Original Full Length Article

Utilizing time-lapse micro-CT-correlated bisphosphonate binding kinetics and soft tissue-derived input functions to differentiate site-specific changes in bone metabolism in vivo

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A B S T R A C T

The turnover of bone is a tightly regulated process between bone formation and resorption to ensure skeletal homeostasis. This process differs between bone types, with trabecular bone often associated with higher turnover than cortical bone. Analyses of bone by micro-computed tomography (micro-CT) reveal changes in structure and mineral content, but are limited in the study of metabolic activity at a single time point, while analyses of serum markers can reveal changes in bone metabolism, but cannot delineate the origin of any aberrant findings. To obtain a site-specific assessment of bone metabolic status, bisphosphonate binding kinetics were utilized. Using a fluorescently-labeled bisphosphonate, we show that early binding kinetics monitored in vivo using fluorescent molecular tomography (FMT) can monitor changes in bone metabolism in response to bone loss, stimulated by ovariectomy (OVX), or bone gain, resulting from treatment with the anabolic bone agent parathyroid hormone (PTH), and is capable of distinguishing different, metabolically distinct skeletal sites. Using time-lapse micro-CT, longitudinal bone turnover was quantified. The spine showed a significantly greater percent resorbing volume per bone surface in response to OVX, while mice treated with PTH showed significantly greater resorbing volume per bone surface in the spine and significantly greater forming surfaces in the knee. Correlation studies between binding kinetics and micro-CT suggest that forming surfaces, as assessed by time-lapse micro-CT, are preferentially reflected in the rate constant values while forming and resorbing bone volumes primarily affect plateau values. Additionally, we developed a blood pool correction method which now allows for quantitative multi-compartment analyses to be conducted using FMT. These results further expand our understanding of bisphosphonate binding and the use of bisphosphonate binding kinetics as a tool to monitor site-specific changes in bone metabolism in vivo.

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Introduction

Bone turnover is tightly regulated by osteoblasts and osteoclasts, and is essential for maintaining the integrity and adaptation of the skeleton. This process is both spatially and temporally regulated, ensuring equal bone resorption and new bone formation, in order to maintain bone mass [13,29]. Disruption of this coupled process can lead to abnormal bone loss or gain, both with severe ramifications. Remodeling is also essential for the turnover of aged bone, which removes microfractures and maintains the mechanical competence of the bone tissues throughout the body [20]. Long and vertebral bones differ in their composition of trabecular and cortical bone, and in their levels of metabolic activity. While both cortical and trabecular bones require constant remodeling to maintain the integrity of the skeleton, trabecular bone resorption additionally contributes, to a greater extent, to blood calcium homeostasis, resulting in a higher level of metabolic activity [5,24].

While longitudinal analyses of bone by micro-computed tomography (micro-CT) provide detailed insights into structural adaptations, as well as changes in bone mineral content over time, such studies require weeks or months in mice before changes are observed and the assessment of the short-term changes in metabolic status of these bones remains difficult. Recent method developments have allowed the assessment of bone formation and resorption rates in vivo [15,28], but fail to show the instantaneous state of bone metabolism. In contrast, analyses of serum markers can provide insight into the current metabolic status of the skeleton, but fail to differentiate the individual...
metabolic statuses of different skeletal regions [7]. In humans, (18)F-fluoride positron emission tomography (PET) has proven to be a feasible tool for metabolic monitoring [22]. However, in mice, only cases of extreme changes in bone activity (complete fracture or cancer-induced severe osteolysis) have been successfully documented [1] and require the repeated injection of radioactive material. To this end, new methods are required for the site-specific monitoring of bone metabolic status.

Because of their anti-resorptive properties, bisphosphonates are in widespread clinical use in patients with excessive bone loss (i.e. osteoporosis, tumor osteolysis) [17]. Recent research has also exploited the bisphosphonates’ ability to bind bone mineral with high affinity as a bone metabolic marker rather than as a therapeutic agent. Conjugation of bisphosphonates with fluorescent dyes is currently under great scrutiny to help characterize bisphosphonate binding [11,27], as well as in the use as a bone-specific turnover marker [11,14]. While early works focused on bisphosphonate binding days after injection, new data suggests that monitoring the kinetics of early binding (as little as 5 min after injection) may provide additional information about the metabolic status of the bone [30]. One limitation in previous in vivo kinetic assessments has been the lack of indicators of blood pool bisphosphonates available for bone binding, which are used in more refined, quantitative, multi-compartment analyses commonly seen in the context of radiolabeled bisphosphonates [18]. Recent radionuclide studies in humans have also suggested that significant overall changes in skeletal activity, such as those seen in the case of treatment with parathyroid hormone (PTH), may alter the plasma time-activity curve [2]. This effect would alter both the rate and total binding values of a fluorescently-labeled bisphosphonate as discussed previously [30].

The purpose of this study was to assess whether the binding kinetic characteristics previously observed in the proximal tibia could be distinguished from other, metabolically distinct skeletal sites and to compare the relationship between observed binding kinetic parameters and longitudinal changes in bone structure, as assessed by time-lapse micro-CT [15]. Additionally, we have generated a blood pool correction factor allowing multi-compartment, quantitative kinetic analyses to be conducted using a modified Patlak’s method [18], accounting for changes in the quantity of bisphosphonate probe available for binding.

**Materials and methods**

**Animals**

12 week old female, CD-1 nude mice were purchased from Charles River (Wilmington, MA). All animals were kept in a temperature and humidity-controlled environment, with a 12 h light/dark cycle, and access to food and water ad libitum. Animal experiments and care were in accordance with the guidelines of institutional authorities and approved by the Ethics Committee for Animal Experiments at the Christian-Albrechts-Universität zu Kiel [V 312-72241.121-33]. Mice were anesthetized with intraperitoneal injections of 80 mg/kg ketamine (Aveco Pharmaceutical, IA) and 10 mg/kg xylazine (Rugby Laboratories, GA). For long-term anesthetization, additional administration of ketamine and xylazine at half dose was administered upon initial signs of mouse waking. Animals were separated into 3 groups (n = 9/group): i) non-operated, control animals, ii) ovariectomized (OVX) animals, imaged 3 days (short-term) and 14 days (long-term) after OVX, and iii) PTH-treated mice, which were subjected to OVX, then received daily PTH injections for 3 days beginning 11 days post-surgery (total of 14 days OVX and 3 days PTH treatment) as previously described [30]. Animals were ovariectomized via their dorsal side. Human parathyroid hormone fragment 1–34 (Sigma-Aldrich, MO) was given subcutaneously at a dose of 100 μg/kg daily.

**Bisphosphate binding kinetics**

Anesthetized mice were injected intravenously with 100 μl PBS containing 2 nmol of dissolved OsteoSense750, a fluorescently-conjugated pamidronate derivative [32]. For all in vivo, quantitative assessments of bisphosphate binding, anesthetized mice were imaged immediately following injection and every subsequent ~15 min interval for 210 min by FMT using the 750 nm channel of the FMT2500LX (Perkin Elmer, MA, USA). Images were reconstructed and VOIs of equal dimensions were positioned using the photographic image around the proximal tibia region, as well as the L1 and L2 vertebrae, and quantified using the TrueQuant software. Kinetic curves were generated using the average fluorescent intensity of either the proximal tibiae or vertebral regions over time using one-phase association curves, with \( Y_0 \) values constrained to zero, generated from Prism (version 5, GraphPad Software, CA). Graphs show only the first 100 min of imaging to more clearly illustrate changes in initial binding kinetics, though all time points were used to generate binding kinetic curves. Due to the low resolution of FMT, proximal tibia VOIs may also contain fluorescence of the distal femur. For this reason, this region is henceforth referred to as the knee region.

**Micro-CT analysis**

All animals were scanned by micro-CT prior to treatment to establish baseline bone parameters. Changes in bone mineral and structure resulting from OVX or PTH treatment were characterized from micro-CT assessment of skeletal changes in vivo. Anesthetized mice were placed in full-body holders and the tibiae aligned by visual inspection. Scans were made using a Scanco vivaCT 40-micro-CT (Brüttisellen, Switzerland) at an isotropic voxel size of 19 μm (70 kVp, 114 μA, 250 ms integration time, 1000 projections on 180° 2048 CCD detector array, cone-beam reconstruction with a radiation dose of approximately 520 mGy (CTD\(_{100}\)). PTH-treated mice were additionally scanned by micro-CT after 14 days of intermittent PTH treatment to capture long-term bone changes. Three volumes of interest (VOIs) were selected, one for the vertebrae, as well as one each for the epiphyseal and metaphyseal region of the proximal tibia (to exclude the growth plate region). Contours along the periosteal surfaces were drawn encompassing either 60 slices (1.05 mm) of the L1 vertebra, starting at the beginning of trabecular bone within the spinal body, 50 slices (0.875 mm) beginning at the proximal tip of the tibia epiphyseal trabecular bone, or 50 slices (0.875 mm) beginning just distal to the tibial growth plate, all extending in the distal direction. Baseline VOIs were transferred to the follow-up scans using an image registration approach to ensure analysis of consistent VOIs at each time point [3]. Bone mineral density (BMD) was calculated from the grey-scale micro-CT images (Image Processing Language (IPL) v5.15, ScancoMedical, Brüttisellen, Switzerland) as the total bone mineral content within the contour divided by the contour volume. Localized bone formation and resorption was determined from the time-lapsed micro-CT images after registration using programs written in IPL for the registration, and Matlab (R2010b; Mathworks, Natick, MA, USA) for the quantification of bone turnover, following similar procedures as previously described [28]. Briefly, a three-color image was produced from the overlaid follow-up and baseline images after a threshold (23% of maximal greyscale value) was applied. From this image, the volume of formed, resorbed and quiescent bone could be determined. The contact surfaces between the colored regions were used to calculate the formed and resorbed surface area.

**Soft tissue–blood pool correlation**

VOIs were placed over soft tissue regions in the abdomen and the corresponding fluorescence quantified. Kinetic curves for the soft tissue analysis were generated in Prism using one-phase exponential decay
curves, with the non-specific binding at infinite times (NS) values constrained to >0. A standard curve was generated by direct addition of known concentrations of OsteoSense750 to serum collected from non-injected mice and subsequently scanned by the Odyssey fluorescence scanner. For comparison of soft tissue fluorescence (assessed by FMT) and serum bisphosphonate levels, mice were imaged by FMT 1, 15, 30, 45 and 60 min after injection, followed by immediate blood collection from the tail vein. Whole blood was separated at 3000 g for 10 min and serum isolated. Serum was scanned on the Odyssey fluorescence scanner and compared to standard curve fluorescent readings to determine bisphosphonate concentrations.

Statistical analysis

All statistical analyses were conducted using Prism. Binding rate constants (k) and plateau values were calculated for curves from the line of best fit for each mouse using the formula: \[ Y = Y_0 + (\text{Plateau} - Y_0) (1 - \exp^{-kx}), \] where \( Y = \) fluorescence at time \( x \), \( Y_0 = \) the fluorescence at time 0, and \( x = \) time in minutes. The plateau, reflecting the maximum binding fluorescence expected as time approaches infinity, and the rate constant, reflecting the rate at which the curve approaches its plateau value, was multiplied to generate a plateau-weighted (Pw) rate constant for each mouse representing overall curve characteristics. For soft tissue analysis, \( Y_0 \) (fluorescent signal at time zero), NS (fluorescent signal as time approaches infinity) and K (clearance rate) values were calculated for curves from the line of best fit for each mouse using the formula: \[ Y = (Y_0 - \text{NS})^{-kx} + \text{NS}. \] Comparison between groups was made using two-sample \( t \)-tests using the Welch–Satterthwaite method to avoid the assumption of equal variabilities. Comparison between knee and spine values within the same mouse was made using paired \( t \)-tests. Time-lapse micro-CT statistics were calculated using a two-way ANOVA. Interaction between binding kinetic and time-lapse micro-CT parameters was assessed by the probability of the slope of the linear correlation being equal to zero using Prism. \( p \) values of <0.05 were considered to be statistically significant.

\( k_{\text{Bone}} \) was determined using a modified Patlak’s method as previously described [18]. In short, average \( k_{\text{Bone}} \) values were determined by calculating changes in area-under-the-plateau-clearance-curve (AUC) and whole-body retention (WBR) values generated for each mouse between each FMT imaging time point. Plasma concentrations and bone uptake (BU) values at each time point were determined by the formulas:

\[
[\text{plasma}] = \frac{\text{injected fluorescence (pmol)} \times \text{VOI volume (ml)}}{\text{soft tissue fluorescence (pmol)}}
\]

\[
\text{BU} = \frac{\text{bone VOI fluorescence (pmol)} \times \text{mass of mouse (kg)}}{\text{injected fluorescence (pmol)} \times \text{VOI volume (ml)}}.
\]

Results

Vertebrae bisphosphonate uptake kinetics are altered in ovariectomized and PTH-treated mice

Average fluorescence of the L1 and L2 vertebrae was quantified from injected mice and subjected to nonlinear regression analysis (Fig. 1A). Vertebral fluorescence showed significant reductions in binding plateaus, rate constants and Pw rate constants after short-term OVX when compared to control groups, with further significant reductions in long-term OVX mice (Figs. 1B–D), while PTH-treated mice showed significant increases in binding plateau, rate constant and Pw rate constant values compared to untreated OVX controls.

Fluorescent bisphosphonate binding kinetics reveal differential changes in uptake parameters in the knee and spine

To examine region-specific differences in uptake, changes in fluorescent bisphosphonate kinetic parameters obtained for the knee (as previously published [30]) and spine for each group were compared. Overall, changes in kinetic parameters were similar for both the knee and spine in response to OVX. No significant differences in percent change in plateau values were observed between short and long-term OVX, relative to control, for either the knee or the spine (Fig. 2A). In contrast, while both the spine and the knee showed significant reductions in the rate constant, the changes observed in the knee were slightly but significantly greater than those observed in the spine after long-term OVX (Fig. 2B). Both the knee and the spine showed reductions in the Pw rate constant, with significantly greater reductions after long-term OVX as compared to short-term (Fig. 2C), but no significant differences were observed between regions. In response to PTH treatment of OVX mice, clear differences were observed in the rate constant and plateau values between the knee and spine regions. The spine showed a significantly greater increase in the binding plateau, while the knee showed a significantly greater increase in rate constant values in OVX mice treated with PTH, relative to untreated OVX controls (Figs. 2D and E). Overall, no significant changes in the Pw rate constants were observed between the knee and the spine in response to PTH treatment (Fig. 2F).

To delineate the biological significance of the observed changes in binding plateau and rate constant values, micro-CT analyses were conducted to monitor changes in BMD, bone microstructure and turnover in the knee and spine. Bone loss expected as a result of OVX was confirmed by BMD assessment which showed significantly reduced values in both the spine and knee after long-term OVX (data not shown) and serum marker analyses [30]. Baseline and follow-up scans were overlaid to ensure analyses of similar regions and to visualize regions of bone formation and resorption which occurred between imaging time points and pre-OVX baseline scan (Fig. 3A). Images were used to quantify bone forming and resorbing surfaces (Figs. 3B and D) and volumes (Figs. 3C and E). Time-lapse micro-CT showed a trend towards increased resorbing surfaces (\( p = 0.0683 \)) and significantly greater resorbed bone volume in the tibia between short-term and long-term OVX. Relative to the tibia, the spine showed a trend towards an increased percent resorbing surface after short-term (\( p = 0.0926 \)) and long-term (\( p = 0.0771 \)) OVX, as well as significantly greater resorbed bone volume after short-term and long-term OVX. A two-way ANOVA was conducted to determine overall effects of skeletal site and length of OVX on time-lapse micro-CT parameters (Table S1). Neither the skeletal site (tibia vs spine), nor the duration of OVX (day 3 vs day 14) had a statistical impact on bone forming surfaces or volumes. In contrast, resorbing surfaces and volumes both showed a significant dependence on both the skeletal site assessed, as well as the duration of OVX.

Micro-CT analyses were next conducted on mice treated with PTH. Because BMD values failed to show any significant changes after 3 days of PTH treatment, an additional follow-up scan was taken after 14 days of PTH treatment, which showed a significant increase in knee BMD (data not shown) and significantly increased bone metabolic serum markers [30] and likely represents changes in bone structure which would result from the day 3 metabolic bone statuses. Baseline and follow-up scans were overlaid to ensure analyses of similar regions and to visualize regions of localized bone formation and resorption in the vertebral and tibial VOIs (Fig. 4A) and to quantify bone forming and resorbing surfaces (Figs. 4B and D) and volumes (Figs. 4C and E) which occurred relative to pre-treatment baseline scan. Both the tibia and spine showed significantly greater bone forming surfaces and volumes between early and long-term PTH treatment, with the tibia containing significantly greater forming surfaces than the spine after long-term PTH treatment. In contrast, the spine contained significantly greater bone resorbing surfaces and volumes than the tibia.
after both early and long-term PTH treatment. Two-way ANOVA analyses of time-lapse micro-CT parameters revealed that both the skeletal site and the duration of PTH treatment (day 3 vs day 14) significantly affected the bone forming surfaces, while only the length of PTH treatment affected bone formation volumes (Table S2).

**Binding kinetic parameters correlate with changes in bone formation and resorption**

In order to determine which aspects of bone formation and resorption are reflected in each bisphosphonate binding kinetic parameter, correlation studies were conducted between rate constant, plateau and Pw rate constant values determined by FMT and time-lapse micro-CT-derived bone forming and resorbing surfaces (Figs. 5A and C) and volumes (Figs. 5B and D), and their interaction assessed (Table 1). Percent forming surfaces, assessed by time-lapse micro-CT, showed significant correlations with rate constant and Pw rate constant values, assessed by FMT binding kinetics, while percent forming and resorbing bone volumes significantly correlated with plateau values.

**Soft tissue fluorescence of the abdominal region correlates with serum levels of bisphosphonate**

To assess the blood pool levels of bisphosphonate available for binding over time, abdominal soft tissue fluorescence was measured (Fig. 6A) and subjected to nonlinear regression analysis (Fig. 6B). Both short and long-term OVX mice showed significantly greater maximum tissue fluorescence than control mice, while treatment with PTH significantly reduced maximum fluorescence back to levels comparable with control mice (Fig. 6C). Ovariectomy also resulted in increased minimum fluorescence values with long-term OVX mice having significantly greater values than that of control mice. Treatment with PTH resulted in a significant decrease in minimum fluorescence values to levels consistent with control mice (Fig. 6D). Both short and long-term ovariectomy resulted in significantly decreased soft tissue clearance from control mice, while PTH treatment resulted in a significant increase in clearance rate compared to untreated long-term OVX (Fig. 6E). A standard curve (Fig. 6F) was used to calculate serum level bisphosphonates in imaged mice and compared to soft tissue fluorescence detected by FMT. A strong correlation (R² = 0.8621) was found between bisphosphonate concentrations, determined by FMT soft tissue fluorescence, and ex vivo serum analysis (Fig. 6G). Using a modified Patlak’s method, ovariectomized mice showed significantly reduced K_{Bone} values as compared to control mice in both the knee and spine regions, while PTH-treated mice showed significantly increased K_{Bone} values from untreated OVX controls (Fig. 6H). Analyses also show significantly greater K_{Bone} values in the spine for each test group relative to the corresponding knee values.

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**Fig. 1.** Ovariectomy and treatment with PTH results in significantly altered binding kinetic parameters of a fluorescently-conjugated bisphosphonate. Time resolved bone binding pattern of fluorescently-labeled bisphosphonate in L1 and L2 (A). Long-term ovariectomized mice showed significantly reduced binding plateaus (B), rate constants (C) and plateau-weighted (Pw) rate constants (D) compared to short-term OVX mice, which in turn showed significant reductions in all parameters compared to control mice, while treatment of mice with PTH for 3 days resulted in values comparable to control mice and binding kinetic parameters significantly greater than OVX mice. Dotted lines represent 95% confidence interval of fitted curve. Graphs represent average values ± SD. (**p < 0.01, ***p < 0.001) (n = 9).
Fig. 2. Spine and knee regions show differential changes in bisphosphonate binding kinetics in response to ovariectomy and PTH treatment. Analyzing changes in binding kinetic parameters in response to ovariectomy, both the knee and spine showed no significant differences in the change between short and long-term OVX (A), but did show a significant increase in rate constant values (B). Both regions showed significant changes in Pw rate constant values between short and long-term OVX mice (C). In response to PTH treatment in OVX mice, the spinal region showed significantly greater changes in plateau binding values (D), while the knee region showed significantly greater changes in rate constant values (E), relative to untreated OVX controls. Pw rate constant values showed no significant differences between the knee and spine region after PTH treatment (F). Dotted lines represent 95% confidence interval. Graphs represent average values ± SD. (*p < 0.05, **p < 0.01, ***p < 0.001) (n = 9).
Discussion

In this study, we demonstrate that fluorescent bisphosphonates show consistent patterns of binding at both the vertebra and long bones in response to OVX or PTH treatment. Furthermore, we show that changes in binding kinetic parameters were found to mirror later changes observed by micro-CT methods. These binding kinetic parameters reflect the site-specific, variations observed in response to changes in metabolic status. We have additionally shown that soft tissue fluorescence, as assessed by in vivo FMT, correlates with serum levels of free bisphosphonate and can be used as a tool to monitor the changes in blood pool levels of bisphosphonates over time. Combining our uptake parameters of both the knee and spine regions with our soft tissue blood pool measure, we have generated $K_{Bone}$ values for each mouse using a modified Patlak’s method. Values obtained from this method show significant reductions in bisphosphonate uptake by the bone in

Fig. 3. Bone loss associated with ovariectomy preferentially affects the spine. (A) In vivo micro-CT was used to analyze changes in bone microstructure in the L1 vertebrae and the tibia. Color-coded subtraction images show regions of bone formation (orange) and bone resorption (blue). Time-lapse micro-CT was used to quantify bone forming surfaces and volumes (B and C), as well as resorbing bone surfaces and volumes (D and E) at each time point relative to its baseline scan prior to OVX. The spine contained consistent levels of bone resorbing volumes after both short-term and long-term OVX, significantly greater than resorbing volumes observed in the tibia, while the tibia showed a significant increase in resorbing bone volume from short-term to long-term OVX. Two-way ANOVA analyses (Table S1) revealed that, although neither skeletal site nor duration of OVX significantly affected bone forming surfaces or volume, both bone region and length of OVX significantly affected bone resorption surfaces and volume. Graphs represent average values ± SD. (*p < 0.05) (n = 9).
Fig. 4. Treatment with PTH results in significant changes in bone microstructure. *In vivo* micro-CT of the L1 vertebra and the knee over 14 days of PTH treatment (A). Time-lapse micro-CT was used to quantify bone forming surfaces and volumes (B and C), as well as resorbing bone surfaces and volumes (D and E) at each time point relative to its baseline scan before commencement of PTH treatment. Both the tibia and spine showed significant increases in bone forming surfaces and forming volumes after prolonged PTH treatment, with the tibia containing significantly greater bone forming surfaces than the spine after long-term PTH treatment. In contrast, the spine showed significantly greater levels of bone resorbing surfaces and volumes compared to the tibia. Two-way ANOVA analyses (Table S2) revealed that while only duration of PTH treatment affected bone formation volumes, both skeletal site and treatment length significantly affected bone forming surfaces. In regard to bone resorption, only skeletal site, and not duration of PTH treatment, was found to significantly affect resorbing volumes or surfaces. Graphs represent average values ± SD (*p < 0.05, **p < 0.01, ***p < 0.0001) (n = 9).
response to ovariectomy and significant increases in bone uptake after PTH treatment.

As has been previously described for the knee region, short-term ovariectomized mice showed significantly reduced plateau, rate constant and \( P_w \) rate constant values compared to control mice, with further significant reductions observed in all parameters after long-term OVX in the spine. Conversely, treatment with PTH was found to significantly increase all binding parameters compared to untreated controls. Based on micro-CT morphometry, combined with the observed changes in binding kinetics of the spine and knee region in response to OVX and PTH treatment, bone surface and metabolic status play an important role in both plateau and rate constant binding parameters. In response to treatment with PTH, we observed a preferential increase in rate constant within the knee and binding plateau within the spine. This corresponded to preferential increases in bone forming surfaces in the knee and elevated levels of bone resorption surfaces and volumes in the spine. This data suggests a relationship between rate constant and plateau values and changes in metabolic activity which ultimately gives rise to bone loss/apposition. Though rate constant and plateau values are interdependent, correlation studies with time-lapse micro-CT, which assesses localized changes in bone microstructure [12,15], support a preferential interaction between bone apposition surfaces and rate constant values, as well as bone loss and apposition volumes and plateau values. The fact that binding kinetics in the knee and spine differ in response to PTH treatment suggest that this kinetic monitoring has the ability to distinguish between these metabolically distinct sites with a high degree of sensitivity. It is important to note that, because micro-CT provides only a snap shot of bone structure, time-lapse micro-CT analyses may underestimate the amount of bone forming volumes and surfaces in regions where new bone formation occurs over top of resorbed areas, but has not deposited sufficient mineral to overcome the mineral lost by previous resorption. Histological confirmation will be required to correlate metabolically actively forming and resorbing surfaces with bisphosphonate uptake. Nevertheless, micro-CT has previously been shown to correlate with dynamic histomorphometry in mice [28].

Previous works have demonstrated a preferential uptake of bisphosphonates by newly mineralized bone, and by active remodeling surfaces compared to quiescent bone surfaces [27,30]. Taken together, we propose a hierarchical model of bone bisphosphonate uptake from high uptake, newly mineralized bone, to medium uptake, newly exposed/resorbing bone, to low uptake, quiescent bone surfaces. Using these criteria, treatment with PTH results in increased high uptake bone surfaces within the knee region, resulting in rapid uptake shortly after injection, while the spine shows increased accumulation of both high and medium uptake bone, resulting in increased uptake rates and plateau values. While the amount of high uptake, newly mineralized bone is dependent on the osteoblast activity, the amount of newly exposed mineral will be dependent on the osteoclasts and will be affected both by the number of osteoclasts as well as the depth of the resorbing pits. Though the preferential uptake of bisphosphonates by newly deposited minerals has previously been suggested to reflect the presence of amorphous, higher affinity calcium phosphate present in newly forming bone as compared to the lower affinity crystalline minerals present in fully mineralized bone [8,30], both quiescent and resorbed surfaces are comprised of fully mineralized bone. Reduced exposure and/or binding of the bone mineral, potentially limited by bone lining cells or secreted extracellular matrix components present on quiescent

![Figure 5](image.jpg)

**Figure 5.** Linear correlations between binding kinetic and time-lapse micro-CT parameters. Binding kinetic parameters, assessed by in vivo FMT, for both the spine and knee regions, were subjected to linear regression analyses with \% forming (A) and resorbing (C) surfaces and \% forming (B) and resorbing (D) volumes as assessed by time-lapse micro-CT.

<table>
<thead>
<tr>
<th>Rate constant (k)</th>
<th>Plateau (μmol)</th>
<th>( P_w ) (μmol/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>% forming surface</td>
<td>0.05/0.0174</td>
<td>ns/ns</td>
</tr>
<tr>
<td>% resorbing surface</td>
<td>ns/NS</td>
<td>ns/NS</td>
</tr>
<tr>
<td>% forming volume</td>
<td>ns/NS</td>
<td>0.05/0.0430</td>
</tr>
<tr>
<td>% resorbing volume</td>
<td>ns/NS</td>
<td>0.01/0.0066</td>
</tr>
</tbody>
</table>

Table 1

Rate constant values reflect changes in forming surfaces while plateau values reflect changes in bone formation and resorption volumes. Linear correlations between binding kinetic and time-lapse micro-CT parameters shown in Fig. 5 were assessed for their interaction. Values represent: Significance of the linear correlation/probability that slopes are equal to zero. ns = not significant.
bone surfaces but not on resorbing surfaces, may explain their differential uptake of bisphosphonates from the blood. It has also been suggested that crystal size inversely correlates with the specific area available for bisphosphonate binding, with newly deposited minerals being smaller than those found in fully mineralized bone [10,27]. As a result, high turnover regions will also be associated with smaller crystal sizes, greater specific area available for binding, and subsequently, higher bisphosphonate uptake than low turnover bone.

Finally, we have demonstrated that time-related reductions in bisphosphonate fluorescence from within the abdominal soft tissue show significant reductions in both the extent and rate of clearance in response to OVX and significantly greater clearance kinetics in PTH-treated mice compared to untreated controls. These results are consistent with the observed changes in bisphosphonate bone uptake in that decreased bone uptake, in the case of OVX, would result in delayed blood clearance while increased bone uptake, observed in PTH-treated mice, would result in increased blood pool clearance. We also show a strong correlation between soft tissue fluorescence assessed by in vivo FMT and serum levels of free bisphosphonate. Using soft tissue fluorescence as a blood pool correction, modified compartment model Patlak’s calculations were conducted to assign quantitative values of bisphosphonate bone uptake. Results show that ovarectomy gives rise to significant reductions in \( K_{bone} \) values while treatment with PTH shows significant increases in this parameter. Additionally, Patlak’s calculations also showed significantly greater \( K_{bone} \) values for the spine in each test group relative to corresponding values observed in the knee, similar to results observed in humans using 18F-fluoride PET comparing the vertebra to the humerus [6]. This has previously been explained by greater tracer delivery to the spine, accounted for by greater regional blood flow [22]. Because these values are derived from both blood and bone values, \( K_{bone} \) values reflect the accumulation of bisphosphonates on the bone adjusted for the clearance of available unbound probe from the blood and thus, \( K_{bone} \) values should better approximate changes in bone metabolism. In our models of relatively short term OVX or treatment with PTH, minimal changes in body weight, renal function or blood flow are expected to occur, minimizing the overall benefit received with this modified multi-compartment analysis. However, while the resolution of FMT does not permit the evaluation of site-specific blood flow, this general blood pool correction factor should prove useful in mouse models in which significant changes in body mass or renal function occur, such as the case in tumor models and some therapeutic treatments, and help overcome some bias in bone binding kinetics resulting from the uptake of bisphosphonates by other skeletal sites as has previously been observed in treated patients [2].

It remains unclear why micro-CT results demonstrated a greater response to PTH in the knee compared to the spine. Previous works have suggested competing perspectives on the effects of PTH treatment on spine and tibia bone mineral changes [9,33]. It is possible that because of the site of injection and the dosing administered in this study, bone changes in the spine reflect more a continuous PTH-dosing model in which the anabolic effects are replaced with significant upregulation in bone remodeling with no net bone mineral increase [16]. Other works have also suggested that different skeletal sites may be comprised of functionally distinct cell populations which respond at different rates and to different extents to anabolic therapy [23]. This idea is supported by the fact that, while both the knee and spine regions show relatively similar increases in bone forming surfaces and volumes, relative to OVX controls, the knee shows substantially greater reductions in bone resorbing volumes and surfaces than were observed in the spine. As a result, the overall bone metabolic activity (bone formation and resorption) is greater in the spine than the knee. This is consistent with micro-CT and \( K_{bone} \) values in which the spine shows significantly greater bone turnover (forming and resorbing bone volume) and greater uptake of bisphosphonate relative to the knee region. It has also been noted that PTH treatment tends to enhance bone formation at sites of stress [26] and this effect may be mediated through sclerostin [21]. Nude mice used in this study are also T cell-deficient, which have previously been shown to play a role in stimulating both osteoclastogenesis and osteoblast differentiation [4,19,25,31]. It would be of great interest to determine which, if any, of these factors contribute to the differential responses observed to PTH treatment between the knee and the spine in this study and other studies published in mice, rats and humans. It is also possible that, because kinetic analyses were done after a relatively short time period after the beginning of PTH treatment, the knee region, and predominantly the actively-modeling trabecular regions distal to the growth plate, are more rapidly responsive and thus, show more significant changes in BMD values relative to the spine.

Overall, these methods show great utility in using bisphosphonate binding kinetics and time-lapse micro-CT as in vivo tools for assessing metabolic activity in a range of potential bone-loss and bone-gain models. We were able to show bone metabolism, as assessed by binding kinetics, varies between skeletal sites. We also provide evidence that these differences may be due to changes in bone surface properties, as illustrated by correlation with time-lapse micro-CT analyses. Both methods were also able to distinguish differential responses to bone anabolic treatment suggesting utility in monitoring site-specific responses to novel therapeutic interventions. With the addition of a blood pool correction and multi-compartment analyses, the binding kinetics assay now accounts for biases introduced by off-site uptake (both soft tissue and other, non-measured skeletal sites) as well as conditions which may result in delay clearance of free bisphosphonates from the blood pool (i.e. renal impairment).

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Fig. 6. Abdominal soft tissue fluorescence correlates with serum bisphosphonate levels and shows altered clearance in OVX and PTH-treated mice. Soft tissue fluorescence of reconstructed FMT images was quantified in regions devoid of bone (A) and subjected to nonlinear regression analysis (B). OVX resulted in significantly increased maximum (C) and minimum (D) soft tissue fluorescence compared to control mice, while treatment with PTH significantly reduced maximum and minimum fluorescence compared to untreated OVX controls. OVX also resulted in significantly decreased clearance rates of soft tissue fluorescence while treatment with PTH significantly increased the clearance rate of soft tissue fluorescence relative to untreated OVX controls. (F) A standard concentration curve of fluorescent bisphosphonates in serum was generated and used to quantify serum levels of bisphosphonate in intravenously injected mice. Fluorescence in the soft tissue region, determined by FMT imaging, and serum levels of fluorescent bisphosphonate show a strong correlation between soft tissue fluorescence and actual serum levels of bisphosphonate (G). Using a modified Patlak’s method (H) analysis shows significant reductions in \( K_{bone} \) values in response to OVX and significant increases in response to PTH treatment for both the knee and spine regions (*). Analyses also show significantly greater \( K_{bone} \) values for the spine relative to knee values for each group (#). Dotted lines represent 95% confidence interval. Graphs represent average values ± SD, (*p < 0.05, **p < 0.01, ***p < 0.001) (n = 9).
References


