# BASIC RESEARCH ARTICLE

# Part I: Metabolic bone disease: histomorphometry as a diagnostic aid

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# **Abstract**

Metabolic diseases affecting bone are generally diagnosed late when patients present clinically with end stage skeletal debilitation. This is the result of their subclinical progression and the lack of unequivocal biochemical-or radiological techniques to identify bone changes at an early stage. This manuscript presents histomorphometry as a quantifiable method for the accurate assessment of metabolic bone disease at cellular level.

## Introduction

Factors impacting on normal generalised skeletal growth and maturation are conveniently grouped as metabolic diseases of bone. These systemic states, which are discussed in greater detail in Part II (which will be published in a future issue of SAOJ), are unrelated, complex and involve genetic factors, nutrition, hormones, physical activity, lifestyle, environmental variables and organic health. Although most manifest with late stage skeletal debilitation, the aetiology may be associated with clinical signs not related to the skeleton. For instance, hypercortisonism may manifest with a characteristic fat distribution, moon-face and abdominal striae before symptoms related to bone occur. Several lifestyle factors, which are discussed in Part II, may alert a clinician to the potential of skeletal wasting long before the patient presents with fractures of long bones or other late skeletal manifestations of metabolic bone disease.

Although it is beyond the scope of this article to present a review on skeletal growth and maturation, a basic understanding of skeletal changes in a healthy subject throughout life is pivotal in order to appreciate the impact of metabolic diseases on bone. During normal

skeletal ageing, between 60% and 80% of variation in bone mass at any age is genetically determined and lifestyle factors determine the remaining (these factors will be discussed in Part II). With the advent of puberty and under the influence of anabolic gonadal and growth hormones, the mass of the skeleton increases about three-fold over a few years.1 Peak skeletal bone mass is achieved between 25 and 35 years of age.2 It is generally agreed that the height of this peak will determine the fracture susceptibility during later life as a gradual decline of bone occurs with advancing age, despite optimal metabolic and environmental conditions. This decline in bone mass is aggravated in females by the rapid reduction of gonadal hormones after menopause, an important factor contributing to their greater susceptibility to fractures in old age.

The organic matrix of all types of bone is generally referred to as osteoid and only after mineralisation thereof can it be designated as bone. The formation of osteoid and the complex mineralisation process are mediated by osteoblasts and can only be viewed in non-demineralised resin embedded microscopic sections. Active osteoblasts are plump and arranged in a linear fashion on the metabolically active bone surface.

The volume and nature of osteoid is a reflection of the effi-

ciency of osteoblastic recruitment and function and are important variables in the establishment of a diagnosis and prognosis of a patient suffering a generalised bone state. Mineralisation of osteoid proceeds in a linear pattern. The mineralisation line is present at the osteoid-bone interface and can either be sharp (indicating slow mineralisation activity) or broad (indicating enhanced mineralisation activity). The initial bone formed is of a woven type and the complex process of lamellation that follows is still shrouded in much controversy.3 Woven bone generally constitutes less than 10% of any bone surface area in a microscopic section. The arrest of bone maturation in the woven stage is indicative of disease. Bone is continuously metabolised through a system of interconnected osteocytes and osteoclasts. Cortical and trabecular (or cancellous) bone are two distinct metabolic compartments which are metabolised differently. Cortical bone constitutes 85% of the skeletal mass and is metabolised externally through the periosteum and internally through an extensive network of Haversian and Volkmann's canals. Trabecular bone, constituting the remaining 15 %,4 is enveloped in fat and haemopoietic marrow, avascular internally and remodelled on its surface. Resorption by osteoclasts, whose recruitment and activities are controlled among others by parathyroid hormone (PTH) under the influence of serum calcium, creates a shallow depression (referred to as a Howship's lacuna) on the surface of bone. The number and distribution of osteoclasts on bone surfaces vary in different disease settings. During several metabolic bone disease states their resorptive activity could either lead to the thinning of bony trabeculae or the removal of complete trabeculae, the latter often associated with compensatory thickening of the remaining trabeculae.5 Pathological conditions in which osteoid covers more than 90% of all bone surfaces may lead to a hypocalcaemic crisis6 as osteoclasts recruited through the release of PTH may have insufficient metaboliseable bone surfaces to resorb. Under normal metabolic circumstances the osteoclasts are shed from the Howship's lacunae and the lacunae subsequently fill up with osteoid deposited by newly recruited osteoblasts. The reparative osteoid is separated from residual bone by a cemental line and follows through the processes of mineralisation and ultimately lamellation. The number of vacant Howship's lacunae is indirectly related to the recruitment of new osteoblasts - the more effective the osteoblastic recruitment, the less empty lacunae are counted microscopically on bone surfaces (Figure 1). Cemental lines within bone, as well as fibrous marrow scars, provide valuable information on past sites of osteoclastic activity. The interaction between osteoblasts and osteoclasts is referred to as bone remodelling, and the efficiency thereof is important in maintaining serum calcium concentrations and structural integrity of the skeleton. Under physiological circumstances, bone cell differentiation and activities of both osteoblasts and osteoclasts are linked and mediated through complex signalling pathways<sup>7</sup> (Figure 2).

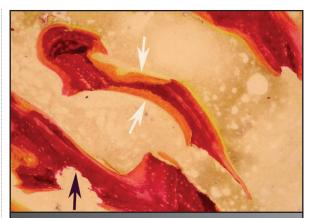


Figure 1: Microscopic view of hypermetabolised trabecular bone. Note the empty resorptive facet devoid of osteoclasts indicating a suspension of osteoclastic activity and lack of osteoblast recruitment (black arrow). The white arrows indicate resorptive facets filled with reparative osteoid, which stains light pink indicating successful osteoblastic recruitment in these foci (non-demineralised section, Picrosirius stain ×100)

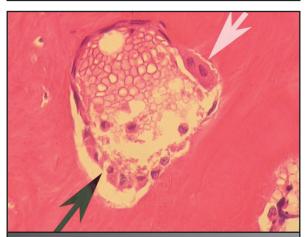


Figure 2: Micrograph demonstrating plump osteoblasts (black arrow) and one osteoclast in a shallow Howship's lacuna (white arrow) in woven bone (non-decalcified section, H&E stain, magnification ×200)

Homeostasis between osteoblasts and osteoclasts is such that both upregulation (hyperdynamic bone states) and downregulation (hypodynamic bone states) of their activities lead to loss of mineralised bone. Skeletal metabolism is however not as simple as depicted above. Other mechanisms of bone resorption include osteocytic induced osteolysis and delitescence, or bone loss not related to osteoclastic activity from quiescent lamellar bone surfaces. The remodelling of bone is sensitive to, among others, the provision of nutrients, anabolic hormones, organic disease and several lifestyle factors that will be discussed in greater detail in Part II.



Figure 3: Radiograph of an 8 mm trephine from the iliac crest. Note the soft tissue on the external cortical plate



Figure 4: Non-decalcified section demonstrating the outer cortical plate (left), trabecular bone and inner cortical plate (non-decalcified H&E stain, magnification ×10)

# Monitoring of bone metabolism

It is not the purpose of this article to provide a detailed account of biochemical and radiological techniques employed in the study of bone metabolism. However, limitations in the interpretation of biochemical markers for bone metabolism often negate early diagnosis and frequently complicate the establishment of a final diagnosis and a treatment plan in patients suffering from metabolic bone disease. These markers are generally assessed on peripheral body fluids (serum and urine) which are subjected to the efficiency of metabolic pathways and modifications related to volume distribution. The minimum recommended biochemical tests advised are thyroid function tests, s-PTH, s-total protein, s-albumin, s-calcium (both ionised and total), s-phosphate, s-alkaline phosphatase, s-magnesium, u-albumin, u-calcium, u-phosphate and u-phosphate excretion index. The availability of s-osteocalcin concentrations and urinary metabolites of bone collagen are often contributory to the diagnosis and management of a case. These tests exclude those that may be dictated by specific clinical situations (e.g. blood glucose concentrations, cortisol, gonadal hormonal status, growth hormone, renal function tests and others). Neither serum alkaline phosphatase (for the monitoring of osteoblastic bone formation) nor urinary hydroxyproline (for osteoclastic bone resorption) are specific to bone metabolism and neither can detect small changes in bone turnover.

Table I: Terms, definitions and reference values <sup>14</sup>			
Measurement	Abbreviation	Description	Ref value (SD)
Static histomorphometry: Indices of osteoid			
Trabecular osteoid surface Trabecular osteoid volume Mean osteoid seam width	TOS TOV MOSW	% Bone surface covered by osteoid Osteoid expressed as % TBV Average width of trabecular osteoid seam	18.9% (5.0) 1.9% (0.4) 9.7 microns (0.4)
Static histomorphometry: Indices of bone mass			
Trabecular bone volume	TBV	% Medullary cavity occupied by mineralised and non-mineralised bone	22.5% (3.5)
Mean trabecular width Mean cortical width MCW	MTW Mean thickness of	Average width of all trabecular bone spicules	213 microns (65) 909 microns (98)
Static histomorphometry: Indices of bone resorption			
Trabecular resorptive surface Osteoclast resorptive surface	TRS ORS	% Bone surface showing Howships lacunae % Bone surface lined by osteoclasts	5.1% (0.6) 0.13% (0.6)
Dynamic histomorphometry: Indices of mineralisation			
Calcification rate	CR	Distance between the two tetracycline lines divided by the number of days between the tetracycline labels	0.64 microns/day (0.1)
Percentage trabecular surface labelled Bone formation rate	TSL BFR	% Bone surfaces labelled by tetracycline Calcification rate times the % trabecular surfaces labelled	12.8% (2.3) 0.44 microns/day (0.04)

The only protein unique to bone and dentin is Bone GLA-Protein (BGP) or osteocalcin.9 BGP is produced by osteoblasts, plays a role in the recruitment of osteoclasts and can be measured accurately with radio-immunoassay in serum specimens. The metabolism and clearance of BGP is as yet unresolved and BGP measurements should be interpreted with caution. The most tangible achievement in the search for biochemical markers for bone metabolism is the urinary excretion of metabolites of bone collagen. Hydroxylysylpyrinoline (HP) is a subunit of type I collagen of bone and type II collagen of cartilage, and lysylpyrinoline (LP) is present only in type I collagen of bone.10 The determination of urinary HP and LP has limited application in assessing bone resorption, 9,11,12 but provides no information on bone formation. The unavailability of a sensitive and specific biochemical marker for bone formation leaves a clinician with the option of advanced radiological imaging or microscopy for the assessment of generalised skeletal bone volume and quality. The former has the advantage of being noninvasive but lacks sensitivity to detect minor changes in bone metabolism. The lack of standardisation of bone densitometry instruments often contributes to variable results and confusion in this field of study.<sup>13</sup>

# Bone histomorphometry

The advent of bone histomorphometry established microscopy as the gold standard in the early identification of bone changes and monitoring of metabolic bone disease at a cellular level. 14,15 Static bone histomorphometry facilitates the establishment of accurate reproducible microscopic measurements of osteoid, mineralised bone and its cellular constituents at the time of the biopsy. The *in vivo* labelling of bone with a marker (such as tetracycline) that competes for incorporation in the mineralisation front in osteoid, makes accurate measurements on the rate of mineralisation possible (dynamic histomorphometry).



Figure 5: Note the two fluorescing tetracycline lines in the mineralisation front of the newly formed bone (non-demineralised unstained section viewed with ultraviolet light, magnification ×200)

Although other markers for bone mineralisation are available, 16 tetracycline is the drug of choice, as the use thereof is relatively free of complications. The biopsy is performed two days after the last of two 3-day cycles of tetracycline administration, which are separated by at least 10 days during which the patient receives no tetracycline.17 A transcortical biopsy of a non-weight bearing bone, like the anterior iliac crest, is advised14 in order to exclude secondary bone changes brought about by mechanical loading of other parts of the skeleton (Figure 3). The disadvantage of this technique is that it is potentially painful with the possibility of infection. However, the development of the bone trephine and the choice of the biopsy site beneath the superior aspect of the anterior iliac spine away from muscle attachments have contributed significantly to a relatively pain-free technique.16 The trephine size may vary between 5 mm and 8 mm. Mechanical disturbance of the internal architecture of the bone may become a problem with smaller biopsies, affecting the accuracy of measurements. The biopsy should be taken with care as any disturbance in the relationship between the cortex and medulla could influence the outcome of the measurements. The presence of both cortical and trabecular bone in the biopsy specimen is important.

A consensus diagnosis on each case should be established by a panel consisting of an experienced clinician and experts in radiology, biochemistry and histomorphometry

The biopsy is transported to the laboratory in 90% ethanol which minimises the loss of tetracycline from the mineralised compartment. The tissue must not be subjected to acid demineralisation as the information on mineral (and tetracycline) incorporation will be lost. With the aid of a rotating diamond saw, care is taken in the laboratory to prepare bone samples that are adequately sized for resin imbedding and non-demineralised sectioning. The selected samples are embedded in resin under vacuum which is a lengthy process that takes 3-4 weeks to complete. Non-decalcified sections are meticulously prepared and stained with haematoxylin and eosin (Figure 4) (for cell architecture) and the Von Kossa and Picrosirius techniques for the evaluation of mineral and osteoid respectively.<sup>17</sup> The measurements are performed with the aid of image analysis software and compared with published normal reference values (static histomorphometry,14 Table I). Unstained sections are viewed with ultraviolet illumination to determine the linear extent of tetracycline incorporation and the distance between the two tetracycline lines (Figure 5). These measurements reflect the extent of new bone formed between the administrations of the tetracycline labels. The results are compared with standard reference values for optimal peak skeletal bone formation (dynamic histomorphometry, <sup>14</sup> Table I).

Tetracycline is frequently used to treat or prevent infections and the presence of multiple tetracycline lines in bone may be difficult to distinguish from the markers administered by the investigating physician. Values obtained in the dynamic and static categories are correlated with clinical, biochemical and radiological findings thereby including all parameters of bone metabolism in achieving an accurate assessment of the state of the skeleton at the time of biopsy and bone formation activity between the administrations of the labels. It is important to carefully examine the state of the bone marrow, for which non-demineralised sections are ideal, with the proviso that the biopsy reaches the laboratory immediately in order to prevent cytological changes induced by prolonged alcohol fixation. The cytology of the bone marrow may assist, among others, in the identification of myeloproliferative disorders. In the author's experience the presence of mature bone marrow eosinophils have often guided a diagnosis of intestinal parasitic infestation or gluten sensitive enteropathy, both factors of which could contribute to malabsorption of bone metabolites from the intestine. A final diagnosis should never be attempted on one modality only. A consensus diagnosis on each case should be established by a panel consisting of an experienced clinician and experts in radiology, biochemistry and histomorphometry. It is important to emphasise that the accuracy of the interpretation of the microscopic appearance and histomorphometric data is complimented by the availability of a detailed clinical history, blood and urinary biochemistry, and skeletal radiographs.

# Conclusion

Bone histomorphometry is a quantifiable technique that facilitates the accurate assessment of metabolic bone disease at cellular level. It suffers the disadvantage of being invasive as it is based on the microscopic analyses of a transcortical biopsy of the iliac crest.

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