

## REVIEW

# Histomorphometric interpretation of bone biopsies for the evaluation of osteoporosis treatment

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**Bone histomorphometry is a valuable tool in the evaluation of bone safety and the mechanism of action of drugs used in the treatment of osteoporosis. Recent studies in patients treated with anti-resorptive agents have highlighted technical issues, in particular, related to the calculation of dynamic indices of bone turnover using fluorochrome labelling. This review addresses the need for standardised approaches for overcoming these problems in order to enable valid comparison of the effects of different interventions on bone remodelling.**

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### Introduction

Histological examination of bone biopsies provides unique information about the safety and mechanism of action of drugs used in the treatment of osteoporosis. The available options for treatment, and some of those currently undergoing development, have diverse effects on bone remodelling that may impact both on safety and efficacy. This review addresses the use of bone histomorphometry in evaluating the effects of non-anabolic agents on bone remodelling, and summarises the data available for different interventions.

### Strengths and Limitations of Bone Histomorphometry in the Evaluation of Treatment Effects

Histomorphometric assessment of bone biopsy sections provides detailed information about a variety of indices related to bone remodelling and structure. Some of these cannot be evaluated by other approaches, for example, active bone formation, cell number and morphology and remodelling balance. In addition, the presence of lamellar and woven bone and of subtle mineralisation defects requires direct examination of bone histology.

Nevertheless, there are limitations. The procedure of bone biopsy samples only a small amount of bone at a site that may not be fully representative of bone elsewhere, for example, in the spine and proximal femur. The inter-observer and intra-observer variations in measurement are considerable, in part, because there is a subjective element to most of the measurements

performed, even when these are done on sophisticated image analysis systems.<sup>1,2</sup> Performance of bone histomorphometry is labour intensive and the sample size is further constrained by the reluctance of some subjects to undergo a biopsy. Although paired biopsy samples (before and after treatment) provide the ideal design, a single biopsy design of post-treatment samples may provide the most cost-effective approach.<sup>3</sup> The timing of post-treatment biopsies depends on whether the main outcomes of interest reflect early transient, or steady state changes. For investigation of the latter, treatment periods in excess of 3 years may be required.

### Assessment of Bone Safety

Assessment of bone safety using histological examination of bone biopsies is a regulatory requirement for all approved agents. Adverse effects include the presence of woven bone, osteomalacia, marrow fibrosis, or malignant transformation of marrow cells. These can be documented using qualitative assessment of bone biopsies. The uptake of tetracycline labels is also an important component of bone safety.

The optimal timing of bone safety studies with respect to duration of treatment depends on the main outcomes of interest. For example, anabolic agents might result in the early formation of woven bone, with subsequent conversion by remodelling to lamellar bone. To detect this, biopsies should be obtained in the early months of treatment. However, for most safety outcomes and for the purposes of regulatory requirements examination of biopsies obtained after several years of treatment is accepted practice.

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## Assessment of Effects on Bone Remodelling

**Bone turnover.** Bone turnover is defined as the amount of bone removed and replaced in a given volume in a given time. It is determined both by the number of remodelling units and the focal balance within individual remodelling units. Following double tetracycline labelling, the surface extent of tetracycline fluorescence, or mineralising surface (MS/BS), indicates sites of active bone formation and is used as a measure of remodelling rate based on the assumption that there is temporal and spatial coupling of resorption and formation.<sup>4</sup> Bone formation rate (BFR) is calculated as MS/BS multiplied by the mineral apposition rate (MAR); the latter is estimated from the distance between double tetracycline labels divided by the number of days between administration of the two labels. Activation frequency, which is also used as an index of remodelling rate, is calculated as BFR divided by wall width, based on the assumption that remodelling rate and balance are co-regulated.<sup>5</sup> However, this assumption has been challenged and MS/BS provides a more reliable measure of remodelling rate.<sup>6</sup>

All interventions currently used in the treatment of osteoporosis have effects on bone turnover, and reduction in the remodelling rate is the predominant effect of anti-resorptive drugs such as the bisphosphonates and denosumab. Because of potential concerns about long-term suppression of bone turnover, much attention has focused on the degree of suppression induced by different anti-resorptive agents. However, there have been few head-to-head studies of different anti-resorptives and comparison between agents is further complicated by non-uniformity in the reporting and analysis of tetracycline labelling-based data.

In the majority of bone biopsies from normal subjects both double and single tetracycline labels are seen in cortical and trabecular bone following standard labelling regimens. With increasing suppression of bone turnover, the amount of label decreases and variable percentages of biopsies from treated patients may show little or no label. This may not only reflect true absence of bone turnover but may also arise because the sampling procedure fails to detect labels that are present elsewhere in the biopsy or because of failure of the patient to take or absorb the tetracycline; in this context it should be noted that labels have not been detected in all placebo-treated women in some studies.<sup>7,8</sup> In the absence of label, MS/BS could either be treated as a missing value or included as a zero value in the calculation of the mean MS/BS value.<sup>9</sup> In situations in which a high proportion of biopsies do not have detectable label, exclusion of these biopsies from calculation of the mean MS/BS will generate considerably higher values for mean MS/BS than if they are included. However, if zero values are included in the calculation then the mean value may be underestimated by an unknown amount because of the likely presence of active bone formation, albeit not detected for the reasons noted above, in some biopsies. There is no clear consensus on which approach should be adopted but if the aim is to assess the magnitude of reduction in remodelling rate, inclusion of zero values in the calculation is a more rational approach.

There is also some inconsistency in the qualitative reporting of tetracycline labelling in bone that may be relevant to the quantitatively assessed data. In some studies, the presence of double and single label is not clearly distinguished, labelling

simply being described as present or absent. Furthermore, labelling in cortical and trabecular bone are not always separately described. In most reported studies of anti-resorptive drugs, histomorphometric analysis of bone remodelling (although not structure) has been restricted to trabecular bone, and as drugs may have differential effects in cortical and trabecular bone it is important to distinguish clearly where label is taken up. From the available data it appears that labelling is more commonly reported in cortical than trabecular bone in biopsies from patients exposed to potent anti-resorptive drugs.<sup>7,10</sup> Cortical label should not be used to derive dynamic indices in trabecular bone in biopsies where trabecular label is absent because of the differences in remodelling between the two bone compartments.

MAR can only be measured when double tetracycline labels are present. However, apposition may also be occurring in biopsies in which only single label is present because of intermittent pauses in remodelling (the 'on-off' phenomenon), label escape due to formation starting before the first label is given or completed in the period between administration of the two labels, sampling variance or other technical issues. On the basis of the frequency distribution of average values for MAR in biopsies from normal subjects and women with postmenopausal osteoporosis, Foldes *et al.*<sup>11</sup> suggested that MAR has a lower finite value of 0.3  $\mu\text{m}$  per day and that this value should be imputed if only single labels are present. Alternatively, Hague *et al.*<sup>9</sup> recommended that the value imputed in such cases should be 0.1  $\mu\text{m}$  per day, which corresponds to the minimum MAR in individual biopsies. Where no label is present in the biopsy, MAR could be treated as missing data<sup>11</sup> or assigned a value of zero and either included in the calculation of mean values or reported separately.<sup>9</sup> As in the case of MS/BS, if zero values for MAR are excluded and/or biopsies with single label only are treated as missing data, the mean value will be overestimated.<sup>12</sup>

The approach taken for reporting and estimating MS/BS and MAR in biopsies with low turnover has an important impact on other derived dynamic variables. In particular, exclusion of zero values for MS/BS and MAR and/or failure to impute values for MAR in biopsies with single label only will result in higher mean values for BFR and activation frequency. These issues have been highlighted by recent studies of potent anti-resorptive agents, such as zoledronic acid and denosumab, in which label was not detected in a significant proportion of biopsies.<sup>7,10</sup>

**Remodelling balance.** The focal balance between bone resorption and formation in the individual bone multicellular unit is assessed from measurements of erosion depth and mean wall width. Erosion depth is difficult to measure accurately because of technical issues and uncertainty about when resorption has been completed.<sup>13–16</sup> Wall width measurements are more robust, but because of the length of time required for completion of individual remodelling units and difficulties associated with identifying recently formed bone, treatment-induced changes in wall width may not be evident for several years.<sup>17,18</sup> However, in the presence of bone turnover suppression, especially that induced by the more potent anti-resorptive drugs, effects on remodelling balance are unlikely to be significant given the very small number of remodelling units present. In contrast, if the remodelling rate is increased as, for example, in response to parathyroid hormone peptides, effects of changes in remodelling balance on bone mass will be more prominent.<sup>19</sup>

## Effects of Anti-resorptive Agents on Bone Remodelling

**Bone turnover.** Decrease in MS/BS, BFR and activation frequency has been reported in biopsies from patients treated with bisphosphonates, oestrogen, raloxifene and denosumab.<sup>7,8,10,20–28</sup> Although head-to-head comparisons are not available for all drugs, it appears that denosumab and zoledronic acid produce the greatest suppression of remodelling. In studies of risedronate, ibandronate, and pamidronate double labels have been reported in all treated patients or in a similar proportion to placebo-treated women, sometimes after the examination of additional sections. Tetracycline labels were also observed in similar proportions of women treated with alendronate or placebo, although it was not stated whether this was double or single labelling. For zoledronic acid and denosumab, more detailed information is available. Of 59 women treated with zoledronic acid for 3 years, double label was present only in cortical bone in 16 and single label only in cortical or trabecular bone in 3, whereas in 52 placebo-treated women double label was present in cortical and trabecular bone in all biopsies except two, in which it was present only in cortical bone.<sup>10</sup> Histomorphometric data from the FREEDOM and STAND studies<sup>7</sup> revealed that label (either single or double) was absent from trabecular and cortical bone in 66% and 43%, respectively, in women treated with denosumab for 24 or 36 months. In women who had transitioned from alendronate to denosumab, the corresponding figures after 1-year treatment with denosumab were 40% and 27%, respectively. In contrast, label was present in all women in the STAND study who continued to take alendronate (total duration at least 18 months), 90% showing double label in trabecular bone and 100% in cortical bone. Interestingly, in this study serum levels of bone formation markers (bone-specific alkaline phosphatase and P1NP) were no lower in subjects without tetracycline label than in those with double label, suggesting that the bone sampled for the biopsy may not be representative of bone turnover in the entire skeleton.

Differences between drugs in the extent of tetracycline labelling are supported by the reported reductions in BFR and activation frequency. Thus, after 3 years of zoledronic acid treatment, the median activation frequency was reduced by 67%,<sup>10</sup> whereas 24 or 36 months treatment with denosumab was associated with a 97% reduction in BFR;<sup>7</sup> it should be noted that these figures were derived after exclusion of biopsies without double label in trabecular bone. In biopsies from women treated with risedronate, ibandronate, hormone replacement therapy or raloxifene, the reported median reductions in activation frequency have been around 40–50%.<sup>21,22,24–28</sup> However, in the study of Chavassieux *et al.*,<sup>8</sup> despite the presence of label in similar proportions of alendronate and placebo-treated women, reductions in activation frequency at 24 and 36 months were 88% and 93%, respectively, in the active treatment group.

Evidence supporting the reversibility of changes in bone remodelling after cessation of denosumab has recently been reported in a cross-sectional study of 15 postmenopausal women who had discontinued treatment for 21–29 months. Double tetracycline labelling was present in cortical and cancellous bone in 93% and 87% of biopsies, respectively, and indices of resorption and formation were similar to those of an untreated group of women.<sup>29</sup> However, definitive evidence of

reversibility requires paired biopsy studies in which bone samples are obtained during treatment and following withdrawal.

**Remodelling balance.** The decrease in BFR induced by anti-resorptive drugs predominantly results from a decrease in remodelling rate. Although remodelling balance changes are less certain, a non-significant reduction in erosion depth was reported in biopsies from women treated with oestrogen<sup>26</sup> or risedronate,<sup>21</sup> whereas wall width was unchanged, suggesting a small improvement in remodelling balance. Data on erosion depth are not available for other bisphosphonates, and significant changes in wall width have not been reported. However, as noted above, the duration of treatment required to detect significant changes in wall width may extend to more than 3 years, particularly in the presence of a low remodelling rate. Measurement of MAR may provide earlier clues as to the effects of drugs on osteoblast activity. No consistent effect of bisphosphonates on MAR has been reported in humans; a significant increase was observed in women treated with zoledronic acid but the value achieved is likely to have been overestimated because of exclusion of biopsies with single label only from the analysis.<sup>10,12</sup> In women treated with denosumab for 24 or 36 months, MAR was significantly lower than in the placebo-treated group, but measurement was only possible in seven biopsies in the treatment group; a value of 0.3  $\mu\text{m}$  per day was imputed in five of these biopsies because only single label was present.<sup>7</sup>

The effects of anti-resorptives on remodelling balance thus remain to be clearly established. It would not be surprising if they reduced erosion depth and this merits further exploration. The transient increase in parathyroid hormone production following dosing of an anti-resorptive provides a possible biological mechanism for increased osteoblast activity and increased bone formation at bone multicellular unit level.<sup>30</sup> Direct evidence for such an effect is lacking, but the inverse relationship between the magnitude of increase in parathyroid hormone and cortical porosity demonstrated in women treated with denosumab or alendronate provides some indirect evidence.<sup>31</sup> However, although the effects of anti-resorptives on remodelling rate in cortical bone have not been specifically investigated, in trabecular bone the effect of a positive remodelling balance would be small because of the accompanying reduction in remodelling rate, particularly for the most potent anti-resorptive drugs.

**Effects on osteoclast number and eroded surface.** Although the nitrogen-containing bisphosphonates are widely thought to increase osteoclast apoptosis, this is not a prerequisite for inhibition of resorption,<sup>32</sup> and studies in animals or humans treated with these drugs do not show the expected reduction in osteoclast number.<sup>8,23,33,34</sup> Weinstein *et al.*<sup>34</sup> reported an increase in number of osteoclasts in women treated with alendronate, with a positive association between osteoclast number and cumulative dose. Some of the osteoclasts present were of normal appearance but just over 25% were giant cells with pyknotic nuclei that appeared to be undergoing protracted apoptosis. Although lying adjacent to resorption cavities these cells were detached and did not appear to be involved in active resorption. Further evidence that the increased number of osteoclasts present in subjects treated with nitrogen-containing bisphosphonates do not resorb bone normally is provided by the lack of any significant change in eroded surface reported in most studies.<sup>10,19,21–23</sup> Whether the osteoclasts present in

bisphosphonate-treated bone retain some or all of their signalling functions is unknown. In contrast, decrease in osteoclast number has been reported in women treated with raloxifene and oestrogen,<sup>27</sup> whereas denosumab therapy was associated with absence of osteoclasts in over 50% of biopsies.<sup>7</sup>

**Strontium ranelate.** In animals there is some evidence that strontium ranelate inhibits bone resorption and stimulates bone formation,<sup>35,36</sup> although this finding has not been universal.<sup>37</sup> In postmenopausal women treated with strontium ranelate, however, only weak effects on bone remodelling have been demonstrated. Arlot *et al.*<sup>38</sup> studied biopsies obtained from 141 postmenopausal women assigned to strontium ranelate or placebo, including 8 women who underwent paired biopsies. Pooled data for biopsies obtained after 1–5 years treatment with strontium ranelate were compared with pooled data from the placebo group over the same time period together with baseline data from the group assigned to strontium ranelate. No significant difference was seen in MS/BS, BFR, activation frequency, number of osteoclasts, osteoclast surface or eroded surface in either trabecular bone alone or combined trabecular + endocortical bone between the treatment and control group. However, MAR in trabecular (but not in combined trabecular + endocortical bone) was slightly but significantly higher in the treatment group (median 0.62 vs 0.57  $\mu$  per day;  $P=0.019$ ) and osteoblast surface was significantly higher in trabecular + endocortical bone (median 5.59 vs 3.55%;  $P=0.047$ ), but not in trabecular bone alone.

Although the authors concluded that these results were consistent with a stimulatory effect of strontium ranelate on bone formation, the lack of consistency in the differences in MAR and osteoblast surface in combined endocortical + trabecular bone and trabecular bone alone together with the lack of difference in MS/BS in either trabecular or combined trabecular + endocortical bone suggests that the effect, if any, is small. Furthermore, the use of placebo biopsies obtained over the course of 5 years is a possible confounder, as age-related changes may have contributed to the differences. A more valid analysis would have been comparison of baseline and post-treatment changes in the strontium ranelate-treated group or of treatment and placebo biopsies at each time point. In a comparative study of the effects of teriparatide and strontium ranelate on bone formation after 6 months of treatment, Recker *et al.*<sup>39</sup> reported values for MS/BS and osteoblast surface within the normal range in strontium ranelate-treated women. Finally, a large biopsy study so far reported only in abstract form compared the effects of alendronate and strontium ranelate on bone formation in postmenopausal women at 6 and 12 months.<sup>40</sup> Although this was a paired biopsy study, baseline values were not reported. At both time points MS/BS and BFR were significantly lower in alendronate-treated women but the values obtained in strontium ranelate-treated women were within the normal range, indicating, as expected, greater suppression of bone turnover with alendronate than with strontium ranelate but not providing convincing evidence for a stimulatory effect of the latter on bone formation. MAR at 6 and 12 months was significantly higher in women treated with strontium ranelate than in those treated with alendronate; however, the approach taken to calculating mean values in women with no label or single label only (likely to be greater in the alendronate-treated group) was not stated. Overall, therefore, the results of these

histomorphometric studies are in accordance with the small and inconsistent changes in biochemical turnover markers demonstrated in several studies<sup>41–43</sup> and suggest that the effects of strontium ranelate on bone strength are likely to be mediated by mechanisms other than changes in bone remodelling.<sup>44,45</sup>

## Conclusions

Histomorphometric analysis of bone biopsies provides valuable information about how drugs used in the treatment of osteoporosis affect bone remodelling. Of the anti-resorptive interventions studied, denosumab produces the greatest suppression of bone turnover followed by zoledronic acid and alendronate. Effects on remodelling balance have not been convincingly demonstrated. For strontium ranelate only weak effects on bone remodelling have been demonstrated and robust evidence for an anabolic effect in humans is lacking.

Comparison of the effects of different interventions on bone remodelling is difficult because of the scarcity of head-to-head studies, inconsistencies in the qualitative reporting of tetracycline labelling, and different handling of quantitative data when no label or only single label is present.<sup>46</sup> The presence of tetracycline label should be separately reported for single and double labelling both in trabecular and cortical bone, and the number of sections examined to detect labelling should be stated. In the absence of detectable label, zero values may either be included in calculation of the mean values for MS/BS and MAR or reported separately and excluded from estimation of mean values. The latter approach avoids possible bias from having a greater number of values for MS/BS than for MAR but is likely to lead to overestimation of mean values in non-excluded biopsies. Presentation of data both with and without zero values provides the most complete information and should become standard procedure to understand and compare the effects of bone active drugs in a better manner.

## Conflict of Interest

The author declares no conflict of interest.

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