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Effects of Bisphosphonate Treatment on Circulating Osteogenic Endothelial Progenitor Cells in Postmenopausal Women

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Abstract

Objective—To evaluate whether bisphosphonates modulate vascular calcification by a modification in endothelial progenitor cells (EPCs) co-expressing osteoblastic surface markers and genes.

Patients and Methods—Double blind, randomized study including 20 healthy early postmenopausal women (from February 1, 2008 through July 31, 2008) treated either with placebo or risedronate (35 mg/week) for 4 months. CD34+/KDR+ cells were isolated and gene expression was studied. Peripheral blood was collected at baseline and at 4 months to determine serum inflammatory markers, osteoprotegerin (OPG) and RANKL levels and bone turnover markers. Peripheral blood mononuclear cells were stained for EPC surface markers (CD34, CD133, and VEGF receptor [KDR]) as well as osteoblast markers (osteocalcin [OCN], alkaline phosphatase [AP], and Stro-1).

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Results—Risedronate treatment resulted in a significant downregulation of gene sets for osteoblast differentiation and proliferation in EPCs with a trend of decreasing EPCs coexpressing OCN.

Conclusion—Our findings indicate that bisphosphonate treatment downregulates the expression of osteogenic genes in EPCs and suggest a possible mechanism by which bisphosphonates may inhibit vascular calcification.

Keywords

endothelial progenitor cells; inflammation; vascular calcification; bisphosphonate

Introduction

There has recently been considerable interest in the mechanisms of vascular calcification and the possible role of endothelial or endothelial progenitor cells (EPCs) in this process. In previous studies, we found that a higher percentage of EPCs (identified by the surface expression of CD34, CD133, and the vascular endothelial growth factor 2/kinase insert domain receptor [KDR]) from patients with coronary atherosclerosis expressed the bone-related protein, osteocalcin (OCN) compared to control subjects¹. More recently, simultaneous samples from the proximal aorta and coronary sinus demonstrated that even patients with early coronary atherosclerosis were characterized by retention of OCN+ EPCs within the coronary circulation². These data suggest that EPCs expressing osteogenic proteins may contribute to vascular calcification as opposed to initiating normal vascular repair. These findings in patients with coronary atherosclerosis are of particular interest given the recent demonstration that, under certain conditions (e.g., exposure to TGF- β 2 or BMP4), endothelial cells can undergo an endothelial-to-mesenchymal transition³ which may play a critical role in the pathogenesis of a number of conditions, including not only atherosclerosis, but also pulmonary hypertension, wound healing, and cancer progression⁴.

Of interest, increased bone turnover has been associated with vascular calcification as well as increased cardiovascular mortality⁵⁻⁸, but the underlying mechanism(s) for these associations remain unclear. EPCs, which reside at least in part in the bone marrow, are a potential candidate for providing a link between bone metabolism and the vascular system since they are mobilized in response to vascular injury and contribute to vascular repair^{9, 10} but, as noted above, they may also contribute to vascular calcification. In addition, many of the same factors that modulate bone turnover, including certain cytokines, hormones and lipids, also modulate the development of atherosclerosis and vascular calcification. Thus, whereas increased production of cytokines such as IL-1 β , IL-6, and IL-8 has been associated with bone loss in the skeletal system, in the vascular system these same cytokines have been associated with atherosclerotic plaque formation and vascular calcification^{8, 11}. In addition, IL-8, as well as other chemokines and proteolytic enzymes, may play a major role in the mobilization of progenitor cells from the bone marrow¹², with IL-8 also recently being linked to the homing of EPCs to vascular tissue¹³.

Interestingly, experimental and clinical studies in postmenopausal women have suggested that bisphosphonates, which are commonly used to treat osteoporosis by reducing bone turnover, may also reduce arterial inflammation and calcification^{8, 14-18}. A recent analysis of a longitudinal cohort study further demonstrated that treatment with bisphosphonates resulted in a lower prevalence of cardiovascular calcification in women older than 65 years of age¹⁹. Although the exact mechanisms by which bisphosphonates inhibit vascular calcification are not entirely understood, several hypotheses have been suggested, including an indirect effect through inhibition of bone remodeling and a direct effect of these drugs on

the vascular wall^{20, 21}. To further evaluate the possible mechanisms by which bisphosphonates may regulate vascular calcification, in this study we tested whether treatment of healthy postmenopausal women with the bisphosphonate, risedronate, resulted not only in a decrease in bone turnover but also in a reduction in EPCs co-expressing osteoblastic cell surface markers and genes. In addition, we analyzed the effect of risedronate on circulating levels of inflammatory cytokines as well as OPG and RANKL levels.

Patients and Methods

For this double blind, randomized study we recruited 20 healthy postmenopausal women who had cessation of menses for more than a year but who were within 5 years of their last menstrual period. Patients were recruited through an institutional classified advertisement seeking research participants at the Mayo Clinic in Rochester, MN, and were included during a 6-month enrollment period (from February 1, 2008 through July 31, 2008). Screening laboratory studies included a complete blood count, serum levels of 25-hydroxyvitamin D (25OHD), follicle stimulating hormone (FSH), parathyroid hormone (PTH), creatinine, calcium, and phosphorus. Exclusion criteria were: use of bisphosphonates or other bone-active drugs in the previous 3 years; history of metabolic bone disease, diabetes, or significant cardiac, renal, or liver disease; history of fracture within the last 5 years; hysterectomy; history of esophageal reflux/stricture; abnormalities in the screening laboratory studies. The study was approved by the Mayo Institutional Review Board and all subjects provided written, informed consent to participate.

The study subjects received placebo or 35 mg weekly risedronate for 4 months (n=10 per group). All patients were instructed to take the drug with water on an empty stomach at least 30 minutes before breakfast. Patients complied to the treatment as assessed by interview of the patients at the end of the study. Peripheral blood was collected to determine serum bone turnover markers, levels of IL-8, hsCRP, OPG, RANKL and to obtain peripheral blood mononuclear cells (PBMCs) for flow cytometry. After 4 months the measurements were repeated and CD34⁺/KDR⁺ cells isolated for gene expression analysis.

Flow cytometry

PBMCs were stained with fluorescent conjugated antibodies: CD34 (Beckton-Dickinson), CD133 (Miltenyi Biotec GmbH) and KDR (R&D Systems). Co-staining for osteoblast markers (OCN, Stro-1, and alkaline phosphatase [AP]) was performed using anti-human OCN (Santa Cruz), anti-human Stro-1 (R&D Systems), biotinylated anti-human AP (R&D Systems) antibodies. Cell fluorescence was measured immediately after staining (Becton Dickinson, FACS Calibur) and data were analyzed using the CellQuest software (Becton Dickinson). Based on the surface antibody expression and in order to subclassify the cells EPCs were divided into four different populations 1) CD34⁺/KDR⁺; 2) CD34⁺/CD133⁺/KDR⁺; 3) CD34⁺/CD133⁺/KDR⁺; and 4) CD34⁺/CD133⁻/KDR⁺^{1, 9}.

Gene expression analysis

WT-OvationTM Pico RNA linear amplification (NuGEN, Technologies, Inc) was used to synthesize cDNA from total RNA of CD34⁺/KDR⁺ cells. Primers for bone and stem cell-related genes (apoptosis genes: Bax, Bcl-2, Bcl-XL, Caspase 3, Caspase 8, Fas, P53; BMP targets: Id2, Smad 1, Smad 5, Sox 4, TIEG; osteoblast differentiation: BSP, Col1 α 2, OCN, Osteonectin, Runx 2; proliferation genes: Cyclin B1, Cyclin C, Lef 1; Wnt signaling: Axin, b-Catenin, Tcf-7, Veriscan, Wnt 4; others: OPG, RANKL) were used in QPCR. Sample normalization was performed using the ribosomal protein, L13. Individual gene expression was determined by 2^{-8CT}.

Biochemical assays

Venous blood was drawn at 8 am at baseline and after 4 months following overnight fasting. Bone formation was assessed by measuring serum amino-terminal propeptide of type I procollagen (P1NP) by radioimmunoassay (Immunodiagnostic Systems [IDS] Ltd), interassay CV<9%). Bone resorption was assessed using serum carboxy-terminal telopeptide of type I collagen (CTX) (interassay CV<10%) and tartrate-resistant acid phosphatase 5b (TRAP5b) (interassay CV<4%), both measured by ELISA (IDS). Serum OPG and RANKL were measured using quantitative immunoassays (ALPCO Diagnostics, Windham, NH) (interassay CV8% and 9%, respectively). IL-8 was measured with the Human Ultrasensitive Cytokine 10-plex Assay using the Luminex® xMAP® platform and ELISA assays (Invitrogen), interassay CV<10%. Hs-CRP levels were measured using a high-sensitivity solid phase direct sandwich ELISA (Calbiotech, Spring Valley, CA, interassay CV<8.5%).

Screening laboratory tests (including complete blood count, creatinine, calcium, and phosphorus) were analyzed using standard procedures.

Statistical analyses

The primary aims of the study were to evaluate the effect of risedronate on EPCs co-expressing osteoblast surface markers and genes. The secondary aims were to evaluate the effect of risedronate in inflammatory and bone turnover markers. Based on our experience with qPCR²², 10 subjects per group has been sufficient for detecting differences of between 1.5-3 fold in the expression of most genes.

The serum bone markers and flow cytometry data measured at four months were compared using a linear model, testing for a treatment group difference after adjusting for the baseline measurement. Based on model diagnostics, the log transformation was used for the flow cytometry variables. The data is summarized as mean $\pm$ SEM. Percent changes of each variable between baseline and 4 months was calculated, and comparisons of these values were made using the Spearman Rank correlation ignoring treatment status. Gene cluster analysis was performed using the O'Brien Umbrella method²³, with data presented as medians and 25th-75th percentiles (interquartile range [IQR]). The data was analysed using an intent-to-treat approach. $P < 0.05$ was considered statistically significant.

Results

Patient characteristics

The relevant clinical and biochemical data of the study subjects are shown in Table 1. At entry, all subjects had normal serum calcium, phosphorus, creatinine, 25OHD and PTH values. As expected, FSH serum levels were elevated (84 ± 19 U/L) in all patients, consistent with the postmenopausal status of the study subjects. Even though baseline serum phosphorus and creatinine values were slightly higher in the risedronate group, they were within the normal range. All patients reported compliance with the treatment during the study as assessed by an interview at the end of the study.

Bone turnover and inflammatory markers and OPG and RANKL

Figure 1 shows the mean values of bone formation (P1NP) and bone resorption (TRAP5b and CTx) markers in patients treated with placebo and risedronate at baseline and after 4 months of treatment. Serum P1NP and TRAP5b levels decreased significantly after risedronate treatment, with mean decreases of 38% and 22%, respectively. Patients treated with placebo also showed a significant decrease in both markers but of a smaller magnitude: serum P1NP decreased by 14% and TRAP5b levels by 9.7%, a change attributed to the seasonal variation in bone markers²⁴. There was a significant difference in the change of

P1NP from baseline to 4 months between the risedronate and the placebo group ($p=0.01$) and similarly for TRAP5b ($p=0.03$). CTx levels decreased with risedronate treatment, albeit, not significantly (Figure 1), a finding that was attributed to the higher variability of this marker²⁴.

Serum IL-8, OPG, RANKL and hsCRP values were not significantly different either at baseline or after risedronate treatment (Table 2). Spearman rank correlation was used to assess the association between percent change from baseline to 4 months in serum OPG and percent change from baseline to 4 months in hsCRP ignoring treatment status ($r=.4$, $p=0.01$). Likewise $r=.3$, $p=0.03$ for the association between percent change from baseline to 4 months in RANKL and percent change to 4 months in IL-8. $Rho=.5$, $p=0.04$ for the association between percent change from baseline to 4 months in RAKL and percent change from baseline to 4 months in hsCRP.

Gene expression analysis of CD34+/KDR+ cells

On average we isolated 8630 ± 995 CD34+/KDR+ cells in the groups. The gene set analysis for osteoblastic differentiation (bone sialoprotein [BSP], collagen 1 alpha 2 [Col 1 α 2], OCN, osteonectin [ON], runx 2) and proliferation (cyclin C, cyclin B1, Lef 1) demonstrated a significant downregulation of all these genes after risedronate treatment (Figure 2).

EPC populations and EPCs co-expressing osteogenic phenotypes

CD34-/CD133+/KDR+ decreased after risedronate therapy (119 [IQR 25th: 59; 75th: 142] to 51 [IQR 25th: 37; 75th: 78] but this decrease was not significantly different from the placebo group decrease (109 [IQR 25th: 49; 75th: 159] to 71 [IQR 25th: 53; 75th: 95]). Neither treatment resulted in significant changes in any of the other EPC subpopulations.

Figure 3 (A-D) shows that all of the EPC subpopulations co-staining for OCN tended to decrease in the risedronate group and either increase or remain unchanged in the placebo group, but this pattern was not significantly different between the two groups. Analysis of the additional cell surface markers, AP and Stro1 was performed in all EPC populations in both groups of patients at baseline and at 4 months. Overall, risedronate treatment resulted in a ~59% reduction in CD34+/KDR+ EPCs co-expressing osteoblastic surface markers (-63% in EPCs co-stained with Stro-1, -54% with AP), whereas placebo treatment showed an average reduction of 4%. These changes, however, were not significantly different between the two groups.

Correlations between osteoblastic cell surface markers in EPCs populations

EPCs co-staining for AP and OCN were significantly correlated in CD34/KDR/OCN versus CD34/KDR/AP populations ($r=0.61$, $P=0.005$) and CD34-/CD133-/KDR+ populations ($r=0.47$, $P=0.03$). Conversely, EPCs co-expressing Stro-1 did not correlate with either AP or OCN expressing EPCs

Correlations between serum bone turnover/inflammatory markers, OPG, RANKL and EPCs co-expressing osteoblast markers

We observed a direct correlation between the bone resorption markers (TRAP5b and CTx) and CD34+/CD133+/KDR+ EPCs co-expressing AP ($r = 0.54$, $P = 0.01$ for TRAP5b; $r = 0.62$, $P = 0.003$ for CTx). Moreover, on analyzing the correlations between the percentage changes of EPCs co-expressing osteoblastic markers and those of OPG, RANKL and the inflammatory markers, we observed a direct correlation between changes in hsCRP levels and changes in CD34/KDR EPCs co-expressing OCN ($r = 0.5$, $P = 0.04$).

Discussion

The results of our study demonstrate that bisphosphonate therapy in healthy postmenopausal women not only results in a decrease in bone turnover but also lower expression of osteoblast-related genes, with a trend of decreasing the expression of osteoblastic cell surface markers by circulating EPCs. The current results expand our previous findings^{1, 2} and further generate the hypothesis that bisphosphonates may inhibit vascular calcification by preventing EPCs from developing an osteogenic phenotype. In a broader context, our work also raises the likelihood that bisphosphonates may modulate the process of endothelial-to-mesenchymal transition^{3, 4} and point to the need for further studies to address these intriguing possibilities.

We observed that the expression of genes related to cell proliferation such as cyclin C, cyclin B1, and *lef 1* in CD34+/KDR+ cells was significantly lower in women receiving risedronate as was the expression of genes related to osteoblastic differentiation, such as BSP, collagen type 1, OCN, ON and Runx2, further supporting an anti-osteogenic effect of this therapy on EPCs. Although previous studies have indicated that bisphosphonates can enhance the differentiation and proliferation of osteoblastic cells²⁵, the effect of these agents likely depends on the cell type and the bisphosphonate used²⁶. Indeed, recent studies have found that zoledronic acid and other compounds have an inhibitory effect on the proliferation and differentiation of endothelial cells^{16, 27, 28}. It is likely that bisphosphonate treatment decreases the clonal expansion capacity of EPCs. Furthermore, we also observed a consistent downregulation of genes related to apoptosis as well as Wnt and BMP targets in CD34+/KDR+ cells following risedronate treatment, although without statistical significance. In agreement with our results, recent data suggest that the use of nitrogen-containing bisphosphonates, especially in older women, is associated with decreased prevalence of vascular calcification^{19, 27}.

We also observed that risedronate treatment tended to decrease the number of different EPC populations co-expressing OCN. Furthermore, analysis of additional osteoblastic cell surface markers (AP and Stro-1) showed that following risedronate treatment, fewer cells of the different EPC subpopulations co-expressed, albeit not significantly, these markers. Despite previous studies showing a relationship between circulating EPC levels and cardiovascular outcomes, with higher risk related to lower EPC levels²⁹, recent data indicate that the EPC phenotype may play an important role in endothelial repair¹⁰. Our group has shown that EPCs co-expressing an osteogenic phenotype are significantly increased in patients with severe coronary artery disease or endothelial dysfunction and that these cells can mineralize, at least *in vitro*¹. Indeed, although in most studies EPCs are identified by flow cytometric characteristics, specifically by the expression of CD34, CD133, or KDR, the origins and functions of EPCs remain controversial. Moreover, EPCs seem to fulfill varying roles at different stages of their development, e.g. late EPCs seem to have a higher proliferative capacity *in vitro*, whereas early EPCs may act to secrete angiogenic growth factors¹⁰. Furthermore, the concept of “osteogenic” versus “non-osteogenic” EPCs could partially explain, previous discordant results in experimental studies where treatment with EPCs or with bone marrow mononuclear cells may accelerate atherosclerotic plaque formation instead of improving vascular function^{30, 31}. Also relevant to our findings is that bisphosphonates have been associated with anti-angiogenic effects, a finding that has been related to the anticancer activities of these agents and also to the development of jaw osteonecrosis, especially with the most potent amino-bisphosphonates such as zoledronic acid. Therefore, we cannot exclude effects of risedronate on either an inhibition of EPCs migration or increased cell apoptosis³³.

Although the evidence that EPCs co-expressing osteogenic markers are involved in vascular calcification is indirect, the *in vitro* capacity of these cells to calcify leads to the hypothesis that EPCs may contribute to vascular calcification. Interestingly, a recent study identified a novel type of blood-derived procalcific cell potentially involved in vascular calcification of diabetic patients. These cells had a myeloid origin but also expressed OCN and AP³².

We also found that the bone resorption markers, TRAP5b and CTx, were positively correlated with the number of CD34+/CD133+/KDR+ EPCs co-expressing osteoblast surface markers. These findings indicate a possible relationship between the bone turnover rate and circulating osteogenic EPCs and suggest the possibility that increased bone turnover may contribute to the development of an osteogenic phenotype by circulating EPCs. Indeed, a recent study has reported a positive relationship between bone turnover markers and the number of circulating CD34 cells co-expressing AP or OCN³⁴. All these findings coincide with the previously reported association between increased bone turnover and vascular calcification, linking bone metabolism with the vascular system^{5, 6, 8, 35, 36}. Conversely, Stro-1 expression by EPCs was not correlated with AP or OCN EPC populations or with bone turnover markers. It has previously been reported that, compared to osteoblastic cells expressing AP, those only expressing the Stro-1 antigen represent a less differentiated osteoblast population with reduced capacity for mineralization and lack of expression of various bone-related markers³⁷. Our data thus suggest that EPC subpopulations co-expressing AP and OCN may be more differentiated and consequently, may have a higher capacity for mineralization and vascular calcification. Furthermore, since our previous work had only stained EPC populations with OCN^{1, 2}, the significant correlation we noted between EPCs co-staining for OCN and AP provides greater confidence that staining for OCN identifies EPC populations also likely to express other bone-related proteins.

Several lines of evidence suggest that inflammation plays a major role in the development of vascular calcification^{11, 38, 39} and that various cytokines and proteolytic enzymes regulate the release, migration and homing of progenitor cells from the bone marrow¹². Nonetheless, despite evidence for an immunomodulatory effect of bisphosphonates¹⁶, most data are based on animal or *in vitro* studies^{40, 41} or on patients with inflammatory or metabolic bone disease^{16, 42, 43}. In the present study, inflammatory markers, such as IL-8 and hsCRP, and bone regulatory factors, such as OPG and RANKL, tended to decrease, albeit not significantly, with bisphosphonate therapy, likely due to the relatively small number of subjects included. Moreover, serum hsCRP levels were directly correlated with OPG concentrations and changes in hsCRP levels were also correlated with changes in RANKL and in CD34+/KDR+ EPCs co-expressing OCN.

This study has several limitations. Our subjects were healthy postmenopausal women. Nonetheless, it remains unknown whether the presence of associated vascular disease or osteoporosis may modify the changes we observed following bisphosphonate therapy. Furthermore, we did not assess parameters of vascular calcification and the number of individuals was low, limiting the strength of our results. The biological effects of bisphosphonates may also vary depending on the bisphosphonate used¹⁶; therefore, different response with other bisphosphonates cannot be ruled out. However, despite the small sample size of the study the changes in the osteoblastic potential of the EPC populations were consistent with downregulation of the osteoblastic differentiation genes in EPCs after risedronate treatment.

In conclusion, our data indicate that treatment with a bisphosphonate in healthy postmenopausal women may modulate cellular pathways leading to vascular calcification by downregulating the expression of osteogenic genes in EPCs. However, these preliminary findings need further validation in larger studies and additional evidence to define a role for

EPCs in inducing vascular calcification. Nonetheless, our results provide a rationale for further studies examining the possible effects of bisphosphonate therapy on expression of osteogenic markers by EPCs as well as on vascular calcification.

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Alphabetical list of abbreviations

25OHD	25-hydroxyvitamin D
AP	alkaline phosphatase
BL	baseline
BMI	body mass index
BSP	bone sialoprotein
Col 1α2	collagen 1 alpha 2
CTx	carboxy-terminal telopeptide of type I collagen
EP	endpoint
EPCs	endothelial progenitor cells
FSH	follicle stimulating hormone
Hs-CRP	high sensitive-C reactive protein
IL-8	interleukin-8
IQR	interquartil range
KDR	vascular endothelial growth factor 2/kinase insert domain receptor
Lef1	lymphoid enhancer-binding factor1
OCN	osteocalcin
ON	osteonectin
OPG	osteoporotegerin
PBMNC	peripheral blood mononuclear cells
P1NP	amino-terminal propeptide of type I procollagen
PTH	parathyroid hormone
RANKL	receptor activator of nuclear factor kappa-B ligand
Runx2	runt-related transcription factor 2
TRAP5b	tartrate-resistant acid phosphatase 5b

References

- Gossel M, Modder UI, Atkinson EJ, Lerman A, Khosla S. Osteocalcin expression by circulating endothelial progenitor cells in patients with coronary atherosclerosis. *J Am Coll Cardiol*. 2008; 52(16):1314–1325. [PubMed: 18929243]
- Gossel M, Modder UI, Gulati R, et al. Coronary endothelial dysfunction in humans is associated with coronary retention of osteogenic endothelial progenitor cells. *Eur Heart J*. 2010; 31(23):2909–2914. [PubMed: 20935001]
- Medici D, Shore EM, Lounev VY, Kaplan FS, Kalluri R, Olsen BR. Conversion of vascular endothelial cells into multipotent stem-like cells. *Nat Med*. 2010; 16(12):1400–1406. [PubMed: 21102460]
- Arciniegas E, Frid MG, Douglas IS, Stenmark KR. Perspectives on endothelial-to-mesenchymal transition: potential contribution to vascular remodeling in chronic pulmonary hypertension. *Am J Physiol Lung Cell Mol Physiol*. 2007; 293(1):L1–L8. [PubMed: 17384082]
- Hofbauer LC, Brueck CC, Shanahan CM, Schoppet M, Dobnig H. Vascular calcification and osteoporosis-from clinical observation towards molecular understanding. *Osteoporos Int*. 2007; 18(3):251–259. [PubMed: 17151836]
- Sambrook PN, Chen CJS, March LM, et al. High bone turnover is an independent predictor of mortality in the frail elderly. *J Bone Miner Res*. 2006; 21(4):549–555. [PubMed: 16598375]
- Chow JT, Khosla S, Melton LJ, Atkinson EJ, Camp JJ, Kearns AE. Abdominal aortic calcification, BMD, and bone microstructure: a population-based study. *J Bone Miner Res*. 2008; 23(10):1601–1612. [PubMed: 18466072]
- Anagnostis P, Karagiannis A, Kakafika AI, Tziomalos K, Athyros VG, Mikhailidis DP. Atherosclerosis and osteoporosis: age-dependent degenerative processes or related entities? *Osteoporos Int*. 2009; 20(2):197–207. [PubMed: 18509713]
- Urbich C, Dimmeler S. Endothelial progenitor cells: characterization and role in vascular biology. *Circ Res*. 2004; 95(4):343–353. [PubMed: 15321944]
- Zampetaki A, Kirton JP, Wu Q. Vascular repair by endothelial progenitor cells. *Cardiovasc Res*. 2008; 78(3):413–21. [PubMed: 18349136]
- Smith BJ, Lerner MR, Bu SY, et al. Systemic bone loss and induction of coronary vessel disease in a rat model of chronic inflammation. *Bone*. 2006; 38(3):378–386. [PubMed: 16256450]
- Lapidot T, Petit I. Current understanding of stem cell mobilization: the roles of chemokines, proteolytic enzymes, adhesion molecules, cytokines, and stromal cells. *Exp Hematol*. 2002; 30(9):973–981. [PubMed: 12225788]
- Chavakis E. IL-8: a new player in the homing of endothelial progenitor cells to ischemic myocardium. *J Mol Cell Cardiol*. 2006; 40(4):442–445. [PubMed: 16516915]
- Skolnick AH, Osranek M, Formica P, Kronzon I. Osteoporosis treatment and progression of aortic stenosis. *Am J Cardiol*. 2009; 104(1):122–124. [PubMed: 19576331]
- Koshiyama H, Nakamura Y, Tanaka S. Decrease in carotid intima-media thickness after 1-year therapy with etidronate for osteopenia associated with type 2 diabetes. *J Clin Endocrinol Metab*. 2000; 85(8):2793–2796. [PubMed: 10946883]
- Corrado A, Santoro N, Cantatore FP. Extra-skeletal effects of bisphosphonates. *Joint Bone Spine*. 2007; 74(1):32–38. [PubMed: 17196868]
- Price PA, Faus SA, Williamson MK. Bisphosphonates alendronate and ibandronate inhibit artery calcification at doses comparable to those that inhibit bone resorption. *Arterioscler Thromb Vasc Biol*. 2001; 21(5):817–824. [PubMed: 11348880]
- Lomashvili KA, Monier-Faugere MC, Wang X, Malluche HH, O'Neill WC. Effect of bisphosphonates on vascular calcification and bone metabolism in experimental renal failure. *Kidney Int*. 2009; 75(6):617–625. [PubMed: 19129793]
- Elmariah S, Delaney JA, O'Brien KD, et al. Bisphosphonate use and prevalence of valvular and vascular calcification in women: MESA (The Multi-Ethnic Study of Atherosclerosis). *J Am Coll Cardiol*. 2010; 56(21):1752–1759. [PubMed: 21070928]

20. Luckish A, Cernes R, Boaz M, et al. Effect of long-term treatment with risedronate on arterial compliance in osteoporotic patients with cardiovascular risk factors. *Bone*. 2008; 43(2):279–283. [PubMed: 18515205]
21. Neven EG, De Broe ME, D'Haese PC. Prevention of vascular calcification with bisphosphonates without affecting bone mineralization: a new challenge? *Kidney Int*. 2009; 75(6):580–2. [PubMed: 19247382]
22. Mödder UI, Roforth MM, Nicks KM, et al. Characterization of mesenchymal progenitor cells isolated from human bone marrow by negative selection. *Bone*. 2012; 50(3):804–810. [PubMed: 22226689]
23. O'Brien PC. Procedures for comparing samples with multiple endpoints. *Biometrics*. 1984; 40(4): 1079–1087. [PubMed: 6534410]
24. Seibel MJ. Biochemical markers of bone turnover. Part I: Biochemistry and variability. *Clin Biochem Rev*. 2005; 26(4):97–122. [PubMed: 16648882]
25. Xiong Y, Yang HJ, Feng J, Shi ZL, Wu LD. Effects of alendronate on the proliferation and osteogenic differentiation of MG-63 cells. *J Intern Med Res*. 2009; 37(2):407–416.
26. Reinholz GG, Betz B, Pederson L, et al. Bisphosphonates directly regulate cell proliferation, differentiation, and gene expression in human osteoclasts. *Cancer Res*. 2000; 60(21):6001–6007. [PubMed: 11085520]
27. Hamma-Koutbali Y, Benedetto M, Ledoux D, et al. A novel non-containing-nitrogen bisphosphonate inhibits both in vitro and in vivo angiogenesis. *Biochem Biophys Res Comm*. 2003; 310(3):816–823. [PubMed: 14550277]
28. Yamada J, Tsuno NH, Kitayama J, et al. Anti-angiogenic property of zoledronic acid by inhibition of endothelial progenitor cell differentiation. *J Surg Res*. 2009; 151(1):115–120. [PubMed: 18619615]
29. Werner N, Kosiol S, Schiegl T, et al. Circulating endothelial progenitor cells and cardiovascular outcomes. *N Engl J Med*. 2005; 353(10):999–1007. [PubMed: 16148285]
30. Silvestre JS, Gojova A, Brun V, et al. Transplantation of bone marrow-derived mononuclear cells in ischemic apolipoprotein E-knockout mice accelerates atherosclerosis without altering plaque composition. *Circulation*. 2003; 108(23):2839–2842. [PubMed: 14656923]
31. George J, Afek A, Abashidze A, et al. Transfer of endothelial progenitor and bone marrow cells influences atherosclerotic plaque size and composition in apolipoprotein E knockout mice. *Arterioscler Thromb Vasc Biol*. 2005; 25(12):2636–2641. [PubMed: 16195475]
32. 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(9):1112–1121. [PubMed: 21393578]
33. Ziebart T, Pabst A, Klein MO, et al. Bisphosphonates: restrictions for vasculogenesis and angiogenesis: inhibition of cell function of endothelial progenitor cells and mature endothelial cells in vitro. *Clin Oral Investig*. 2011; 15(1):105–11.
34. Pirro M, Leli C, Fabbriani G, et al. Association between circulating osteoprogenitor cell numbers and bone mineral density in postmenopausal osteoporosis. *Osteoporos Int*. 2010; 21(2):297–306. [PubMed: 19484167]
35. Schulz E, Arfai K, Xiaodong L, Sayre J, Gilsanz V. Aortic calcification and the risk of osteoporosis and fractures. *J Clin Endocrinol Metab*. 2004; 89(9):4246–4253. [PubMed: 15356016]
36. Pirro M, Schillaci G, Mannarino MR, et al. Circulating immature osteoprogenitor cells and arterial stiffening in postmenopausal osteoporosis. *Nutr Metab Cardiovasc Dis*. 2011; 21(9):636–642. [PubMed: 20554181]
37. Gronthos S, Zannettino ACW, Graves SE, Ohta S, Hay SJ, Simmons PJ. Differential cell surface expression of the STRO-1 and alkaline phosphatase antigens on discrete developmental stages in primary cultures of human bone cells. *J Bone Miner Res*. 1999; 14(1):47–56. [PubMed: 9893065]
38. Mahmoudi M, Curzen N, Gallagher PJ. Atherogenesis: the role of inflammation and infection. *Histopathology*. 2007; 50(5):535–546.
39. Liehn EA, Zernecke A, Postea O, Weber C. Chemokines: inflammatory mediators of atherosclerosis. *Arch Phys Biochem*. 2006; 112(4-5):229–238.

40. Giuliani N, Pedrazzoni M, Passeri G, Girasole G. Bisphosphonates inhibit IL-6 production by human osteoblast-like cells. *Scand J Rheumatol.* 1998; 27(1):38–41. [PubMed: 9506876]
41. Dunn CJ, Galinet LA, Wu H, et al. Demonstration of a novel anti-arthritic and anti-inflammatory effects of diphosphonates. *J Pharmacol Exp Ther.* 1993; 266(3):1691–1698. [PubMed: 8371167]
42. Dunder U, Kavunku V, Ciftci IH, Evcik D, Solak O, Cagir T. The effect of risedronate treatment on serum cytokines in postmenopausal osteoporosis: a 6-month randomized and controlled study. *J Bone Miner Metab.* 2009; 27(4):464–470. [PubMed: 19301089]
43. Cantatore FP, Acquista CA, Pipitone V. Evaluation of bone turnover and osteoclastic cytokines in early rheumatoid arthritis treated with alendronate. *J Rheumatol.* 1999; 26(11):2318–2323. [PubMed: 10555884]

Figure 1.

Markers of bone turnover. The figure shows the values of bone formation (P1NP) and bone resorption (TRAP5b and CTx) markers in patients treated with risedronate and placebo at baseline (BL) and after 4 months (EP). P values compare the group difference of the 4 month values after adjusting for the baseline measurement.

Figure 2.

Gene expression analysis of FACS-sorted CD34+/KDR+ cells from peripheral blood. The cluster analysis of the gene sets (see Methods) for osteoblast differentiation and proliferation showed a significant downregulation after risedronate (R) treatment compared to placebo (PL) treatment. Results are expressed as medians and IQRs.

Figure 3.

Number of EPCs from the different subpopulations co-expressing the osteoblastic marker, OCN. CD34+/KDR+/OCN+ (A), CD34-/CD133+/KDR+/OCN+ (B), CD34+/CD133+/KDR+/OCN+ (C) and CD34+/CD133-/KDR+/OCN (D). The data are expressed as absolute counts per 100,000 events. Results are expressed as medians and IQRs.

Table 1

Clinical characteristics and biochemical measurements in the subjects at baseline (data are mean $\pm$ SEM).

Variable ^b	Placebo	Risedronate
N	10	10
Age (years)	55 $\pm$ 0.8	55 $\pm$ 0.6
Height (cm)	163 $\pm$ 1.5	168 $\pm$ 1.6
Weight (kg)	78 $\pm$ 3.5	78 $\pm$ 4.3
BMI ^a (kg/m ²)	29 $\pm$ 1.5	28 $\pm$ 1.5
Calcium (mg/dL)	9.7 $\pm$ 0.07	9.8 $\pm$ 0.07
Phosphorus (mg/dL)	3.6 $\pm$ 0.08	3.9 $\pm$ 0.1 *
Creatinine (mg/dL)	0.72 $\pm$ 0.01	0.78 $\pm$ 0.02 *
25-hydroxyvitamin D (ng/mL)	28.7 $\pm$ 2.4	32.2 $\pm$ 2.9
PTH ^a (pg/mL)	51.5 $\pm$ 4.42	8 $\pm$ 4.6

^aBMI: body mass index; PTH: parathyroid hormone. T-test

* P < 0.05

^bSI conversion factors: To convert calcium levels to mmol/L, multiply by 0.25; to convert phosphorus levels to mmol/L, multiply by 0.32; to convert creatinine levels to μ mol/L, multiply by 88.4; to convert 25-hydroxyvitamin D levels to nmol/L, multiply by 2.5; to convert PTH levels to pmol/L, multiply by 0.11.

Table 2

Serum levels of OPG, RANKL and inflammatory markers in subjects treated with placebo or risedronate at baseline and after treatment (data are mean $\pm$ SEM).

Variable ^b	Placebo			Risedronate		
	Baseline	4 months	p [†]	Baseline	4 months	p [†]
OPG ^a (pmol/L)	3.02 $\pm$ 0.2	3.32 $\pm$ 0.35	0.22	2.86 $\pm$ 0.17	2.65 $\pm$ 0.18	0.12
RANKL ^a (pmol/L)	2808.8 $\pm$ 1356	2819.9 $\pm$ 1252	0.99	4422.9 $\pm$ 2200	3544.9 $\pm$ 1693	0.82
RANKL/OPG	848.6 $\pm$ 341	822.8 $\pm$ 373	0.95	1646.2 $\pm$ 825	1538 $\pm$ 792	0.94
IL-8 ^a (pg/mL)	3.02 $\pm$ 0.45	2.46 $\pm$ 0.46	0.15	4.36 $\pm$ 0.71	3.7 $\pm$ 0.58	0.53
Hs-CRP ^a (mg/L)	1.9 $\pm$ 0.8	2.1 $\pm$ 0.5 0.	77	2.4 $\pm$ 1.1 1.	9 $\pm$ 0.6	0.43

^aHs-CRP: high sensitive-C reactive protein, IL-8: interleukin-8, OPG: osteoprotegerin, RANKL: receptor activator of nuclear factor kappa-B ligand.

^bSI conversion factors: To convert hs-CRP levels to nmol/L, multiply by 9.52

[†]P-values denote statistical significance for the difference between baseline and 4 month values.