ORIGINAL RESEARCH ARTICLE

Cardiovascular Therapeutics

Atorvastatin Reduces Circulating Osteoprogenitor Cells and T-Cell RANKL Expression in Osteoporotic Women: Implications for the Bone–Vascular Axis

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SUMMARY

Aim: Circulating osteoprogenitors and receptor activator of nuclear factor kappa-B ligand (RANKL) expression in immune cells have been implicated in the pathogenesis of osteoporosis and vascular calcification. The role played by statin therapy in the bone–vascular axis is unknown. **Methods:** Twenty naïve postmenopausal osteoporotic hypercholesterolemic women were treated with Atorvastatin 40 mg/day for 3 months. Gene expression analysis was performed to assess modification in osteoprotegerin (OPG)/RANK/RANKL expression in isolated T cells and monocytes. A flow cytometry analysis was used to study changes in the levels of circulating osteoprogenitor cells. **Results:** After 3 months of treatment, Atorvastatin significantly reduced total cholesterol and LDL-C, without affecting HDL-C and triglycerides. Among circulating bone and phosphocalcium homeostasis markers, we found a significant increase in OPG levels ($P < 0.01$) and a modest reduction in osteocalcin (OCN) ($P < 0.05$). We also observed a significant reduction in RANKL expression in T cells ($P < 0.05$). No differences were found in the expression of RANK in T cells and RANKL and RANK in monocytes. OPG expression was low in both immune cell types and was not affected by the treatment. As for circulating osteoprogenitors, we found a significant reduction of $CD34^+BAP^+$ ($P < 0.05$) and $CD34^+OCN^+BAP^+$ ($P < 0.05$) cells. *In vitro* studies showed that Atorvastatin reduced RANKL expression in activated human T-lymphoblastoid cells (Jurkat cell line). **Conclusions:** Three-month Atorvastatin treatment leads to a reduction in circulating osteoprogenitor cells and RANKL expression in T cells, as well as increase in OPG serum levels. These data suggest that statins could have protective effects in the bone–vascular axis.

Introduction

A considerable number of clinical studies have shown that osteoporosis carries a significant increase in cardiovascular risk [1]. In addition, loss of bone mineral density and vertebral fractures has been associated with accelerated atherosclerotic and calcific degeneration of the arterial wall in humans [2,3]. Although the pathophysiological substrate of the so-called bone–vascular axis is still elusive, an important role is attributed to the OPG/RANK/RANKL triad, a master regulator of bone remodeling [4]. In addition, circulating osteoprogenitors have been recently described as a new subset of immature cells harboring pro-calcific potential in the vasculature and heart valves [5].

The interaction between RANKL (expressed on osteoblasts and stromal cell surface) and RANK (expressed by osteoclast precursors) activates an intracellular cascade leading to osteoclast differentiation and maturation. Osteoprotegerin (OPG), which acts as a decoy receptor for RANKL, prevents osteoclast activation and favors bone anabolism [4]. On the contrary, RANKL upregulation in osteoblasts and immune cells enhances pathological bone resorption, thus leading to osteoporosis [6,7]. In parallel, elevated RANKL in vascular and immune cells can participate in the pathogenesis of atherosclerosis and vascular calcification [8,9]. In particular, increased RANKL expression in T cells has been reported in patients with bone disorders [10,11] and clinical atherosclerotic disease [9]. On the other hand, reduced availability of OPG has been associated with the development of osteoporosis, atherosclerosis, and vascular calcification [12].

The impact of hypercholesterolemia in the pathogenesis of osteoporosis has been so far controversial [13]. Nevertheless, a number of studies indicated that the accumulation of lipid oxidation products within the skeleton may contribute to pathological bone resorption, mainly through the inhibition of osteoblast differentiation and the induction of osteoclast maturation/activation [14]. Of interest, data are available showing that oxidized lipids can induce RANKL expression in T cells [15] and that RANKL is

increased in bone marrow and splenic T cells of mice with hyperlipidemia-induced bone loss [16]. No data are available so far showing whether lipid-lowering strategies can modulate *in vivo* OPG/RANK/RANKL expression in immune cells.

Recently, the existence in the bloodstream of circulating cells harboring an osteogenic potential has been demonstrated [5]. These so-called osteoprogenitor cells, which include both myeloid and mesenchymal-derived subtypes, are elevated in pathological conditions such as diabetes, atherosclerosis, calcific aortic valve stenosis, bone fractures, and osteoporosis [5,17]. Increased circulating levels of osteoprogenitor cells have been also linked to the presence of vascular damage, such as arterial stiffness and aortic calcification [18,19]. In addition, animal studies provided evidence of a direct involvement of these cells in vascular disease progression [20]. Whether statin treatment can modulate the levels of circulating calcifying cells is currently unknown.

Starting from these notions, we sought to investigate whether treatment with Atorvastatin can affect circulating levels of osteoprogenitor cells and OPG/RANK/RANKL expression in T cells and monocytes in hypercholesterolemic postmenopausal osteoporotic women.

Methods

Population

We enrolled 20 consecutive hypercholesterolemic (LDL-C $\geq$ 130 mg/dL) women with newly diagnosed osteoporosis who referred to the Osteoporosis and Bone Metabolism Unit of the Cà Foncello Hospital in Treviso. Diagnosis of osteoporosis was based on T-score ≤ -2.5 SD at either the lumbar spine or femoral neck. We did not enroll patients with a previous history of bone fractures, clinical evidence of atherosclerotic disease and anamnestic history of CKD, liver disease, COPD, and rheumatic disorders. We also excluded subjects with current or previous treatment with statins, steroids, hormonal replacement therapies, and antiosteoporotic drugs (including vitamin D and calcium supplementation). All patients received Atorvastatin 40 mg/day for 3 months. Blood samplings were performed at the time of enrollment and at the end of the treatment period. During the study, the patients were not treated with calcium, vitamin D, and bisphosphonates. We excluded women with clinical judgment of high risk of bone fracture. The study has been carried out in accordance with the Declaration of Helsinki (2008) of the World Medical Association and was approved by the local Ethical Committee (the study was registered on ClinicalTrials.gov after its completion, NCT02342015). All the subjects enrolled gave written informed consent.

Data Collection and Biochemical Analysis

Data collected from patients at the enrollment included: age, menopause age, BMI, smoking status, known arterial hypertension, known diabetes, ongoing medications. The latter remained unchanged during the study period (calcium channel blockers: n = 2; sartans: n = 1, proton pump inhibitors: n = 4, COX-2 selective inhibitors: n = 1). At the beginning of the study and after 3 months, serum samples were collected to assess the lipid profile (total cholesterol [TC], LDL-C, HDL-C, triglycerides [TG]), level of hs-CRP (sensitive immunonephelometric method, DADE Behring), osteocalcin (OCN, immunochemiluminescent assay LIAISON®; DiaSorin, Vercelli, Italy), bone alkaline phosphatase (BAP, immunochemiluminescent assay, automatized Beckman Coulter DxI 800; Brea, CA, USA), cross-linked carboxy-terminal telopeptide of type I collagen (CTX-I, immunochemiluminescent assay, automatized Modular Analytics E170, Roche Diagnostics, Basel, Switzerland), and OPG (DuoSet ELISA, R&D Systems, Minneapolis, MN, USA).

Flow Cytometry Analysis

Blood samples for WBC count and flow cytometry analysis were processed within 2 h. For each patient, a detailed description of the WBC types was obtained by automated analyzer (ADVIA 2120 Hematology System, Siemens Healthcare Diagnostics, Erlangen, Germany), including the count of circulating neutrophils, lymphocytes, monocytes, basophils, and eosinophils.

Identification and quantification of circulating osteoprogenitor cells were performed using polychromatic flow cytometry. Briefly, after red blood cell lysis, peripheral blood progenitor cells were analyzed for the surface expression of CD34, OCN and BAP using Fitc-conjugated anti-human CD34 (Becton Dickinson), PE-conjugated anti-human OCN (R&D Systems), and APC-conjugated anti-human BAP (clone B7/48, R&D System). First, we gated $CD34^+$ cells in the morphologic mononuclear cell fraction and then examined the dual or triple expression of OCN and BAP, relative to the respective isotype controls (R&D Systems). In parallel, total OCN^+, BAP^+ cells, and OCN^+/BAP^+ cells were also enumerated. In all samples, 1×10^6 events were acquired, scored using FACSCanto flow-cytometer (Becton Dickinson), and analyzed using the FACSDIVA software program (Becton Dickinson).

Monocytes/T-Cell Isolation and Gene Expression Analysis

Blood samples were layered onto Histopaque-1077 to obtain a suspension of mononuclear cells (PBMC). Pure and viable monocytes and T cells were obtained by negative isolation using Dynabeads Untouched Human Monocytes and Dynabeads Untouched Human T cells (Invitrogen Dynal AS, Oslo, Norway), respectively. Total RNA from both cell types was collected using QiaAmp RNA Blood Mini kit (Qiagen, Hilden, Germany) and stored at $-80°C$ until analysis. Quantification and purity of total RNA was assessed after DNase I digestion (Ambion, Foster City, CA, USA) using a Nanodrop 2000c spectrophotometer (Thermo Scientific, Rockford, IL, USA). Total RNA (1 μg) was converted to cDNA using iScript cDNa Synthesis kit (Bio-Rad, Hercules, CA, USA). The levels of RANK, RANKL, and OPG transcripts were quantified by real-time PCR with universal probeLibrary probes labeled with fluorescein (FAM) at the 5′-end and with a dark quencher dye near the 3′-end (Roche, Basel, Switzerland) in the Bio-Rad CFX96 instrument (Bio-Rad). Amplification primers and hydrolysis probes were designed using Probe Finder software (www.universalprobelibrary.com, Roche) and synthesized by Roche (see Table S1). Expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as reference gene, and the expression ratios

were computed utilizing the comparative cycle threshold method ($2^{-\Delta\Delta Ct}$).

In Vitro Experiments

Jurkat human T-cell leukemia cells were cultured in RPMI-1640 (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% heat inactivated fetal bovine serum (Sigma-Aldrich), 2 mM L-glutamine (Sigma-Aldrich), 100 U/mL penicillin (Sigma-Aldrich), and 100 μg/mL of streptomycin (Sigma-Aldrich) in 5% CO_2 at 37°C. After overnight starvation, T cells (8×10^5 cell/mL) were treated for 12 h with TNF-alpha 20 ng/mL (Milteny Biotec, Bergisch Gladbach, Germany), 10 μM Atorvastatin (Pfizer, New York, NY, USA), TNF-alpha 20 ng/mL plus 10 μM Atorvastatin (Pfizer), or control medium. Total RNA was extracted using the RNeasy mini kit (Qiagen GmBh), and RANKL expression was quantified by real-time PCR following the same protocol described above for the *in vivo* study.

Statistical Analysis

Data are expressed as mean ± standard deviation (SD) or as percentage, where appropriate. Comparison for continuous variables was performed using paired Student's *t*-test or Wilcoxon signed-rank test, on the basis of data distribution. Due to a skewed distribution of gene expression values in immune cells, the analysis was performed after data log-transformation. Patients with ΔCT values for RANKL equal or below the median of the study population were classified as RANKL_high. ANOVA followed by post hoc Fisher LSD test was used for the *in vitro* experiments. Statistical significance was accepted at $P < 0.05$. All statistical analyses were performed with IBM SPSS statistics 21.0 (Armonk, NY, USA).

Results

Population Characteristics and Effect of Atorvastatin on Biochemical Parameters

The clinical characteristics of the population under investigation are summarized in Table 1. We enrolled 20 women with a mean age of 64 ± 5.3 years and a mean lumbar spine T-score of −3.09 ± 0.5. As expected, the 3 months of treatment with Atorvastatin 40 mg/day resulted in a significant reduction of TC and LDL-C, whereas HDL-C and TG levels were not affected (Table 2). Treatment was not accompanied by significant changes in the level of calcium, phosphate, vitamin D, and PTH. As for other bone-related parameters under investigation, we observed a significant increase in circulating OPG levels ($P = 0.005$), whereas OCN showed a modest but significant reduction ($P = 0.022$). No significant changes were observed for BAP and CTX-I levels, although the latter showed a reduction trend. We also observed a significant lowering in hs-CRP levels ($P = 0.047$). Overall, the treatment was well-tolerated and we did not record any clinically relevant side effect or major adverse events.

Table 1 Population characteristics at enrollment

N	20
Mean age, years (SD)	64 (5.3)
BMI (SD)	23.9 (3.1)
Mean menopause age, years (SD)	50.7 (4.1)
Mean T-score lumbar spine (SD)	−3.09 (0.5)
Active/previous smokers, n (%)	2 (10)
Mean SBP, mmHg (SD)	140 (13)
Mean DBP, mmHg (SD)	83 (9)
Mean eGFR, (SD)	110.4 (18.4)

SD, standard deviation; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; eGFR, estimated glomerular filtration rate.

Analysis of OPG/RANK/RANKL Expression in Immune Cells

Isolated T cells and monocytes were obtained at baseline and at the end of the treatment period. No significant changes were observed in the count of all the leukocytes subtypes (data not shown). Gene expression analysis was performed to assess the expression level in each cell type of OPG, RANK, and RANKL.

Major finding of our analysis was that Atorvastatin treatment induced a significant reduction in T-cell RANKL

Table 2 Effects of Atorvastatin (40 mg/day) on biochemical parameters

	Baseline	3 months	*P*
Mean total cholesterol, mg/dL (SD)	257.7 (24.1)	170.2 (38.2)	**<0.0001**
Mean HDL-C, mg/dL (SD)	71.6 (16.1)	72.0 (17.5)	0.873
Mean LDL-C, mg/dL (SD)	162.2 (22.9)	82.6 (31.6)	**<0.0001**
Mean triglycerides, mg/dL (SD)	92.8 (38.1)	77.3 (24.2)	0.096
Median hs-CRP, mg/dL (IQR)	1.00 (0.64–1.81)	0.57 (0.42–1.21)	**0.047**
Mean calcium, mmol/L (SD)	2.36 (0.06)	2.33 (0.07)	0.080
Mean phosphorus, mmol/L (SD)	1.13 (0.10)	1.19 (0.14)	0.061
Mean vitamin D3, nmol/L (SD)	74.8 (45.9)	77.2 (31.1)	0.822
Mean PTH, pg/mL (SD)	42.4 (26.1)	41.6 (13.0)	0.87
Mean OCN, ng/mL (SD)	25.4 (5.7)	23.4 (5.6)	**0.022**
Mean BAP, µg/L (SD)	14.5 (2.5)	14.6 (3.5)	0.861
Mean CTX-I, ng/mL (SD)	529.3 (178.6)	472.9 (180.9)	0.099
Mean OPG, pg/mL (SD)	644.5 (271.8)	814.5 (298.8)	**0.005**

SD, standard deviation; IQR, interquartile range; hs-CRP, high-sensitivity C-reactive protein; PTH, parathyroid hormone; OCN, osteocalcin; BAP, bone alkaline phosphatase; CTX-I, cross-linked carboxy-terminal telopeptide of type I collagen; OPG, osteoprotegerin. Bold values indicate significant results ($P < 0.05$).

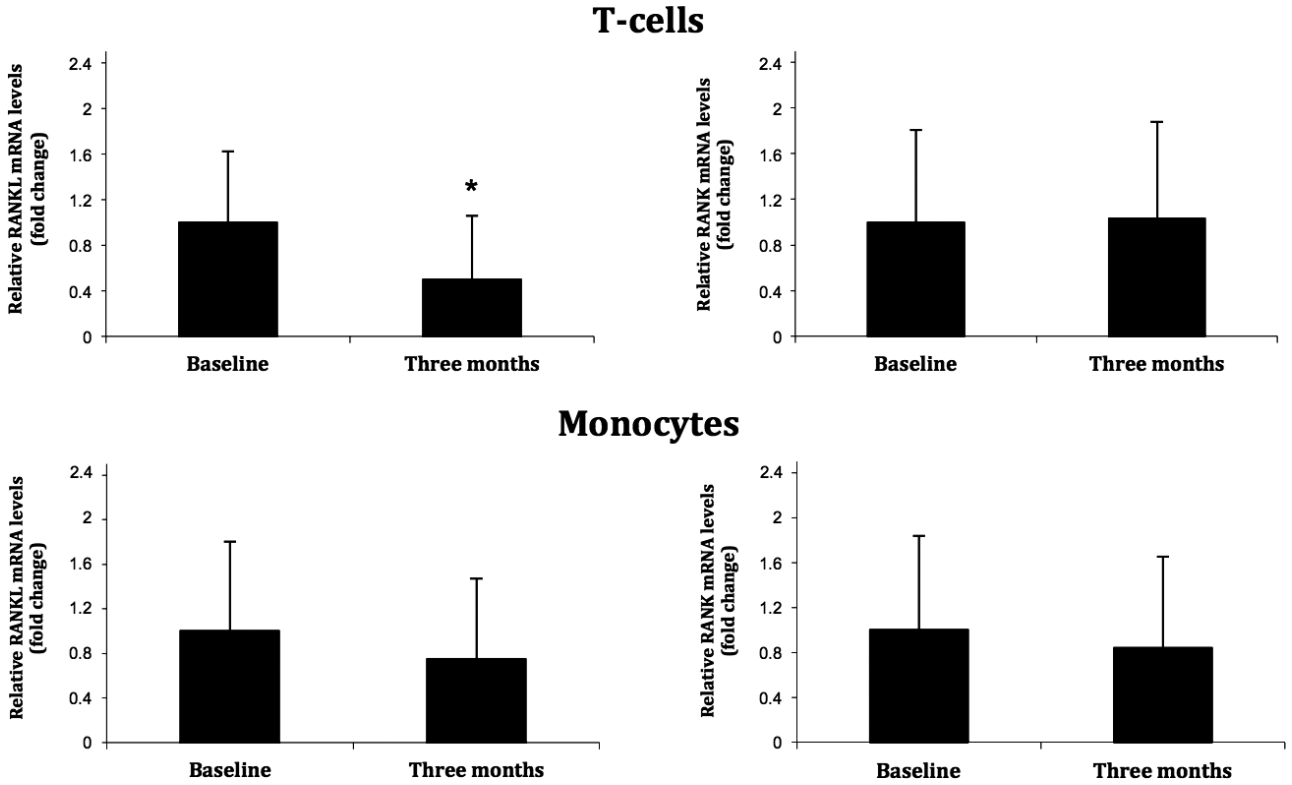

Figure 1 Effect of Atorvastatin on RANK/RANKL expression in T cells and monocytes. RANK and RANKL mRNA expression was quantified in isolated T cells and monocytes at baseline and after 3 months of treatment with Atorvastatin 40 mg/day. A significant reduction in RANKL expression was observed in isolated T cells at the end of the treatment period (*$P < 0.05$). No significant changes were seen in the expression of RANK in T cells and RANK and RANKL in monocytes.

expression (Figure 1) ($P = 0.047$). Of note, the most sizeable effect of Atorvastatin was seen in the subjects with higher basal expression level of RANKL (RANKL_high) ($P = 0.004$). In fact, patients with T cells displaying low expression of RANKL at baseline (RANKL_low) underwent only modest, nonsignificant changes following Atorvastatin treatment ($P = 0.147$). RANK expression in lymphocytes was low and unaffected by Atorvastatin. Isolated circulating monocytes showed low expression of both RANK and RANKL either at baseline or at the end of the study and were not significantly affected by the treatment (Figure 1). OPG was expressed at a very low level (close to the detection limit) in both monocytes and T cells, without changes following Atorvastatin administration.

Effect of Atorvastatin on Circulating Osteoprogenitor Cells

Circulating levels of osteoprogenitor cells were measured by FACS analysis. After 3 months of treatment with Atorvastatin, we observed a significant reduction in the number of $CD34^+BAP^+$ ($P = 0.041$) and $CD34^+OCN^+BAP^+$ cells ($P = 0.045$). A reduction, although not significant, was also seen in the levels of the OCN^+BAP^+ and $CD34^+OCN^+$ populations (Figure 2).

In Vitro Effect of Atorvastatin on RANKL Expression in T Cells

In vitro experiments were performed to investigate the existence of a lipid-lowering independent effect of Atorvastatin in modulating RANKL expression in T lymphocytes. To this purpose, Jurkat T cells were activated with TNF-alpha (20 ng/mL), which has been previously shown to induce RANKL expression in this cell type [21]. In agreement, we found a 2-fold increase in RANKL transcripts in the T cells treated with TNF-alpha as compared to control. Of note, this response was significantly blunted by adding Atorvastatin 10 μM to the culture condition ($P = 0.018$ vs. TNF-alpha alone) (Figure 3).

Discussion

In the present study, we show that treatment of hypercholesterolemic osteoporotic women with Atorvastatin is accompanied by a significant reduction in circulating levels of osteoprogenitor cells and RANKL expression in T cells.

Several clinical and basic science studies demonstrated that lowering of the OPG/RANKL ratio is associated with activation of bone resorption programs within the skeleton [4] and pro-atherosclerotic/pro-calcific pathways in the vessel wall [12]. Increase in RANKL and/or decrease of OPG expression in the bone results in

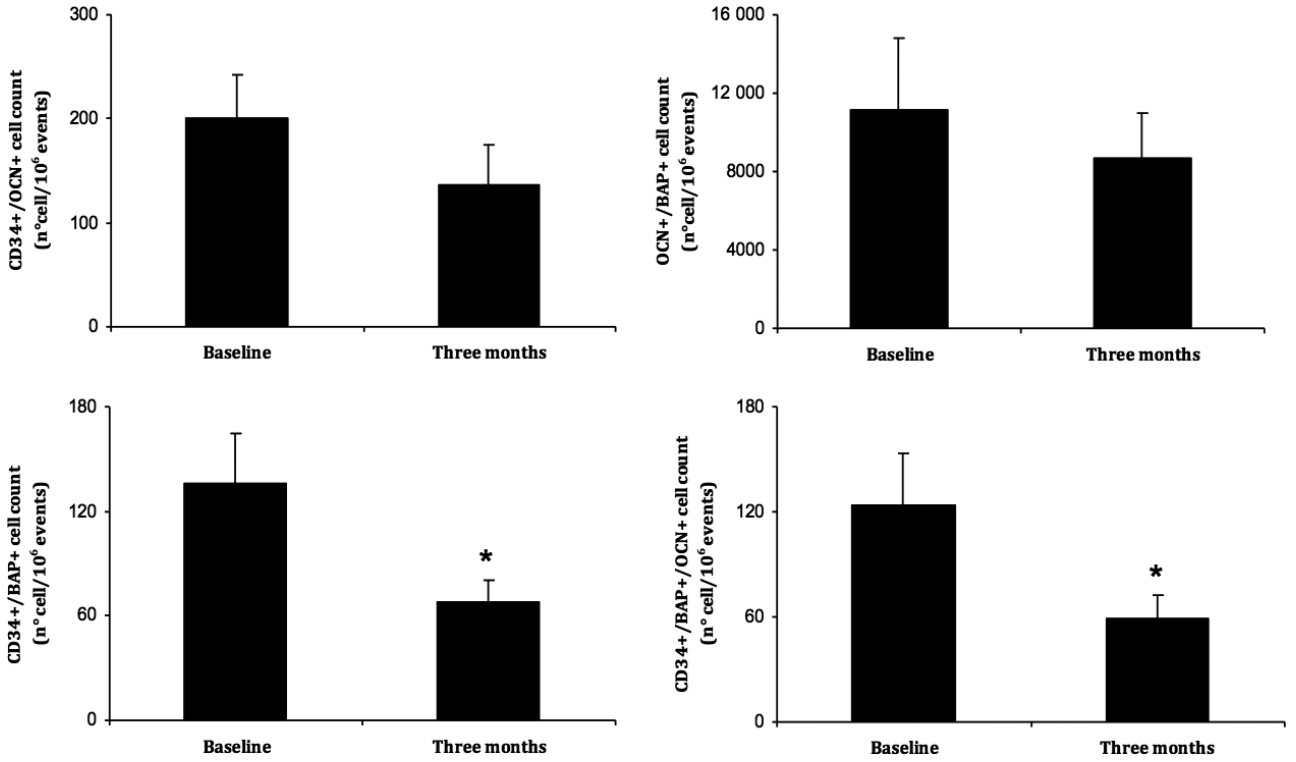

Figure 2 Effect of Atorvastatin on circulating osteoprogenitor cells. Circulating levels of osteoprogenitor cells were quantified using FACS analysis at the baseline and after 3 months of treatment with Atorvastatin 40 mg/day. At the end of the study period, we observed a significant reduction in the count of CD34+/BAP+ and CD34+/OCN+/BAP+ cells (*$P < 0.05$). A nonsignificant lowering was also found in the count of CD34+/OCN+ and OCN/BAP+ cells.

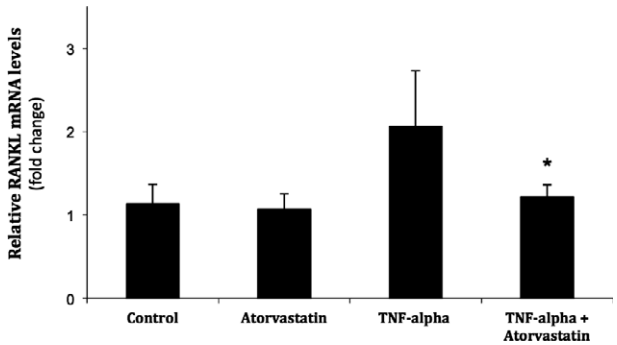

Figure 3 Atorvastatin reduces RANKL expression in activated T cells. Jurkat T cells were treated for 12 h with TNF-alpha 20 ng/mL alone or with the addition of Atorvastatin 10 μM. A significant reduction in RANKL mRNA expression was found in T cells treated with Atorvastatin as compared to those treated with TNF-alpha alone (*$P < 0.05$ vs. TNF-alpha, n = 6).

the maturation/activation of osteoclasts which drive the resorption processes [4]. In parallel, reduction in OPG expression and upregulation of RANKL in vascular cells is followed by accelerated atherogenesis and increase in calcium deposition [8,12]. Recruitment and inflammatory activation of immune cells (lymphocytes and monocytes/macrophages) is now considered a crucial step in the pathogenesis of both bone loss and atherogenesis [7]. RANKL expression in T cells, either as membrane-bound or secreted form, can be induced by different pro-inflammatory mediators and is a potent trigger of osteoclastogenesis [6,7]. Of interest, upregulation of RANKL was found in circulating T cells of patients with acute coronary syndrome (ACS) (unstable angina/myocardial infarction) as compared to subjects with stable coronary artery disease [9]. More recently, researches by Graham et al. [15] demonstrated that exposure of T cells to oxidized lipids is accompanied by increased expression of RANKL in the cells. The same group also showed that RANKL was upregulated in the bone marrow and splenic T cells of mice that develop osteopenia when fed high-fat diet [16]. Overall, these findings suggest that hypercholesterolemia-induced activation of immune cells could represent a pathophysiological substrate connecting osteoporosis and vascular disease. In our investigation, conducted in 20 naïve osteoporotic women (never treated with lipid-lowering and antiosteoporotic drugs) we observed that a 3-month regimen with Atorvastatin was followed by significant reduction in RANKL expression by T cells. Of interest, we also noticed that this response was more evident in patients showing higher basal expression of RANKL, suggesting that the treatment was more effective in the subgroup of osteoporotic women with activated lymphocytic cells. These findings are strengthened by data coming from the *in vitro* investigations, where we found that Atorvastatin was effective in reducing RANKL expression in activated T cells. These *in vitro* data also suggested the existence of a direct effect of Atorvastatin in modulating RANKL transcripts in immune cells. Thus, it appears that both lipid-lowering and cellular effects of Atorvastatin might cooperate *in vivo* in reducing RANKL expression by circulating T cells.

RANK is the main surface receptor of RANKL, and it activates the intracellular cascade leading to maturation of pre-osteoclasts

(monocytic-derived cells) into active osteoclasts [4]. Previous data in humans showed that RANK expression is increased in circulating monocytes from patients with ACS [9]. For these reasons, we hypothesized that modification in RANK expression in monocytes could represent another interesting target of statin treatment. Nevertheless, in our population, we found that RANK is expressed at a low level in isolated monocytes and is not significantly modified by Atorvastatin treatment.

Osteoprotegerin mRNA was almost undetectable in isolated T cells and monocytes both at baseline and after 3 months. Nevertheless, we observed a significant increase in OPG serum levels following treatment with Atorvastatin. OPG is glycoprotein that can be produced by several cell types including endothelial cells, smooth muscle cells osteoblasts, and stromal bone cells [4,22]. The relative contribution of the different cell types in determining OPG circulating levels is still unknown and might vary according to different pathological conditions. In fact, epidemiological studies showed that increased OPG levels are associated with negative cardiovascular prognostic value in different clinical settings [23]. Nevertheless, the majority of basic science researches indicated a protective effect of OPG toward vascular calcification and atherosclerosis progression [12,22]. *In vitro* studies also indicated that statin treatment, including Atorvastatin, can upregulate OPG expression in bone marrow-derived stromal cells [24] and osteoblasts [25]. Therefore, the increase in OPG serum levels observed in our osteoporotic population treated with Atorvastatin might represent an ancillary protective effect of statin therapy on both vascular and bone disease progression. Nevertheless, data in the literature about the effect of statins on OPG circulating levels are discordant and might vary according to the population under investigation [26]. Thus, additional studies are needed to fully elucidate the molecular basis of OPG modulation by statins as well as the actual significance of OPG level modifications in the different clinical settings.

The existence of circulating cells harboring a calcifying profile is a relative new concept that can provide novel insights for the comprehension of bone and vascular pathology [5]. Several distinct phenotypes of these circulating calcifying cells have been so far identified and characterized [5]. A specific subpopulation of circulating calcifying cells of myeloid origin, expressing OCN and BAP is increase in the bloodstream of diabetic subjects [27] and actively participate in the amplification of vascular damage [20]. In our population of osteoporotic women treated with Atorvastatin, we found a trend reduction in the number of OCN^+BAP^+ myeloid calcifying cells. On the other hand, a significant lowering in cell count was observed in the subset of more immature cells, such as $CD34^+OCN^+BAP^+$ and $CD34^+BAP^+$ cells. Of interest, circulating levels of $CD34^+$ osteoprogenitors were previously shown to be elevated in postmenopausal osteoporotic women [17] and to correlate with the presence of arterial stiffness in this clinical condition [19]. Our findings are line with these observations and suggest that Atorvastatin-induced reduction in circulating osteoprogenitor cells might have a positive impact in the pathology of the bone–vascular axis. The mechanisms that drive an excess of circulating pro-calcific cells are still unknown. Based on the results of the present study, we speculate that statin-sensitive mechanisms may be implicated. Additional studies are needed to confirm this concept and to demonstrate that reducing circulating osteoprogenitor cells translate into a positive clinical outcome.

The efficacy of statin therapy in preventing osteoporosis and bone fractures is still controversial [28]. *In vitro* studies showed that statins can promote osteoblast differentiation [29] and inhibit osteoclast maturation, [30] thus suggesting a protective role of these drugs on bone metabolism. Most of these anabolic effects were due to induction of OPG expression by bone/stromal cells [24,25] and blockade of RANKL-induced osteoresorptive processes [30–32]. On the other hand, despite a number of clinical studies showing lower fracture risk in users of statin vs. nonusers [33] prospective investigations did not support an independent effect of statins in preventing bone fractures [34]. Explanation for these discrepancies probably can be found in the pathological complexity of osteoporosis, which is not just a lipid-driven disorder, but instead involves several endocrine pathways including sex hormones, vitamin D, and phosphocalcium metabolism. Nevertheless, our findings of reduced RANKL expression in T cells and increase in OPG serum levels might represent positive ancillary effects of statin therapy, and advice for aggressive lipid-lowering treatment of hypercholesterolemic osteoporotic patients.

Limitations of the present study are the relative low number of patients and the lack of a control group. Nevertheless, we would like to underscore the effort in recruiting a relatively healthy and homogenous population of women with recent diagnosis of hypercholesterolemia and osteoporosis, without history of previous treatment for these conditions. Due to the low presence of comorbidities, the used of additional drugs was minimal (see Methods for details). In addition, concomitant medications were left unchanged during the study period. This strict approach in the selection of the patients allowed us to minimize the impact of confounding factors over Atorvastatin effects on both osteoprogenitors and OPG/RANK/RANKL expression in isolated immune cells. For these reasons, our findings cannot be at the present generalized to other clinical contexts, but provide the rationale to pursue additional studies in different pathological conditions (such as ACS and other atherosclerotic disorders). Moreover, even if both RANKL expression in immune cells and circulating osteoprogenitors should be considered as surrogate markers of bone–vascular pathology, our data can offer the basis to test these markers in longitudinal studies coupled with harder clinical endpoints (such as changes in bone mineral density and/or progression of vascular disease).

In conclusion, we found that Atorvastatin treatment of hypercholesterolemic osteoporotic women is followed by lowering in circulating levels of osteoprogenitor cells, reduction in T-cell RANKL expression, and increase in OPG serum levels. These entire changes can positively affects both osteoporotic disorders and atherosclerotic disease progression, suggesting a protective role of statin therapy in the pathology of the bone–vascular axis.

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Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

Marcello Rattazzi and Paolo Pauletto conceived the study, performed analysis of the data, and wrote the manuscript; Roberta Buso and Roberto Di Virgilio enrolled the patients and collected clinical data; Martina Zaninotto and Mario Plebani performed laboratory analyses; Elisabetta Faggin, Elisa Bertacco, and Tiziana Palmosi performed gene expression analyses and *in vitro* investigations; Gian Paolo Fadini performed flow-cytometer analyses.

References

1. Tanko LB, Christiansen C, Cox DA, Geiger MJ, McNabb MA, Cummings SR. Relationship between osteoporosis and cardiovascular disease in postmenopausal women. *J Bone Miner Res* 2005;**20**:1912–1920.
2. Varma R, Aronow WS, Basis Y, et al. Relation of bone mineral density to frequency of coronary heart disease. *Am J Cardiol* 2008;**101**:1103–1104.
3. El Maghraoui A, Rezqi A, Mounach A, Achemlal L, Bezza A, Dehhaoui M, Ghozlani I. Vertebral fractures and abdominal aortic calcification in postmenopausal women. A cohort study. *Bone* 2013;**56**:213–219.
4. Hofbauer LC, Schoppet M. Clinical implications of the osteoprotegerin/RANKL/RANK system for bone and vascular diseases. *JAMA* 2004;**292**:490–495.
5. Fadini GP, Rattazzi M, Matsumoto T, Asahara T, Khosla S. Emerging role of circulating calcifying cells in the bone-vascular axis. *Circulation* 2012;**125**:2772–2781.
6. Kong YY, Feige U, Sarosi I, et al. Activated T cells regulate bone loss and joint destruction in adjuvant arthritis through osteoprotegerin ligand. *Nature* 1999;**402**:304–309.
7. Caetano-Lopes J, Canhao H, Fonseca JE. Osteoimmunology—The hidden immune regulation of bone. *Autoimmun Rev* 2009;**8**: 250–255.
8. Byon CH, Sun Y, Chen J, et al. Runx2-upregulated receptor activator of nuclear factor kappaB ligand in calcifying smooth muscle cells promotes migration and osteoclastic differentiation of macrophages. *Arterioscler Thromb Vasc Biol* 2011;**31**:1387–1396.
9. Sandberg WJ, Yndestad A, Oie E, et al. Enhanced T-cell expression of RANK ligand in acute coronary syndrome: Possible role in plaque destabilization. *Arterioscler Thromb Vasc Biol* 2006;**26**:857–863.
10. Eghbali-Fatourechi G, Khosla S, Sanyal A, Boyle WJ, Lacey DL, Riggs BL. Role of RANK ligand in mediating increased bone resorption in early postmenopausal women. *J Clin Invest* 2003;**111**:1221–1230.
11. D'Amelio P, Grimaldi A, Di Bella S, et al. Estrogen deficiency increases osteoclastogenesis up-regulating T cells activity: A key mechanism in osteoporosis. *Bone* 2008;**43**:92–100.
12. Bennett BJ, Scatena M, Kirk EA, et al. Osteoprotegerin inactivation accelerates advanced atherosclerotic lesion progression and calcification in older ApoE-/- mice. *Arterioscler Thromb Vasc Biol* 2006;**26**:2117–2124.
13. Samelson EJ, Cupples LA, Hannan MT, et al. Long-term effects of serum cholesterol on bone mineral density in women and men: The Framingham Osteoporosis Study. *Bone* 2004;**34**:557–561.
14. Parhami F. Possible role of oxidized lipids in osteoporosis: Could hyperlipidemia be a risk factor? *Prostaglandins Leukot Essent Fatty Acids* 2003;**68**:373–378.
15. Graham LS, Parhami F, Tintut Y, Kitchen CM, Demer LL, Effros RB. Oxidized lipids enhance RANKL production by T lymphocytes: Implications for lipid-induced bone loss. *Clin Immunol* 2009;**133**:265–275.
16. Graham LS, Tintut Y, Parhami F, Kitchen CM, Ivanov Y, Tetradis S, Effros RB. Bone density and hyperlipidemia: The T-lymphocyte connection. *J Bone Miner Res* 2010;**25**:2460–2469.
17. Pirro M, Leli C, Fabbriciani G, et al. Association between circulating osteoprogenitor cell numbers and bone mineral density in postmenopausal osteoporosis. *Osteoporos Int* 2010;**21**:297–306.
18. Pal SN, Rush C, Parr A, Van Campenhout A, Golledge J. Osteocalcin positive mononuclear cells are associated with the severity of aortic calcification. *Atherosclerosis* 2010;**210**:88–93.
19. Pirro M, Schillaci G, Mannarino MR, et al. Circulating immature osteoprogenitor cells and arterial stiffening in postmenopausal osteoporosis. *Nutr Metab Cardiovasc Dis* 2011;**21**:636–642.
20. Albiero M, Rattazzi M, Menegazzo L, et al. Myeloid calcifying cells promote atherosclerotic calcification via paracrine activity and allograft inflammatory factor-1 overexpression. *Basic Res Cardiol* 2013;**108**:368.
21. Wu L, Lin JH, Bao K, Li PF, Zhang WG. In vitro effects of erythromycin on RANKL and nuclear factor-kappa B by human TNF-alpha stimulated Jurkat cells. *Int Immunopharmacol* 2009;**9**:1105–1109.
22. Buso G, Faggin E, Pauletto P, Rattazzi M. Osteoprotegerin in cardiovascular disease: Ally or enemy? *Curr Pharm Des* 2014;**20**:5862–5869.
23. Venuraju SM, Yerramasu A, Corder R, Lahiri A. Osteoprotegerin as a predictor of coronary artery disease and cardiovascular mortality and morbidity. *J Am Coll Cardiol* 2010;**55**:2049–2061.
24. Tsubaki M, Satou T, Itoh T, et al. Bisphosphonate- and statin-induced enhancement of OPG expression and inhibition of CD9, M-CSF, and RANKL expressions via inhibition of the Ras/MEK/ERK pathway and activation of p38MAPK in mouse bone marrow stromal cell line ST2. *Mol Cell Endocrinol* 2012;**361**:219–231.
25. Viereck V, Grundker C, Blaschke S, Frosch KH, Schoppet M, Emons G, Hofbauer LC. Atorvastatin stimulates the production of osteoprotegerin by human osteoblasts. *J Cell Biochem* 2005;**96**:1244–1253.
26. Celinska-Lowenhoff M, Lowenhoff T, Undas A, Gluszko P. Effects of hypolipemic drugs on the osteoprotegerin—sRANKL system in patients with coronary artery disease. *Thromb Haemost* 2007;**97**:868–870.
27. Fadini GP, Albiero M, Menegazzo L, et al. Widespread increase in myeloid calcifying cells contributes to ectopic vascular calcification in type 2 diabetes. *Circ Res* 2011;**108**:1112–1121.
28. Rizzo M, Rini GB. Statins, fracture risk, and bone remodeling: What is true? *Am J Med Sci* 2006;**332**:55–60.
29. Mundy G, Garrett R, Harris S, et al. Stimulation of bone formation in vitro and in rodents by statins. *Science* 1999;**286**:1946–1949.
30. Woo JT, Nakagawa H, Krecic AM, Nagai K, Hamilton AD, Sebti SM, Stern PH. Inhibitory effects of mevastatin and a geranylgeranyl transferase I inhibitor (GGTI-2166) on mononuclear osteoclast formation induced by receptor activator of NF kappa B ligand (RANKL) or tumor necrosis factor-alpha (TNF-alpha). *Biochem Pharmacol* 2005;**69**:87–95.
31. Ahn KS, Sethi G, Chaturvedi MM, Aggarwal BB. Simvastatin, 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor, suppresses osteoclastogenesis induced by receptor activator of nuclear factor-kappaB ligand through modulation of NF-kappaB pathway. *Int J Cancer* 2008;**123**:1733–1740.
32. Nakashima Y, Haneji T. Stimulation of osteoclast formation by RANKL requires interferon regulatory factor-4 and is inhibited by simvastatin in a mouse model of bone loss. *PLoS One* 2013;**8**:e72033.
33. Chan KA, Andrade SE, Boles M, et al. Inhibitors of hydroxymethylglutaryl-coenzyme A reductase and risk of fracture among older women. *Lancet* 2000;**355**:2185–2188.
34. Yue J, Zhang X, Dong B, Yang M. Statins and bone health in postmenopausal women: A systematic review of randomized controlled trials. *Menopause* 2010;**17**:1071–1079.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Primers and probes used for real time amplification.