MICRO-COMPUTED TOMOGRAPHIC ANALYSIS OF BONE HEALING SUBSEQUENT TO GRAFT PLACEMENT

by

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Micro-Computed Tomographic analysis of bone healing subsequent to graft placement.

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ABSTRACT

A MicroCT analysis of bone healing subsequent to graft (tri calcium phosphate/TCP) placement in the maxillary sinus prior to dental endosteal implant placement was the objective of the current study. Ten trephined rod shaped human bone cores were obtained three months after the placement of particulate graft material and the samples were stored immediately in 10% neutral buffered formalin. Using a Micro-computed tomography, the samples were evaluated at resolutions of 6 and 20µm. The thresholds for bone and graft material were determined using visual image, intensity (grey level) and histogram analyses. The graft and bone threshold was 235 greylevel and only graft threshold was 600 greylevel magnitudes. The volume, density and three-dimensional micro-architecture like trabecular thickness (Tb Th), trabecular separation (Tb sp), structural model index (SMI), trabecular number (Tb no), and connectivity density of the bone and graft material were analyzed using the instrument software. The results were analyzed statistically, using the paired Student’s T-test, with p<0.05 indicating statistical significance. The graft threshold at 20µm was altered to a 500 greylevel magnitude and the results compared to results obtained at 6µm resolution (graft greylevel magnitude being 600). Eight of the ten samples were prepared as non-decalcified sections and stained with toluidine blue and methylene blue/ basic fuchsin stain, for histological examination.
The MicroCT images at 6 and 20µm resolutions exhibited different grey scale magnitudes for both bone and graft allowing a definitive differentiation and quantification of the two sample regions. The mean bone volume was more than mean graft volume, that is, 23.8mm³ and 25.5mm³ bone volume versus 2.2mm³ and 2.0 mm³ graft volume at 6 and 20 µm resolutions respectively. Comparison of 6 and 20 µms, showed a significant difference in the graft volume (p=0.0120) and density (p=0.0001). Altering the graft threshold at 20µm to 500 and comparing the results to 6 µm showed a significant difference in graft density. (p=.0001) but difference were not significant for graft volume. The structural data from 2 and 3 dimension images provided a valuable assessment of the graft distribution, its relation to modeling bone and also the nature of the healing bone. The histological examinations showed anticipated cellular activity, osteoid formation and presence of uniformly mineralized trabecular bone, without any significant foreign body response.
DEDICATION

I dedicate my thesis to my parents, Enakshi and Tilak Raj Mahajan.
ACKNOWLEDGMENTS

I would like to express my sincere appreciation and thanks to my mentor Dr. Jack Lemons for his constant guidance, patience and encouragement. I also express my thanks to my committee members for their advice over the course of my thesis work.

I would also like to thank Dr Maria Johnson, Dr Sandre McNeal and Mr. Preston Beck for their constant guidance and valuable inputs. I would have not done this without the support of the lab, Patty, Annie, Mark and Leigh, the biomaterials residents and Deniz Cakir and Mona Anabtawi. Special thanks to my roommate K. Ghosh and lastly I would express my heart felt thanks to my family and my husband, Sumit.
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Introduction

General Background:

Replacement of bone at localized alveolar ridge deformities caused by various reasons such as tooth loss, periodontal disease trauma, and cystic and tumorous lesions has always been a focus of interest. This replacement should be such that the regenerated bone has adequate strength to bear the load of the prosthesis (a denture or an implant), other masticatory forces and also be able to fulfill the esthetic, functional and physiological roles of the alveolar ridge.

Bone augmentation of the residual alveolar ridge has been done by using bone grafts. The bone grafts can be autogenic, allogenic or alloplastic, with alloplastic including many different bone substitute materials. An autograft is generally the gold standard, however limitations like donor site morbidity, inadequate amount and the inability to mould the autograft sometimes necessitates the need for additional options. Allografts which can be a cortical, cancellous or a combination of both have been utilized; however they have been related to increased risk of infection, immunogenicity and cost of processing. Hence the need for an alternative that does not have the above mentioned limitations of cost, availability and immune response is warranted.

The most extensively explored options within the alloplastic group are the calcium phosphate biomaterials; hydroxyapatite and tricalcium phosphate.\(^1\) This group most closely simulates the bone mineral compared to other types of graft biomaterials\(^1\,3\). They
have been shown to be biocompatible, osteocondutive materials, and their bone regeneration depends upon factors like their specific chemistry, form (solid or particulate), pore size (macro or micro), and interconnectivity. Considering alveolar bone augmentation, the solid form in particular allows limited incorporation (up to 2mm of the maximum bone growth) as augmentations onto the host bone. A porous architecture has shown direct osseous bone in-growth into the bone graft substance and a direct bone contact without intervening fibrous tissue. It is suggested that porosity with interconnectivity is important for optimal bone growth. The pore size varies anywhere from 100 to 500 micrometers and the interconnections must be larger than 100 micrometers. Other factors that affect the bone substitute resorption and bone regeneration depend upon chemistry, density, and purity of the biomaterial plus the critical size of the bone cavity, and the osteogenetic potency of the bone site.

Various methods have been employed to study the quality and quantity of the new (cancellous) bone. Quantity and quality includes the new bone volume and density plus the parameters that describe the micro-architecture of the bone formed. This is important since research has shown that in addition to bone mineral density, the three dimensional bone micro-architecture strongly influences the physical/mechanical and biological properties of the cancellous bone.

For the assessment of bone formed, various techniques have been used to obtain and to study the bone sample. In the present study, to obtain the sample a two-stage method was used. The preliminary step of which was the graft insertion in the maxillary sinus during surgical manipulation which was then followed by implant placement into the grafted site after several months (this period could be ranging from 3 to 12 months). At the time of
implant insertion, staged biopsy specimens from the regenerating bone were taken for analysis. This type of procedure has been used previously.\textsuperscript{12}

These bone biopsies have traditionally been analyzed using two dimensional histological sections. (For details refer to Appendix A on histology and histomorphometry of bone biopsies).

The histological and histomorphometric analyses are not only tedious and destructive and many of the analyses have been based on simplified models of trabecular shape as an estimate of three dimensional anatomy. These models estimate the three dimensional structure from the two dimensional images assuming that all trabeculae have similar geometrical shapes and spatial distributions. This is however not entirely true because the trabecular bone has both rod and plate like structures\textsuperscript{13} and the architecture varies with anatomical location. Further, the analysis of a few sections cannot be fully representative of an entire sample. Serial sectioning has also been done but that technique is often too destructive and labor intensive\textsuperscript{14}.

Newer imaging techniques like Microfocus Computed Tomography (\(\mu\)CT) have made it possible to obtain the high resolution three dimensional images necessary to directly examine the bone architecture.\textsuperscript{15} This technique uses x-rays to create cross-sections of a 3-D-object that later can be used to recreate 3-D models without destroying the original sample. No specimen preparation is required and testing is non destructive and completely shielded. The resolutions of locally available MicroCT systems are in the order of 6-72 \(\mu\)m for nominal isotropic substances, depending on the size of the sample\textsuperscript{16}.

The term \textit{micro} is used to indicate that the pixel sizes of the cross-sections that can be in the micrometer range. The complete data can directly calculate histomorphometric
parameters\textsuperscript{17} including bone surface (BS), bone volume (BV), mean trabecular thickness (Tb.Th), trabecular separation (Tb.Sp), and trabecular number (Tb.N) and non-metric parameters like connective density (Conn.D), and SMI (structure model index for shape). These parameters describing the microarchitecture of bone have been shown to be important as it has been repeatedly shown by literature that quantity and quality of the bone collectively provides an estimate of the physical and mechanical properties of the bone.\textsuperscript{7,9-11,15,18}

It also has been shown that these calculated parameters from the MicroCT of bone images can be correlated with the values obtained from the traditional histological methods\textsuperscript{18-20}.

Objectives and Hypothesis:

The present study using MicroCT and the traditional histological analyses, focused on the structural and quantitative parameters of regenerating bone three months after graft placement in maxillary sinuses prior to implant surgery.

The MicroCT analysis using the SCANCO\textsuperscript{\textregistered}CT40 machine was used with an initial purpose of determining whether or not it would be possible to differentiate between graft and bone material separately, on basis of grey-level values, (thresholds) and if the answer was yes, then at what resolution?

Establishing the optimal image resolution for structural dimensions was not the only purpose in that also achieving a resolution at which there was economical usage of machine time and the data generated could be correlated with results obtained at the best resolution achievable.
Comparisons of the quantitative data such as bone/graft volume, bone/graft density were calculated and analyzed at two resolutions, 6 and 20 $\mu$m. The 6$\mu$m was the maximum resolution possible with current machine settings and the sample size.

The hypotheses tested were:

1. The bone and the graft material will show different magnitudes of grey-level intensities thereby permitting visual and quantitative analysis of each. Also, histogram analysis would further corroborate the differences in grey-level magnitudes.

2. The quantitative measurements of bone and graft material density and volume would be significantly similar at 6 and 20$\mu$m resolution magnitudes.

3. The data describing the micro-architecture of the alveolar trabecular bone would provide a structural characterization for the bone at three months time period of graft placement.
Materials and Methods

Sample:
Ten (10) rod shaped bone cores obtained by trephine osteotomy, three months after the placement of FDA approved synthetic particulate tricalcium phosphate bone graft material for maxillary sinus interior augmentation prior to endosteal dental implant placement. These samples were obtained as a part of the Dental Implant retrieval program, UAB, (IRB approval no XO50823001).

The specimens after removal were placed in 10% neutral buffered formalin. Each bone core varied in size ranging from 3mm to 9mm in length with a consistent diameter of approximately 4mm.

For the surgical procedure details refer to Appendix B on the sinus lift dental procedure.

MicroCT Machine:
A high resolution, desktop Microtomographic imaging system (µCT40.scanco Medical AG, Bassersdorf, Switzerland), was used for the major portion of the current study.

For more details refer to Appendix C on the MicroCT machine.

Methods:
The samples were removed from the trephine by mechanical “push out” and initially scanned at a resolution of 37µm. This pilot investigation showed that this resolution and machine program used were inadequate for the present study. The details of the pilot study are summarized in Appendix D.
A high resolution, desktop Micro-tomographic imaging system (µCT40.scanco Medical AG, Bassersdorf, Switzerland) was then utilized. The samples were snugly fit and longitudinally oriented in a sample holder that was sized to 10 mm length with 10% neutral buffered formalin. The specimens were scanned at the machine voltage of 70 kVp and amperage 114 uA. The resolution and number of sections depended upon the size of the sample. Since the samples were relatively small, a uniform resolution of up to 6 micrometers (µms) was possible. Once scanned, two dimensional digitalized sections (tomographs) were obtained. The number of tomographs/slices obtained varied dependent on the overall length of the specimen however each slice thickness was 6 or 20 µm, (dependent on the scanning resolution). An example of one 6µm slice is shown

Figure 1

![Figure 1: A 2-D section/ slice/ tomograph obtained at 6µm showing the bone, graft material and the assigned Region of Interest.](image)

From the tomographs a **Region of Interest (ROI)** was defined; which in this case, was the entire slice containing the graft (TCP) and the bone as shown in **Figure 1**

Segmentation of the image region was based upon the visual and greylevel differences between the bone and graft. Separation on basis of grey level intensities is also called **thresholding**.
Threshold or segmentation as shown in Figures (2a-2f) for 6µm resolution was used to differentiate between bone and graft substances.

Figure 2a: A 2-D section, showing ROI including graft and bone

2b- Within the given ROI, at 235 threshold value both bone and graft was selected in the given ROI

2c- At 600 threshold only graft material was selected excluding the bone in the given ROI
2d- ROI defining exclusively the bone without adjacent bone graft region

2e- At 235 all the region of exclusive bone was selected in the given ROI, excluding the background

2f- In the ROI containing only regional bone at 600, no material was selected, indicating that no bone has a threshold of or above 600
Histogram Analysis:

For further corroboration of these values a histogram analysis of the intensity levels was also conducted.

As a control, TCP alone was scanned, however it was determined that the threshold obtained could not be used directly in the study as the combination of bone and graft was unique in the CT imaging and TCP powder alone did not emulate the TCP in this in vivo sample combination.

The bone, graft volume, densities and various histomorphometric parameters were then determined by the three dimensional evaluation of the entire sample from the system software program.

The scanning at 6µm resolution was very time consuming of the available CT machine time.

Scan at 20µm resolution

The samples were oriented horizontally and subsequently scanned at 20µm resolution, keeping all other machine parameters the same.

The results at 6 and 20 µm resolutions were then compared at the two different magnitudes for significant similarities or differences. As an expansion of the study, the threshold for graft was changed from 600 to 500 magnitude and the results were computed again keeping all other parameters the same.
All the results were analyzed statistically in consultation with the statistician and compared by SPSS data analysis software program using a paired Student’s T-Test for comparing the means between the two sets of measurements.

Histological analysis:

The histological analysis included eight bone cores since two out of ten could not be processed and used for histology as they were not fully intact. These eight samples were embedded for standardized non decalcified tissue preparation using methyl methacrylate (MMA) material which was then polymerized (PMMA). During embedding care was taken to orient and place the samples as they were placed for the MicroCT scan. This was intended to duplicate, as best as possible, the CT and histological section planes. Plastic, non de-calcified ground two dimensional sections of thickness ranging from 20 to 40µm were prepared, one stained with toluidine blue and the other stained with methylene blue/basic fuchsin stain. (See Appendix A for details, on histology and histomorphometry plus staining procedure.)

The stained sections were examined and a region of interest was selected which in this case was the section area including the bone and graft material. A Bioquant Nova Image Analysis Software System (BIOQUANT Image Analysis Corporation, Nashville, TN), was selected for standard histomorphometric analysis which was conducted to determine the total graft and bone area. From these data the total material volume and graft to bone ratio and graft and bone percentage area were determined for the eight samples.
Results and Discussion

The initial pilot study done at 37µm resolution, yielded results that were inconclusive and discreet imaging or differentiation of the graft and bone regions was not possible. These results are explained in detail in the (Appendix D on Pilot study.)

In contrast to the results at 37µm, when using the high resolution, desktop microtomographic imaging system (µCT40.Scanco Medical AG, Bassersdorf, Switzerland); scanning and analyzing was possible at 6µm. This MicroCT method was instrumental for studying the nature of healing bone subsequent to graft placement in the present study. During scanning of bone samples orientation was kept such that the 2-D digitalized sections could be directly compared with the histological sections obtained in the future part of the study.

MicroCT analysis:

The Micro-computed tomographic analysis provides visual information in two and three dimension; in addition to that images provided quantitative data and also included parameters that explicitly described the microarchitecture of trabecular bone.

Structural features in two and three dimensions:

A 2-D digitalized section (tomograph/slice) is shown in Figure 3 where both bone and graft are discreetly visualized on a basis of their structure and differences in grey level values. The grey level threshold magnitudes at 6µm, 20µm resolutions were determined
to be less than 235 for organic and fluid, 235-450 for bone, 400-600 for bone and graft and 600 for the graft material.

The three dimensional reconstruction of the bone core at 6µm is shown in Figure 4 (a & b). Bone and graft were segmented where it was possible to visualize the graft and its distribution over the bone sample and its relation with regenerating bone was possible.

Figure 3: Image of a 6µm section where bone and graft can be defined seen separately in a 2-D digitalized section
The reconstruction below clearly explains the distribution of the graft in relation to the bone in the given study.

Figure 4

**a:** Image of a 3-D reconstruction of an entire sample at 6µm

**b:** Image of a 3-D reconstruction of the graft above

The CT analysis at 6 µm showed a consistent grey-level shade for the bone over the entire sample. Detailed analysis demonstrated the presence of well formed regenerating trabecular bone having a relatively uniform grey level (proportional to density), with minimal variation. However, as shown by the previous literature, through analysis of the MicroCT it was not possible to demonstrate the active cellular activity and osteoid
formation that takes place in a newly forming bone (the organic phases and fluids could not be defined).

As an example, as shown in Figure 1, visual differentiation between the bone and graft material was clear on the tomograph (2-D), however segmentation on the basis of grayscale magnitude (threshold) raised questions, suggesting a narrow region of possible overlap. The threshold has been shown to be dependent upon the difference in mineral content of the materials. The region of overlap in the grayscale value for both graft and bone may be explained due to the consideration that bone and graft (TCP) could have similar mineral contents but also they are undergoing changes in their mineral composition with in vivo exposure. This would be due to the continuous bone regeneration and remodeling coupled with the graft resorption within the inter-positional location of the alveolar sinus. Also, any original or progressive porosity with the TCP bone graft could influence the bulk density as noted on grey level magnitudes. The manufacturer and independent sections showed that the original TCP graft materials was non porous. In the present study the threshold for graft and bone were kept the same over the entire sample size at the given resolutions for this time period of healing. This was done to reduce sampling variability. Subsequent studies at different time periods of healing may warrant an adjustment in the threshold magnitudes.

Histogram Analysis:

To further analyze the details of overlap of data a histogram analysis was done (at 20µm) for all ten samples. The histogram was found to be bimodal, and on calculation of the thresholds it was evident that the histogram exhibited two distinct peaks representing the
background (marrow, collagen etc) and the bone and graft combination, as shown in Figure 5.

The data shown in Figure 5, is a sample histogram for one of the 10 samples, it exhibits two distinct peaks, each representing collagenous matrix and bone and graft complex respectively. The darker pointer marks the point which segments the two peaks. Therefore from the histogram, it was corroborated that the combined threshold for bone and graft was 235.

![Sample Histogram](chart.png)

**Figure 5:** A sample histogram for one of the 10 samples at 20μm

Three dimensional reconstructions with a representative midline 2-D section for all the ten samples are shown in Figures 6-15(L & R). These images show the graft distribution, bone structure, graft to bone relationship, and to an extent an overall aspect of the graft placement and trephine sampling procedure.

In Figures 6R, 7R the graft was localized at one end of the sample while the images in Figures 12 R, 13 R, and 14 R; show the graft in relation to the regenerating bone. In
Figure 10 R the graft is only present in relation to the well mineralized bone and it could be speculated that in this case the trephine may have been positioned differently as opposed to the examples mentioned above. The corresponding 3-D reconstruction also shows bone structure that is more plate-like and has a well formed mineralized bone at one end. This deviation in the osteotomy procedure can be anticipated while performing any clinical procedure.

The images shown in Figures 8 R, L indicates a high rate of bone formation as opposed to others as most bone is trabecular in nature and associated with resorbing graft material. In addition to greater bone formation as noted in figures 12 L 13 L and 14 L the plate and rod like structure of the trabecular bone was also noted. This structural analysis of whether the bone is plate or rod like can provide information about how much into the bone formation the new bone is; since newly formed bone would be more rod-like as opposed to bone formed later. The images in Figures 9 R, L provide a representation of a sample with mostly powder and minimal bone structure.
Figures 6-15: The 3-D reconstruction with the midline section (R) of the individual bone core (L)
Quantitative analysis of structural parameters from the MicroCT: 6 and 20µm resolutions.

In addition to the structural reconstruction of the image in 3-D, the MicroCT program also quantifies the structural parameters which in this case were in terms of bone and graft volumes and densities, total volume and the ratio of the bone to graft volume.

The structural measures for all the 10 samples at 6µm are provided in Table 1. There was variability among the samples, the graft volume ranges from 0.15mm³- 5.86 mm³, and the bone volume ranges from 9.15mm³-39.36mm³. However there was minimal variation in graft density and very little variation in the bone densities.

Table 1: The quantitative data describing the bone, graft volume and densities, obtained at 6 µm. Bone & Graft Threshold: 235, Only Graft Threshold: 600

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total volume</th>
<th>Graft volume</th>
<th>Bone volume</th>
<th>Graft/Bone Volume Ratio</th>
<th>Graft density</th>
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The sample was also scanned at 20µm and these scans were equally informative, as the ones at 6 µm; however the 20µm scans were less time consuming and allowed direct
comparison with histological sections of thickness of 20µm which was the next step of the study.

**Table 2:** The quantitative data describing the bone, graft volume and densities, at 20µm resolution. Bone & Graft Threshold: 235, Only Graft Threshold: 600

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total volume</th>
<th>Graft volume</th>
<th>Bone volume</th>
<th>Graft/Bone Volume Ratio</th>
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<td>3</td>
<td>29.62</td>
<td>0.40</td>
<td>29.22</td>
<td>0.0138</td>
<td>1952.37</td>
<td>1083.71</td>
</tr>
<tr>
<td>4</td>
<td>17.68</td>
<td>0.18</td>
<td>17.50</td>
<td>0.0105</td>
<td>1984.33</td>
<td>1037.19</td>
</tr>
<tr>
<td>5</td>
<td>38.01</td>
<td>0.70</td>
<td>37.31</td>
<td>0.0187</td>
<td>2033.82</td>
<td>1108.41</td>
</tr>
<tr>
<td>6</td>
<td>49.07</td>
<td>1.17</td>
<td>47.9</td>
<td>0.0244</td>
<td>1958.38</td>
<td>1153.82</td>
</tr>
<tr>
<td>7</td>
<td>21.51</td>
<td>0.34</td>
<td>21.17</td>
<td>0.0162</td>
<td>1993.77</td>
<td>1083.73</td>
</tr>
<tr>
<td>8</td>
<td>24.53</td>
<td>0.18</td>
<td>24.35</td>
<td>0.0075</td>
<td>2002.38</td>
<td>1033.62</td>
</tr>
<tr>
<td>9</td>
<td>23.02</td>
<td>0.84</td>
<td>22.18</td>
<td>0.0380</td>
<td>1981.09</td>
<td>1161.63</td>
</tr>
<tr>
<td>10</td>
<td>25.31</td>
<td>0.34</td>
<td>24.98</td>
<td>0.0134</td>
<td>1991.13</td>
<td>1027.87</td>
</tr>
</tbody>
</table>

Interpretation of data from **Tables 1 and 2 shows**, that at both resolutions (6 and 20 µm) the bone volume was substantially more that the graft volume; and the graft density was more than the bone density at both resolutions. Also both graft and bone can be differentiated on basis of greylevel variations which thereby provide a method to segment the substance studied. Both the above analysis at 6 and 20µm were done keeping the lower threshold value for bone and graft at 235 while 600 was for exclusively for the graft material. The data obtained at both resolutions was then compared statistically using the paired Student’s T-Test, keeping all other machine program parameters the same. The standard test results are presented in **Table 3**.
**Table 3:** The standard test results, comparing 20 and 6µm resolutions;

Bone & Graft Threshold: 235, Only Graft Threshold: **600**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Resolution (µm)</th>
<th>Mean (SD)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6µm</td>
<td>20µm</td>
<td></td>
</tr>
<tr>
<td>Graft Volume</td>
<td>2.25 (1.83)</td>
<td>0.42 (0.37)</td>
<td><strong>0.0120</strong></td>
</tr>
<tr>
<td>Bone Volume</td>
<td>23.77 (8.46)</td>
<td>25.50 (11.28)</td>
<td>0.7019</td>
</tr>
<tr>
<td>Graft Density</td>
<td>2069.2 (31.82)</td>
<td>1992.3 (28.56)</td>
<td><strong>0.0001</strong></td>
</tr>
<tr>
<td>Bone Density</td>
<td>1076.4 (80.87)</td>
<td>1056.3 (81.99)</td>
<td>0.5871</td>
</tr>
<tr>
<td>Total Volume</td>
<td>26.02 (9.85)</td>
<td>20.93 (11.57)</td>
<td>0.9853</td>
</tr>
</tbody>
</table>

The mean and standard deviations in bone/graft volume and densities at resolutions of 20 and 6µm, showed a significant difference in graft volume and density at 6 and 20 µm.

Considering the above mentioned significant difference in graft volume and density\(^{21}\) a subsequent analysis of the threshold for graft utilized a change from 600 to 500 magnitudes. This altered resolution leads to a change in the pixel size, therefore less sensitivity to changing shades of grey.\(^{22}\) The results were then compared to the results at 6 µm using a paired Students t-test as provided in **Table 4**.
Table 4: The standard test results, comparing 20 and 6µms resolutions;

Bone & Graft Threshold: 235, Only Graft Threshold: 500

<table>
<thead>
<tr>
<th>Variable</th>
<th>Resolution (µm)</th>
<th>Mean (SD)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6µm</td>
<td>20µm (Threshold for Graft 500)</td>
<td></td>
</tr>
<tr>
<td>Graft Volume</td>
<td>2.25 (1.83)</td>
<td>1.95 (1.94)</td>
<td>0.7321</td>
</tr>
<tr>
<td>Bone Volume</td>
<td>23.77 (8.46)</td>
<td>23.97 (9.99)</td>
<td>0.9613</td>
</tr>
<tr>
<td>Graft Density</td>
<td>2069.2 (31.82)</td>
<td>1879.7 (35.57)</td>
<td>0.0001</td>
</tr>
<tr>
<td>Bone Density</td>
<td>1076.4 (80.87)</td>
<td>1015.9 (60.39)</td>
<td>0.0742</td>
</tr>
<tr>
<td>Total Volume</td>
<td>26.01 (9.85)</td>
<td>25.93 (11.57)</td>
<td>0.9853</td>
</tr>
</tbody>
</table>

Interpretation of results in Table 4 shows that there was a significant difference in the graft densities however a significant difference in graft volume was not found in this analysis. However the difference in bone density was almost significant at p=.0742.

There was a significant difference in the graft density at both the resolutions however no significant difference was noted for the graft volume. This difference in the graft density could be explained due to the reduction in set threshold as threshold and density depend on one another.\textsuperscript{21, 22} One possibility considered was that at 20 µm pixel sampling of the graft included more dark regions due to the larger quantity of particulate interfacial (or edge) regions. This could have reduced the grey level magnitude for the graft material thereby making it similar to bone. The gray scale in the current study ranges from 0-1000; with 0 representing air (black) and the 1000 representing dense opaque white material.
Bone Micro-architecture analysis:

The MicroCT analysis includes the assessment of trabecular bone parameters\textsuperscript{17} that are directly assessed by actually measuring various distances in the 3-D space. There are no assumptions made regarding the trabecular bone model. These parameters are defined as follows.

\textit{Tb.Th\textsuperscript{*}: Trabecular thickness:} This is determined by filling maximal spheres into the structure with the distance transformation. The average thickness of all bone voxels is calculated to give Tb.Th

\textit{Tb.Sp\textsuperscript{*}: Trabecular separation:} This parameter is measured by filling the maximum spheres in the voxels representing non bone regions.

\textit{Tb.N\textsuperscript{*}: Trabecular number:} The average of the number of trabeculae in a given volume using a 1mm diameter sphere. (Tb.N) (1/mm)

The structure model index (SMI) is an estimation of the plate-rod characteristic of the structure. For an ideal plate and rod structure the SMI value is 0 and 3 respectively; 0 being for only plate-like and 3 being for only rod-like.

When evaluating new bone formation one can predict the maturity and therefore estimate the time into bone formation by evaluating the above mentioned parameters. For a newly forming bone the trabecular number and connectivity density would be more on a relative basis. Also the SMI would indicate a more rod-like structure as opposed to a plate-like structure.
Table 5: Micro architectural parameters for individual bone cores at a fixed threshold of 235, 20 µm resolution:

<table>
<thead>
<tr>
<th>Sample</th>
<th>Tb.N</th>
<th>Tb.Th</th>
<th>Tb.Sp</th>
<th>SMI</th>
<th>Con density</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.08</td>
<td>0.14</td>
<td>0.32</td>
<td>3.82</td>
<td>16.6</td>
</tr>
<tr>
<td>2</td>
<td>2.14</td>
<td>0.35</td>
<td>0.43</td>
<td>1.01</td>
<td>15.41</td>
</tr>
<tr>
<td>3</td>
<td>2.53</td>
<td>0.30</td>
<td>0.37</td>
<td>3.33</td>
<td>24.63</td>
</tr>
<tr>
<td>4</td>
<td>2.93</td>
<td>0.23</td>
<td>0.30</td>
<td>0.06</td>
<td>47.71</td>
</tr>
<tr>
<td>5</td>
<td>3.49</td>
<td>0.16</td>
<td>0.30</td>
<td>1.26</td>
<td>38.01</td>
</tr>
<tr>
<td>6</td>
<td>3.52</td>
<td>0.25</td>
<td>0.26</td>
<td>2.53</td>
<td>34.72</td>
</tr>
<tr>
<td>7</td>
<td>2.45</td>
<td>0.25</td>
<td>0.38</td>
<td>0.09</td>
<td>29.24</td>
</tr>
<tr>
<td>8</td>
<td>2.01</td>
<td>0.21</td>
<td>0.48</td>
<td>1.63</td>
<td>22.84</td>
</tr>
<tr>
<td>9</td>
<td>2.66</td>
<td>0.23</td>
<td>0.38</td>
<td>2.24</td>
<td>25.17</td>
</tr>
<tr>
<td>10</td>
<td>1.81</td>
<td>0.20</td>
<td>0.59</td>
<td>0.92</td>
<td>20.57</td>
</tr>
</tbody>
</table>

The results shown in Table 5 were judged not be very useful for the current study as the parameters were not calculated exclusively for the bone but collectively for graft and bone. Future studies should include separation of the bone and graft for these calculations. This was not possible in the current study due to the bone and graft being integrated or contiguous and also due to the lack of definite gray level values for exclusively the graft and bone.

However this information could be used to compare samples from different time periods; future bone cores would be compared at 3, 6 and 12 months time periods of graft/bone healing. It can be argued that changing the ROI to include only the bone could have been done however since the graft material was closely incorporated in the bone this was not possible to do for all the slices over the ten samples.

Histological Analysis:
Histological analysis was included for 8 samples because the other two samples were not in a single piece. Midline ground, plastic, non decalcified sections of thickness ranging from 20-40µm were made. The 2-D histological sections stained with toluidine blue and methylene blue/ basic fuchsine, H&E like stain are shown in Figures 16.

**Figure 16:** Bright field 40x image, showing graft incorporated into the remodeling bone.

**Figure 17** Image of the new bone formation and the lining of the osteoblasts along the forming bone.
Figure 18: Image (20x) showing bone formation, with the presence of osteocytes in the lacuna.

Figure 19: Image (10x) showing the cement lines and adjacent woven bone as a polarized image.

Analysis at this given time period of three months demonstrated an active bone formation with presence of early osteiod and bone in relation to graft material. Also seen was definitive cellular activity, again corroborating bone formation. Multiple regions also showed bone in relation to graft materials where they exhibited integration. Over time
graft dissolution and bone remodeling phenomena should result in decreased quantities of the graft material.
Summary and Conclusions

The present study was an initial investigation done with a primary intention of examining whether or not the MicroCT could be used for evaluating the nature of healing bone subsequent to graft (TCP) placement in the dental maxillary sinus.

The samples were scanned at 6µm, the maximum resolution with the present MicroCT machine and the analysis was conducted using bone and graft threshold as 235 and for graft only at 600 greylevel magnitudes. All MicroCT parameters were kept the same and samples were also scanned at 20µm resolution and results were compared statistically. Considering the statistical results, the samples were then scanned again at 20µm keeping the bone and graft threshold as 235, but changing the exclusive graft threshold to a 500 magnitude. Eight out of these ten samples were sectioned non-decalcified and stained with toiludine blue and methylene blue/ basic fuchsin for histological analysis.

The conclusions drawn from the study are enumerated as follows.

- Images from the MicroCT at 6µm and 20 µm resolutions exhibited different grey scale magnitudes for both bone and graft which allowed a definitive differentiation and quantification of the two sample regions. The global threshold method was used to set the absolute grey level value for the threshold of bone and graft combination (excluding the organic matter) at 235. The histogram analysis provided a method for segmenting the bone graft complex from the background
therefore further corroborating the absolute threshold value chosen for the bone and graft complex (235).

- The threshold value for the graft exclusively was determined to be 600 however there was a region of overlap of the bone and graft from 450 to 600 magnitudes. Results from the comparison of the 6 and 20 µm resolutions showed that changing the threshold at 20µm was warranted. Changing the threshold for the graft exclusively at 20µm from a 600 to 500 magnitude was examined thereby resulting in a statistical comparison that was similar to 6µm resolution analyses.

- Considering the clinical information that the graft was placed in a space without bone, the parametric measures showed a mean bone volume of 23.8mm³ and a mean graft volume of 2.2mm³ at 6µm resolution. Studies at 20µm (with graft threshold magnitude: 500) show a mean bone volume, 25.5mm³ and a mean graft volume of 2.0 mm³. The results show that the bone volume was more than graft volume, that is, 23.8mm³ and 25.5mm³ bone volumes versus 2.2mm³ and 2.0 mm³ graft volumes.

- The histological examinations showed anticipated cellular activity, osteoid formation and presence of uniformly mineralized trabecular bone without any significant foreign body response. Histology results from staining also showed that the bone was relatively uniform related to mineralization. This staining is an indication that the bone thus formed was in the same phase of mineralization. Analyses of MicroCT images also showed minimal grey level variability in the bone.
• Overall comparisons of methodologies showed that a 20µm resolution magnitude was more optimum in that the results were similar to 6µm resolution, with the advantage of less machine time usage and with possible future correlations with histology.

• The structural data from 2 and 3 dimension images provided a valuable assessment of the graft distribution, its relation to modeling bone and also the nature of the healing bone. The parameters describing the micro-architecture of the bone graft complex like the SMI, trabecular number, separation, thickness, and connectivity density, were useful and can be used in futures studies when analyzing the trephine bone cores over different time periods.
Recommendations for Future Studies

This being an initial study, in the future, the MicroCT could be used to evaluate samples over different time periods and then the analysis could be used to judge the relative performance of the given graft material. Also evaluation of the performance of the graft material should be compared to a control which in this case might be autogenous bone grafts (the gold standard).

Another possibility would be to study the bone around and at the bone implant interface. The present information like bone volume/density would be beneficial and of more importance would be the parameters that describe the trabecular number, separation, connectivity density and the SMI since these describe the micro-architecture of the forming bone. This information may be used to evaluate the nature (structural and quantitative) of healing bone without sample destruction and without assuming the 3-D model of the trabecular bone.
APPENDIX A

Histology and Histomorphometry
1. A bone biopsy

![Bone biopsy image]

2. Bone sectioning

Techniques and types of Bone Sectioning
- Decalcified Sections
  - Acid Decal
  - EDTA decal
- Frozen Sections
- Ground Sections
- Plastic Sections

In the present study the sections were non-decalcified, ground sections

![Bone section image]

3. Staining of the section to visualize the tissue and the cellular components

In the present study the two sections were stained with Toluidine Blue and Methylene Blue/basic fuchsin Stain, H&E like stain respectively.
**Toluidine Blue Stain**

**Procedure:**
- Etch in 1% formic acid for 1 min
- Stain in Toluidine blue/EDTA for 3 min; see under the microscope before doing more
- Air dry, Rinse in xylene, Mount

**Results**
- Nuclei: blue
- Mineralized bone: light purple
- Osteoid: colorless, pale blue
- Mineralized front: light blue
- Reversal line: dark blue/purple

---

![2-D bone section stained with Toluidine Blue](image)

**Methylene Blue/basic fuchsin Stain**

**Procedure**
- Etch in 1% formic acid for 1 min, Rinse in deionized water. Remove excess water
- Sonicate in 50% ethanol for 1 min, Rinse in deionized water, Remove excess water
- Stain in methylene blue for up to 1 min, Rinse in deionized water, Remove excess water
- Stain in working solution of basic fuchsin for 2 min
- Rinse well in deionized water and allow air drying

**Results**
- Nuclei: Blue
- Bone: varying shades of pink
**Histomorphometry**

Histomorphometry is the measurement of the biological samples by means of image analysis, topographic morphometry and 3-d visualization. It estimates the various parameters like bone volume, trabecular number, connectivity density in 3-D by evaluating a 2-D section and assuming the rest of the 3-D structure of the bone sample. The diagram below is adapted from (Handbook of histology Methods for Bone and Cartilage.) It shows that in Histomorphometry we assume the three dimensional structure based on the data available from the 2-D section of biological tissue.

On the stained sections a region of interest was selected which in this case was the entire section including the bone and graft material and then using the Bioquant Nova Image
Analysis Software System (BIOQUANT Image Analysis Corporation, Nashville, TN), standard histomorphometric analysis was done to determine the total graft and bone area.
APPENDIX B

LATERAL MAXILLARY SINUS LIFT PROCEDURE

(Graft placement and bone core trephine surgery)*
1. **Name of grafting procedure:** Maxillary Antroplasty using b-TCP enriched with patient’s blood

2. **Explanation of the approach:** Under local anesthesia a full thickness flap was reflected extending from maxillary canine premolar region up to the maxillary tuberosity. With a Brasseler laboratory bur, rotating at about 1000 rpm and with external irrigation, an osteotomy was made in the lateral wall of the sinus producing an oval shape. The base of the osteotomy extended to about 3-4mm from the sinus floor. Once the membrane was exposed using proper curettes, initially the floor and then the mesial and distal aspects of the membrane were reflected until the mesial wall of the sinus was exposed. The patient’s blood and SynthoGraft (TCP) were mixed to obtain a putty-like consistency, and using a metallic bone syringe, the material was collected and injected into the sinus cavity first mesially, then distally until it has been densely compacted. *(picture provided)* Then, the rest of the site was packed compressing the material to the mesial wall of the sinus. A Bicon Collagen Membrane is then placed so that it completely covered the osteotomy site and extended over the crest to the palatal aspect. The flap was closed with 3.0 silk using a continuous suture.

3. **Site from which bone cores were trephined:** Repeated the flap in the same manner as the initial flap, but with a smaller extension. Using a 4.75mm or 5.25mm trephine depending on the thickness of the crest. The trephine is placed from the crest to the apical at the osteotomy level of the first molar region *(picture provided)*. The osteotomies were extended to 10 mm into the graft.
Bone cores were placed in a 10% Neutral Buffered Formalin solution. An implant was placed immediately after the core was removed. The last diameter hand reamer was used to prepare the calibrated osteotomy for the implant. The graft implants were sampled three months after placement and the implants were restored after an additional three months of passive bone healing.

For the diagrammatic representation of the procedure kindly refer to pictures provided:

A full thickness flap was reflected extending from maxillary canine premolar region up to the maxillary tuberosity.

The osteotomy was made in the Lateral wall of the sinus producing an oval shape.
Raising the maxillary sinus floor membrane, and placement of TCP mixed with blood. (MF), maxillary sinus floor, (SM), Schneiderian membrane, (G), graft, (T), trapped door of lateral sinus wall


Using a 4.75mm or 5.25mm trephine, depending on the thickness of the crest, the trephine was placed from the crest to apical, at the osteotomy level of the first molar region.
Image after trephine and before implant placement
APPENDIX C

MicroCT40 SCANCO machine
MicroCT40 SCANCO machine

Microfocus computer tomography (µCT) uses analysis of x-rays to create cross-sections of a 3-D object that later can be used to recreate 3-D models without destroying the original sample. The term micro is used to indicate that the pixel sizes of the cross-sections that can be in the micrometer range.

For micro tomography, x-ray scanners typically use 2 methods for scanning; Fan beam reconstruction or Cone beam reconstruction.

The machine used in the present study was a Desktop Cone-Beam MicroCT Scanner (This means the system is based on a 2-dimensional x-ray detector (camera) and an electronic x-ray source, creating projection images that subsequently were being used to reconstruct the image cross-sections).

The µCT 40 normally produces a maximum resolution of 9 µm @ 10 mm object size, 10% MTF. In addition to the high resolution capabilities, it also offers opportunities for a larger specimen size (36 mm diameter, 8 cm specimen length. The exact specification are given as follows: 16

The X-Ray : Microfocus X-Ray-source with 5 or 7 µm spot size, 30-70 kVp / 20-50 keV (160 µA)

Detector: 2048x256 elements, 24 µm pitch

Resolution: 6-72 µm nominal isotropic, 9 µm (10% MTF)

Image matrix 512x512, 1024x1024 or 2048x2048 Pixels

Specimen Size: FOV 12 to 36.9 mm, max. Scan Length 80 mm

Scan Time: Reconstruction 3 sec/section for 1k x 1k, 0.72° angular increment
24 sec/section for 2k x 2k, 0.36° angular increment
48 sec/section for 2k x 2k, 0.18° angular increment

**Computer:** HP AlphaStation 64-bit 1 or 2 processors

- Memory: min. 1 GB, max. 16 GB
- Disk: 18, 36, 72 or 180 GB disk drives or Terabyte Disktower
- 18" or 19" TFT-Monitor
- 40/80 GB DLT or 160/320 GB SDLT Tape drive (external)
- Optional PostScript printers

**Software:** Complete imaging and evaluation solution with data acquisition,
Online/Offline reconstruction, sophisticated 2D/3D evaluation, 3-D visualization/animation and Archiving And FE analysis. A 64bit Software (only 64bit Software allows the handling of a large dataset

**Electrical:** 100-240 V / 50-60 Hz 300W

AS adapted from SCANCO Medical\(^{16}\).
APPENDIX D

Results of scan at 37 µm (Pilot study)
Results of scan at 37 µm (Pilot study)

In the pilot study a MicroCAT II (IMTEK) was used and the samples were scanned at a resolution of 37µm. The gave inconclusive results for the present study as at 37µm resolution the unique separation between the bone and graft material in the give bone core was not achieved. The attached picture shows a 3-D reconstruction and it is evident that the separation of the two materials on basis of geometry and density was not achieved.

3-D sample reconstruction at 37µm, differentiating between graft and bone was difficult

A 3-D reconstruction at 6µm and it is demonstrated the µCT 40 system is able to segment the bone and graft material as shown below.

A 3-D sample reconstruction at 6µm, graft and bone can be differentiated and easily segmented.
The graft (TCP) material was scanned separately, at 37 µm resolution, and a unique separation of the particulate structure of the graft was not possible. In contrast, the graft when scanned separately at 6µm using the µCT 40 machine showed a unique separation of the particulate structure of the graft.

TCP/Graft at 37µm: The particulate structure of the TCP was not possible

TCP/ Graft material: 6µm, the particulate structure was defined
APPENDIX E

IRB Approval Form
## Protection of Human Subjects

### Assurance Identification/IRB Certification/Declaration of Exemption

**(Common Rule)**

Policy: Research involving human subjects may not be conducted in cooperation with the Department and agencies adopting the Common Rule (45CFR46, 40 CFR 93), unless the policies are exempt from or approved in accordance with the Common Rule. See section 109 of the Common Rule for exemptions. Institutions submitting applications or proposals to support materials, a certificate of appropriate institutional review board (IRB) review and approval to the Department of Agency in accordance with the Common Rule.

### Request Type

- [ ] ORIGINAL
- [ ] GRANT
- [ ] CONTRACT
- [ ] FELLOWSHIP
- [ ] COOPERATIVE AGREEMENT
- [ ] EXEMPTION
- [ ] OTHER:

### Title of Application or Activity

Analyses of In Situ and Explanted Surgical Implant Devices

### Name of Federal Department or Agency and, if known, Application or Proposal Identification No.

LEMONS, JACK E.

### Assurance Status of this Project (Respond to one of the following)

- [ ] This Assurance, on file with Department of Health and Human Services, covers the activity:
  - Assurance Identification No.: IRB0000009
  - Expiration Date: 01/15/99

- [ ] This Assurance, on file with (agency name), covers this activity:
  - Assurance No.: IRB0000009
  - Expiration Date: 01/15/99

### Exemption Status: human subjects are involved, but the activity qualifies for exemption under Section 101(b), paragraph:

### Certificate of IRB Review (Respond to one of the following if you have an Assurance on file)

- [ ] This activity has been reviewed and approved by the IRB in accordance with the Common Rule and any other governing regulations.

### IRB Approval:

- [ ] Full IRB review
- [ ] Expedited review

### Comments

- Protocol subject to Annual continuing review.

### Title

Analyses of In Situ and Explanted Surgical Implant Devices

### IRB Approval:

- Yes

### Name and Address of Institution

University of Alabama at Birmingham
702 20th Street South
Birmingham, AL 35294

### Name of Official

Marilyn Davis, M.A.

### Signature

Marilyn Davis, M.A.

### Date

5/10/99

### Authorizer for Local Reidentification

Sponsored by IRB

Public reporting burden for this collection of information is estimated to average 1 hour per response. An agency may not conduct or sponsor, and a person is not required to respond to, a collection of information unless it displays a currently valid OMB control number. The National Academy of Sciences is conducting this study under contract to OMB. For more information see OMB control numbers: 0557-0000.
UAB IRB Approval of Waiver of Informed Consent and/or Waiver of Patient Authorization

Approval of Waiver of Informed Consent to Participate in Research. The IRB reviewed the proposed research and granted the request for waiver of informed consent to participate in research, based on the following findings:
1. The research involves no more than minimal risk to the subjects.
2. The research cannot practicably be carried out without the waiver.
3. The waiver will not adversely affect the rights and welfare of the subjects.
4. When appropriate, the subjects will be provided with additional pertinent information after participation.

Check one:
☑ Waiver of Authorization below
☑ Waiver of Authorization below
☑ Waiver of Authorization not applicable

Approval of Waiver of Patient Authorization to Use PHI in Research. The IRB reviewed the proposed research and granted the request for waiver of patient authorization to use PHI in research, based on the following findings:
1. The use and disclosure of PHI involves no more than minimal risk to the privacy of individuals.
   i. There is an adequate plan to protect the identity of the research subjects.
   ii. There is an adequate plan to destroy the identifiers at the earliest opportunity consistent with the conduct of the research, unless there is a health care requirement for retaining the identifiers or such retention is otherwise required by law.
   iii. There is an assurance that the PHI will not be reused or disclosed to any other person or entity, except as required by law, for authorized oversight of the research study, or for other research for which the use or disclosure of PHI would be permitted.
2. The research cannot practicably be conducted without the waiver or alteration.
3. The research cannot practicably be conducted without access to and use of the PHI.

Full Review
The IRB reviewed the proposed research at a convened meeting at which a majority of the IRB members was present, including one member who is not affiliated with any entity conducting or sponsoring the research, and not related to any person who is affiliated with any such entity. The partial waiver of authorization for screening was approved by the majority of the IRB members present at the meeting.

Date of Meeting

Signature of Chair, Vice-Chair or Designee

Expedited Review
The IRB used an expedited review procedure because the research involves no more than minimal risk to the privacy of the individuals who are the subject of the PHI for which use or disclosure is being sought. The review and approval of the partial waiver of authorization for screening was carried out by the Chair of the IRB or by one of the Vice-Chairs of the IRB as designated by the Chair of the IRB.

Date of Expedited Review

Signature of Chair, Vice-Chair or Designee

Date
References


