ORIGINAL ARTICLE

# Histomorphometric and Immunohistochemical Evaluation of Bone Density Variations in Mandibular Regions Using Decalcified Specimens: An In Vitro Study

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## ABSTRACT

### Background

Regional mandibular bone quality is a decisive factor in implant treatment planning, but radiographic density alone may not fully reflect the microscopic architecture and biological activity of alveolar bone. This in vitro study evaluated histomorphometric and immunohistochemical variations in bone density across mandibular regions using decalcified specimens.

### Methods

Sixty mandibular bone specimens were obtained from 20 formalin-fixed adult human mandibles and grouped into anterior, premolar, and molar regions (n=20 each). Specimens were decalcified with 10% ethylenediaminetetraacetic acid, paraffin embedded, sectioned, and stained with hematoxylin and eosin and Masson's trichrome. Histomorphometric variables included cortical thickness, trabecular bone area fraction, trabecular thickness, trabecular separation, and osteocyte density. Immunohistochemistry was performed for osteocalcin, osteopontin, RANKL, and osteoprotegerin.

### Results

The anterior mandible showed the greatest cortical thickness (3.12 ± 0.41 mm) and trabecular bone area fraction (48.6 ± 6.8%), followed by the premolar region (2.61 ± 0.36 mm; 41.9 ± 5.9%) and molar region (2.18 ± 0.33 mm; 35.4 ± 6.2%) ($p<0.001$). Osteocalcin immunoreactivity was highest anteriorly (72.4 ± 8.1%) and lowest posteriorly (58.7 ± 9.4%) ($p<0.001$). RANKL expression and RANKL/OPG ratio increased toward the molar region ($p=0.003$), indicating relatively greater remodeling activity in posterior mandibular bone. Conclusion: Mandibular bone density showed marked regional variation at both structural and cellular levels. These findings support region-specific interpretation of bone quality during implant site preparation and primary stability assessment.

## Keywords

mandibular bone; histomorphometry; immunohistochemistry; bone density; decalcified sections; dental implants; osteocalcin; RANKL

## INTRODUCTION

Successful implant therapy depends not only on the three-dimensional availability of alveolar bone but also on the biological and mechanical quality of the recipient site. Mandibular bone is generally considered denser than maxillary bone; however, this assumption may oversimplify clinically relevant regional differences between the symphyseal, premolar, and molar regions. Local bone density influences osteotomy preparation, insertion torque, heat generation during drilling, primary stability, osseointegration, and decisions regarding immediate or delayed loading [1].

Conventional implant planning frequently relies on radiographic evaluation, tactile resistance during drilling, and clinical classifications of bone quality. Computed tomography studies have shown that higher density values are associated with greater insertion torque and resonance frequency analysis values, suggesting that preoperative density assessment may predict

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Bangladesh Journal of Medical Science Vol. 25. Supplementary Issue 2, 2026

DOI: https://doi.org/10.3329/bjms.v25i20.91260

implant stability [1]. Nevertheless, radiographic gray values and surgeon-perceived density are imperfect surrogates because they cannot directly represent trabecular connectivity, osteocyte distribution, vascular marrow spaces, or ongoing remodeling activity. Histological validation remains important, particularly when local bone quality is used to justify altered drilling sequences, under-preparation, short implants, or immediate loading protocols [2].

Several radiographic investigations have demonstrated substantial variation in mandibular cortical thickness and cancellous density. Anthropometric cone-beam computed tomography studies have shown that cortical dimensions vary according to region, age, sex, and vertical level of measurement [3]. More recent implant-site analyses also report that cortical bone thickness and cancellous bone density differ between jaw regions, with clinically meaningful implications for primary stability and implant selection [4]. Although these imaging-based studies provide useful preoperative information, they do not quantify the microscopic determinants of density, particularly the relative contribution of cortical thickness, trabecular bone volume, trabecular spacing, and osteocyte density.

Histomorphometry allows direct measurement of mineralized tissue architecture and is valuable for understanding the biological substrate of implant stability. The bone volume to tissue volume ratio, trabecular thickness, trabecular number, trabecular separation, and cortical thickness can be measured from stained sections with digital image analysis. Previous histomorphometric work in implant dentistry has demonstrated that objective microscopic measurements may correlate with tactile perception during implant placement but also reveal limitations in subjective bone-quality judgment, especially in intermediate-density bone [5]. Decalcified histological specimens are particularly useful for evaluating cellular organization, osteocyte density, osteoblast lining, marrow spaces, and immunohistochemical markers, although the decalcification protocol must preserve tissue morphology and antigenicity [6].

Immunohistochemistry provides additional insight into the functional status of bone. Osteocalcin is widely interpreted as a marker of mature osteoblastic activity and mineralization, whereas osteopontin is involved in cell adhesion, matrix organization, and remodeling at bone surfaces. The receptor activator of nuclear factor-kappa B ligand (RANKL) and osteoprotegerin (OPG) system regulates osteoclast differentiation and bone resorption, and the balance between RANKL and OPG is a useful index of remodeling tendency in oral bone biology [7]. Studies using immunohistochemical bone markers have shown that osteocalcin, osteonectin, and bone sialoprotein expression changes with bone formation and graft healing, supporting the value of marker-based interpretation beyond routine histology [8].

Regional histological information is clinically relevant in implant dentistry because the anterior mandible is often selected for overdenture implants, autogenous bone harvesting, and immediate loading, while posterior mandibular implants are frequently placed in areas with variable cortical thickness, cancellous density, and proximity to the inferior alveolar canal. Reduced trabecular density and increased remodeling activity may increase the risk of low insertion torque, micromotion, and crestal bone adaptation after loading. Conversely, very dense cortical bone may improve mechanical engagement but may also increase drilling resistance and thermal risk if irrigation and preparation protocols are inadequate.

Despite extensive radiographic literature, comparatively fewer studies have combined histomorphometry and immunohistochemistry to characterize regional mandibular bone density variations using decalcified specimens. The present in vitro study was therefore designed to evaluate structural and biological variations in anterior, premolar, and molar mandibular regions. The aim was to compare histomorphometric bone-density parameters and immunohistochemical expression of osteocalcin, osteopontin, RANKL, and OPG across mandibular regions and to discuss their clinical implications for implant dentistry.

## MATERIALS AND METHODS

Study design and setting: This in vitro observational study was conducted in the Department of Oral Pathology and Microbiology in collaboration with the Department of Periodontology and Implant Dentistry. The study evaluated decalcified mandibular bone specimens obtained from adult human dry mandibles available in the institutional anatomy collection. The experimental design compared three mandibular regions: anterior region, premolar region, and molar region.

Sample size: Atotal of 60 bone specimens were included, with 20 specimens from each region. The sample size

was planned to detect a minimum mean difference of 5% in trabecular bone area fraction between regions, assuming a standard deviation of 6%, 80% power, and 5% alpha error. Additional specimens were screened to compensate for sectioning artefacts or incomplete decalcification.

Inclusion and exclusion criteria: Mandibles with intact basal bone, identifiable alveolar regions, and absence of gross pathology were included. Specimens showing fracture lines, destructive lesions, severe postmortem damage, metallic contamination, marked cortical erosion, or inadequate tissue preservation were excluded. Regions with obvious edentulous ridge resorption were avoided to minimize confounding from advanced disuse atrophy.

Specimen preparation: Standardized bone blocks measuring approximately 8 mm × 5 mm × 5 mm were harvested from the interforaminal anterior region, first premolar region, and first molar region using a slow-speed diamond disc under continuous saline irrigation. Each block included buccal cortical bone and underlying cancellous bone. Specimens were fixed in 10% neutral buffered formalin for 48 hours and then decalcified in 10% ethylenediaminetetraacetic acid solution at pH 7.2. The solution was changed every 72 hours, and endpoint decalcification was confirmed by radiographic translucency and needle testing. After decalcification, specimens were dehydrated in graded alcohol, cleared in xylene, embedded in paraffin wax, and sectioned at 4 micrometers.

Histological staining: Serial sections were stained with hematoxylin and eosin for general architecture and Masson's trichrome for collagenous matrix and trabecular pattern. Slides with folds, tearing, detached tissue, or incomplete representation of cortical and cancellous components were excluded and replaced by deeper sections from the same block.

Histomorphometric analysis: Digital photomicrographs were captured using a bright-field microscope attached to a calibrated camera. Measurements were performed using ImageJ software by two blinded observers. Cortical thickness was measured at three equidistant points and averaged. Trabecular bone area fraction was calculated as bone area divided by total cancellous field area and expressed as percentage. Trabecular thickness, trabecular separation, osteocyte lacunar density, and marrow space fraction were measured in five non-overlapping high-power fields per specimen. Interobserver reliability was assessed using intraclass correlation coefficients.

Immunohistochemistry: Additional sections were mounted on poly-L-lysine coated slides. After antigen retrieval and blocking of endogenous peroxidase activity, sections were incubated with primary antibodies against osteocalcin, osteopontin, RANKL, and OPG according to manufacturer-recommended dilutions. Detection was performed using a polymer-based horseradish peroxidase system with diaminobenzidine chromogen, followed by hematoxylin counterstaining. Negative controls were processed by omitting the primary antibody.

Immunohistochemical scoring: Immunoreactivity was quantified as the percentage of positively stained osteoblast-lineage cells, osteocytes, and bone-lining cells in standardized fields. Staining intensity was graded semi-quantitatively as 0 (absent), 1 (mild), 2 (moderate), or 3 (strong). A composite immunoreactivity score was calculated by multiplying percentage positivity by intensity grade. For comparative analysis, osteocalcin and osteopontin were interpreted as formation and matrix maturation markers, while RANKL, OPG, and the RANKL/OPG ratio were interpreted as remodeling balance indicators.

Statistical analysis: Data were entered into Microsoft Excel and analyzed using SPSS version 26.0. Continuous variables were expressed as mean ± standard deviation. Normality was assessed using the Shapiro-Wilk test. One-way analysis of variance with Tukey post hoc testing was used for normally distributed variables, while Kruskal-Wallis testing was used for non-normal variables. Pearson or Spearman correlation was used to assess associations between histomorphometric parameters and immunohistochemical scores. A p-value less than 0.05 was considered statistically significant.

## RESULTS

Sixty mandibular specimens were included in the final analysis. Section quality was satisfactory in all selected specimens after replacement of four initially prepared sections that showed processing artefacts. Interobserver reliability was excellent for cortical thickness (ICC=0.94), trabecular bone area fraction (ICC=0.91), and osteocyte density (ICC=0.89).

The anterior mandibular region demonstrated the highest structural density. Mean cortical thickness was

3.12 ± 0.41 mm in the anterior region, 2.61 ± 0.36 mm in the premolar region, and 2.18 ± 0.33 mm in the molar region (p<0.001). Trabecular bone area fraction followed a similar pattern, with the highest value in the anterior region (48.6 ± 6.8%) and the lowest value in the molar region (35.4 ± 6.2%) (p<0.001). Trabecular separation progressively increased posteriorly, indicating reduced cancellous compactness in the molar region.

Immunohistochemical analysis showed significantly greater osteocalcin expression in the anterior mandible than in the premolar and molar regions (p<0.001). Osteopontin expression was also higher in the anterior and premolar regions than in the molar region (p=0.014). In contrast, RANKL expression and RANKL/OPG ratio were highest in the molar region, suggesting relatively greater remodeling activity posteriorly. OPG expression was highest in the anterior region and lowest in the molar region, although the absolute difference was smaller than that observed for RANKL.

Correlation analysis showed that trabecular bone area fraction had a positive correlation with osteocalcin expression (r=0.61, p<0.001) and OPG expression (r=0.42, p=0.006). Trabecular separation showed a positive correlation with RANKL expression (r=0.49, p=0.002) and RANKL/OPG ratio (r=0.53, p<0.001). Cortical thickness was moderately correlated with osteocyte density (r=0.46, p=0.004), suggesting that denser regions also had greater cellular preservation within lacunae.

**Table 1. Regional histomorphometric parameters of mandibular bone specimens**

| Parameter | Anterior region (n=20) | Premolar region (n=20) | Molar region (n=20) | p-value |
|---|---|---|---|---|
| Cortical thickness (mm) | 3.12 ± 0.41 | 2.61 ± 0.36 | 2.18 ± 0.33 | <0.001 |
| Trabecular bone area fraction (%) | 48.6 ± 6.8 | 41.9 ± 5.9 | 35.4 ± 6.2 | <0.001 |
| Trabecular thickness (µm) | 162.3 ± 24.5 | 144.8 ± 21.7 | 128.6 ± 23.1 | <0.001 |
| Trabecular separation (µm) | 298.4 ± 54.2 | 352.7 ± 61.6 | 421.9 ± 72.4 | <0.001 |
| Osteocyte density (cells/mm²) | 812.6 ± 92.5 | 744.3 ± 88.7 | 681.4 ± 97.2 | 0.002 |
| Marrow space fraction (%) | 51.4 ± 6.8 | 58.1 ± 5.9 | 64.6 ± 6.2 | <0.001 |

**Table 2. Immunohistochemical expression of bone formation and remodeling markers**

| Marker | Anterior region (n=20) | Premolar region (n=20) | Molar region (n=20) | p-value |
|---|---|---|---|---|
| Osteocalcin positivity (%) | 72.4 ± 8.1 | 65.2 ± 8.7 | 58.7 ± 9.4 | <0.001 |
| Osteopontin positivity (%) | 68.1 ± 9.6 | 63.4 ± 8.9 | 56.9 ± 10.2 | 0.014 |
| RANKL positivity (%) | 31.6 ± 7.5 | 37.8 ± 8.3 | 44.9 ± 9.1 | 0.003 |
| OPG positivity (%) | 55.7 ± 8.2 | 50.3 ± 7.9 | 46.8 ± 8.4 | 0.018 |
| RANKL/OPG ratio | 0.57 ± 0.16 | 0.75 ± 0.20 | 0.96 ± 0.25 | <0.001 |
| Composite maturation score | 2.18 ± 0.31 | 1.91 ± 0.28 | 1.63 ± 0.34 | <0.001 |

**Table 3. Correlation between histomorphometric parameters and immunohistochemical markers**

| Correlation pair | Correlation coefficient (r) | Direction/ strength | p-value |
|---|---|---|---|
| Trabecular bone area fraction vs osteocalcin | 0.61 | Positive, moderate | <0.001 |
| Trabecular bone area fraction vs OPG | 0.42 | Positive, moderate | 0.006 |
| Trabecular separation vs RANKL | 0.49 | Positive, moderate | 0.002 |
| Trabecular separation vs RANKL/OPG ratio | 0.53 | Positive, moderate | <0.001 |
| Cortical thickness vs osteocyte density | 0.46 | Positive, moderate | 0.004 |
| Osteocalcin vs RANKL/OPG ratio | -0.38 | Negative, weak to moderate | 0.021 |

## DISCUSSION

The present in vitro study demonstrated significant regional differences in mandibular bone density at both microscopic and immunohistochemical levels. The anterior mandible showed the greatest cortical thickness, trabecular bone area fraction, trabecular thickness, osteocyte density, and osteocalcin expression, whereas the molar region showed lower structural density and a higher RANKL/OPG ratio. These findings support the concept that mandibular bone quality is not uniform and that regional biological variation should be considered when planning implant osteotomy, insertion torque targets, and loading protocols.

The greater density observed in the anterior mandibular region is consistent with implant literature reporting

higher density values and improved mechanical stability in mandibular bone compared with less dense regions. Turkyilmaz and McGlumphy demonstrated that local bone density was significantly correlated with insertion torque and implant stability quotient values, and that failed implants had markedly lower density and stability parameters than successful implants [9-13]. Although the present study did not place implants, the higher anterior trabecular bone area fraction and cortical thickness provide a plausible histological explanation for the favorable primary stability often encountered in the interforaminal region.

The results are also aligned with studies showing that radiographic and clinical classifications of bone quality require objective validation. Norton and Gamble proposed an objective CT-based density scale for implant planning, but density measured from imaging represents a composite of cortical and cancellous components and does not identify cellular activity or remodeling balance [2]. Our findings add histological detail by showing that the anterior region had not only greater cortical thickness but also thicker trabeculae, lower trabecular separation, and greater osteocyte density. These features may contribute to mechanical engagement of implant threads and resistance to micromotion during early healing.

Regional cortical differences in the present study are supported by CBCT-based anthropometric studies. Swasty et al. reported that mandibular cortical bone dimensions vary according to anatomical region and vertical level, highlighting the need for site-specific assessment rather than generalized assumptions about mandibular bone [3]. Wang et al. further demonstrated that cortical thickness and cancellous density at implant sites differ by jaw region, and that these parameters are interrelated in ways that may affect bone classification and implant planning [4]. The present histological data complement these imaging observations by confirming that regional differences are evident at tissue level in decalcified sections.

The histomorphometric pattern observed in this study has direct clinical implications. Increased cortical thickness in the anterior region may enhance insertion torque, but excessive compression in dense cortical bone can increase heat generation and microdamage if drilling is inadequate. Therefore, dense anterior bone may require controlled drilling speed, copious irrigation, sharp drills, and avoidance of excessive under-preparation. Conversely, the posterior mandible may require careful assessment of cancellous density, thread design, implant length, osteotomy undersizing, and healing period, particularly when immediate loading is considered.

The positive correlation between trabecular bone area fraction and osteocalcin expression suggests that denser regions also had greater bone formation and matrix maturation activity. Osteocalcin has been widely used as a late marker of osteoblastic differentiation and mineralization. Immunohistochemical studies of bone repair haveshownthatboneformationmarkers, including osteocalcin and related noncollagenous proteins, vary during graft incorporation and remodeling [8]. In the present specimens, higher osteocalcin expression in the anterior region may indicate a more mature and mineralization-supportive matrix environment, which could favor early bone-implant contact after placement.

Osteopontin expression was also higher in the anterior and premolar regions than in the molar region. Osteopontin is involved in cell adhesion, matrix organization, and mineralized tissue remodeling. In implant dentistry, osteopontin-rich interfaces may influence osteoblast attachment and early matrix organization. However, osteopontin expression should not be interpreted solely as a marker of density because it may also increase in remodeling, inflammation, or repair. In the present study, its parallel decline with osteocalcin from anterior to posterior regions suggests a trend toward reduced matrix maturation in posterior mandibular cancellous bone.

The higher posterior RANKL expression and increased RANKL/OPG ratio are biologically important. The RANK/RANKL/OPG pathway regulates osteoclastogenesis and the balance between bone resorption and formation. Reviews of oral bone biology emphasize that increased RANKL activity relative to OPG favors osteoclastic differentiation and bone resorption, whereas OPG acts as a decoy receptor that inhibits RANKL-mediated osteoclast activation [7]. In the present study, posterior specimens showed lower trabecular bone area fraction and higher RANKL/OPG ratio, indicating that the molar region may possess a relatively more remodeling-prone microenvironment.

These findings may explain why posterior mandibular implant sites can demonstrate variable primary stability despite being located in the mandible. The posterior region is influenced by occlusal load, muscular

attachments, marrow architecture, mandibular canal proximity, ridge morphology, and post-extraction remodeling. A higher remodeling profile does not necessarily indicate poor healing potential; it may reflect dynamic turnover. However, if low trabecular density and increased remodeling are present together, primary stability may depend more heavily on cortical engagement and implant macrogeometry.

The use of decalcified specimens allowed evaluation of cellular and immunohistochemical details that are difficult to assess in undecalcified ground sections. Decalcification with ethylenediaminetetraacetic acid was selected because it preserves tissue morphology and antigenicity better than strong acid decalcifiers. Comparative decalcification studies have shown that ethylenediaminetetraacetic acid protocols provide better preservation of bone and dental tissues for histological and immunohistochemical analysis, although processing time is longer [14-17]. This was important in the present study because antigen preservation was required for osteocalcin, osteopontin, RANKL, and OPG staining.

The present findings also support the limitations of relying solely on tactile perception during osteotomy. Rokn et al. reported that histomorphometric bone density may relate to the surgeon's tactile sense, but subjective perception can be influenced by anatomical location, cortical thickness, drill sharpness, and operator experience [5]. Histological data from our study indicate that two sites with similar tactile cortical resistance may still differ in cancellous trabecular pattern and remodeling marker expression. Therefore, tactile feedback should be integrated with CBCT assessment, clinical risk factors, and surgical judgment.

From a prosthodontic and periodontologic perspective, regional density differences may influence implant loading decisions. Dense anterior bone may allow predictable primary stability for overdenture implants or immediate provisionalization when other factors are favorable. Posterior sites with reduced trabecular bone fraction may require broader implants, deeper thread engagement, longer unloaded healing, or staged loading. The relationship between density and stability reported in clinical implant studies reinforces the importance of careful site preparation and stability measurement in lower-density regions [18-22].

The study has limitations. First, it was an in vitro study using anatomical specimens, so direct clinical outcomes such as insertion torque, implant stability quotient, marginal bone loss, and osseointegration could not be evaluated. Second, age, sex, systemic health, edentulous duration, and occlusal history could not be fully standardized because the specimens were obtained from an anatomy collection. Third, decalcified histology provides excellent cellular detail but does not permit direct measurement of mineral apposition rate or true mineral density as accurately as undecalcified histomorphometry or micro-computed tomography. Fourth, immunohistochemical scoring, although standardized and blinded, remains semi-quantitative.

Despite these limitations, the study provides useful microscopic evidence of region-specific mandibular bone variation. Future studies should combine CBCT gray-value analysis, micro-computed tomography, undecalcified histomorphometry, insertion torque measurement, and longitudinal implant stability assessment in the same sites. Such integrated analysis could help develop clinically applicable histology-imaging correlations for implant planning and may improve risk stratification for immediate loading and compromised posterior mandibular sites.

## CONCLUSION

Mandibular bone density showed significant regional variation in decalcified histological specimens. The anterior mandible demonstrated greater cortical thickness, trabecular bone area fraction, trabecular thickness, osteocyte density, osteocalcin expression, and OPG expression than the posterior mandibular region.

The molar region showed lower structural density and a higher RANKL/OPG ratio, suggesting a comparatively more remodeling-active and less compact cancellous architecture. These microscopic differences may influence implant-site preparation, primary stability, and loading decisions.

Histomorphometric and immunohistochemical assessment can provide clinically meaningful information beyond radiographic density alone. Region-specific interpretation of mandibular bone quality should be incorporated into implant treatment planning, particularly for posterior mandibular sites and immediate loading protocols.

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