

• U



C •

FMUC

FACULDADE DE MEDICINA  
UNIVERSIDADE DE COIMBRA

## INTEGRATED MASTER IN DENTISTRY

### **BONE HISTOMORPHOMETRY**

José Francisco de Jesus Fernandes Basto\*

Advisor: Fernando Guerra\*, DMD MS PhD

Co-advisor: Rui Isidro Falacho\*, DMD MSci

\*Faculty of Medicine, University of Coimbra

Coimbra, 2014

## Bone Histomorphometry

Basto, JF ; Guerra, F ; Falacho, RI

Área de Medicina Dentária da Faculdade de Medicina da Universidade de Coimbra

Av. Bissaya Barreto, Bloco de Celas

3000-075 Coimbra

Portugal

Tel: +351 239 484 183

Fax: +351 239 402 910

e-mail: franciscobasto.md@gmail.com



**INDEX**

<b>AKNOWLEDGEMENTS</b>	<b>6</b>
<b>SUMMARY</b>	<b>7</b>
<b>KEYWORDS</b>	<b>7</b>
<b>INTRODUCTION</b>	<b>8</b>
<b>Osteoblastic lineage cells</b>	<b>8</b>
<b>Osteoclastic lineage cells</b>	<b>10</b>
<b>Normal bone anatomy</b>	<b>11</b>
<b>Macroscopical level</b>	<b>11</b>
<b>Bone tissue dynamics</b>	<b>12</b>
<b>Bone remodeling</b>	<b>13</b>
<b>Bone tissue study</b>	<b>14</b>
<b>MATERIAL AND METHODS</b>	<b>15</b>
<b>RESULTS</b>	<b>16</b>
<b>DISCUSSION</b>	<b>17</b>
<b>Specimen collection</b>	<b>17</b>
<b>Specimen preparation</b>	<b>18</b>
<b>Bone histomorphometry</b>	<b>24</b>
<b>Section description</b>	<b>26</b>
<b>Region of interest (ROI)</b>	<b>26</b>
<b>Methods of measurement</b>	<b>26</b>
<b>Measurements</b>	<b>27</b>
<b>Dimensional extrapolation</b>	<b>27</b>
<b>Variables</b>	<b>28</b>
<b>Referents</b>	<b>29</b>

<b>Bone tissue</b>	<b>29</b>
<b>Cell number and activity</b>	<b>30</b>
<b>Hystomorphometric parameters</b>	<b>30</b>
<b>Units and dimensions</b>	<b>32</b>
<b>Limitations</b>	<b>32</b>
<b>Complementary Techniques to Histomorphometry</b>	<b>33</b>
<b>Quantitative Histomorphometric Analysis In Dentistry</b>	<b>33</b>
<b>Proposed protocol:</b>	<b>36</b>
<b>CONCLUSION</b>	<b>42</b>
<b>BIBLIOGRAPHY</b>	<b>43</b>

## **ACKNOWLEDGEMENTS**

I want to thank my family, who supported me through my academic path and made this work possible.

I would also like to express my gratitude to all my teachers. Particularly, I want to thank my advisors, Professor Doutor Fernando Guerra and Dr. Rui Isidro Falacho, for all the help, advice, collaboration and promptitude, not only during the elaboration of this thesis but throughout my graduation. They have triggered my interest in Fixed Prosthodontics and inspired me to learn and to keep on improving.

I also want to express my gratitude to Professor Paulo Palma for his promptness, help and wisdom and to D. Claudia Brites for her collaboration and insights.

Finally, I am also grateful to all my colleagues and friends whom I shared five years with and to my closest friends whom I have learned to be a better person with.

## **SUMMARY**

This paper addresses bone tissue formation describing its structure and the cells that are in its origin. Bone formation, resorption and remodeling processes are discussed and briefly explained.

A summary revision about the different methods to study bone tissue was made and different methods' advantages and disadvantages were presented.

The main objective of this work was to critically examine the protocol used for histomorphometric analysis with BioQuant software in our investigation group and to propose a new and optimized one in order to improve results quality, reduce analysis time and standardize protocols for bone regeneration studies.

A secondary objective of this paper was to review every step from specimen collection to slide analysis using histomorphometry. The protocols used in our investigation group for slide preparation were presented. Bone histomorphometry was also extensively discussed throughout the paper.

## **KEYWORDS**

Bone; Bone histomorphometry; Histological sample processing; BioQuant protocol

## INTRODUCTION

Bone tissue is a highly mineralized multifunctional tissue with important mechanical support and protection functions. It is also involved in mineral homeostasis, hematopoiesis and endocrine functions. Due to its high mineral content, this tissue is extremely resilient but its organic portion also allows a certain degree of flexibility and elasticity improving its behavior under mechanical forces. [1-4]

Osseous matrix represents the largest mineral ions reservoir, particularly calcium and phosphorus, of the human body, thus having an important and active role in calcium blood levels and, therefore, in tissue fluids. Structural and metabolic functions, when in normal conditions, are in a dynamic equilibrium. When this equilibrium is disturbed metabolic function is always favored over structural function. [4, 5]

One of bone tissue's most notable features is its ability to renew itself when harmed. Bone tissue is a highly dynamic tissue, which can grow, remodel or model itself throughout life. This ability leaves structural marks, which can be a unique source of information. [2, 4]

Bone tissue formation, modeling and remodeling are due to the activity of specific cells constituting the osteoblastic lineage, responsible for tissue formation, and osteoclastic lineage, responsible for tissue resorption. Their activity is under regulation from a variety of different factors, both local and systemic. [1, 6-9]

In order to keep the balance between those different cells and to preserve the dynamic equilibrium between functions, it is necessary that every aspect of bone formation and resorption are synchronously controlled. Molecular control relies on cytokines from the RANKL/RANK/OPG pathway. [1, 6-10]

Bone tissue cell populations have different presentations and designations, based on their morphology, activity and localization. Those cells can be grouped in two different cell lineages: osteoclastic lineage, related to bone resorption, and osteoblastic lineage, responsible for bone tissue formation. [1, 10-12]

### **Osteoblastic lineage cells**

Cells from osteoblastic lineage derive from pluripotent undifferentiated mesenchymal cells from bone marrow and periosteum. This occurs due to local messengers, mechanical stimuli and blood supply conditionants. Blood supply can control differentiation whether in osteoblast, when tissues are abundantly supplied, or condroblasts, when low oxygen tension exists. [1, 4, 7, 8]



There are four sub-populations to osteoblastic lineage: pre-osteoblasts, osteoblasts, bone lining cells and osteocytes.<sup>[1]</sup>

Pre-osteoblasts are considered osteoblasts precursor cells yet presenting themselves as an intermediary stage in osteoblasts differentiation. They are usually found as elongated cells nearby bone formation sites.<sup>[5]</sup> Osteoblasts differentiation is controlled by growth factors such as bone morphogenetic proteins (BMP's) and tumor growth factor beta (TGF- $\beta$ ), which activate specific bone formation genes.<sup>[11]</sup> Pre-osteoblast also express determined proto-oncogenes (8c-myc, c-fos, c-jun) actively involved in young osteoblastic cell proliferation and differentiation.<sup>[11, 13]</sup>

When osteoblasts reach bone surface they become mature. At that point, they are highly polarized cuboid shaped cells with a palisade arrangement controlling ions flux in and out of bone tissue. Gap cells are formed between these cells with vital importance in molecule movement and therefore in the communication.<sup>[1, 7]</sup>

Mature osteoblasts do not present cell division potential but are still metabolically very active. Due to its protein synthesis and production activities osteoclasts present highly developed Golgi apparatus and rugous endoplasmic reticulum and numerous mitochondria.<sup>[9]</sup> Osteoblasts synthetize a large number of proteins among which are type I collagen and non-collagenic proteins such as osteopontin, osteocalcin and osseous sialoprotein.<sup>[5, 9, 14]</sup>

Osteoblasts activity is responsible for matrix synthesis and mineralization and for angiogenesis. Osteoblasts products are also of great importance in osteoclasts differentiation and activity. Actual evidence suggests that osteoblasts cells are responsible for bone resorption regulation.<sup>[5-7, 14]</sup>

When secretion ends, osteoblasts can transform in lining cells, osteocytes or disappear from the formation site, probably through an apoptotic mechanism.<sup>[15, 16]</sup>

Bone lining cells form a continuous cell layer, which covers most of the calcified portion along the endosteous surface. These cells have little activity and, therefore, are considered inactive.<sup>[4]</sup>

When fully surrounded by calcified bone matrix, trapped osteoblasts differentiate into osteocytes.<sup>[15, 16]</sup>

Osteocytes are highly branched cells located inside osseous lacunae, communicating among themselves and with superficial cells through a wide net of canals called canaliculi. Those osteocyte branches form gap junctions becoming similar to a spider web, the "spider's web of bone". Osteocytes are likely to orchestrate focal modeling and remodeling by detecting strain so that bone can be added by modeling

and removed by remodeling. Osteocytes are capable of bone matrix resorption through a process known as osteocytic osteolysis.<sup>[1, 12, 16]</sup>

Seeman even says, “in the cellular symphony of bone remodeling the osteocytes are one of the most important members of the orchestra, if not the first violinist or conductor”.<sup>[17]</sup>

### **Osteoclastic lineage cells**

Osteoclasts were discovered in 1873 and provoked great interest due to their unique ultra-structural characteristics. One of their most curious features is the presence of a “ruffled border”, a complex structure of interfolding finger-like projection of the plasma and cytoplasmic membranes adjacent to the bone surface.<sup>[10, 18]</sup>

Tonna, in 1960, hypothesized that osteoclasts arose from osteoblasts fusion and could dissociate again into osteogenic precursors cells. Later, in 1974, Rasmussen proposed that osteoclasts dissociation resulted in osteoblasts and osteocytes. Nijweide findings, in 1986, strongly suggested that osteoclast derived from the hematopoietic stem cell, and therefore, had a different lineage than osteoblasts.<sup>[10][19]</sup>

Osteoclasts play a key role in bone remodeling by removing the osseous matrix, allowing new tissue formation or maintenance of normal serum calcium levels. These cells can be observed by the endosteous surface, in the haversian canals, or, occasionally, by the periosteum surface of tissue regions about to be resorpted.<sup>[7, 18]</sup>

The osteoblastic lineage is composed of circulating monocytes, bone marrow monocytes, pre-osteoclasts and osteoclasts. Osteoclastogenesis is controlled by monocytes colony stimulating factor (M-CSF) which is produced by osteoblasts and bone marrow cells. M-CSF also plays an important role in pre-osteoclasts survival and proliferation.<sup>[10, 20, 21]</sup>

Osteoclasts most important functional features are: their close adhesion to bone matrix and the creation of the sealing zone that contains bone resorption to a specific site, the acidification of that zone leading to mineral dissolution and the proteolythic enzymes secretion.<sup>[10]</sup> Osteoclasts adhesion is intermediated by integrins, which specifically bond to certain matrix proteins such as trombospondin, osteopontin and sialoprotein.<sup>[7, 18, 22]</sup>

A known osteoclasts marker is tartaric resistant acid phosphatase (TRAP) which will be addressed further ahead in this paper.<sup>[23]</sup>

It is not yet known what occurs when osteoclasts complete their activity. Their mobile nature cannot be overlooked but some papers also suggest that they can go through an apoptotic process.<sup>[15]</sup>

### **Normal bone anatomy**

Primary bone is *de novo* formed bone and can be differentiated under the microscope in three types of bone: primary lamellar bone, plexiform bone and primary osteons. They are morphologically distinct with different mechanical and physiological properties according to their function. Primary bone, by definition, has to be deposited directly onto a substrate, without resorption of previously formed bone.<sup>[1, 2, 4]</sup>

Primary lamellar bone is the principal type formed on the periosteal surface. It has few vascular channels and it is quite dense being, therefore, very strong.<sup>[1, 2, 4]</sup>

Plexiform bone is typically found in rapid growth animals and not very common in humans. It is a combination of non-lamellar and lamellar bone. It has more vascular supply than primary lamellar bone but retains some of its physical properties.<sup>[1, 2, 4]</sup>

Secondary bone is the product of bone deposition in a place where previously formed bone has been through a resorptive process. Its deposition is far more complex than primary bone deposition as it requires a coordinated series of processes to replace the already existing bone. The result of this process is called secondary osteon, or Haversian system.<sup>[1, 2, 4]</sup>

Interstitial bone is the remaining of either primary or secondary bone and fills the gap between adjacent secondary osteon. Because it has not undergone remodeling processes, it is an older tissue and therefore more susceptible to accumulation of micro cracks.<sup>[1, 2, 4]</sup>

### **Macroscopical level**

At a macroscopical level, bone tissue can be divided into dense cortical bone and in more porous cancellous bone. These types of bone are also distinguished by its location and function. The human skeleton is composed of about 80% cortical bone and 20% cancellous bone. Cancellous bone is dense and solid whereas trabecular bone is composed of a honeycomb-like network of trabecular plates and rods. Both trabecular and cancellous bone are composed of osteons.<sup>[5]</sup>

Cortical osteons are denominated Haversian systems, which form a branching network within the cortical bone. Haversian system's wall are arranged concentrically around individual vascular channels (concentric lamellae). Cortical bone is usually more

static than cancellous bone. Aging leads to thinner and more porous cortical bone. Bone formation, on the periosteal side, usually exceeds bone resorption leading to increased diameter with age. On the other side, as bone resorption outweighs bone formation on the endosteal surface, bone marrow space tends to expand with age.<sup>[2, 5, 13]</sup>

Cancellous bone osteons are called packets and are semilunar shaped. Cortical and cancellous bone are normally formed in a lamellar pattern with collagen being laid down in altering orientation, which provides a microscopic characteristic aspect under polarized light. Due to this architecture, lamellar bone is extremely tough.<sup>[2-4, 21]</sup>

Woven bone on the other hand has no collagen organization and is weaker than lamellar bone. Woven bone is normally produced during primary bone formation or as a repair tissue, being usually, but not always, deposited *de novo*, without any previous hard tissue or cartilaginous model.<sup>[2-4, 21]</sup>

There is a fibrous connective tissue sheet covering bone tissue called periosteum, which is tightly attached to bone via Sharpey's fibers. The inner surface of bone is also covered by a membranous structure called endosteum.<sup>[2-4, 21]</sup>

### **Bone tissue dynamics**

Bone is a highly dynamic plastic structure that undergoes constant remodeling throughout life in order to maintain its metabolic and mechanical properties adapted to the different stimuli it is exposed to. Bone remodeling stands as one of the most important physiology principles to withstand mechanical solicitation.<sup>[2, 4, 22]</sup>

This complex process cannot be summed to osteoblasts and osteoclasts interactions to promote bone resorption or formation. It is in fact the result of a complex process influenced by mechanical forces, systemic hormone levels, cytokines, prostaglandins and local growth factors. The latter ones, synthesized mostly by bone cells, are gradually release as remodeling progresses.<sup>[6, 9, 15]</sup>

The specific and restrict bone remodeling location must be accounted for. Local bone remodeling is independent of any other remodeling event in nearby areas. This leads to the acceptance that bone remodeling is mainly under regulation from local factors.<sup>[6, 9, 15]</sup>

## Bone remodeling

Bone alterations occur in well-defined areas under the action of a specific set of cells denominated “basic multicellular unit” (BMU) constituting a functional response. [24]

In 1892, Wolf stated that, in terms of bone tissue, “form follows function”. Bone geometric distribution and form is determined by functional stress and magnitude. [25]

Bone remodeling, in simple terms, is the process by which bone tissue is eliminated and replaced by new tissue without mass alteration. Therefore, remodeling processes comprise an encompassed series of events, activation – resorption - reversion – formation, by which inactive bone surface is converted into active remodeling surface. [6, 26]

Activation involves bone surface reckoning, its preparation for remodeling as well as osteoclasts precursor’s cells recruiting. In the bone surface that will undergo remodeling it is verified that lining cells draw back so that enzymatic digestion can expose mineralized matrix and osteoclasts perform their functions. [7, 9]

Resorption stage is characterized by the formation of cutting cones, small surface bone cavities (about 200 µm deep), due to osteoclasts action. That will be the approximate size of the future Haversian system. Cancellous bone’s howship lacunae will be smaller in size (about 50 µm deep). Resorption is faster than formation as it can take up to three months to form the same amount of bone resorpted in three weeks. [2, 4]

This stage is characterized by the appearance of mononuclear cells with similar characteristics to macrophages and pre-osteoblasts responsible for the apparition of a collagen poor line, which demarks the boundary between old bone and new one. Reversion lines are indelible marks, which characterize bone tissue. They represent bone tissue’s dynamic past. [2, 4]

At last, bone resorption cavities will be filled with new bone and more osteoblast lineage cells appear. Osteoblasts proliferation and differentiation is due to the release of osseous matrix’s factor during resorptive stage. Among those factors are TGF-β, IGF, BMPs, PDGF e FGF. [6, 7]

This stage consists in the formation of a osteoid matrix and posterior mineralization resulting in a filling cone with new bone tissue. [2, 4]

## Bone tissue study

In the 1950s, with the development of plastic embedding for microscopic analysis for undemineralized bone, the understanding of bone physiology evolved. Before this point, it was only possible to study demineralized samples.<sup>[27]</sup> Since then we have advanced in understanding bone biology, from scarcely appreciating that there are cells in bone, to now seeing a wonderful complex of intercellular communication, involving not only the cells of bone but also those of the hematopoietic and immune systems, and control systems far beyond the early simple ideas of hormone regulation.<sup>[10]</sup>

In 1960, Frost's publication introduced the dynamic bone study with the use of tetracycline.<sup>[28, 29]</sup> That allowed for a better understanding of the bone tissue dynamic nature in the 1980s various publications about histomorphometry denoted concern with the standardization of techniques and data publication.<sup>[27]</sup> In 1983, Parfitt published a report, which remains a reference in this area.<sup>[27]</sup> Bone histomorphometry is the assessment of cellular and structural variables through histological sections observation.<sup>[4]</sup>

Other methods have also boosted bone knowledge. The discovery that different stains could identify different cells, by Bradbeer in 1992, and certain apoptotic cells, Weinstein and Verborg in 2000, were of great importance.<sup>[30, 31]</sup>

The appearance of scanning electron microscopy allowed further examination of three-dimensional study.<sup>[32, 33]</sup> On the other hand back scattered electron photomicrographs and radiodensitometry are the only methods that show the differential mineralization stages and allow determination of bone ages.<sup>[33]</sup>

One fundamental way to study bone is through imaging. Bone imaging issued clinically to assess bone fractures or to diagnose and classify bone pathologies. It can also be used to determine intervention efficacy and metabolic activity.<sup>[33]</sup>

Several options exist for bone imaging. Radiography is the standard for assessing bone morphology and its alteration whether due to external mechanical factors or systemic diseases.<sup>[34, 35]</sup>

Dual-energy X-ray absorptiometry (DXA) can differentiate bone tissue from soft tissue and is the gold standard to measure bone mineral content (BMC). It is an important tool for osteopenia or osteoporosis diagnosis. It allows integrated view of remodeling effects but cannot predict bone microstructure or differentiate between increased formation and decreased resorption.<sup>[32, 33]</sup>

Computed tomography (CT) is a powerful tool to assess bone morphological features. With CT, it is possible to estimate volumetric bone density (vBMD). CT drawback is related to higher radiation exposure.<sup>[32, 33, 36, 37]</sup>

Magnetic resonance imaging (MRI) is an emerging modality in noninvasive bone study. It does not involve radiation exposure and can be used to study bone microarchitecture.<sup>[32]</sup>

Ultrasound has limited applications in this area. It can indirectly assess bone mass but can only do so for bone heels and fingers.<sup>[4]</sup>

Radionuclide imaging represents a powerful tool as it can also provide information into tissue level activity.<sup>[2]</sup>

Bone histologic study is of vital importance to understand tissue-level mechanisms of bone physiology and to provide detailed information about bone tissue constitution.<sup>[4]</sup>

Histomorphometry on a bone biopsy is the gold standard for the clinical assessment of tissue-level bone activity and will be extensively addressed further ahead in this paper.<sup>[38]</sup>

## **MATERIAL AND METHODS**

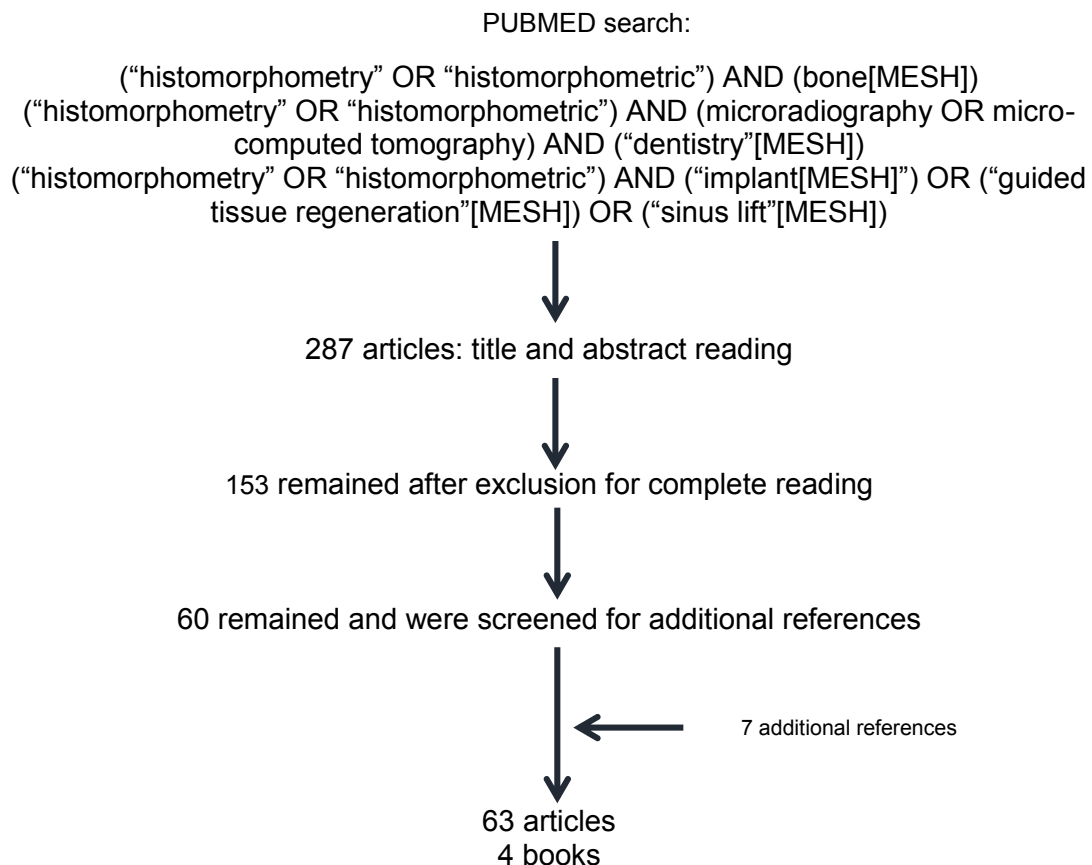
A literature search was performed in PubMed database. The search included the following combinations: (“histomorphometry” OR “histomorphometric”) AND (bone[MESH]) limited to English language, review articles in humans with full text available; (“histomorphometry” OR “histomorphometric”) AND (microradiography OR micro-computed tomography) AND (“dentistry”[MESH]) limited to reviews in English language; (“histomorphometry” OR “histomorphometric”) AND (“Dental Implants”[Mesh]) OR (“guided tissue regeneration”[MESH]) OR (“sinus lift”)) which were limited to English articles published between 2004 and 2014 with full text available. Furthermore, these studies had to meet the following criteria: correct study design with detailed histomorphometry protocol description. The titles and abstracts were screened. The full texts of all the articles that met the requirements and were accessible were collected and reviewed. Their reference was screened for additional publication that might have been missed by the electronic search.

The second part of this work consisted in histomorphometric analysis of existing slides with Bioquant® Nova (Bioquant – Image Analysis Corporation, Nashville, United States of America) software. Images were acquired from a Nikon® SMZ 1500 (Nikon®, Japan) stereoscope with Optronics DEI 750D CE (Optronics, Goleta, California, United States of America) digital video camera. Calibration and analysis were performed by the same operator.

## RESULTS

A total of 287 abstracts were identified through the electronic database with the selected terms. After screening 134 publications were excluded because they did not meet the inclusion criteria. The full text of the 153 remaining studies were read and 93 were also excluded because they also did not meet the criteria. The citations of the 6 remaining citations were screened for additional ones that might have been missed by the electronic search. From this extended search, 7 additional cross references were included. Finally, 63 papers and 4 books remained. Our analysis for the case reports focused on the protocol parameters and the assessment methods.

The electronic search is resumed in the following diagram:



For the second part of this work, existing protocol was analyzed and, after existing slides histomorphometric analysis, a new protocol was suggested.



## DISCUSSION

Bone histomorphometry, the assessment of cellular and structural variables on histologic sections, is an essential technique for understanding tissue-level mechanisms of bone physiology. It is the gold standard for tissue-level bone activity.<sup>[38]</sup>

However, before histomorphometric analyses takes place, it is necessary that study samples undergo a complex preparation protocol, from specimen collection to microscopical observation of the prepared slide. Those steps are:

### Specimen collection

In humans, clinical samples are acquired via a core biopsy in order to obtain an intact bone. This kind of biopsy can be attained through either a needle biopsy or an open biopsy. Open biopsy is, most commonly, performed when the biopsy site is not readily accessible to a biopsy needle or when surgical treatment is already taking place. The minimum acceptable tissue to be sampled is 30 mm<sup>2</sup> and the minimum perimeter is 60mm.<sup>[28, 29]</sup>

Animal models for bone study are an essential step to study implants or bone regeneration materials prior to clinical use in humans. Animal model choice depends on bone specific features such as bone macrostructure, microstructure, composition and remodeling, with emphasis being placed on the similarity between the animal model and the human clinical situation.<sup>[39, 40]</sup> It should also be taken under consideration the cost to acquire and care for animals, availability, acceptability to society, tolerance to captivity and ease of housing.<sup>[41]</sup> Other factors include ease of handling, resistance to infection and disease, inter-animal uniformity, tolerance to surgery, adequate facilities and support staff. In addition to this, the lifespan of the species chosen should be suitable for the duration of the study.<sup>[39]</sup>

The most frequently used animal models are the dog, sheep, goat, pig and rabbit models for the evaluation of bone-implant interactions<sup>[39, 42-44]</sup> and primates, dogs, rats, rabbits, pigs, hamsters and ferrets for bone regeneration and bone response to periodontal treatment.<sup>[40, 43, 45-47]</sup>

There are only minor differences in bone composition between the various species and humans. The pig demonstrates a good likeness with human bone, however difficulties may be encountered in relation to their size and ease of handling.<sup>[39]</sup>

Within a field of study, no single animal model will be appropriate for all purposes, nor can a model be dismissed as inappropriate for all purposes. Furthermore, multiple model systems are likely required to establish a broad body of knowledge.<sup>[48]</sup>

Ideally the biopsy should be obtained from a standard site<sup>[30]</sup> and it is advised to collect at least from two and preferably three different regions<sup>[28, 29]</sup> and the number of sections should be reported.

Until preparation, the biopsy can be fixed in formalin, 70% ethanol or methanol. The latter two give better preservation of tetracycline preservation.<sup>[30]</sup>

### **Specimen preparation**

There are two different kinds of histologic samples, one in which the mineral part is left intact – undecalcified technique – and the other in which the histologic sample are processed after a period of decalcification. The latter one involves the use of an acidic solution (such as ethylenediaminetetraacetic acid) to remove the mineral component and to allow embedding in paraffin wax which is the standard process performed in most histopathology laboratories. Once embedded in paraffin thin sections can be cut using a microtome.<sup>[30]</sup>

Decalcified samples can be used to study structure and cellular detail but fluorochrome labels, which are bound to the mineral portion, is lost in the process, impeding bone remodeling study.<sup>[27]</sup> For immunohistochemistry, paraffin is also preferred for it is less harmful to proteins than those substances used in the undecalcified technique. Depending on the size of the specimen, complete decalcification can take from weeks to months. Decalcifying time can be shortened by increasing the acidic concentration but this can alter specimen integrity. Other than that paraffin embedding can present a second limitation. Embedded sections can suffer dimensional alterations and shrinkage of up to 15%. Another limitation is due to the usually incomplete sample decalcification.<sup>[4, 14]</sup>

Undecalcified samples can be embedded into a hard plastic like methyl methacrylate. Specimen deformation is lower with this technique. This process is faster and allows fluorochrome labeling assessment. Plastic embedded specimen can also be

sectioned using a microtome but are more frequently sectioned with a wafer or wire saw resulting in a thicker sample. Immunohistochemistry techniques can also be used but protocols must often be adapted. Cryotomy can also be used to obtain undecalcified sections. Using liquid nitrogen specimens are flash frozen embedded in optical cutting temperature compound (OCT) and cut with a cryostat. This method is more technically rigorous than plastic embedding.<sup>[49]</sup>

For undecalcified specimen our investigation group follows the protocol by Karl Donath & Breuner in 1982 and revised by Donath in 1995. Hard tissues processing is based in the Exakt-cutting-grinding system (Exakt-Apparatebau, GmbH&CO, Norderstedt, Hamburg, Germany) and encompasses the following steps: <sup>[25, 49-51]</sup>

- Fixation and dehydration

Immediately after specimen collection, it must undergo a fixation process. Fixation consists in killing cells while conserving their biochemical structure and preserving it from decay and putrefaction. Fixation procedure avoids autolytic cell destruction and consequent cellular alteration. It also prevents bacterial contamination while increasing specimen strength and improving specimen stains affinity.<sup>[25, 49-51]</sup>

Fixation can be achieved by chemical procedures, chemical fixation, or by physical procedures, physical fixation. Chemical fixation is based in the reaction between simple fixation agents, such as ethanol or acetone, or compound agents, such as carnoy's solution or flemming's solution. Physical fixation can be achieved through specimen heating or specimen cooling. This kind of fixation is more exact because it induces less dimensional alterations. Finally, fixation can also be achieved by immersion in a fixative volume fifteen to twenty times greater than the tissue volume to be fixated or by perfusion where the fixative is inject, in vivo, through the vascular system and spreads to the entire organism.<sup>[25, 49-51]</sup>

Fixation procedures duration is related to the specimen size and can vary from twenty-four hours, when thickness is smaller than 3mm, to over a week, when specimen thickness is higher than 5 mm.<sup>[25, 49-51]</sup>

The procedure most often adapted by our investigation group is chemical fixation by immersion in 10% formalin solution with a phosphate buffer. It is vital that this concentration is respected because higher percentages can lead to superficial decalcification. This could also result in staining errors. After fixation, water must be removed from the specimen through a process called dehydration. The tissue must not be dehydrated rapidly because this will cause distortion. Rather, rehydration is carried

out in a slow, step-wise manner by passing the tissue block through a series of solutions of increasing Ethanol concentration (60%, 80%, 96% and twice at 100%). EXAKT® 510 Dehydration & Infiltration System provides agitation and vacuum for the process. In this way the water is fully leached out and replaced with ethanol.<sup>[25, 49-51]</sup>

- Infiltration and embedding

Infiltration step is also performed with continuous agitation in EXAKT® 510, which reduces in half the required time for this step. Solutions with increasing percentages of resin (in our group it is used methyl methacrylate – Technovit® 7200 VLC, Kulzer) and ethanol (resin/alcohol; 30/70%, 50-50%, 70/30% and twice with 100%). It is important that the maximum possible specimen surface is in contact with the infiltrative solution.

Embedding is performed with the same resin followed by polymerization with Exact® 520 for 8 hours.

- Initial sectioning

After embedding, resin blocks are saw cutted according to the desired slide orientation within our investigation group Exact® 310 CP is used.

- Section mounting

After specific preparation, previously sectioned blocks, are prepared and vacuum mounted in a first acrylic slide (slide A) with a different, specific, methyl metacrilate (Technovit® 4000 VLC, Kulzer).

- Initial grinding – facing

This step aims for the obtainment of parallel surfaces to allow further cutting and polishing techniques. This is achieved with the Exact® 400 grinder.

- Final slide mounting – sandwich

Final slides are prepared with a “sandwich” technique where the slide free surface is glued to a second acrylic slide (slide B). A specific photopolymerizable resin, Technovit®7210 VLC, is used in a vacuum press under polymerization light.

- Sectioning and final grinding

Sectioning is done using an high precision contact point cutting unity, with speed control and irrigation, using 100µm saw (Exakt® 310 CP), and a grinding unity (Exact® 400 CS). Both units use vacuum fixation.

The cutting unity possesses a parallelism system, where slides are fixated, and a laser orientation pointer to ensure accurate parallelism between successive cuts and correct slide thickness.

The grinder unity uses an electronic sensor (AW 100 Electronic Measuring System) to ensure uniform final slide thickness.

In this step, the “sandwich” is cutted nearest from the slide B side. Final thickness of about 50µm is obtained using a decreasing granulometry diamond layered disks (800, 1000, 1200, 2500, 4000; Hermes®, Germany). A control measurement is performed with a digital micrometer (Mitutoyo® 2093, Digimatic Micrometer, Japan) to confirm slide thickness.

- Staining techniques

Tissue staining is a technique used to better visualize different tissues, tissue morphology and cells under a microscope. By using different stains, it is possible to preferentially stain certain cell components, such as a nucleus or a cell wall, or the entire cell.<sup>[52]</sup>

For important routine diagnostics, established standard histological staining methods are discussed here. Basic information on techniques and on the best-practice use are mentioned only briefly and therefore do not substitute reference to the specialist literature.

#### *Conventional histologic staining methods*

Staining methods differs whether for calcified or undecalcified specimen. For calcified samples all the necessary details are detectable in hematoxilyn and eosin stained sections. Even tough, it is preferable to use other stains to highlight particular features.<sup>[30, 53]</sup> It is common to stain a separate section with tartrate-resistant acid phosphatase (TRAP) which is specific for osteoclasts.<sup>[1, 22, 53]</sup>

In our investigation group, for calcified samples, toluidine blue and hematoxilyn and eosin are used.<sup>[25, 50, 51]</sup> and the protocol adopted is described ahead:

**I. Toluidine blue in slides with bone specimen**

1. Stirrer slide for 5 minutes in 10% hydrogen peroxide solution
2. Wash slide under running water
3. Dry with paper
4. Dye with toluidine blue for 20 minutes
5. If necessary (excessively blue slide) use alcohol to clean the slide
6. After 5 minutes apply Technovit® 7200 VLC and let it rest for 12 hours.
7. Polymerization for final protection. Slide placed on top of the polymerization table with a drop of Technovit® 7200 VLC place the cover slip and polymerize

**II. Surface technique with toluidine blue in slides with bone specimen**

1. Slides placement in a flat surface
2. Place slide label in the flat surface
3. Degrease slide with 100% alcohol
4. Apply formic acid for 4 minutes
5. Wash slides under running water
6. Dry with paper
7. Place slides in methanol
8. Wash slides under running water
9. Dry slides with paper
10. Dye with toluidine blue for 3 minutes
11. Wash slides under running water
12. Wash slides with distilled water
13. Dry slides with paper
14. Label slides

**III. Hematoxylin and eosin staining**

1. Stirrer slide for 10 minutes in 10% hydrogen peroxide solution
2. Wash slide under running water
3. Dry slide with paper
4. Stain with hematoxylin and eosin for 30 minutes
5. If excessively stained wash with 70% alcohol solution
6. Wait 5 minutes and place in Technovit® 7200 VLC for 12 hours
7. Surface polymerization and cover slip placement.

For undecalcified samples, Goldner's trichrome dyes mineralized bone green and osteoid red and gives good contrast between them.<sup>[30]</sup> Using von Kossa differentiation of osteoid from mineralized bone is easily achieved, as mineralized tissue stains black and osteoid tissue blue.<sup>[30, 53]</sup> Von Kossa and solochrome cyanin provide similar results.<sup>[30, 53]</sup> It is also common to stain a separate section with TRAP, which will red-stain osteoclasts. Safranin O binds to proteoglycans and so it is used to assess cartilage in fracture repair studies. Toluidine blue permits visualization of cement lines surrounding osteons and hemiosteons. It provides good definition of cellular details. Aniline blue results in a blue staining of the bone tissue.<sup>[53]</sup>

### I. Hematoxylin and eosin

1. Stain with hematoxylin for 20 minutes
2. Wash with distilled water twice
3. Wash under running water for 10 minutes
4. Wash with distilled water
5. Stain with eosin for 5 minutes
6. Wash with 96% alcohol solution
7. Wash with 100% alcohol for one to two minutes
8. Xylol II for 5 minutes
9. Xylol for 10 to 15 minutes
10. Immediate placement in the mounting solution
11. Slides in the hothouse for 24 to 48 hours

Even though this protocol is similar to a wide variety of staining agents, trichrome dyes usually require a different and specific protocol.

#### *In vivo labelling:*

All up-mentioned staining techniques can only provide limited temporal information. They are static and, therefore, require at least two samples collected at different moments to study changes over time. In the 1960s, Harold Frost introduced the use of fluorochrome labels, which allow for dynamic analysis of bone change over a defined period. They bind to calcium during active mineralization and are incorporated into the bone. Tetracycline is the most used fluorochrome in humans although doxycycline is also sometimes used. Fluorochrome are administered on two separate

occasion. Tetracycline is administered clinically as an oral dose usually for three consecutive days with a two or three week administration interval.<sup>[27, 30, 54]</sup> The biopsy is then taken three to fourteen days after.<sup>[30, 54]</sup>

Too long interlabel duration can result in an underestimation of double-labeled surfaces, as regions will not be engaged in active bone formation at the time of both label administration. This phenomenon is often referred to as “label escape”. On the other hand, if too little time separates each administration bone formation between labels will be insufficient to allow differentiation during analysis.<sup>[28, 29]</sup> The time after last fluorochrome administration is of less importance but should be at least of 3 days to allow the label to bind sufficiently to the mineral in order to endure histologic preparation.<sup>[54]</sup>

### **Bone histomorphometry**

One of the first addressed issues with histomorphometric analysis was the need to standardize the terminology used in order to improve communication among the scientific community.<sup>[29]</sup> Parfitt was given the task to develop a new and unified system of terminology which was promptly adopted and markedly improved the ability to communicate within histomorphometrists and with nonhistometrists.<sup>[28]</sup> That report was revised in 2013 by David Dempster.

The meaning of the term “bone” is one of the first addressed issues. It has at least three meanings: mineralized bone matrix excluding osteoid, which restricts bone to hard tissue; bone matrix, including both mineralized and non-mineralized tissue; and as a tissue that includes bone, marrow and other soft tissue.<sup>[28, 29, 55]</sup> The term bone shall refer to the second definition and the third shall be referred to as bone tissue.<sup>[28, 29]</sup>

The term “trabecular” bone, often used, should also be substituted by “cancellous” bone, which is most usually used in textbooks.<sup>[28, 29]</sup>

“Osteoblast” is also a source of confusion since it is defined differently in clinical and experimental literature. That term should be restricted to cells that are assumed to be currently making bone. Inactive osteoblasts are called lining cells and are not accounted as osteoblasts. It is also advised to avoid terms that embody assumptions as resorpting and formation surfaces. Instead osteoid and eroded surface shall be used. Due to the different existing methods to identify osteoblasts and osteoblast, the used criteria should always be stated.<sup>[28, 29]</sup>

The recommend terms are listed in table I. It is strongly advised that those abbreviations are used in order to uniformize reported data.



Although all bone journals require articles to be submitted accordingly to the standard terminology described below, some of the recommendations are not always followed. Common omissions are the referents for bone formation rates and the specification of the width of osteoid tissue that is measured. Other frequent issues are the reporting of derived indices without the primary data, the use of improper referents for histomorphometric measurements and the lack of utility in certain measurements and endpoints.<sup>[27, 56]</sup>

**Table I** – List of recommended terms

A	Apposition(al)	f	Frequency	Pf	Profile
Ad	Adipocyte	G	Grow (th)(ing)	Pl	Plate
Ab	Absolute	H	Haversian	Pm	Perimeter
Ac	Activation	Hm	Hematopoietic	Po	Por(e)(ous)(osity)
Aj	Adjusted	Hp	Hypertrophic	Ps	Periost(eal)(eum)
Al	Aluminium	Ht	Height	Pt	Point
Ar	Area	Hz	Horizontal	Q	Quiescent
a	Activ(e)(ity)	H	Hit	R	Rate
B	Bone	I	Interface	Rd	Radi(al)(us)
BMU	Basic multicellular unity	Ia	Intra	Rf	Referen(ce)(t)
Bd	Boundary	Ic	Intercept	Rm	Remodeling
C	Core	Ii	Initial	Rs	Resorption
Ca	Canal(icula)(r)	In	Internal	Rv	Reversal
Cd	Corrected	Ir	Inter	S	Surface
Ce	Cell	Is	Instantaneous	Sa	Sample
Cg	Cartilage	It	Interstitial	Se	Section
Cm	Cement	L	Label(led)	Sg	Sigma
Cn	Cancellous	Lc	Lacuna(r)	Sm	Seam
Cp	Cytoplasmatic	Le	Length	Sn	Spongiosa
Ct	Cort(ex)(ical)	Li	Lining	Sp	Separation
Cy	Cycle	Lm	Lamella(r)	St	Struct(e)(al)
D	Dimensio(al)	Lo	Longitudinal	s	Single
De	Depth	L	Lag	T	Tissue
Dg	Degenera(tive)(tion)	M	Mineral(iz)(ing)(ation)	Tb	Trabecula(r)
Dm	Diameter	Ma	Marrow	Th	Thickness
Dn	Density	Md	Mineralized	Tm	Termin(al)(us)
Do	Domain	Me	Medullary	Tr	Transitional
Dp	Diaphy(is)(eal)	Ml	Modeling	Tt	Total
Dt	Delta	Mo	Mononucle(ar)(ated)	t	Time
d	Double	Mp	Metaphys(is)(eal)	U	Unit
E	Ero(ded)(sion)	Mu	Multinucle(ar)(ated)	V	Volume
Ec	Endocortical	Mx	Matrix	Vd	Void
En	Envelope	N	Number	Vk	Volkman
Ep	Epiphys(si)(eal)	Nc	Nucle(us)(ar)	Vt	Vertical
Es	Endost(eal)(eum)	Nd	Node	W	Wall
Ex	External	O	Osteoid	Wi	Width
F	Formation	Ob	Osteoblast(ic)	Wo	Woven
Fa	Fat(ty)	Oc	Osteoclast(ic)	y	Year
Fb	Fibro(sis)(us)	On	Osteon(al)	Z	Zone
Fe	Iron	Ot	Osteocyt(e)(ic)		
Fr	Front	P	Period		

## Section description

Data report should be presented as follow: source-measurement/referent. Source refers to the structure on which measurements were made. Most common sources and referents are listed bellow. Referents importance will be addressed latter on.

**Table II** – List of Sources and Referents

Sources		Referents	
Name	Abb.	Name	Abb.
Total core	Tt	Bone surface	BS
Cortical bone tissue	Ct	Bone volume	BV
Cancellous bone tissue	Cn	Tissue volume	TV
Endocortical surface	Ec	Core volume	CV
Periosteal surface	Ps	Osteoid surface	OS
Transitional zone	Tr.Z	Bone interface	BI
Diaphyseal bone	Dp	Eroded surface	ES
Metaphyseal bone	Mp	Mineralized surface	Md.S
Epiphyseal bone	Ep	Osteoblast surface	Ob.S
Medullary bone	Me	Osteoclast surface	Oc.S

## Region of interest (ROI)

Region of interest is defined as the area of the slide to be studied. It varies in shape and dimension between studies and specimens but should be similar for different slides in the same study.

In bone regeneration histomorphometry it should encompass the entire area of the created defect. It should also have minimal surrounding tissue in order to avoid errors and measure bias.

ROI delimitation is somewhat subjective and should be done by the same operator for every slide in study to minimize *bias*.

## Methods of measurement

Initially, histomorphometry relied mostly in the application of probability theories by the use of estimates. Repeated counting was used to make the estimate as accurate as possible. The use of different eye-piece graticules attached to the microscope. Point counting was performed by the superimposition of a series of points on the microscope and a percentage was calculated from the number of hit points for a particular feature from the total possible points. Another method used for measurement of surface areas or lengths was the linear intercept method in which an eye-piece graticule enabled the superimposing of lines. The number of interceptions with the tissue studied was then related with the total interceptions with the reference surface.<sup>[30]</sup>

Later, the semiautomatic and automatic methods appeared. The semiautomatic method involves the use of a microscope equipped with a drawing tube through which the image of the digitizing platen is projected over the optical field. The investigator selects and traces all histologic structures to be measured by moving a cursor on the digitizing platen which is visible by its projection over the histologic field.<sup>[30, 57]</sup> [58]

Nowadays the sections are analyzed digitally with image analysis software. The system consists of a digitizing tablet, a high resolution image obtained through the microscope and a computer with customized image analysis software.<sup>[54, 59, 60]</sup>

### **Measurements**

It is possible to distinguish two kinds of measurements: static and dynamic. Static measurements do not provide any temporal information, they do not take into consideration the dynamic nature of bone and cannot describe bone processes such as bone resorption or formation. Trabecular thickness, number and separation are examples of static measurements that characterize trabecular bone structure and bone volume, cortical area and porosity are also static measurements that describe the amount of tissue. Osteoblasts, osteoclasts and osteoid surfaces are also static.<sup>[28, 29]</sup>

Dynamic measurements are obtained through bone labeling with fluorochrome markers in order to assess rates and magnitudes of change in bone tissue in various moments in time. With this information it is possible to assess changes in normal bone physiology. They can also be used to determine the effect of a treatment or intervention over time. Dynamic histomorphometry will be addressed further ahead in this paper.<sup>[28, 29]</sup>

Measurements can also be differentiated in direct measurements or indirect measurements. The direct method is more precise but requires randomly chosen measurement locations whereas the indirect method is less laborious and requires less subject sampling.<sup>[28, 29]</sup>

### **Dimensional extrapolation**

Histological sections are bi-dimensional representations of three-dimensional structures. It is possible to measure area, length, distance between points or lines and number. However, the ultimate goal is to understand the three dimensional reality. Stereology, a mathematical discipline, offers the possibility to extrapolate those two-dimensional measurements to three-dimensional data.<sup>[61]</sup> The application of stereologic

theorems may be used with acceptable error but the sampling is required to be random and unbiased.<sup>[61]</sup>

It is recommended that data reporting should be consistently two-dimensional (perimeter, area and width) or presented only in the corresponding three-dimensional terms (surface, volume, thickness).<sup>[28, 29]</sup>

### Variables

There are two types of variables in histomorphometric analysis: primary and derived variables. Primary variables are those measured directly through histological observation. Those variables, which require calculation and mathematical formulas, are entitled derived variables. The most useful variables are frequently derived because they allow comparisons between groups and calculation of information impossible to obtain through histological observation. Derived variables present a difficulty related to accumulation of possible inaccuracies. Each primary variable measurement is associated with its own standard deviation and consequently it can affect the reliability of derived variables. Nevertheless, the importance of those variables outweighs their limitations.<sup>[28, 29]</sup>

**Table III – List of primary variables**

Type	Name	Abbreviations		
		2D	3D	
Area	Bone volume	BV	B.Ar	
	Osteoid volume	OV	O.Ar	
	Mineralized volume	Md.V	Md.Ar	
	Void volume	Vd.V	Vd.Ar	
	Marrow volume	Ma.V	Ma.Ar	
	Fibrosis volume	Fb.v	Fb.Ar	
	Canal Volume	Ca.V	Ca.Ar	
	Cell volume	Ce.v	Ce.Ar	
	Cytoplasmic volume	Cy.V	Cy.Ar	
	Nuclear volume	Nc.v	Nc.Ar	
	Length	Bone interface	BI	B.Bd
		Bone surface	BS	B.Pm
Osteoid surface		OS	O.Pm	
Eroded surface		ES	E.pm	
Quiescent surface		QS	Q.pm	
Mineralized surface		Md.S	Md.Pm	
Osteoblast Surface		Ob.S	Ob.Pm	
Single-labeled surface		sLS	sL.Pm	
Double-labeled surface		dLS	dL.Pm	
Osteoclast surface		Oc.S	Oc.Pm	
Reversal surface		Rv.S	Rv.Pm	
Distance		Cortical thickness	Ct.Th	Ct.Wi
	Wall thickness	W.Th	W.Wi	
	Mineralized surface	Md.Th	Md.Wi	
	Osteoid thickness	O.Th	Md.Wi	
	Label thickness	L.Th	O.Wi	
	Trabecular thickness	Tb.Th	L.Wi	
	Interstitial thickness	It.Th	Tb.Wi	
	Trabecular diameter	Tb.Dm	It.Wi	
	Canal Radius	Ca.Rd	-	
	Cell height	Ce.Ht	-	
	Nuclear height	Nc.Ht	-	
	Erosion depth	E.De	-	
Number	Osteoblast number	-	N.Ob	
	Osteoclast number	-	N.Oc	
	Osteocyte number	-	N.Ot	
	Adipocyte number	-	N.Ad	
	Nuclear number	-	N.Nc	
	Canal number	-	N.Ca	
	Seam number	-	N.Sm	
	Erosion number	-	NE	
Profile number	-	N.Pf		

## Referents

Using a referent to normalize results is one of the most important steps when publishing information from histomorphometric analysis. When absolute values are used there is no way of comparing data because there is no information about the sample in study. For example the comparison of osteoclasts perimeter between two samples is only fairly produced if results are presented as a relative number to a common referent as the bone surface of that sample.<sup>[28, 29]</sup>

The referent should be some clearly defined area or perimeter within the section. Different referents serve different purposes and lead to different interpretation. It is necessary to do so and it is important to clearly distinguish between them.<sup>[28, 29]</sup>

Commonly used examples are tissue volume (TV), bone volume (BV), bone surface (BS) and osteoid surface (OS).

## Bone tissue

Assessing the type of bone can provide information on whether bone formation is occurring normally or not. Some stains or observation under polarized light can make this differentiation. Due to its constitution, lamellar bone patterns are easily recognized under that kind of light. Its thickness or type of organization can also be observed. There are also stains for osteoid bone differentiating mineralized from non-mineralized bone in order to evaluate bone processes.<sup>[4, 5, 14]</sup>

When under the microscope, cortical bone tissue seems solid and flat, with an homogenous surface. On the other hand, cancellous bone has a sponges' appearance.<sup>[4, 5, 14]</sup>

Even tough, cancellous bone and cortical bone have the same constitutive elements (cells and osseous matrix) they present important functional and structural differences.<sup>[4, 5, 14]</sup>

Cancellous bone is formed by thin trabeculae with 100 to 150  $\mu\text{m}$  constituted by parallel rod, which delimit wide cavities occupied by bone marrow. Trabeculae organization forms a tridimensional net, according to mechanical force lines, providing its optimal force resistance. Cortical bone is constituted by cylindrical columns, called osteons or Havers' systems, with a diameter that ranges from 150 to 300  $\mu\text{m}$ , therefore having the appearance of concentric lamellae surrounding a central canal, the Havers'

canal. Between osteons' lamellae there are lacunae called osteoplasts. Radially disposed canals between osteons can, sometimes, be seen and represent osteocytes branches. The peripheric osteons' layer is often marked by a hyper mineralized line, the cement line. This defines the boundary between deferent Havers' systems.<sup>[4, 5, 14]</sup>

### **Cell number and activity**

Knowing the surface occupied with osteoblasts can be a useful tool to predicting bone processes. Osteoblasts are often identified by their morphology. It is possible to measure their number and surface, usually in relation to bone surface. It is challenging to identify osteoblast activity therefore, their assessment is often restricted to osteoid regions. In this situations, osteoid is divided in osteoid with osteoclasts (active formation) and osteoid without osteoclasts (inactive formation)<sup>[28, 29]</sup>. It is also possible to use immunohistochemistry to identify these cells. The most accurate form to determine osteoblasts function is trough fluorochrome labeling. In regions with double label dividing the distance between those labels by the number of days between administrations, represents the mineral apposition rate (MAR). MAR is considered to be an indicator of osteoblast activity.<sup>[28, 29]</sup>

Osteoblasts can also be identified by their morphology but are more often studied in TRAP stained sections. Osteoclast number and surface are most commonly normalized to bone surface as well. Osteoclasts static measurements cannot reflect their activity. To predict it is often assessed their presence in eroded surfaces, which is indicative of an active resorption site whereas their absence points at an inactive resorption site.<sup>[3, 6, 62]</sup>

### **Hystomorphometric parameters**

Hystomorphometric parameters are generally divided into two categories: structural and remodeling with the latter being subdivided into static and dynamic parameters.<sup>[28, 29, 54, 55]</sup>

Structural parameters provide information about bone and mass and structure. These parameters are related to three-dimensional geometry of the bone and are calculated from measurements of total bone area and the total bone perimeter. The assessment of bone structure is important due to its relationship with bone strength.<sup>[54]</sup>

Remodeling parameters provide information about the amount of unmineralized bone and extent of resorption cavities.<sup>[28, 29]</sup>

Dynamic remodeling parameter yields information on bone formation rate and can only be measured when patients undergone in vivo labeling or, when conducting an animal model study, by comparing samples from studies different timeline moments.<sup>[28, 29]</sup>

**Table IV** – List of histomorphometric parameters

Type of index	Name of index	Abbreviation	Formula
Structural	Trabecular number	Tb.N	$(BV/TV)/TB.Th$
	Trabecular separation	Tb.Sp	$(1/Tb.N) - Tb.Th$
	Trabecular width	Tb.Wi	$(BV/TV)Tb.N$
Dynamic	Mineralizing surface	MS	$(dLS+sLS/2)/BS$
	Mineral apposition rate	MAR	$Ir.L.Th/Ir.L.t$
	Adjusted apposition rate	Aj.AR	$MAR(MS/OS)$
	Osteoid apposition rate	OAR	-
	Mineral formation rate	MFR	$MAR(MS/BS)$
	Bone formation rate	BFR	-
	Bone resorption rate	BRs.R	-
	Mineralization lag time	Mlt	$O.Th/Aj.AR$
	Osteoid maturation time	Omt	$O.Th/MAR$
	Formation period	FP	$W.Th/Aj.AR$
	Resorption period	Rs.P	$FP(Oc.S/OS)$
	Reversal point	Rv.P	$FP(ES-Oc.s)/OS$
	Remodeling period	Rm.P	$FP(ES+OS)/OS$
	BMU life span	Sg	-
	Quiescent period	QP	$FP(QS/OS)$
	Total period	Tt.P	$FP(BS/OS)$
Activation frequency	Ac.f	$1/Tt.P$	

### ***Dynamic histomorphometry***

Dynamic bone histomorphometry is the standard method for evaluating alterations in bone remodeling at the level of the basic multicellular unit.<sup>[38]</sup> Dynamic bone histomorphometry allows various parameters measuring such as mineral apposition rate, bone formation rate and activation frequency.<sup>[28, 29, 38]</sup>

There are a few limitations to this technique such as the impossibility to make other measurements, which require the use of stain, incompatible with fluorochrome label analysis. Therefore, it is impossible to identify osteoblasts and osteoclasts.<sup>[28, 29, 38]</sup>

The presence of only one or no label can be related to low bone turnover or inactive bone surface. This can be supported by low values of bone formation parameters such as osteoid or osteoblast surface. An extended label search should be done to assess if tetracycline is found in other places to rule out improper labels taking or deficient absorption.<sup>[28, 29]</sup>

## Units and dimensions

Micrometer and millimeter, to express length, and days and year, to express time, are the chosen units to be used. It will depend on convenience, consistency and the principle to provide the most important information. Possible ratios should be presented in percentage rather than decimal fractions. This simplifies data presentation and makes it easier to be interpreted.<sup>[28, 29]</sup>

**Table V** – List of units and dimensions

Parameter	Abbreviation	Units
Tissue area	T.Ar	mm <sup>2</sup>
Bone area	B.Ar	mm <sup>2</sup>
Bone perimeter	B.Pm	mcm
Bone volume	BV/TV	%
Wall thickness	W.Th	mcm
Osteoid surface	OS/BS	%
Osteoid volume	OV/BV	%
Osteoblast surface	Ob.S/BS	%
Osteoblast number	N.Ob/BS	/mm
Osteoid thickness	O.Th	mcm
Eroded surface	ES/BS	%
Osteoclast surface	Oc.S/BS	%
Osteoclast number	N.Oc/T.A	/mm <sup>2</sup>
Bone surface	BS/TV	mm <sup>2</sup> /mm <sup>2</sup>
Double-labeled surface	dLS/BS	%
Single-labeled surface	sLS/BS	%
Mineralizing surface	MS/BS	%
Mineralizing surface	MS/OS	%
Mineral apposition rate	MAR	mcm/d
Adjusted apposition rate	Aj.AR	mcm/d
Mineralization lag time	Mlt	D
Osteoid maturation time	Omt	D
Activation frequency	Ac.F	N/y
Cortical thickness	Ct.Th	mcm
Cortical porosity	Ct.Po	%
Bone formation rate	BFR/BS	mcm <sup>3</sup> /mcm <sup>2</sup> /d
Bone formation rate	BFR/BV	%/y

## Limitations

Bone histomorphometry limitations relate to technical limitations, protocol complexity, costs and required time. Cutting and grinding bone blocks may damage the interface and influence results.<sup>[63]</sup> On the other hand, thicker slices result in an overestimation of measurements and therefore, sections over 30 mm should be avoided.<sup>[64]</sup>



Main disadvantages are the high cost and delayed time for analysis<sup>[65]</sup> but the biggest limitation is that histology only presents 2D information.<sup>[63]</sup>

### **Complementary Techniques to Histomorphometry**

Bone formation techniques and materials are always tested in animals before human application. Histomorphometry has been used to evaluate bone response under different conditions. Due to its cost, technical demands and time consumption, alternative methods have been studied and tested.<sup>[32, 35]</sup>

Microradiography is a successful alternative for quantitative evaluation of bone defects and bone formation. It is fast, accurate and simple. Its main disadvantage is that it can only obtain a bi-dimensional technique and cannot be used to make three-dimensional volumetric calculation. Recent studies report that microradiography tends to underestimate bone defects.<sup>[34, 35]</sup>

More recently micro-computed tomography appeared. It is capable of forming both bi and tridimensional images with applications in bone formation quantification, as well as in bone modeling in bone grafts. It is also fast and allows nondestructive analysis without a specific preparation protocol. Even though it has more advantages than microradiography, micro-computed tomography is not widely used due to its high costs.<sup>[35-37, 63, 66, 67]</sup>

Micro-computed tomography presents advantages over histomorphometry but only the latter one can make cellular level observation.<sup>[35]</sup> It is, therefore, useful to use a combination of both histomorphometry and micro-computed tomography to maximize obtained information.<sup>[63, 67, 68]</sup>

### **Quantitative Histomorphometric Analysis In Dentistry**

In the field of dentistry the most common histomorphometry applications are in sinus lift techniques success assessment<sup>[69, 70]</sup>, guided tissues regeneration agents<sup>[25, 43, 45-47, 50, 51]</sup> and bone to implant contact.<sup>[42-44]</sup>

In our investigation group this procedure is most often used to study and evaluate regenerative bone procedures and the most relevant parameters to quantify are:

1. New bone formation:  $\text{New bone area} / \text{Defect area} \times 100$
2. Particles percentage:  $\text{Regenerative material area} / \text{Defect area} \times 100$
3. Proportion between newly formed bone and regeneration material:  $\text{New bone} \% / \text{Regenerative material} \%$

#### 4. Defect resolution: $(\text{New bone \%} + \text{Regenerative particles \%}) / \text{Total area}$

The main aim of this work was to review the protocol at use in our investigation group and to propose a new, improved and systematized protocol to optimize results and to diminish dispended time.

It is important to know that this protocol covers only a few features of the BioQuant software, which are the histomorphometry procedure for bone regeneration analysis.

It should also be noted that the system files (e.g., data volume file, array list file, and calculation set file) used in this protocol were customized for routine bone regeneration analysis. They were manually defined by the author and will not be found in other BioQuant systems.

It is recommended to follow the order of the procedures in Analysis section shown below because of the following reasons: (1) some parameters are used to calculate some of the later parameters; (2) you can only undo some of the procedures; (3) the program may freeze from messy procedures. Once you find you cannot draw anything in the image, you have to exit and restart the program.



## Proposed protocol:

### Launch BioQuant OSTEO 2009 from desktop

**Set up your environment** to study bone regeneration it is advised to set up an environment with the following windows: *Shelf*; *ROI Tools*, *Select Arrays*; *Parameters*; *Threshold*; *Auto Measure*; *Calculations and Overview*. If any of these are not visible, click the corresponding button:



Figure 1 – Bioquant environment

**Save tracings on image** - For some situations, it's better to show the bone slice picture along with its markings/tracings. For example, in a bone resorption experiment, as the staining backgrounds are usually very strong, they are much easier to see if the edges of pits are highlighted.

To do this, choose *Option* -> *Save Tracings On Image* from main menu before making any markings/tracings. Once you done with drawings, choose *File* -> *Save Image(s)* -> *Save Single Image...*)

**Get image from microscope:** In the main menu of BioQuant, go to *Image* -> *live*. If microscope is on, this should turn the *Image Window* white instead of black.

**Adjust image:** place your slide on the scope with X0.75 magnification. In the Optronics® unit keyboard click *Auto Expose* button. Now your tissue should show. Focus to screen and put a field with some or all white (no tissue) on the screen and choose *White Balance* button. If you prefer it is possible to adjust several image parameters manually using: *Image* -> *Video Setup* -> *Image*.

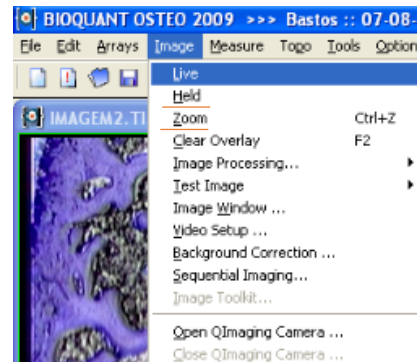



Figure 2 – Image menu

After this, click *Image* -> *Held* to make a still image. Then select *File* -> *Save* -> *Single Image* select desired destination folder and save your file.

A common related issue with this technique is the poor quality of the acquired image. This could be improved if several images were acquired with higher magnification and then stitched together using a postproduction software to create a higher resolution image.

**Create data set:** click *File -> New Data Set* which will be your “main file” and for each new slide it will be necessary to create a new file in *Quick Data Set* button on main toolbar or *File -> New -> Quick Data Set...* from main menu. Name your data set and leave *Current Data Set* selected. Click *Create* button.

**Choose arrays for measurement:** in *Select Arrays* window, under *Select* menu, *Select All Arrays* if you need all of them or *Load Selected List* then choose *teseJB.bqa* and double click *D3, D4 and D5* if your purpose is bone regeneration analysis. If the *Image Window* screen turns solid yellow after you load the arrays, use the *Clear Overlay* button  in *Shelf* window to see your image. Selected arrays here define what kind of parameters you want for the following measurements. The number in the parenthesis at the end of every array denotes how many values are measured by it.

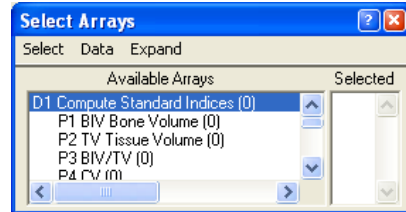


Figure 2 – Select array window

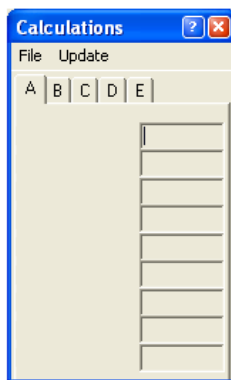


Figure 3 – Calculation windows

**Choose calculation results:** Find *Calculations* window, click *File -> Load Set from File...*, and choose *teseJB.bqc* in the *BQ Open File* window. The items in the tabs of *Calculations* window are the items you will see in your final results spreadsheet. Each item equals a parameter of measurement.

**Note:** *teseJB.bqa* and *teseJB.bqc* was manually created by users to make bone regeneration analysis. It is possible to make custom system files to match operator needs and facilitate the analysis:

**Define custom array list** in *Select Arrays* window, *Select -> UnSelect All Arrays ->* double click the arrays you want in the *Available Arrays* box -> repeat until all the arrays you want are in the *Selected* box -> *Save Selected List ->* name your bqa file. By default, your own array list file will be saved to *C:\BQOSTEO\Selected Lists*.

**Define custom calculation set** in *Calculations* window, choose *File -> Select New Arrays...* A window called *Select Calculation Arrays* shows up, put the parameters you want in to different pages. There are 5 pages you can choose, and normally 3 pages are enough. The parameters in page A will show in tab A in the *Calculations* window. Once you are done with selecting, save your own calculation arrays by choosing *File -> Save Set to File...* -> name your bqc file. By default, your own calculation set file will be saved to *C:\BQOSTEO\Calculation Lists*.

## ANALYSIS

**Set magnification and topography:** In *Parameters* window, make sure the microscope objective in use matches the magnification selected. Check the box for *Save Topo* as all the topographic info will be saved and showed in *Overview* window.

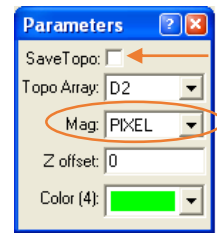



Figure 4 – Parameter windows

**Set a consistent ROI** for tissue volume measurement. Click *wrench* icon  on main toolbar if *ROI Tools* window is not there. In *Create* tab of *ROI Tools* window, choose a custom shape from *Type* drop-down menu if desired. Set the height and width or radius. Click *Define ROI* button, cursor will show up in the *Image Window* in the shape of a cross with a dark-blue shape box round it. When you are satisfied with the position of ROI, left click to fix it and right click to get out. A consistent ROI for all the bone slices is critical.

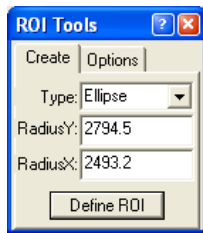


Figure 4 – ROI tools windows

After this is done, it is necessary to go to *Options* -> *Save tracing on image* and then select *File* -> *Save* -> *Single Image* select desired destination folder and save your file with the ROI. Also save ROI file *file*-> *save roi*.

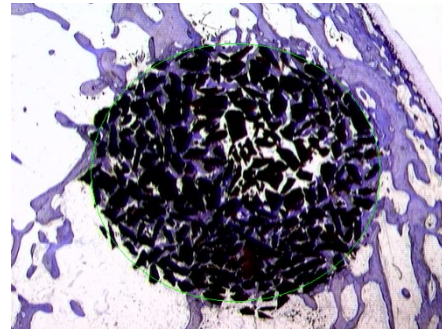



Figure 5 – ROI over slide image

**Measure tissue volume** Highlight *D3* in the *Selected* box of the *Select Arrays* window. Now, your ROI turns solid yellow with a green edge. Find *Auto Measure* window (or you can click the *compass* icon on the main  toolbar if it is not there), click *Preview* button in *General* tab.

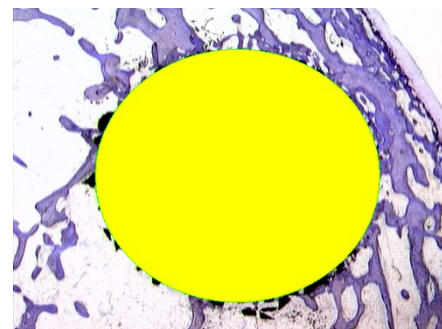


Figure 6 – Tissue volume measurement

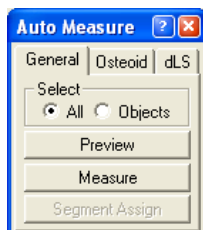


Figure 7 – Auto measure windows

Then click *Measure* button to obtain tissue volume (TV). Now ROI turns light blue. You will see a box that appears in the *Overview* window. The TV value will appear in the *List Current Array* window and the number in *D3* parenthesis will turn to 1.

### Measure new bone volume

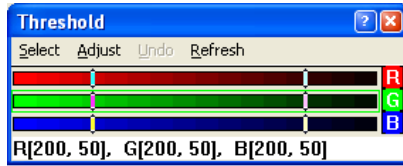



Figure 8 – Threshold window

Highlight *D4* in the *Selected* box of the *Select Arrays* window. It is advised to choose a different color Now, in *threshold* press *Select* and adjust the color range of the desired tissue. It is possible to pick the color in the image or to adjust the sliders in the threshold box. After this it is important to manually refine using *Toggle Display Threshold* can be useful to  confirm selection. When an accurate selection is made click the *Preview* button in *General* tab. Then click the *Measure* button to obtain new bone volume (TV). In this step it is necessary to select *Memorize to comment* in *Options* menu before saving. It is advised to save a new image for each measurement step.

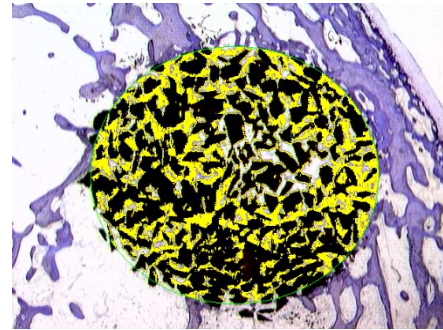





Figure 9 – New bone selected

**Measure particle volume** Highlight *D5* in the *Selected* box of the *Select Arrays* windows and repeat the last step procedures.

If necessary it is possible to manually select the particles boundaries using the *draw threshold* tool  After that use the *fill closed object* tool  to fill the selection. If needed, the *erase threshold* tool  can also be used. Then click in *Auto-Measure* -> *Preview* and *Measure*.

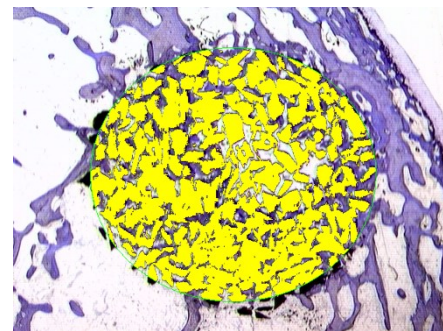


Figure 10 – Particles selected

**New slide analysis** go to *File* -> *New* -> *Quick Data Set...* and repeat every step from the beginning.

To **export the results into an Excel file**, choose *File* -> *Save Data*, then *File* -> *Data Manager*. In the *BIOQUANT Data Manager* window, highlight *BQDATA* folder in the left box as shown below

Click *Search for BIOQUANT Volumes* button on the main toolbar, you will see all the data sets in the middle box. Highlight them, and you'll see the results appear in the right box.

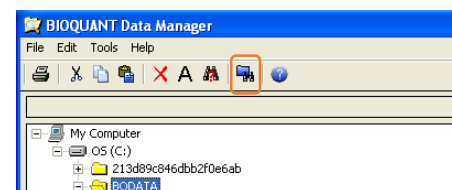


Figure 11 – Data Manager window

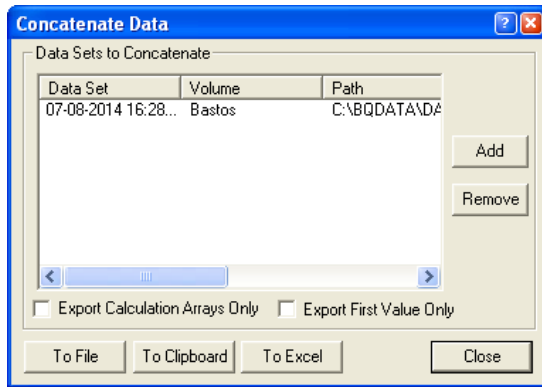


Figure 12 – Concatenate Data window

Click *File -> Concatenate Data...*, when you see the *Concatenate Data* window appears, highlight the data set you want to export in the middle box, click *Add* button in the *Concatenate Data* window. You'll see your data set show up in the *Data Sets to Concatenate* box. Click *To Excel* button to export the data to excel spreadsheet file. If you do not want to see

blank arrays, check *Export Calculation Arrays Only* box.

**Topography** if desired, it is possible to create a topographic image of the slide. Go to *new atlas window* then *resize to fit* and *black box ground*.

You can export your topographic image under *File -> Export atlas*.





## CONCLUSION

Histomorphometry on a bone biopsy is the gold standard for the clinical assessment of tissue-level bone activity.

Bone histomorphometry limitations relate to technical limitations, protocol complexity, costs, required time and the impossibility to study a real 3D specimen. It could, therefore, be useful to combine histomorphometry analysis with other tri-dimensional image study methods such as micro-CT to maximize obtainable information.

Bone histomorphometry protocol relies on a still image and its conditioned by its quality. It is, therefore, essential to strictly follow every step from specimen collection to slide staining in order to improve slide quality.

In this protocol, a magnification of 0,75X is used. The author suggests that if several images with higher magnification were stitched together with digital image processing software it would be possible to obtain a better image quality and, perhaps, more accurate results. This conclusion presents an important improvement to the technique described and used worldwide in histomorphometry.

There is some subjectivity during analysis, such as choosing ROI, outlining new bone, and regeneration particles. The position at which the specimen and sample are prepared, adds variance too. Those factors are not a big issue as long as the operator remains consistent during the measurements and has enough samples. In order to be more objective, BioQuant users should not know which slice they are working on.

The array file *teseJB.bqa* and calculation file *teseJB.bqc* mentioned in this protocol were defined by the author and can be used in future scientific work to simplify the procedure. They allow for the percentual study of new bone formation and regeneration particles.

Due to the low accuracy of bioquant software in particle selection it is strongly advised that the particles are manually delimited or refined.

## BIBLIOGRAPHY

1. Rodan, G.A., *Introduction to bone biology*. Bone, 1992. **13 Suppl 1**: p. S3-6.
2. Bilezikian, J.P., L.G. Raisz, and G.A. Rodan, *Principles of bone biology*. 2nd ed. 2002, San Diego: Academic Press.
3. Clarke, B., *Normal bone anatomy and physiology*. Clin J Am Soc Nephrol, 2008. **3 Suppl 3**: p. S131-9.
4. Burr, D.B. and M.R. Allen, *Basic and applied bone biology*. 2014. xv, 373 pages.
5. Junqueira, L.C.U.a., J. Carneiro, and A.N. Contopoulos, *Basic histology*, in *A Concise medical library for practitioner and student*. 1975, Lange Medical Publications: Los Altos, Calif. p. v.
6. Theoleyre, S., et al., *The molecular triad OPG/RANK/RANKL: involvement in the orchestration of pathophysiological bone remodeling*. Cytokine Growth Factor Rev, 2004. **15**(6): p. 457-75.
7. Sims, N.A. and J.H. Gooi, *Bone remodeling: Multiple cellular interactions required for coupling of bone formation and resorption*. Semin Cell Dev Biol, 2008. **19**(5): p. 444-51.
8. Rose, T., et al., *The role of cell type in bone healing mediated by ex vivo gene therapy*. Langenbecks Arch Surg, 2003. **388**(5): p. 347-55.
9. Lemaire, V., et al., *Modeling the interactions between osteoblast and osteoclast activities in bone remodeling*. J Theor Biol, 2004. **229**(3): p. 293-309.
10. Martin, T.J., *Historically significant events in the discovery of RANK/RANKL/OPG*. World J Orthop, 2013. **4**(4): p. 186-197.
11. Deng, Z.L., et al., *Regulation of osteogenic differentiation during skeletal development*. Front Biosci, 2008. **13**: p. 2001-21.
12. Bonewald, L.F. and M.L. Johnson, *Osteocytes, mechanosensing and Wnt signaling*. Bone, 2008. **42**(4): p. 606-15.
13. Kierszenbaum, A.L., *Histology and cell biology : an introduction to pathology*. 2nd ed. 2007, Philadelphia, PA: Mosby Elsevier. xiv, 671 p.
14. Kierszenbaum, A.L. and L.L. Tres, *Histology and cell biology : an introduction to pathology*. 3rd ed. 2012, Philadelphia, PA: Saunders. xiv, 701 p.
15. Xing, L. and B.F. Boyce, *Regulation of apoptosis in osteoclasts and osteoblastic cells*. Biochem Biophys Res Commun, 2005. **328**(3): p. 709-20.
16. Noble, B.S. and J. Reeve, *Osteocyte function, osteocyte death and bone fracture resistance*. Mol Cell Endocrinol, 2000. **159**(1-2): p. 7-13.
17. Seeman, E., *Osteocytes--martyrs for integrity of bone strength*. Osteoporos Int, 2006. **17**(10): p. 1443-8.
18. Geddes, A.C., *The Origin of the Osteoblast and of the Osteoclast*. J Anat Physiol, 1913. **47**(Pt 2): p. 159-76.
19. Tonna, E.A., *Periosteal osteoclasts, skeletal development and ageing*. Nature, 1960. **185**: p. 405-7.
20. Gupta, A., et al., *Identification of the type II Na(+)-Pi cotransporter (Npt2) in the osteoclast and the skeletal phenotype of Npt2<sup>-/-</sup> mice*. Bone, 2001. **29**(5): p. 467-76.
21. Cohen, M.M., Jr., *The new bone biology: pathologic, molecular, and clinical correlates*. Am J Med Genet A, 2006. **140**(23): p. 2646-706.
22. Datta, H.K., et al., *The cell biology of bone metabolism*. J Clin Pathol, 2008. **61**(5): p. 577-87.
23. Schneider, A., et al., *Skeletal homeostasis in tissue-engineered bone*. J Orthop Res, 2003. **21**(5): p. 859-64.
24. Seeman, E., *Structural basis of growth-related gain and age-related loss of bone strength*. Rheumatology (Oxford), 2008. **47 Suppl 4**: p. iv2-8.
25. Palma, P., *Estudo de novas formulações para regeneração óssea em defeitos de dimensão crítica*. 2009.
26. Tsangari, H., D.M. Findlay, and N.L. Fazalari, *Structural and remodeling indices in the cancellous bone of the proximal femur across adulthood*. Bone, 2007. **40**(1): p. 211-7.
27. Recker, R.R., et al., *Issues in modern bone histomorphometry*. Bone, 2011. **49**(5): p. 955-64.
28. Dempster, D.W., et al., *Standardized nomenclature, symbols, and units for bone histomorphometry: a 2012 update of the report of the ASBMR Histomorphometry Nomenclature Committee*. J Bone Miner Res, 2013. **28**(1): p. 2-17.

29. Parfitt, A.M., et al., *Bone histomorphometry: standardization of nomenclature, symbols, and units. Report of the ASBMR Histomorphometry Nomenclature Committee*. J Bone Miner Res, 1987. **2**(6): p. 595-610.
30. Revell, P.A., *Histomorphometry of bone*. J Clin Pathol, 1983. **36**(12): p. 1323-31.
31. Moore, P.R., R. Gokal, and J.A. Hoyland, *Bone biopsy--beyond light microscopy and histodynamics*. Nephrol Dial Transplant, 1997. **12**(4): p. 659-63.
32. Cano, J., et al., *High resolution image in bone biology II. Review of the literature*. Med Oral Patol Oral Cir Bucal, 2008. **13**(1): p. E31-5.
33. Chappard, D., et al., *New laboratory tools in the assessment of bone quality*. Osteoporos Int, 2011. **22**(8): p. 2225-40.
34. Schortinghuis, J., et al., *Microradiography to evaluate bone growth into a rat mandibular defect*. Arch Oral Biol, 2003. **48**(2): p. 155-60.
35. Gielkens, P.F., et al., *A comparison of micro-CT, microradiography and histomorphometry in bone research*. Arch Oral Biol, 2008. **53**(6): p. 558-66.
36. Marechal, M., et al., *Histomorphometry and micro-computed tomography of bone augmentation under a titanium membrane*. Clin Oral Implants Res, 2005. **16**(6): p. 708-14.
37. Tamminen, I.S., et al., *Reproducibility and agreement of micro-CT and histomorphometry in human trabecular bone with different metabolic status*. J Bone Miner Metab, 2011. **29**(4): p. 442-8.
38. Slyfield, C.R., et al., *Three-dimensional dynamic bone histomorphometry*. J Bone Miner Res, 2012. **27**(2): p. 486-95.
39. Pearce, A.I., et al., *Animal models for implant biomaterial research in bone: a review*. Eur Cell Mater, 2007. **13**: p. 1-10.
40. Struillou, X., et al., *Experimental animal models in periodontology: a review*. Open Dent J, 2010. **4**: p. 37-47.
41. Schimandle, J.H. and S.D. Boden, *Spine update. Animal use in spinal research*. Spine (Phila Pa 1976), 1994. **19**(21): p. 2474-7.
42. Kim, S., et al., *Reosseointegration of mechanically disintegrated implants in dogs: mechanical and histometric analyses*. Clin Oral Implants Res, 2014. **25**(6): p. 729-34.
43. Friedmann, A., et al., *Convergent methods assessing bone growth in an experimental model at dental implants in the minipig*. Ann Anat, 2014. **196**(2-3): p. 100-7.
44. Alghamdi, H.S., et al., *Synergistic effects of bisphosphonate and calcium phosphate nanoparticles on peri-implant bone responses in osteoporotic rats*. Biomaterials, 2014. **35**(21): p. 5482-90.
45. Raposo-Amaral, C.E., et al., *Is bone transplantation the gold standard for repair of alveolar bone defects?* J Tissue Eng, 2014. **5**: p. 2041731413519352.
46. Park, J.W., et al., *Bone healing with oxytocin-loaded microporous beta-TCP bone substitute in ectopic bone formation model and critical-sized osseous defect of rat*. J Clin Periodontol, 2014. **41**(2): p. 181-90.
47. Alaei, F., et al., *Evaluation of osteogenic cell differentiation in response to bone morphogenetic protein or demineralized bone matrix in a critical sized defect model using GFP reporter mice*. J Orthop Res, 2014.
48. Hazzard, D.G., et al., *Selection of an appropriate animal model to study aging processes with special emphasis on the use of rat strains*. J Gerontol, 1992. **47**(3): p. B63-4.
49. Donath, K., *The diagnostic value of the new method for the study of undecalcified bones and teeth with attached soft tissue (Sage-Schliff (sawing and grinding) technique)*. Pathol Res Pract, 1985. **179**(6): p. 631-3.
50. Matos, S., *Aplicação de matrizes enriquecidas com moduladores biológicos na regeneração de tecidos periodontais e defeitos ósseos*. Tese de Doutorado, 2008.
51. Guerra, F., *Biomimética - Formulações haptóticas para aplicação em regeneração óssea*. Tese de Doutorado, 2003.
52. Dettmeyer, R.B., *Forensic Histopathology*. 2011: Springer. 20.
53. Dettmeyer, R.B., *Forensic Histopathology*. 2011: Springer. 454.
54. Kulak, C.A. and D.W. Dempster, *Bone histomorphometry: a concise review for endocrinologists and clinicians*. Arq Bras Endocrinol Metabol, 2010. **54**(2): p. 87-98.
55. Rauch, F., *Watching bone cells at work: what we can see from bone biopsies*. Pediatr Nephrol, 2006. **21**(4): p. 457-62.

56. Ott, S.M., *Histomorphometric measurements of bone turnover, mineralization, and volume*. Clin J Am Soc Nephrol, 2008. **3 Suppl 3**: p. S151-6.
57. Malluche, H.H., et al., *A new semiautomatic method for quantitative static and dynamic bone histology*. Calcif Tissue Int, 1982. **34**(5): p. 439-48.
58. Jager, A., W. Doler, and G. Sattler, *Semiautomatic method for histomorphometry*. Comput Methods Programs Biomed, 1990. **33**(1): p. 49-55.
59. Egan, K.P., T.A. Brennan, and R.J. Pignolo, *Bone histomorphometry using free and commonly available software*. Histopathology, 2012. **61**(6): p. 1168-73.
60. Doube, M., et al., *BoneJ: Free and extensible bone image analysis in ImageJ*. Bone, 2010. **47**(6): p. 1076-9.
61. Schwartz, M.P. and R.R. Recker, *Comparison of surface density and volume of human iliac trabecular bone measured directly and by applied stereology*. Calcif Tissue Int, 1981. **33**(6): p. 561-5.
62. Turner, C.H. and F.M. Pavalko, *Mechanotransduction and functional response of the skeleton to physical stress: the mechanisms and mechanics of bone adaptation*. J Orthop Sci, 1998. **3**(6): p. 346-55.
63. Vandeweghe, S., et al., *Utilizing micro-computed tomography to evaluate bone structure surrounding dental implants: a comparison with histomorphometry*. J Biomed Mater Res B Appl Biomater, 2013. **101**(7): p. 1259-66.
64. Morberg, P.H., et al., *Improved long-term bone-implant integration. Experiments in transgenic mice overexpressing bovine growth hormone*. Acta Orthop Scand, 1997. **68**(4): p. 344-8.
65. Gonzalez-Garcia, R. and F. Monje, *Is micro-computed tomography reliable to determine the microstructure of the maxillary alveolar bone?* Clin Oral Implants Res, 2013. **24**(7): p. 730-7.
66. Park, C.H., et al., *Three-dimensional micro-computed tomographic imaging of alveolar bone in experimental bone loss or repair*. J Periodontol, 2007. **78**(2): p. 273-81.
67. Particelli, F., et al., *A comparison between micro-CT and histology for the evaluation of cortical bone: effect of polymethylmethacrylate embedding on structural parameters*. J Microsc, 2012. **245**(3): p. 302-10.
68. Schouten, C., et al., *The quantitative assessment of peri-implant bone responses using histomorphometry and micro-computed tomography*. Biomaterials, 2009. **30**(27): p. 4539-49.
69. Xavier, S.P., et al., *Maxillary sinus grafting with autograft vs. fresh frozen allograft: a split-mouth histomorphometric study*. Clin Oral Implants Res, 2014.
70. de Lange, G.L., et al., *A histomorphometric and micro-computed tomography study of bone regeneration in the maxillary sinus comparing biphasic calcium phosphate and deproteinized cancellous bovine bone in a human split-mouth model*. Oral Surg Oral Med Oral Pathol Oral Radiol, 2014. **117**(1): p. 8-22.





UNIVERSIDADE DE COIMBRA