Original Article

Intravenous treatment with ibandronate normalizes bone matrix mineralization and reduces cortical porosity after two years in male osteoporosis: a paired biopsy study

Barbara M Misof1*, Janina M Patsch2,4, Paul Roschger1, Christian Muschitz3, Sonja Gamsjaeger1, Eleftherios P Paschalis1, Eva Prokop1, Klaus Klaushofer1, Peter Pietschmann4, Heinrich Resch3

1 Ludwig Boltzmann Institute of Osteology at the Hanusch Hospital of WGKK and AUVA Trauma Centre Meidling, 1st Medical Department, Hanusch Hospital, Vienna, Austria
2 Department of Radiology, Medical University of Vienna, Vienna, Austria
3 Medical Department II with Osteology/Rheumatology & Gastroenterology, KH Barmherzige Schwestern (St. Vincent Hospital) Vienna, Academic Teaching Hospital of the Medical University Vienna, VINFORCE study group, Vienna, Austria
4 Department of Pathophysiology and Allergy Research, Center for Pathophysiology, Infectiology and Immunology, Medical University of Vienna, Vienna, Austria

* Corresponding author: Dr. Barbara Misof, Ludwig Boltzmann Institute of Osteology, UKH Meidling, Kundratstr. 36, A-1120 Vienna, Austria; E-mail: barbara.misof@osteologie.at

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Abstract

The spectrum of therapeutic options and the amount of clinical trials for male osteoporosis (mOP) is lower than those for postmenopausal osteoporosis. Therefore we examined the effects of 24 months ibandronate (IBN) treatment (3mg/3ml intravenously every 3 months) on bone material quality in 19 subjects with mOP within an open-label, single-center, prospective Phase III study (Eudract number 2006-006692-20). Patients (median age [25th, 75th percentiles] 53.0 [44.5; 57.0] years) were included if they had low BMD and/or at least one low trauma fracture and no secondary cause of osteoporosis. Primary endpoint was to evaluate IBN effects on cancellous (Cn.) and cortical (Ct.) bone mineralization density distribution (BMDD) based on quantitative backscattered electron imaging (qBEI) of paired transiliacal bone biopsies (baseline, 24 months). Secondary endpoints included changes in areal bone mineral density (BMD by DXA) and serum markers of bone turnover including type I collagen peptides CrossLaps (CTX), procollagen type 1 amino-terminal propeptide (P1NP), and osteocalcin (OC).

At baseline, cancellous bone matrix mineralization from mOP was lower than published reference data (mean degree of mineralization Cn.CaMean -1.8%, p<0.01). IBN treatment increased calcium concentrations versus baseline (Cn.CaMean +2.4%, Ct.CaMean, +3.0% both p<0.01), and reduced heterogeneity of mineralization (Cn.CaWidth -14%, p=0.044; Ct.CaWidth, -16%, p=0.001) leading to cancellous BMDD within normal range. IBN treatment was associated with a decrease in porosity of mineralized cortical tissue (-25%, p=0.01), increases in BMD at the lumbar spine, the femoral neck and the total hip (+3.3%, +1.9%, and +5.6%, respectively, p≤0.01) and reductions in CTX (-37.5%), P1NP (-44.4%), and OC (-36.3%, all p<0.01). Our BMDD findings are in line with the reduction of bone turnover markers and the increase in BMD by IBN in our patients and suggest that latter mainly reflect the increase in matrix mineralization and the reduction of cortical porosity in this cohort with mOP.
Introduction

Osteoporosis in men is an important public health problem and a frequent cause of morbidity and mortality. One in five men over the age of 50 years will sustain an osteoporotic fracture (1). Male osteoporosis is considered as a multifactorial disease, and hyperparathyroidism, hyperthyroidism, hypogonadism, glucocorticoid excess, hypercalciuria, diabetes mellitus or alcohol abuse are often identified as secondary causes (1, 2, 3, 4). Less than half of the male osteoporotic patients have primary osteoporosis including age-related and idiopathic osteoporosis.

Compared with female patients there is considerably less information available on therapeutic options for men with osteoporosis. However, several therapeutic agents including bisphosphonates (alendronate, risedronate, ibandronate and zoledronic acid), strontiumranelate and teriparatide treatment were shown to increase areal bone mineral density (BMD) in osteoporotic men (5, 6, 7, 8, 9, 10, 11, e.g.). With few exceptions (12, 13, 14, 15), most of these studies have focused on BMD and serum markers of bone turnover as surrogate outcomes. However, it is well-accepted that fracture risk reduction by established anti-osteoporotic drugs is not only attributable to increases in BMD.

Additional parameters beyond BMD such as bone structural and material properties provide important information on treatment effects and changes in mechanical competence (16, 17, 18). In particular, bone mineralization density distribution (BMDD) is an important parameter reflecting material stiffness of bone. Noteworthy, in healthy adults, BMDD of trabecular bone was observed to have little biological variance with age, sex, ethnicity, and skeletal site (19) likely pointing toward a relatively constant level of optimal matrix mineralization. The former made it possible to establish a reference BMDD of cancellous bone which can be used as normative data (19, 20).
In order to study the effects of intravenous ibandronate (IBN) treatment on BMDD as assessed by quantitative backscattered electron imaging (qBEI) in a cohort of male patients with osteoporosis, we have conducted a 2-year clinical trial and obtained paired iliac crest biopsies at baseline and 24 months. Our primary hypothesis was that intravenous IBN (3 mg every 3 months) would significantly decrease the percentage of low mineralized bone areas (CaLow) and the heterogeneity of mineralization (CaWidth) in mOP. Secondary hypotheses were to find treatment-induced increases in areal BMD at the hip and the lumbar spine and decreases in serum markers of bone turnover over treatment duration.

Materials & Methods

Patients and Study Design

This study on treatment of male osteoporosis with intravenous IBN (TOMIBA-trial, Eudract number 2006-006692-20) was approved by the local ethics committee. All patients were prospectively recruited. They signed a written informed consent prior to any study related procedures. Medical history details, including the previous use of antiosteoporotic drugs, current medications, lifestyle habits (eg, smoking), previous fractures (including trauma history), and parental fractures were recorded in detail. Inclusion criteria were: (i) age from 20 to 85 years, (ii) T-score of ≤ -2 at the femoral neck and a T-score of ≤ -1 at the lumbar spine OR a T-score of ≤ -1 at the femoral neck and at least one moderate vertebral deformity (meeting the Genant criteria) or at least one non-vertebral low trauma fracture (21, 22), (iii) feasibility of aBMD assessment at the hip and the lumbar spine (i.e. at least two vertebrae between L1 and L4 had to be measurable by DXA; no bilateral hip replacement), (iv) 25-hydroxyvitamin D levels ≥ 10 ng/ml, (v) no metabolic bone disease other than primary osteoporosis/osteopenia OR osteoporosis/osteopenia associated with low testosterone levels. Exclusion criteria were (i) previous use of specific anti-osteoporotic treatments, such as oral bisphosphonates within the last 6 months, i.v. bisphosphonates at any time, calcitonin
and/or SERMs within the last 6 months, fluorides, strontium ranelate, teriparatide or PTH 1-84, or current use of antiepileptic substances, thyroid hormones, or recently initiated androgen replacement therapy, (ii) previous use of anabolic steroids or glucocorticoids (≥ 5 mg prednisone equivalent for more than 2 weeks within the last 6 months), (iii) manifest hyper- or hypothyreoidism (TSH beyond the range of 0.27 – 4.20 μU/ml) and/or (iv) a history of prior malignancy within the last five years (except basal cell carcinoma).

The primary endpoint of the TOMIBA trial was the effect of intravenous IBN treatment on bone matrix mineralization as measured by the changes in BMDD parameters (based on qBEI analysis) in paired transiliacal bone biopsy samples obtained at baseline and at 24 months of IBN treatment. Secondary endpoints included treatment-induced changes in areal bone mineral density (BMD by DXA) and serum markers of bone turnover including type I collagen peptides CrossLaps (CTX), procollagen type 1 amino-terminal propeptide (P1NP), and osteocalcin (OC).

Clinical and biochemical evaluation

All patients underwent physical exams at each clinical visit. Body weight and height were obtained. Fasting blood samples were drawn from between 8:00 and 10:00 a.m. Routine blood and urine analyses were performed at the ISO-certified Vienna-based Central Laboratory of the St. Vincent Group after daily quality protocol procedures. These included a whole blood count, serum potassium, sodium, calcium, and phosphate, parathyroid hormone (PTH), 25-hydroxyvitamin D, thyroid-stimulating hormone (TSH), kidney and liver function parameters, and total testosterone measurements. Calcium and phosphate excretion was measured after 24-hour urine collection. All patients underwent DXA of the hip and spine (GELunar iDXA scanner, software version Encore 13, 50,040; GE LUNAR Corporation, Madison, WI, USA). Vertebral fracture status was determined by anteroposterior and lateral radiographs of the thoracic and lumbar spine. The images were semiquantitatively graded by an experienced physician according to the Genant classification.
(23). Non-vertebral fractures were self-reported, radiology or trauma care reports that confirmed the fractures were available.

For each patient, 2mL of serum were stored in 1-mL voids at -70°C for batched bone marker analyses at the Department of Laboratory Medicine of the Medical University of Vienna. Serum markers of bone turnover were measured using electrochemiluminescence immunoassays [ECLIA; b-CrossLaps (CTX), N-MID Osteocalcin (OCN), total N-terminal type 1 procollagen propeptide (P1NP); all Roche Diagnostics, Mannheim, Germany] on a Modular Analytics E170 device (Roche Diagnostics).

**Bone biopsy**

Paired transiliac biopsies were obtained from the entire study cohort (n=19 male patients) with a Bordier-type trephine with an inner diameter of 7.5 mm (the biopsy at 24 months was obtained from the contralateral site of the iliac crest). Biopsy samples were not labeled because of potential interference of tetracycline and osteogenic gene expression patterns which were the primary endpoints of substudy of the TOMIBA trial (24). Bone samples were immediately fixed and dehydrated in a series of alcohols, embedded in polymethylmethacrylate, and prepared for qBEI evaluation as previously described (19).

**Histomorphometric analysis of static parameters of bone formation and resorption**

Undecalcified bone samples were embedded in polymethylmethacrylate. Consecutively, 3-µm sections were stained with a modified Goldner’s Trichrome method. Bone histomorphometry was performed according to Parfitt et al. (25) by means of light microscopy equipped with a digital camera (Axiophot, AxioCam, Zeiss, Oberkochen, Germany). The bone sections were examined using objective lenses of 10x and 20x magnification. Images were further analyzed on computer
using software ImageJ version 1.46r (Wayne Rasband, National Institute of Health, USA) by applying custom made routines. The parameters analyzed were osteoid volume per bone volume (OV/BV), osteoid thickness (O.Th), osteoid surface per bone surface (OS/BS), osteoblast surface per bone surface (Ob.S/BS), osteoclast surface per bone surface (Oc.S/BS), eroded surface per bone surface (ES/BS), and number of osteoclasts per bone surface (NOc/BS). Outcomes were compared to available reference data from healthy men, aged 51-60 yrs from Rehman et al.(26).

Morphometric indices of mineralized bone tissue

Due to the high contrast between mineralized and soft tissue in the backscattered electron images, the latter were used for the analysis of structural parameters of mineralized tissue utilizing customized evaluation routines (NIH-image software version 1.63 Wayne Rasband, National Institutes of Health, Bethesda, MD). Using the qBEI overview images of the cross-sectional area (spatial resolution of 9 μm), we measured: mineralized trabecular bone volume per tissue volume (md. BV/TV), trabecular width (md. Tb.Th.) and trabecular number (md. Tb.N), and as cortical width (md. Ct.Wi). The two cortices were individually isolated from the trabecular compartment of the biopsy by following a line where the compact bone character was changing to a cancellous one. On the basis of 4 μm pixel resolution images used also for BMDD analysis, the porosity of cortical mineralized tissue (md. Ct.Po.) was measured by dividing total cortical void area (ignoring osteocyte lacunae) by the total cortical tissue area. All morphometric indices from cancellous bone were directly numerically compared to normative data from healthy individuals published by Rehman et al. (26) by T-scores. For cortical bone, normative data were only available for cortical thickness (Ct.Th) (which is the 2-dimensionally measured Ct.Wi transformed to the 3-dimensional cortical thickness) and for cortical bone volume per tissue volume (CV/TV). For comparison, we transformed our measures of md. Ct.Wi to Ct.Th by multiplication with $\pi/4$ and md.Ct.Po to md.CV/TV by subtracting Ct.Po from 100%. It should be noted that our morphometric measures provide information on the mineralized tissue only (osteoid excluded).
Quantitative backscattered electron imaging (qBEI)

Calibrated digital images with a 4 µm pixel resolution were used to obtain cancellous and cortical bone matrix mineralization density distributions (Cn. and Ct. BMDD) using quantitative backscattered electron imaging (qBEI). Full details, including the validation, technical precision and its application to bone tissue have been published previously (20, 27). The intensity of the signal of the backscattered electrons is proportional to the local calcium concentration of the scanned bone section; thus the denser the mineralization the brighter the pixels in the image (Fig. 1). These digital images were used for the evaluation of the grey-level histograms, which were further transformed to weight percent calcium histograms (so called bone mineralization density distributions, BMDD). Five variables were obtained from the BMDD curves: CaMean, the weighted mean Ca-concentration of the bone area; CaPeak, the mode calcium concentration (the peak position of the histogram), which indicates the most frequently occurring calcium concentration of the scanned bone area; CaWidth, the full width at half maximum of the distribution, describing the variation in mineralization density; CaLow, the percentage of mineralized bone with a calcium concentration less than the 5th percentile of the reference BMDD of cancellous bone (less than 17.68 weight percent calcium) which reveals the percentage of bone area undergoing primary mineralization; and CaHigh, the portion of bone areas with a calcium concentration higher than the 95th percentile (higher than 25.30 wt% Ca) of the reference BMDD of cancellous bone (predominantly fully mineralized interstitial bone) (20, 19). The BMDD variables of cancellous bone from the patients were compared to reference cancellous BMDD data from an adult healthy population (n=52) published previously (19). Ct. BMDD outcomes of the patients could not be compared to normal as reference data for cortical bone have not been established yet.

Relative changes in BMD are the sum of relative changes in mineral/hydroxyapatite volume fraction (HA vol) in the bone material and bone volume (28). For the estimation of the contribution
of the former to the changes in the BMD, the Ca weight fraction CaMean as measured by qBEI has to be transformed to values of HA vol volume fractions using the following relation (29):

\[ HA_{vol} = \frac{HA_{wt}}{HA_{wt} + (1 - HA_{wt}) \cdot \frac{\rho_{min}}{\rho_{org}}} \]  

equation (1)

where \( \rho_{min} \) being 3.18 g/cm\(^3\) the density of HA, and \( \rho_{org} = 1.41 \) g/cm\(^3\) being the density of the organic matrix (30). HAwt is the weight fraction of HA, which is calculated from CaMean by the relation \( HA_{wt} = CaMean \cdot 2.51 \), based on the chemical composition of HA.

**Statistical Analysis**

Statistical analysis was performed with Sigma Stat for Windows Version 2.03 (SPSS Inc.). As the study cohort was relatively small, non-parametric tests were used for analysis. Data in tables and figures are presented as median (25\(^{th}\); 75\(^{th}\) percentiles). Comparison to reference BMDD is based on Mann-Whitney rank sum tests. Treatment effects on BMD (by DXA), serum parameters, morphometric and static parameters of bone formation and resorption, Cn. BMDD and Ct. BMDD outcomes as well as potential compartment-specific effects (i.e. cortical versus trabecular changes) were analyzed by Wilcoxon signed rank tests. The dependency of the treatment-induced changes in average calcium concentrations on the baseline values of the latter are based on linear regression analysis, the relationship between the treatment induced changes in cancellous vs. cortical bone are based on Spearman rank order correlations. Statistical significance was assigned to \( p<0.05 \).
Results

(A) mOP patients at baseline

(i) Clinical and biochemical variables
Baseline characteristics of our mOP cohort are given in Table 1 and 2. T-scores at the lumbar spine, femoral neck and total hip were in the osteoporotic or osteopenic range. About 74% of patients had sustained at least one low trauma osteoporotic fracture 32% had at least one vertebral fracture. Serum Ca, phosphate, and PTH were within the normal range, except for two patients who had somewhat lower phosphate levels (0.61 and 0.73mmol/L). Testosterone levels were normal in all except for two patients who had lower levels (1.37 and 1.44 μg/L). Vitamin D levels of the patients were in the range from 10.29 to 44.50 ng/mL. Serum markers of bone turnover markers revealed mean CTX, P1NP, and OC levels within the lower normal male reference range.

(ii) Morphometric parameters and static parameters of bone formation and resorption
Morphometric parameters of mineralized tissue and static bone formation and resorption are summarized in Table 3. Comparison to normative data revealed md.BV/TV and md.Tb.N at the lower range (Z-scores -1 and -1.3, respectively), and normal md. Tb.Th (Z-score +0.3). Z-scores for Ct.Th and CV/TV were -1.7 -1.0, respectively. OV/BV, O.Th, and Ocs/BS were at normal range, while OS/BS and ES/BS were increased (Z-scores of +1.3 and +3.8, respectively). ObS/BS median was decreased compared to reference mean value (Z-score -1.9).

(iii) Bone mineralization density distribution (BMDD)
Cn. BMDD outcomes of the patients at baseline were compared to reference Cn. BMDD data (Table 4). The mOP patients had reduced average and typical calcium concentrations compared to
normal (Cn.CaMean -1.8%, p<0.01 and Cn.CaPeak -2.6%, p<0.05, respectively), increased heterogeneity of mineralization (Cn.CaWidth +16%, p<0.001) and percentage of low mineralized bone areas (Cn.CaLow +29%, p<0.05), while the percentage of high mineralized bone areas (Cn.CaHigh) was not different from the reference BMDD (Fig. 1, Fig. 2).

(B) Effect of IBN treatment (paired iliac crest biopsies)

(i) Changes in clinical and biochemical variables

Two of the patients had sustained new fragility fractures (one vertebral and one peripheral fracture). Group median values of BMD at lumbar spine, femoral neck and total hip significantly increased by +3.3% (p<0.001), +1.9% (p=0.011), and +5.6% (p<0.001), respectively (for intra-individual changes see Table 2). All serum markers were significantly reduced by IBN (group median differences -37.5%, p<0.001, -44.4%, p=0.001, and -36.3%, p=0.003 for CTX, P1NP, and OC, respectively (Table 2). The median values after treatment were lowered compared to normal range.

(ii) Changes in Morphometric Parameters and static bone formation and resorption

The effect of treatment on histomorphometric outcomes are summarized in Table 3. IBN treatment significantly reduced md.Ct.Po by 23% (p=0.01), OV/BV by -86% (p<0.001), O.Th by -55% (p=0.003), OS/BS by -76% (p<0.001), and Ob.S/BS by -93% (p=0.003). All other parameters were unaffected by IBN.

(iii) Changes in BMDD

IBN treatment caused significant changes in cancellous and cortical BMDD variables (Table 4, Fig. 1, Fig. 2). For a representative BMDD curve before and after treatment see Fig. 1. Median group comparison to baseline revealed significant increases in Cn. and Ct.CaMean (+2.4%, p<0.001 and
+3.0%, p=0.002, respectively) as well as in Cn. and Ct. CaPeak (3.1%, p=0.027 and 2.3%, p=0.030). Cn. and Ct.CaWidth (-14%, p=0.044 and -16%, p=0.001, respectively) as well as Cn. and Ct.CaLow were reduced (-28% and -45% respectively, both p<0.001), while Cn. and Ct.CaHigh remained unchanged by treatment (for intra-individual changes see Table 4). After IBN treatment, Cn. BMDD outcomes revealed no statistically significant differences to normal Cn. BMDD.

Linear regression analysis revealed a strong negative dependency of the absolute treatment induced changes in Cn. and Ct. CaMean on the baseline values of Cn. and Ct. CaMean respectively (R=-0.68 for cancellous and R=-0.82 for cortical bone, both p<0.001, see Figure 3A, 3B). Absolute changes in CaMean of cancellous and cortical bone were positively correlated (Spearman rank order correlation R=0.78, p<0.001, Fig. 3C). Paired comparison of the absolute changes within cancellous vs. cortical bone revealed no significant difference: ΔCn.CaMean = 0.63 (0.28; 0.99) wt% vs. ΔCt.CaMean = 0.95 (0.13; 1.51) wt%, (median (IQR), p=0.465.

(iv) Estimation of the contribution of matrix mineralization to BMD changes

For relating changes in cancellous bone matrix mineralization to those of lumbar spine BMD, Cn.CaMean was transformed to HAvol according to equation 1. The median intra-individual change in HAvol was +4.2% which is of similar magnitude as the intra-individual change in lumbar spine BMD (+3.5%, see also Table 2).

Discussion

We conducted the first paired bone biopsy study in osteoporotic men treated with IBN and found treatment-induced increases in bone matrix mineralization, and a reduction in cortical porosity. This was accompanied by decreases in serum markers of bone turnover, decreases in osteoid
related histomorphometric parameters, a decrease in osteoblast surface, and increases in areal hip and spine BMD.

At baseline, the mOP patients presented with low BMD and fragility fractures according to the inclusion criteria. They had lower bone volume and cortical width than healthy men of similar age (26). Their cortical porosity was higher than normal and was comparable with men suffering from idiopathic osteoporosis (31). The most distinct difference between the male patients of our study and women with postmenopausal osteoporosis was that mOP patients did not exhibit signs of high bone turnover. Histomorphometric static indices of bone turnover were numerically within normal range (osteoclast surface) or somewhat decreased (osteoblast surface). Serum turnover markers were within the lower normal male reference range. Our findings of normal to lower bone turnover are in line with several publications on primary male osteoporosis which support the hypothesis of osteoblast dysfunction as a predominant pathomechanism in men (24, 32, 33, 34, 35, 36).

Despite the differences in etiology and turnover status, the deviations from normal bone matrix mineralization of the mOP patients were conform to those found in postmenopausal osteoporosis (37, 38, 39, 40). Moreover, they are in line with previous findings for men and women with idiopathic osteoporosis (41, 42, 43). In general, deviations from normal BMDD suggest alterations in bone turnover (causing altered average tissue age) and/or in the kinetics of mineralization (causing a different time course of the accumulation of mineral in the newly formed bone material) (20, 44). In high-turnover postmenopausal osteoporosis, the increased bone turnover (45) induces reduced tissue ages of the bone structural units (BSUs) which are associated with lowered degree and higher heterogeneity of mineralization. As aforementioned, the serum data in the present study and the previous subgroup analysis of our mOP patients (24) did not reveal any evidence for increased bone turnover suggesting that other factors which are interfering with the mineralization kinetics, in particular altered bone matrix properties due to dysfunctional osteoblasts, might have
been essential for the reduced degree of matrix mineralization and increased fracture risk at baseline (41).

The changes caused by treatment are in agreement with the antiresorptive/anticatabolic effect of IBN. We observed highly significant reductions in osteoid-related histomorphometric indices and in osteoblast surface, and in particular in bone turnover markers after two years of IBN therapy. These changes were accompanied by significant increases in hip and spine BMD. Serum bone turnover marker reductions and increases in BMD are consistent with a previous study on intravenous IBN treatment in another cohort of men with primary osteoporosis (10).

The aforementioned IBN-induced changes in bone matrix mineralization are compatible with the serologic and histomorphometric evidence of treatment-induced reductions in bone turnover. Antiresorptive agents cause that less bone is being formed and resorbed leading to prolonged secondary mineralization in existing bone structural units (i.e. bone packets) (20, 39). This typically causes increases in average and mode mineralization densities along with decreases in the proportion of low mineralized areas and in heterogeneity of local calcium concentrations. Similar changes in BMDD have been reported for patients with postmenopausal osteoporosis after alendronate, risedronate and zoledronic acid treatment (37, 38, 39, 40, 46, 47). The proportion of highly mineralized areas was numerically increased after treatment but this increase did not reach statistical significance, possibly due to the larger inter-individual variation in this parameter. However, none of the bone packets had abnormally high mineral content after treatment. After treatment, the differences in cancellous bone to reference BMDD data were abolished indicating that the BMDD in cancellous bone had been normalized due to IBN treatment and adequate supplementation with vitamin D and calcium. Noteworthy, the BMDD of postmenopausal osteoporotic patients after zoledronic acid treatment achieved values beyond the normal reference range of Cn.BMDD. In particular, the average calcium concentration and the percentage of highly
mineralized bone areas were significantly increased by 3.7% and 194%, respectively (47). Consistent with this observation, the percentage reduction of serum bone turnover markers was greater in the zoledronic acid treated patients (48). Unfortunately, a comparison to normal reference data is not feasible for cortical BMDD because a cortical BMDD reference database has not yet been established. Nevertheless, IBN increased cortical bone matrix mineralization in line with previous findings after zoledronic acid treatment in the aforementioned postmenopausal osteoporotic patients (47) and in liver transplant patients (49). This is important to note as cortical bone is often considered to be less prone to treatment-induced changes.

Interestingly, the absolute changes in average calcium concentrations were dependent on the baseline value of the latter as previously observed in cohort of postmenopausal osteoporosis after risedronate therapy (28). This indicates that the patients with very low mineralization densities at baseline have the largest benefit of the treatment in terms of bone matrix mineralization and is reflecting that the calcium concentration is reaching a plateau level. Further, those patients who have relatively large increases in average mineralization density in cancellous bone do have the largest increases in cortical bone and vice versa. When comparing the increases in cancellous with cortical bone, no significant difference could be detected, another indication that cortical bone reacts to treatment with similar changes as cancellous bone in this cohort of patients.

Although our study was not powered for changes in morphometric parameters of cortical bone, it is worth mentioning that we observed a significant decrease in cortical porosity as previously also described for alendronate (38) and risedronate (50) treated postmenopausal osteoporotic women. It has to be noted that cortical bone micro-structure exhibits relatively large intra- and inter-individual variability (51). Thus larger sample sizes than ours are needed to reach confident conclusions with respect to possible interactions of anti-osteoporotic agents and changes of intra-cortical porosity. Nevertheless, our observation points toward filling of resorption space in cortical bone induced by
treatment and together with the increase in matrix mineralization this likely reflects a characteristic
effect of antiresorptive treatment with bisphosphonates.

In order to gain additional insight to distinct associations of IBN-induced increases in areal BMD and matrix mineralization properties, we used a previously introduced estimation (28) to directly compare the increase in lumbar spine BMD with the increase in matrix mineralization. The comparison revealed that spinal BMD upon 2-year IBN-treatment gain was of similar magnitude than the increases in matrix mineralization. Such a comparison of changes in BMD and BMDD certainly has some limitations as these parameters were not measured at the same skeletal site, and both cancellous and cortical bone compartments contribute to BMD. Nevertheless, this estimation reveals that the increase in lumbar spine BMD is highly attributable to the increase in matrix mineralization in our cohort of male patients. However, an estimation of the contribution of the increase in matrix mineralization and decrease of porosity in cortical bone to DXA outcomes from cortical bone dominated sites (femoral neck e.g.) is not feasible by this method. The magnitude of the treatment induced changes in cortical bone mineralization and porosity might vary among different skeletal sites due to a generally greater variation in these parameters throughout the human skeleton (52).

Our study did not have new fragility fractures as an endpoint and the study cohort was too small to draw valid conclusions on IBN-treatment in men and its effect on fracture risk. At baseline suboptimal matrix mineralization, low bone volume, and relatively high cortical porosity were evident and might have contributed to low BMD and higher fracture risk. Treatment was observed to normalize matrix mineralization and to decrease cortical porosity. These mechanisms might have contributed to the reduction of fracture risk found in larger clinical trials on male osteoporosis (12, 13, 14, 15). It should be noted that it is difficult to relate these ibandronate treatment induced changes in cortical bone from the iliac crest with fracture risk at sites dominated by cortical bone,
as for ibandronate there is insufficient and/or inconsistent evidence of an effect on these fractures (53). However, recent studies are pointing toward benefit effects of this therapeutic agent on hip structure parameters in male patients with low bone density (54) and further reported a non-inferior effect on non-vertebral fractures compared with risedronate (55).

One limitation of our study is the lack of a placebo control group. We refrained from including a placebo-control group for ethical reasons and due to pre-existing data on safety and efficacy of IBN. However, we were able to compare BMDD data with normal reference data (19). Another limitation is that the invasive methods used do not provide the analysis of the treatment effects in the identical bone volume. However, the intra-individual variation in BMDD between the biopsy sites is considered to be relatively small (19). Regarding the evaluation of cortical porosity, it has to be mentioned that the definition of the cortico-trabecular transition zone is a matter of ongoing research (56). We used the method described as “semi-conservative” by others (50). Finally, the sample size of nineteen patients was small, however, the clinical characteristics of these men were relatively homogeneous and the paired biopsy design of our study was a definite strength and allowed us to detect IBN-effects at the tissue and material level.

In conclusion, we were able to demonstrate in this paired-biopsy study that two-year intravenous IBN treatment had positive effects on BMD and bone quality in osteoporotic men. Hip and spine BMD improved significantly, while bone turnover was suppressed. Bone matrix mineralisation properties were restored to normal and cortical porosity was reduced.
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Authors’ roles: All authors made substantial contributions to either the conception and design, acquisition of data or analysis and interpretation of data, participated in drafting the manuscript or revising it critically for important intellectual content, and approved the final version of the submitted manuscript.

BMM accepts responsibility for the integrity of the data analysis.
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**Figure Legend**

**Figure 1**: (A) Cross-sectional area of transiliacal bone biopsies from one patient before (top) and after IBN treatment (bottom) and (B) corresponding BMDD curves (top for cancellous bone, bottom for cortical bone). Dashed lines indicate baseline, solid lines after IBN treatment. Bars indicate 1 mm in the images.

**Figure 2**: Cancellous BMDD results (median (25th, 75th percentile)) for mOP (BL, white = baseline; IBA, dark grey = after 24 months with intravenous IBA). White dotted lines and grey areas in the background indicate mean ± 1SD or median (25th, 75th percentile) of the normal reference range (from 19). ***p<0.001, *p<0.05 paired comparison vs. baseline (treatment effect), and **p<0.01, °p<0.05 vs. reference BMDD.

**Figure 3**: The relationship between baseline matrix mineralization and the effect of intravenous IBA treatment on mineralization: The absolute changes in average calcium concentrations in cancellous bone (ΔCn.CaMean) (A) and those in cortical bone (ΔCt.CaMean) (B) plotted versus the baseline values of Cn.CaMean and Ct.CaMean. Correlation coefficients and p-values are based on linear regression analyses. (C) the absolute change in average calcium concentration of cancellous (ΔCn.CaMean) versus those of cortical bone (ΔCt.CaMean). Correlation coefficient and p-value are based on Spearman rank order correlation.
Table 1: Baseline characteristics of our mOP study cohort (n=19)

<table>
<thead>
<tr>
<th></th>
<th>mOP patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yrs)</td>
<td>53.0 [44.5; 57.0]</td>
</tr>
<tr>
<td>BMI</td>
<td>24.0 [22.3; 27.0]</td>
</tr>
<tr>
<td>at least 1 osteoporotic fx (% of cohort)</td>
<td>14 (74%)</td>
</tr>
<tr>
<td>at least 1 vertebral fx (% of cohort)</td>
<td>6 (32%)</td>
</tr>
<tr>
<td>T-scores</td>
<td></td>
</tr>
<tr>
<td>lumbar spine</td>
<td>-2.6 [-3.1; -2.1]</td>
</tr>
<tr>
<td>femoral neck</td>
<td>-2.4 [-2.8; -2.0]</td>
</tr>
<tr>
<td>total hip</td>
<td>-2.4 [-2.7; -2.0]</td>
</tr>
<tr>
<td>serum calcium (mmol/L)</td>
<td>2.31 [2.20; 2.39]</td>
</tr>
<tr>
<td>serum phosphate (mmol/L)</td>
<td>1.0 [0.9; 1.1]</td>
</tr>
<tr>
<td>25-(OH)-vitamin D (ng/mL)</td>
<td>32.9 [19.6; 38.2]</td>
</tr>
<tr>
<td>PTH (pg/mL)</td>
<td>42.1 [34.7; 47.5]</td>
</tr>
<tr>
<td>Testosterone (μg/L)</td>
<td>3.42 [2.62; 5.39]</td>
</tr>
<tr>
<td>TSH (μU/mL)</td>
<td>1.02 [0.69; 1.60]</td>
</tr>
</tbody>
</table>

Data are median [25th; 75th percentiles] or percentage of cohort.

normal ranges: serum calcium 2.15 – 2.55 mmol/L, serum phosphate 0.8 – 1.6 mmol/L, 25-(OH)-
vitaminD >30ng/mL, PTH 11.1-79.5 pg/mL, testosterone 2-10 μg/L, TSH 0.27 – 4.20 μU/ml.
Table 2: Baseline values and treatment changes in serum bone turnover markers and bone mineral density (DXA) for mOP patients (n=19)

<table>
<thead>
<tr>
<th>markers</th>
<th>Baseline</th>
<th>After IBN</th>
<th>group % diff.²</th>
<th>paired comparison intra-individual % diff.³</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTX ¹ (ng/mL)</td>
<td>0.32 [0.25; 0.46]</td>
<td>0.20 [0.13; 0.26]</td>
<td>-37.5%</td>
<td>-38%</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>P1NP ¹ (ng/mL)</td>
<td>29.5 [24.6; 36.2]</td>
<td>16.4 [11.5; 20.6]</td>
<td>-44.4%</td>
<td>-53%</td>
<td>0.009</td>
</tr>
<tr>
<td>OC ¹ (ng/mL)</td>
<td>19.0 [16.4; 53.5]</td>
<td>12.1 [8.7; 15.0]</td>
<td>-36.3%</td>
<td>-43%</td>
<td>0.003</td>
</tr>
<tr>
<td>LS BMD</td>
<td>0.917 [0.852; 0.978]</td>
<td>0.947 [0.911; 1.026]</td>
<td>+3.3%</td>
<td>+3.5%</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>FN BMD</td>
<td>0.754 [0.701; 0.804]</td>
<td>0.768 [0.725; 0.823]</td>
<td>+1.9%</td>
<td>+2.5%</td>
<td>0.011</td>
</tr>
<tr>
<td>TH BMD</td>
<td>0.780 [0.742; 0.828]</td>
<td>0.824 [0.762; 0.839]</td>
<td>+5.6%</td>
<td>+4.1%</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Data are median (IQR), p-values are based on Wilcoxon signed rank tests.

¹ paired data available for n=16

²Median of group at 24 months minus median of group at baseline (given in percentage of median of group at baseline).

³Median of intra-individual changes (given in % of baseline of each patient).

Normal ranges: CTX 0.08–0.38 ng/mL, P1NP 23.3–64.8 ng/mL, and OC 14.1–34.5 ng/mL
Table 3: Histomorphometric analysis of structure and static bone formation and resorption from mOP patients (n=19) at baseline and after intravenous IBN treatment.

<table>
<thead>
<tr>
<th>mOP patients</th>
<th>Baseline</th>
<th>After IBN</th>
<th>group % diff.</th>
<th>paired comparison</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>intra-individual</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>median % diff.</td>
<td>% diff. 2</td>
</tr>
<tr>
<td>md. BV/TV (%)</td>
<td>15.5 (11.9; 20.8)</td>
<td>13.1 (10.9; 18.5)</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>md. Tb.Th. (μm)</td>
<td>144 (123; 170)</td>
<td>126 (113; 140)</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>md. Tb.N (mm⁻¹)</td>
<td>1.1 (0.9; 1.3)</td>
<td>1.1 (0.9; 1.3)</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>md. Ct.Wi. (μm)</td>
<td>909 (738; 1118)</td>
<td>976 (899; 1286)</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>md. Ct.Po. (%)</td>
<td>6.6 (4.5; 10.2)</td>
<td>5.1 (3.5; 6.6)</td>
<td>-23%</td>
<td>-29%</td>
</tr>
<tr>
<td>OV/BV (%)³</td>
<td>2.2 (0.6; 4.3)</td>
<td>0.3 (0.1; 0.4)</td>
<td>-86%</td>
<td>-80%</td>
</tr>
<tr>
<td>O.Th (μm)³</td>
<td>8.4 (5.7; 12.7)</td>
<td>3.8 (2.3; 4.7)</td>
<td>-55%</td>
<td>-56%</td>
</tr>
<tr>
<td>OS/BS (%)³</td>
<td>25.0 (7.9; 35.5)</td>
<td>5.9 (2.6; 8.6)</td>
<td>-76%</td>
<td>-71%</td>
</tr>
<tr>
<td>Ob.S/BS (%)³</td>
<td>2.7 (0.3; 5.1)</td>
<td>0.2 (0.0; 0.8)</td>
<td>-93%</td>
<td>-96%</td>
</tr>
<tr>
<td>OcS/BS (%)³</td>
<td>0.5 (0.1; 1.2)</td>
<td>0.0 (0.0; 0.8)</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>ES/BS (%)³</td>
<td>7.4 (3.4; 11.7)</td>
<td>7.4 (3.0; 11.7)</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>NOc/BS (mm⁻¹)</td>
<td>0.1 (0.0; 0.3)</td>
<td>0.1 (0.0; 0.1)</td>
<td>---</td>
<td>---</td>
</tr>
</tbody>
</table>

Data are median (25th; 75th percentiles), p-value based on Wilcoxon signed test comparison.

1 Median of group at 24 months minus median of group at baseline (given in percentage of median of group at baseline).

2 Median of intra-individual changes (given in % of baseline of each patient).

³ Reference data (healthy men, aged 51-60 yrs) from Rehman et al. (26): OV/BV 3.0 (1.6), O.Th 8.7(2.0), OS/BS 17.1 (6.1), Ob.S/BS 4.6 (1.0), OcS/BS 0.7(0.3), ES/BS 3.6 (1.0)
Table 4: BMDD outcomes from male patients with osteoporosis (paired biopsies at baseline and after intravenous IBN treatment, n=19), in comparison to a previously published healthy reference cohort (n=52)

| mOP patients | Baseline | After IBN | group % diff. | treatment effect | intra- | individual % diff. | p-value | Ref. BMDD  
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Cn.CaMean (wt%)</td>
<td>21.83 °°</td>
<td>22.36</td>
<td>+2.4</td>
<td>+2.9</td>
<td>&lt;0.001</td>
<td>(22.09; 22.65)</td>
<td>(21.84; 22.50)</td>
<td></td>
</tr>
<tr>
<td>Cn.CaPeak (wt%)</td>
<td>22.36 °</td>
<td>23.05</td>
<td>+3.1</td>
<td>+2.3</td>
<td>0.027</td>
<td>(22.70; 23.22)</td>
<td>(22.70; 23.14)</td>
<td></td>
</tr>
<tr>
<td>Cn.CaWidth (Δwt%)</td>
<td>3.81 °°°</td>
<td>3.29</td>
<td>-14</td>
<td>-10</td>
<td>0.044</td>
<td>(3.16; 3.94)</td>
<td>(3.12; 3.47)</td>
<td></td>
</tr>
<tr>
<td>Cn.CaLow (%)</td>
<td>5.84 °</td>
<td>4.19</td>
<td>-28</td>
<td>-16</td>
<td>&lt;0.001</td>
<td>(3.89; 4.80)</td>
<td>(3.52; 6.48)</td>
<td></td>
</tr>
<tr>
<td>Cn.CaHigh (%)</td>
<td>2.80</td>
<td>6.51</td>
<td>---</td>
<td>---</td>
<td>n.s.</td>
<td>(1.90; 6.65)</td>
<td>(1.30; 2.90)</td>
<td></td>
</tr>
<tr>
<td>Ct.CaMean (wt%)</td>
<td>21.51</td>
<td>22.57</td>
<td>+3.0</td>
<td>+4.4</td>
<td>0.002</td>
<td>(22.34; 22.98)</td>
<td>n.a.</td>
<td></td>
</tr>
<tr>
<td>Ct.CaPeak (wt%)</td>
<td>22.70</td>
<td>23.22</td>
<td>+2.3</td>
<td>+3.0</td>
<td>0.030</td>
<td>(22.88; 23.83)</td>
<td>n.a.</td>
<td></td>
</tr>
<tr>
<td>Ct.CaWidth (Δwt%)</td>
<td>4.33</td>
<td>3.64</td>
<td>-16</td>
<td>-13</td>
<td>0.001</td>
<td>(3.47; 3.94)</td>
<td>n.a.</td>
<td></td>
</tr>
<tr>
<td>Ct.CaLow (%)</td>
<td>6.36</td>
<td>3.50</td>
<td>-45</td>
<td>-35</td>
<td>&lt;0.001</td>
<td>(2.94; 4.48)</td>
<td>n.a.</td>
<td></td>
</tr>
<tr>
<td>Ct.CaHigh (%)</td>
<td>5.76</td>
<td>8.98</td>
<td>---</td>
<td>---</td>
<td>n.s.</td>
<td>(2.82; 8.49)</td>
<td>n.a.</td>
<td></td>
</tr>
</tbody>
</table>

Data are median (25th, 75th percentiles).
°°° p<0.001, °° p<0.01, ° p<0.05 versus reference BMDD
n.a. = reference data for cortical bone are not available
Figure 1
Figure 2
Figure 3