In Vivo Evaluation of Quantitative Percussion Diagnostics for Determining Implant Stability

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Purpose: *To test in a rat model whether quantitative percussion diagnostics provide reliable, reproducible indications of osseointegration.* ***Materials and Methods:*** *Titanium implants were placed in femurs of 36 Sprague-Dawley rats. Each animal was assigned to one of six groups defined by one of three time points (2, 4, or 8 weeks postplacement) and one of two treatments (matrix metalloproteinase [MMP] inhibitor GM6001 or control). Percussion testing was conducted three times per subject at implant placement and before sacrifice at one of the time points. For each time point, there was an experimental group that received daily intraperitoneal injections of GM6001, and a control group that received no MMP inhibitor. The percussion data consisted of loss coefficient (LC) values that characterize energy dissipation. Statistical analysis was performed on the LC values for the two animal groups using the paired Student* t *test to assess differences as a function of time, and the independent t test to compare mean LC for the study groups at sacrifice (α = .05). Histologic evaluation using the osteogenic CD40 protein marker was also performed.* ***Results:*** *A nearly significant difference in mean LC at the 2-week time point was observed between the two treatments with the GM6001 group having the higher value (*P *= .053). There was a greater difference between the mean LC values for the 4-week GM6001 and control groups (*P *= .001). The histologic evidence for subjects in these two groups confirmed reduction of osteogenesis at the implant interface after administration of the MMP inhibitor.* ***Conclusions:*** *Lower control LC values relative to the GM6001 therapeutic group were observed, consistent with the effect MMP inhibition has on matrix remodeling at the implant bone interface. This finding in conjunction with histologic observations confirms that osseointegration can be monitored using percussion diagnostics.* INT J ORAL MAXILLOFAC IMPLANTS 2013;28:1286–1292. doi: 10.11607/jomi.2779

Key words: *implant, in vivo, matrix metalloproteinase, osseointegration, quantitative percussion diagnostics*

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Successful implants must meet long-term mechanical and esthetic needs of patients. An instrument that could provide lifetime quantifiable measurements of implant stability and surrounding bone quality would be an advantage to patients and the dental industry.[1] Current methods used to measure bone quality and stability at implant sites have limitations. Radiography is difficult to standardize for position and is representative of only two dimensions, while dual-energy x-ray absorptiometry (DEXA) scans are cost prohibitive, radiation intensive, and time consuming.[2] The conventional practice of tapping the implant with a metal instrument to make an auditory assessment is not quantitative. Meanwhile, removal torque is problematic for implants in cancellous bone and can precipitate failure in minimally osseointegrated implants.[3] Resonance frequency evaluations are useful, but have limitations related to the need for disassembly and implant geometries.[4] However, it is important to track the stability of implants during healing and loading since even small changes in bone density and structure can significantly affect stability.[5]

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Osseointegration is the "continuing structural and functional coexistence" of an implant and the bone in which it is placed, providing a stable interface to transmit loads without invoking a significant immune response.[5–7] Similar to the natural tooth complex, an implant and its supporting bone exhibit a combination of elastic and anelastic (time-dependent) behaviors. If the implant and supporting bone were to behave with a strictly elastic response, the loss coefficient (LC) would be zero because no energy would be dissipated.[8,9] However, restorative materials and bone are not strictly elastic and therefore provide some energy dissipation during loading so that LC > 0. If the bone becomes damaged or does not properly osseointegrate, additional energy dissipation can occur due to excessive frictional micromotion at defects within the bone or at the bone-implant interface. Thus, it follows that a reduction in osseointegration should result in an increase in loss coefficient for a given implant.

Immediate loading protocols surgically place an implant in the jawbone, which is restored with an immediate provisional restoration allowing transmission of some occlusal forces to the bone. In a two-stage protocol, implants are surgically placed and submerged to allow for bone healing and osseointegration before they are fitted with a provisional restoration. Despite the advantages of fewer surgeries, a quicker return to a normal diet, and possible improved esthetic outcomes, immediate loading has some potential drawbacks. Delayed loading has a success rate of approximately 96% at end points more than a year out. In contrast, immediate loading has been reported by some authors to have a lower (approximately 80%) success rate at 12 to 18 months after placement for randomly selected patients, including those who were parafunctional or had implants placed in extraction sites.[1,10–12] Improvements in protocols may continue to increase overall success rates. However, an increased risk of failure may persist for immediate loading protocols without a reliable method for periodically monitoring the level of osseointegration.[11,13]

Bone matrix turnover is regulated by the extracellular zinc-endopeptidase family of matrix metalloproteinases (MMPs), which includes collagenases, gelatinases, matrilysins, stromelysins, and membrane-type MMPs.[14] It has been shown that MMPs are important to the formation of properly constituted extracellular matrix during the integration of pure titanium threads with newly formed bone.[15–17] Accordingly, the inhibition of MMPs should lead to less osseointegration over a given period of time after an implant is placed in bone.

The purpose of the present study was to evaluate the hypothesis that quantitative percussion diagnostics can provide reliable and reproducible indications of osseointegration. A synthetic small molecule MMP inhibitor, GM6001, was used to interfere with the endogenous determinants of successful osseointegration in vivo. An evaluation was also performed for one timepoint to further verify that histologic indications of osteogenesis were consistent with the percussion results.

MATERIALS AND METHODS

Commercially pure titanium (Ti) implants (Integrum) were chosen for their compatible geometry. The implant screw was grade 4 titanium with a 0.4 mm-long, smooth middle section, a 2 mm diameter by 2 mm-long M2 thread at the mesial end, and a 1.6 mm diameter by 1.6 mm-long M1.6 thread at the distal end. A 0.4 mm-deep slot was machined at the distal end of the implant to facilitate reverse torque removal. A commercially pure Ti abutment was also provided with the implants. The abutment was used for both surgical placement and percussion testing. Ethanol sterilized implants were kept in dry glass containers and handled with titanium instruments to avoid contamination. All surgical implements were commercially pure titanium and a Dremel Moto-Tool Model 395 (Robert Bosch) was used to hold the surgical burs.

Animals and Surgery

Female adult Sprague-Dawley rats (225 to 250 g, Harlan Labs), were used for the animal model. Thirty-six animals were equally divided into groups of six and were assigned into one of six treatment modalities determined by treatment time (2, 4, and 8 weeks) and drug treatment (GM6001 and control). A previously conducted power analysis showed that statistical significance could be verified with four animals, and each group consisted of at least 4 rats for valid data sets. Animals were housed at 22°C under a 12-hour light/dark cycle with ad libitum access to food and water. The animals were exposed to 4% Isofluorane (Baxter) at 1 L per minute airflow by inhalation for the entire procedure. The site preparation was performed with a 1.7 mm diameter hand drill and threaded with an M2 pretapping device to a depth of 2 mm into the widest part of the femur. The abutment was threaded onto the distal end of an implant to facilitate placement into the surgical site. Each implant was placed to a depth of 2 mm and the animal was removed for percussion testing. Once this testing was completed the animal was taken back to the surgical field and the abutment was removed while holding the implant in place with a surgical screwdriver down the hollow throat of the abutment. The wound was closed with 3-0 absorbable sutures using continuous stitches. The animals were allowed to recover in a sensory-enriched environment without restricting their mobility.

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Fig 1 The approach position of the percussion probe to the testing abutment. The supporting arm is not in contact with the animal's head.

Intraoperative percussion testing was performed, as detailed below, at 2, 4, and 8 weeks after implantation. All procedures conformed to NIH Guidelines for the Care and Use of Laboratory Animals and protocols approved by the Institutional Animal Care and Use Committee and the VA San Diego Healthcare System.

Quantitative Percussion Testing

A quantitative percussion instrument (Periometer, Perimetrics) was used to measure the loss coefficient of the femur implants as shown in Fig 1. Loss coefficient measurements determined by this medical device were shown to correlate with simulated bone densities in a previous in vitro study.[18] The system is composed of a hand piece and control unit, power supply, and custom computer software to control the testing, acquire data, and provide analysis and visualization of the results. Instrument calibration was preformed prior to each use to assure data precision. Calibration is accomplished by testing two material standards with known loss coefficient values (aluminum alloy 6061 and polytetrafluoroethylene). Percussion testing was conducted twice on each animal: immediately following implant placement but before suturing soft tissue over the implant, and at the time of sacrifice. Immediately following the first testing session, each animal was returned to the surgical field for incision closure.

Three percussion measurements were performed during each testing session providing a total of 30 percussions, 10 for each measurement. Reproducible accuracy of the data was assured by the requirement that the standard deviation in the loss coefficient had to be below 0.002 (less than 2% of the LC value).

MMP Inhibitor Therapy

A specific, broad-spectrum small-molecule MMP inhibitor GM6001 (Calbiochem, Novabiochem International) in vehicle (ethanol in filter sterilized buffered saline) were prepared according to the manufacturer's instructions and administered at 60 μg per rat per day by intraperitoneal injection. These injections started the day after surgery and were continued until the day before sacrifice. Six animals were in each experimental group with Ti implants and MMP inhibitor, and six animals were in each control group with Ti implants and no MMP inhibition.

Tissue Isolation and Immunohistochemistry

The animal subjects were anesthetized at the desired time point after implant placement. Any periosteum that had formed around the top of the implant was removed and the testing abutment was attached for the second set of percussion tests. The animals were perfused transcardially with fresh 4% paraformaldehyde (PFA) in 0.2 mol/L phosphate buffer. The femurs were then resected, cleaned and post-fixed in 4% PFA solution at 4°C for 48 hours. The bones were rinsed in a phosphate buffer and in deionized water. The vials were filled with Immunocal (Decal) for decalcification and the bones were cut distal and proximal to each implant.

The implant/bone samples were embedded in paraffin and cut into 10 μm sections. The sections were then put into a solution of 1% Sta-On Tissue Selection Adhesive (Surgipath) in a 50°C flotation bath, mounted onto slides and dried on a slide warmer at 60°C for 1 hour. The slides were then baked overnight at 37°C prior to staining. Immunohistochemical staining was performed for CD40 protein, which promotes bone formation, calcification, and osteoclast genesis.[19] In addition, the expression of CD40 has also been shown to decrease with the administration of the MMP inhibitor used in the present work, GM6001.[20] Thus, the expression levels of this protein should be higher in growing bone and lower when bone growth is suppressed by factors such as the inhibition of MMPs. The sections were deparaffinized with xylene and rehydrated in a series of graded ethanol ranging from 100% to 70%, followed by phosphate-buffered saline (PBS) (0.01 mol/L, pH = 7.4).

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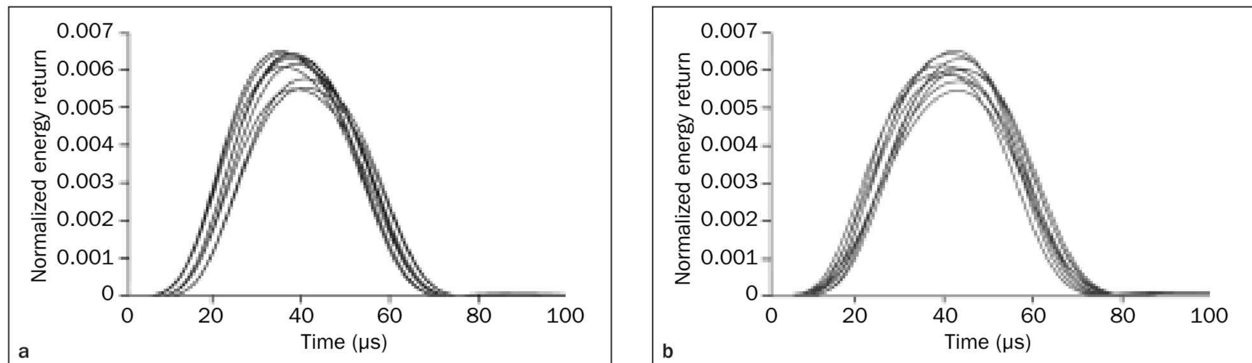

Figs 2a and 2b Representative energy return data corresponding to 10 individual percussions for a 2-week control animal at *(a)* surgical placement and *(b)* sacrifice. The data for all animal subjects were uniformly shaped at each time point similar to those shown here, indicating no significant defects at the bone/implant interface.

Endogenous peroxidase was blocked with 3% hydrogen peroxide, followed by DAKO antigen retrieval application for 300 seconds at 95°C. Nonspecific binding was blocked with 10% horse serum for an hour at room temperature. The sections were incubated overnight with anti-CD40 antibody (Abcam). The sections were then rinsed in PBS, followed by the application of biotinylated goat anti-rabbit (Vector) for an hour at room temperature. The avidin-biotin complex (Vector) was applied for an hour at room temperature. After rinsing with PBS, the sections were developed with 3′3-diaminobenzidine (Vector), counterstained with methyl green (Fisher), dehydrated, and mounted with Entellan medium (Merck). Control sections with nonimmune serum from the rabbit (or mouse) animal source were used as control. The imaging was performed using a Leica DMRB microscope, a Leica DFC 300 camera, a desktop computer, and Openlab 3.1.2 image analysis software (PerkinElmer).

Percussion Data Analysis

The LC was used to characterize the energy dissipation response of the implant and surrounding bone to mild percussion. As stated in the hypothesis, energy dissipation indicated by the LC was expected to decrease as osseointegration progressed. The percussion response, characterized by energy return vs time, was checked for irregularities that can indicate defects in the supporting bone.[18] For each experimental condition within each sacrificial time group, a paired Student *t* test was used to assess the change in LC means between implant placement (baseline) and sacrifice time. For each sacrifice time, the difference between the experimental LC mean and control vehicle mean was assessed using an independent *t* test. In case 1, control sacrifice data vs control implant placement data were analyzed. In case 2, GM6001 sacrifice data vs GM6001 placement data were compared. In case 3, control sacrifice data vs GM6001 sacrifice data were evaluated.

RESULTS

Percussion Testing

Energy return data for typical percussion tests were plotted for each group and individual animal. As represented by the data in Figs 2a and 2b, none of the energy return peaks were overtly skewed nor contained additional peaks that can be indicative of defects in the support structure, implant movement, or loose test abutments.[18] Rather, uniform bell-shaped energy return peaks indicated that all the implants were securely implanted the day of surgery. The implants were in contact with the medullary channel at their distal ends, but the support of the system came from the cortical and cancellous bone in contact with the implant along the majority of its length.

The resulting LC data were analyzed to identify differences between the values of inter- and intratimepoint groups. Figure 3 shows individual LC values, each based on 30 percussions, as a function of weeks after implant placement for both control and GM6001-treated subjects. Each experimental group data set was analyzed to determine if the LC values were normally distributed. Also, the quartile method was used to exclude data that were atypical for three animals. In the 2-week group, one GM6001 data point was excluded for being a low outlier according to this method. In the 8-week data sets, two control data points were excluded, one due to an animal death and another due to an unusually high outlier according to the quartile method. None of the experimental groups showed observable side effects from the daily injections. The animal subjects remained active and docile for the entire study.

Morbidity was limited to four animals. One animal had a nerve irritation from the surgery that caused dragging of the left hind foot, but full dexterity returned by the time of sacrifice. Another animal formed a subdermal pustule slightly distal to the implant that

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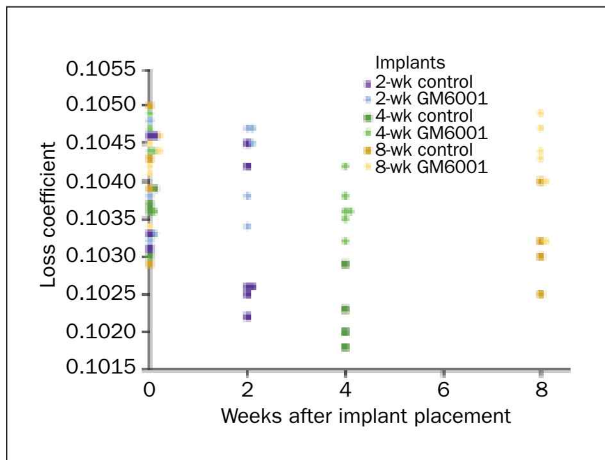

Fig 3 Loss coefficient values at each timepoint with similar values grouped to the right of the corresponding time point. Light and dark blue symbols correspond to animals sacrificed at 2 weeks, light and dark green symbols correspond to animals were sacrificed at 4 weeks, and light and dark orange symbols correspond to animals sacrificed at 8 weeks after implant placement.

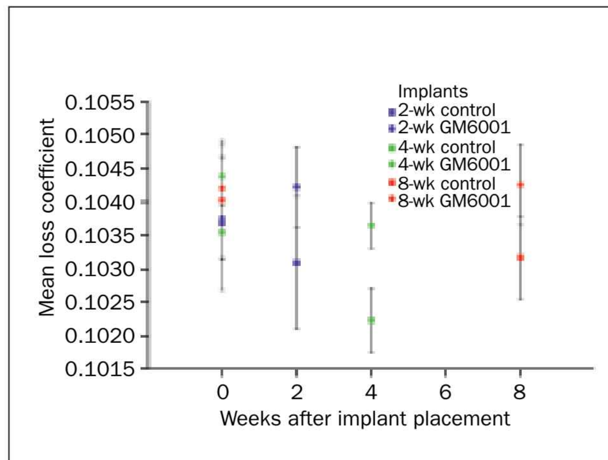

Fig 4 Mean loss coefficient values for all therapeutic groups from placement to sacrifice. Error bars correspond to the standard deviation for each mean.

Table 1 Analysis of Loss Coefficient (Means)

Time to sacrifice	Comparison tested	No. of subjects	*t* Test type	*t* Score	Two-sided *P* value
2 weeks	GM6001 LC: placement vs sacrifice	5	Paired	0.93	.405
	Control LC: placement vs sacrifice	6	Paired	–1.14	.306
	LC at sacrifice: control vs GM6001	11	Independent	2.22	.053
4 weeks	GM6001 LC: placement vs sacrifice	6	Paired	–3.84	.012
	Control LC: placement vs sacrifice	4	Paired	–4.75	.018
	LC at sacrifice: control vs GM6001	10	Independent	5.50	.001
8 weeks	GM6001 LC: placement vs sacrifice	6	Paired	0.16	.881
	Control LC: placement vs sacrifice	4	Paired	–1.29	.288
	LC at sacrifice: control vs GM6001	10	Independent	2.73	.026

did not affect the mobility or energy level of the animal. Finally, two animals experienced lethargy after implant placement, which responded to antibiotic therapy.

Mean LC values for the different week of sacrifice groups are plotted in Fig 4 and the corresponding statistical findings are listed in Table 1. The results indicated measurable differences after just 2 weeks following implant placement. Specifically, a nearly significant difference (*P* = .053) is indicated in Table 1 between the GM6001 and control group means where the former group exhibited the higher value (Fig 4). However, there was no significant change in LC mean from the baseline values for either treatment group for this time point.

The mean LC values for the 4-week sacrifice groups were significantly different from their starting baseline values and from each other as indicated in Table 1. Therefore, the null hypothesis that the mean LC values were the same was rejected for all three cases. The data for both groups at this time point had no significant outliers as defined by the quartile criterion. Both the *P* values in Table 1 and the plotted data in Figure 4 demonstrate clear distinction between the two treatment groups at each time point. In addition, both groups exhibited lower mean LC values than their corresponding initial values, as expected for the osteoblastic phase of bone remodeling. We note that all of the LC values were lower for the control group in Fig 3 at the 4-week time point. Accordingly, the significantly lower control LC means listed in Table 1 relative to the GM6001 therapeutic group indicate that this MMP inhibitor was still effective in altering the matrix remodeling at the implant-bone interface. Further, the overall decrease in LC values for both groups also shown in Table 1 indicates bone growth molecular activity was occurring in all subjects at this time point. Thus, the results for 4 weeks clearly support the hypothesis that percussion diagnostics provide reliable and reproducible indications of osseointegration.

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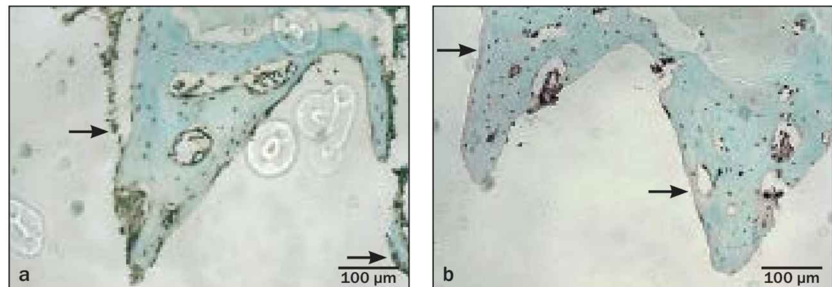

Fig 5 Histologic samples of bone adjacent to an implant at the 4-week time point. Note difference in CD40 localization between *(a)* control group, and *(b)* GM6001-treated group.

The mean LC values after 8 weeks from implant placement returned to near initial values as indicated in Table 1 and Fig 4. This implied decrease in implant stability for both groups suggests that a large number of osteoblasts have become osteocytes by this time point and are maintaining and remodeling as opposed to primarily depositing bone. Accordingly, we hypothesize that the bone at 8 weeks of healing in both groups is primarily undergoing stabilization instead of building activity. Also at this time point, the mean LC value for the GM6001 group was still significantly greater than that for the control group ($P = .026$). Thus, it appears that the MMP inhibitor is still effective at this time point in altering the bone remodeling process for the present animal model.

CD40 Levels at the Implant-Bone Interface

The histologic evaluation and reactivity for the osteogenic CD40[19,20] in bone slices neighboring implants indicated successful titanium osseointegration at 4 weeks after implantation in the control group (Fig 5). While both Figs 5a and 5b show that CD40 is present in the nuclei of cells, the locations of these cells are strikingly different. The control group exhibited a large number of CD40 containing nuclei at the interface of the bone and implant. By contrast, the CD40 protein is not observed directly along the implant/bone interface in the GM6001 treated bone. The staining levels were the greatest at 4 weeks after implant placement, increasing from those for the 2-week group and then were lower at 8 weeks after implant placement (data not shown). To confirm this finding quantitatively, binary images were produced using image analysis software so that the stained nuclei were differentiated from the rest of the bone tissue. Area analysis of the stained nuclei in Figure 5 indicated that the CD40-containing cells corresponded to approximately 10% of the area in the control bone, while they constituted only 2.3% of the GM6001-treated bone area. Thus, the present histologic results are clearly consistent with the percussion results for the 4-week time point in Table 1 indicating that greater osseointegration occurred for the control animals than for the GM6001 administered group.

DISCUSSION

Earlier studies have shown a trend of decreased stability immediately after implant placement followed by a period of significantly increased stability as the osseointegration process continues in human subjects.[21–23] By contrast, the present data did not indicate decreased stability at two weeks after implant placement. However, it is possible that decreasing stability occurred before the 2-week time point due to the fact that osseointegration can occur much earlier in rats than the 18 to 24 weeks that it can take in humans.[24] The large ratio of implant contact surface area to total bone at the implant site may also have contributed. By contrast, the mean LC value is still relatively high after 2 weeks for the GM6001 group. The natural dissolution process appears to be therapeutically affected by GM6001 as a result of initial resorption prior to bone growth. This finding is consistent with several reports, which indicate that the inhibition of MMPs breaks the signaling process that controls the switch from osteoclastic to osteoblastic cellular activity.[25–28] The present study suggests that loss coefficient measurement using percussion diagnostics is an alternative to other methods used for in vivo tracking of osseointegration levels.[8,9,18]

The inhibition effect of MMPs was expected to slow the rate of implant osseointegration resulting in lower levels of stability.[15–17] The hypothesis that LC measurements could detect the altered speed of osseointegration due to the presence of MMP inhibitors was confirmed. Daily MMP inhibitor injections decreased implant stability, as consistently indicated by higher LC means (Fig 4), resulting in a significant difference between the data for the two experimental groups at the 4-week time point (Table 1).

CONCLUSIONS

The results of the present study support the hypotheses that values of the loss coefficient determined by percussion diagnostics provide a reliable, reproducible

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indication of osseointegration as a function of time in vivo. Specifically, a significantly lower control LC means relative to that for the GM6001 group at 4 and 8 weeks after implant placement. These results are consistent with the fact that GM6001 is effective in inhibiting the MMP-influenced control of matrix remodeling at the implant bone interface. Histologic examination of CD40 protein at the bone/implant interface also indicated that the extent of osseointegration can be clinically evaluated from loss coefficient data.

The present findings have implications for the field of bone implants. The LC gives a direct indication of implant stability via an analysis of the response to percussion loading. Further, it is possible to perform percussion tests throughout various stages of the healing process for exposed implants such as dental implants. In doing this, osseointegration can be monitored periodically as needed to assure a successful outcome for the patient.

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