-CSF and RANKL for 24 h; just one cell (black arrow) showed blue, apoptotic nuclei. Fig. 5D shows TUNEL assay on non-starved cells; Fig. 5E shows cells treated with staurosporine 1 μM 3 h before TUNEL assay, as a positive control.

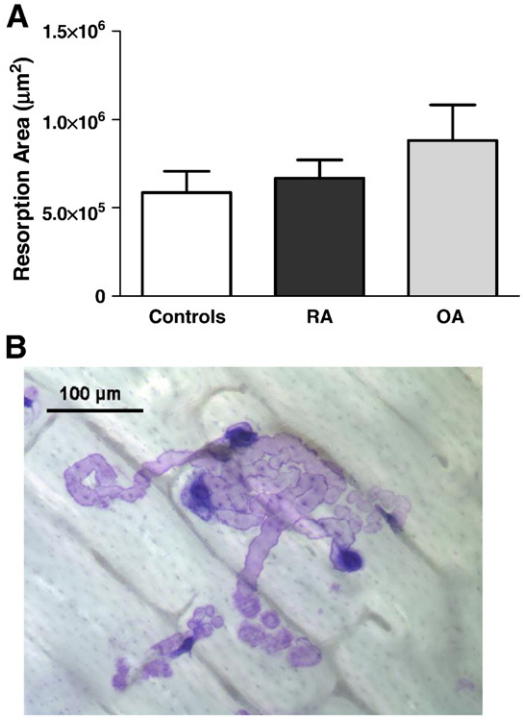


**Fig. 4.** A, Resorption area of bovine cortical bone by *in vitro*-differentiated osteoclasts from self-reported healthy individuals, RA and OA patients. Results are expressed as mean ± SEM of the total surface presenting resorption in $\mu m^2$, from a total disk surface of 27.34 $mm^2$. After 31 days in culture (the last 10 days at 10% $CO_2$), disks were stained with toluidine blue (0.2%) and pit formation was quantified by microscopy. Mann–Whitney test was used with statistical difference set at $p<0.05$. No significant difference was observed between the groups. B, Example of resorption lacunae on bone disk from a patient with RA.

*Multivariate diagnostic models based on significant disease markers*

We next investigated whether some of the OC parameters studied could be used to build a computer-based diagnostic model. We used a logistic regression-based model [22] and nonlinear models (Support Vector Machines and feed-forward neural networks) that we previously found have predictive quality comparable to that of the simpler logistic regression model. The models were validated using two types of tests: 1) the resubstitution test, in which the model is built and tested on the entire cohort; and 2) an out-of-sample test, in which we built the model using a subset of patients and controls and tested it using the remaining members of the cohorts. The latter test verifies the predictive quality for "unseen" individuals, whose data were not used to develop the model, effectively simulating the future use of the model. We applied a 10-fold cross validation test in which the cohort was divided at random into 10 equally sized subsets; nine of these subsets were used to derive the model and the remaining subset was used for testing. This was repeated ten times, each time selecting a different subset to test the model, and these test results were pooled together. We repeated the entire 10-fold cross validation 100 times using different random divisions into subsets to quantify variability among individual cross validations. The results from the resubstitution tests together with averages of the 100 runs of the 10-fold cross validation test are reported in Table 3. We first implemented the models using only the OC apoptosis, which had the strongest significance of the difference between the RA and control groups. Second, we used both the apoptosis and the osteoclastogenesis data to investigate whether combing these two markers would lead to improved predictive quality of our diagnostic model. OC apoptosis alone allowed for predictions with about 73% cross-validated accuracy and the accuracy reaches 76% when we add data on the number of OCs/well. The regression models and their predictions are depicted in Fig. 6.

## Discussion

Our first aim was to study the number of OCs generated *in vitro* from PBMCs of a group of self-reported normal individuals and to investigate possible relationships among demographic characteristics, the percentage of CD14+ cells and the osteoclastogenic capacity. We confirmed our previous unpublished observations that the capacity to generate OCs *in vitro* varies greatly among individuals and showed that these differences are not related to gender, age, or any of the demographic characteristics studied. We also showed that the characteristic of being a high or a low differentiator was stable during the time frame of the study for the great majority of the individuals studied. These results suggest that the capacity of generating high or low numbers of OCs *in vitro* could be an individual characteristic or phenotype. Given the important role of OCs in both local and systemic bone loss in RA, we hypothesized that, if osteoclastogenic capacity is an individual characteristic, it could be a determinant of the presence or severity of RA. This hypothesis led us to compare a cohort of RA patients to a group of controls and patients with knee OA over a period of four years, the "*In vitro* OC Differentiation in Arthritis" (IODA) study. This is the first report on the study, and our primary objective was to compare osteoclastogenesis in RA patients observed at the first visit of the IODA study to that in the control groups.

The demographics of our group of 140 RA patients correspond to those described for a mostly Caucasian population [23] of RA patients, with a 2:1 predominance of females. The female to male ratio was slightly higher in the RA than in the control group, but this difference was not statistically significant. Besides medication that, by definition, was absent from the control group, all other characteristics studied

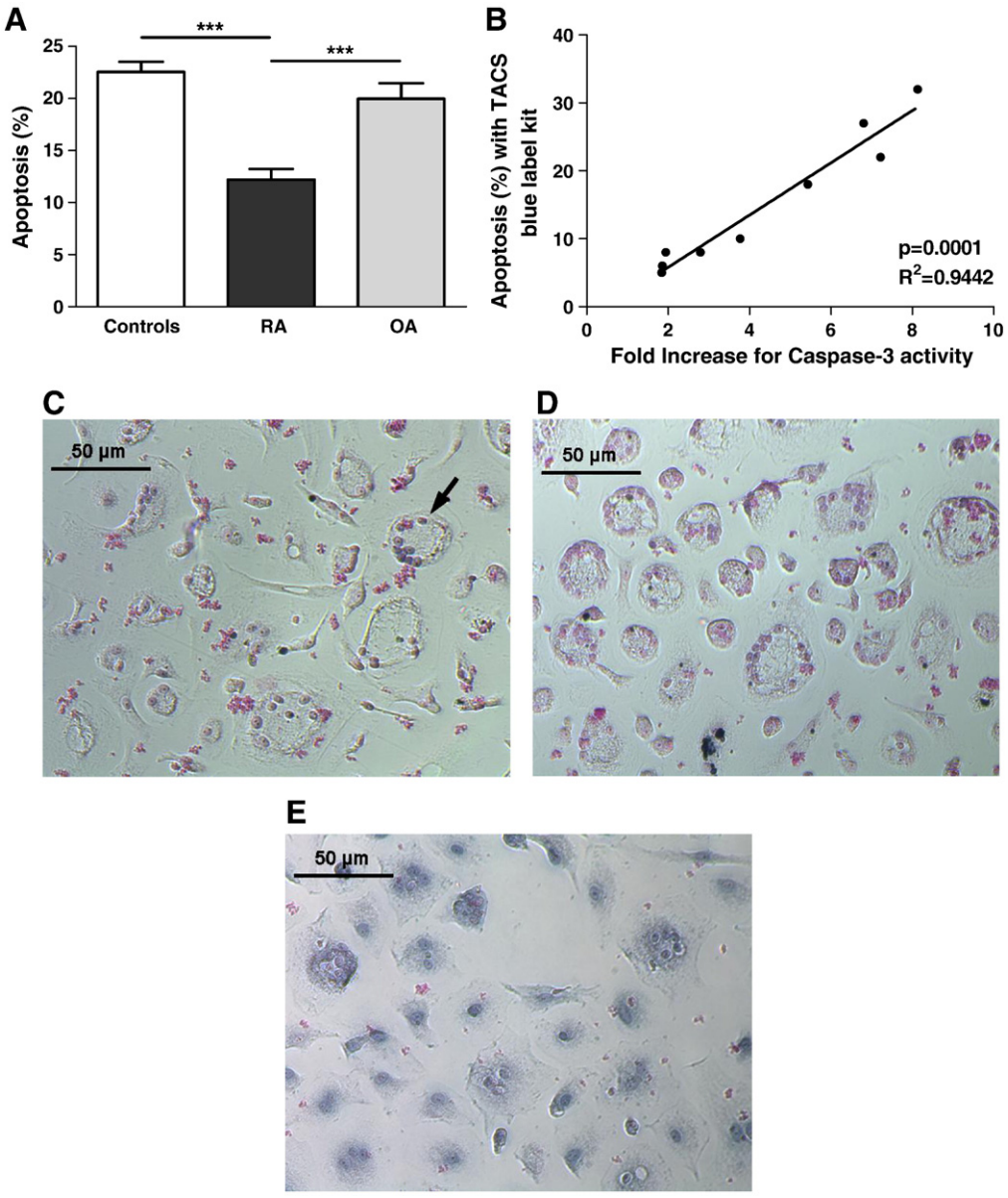


**Fig. 5.** Apoptosis rate in *in vitro*-differentiated OCs from controls and patients with RA or OA. A, Cells were differentiated for 21 days and then RANKL and M-CSF were removed for 24 h. To quantify the number of apoptotic OCs, TACS blue labeling was used. Only cells with three or more nuclei were counted. Results are presented as mean ± SEM of the percentage of cells showing a blue staining for the whole cohorts studied. A lower apoptosis rate was found in cells from RA compared to OA patients or controls (***$p<0.001$, Mann–Whitney test). B, Scatter plot showing the positive, linear, statistically significant correlation of caspase-3 activity and the percentage of RANKL and M-CSF-starved OCs undergoing apoptosis determined using TACS blue labeling n = 9 (5 from patients with RA and 4 from controls). C, TUNEL assay on cells in 5% FBS starved of M-CSF and RANKL for 24 h; black arrow indicates cell with blue nuclei. D, TUNEL on non-starved cells. E, TUNEL on cells treated with staurosporine 1 μM as a positive control for apoptosis. Cells in figures C–D are from a RA patient.

were comparable between the groups except for the characteristic of ever smoking, the frequency of which was higher in the RA group. Tobacco use appears associated with both increased incidence and severity of RA [24], and our data may reflect this effect. Current smoking, however, was comparable in both groups. As expected from the known demographics of OA, this group of patients, used here as a comparator to the RA group, showed a significantly higher age, higher percentage of women in menopause, a higher BMI and a lower percentage of present or past smokers. Even though, as described above, our main outcome in this study (*i.e.* the number of osteoclasts) did not correlate with age, these differences between the groups with RA and OA should be considered when comparing the results in these two groups.

Relative to the control group, patients with RA presented significantly higher numbers of OCs after 21 days in culture, and the OCs in the RA group were larger than in the control group. The difference with the OA group was not statistically significant. Our method of counting the number of TRAP-positive cells with three or more nuclei is representative of the number of OCs, as indicated by a direct and linear correlation between this parameter and cathepsin K activity in the culture. These findings may be of pathophysiological importance since higher numbers of OCs may lead to more bone resorption. Moreover, it is established that larger OCs are more active at bone resorption sites than smaller cells, further accounting for excessive bone loss [25]. It is interesting that the use of methotrexate, bisphosphonates or corticosteroids by the group of RA patients had no impact on the number of OCs generated *in vitro*, as shown by multivariate analysis. Many other medications were taken by the patients in the RA group, such as hydroxychloroquine, sulfasalazine and biologics, but the numbers using each drug were too low to allow

**Table 3**
Assessment of the predictive quality of the logistic regression-based diagnostic models with apoptosis and the combination of apoptosis and osteoclastogenesis (number of OCs per well) as the input variables.

| Inputs | Validation test type | Accuracy (%) | Sensitivity (%) | Specificity (%) | Error reduction (%) |
|---|---|---|---|---|---|
| OC apoptosis | 100 rounds of 10-fold cross-validation | 72.8 ± 1.1 | 74.0 ± 1.1 | 71.6 ± 2.1 | 43.3 ± 2.3 |
| | Resubstitution | 75.5 | 73.0 | 78.3 | 48.9 |
| OC apoptosis and number | 100 rounds of 10-fold cross-validation | 75.9 ± 0.9 | 75.6 ± 0.9 | 76.2 ± 1.6 | 49.7 ± 1.9 |
| | Resubstitution | 76.6 | 76.0 | 77.2 | 51.1 |

The models were assessed using the entire cohort of RA patients and healthy controls using resubstitution tests, in which the model was built and tested on the entire cohort, and out-of-sample cross-validation tests. We applied the 10-fold cross-validation test in which the cohort was divided at random into 10 equally sized subsets; nine of these subsets were used to derive the model and the remaining subset were used for testing. This was repeated ten times, each time selecting a different subset, from among 10 subsets, to test the model and these test results were pooled together. We repeated the entire 10-fold cross-validation 100 times, each time using a different random division into subsets to quantify variability between individual cross validations. We report the averages of the 100 runs together with their standard deviations. We measured *accuracy* = TP + TN/(TP + FP + TN + FN), *sensitivity* = TP/(TP + FN), *specificity* = TN/(TN + FP), and *error reduction* = (100 − accuracy)/(100 − baseline accuracy). True positives (TP) and true negatives (TN) correspond to correctly predicted RA patients and controls, respectively, false positives (FP) denote controls predicted as RA patients, false negatives (FN) denote RA patients predicted as controls, and baseline accuracy denotes the accuracy of a trivial model that always predicts the most frequent predictive outcome, which is that the individual has RA. The accuracy quantifies the overall success rate and the sensitivity/specificity measures the success rate for predicting the RA patients/controls. The error reduction quantifies the percentage of the improvement over the error rate (= 100 − accuracy) when compared with the error rate of the trivial model. Higher values for each of these measurements correspond to better quality of predictions.

any statistically significant conclusion. These results suggest that the higher number of *in vitro*-differentiated OCs in the group of RA patients correlates to the presence of disease and not to its treatment.

The increased number of OCs found in the RA population after 21 days in culture could be a consequence of several different factors. We studied the percentage of CD14+ cells, precursors of OCs, as well as OC apoptosis in both groups. Interestingly the group of RA patients presented with both a higher percentage of CD14+ OC precursors and a lower apoptosis ratio than the control group, suggesting that these two factors contribute to the larger number of OCs found in the RA group. Regulation of OC survival is an important mechanism of physiological bone homeostasis, since a variety of growth factors and cytokines that stimulate bone resorption such as RANKL, IL-1 and TNF-α also prevent OC apoptosis [26,27]. In RA, up-regulation of RANKL in plasma might contribute to the prevention of OC apoptosis, increasing osteoclastogenesis as previously observed [28]. However, our data indicate that RA OCs generated in the presence of standard concentrations of RANKL and M-CSF still exhibit reduced apoptosis compared to control OCs, suggesting significant contribution of intrinsic factors, independent on the presence of cytokines or growth factors.

Despite the higher number of OCs and the higher proportion of large OCs in RA samples, we did not observe significantly higher *in vitro* bone resorption rates in the RA compared to the control or OA groups. This finding is not easy to explain, but it should be noted that

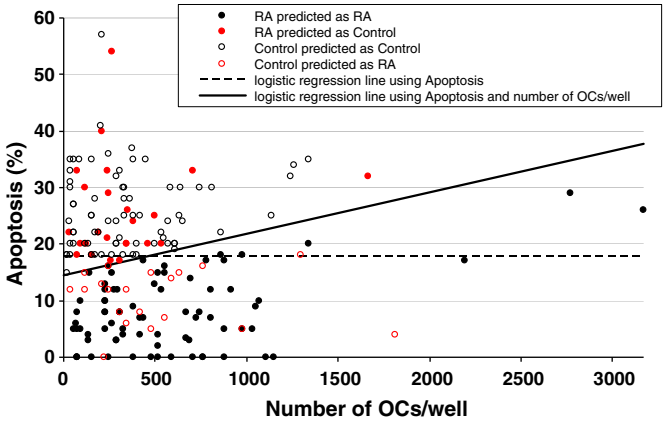


**Fig. 6.** Predictions generated by the logistic regression models built using OC apoptosis (*y*-axis) and osteoclastogenesis data quantified as number of OCs per well (*x*-axis). The lines correspond to decision lines of the regression model based on the OC apoptosis only (dashed line), or based on both the OC apoptosis and number (solid line). Points above a given line are predicted as controls and points below the line are predicted to have RA. The legend gives the annotation of the predictions for the model that used both apoptosis and osteoclastogenesis.

the primary outcome in the study was the number of OCs generated after 21 days of culture and that the study was powered to detect differences in this primary outcome, which has a much smaller variance than that of bone resorption in the present study. Therefore, it is likely that our study lacked the statistical power to detect small differences in bone resorption activity. Indeed, it was previously shown that bone resorption by OC precursors from synovial fluid is increased in patients with RA [29]. In addition, OCs formed from blood precursors from patients with RA have increased bone-resorbing activity compared with those obtained from controls [30]. Although these results differ from ours, it is important to consider that the models are different. In the present study, *in vitro* resorption after 21 days is unlikely to be directly influenced by inflammatory cytokines such as TNF-α and IL-1 [31,32] present in the blood of RA patients, but it is reasonable to speculate that OC precursors may have been primed by these and other cytokines in the circulation before being isolated and seeded in culture.

Decreased apoptosis of the OCs generated *in vitro* could explain, at least in part, the increased number of OCs in the group of patients with RA. Using TACS to detect the number of cells undergoing apoptosis — a method leading to results that correlate linearly with caspase-3 activity in the cells — we showed that starved OCs from RA patients present much lower apoptosis rates than cells from the control or OA groups.

We investigated whether OC apoptosis and *in vitro* osteoclastogenesis could be used to build a diagnostic model for RA using multivariate classification approaches. The accuracy levels of our logistic regression-based diagnostic models are still too low for direct clinical application. However, these models do provide strong diagnostic input, *i.e.*, their predictions are characterized by error reductions of 43% (when using information concerning OC apoptosis) and 50% (when using OC apoptosis together with OC numbers) when compared with error rates of a trivial model that always predicts the most frequent outcome in the cohort (Table 3). In other words, our diagnostic model cuts in half the error rate of the trivial model. We also compared the results of the corresponding 100 runs of the 10-fold cross validations and found that the improvements between the predictions using only OC apoptosis and using both the apoptosis and osteoclastogenesis as inputs are significant ($p<0.001$; we used the Wilcoxon rank sum test since the variables were not normally distributed). These significant improvements concern all quality indices, which are defined in the caption of Table 3, including accuracy (overall rate of correct predictions), sensitivity (rate of correct predictions for the RA patients), specificity (rate of correct predictions for controls), and error reduction (improvement over the error rates of the trivial model). These analyses support our novel finding that OC apoptosis and osteoclastogenesis provide complementary diagnostic

information for RA. It is important to point out that we are not suggesting a new diagnostic test approach for RA, but rather using this computerized procedure as another method testing the association of increased *in vitro* osteoclastogenesis and RA.

The present study corroborates and extends, in a much larger population, the findings of Nose et al. [33], who showed, in a cohort of 10 RA patients and using a different differentiation protocol, a higher number of *in vitro*-differentiated OCs in an RA group compared to controls. Another study in an equally small cohort of RA patients showed an increase in the resorption area by OCs differentiated from RA PBMCs compared to control, even though the number of OCs did not differ between the two groups [30]. These results are difficult to compare with the ones presented here because we did not use dexamethasone in the culture medium, which greatly increases bone resorption by OCs.

In conclusion, our study suggests that the capacity to generate OCs *in vitro* is an individual characteristic or phenotype that tends to be stable over time. It also shows that OC differentiation from PBMCs is enhanced in patients with RA. This result could be explained by both the higher percentage of OC precursors in the peripheral blood of patients with RA and by the reduced apoptosis of the mature OCs. The differences in OC number and apoptosis rate were sufficient to allow implementation of an algorithm capable of distinguishing patients with RA from controls. Further studies are needed to determine if this difference has prognostic impact in the clinical setting. We are presently evaluating the patients of the IODA cohort for other clinical and radiologic characteristics that will allow the determination of subgroups with severe disease and permit us to determine if osteoclastogenesis also relates to disease severity. A prospective evaluation of this cohort during three years, with special emphasis on osteoclastogenesis, periarticular bone erosion and function is also under way.

## Conflict of interest

The authors have no conflict of interest to disclose. This study was supported in part by Pfizer Canada.

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