J Clin Periodontol 2004; 31: 223–228
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Journal of Clinical Periodontology

Local biochemical markers of bone turnover: relationship to subsequent density of healing alveolar bone defects

Richard A. Reinhardt[1], Van J. Sanderfer[1], Trudy A. Meinberg[1], Pirkka Nummikoski[2], Hsi-ming Lee[3] and David B. Marx[4]

[1]Department of Surgical Specialties, University of Nebraska Medical Center College of Dentistry, Lincoln, NE, USA; [2]Department of Dental Diagnostic Science, University of Texas Health Science Center, San Antonio, TX, USA; [3]Department of Oral Biology & Pathology, State University of New York at Stony Brook School of Dental Medicine, Stony Brook, NY, USA; [4]Department of Biometry, University of Nebraska-Lincoln, Lincoln, NE, USA

Reinhardt RA, Sanderfer VJ, Meinberg TA, Nummikoski P, Lee H-M, Marx DB: Local biochemical markers of bone turnover: relationship to subsequent density of healing alveolar bone defects. J Clin Periodontol 2004; 31: 223–228. © Blackwell Munksgaard, 2004.

Abstract

Objectives: This pilot study was designed to test whether biochemical markers of bone turnover in washes of periosteal or trabecular alveolar bone surfaces could be correlated with increases in bone density of an adjacent healing implant socket.

Methods: Ten subjects had a canula inserted into the alveolar crest and sterile phosphate-buffered saline was washed over the periosteal and trabecular surfaces and collected. Surgical flaps were reflected, 5 mm diameter bone cores were removed from the bone wash site, and standardized radiographs were taken. The sites were allowed to heal for 12 weeks, and radiographs were repeated. Bone washes of the healing sites were also collected after 2 and 12 weeks. Washes were analysed for bone turnover markers osteocalcin (OC; radioimmunoassay) and C-terminal telopeptide of Type 1 collagen (ICTP; enzyme-linked immunosorbent assay (ELISA)), and blood component albumin (ALB; ELISA). Changes in bone density during healing were determined by radiographic absorptiometry.

Results: OC/ALB and ICTP/ALB ratios were higher for trabecular than periosteal washes at baseline ($p \leqslant 0.01$). Trabecular OC/ALB and ICTP/ALB were inversely correlated with increasing bone density of the healing bone core socket ($r = -0.72$, $p = 0.03$; Pearson's correlation coefficient).

Conclusions: Biochemical markers of bone turnover in bone washes of specific alveolar bone sites may prove helpful in predicting how the bone density will increase around healing dental implants.

Keywords: bone turnover, bone wash, bone density, osteocalcin, ICTP, radiographic absorptiometry

Accepted for publication 21 May 2003

Assessing bone turnover is critical to understanding the course and treatment of diseases where bone density is important, such as osteoporosis. Protein markers of bone resorption and formation are traditionally measured in serum or urine, and then used to predict future bone density outcomes and response to therapeutic interventions. However, sampling these markers near the involved bone surfaces may be more informative for site-specific oral situations, such as periodontitis and healing dental implant sites.

Osteocalcin (OC) and C-terminal telopeptide of Type I collagen (ICTP) have been used for serum assessment of bone formation/turnover and bone resorption, respectively. In general, serum OC is thought to be a marker of bone turnover when resorption and formation are coupled, and elevated in cases of rapid turnover, such as osteoporosis and fracture repair (Slovik et al. 1984, Yasumura et al. 1987). Where bone resorption is greater than bone formation, such as periodontitis, OC may be a marker of bone formation. OC and ICTP have been evaluated in human gingival crevicular fluid (GCF) (Kunimatsu et al. 1993, Talonpoika & Hamalainen 1994, Golub et al. 1997, Oringer et al. 1998, Palys et al. 1998, Al-Shammari et al. 2001) and found to be correlated with gingival inflammation, past signs of periodontitis, presence of certain periodontal pathogens, and collagenase activity in these studies. Correlations with prospective oral bone changes in humans have not been extensively studied.

The use of GCF markers to test local bone turnover is problematic from several aspects. The site of sample collection is removed from the bone surfaces by several millimeters of gingival tissue, and overlying bacterial and

soft-tissue inflammatory products, including Type I collagen, may obscure the GCF marker contributions from bone. In addition, only bone near the gingival crevice could contribute to GCF, with GCF around dental implants containing low levels of bone turnover markers (Oringer et al. 1998). More distant bone surfaces cannot be evaluated. Finally, protein markers from bone are dramatically diluted in GCF so that multiple measurements are difficult in single samples. We have reported on a novel bone-irrigating (bone wash) device that can penetrate overlying oral soft tissue adjacent to most alveolar bone surfaces and measure multiple biochemical markers of bone turnover directly from washes of the periosteal or trabecular surfaces (Wilson et al. 2003). Can these values be used to predict the nature of future bone turnover and density after bone manipulation? As a first step, the purpose of this pilot study was to measure OC and ICTP in periosteal and trabecular bone wash samples, just prior to removal of a standard bone core with a dental implant trephine bur, and during initial healing of the defect, and then compare these values to prospective changes in bone density after a 3-month healing period.

Material and Methods

Human subjects

Ten human subjects requiring oral surgery in an edentulous region signed an Institutional Review Board-approved informed consent for this study. Inclusion criteria included a 10 mm alveolar ridge height to the maxillary sinus or mandibular canal, and good general health with no previous history of bleeding or healing complications after oral surgery. Subjects were not taking any medications known to affect bone turnover, and pregnant or lactating females were excluded. Six subjects were male and four females, with seven bone core sites taken in the maxilla and three in the mandible. The mean age was 59.4 ± 3.8 (range 40–69) years.

Bone wash and bone cores

Following local anesthesia, two bone wash samples (periosteal and trabecular surfaces) were obtained on the alveolar ridge using techniques previously described (Wilson et al. 2003) at three time points: before the bone core biopsy, and again at 2 and 12 weeks post-biopsy. Briefly, an 18-gauge blunt-end needle (outer canula) penetrated gingiva/mucosa to the bone surface and a 23-gauge blunt-end needle attached to a syringe and suction apparatus was inserted into the 18-gauge needle as an inner canula. Sterile phosphate-buffered saline (PBS; 0.4 ml) was injected onto the isolated periosteal surface through the inner canula to wash the bone surface, and then the fluid was vacuum-suctioned into a sterile collection vial. After the first washing (periosteal), a cortical bone perforator (round bur) was inserted through the outer canula at a slow speed until it perforated the cortical plate. The perforator was inserted 2.5 mm into the underlying trabecular bone as determined by a depth marking on the drill shank. The drill was then removed and a sterile inner canula with the bone washing system attached was reinserted into the outer canula allowing for a trabecular washing (0.4 ml) of the area. Recovery from bone washes typically ranged from 0.3 to 0.4 ml. All of the components of the bone wash apparatus were sterile for each sampling procedure. The vials were frozen at $-70^{\circ}C$ within 5 min of collection pending analysis by commercially available radioimmunoassay and ELISA kits.

In conjunction with a periodontal or oral surgical procedure requiring a flap on the edentulous ridge on the day of the initial bone washes, the ridge was exposed and a bone core was harvested 5 mm deep with a 5 mm outside diameter using a trephine bur (Salvin Dental Specialties, Charlotte, NC, USA). The trephine was powered by a slow-speed dental handpiece further slowed 10–100-fold (through gear reduction and use of contra-angle latch attachments), and cooled with copious sterile water irrigation. At full depth, the bone core was detached from the alveolus with gentle luxation of the trephine in the non-actuated handpiece. Flaps were closed consistent with the primary surgical procedure.

Bone washes at 2 and 12 weeks postoperatively were performed by administering local anesthesia, relocating the bone core biopsy site from previously recorded clinical landmarks, and then repositioning the outer canula to engage what was thought to be the mesial or distal lateral wall of the healing defect. The periosteal and trabecular bone washes were accomplished as described above.

Quantitative radiographic analysis

A radiograph of the bone core site was taken immediately post-biopsy and repeated 3 months post-biopsy. They were analysed using a radiographic absorptiometry method similar to that previously reported (Kuhl & Nummikoski 2000). D-speed film was secured to a vertical bitewing holder containing a bone density reference wedge as a bite block. The calibration reference wedge had the same specific gravity as cortical bone ($1.84\ mg/mm^3$). A single vertical bitewing radiograph was taken of the biopsy site using standardized projection geometry and a cephalometric head positioner (Jeffcoat et al. 1987).

All radiographs were developed with the same automatic film processor (Air Techniques, Inc., Hicksville, NY, USA) and analysed at the Longitudinal Radiograph Assessment Facility (University of Texas Health Science Center at San Antonio) by an evaluator (PN) masked from clinical and biochemical data. Each radiograph was displayed on a computer monitor using a calibrated CCD video camera and the video image was converted into a 640×480 pixel digital image with an 8-bit density range with a framegrabber in the personal computer. After the baseline image was digitized and saved on the computer, the 3-month follow-up radiograph was aligned with the baseline image using a real-time subtraction procedure and digitized in that alignment. Pilot analysis indicated that a $1\ mm^2$ area of interest (AOI) 3 mm apical to the ridge crest was the easiest to locate consistently (Fig. 1), avoiding healing variability at the alveolar crest and the base of the defect. Therefore, relative changes in bone density on the buccal and lingual lateral walls over 3 months of healing were measured in each case. The density was determined by first plotting the digitized pixel values of the profile of the wedge against the thickness, and then calculating a third-degree polynomial between these variables. The derived formula was then used to transform the bone density pixel values to reference device thickness values. The obtained thickness value was divided by the measurement AOI and multiplied by the specific gravity of the reference material ($1.84\ mg/mm^3$) resulting in a final description of density in milligrams per square millimeter. The precision error of this method to measure alveolar bone density is 5.0% (Kuhl & Nummikoski 2000).

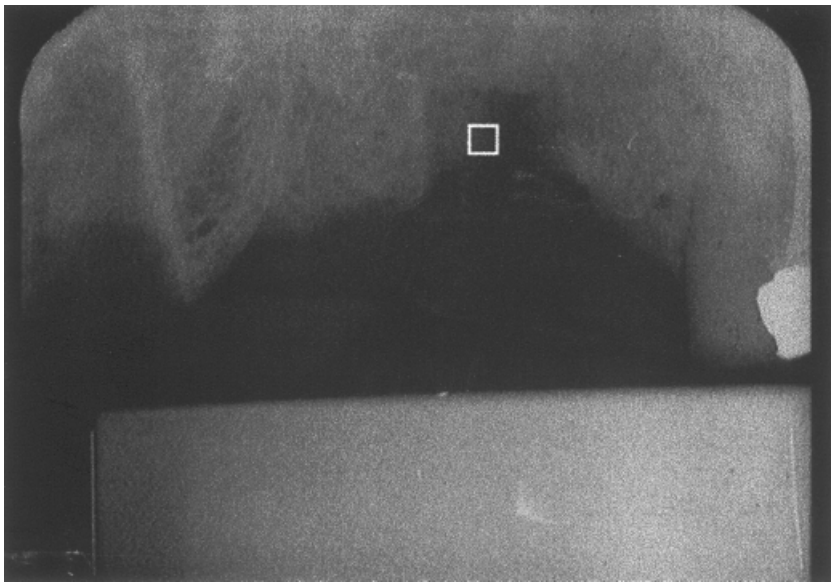

Fig. 1. Digital image of baseline vertical bitewing radiograph using standardized projection geometry. A 1 mm^2 area of interest is shown within the bone core site, located 3 mm apical to the ridge crest. At the inferior edge of the image is the bone density reference wedge used as a bite block.

Analysis of biochemical markers

OC was measured by an immunoradiometric assay (RIA) that detects both the intact bone matrix protein and its large N-terminal mid-fragment (Immutopics, Inc., San Clemente, CA, USA). Thirty microlitres thawed bone wash or 10 μl standard human OC (at concentrations ranging from 0.06 to 60 ng/ml) were incubated with 200 μl of ^{125}I-labeled antibody to OC. The mixture was then vortexed and incubated with OC antibody-coated beads for 3 h at room temperature on a low-speed shaker. The beads were washed with 0.01 M PBS containing 0.05% NaN, counted in a gamma counter, and the OC level was calculated from a standard curve. The RIA has a sensitivity to detect OC at concentrations as low as 50 pg/ml.

ICTP was measured as described earlier (Risteli et al. 1993) using 150 μl of thawed bone wash sample. The radioimmunoassay has a sensitivity to detect ICTP at concentrations as low as 340 pg/ml (DiaSorin, Inc., Stillwater, MN, USA).

Samples were analysed for albumin (ALB) using a commercially available quantitative ELISA ''sandwich'' kit according to the manufacturer's instructions (Wilson et al. 2003; Bethyl Laboratories Inc., Montgomery, TX, USA). Briefly, plates coated with capture antibody were incubated with 1:100 or 1:1000 dilutions of bone washes at room temperature for 60 min. Plates were washed and anti-human ALB-horseradish peroxidase conjugate was added to wells for 60 min. After thorough washing, a 3,3′,5,5′ tetramethyl benzidine/peroxide substrate was added for 20 min, stopped with 2 M H_2SO_4, and read at 450 nm. Unknowns were compared with a linear standard curve. The sensitivity of the assay was 12.5 ng/ml.

Values for OC, ICTP, and ALB were adjusted for dilutions, volume of retrieved bone wash sample, and portion of the sample analysed to yield total nanograms per sample. Finally, OC and ICTP were divided by total nanograms of ALB to normalize samples for varying blood contributions to the bone wash, since ALB is produced in the liver, circulates in the serum, and should be produced minimally in the bone microenvironment (Tew et al. 1985, Wilson et al. 2003). Therefore, all OC and ICTP were expressed as ratios (OC/ALB, ICTP/ALB), and OC/ICTP included ALB adjustments.

Statistical analyses

The primary research outcome, correlation between baseline bone wash biochemical markers of bone turnover, and changes in radiographic bone density in the healing bone defect were analysed with the Pearson and Spearman correlation coefficients. Secondly, biochemical markers were compared according to time points (baseline, 2, 12 weeks), locations (periosteal versus trabecular or maxilla versus mandible), and gender using repeated measures analysis of covariance. Biochemical markers were compared with each other with Pearson's correlation coefficients.

Results

The mean values for biochemical bone turnover markers from bone washes of periosteal and trabecular surfaces are summarized in Table 1. All subjects completed the 12-week study, but one subject did not have bone wash recovery volumes accurately recorded, so all values represent $n = 9$. The data were quite variable from patient to patient, as evidenced by the high standard errors of the mean. However, all baseline biochemical ratios from trabecular bone washes (after cortical perforation) were significantly higher than values from periosteal surfaces. This difference was not evident at either the 2- or 12-week samples. OC/ALB and ICTP/ALB from periosteal washes tended to decrease after 2 weeks and rebound at 12 weeks, while OC/ICTP increased at both 2 and 12 weeks relative to baseline. None of

Table 1. Mean total bone wash amounts of biochemical markers according to location and time

Time	Location	OC/ALB*	ICTP/ALB**	OC/ICTP***
baseline	Periosteum	5.27 ± 3.51	0.63 ± 0.30	7.78 ± 3.92
baseline	Trabecular	965.33 ± 754.31	21.37 ± 16.18	38.75 ± 6.40
2 weeks	Periosteum	1.25 ± 0.53	0.10 ± 0.02	22.02 ± 16.82
2 weeks	Trabecular	5.45 ± 2.46	0.24 ± 0.08	19.70 ± 5.41
12 weeks	Periosteum	33.82 ± 35.23	0.62 ± 0.53	12.19 ± 7.03
12 weeks	Trabecular	5.87 ± 2.77	0.27 ± 0.08	20.96 ± 10.74

*Mean ± standard error of the mean ng/sample of OC/ALB × 10^{-5},
**ng/sample of ICTP/ALB,
***OC/ICTP after normalizing both values for ALB × 10^{-5}, ****$p \leqslant 0.01$.
OC, osteocalcin; ALB, albumin; ICTP, C-terminal telopeptide of Type 1 collagen.

these changes were statistically significant. However, both trabecular OC/ALB and ICTP/ALB were significantly lower than baseline after 2 and 12 weeks. None of the ratios varied according to gender or skeletal location (maxilla versus mandible) of the bone core.

Correlations among the baseline biochemical values and bone density changes are reported in Table 2. The average increase in bone density over the 12-week healing period was $0.45 \pm 0.29\,mg/mm^2$ (range: -1.3 to $+1.8$). Both OC/ALB and ICTP/ALB ratios from trabecular bone washes were significantly, but negatively, correlated with increasing bone density on the facial plus lingual walls of the healing defect. The more conservative Spearman correlations were just outside the significant thresholds of $p = 0.05$ for trabecular washes, with OC/ALB at $r = -0.65$, $p = 0.06$ and ICTP/ALB at $r = -0.65$, $p = 0.07$. Both periosteal and trabecular OC/ALB versus ICTP/ALB were highly correlated with each other, but not with OC/ICTP. None of the other correlations were significant.

Table 2. Correlations among baseline biochemical markers and subsequent bone density changes

Comparison	Pearson correlation
periosteal OC/ALB versus boneΔ	$r = 0.21$, NS
trabecular OC/ALB versus boneΔ	$r = -0.72$, $p = 0.03$
periosteal ICTP/ALB versus boneΔ	$r = 0.14$, NS
trabecular ICTP/ALB versus boneΔ	$r = -0.72$, $p = 0.03$
periosteal OC/ICTP versus boneΔ	$r = 0.17$, NS
trabecular OC/ICTP versus boneΔ	$r = -0.51$, NS
periosteal OC/ALB versus ICTP/ALB	$r = 0.96$, $p = 0.0008$
trabecular OC/ALB versus ICTP/ALB	$r = 0.99$, $p < 0.0001$
periosteal OC/ALB versus OC/ICTP	$r = 0.27$, NS
trabecular OC/ALB versus OC/ICTP	$r = 0.21$, NS
periosteal ICTP/ALB versus OC/ICTP	$r = 0.04$, NS
trabecular ICTP/ALB versus OC/ICTP	$r = 0.19$, NS

Δ = density change in healing defect; NS = $p > 0.05$.
OC, osteocalcin; ALB, albumin; ICTP, C-terminal telopeptide of Type 1 collagen.

Discussion

None of the local biochemical markers of bone turnover (OC/ALB, ICTP/ALB, OC/ICTP) was affected by gender or skeletal location (maxilla versus mandible) in this pilot study. This is in contrast with previous reports of systemic measures (serum or urine) being elevated in young men compared with women (Henry & Eastell 2000). In addition, systemic biochemical markers varied in comparison with local histologic bone turnover in rat studies depending on the part of the skeleton tested (Yamaura et al. 1996). These differences may be due to the age of the study population (the current study had older subjects), model system (animal compared with human studies), and bone type (e.g. jaw versus femur). Time of day for sampling also has been shown to influence serum biochemical markers (Hassager et al. 1992), but variation of local samples taken both in the morning and afternoon, as with this study, has yet to be adequately investigated. In any case, the use of multiple local measures of bone turnover may improve the ability to predict bone remodeling outcomes at a specific site within a bone organ (Bennell et al. 1998).

All of the biochemical markers of bone turnover (OC/ALB, ICTP/ALB, OC/ICTP) were higher at baseline for the trabecular bone washes compared with periosteal washes (Table 1). These findings are generally consistent with a previous report using this bone wash system (Wilson et al. 2003). In that study, OC was used as a marker of bone turnover, cross-linked N-telopeptide of Type I collagen (NTX) was used as a marker of bone resorption, and OC/NTX designated bone turnover normalized relative to bone resorption in the same sample. It was proposed that trabecular washes should result in higher levels of markers of bone turnover since the bone turnover rate of trabecular bone is greater than cortical bone (Parfitt 1988, Ott 1996). Both OC/ALB and OC/NTX were much higher in trabecular bone washes than periosteal washes, with serum values falling in between. However, NTX/ALB was not different between periosteal and trabecular washes, and both were higher than serum, indicating that ICTP/ALB may track more like OC/ALB than the NTX markers of bone resorption at these two sites. This is also supported by the high correlation between OC/ALB and ICTP/ALB at baseline in the current study (Table 2). Synovial fluid ICTP also correlates well with other measures of bone turnover/formation, such as aminoterminal propeptides of Type I procollagen (PINP, Hakala et al. 1995). However, neither OC/ALB nor ICTP/ALB was significantly correlated with OC/ICTP in the current study. This was due to OC/ALB and ICTP/ALB being so highly correlated to each other that when they were combined into OC/ICTP, they canceled each other out, leaving no correlation with bone or with the original variables.

ICTP was chosen in this experiment because of the greater database of local measurements (GCF) available in alveolar bone studies. GCF ICTP has been well correlated with experimental periodontitis in dogs (Giannobile et al. 1995), and with human periodontal collagenase activity (Golub et al. 1997), gingival inflammation, and past signs of periodontitis (Talonpoika & Hämäläinen 1994, Al-Shammari et al. 2001). GCF OC has also preceded bone-seeking radiopharmaceutical uptake during experimental periodontitis in dogs (Giannobile et al. 1995), but was not associated with adult periodontitis sites in human studies (Lee et al. 1999). Significant correlations between local OC or ICTP and alveolar bone turnover in longitudinal human studies are required to determine if these markers have clinical diagnostic potential (McCauley & Nohutcu 2002).

Based on the premise that a healing bone defect should have increased bone turnover during 3 months of healing (Roberts 1988, Ohishi et al. 1998), it was unexpected that trabecular OC/ALB and ICTP/ALB were decreased after 2 and 12 weeks of healing in our bone defects. The chief contributing factor probably centered on problems developing a ''closed'' bone wash system along the lateral wall of the bone core defect compared with the baseline sample. At baseline, the blunt-end outer canula could be inserted onto the periosteal surface forming direct contact and a seal around its entire periphery. Cortical perforation occurred well into the trabecular bone and the PBS irrigation could be injected into and extracted from the trabecular bone without much involvement of the supraperiosteal soft tissue. At the 2- and

12-week samples, the outer canula probably met the lateral defect wall at an angle without the possibility of forming a seal, and cortical perforation was not as deep or predictable, thereby involving more periosteal and supraperiosteal exchange of proteins and blood (ALB) contamination. Garetto et al. (1995) have shown that bone turnover is reduced dramatically in bone as little as 1 mm away from a healing implant surface, a trend that is probably magnified in adjacent soft tissue. Consequently, OC or ICTP would go down and ALB would go up. In fact, total nanograms of ALB went up dramatically in a majority of 2-week samples (data not shown). Even periosteal wash values for OC/ALB and ICTP/ALB after 2 weeks were numerically (not statistically) lower than baseline, suggesting inadequate retrieval of local turnover markers. The decrease in OC and ICTP after 2 weeks was in contrast to an increase in matrix metalloproteinase (MMP)-9 in these samples (data not shown). This marker of late-stage inflammation is produced primarily by cells of inflammatory infiltrate, further suggesting soft-tissue origin. Interestingly, values tended to rebound after 12 weeks of healing despite the sampling problems.

Of primary importance was to determine if the baseline measurements of local bone turnover could be correlated with subsequent changes in bone density at that healing site. Both baseline trabecular OC/ALB and ICTP/ALB were negatively correlated with 3-month changes in bone density of the healing bone defect ($r = -0.72$, $p = 0.03$; Table 2). This finding indicated that the higher the bone turnover values were, the less bone density was increased in the defect during the 3-month healing period. No associations were apparent between trabecular OC/ICTP or periosteal bone turnover values versus changes in bone density. Negative associations between serum OC or ICTP and future changes in bone have been reported. Paimela et al. (1994) found serum ICTP correlated with radiologic progression of bone loss in early rheumatoid arthritis. Cortet et al. (2000) also reported that lumbar spine bone loss correlated with OC ($r = 0.51$, $p < 0.01$) and ICTP ($r = 0.34$, $p < 0.05$) in patients with rheumatoid arthritis. Cheng et al. (2002) found that OC correlated inversely with future calcaneal bone mineral density in a 5-year follow-up, with r values ranging from -0.22 to -0.42. Cruz et al. (2001) also showed that renal transplant patients with high levels of serum OC had high subsequent bone loss in the lumbar spine and hip.

ICTP measured in areas adjacent to the bone studied have shown a similar association with bone loss, except at higher levels than in serum. For example, Aman et al. (1999) reported that synovial fluid ICTP was significantly higher in knee joints with progressive radiological bone loss, but serum ICTP was not. OC and ICTP in GCF were significantly increased during periods of radiographic bone loss during experimental periodontitis in dogs (Giannobile et al. 1995), much higher than in contralateral control sites. The high negative correlation values in the current pilot study between bone wash OC/ALB or ICTP/ALB and subsequent bone density in healing implant-core defects suggested that these values may be helpful in predicting how implant sites might respond post-insertion. That is, sites with high bone turnover may be slow to heal and to increase bone density at the periphery. Larger prospective studies will be necessary to prove this hypothesis. In addition, local bone wash analysis of bone turnover markers could be tested against future changes in alveolar bone density with respect to drug interventions, regeneration procedures, and the oral aspects of systemic conditions like osteoporosis. This approach is more focused than serum biochemical evaluations and much less invasive than the gold standard of histomorphometry. Such information may eventually allow customized bone-augmenting pharmacologic therapies based on the current state of bone turnover at the site of interest.

Acknowledgments

This work was supported by a grant from the University of Nebraska Medical Center College of Dentistry. The authors wish to thank Marian Schmid for her technical assistance and research advice, Dr. Larry Golub for contributions with the ICTP and MMP analyses, Dr. Jeffrey Payne for critical manuscript review, and Andrea Schwartz for preparation of the manuscript.

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Address:
Richard A. Reinhardt
Department of Surgical Specialties
UNMC College of Dentistry
40th and Holdrege Streets
Lincoln, NE 68583-0740
USA
Fax: 402 472 5290
E-mail: rareinha@unmc.edu