Computer-assisted image processing for quantitative measurements of fractional bone area

A methodologic study

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The purpose of this study was to evaluate a method for computer-assisted image processing to assess quantitatively fractional bone volume in sections of bone biopsies (part I). A second purpose was to apply this method in an experimental study to determine bone loss by measuring fractional bone area in sections of calvarial bone of gastrectomized rats and to compare this with a control group. Stained paraffin sections of skull bone of young rabbits (part I) and rats (part II) were examined. The histologic sections were placed on a microscope. A video camera was connected to the microscope, and the image transferred to a display monitor connected to a PC with dedicated software, performing the measurements. In the first part of the study tests of the reproducibility of the method were performed. The influence of factors such as external illumination and light intensity in the microscope was evaluated by measuring the same area several times at different times (= different illumination) and at different microscope light intensity levels. The automatic method was compared with a manual method, using a Merz grid but automatically calculated. The difference between the means of the manual and automatic measurements was determined with the paired *t* test. In the second part of the study the unpaired *t* test was used to determine the differences between the control group and experimental group, both for the manually and the automatically measured values. The data of this study indicate that the automatic image processing method is an efficient tool for determining bone loss by measuring bone area to estimate fractional bone volume in histologic sections. However, the quality of the histologic sections is of paramount importance for precise measurements. □ *Bone biopsy; bone loss; gastrectomy; histomorphometry*

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Bone histomorphometry is used for the diagnosis of metabolic bone diseases and for qualitative evaluation of bone structure and bone remodeling. Bone histomorphometry is an application of stereology—that is, the study of three-dimensional structures from two-dimensional samples (1). A slice of tissue from a histologic section is a three-dimensional object, but it produces a two-dimensional image when looked at through the microscope. This image consists of profile areas that are the projections of three-dimensional structures.

The extrapolation of two-dimensional quantities measured in a section to three-dimensional quantities existing in the sample is inevitably subject to error and uncertainty. However, if bone cell function at different sites is to be compared and if a proper understanding of bone remodeling is to be arrived at, histomorphometric measurements must be expressed in three-dimensional terms (2). Densitometric analysis of bone samples has been important in many biomechanical studies because of the relationship between density and bone strength (3–5). Bone densitometry techniques fall into two categories, the non-invasive and the invasive techniques. The latter have been most frequently used because they do not require sophisticated scanners and detectors.

Two invasive approaches are available. For the first and more direct method, defatted bone is weighed and its total volume measured to determine its apparent density (4, 6). Apparent density is the bone mass per total volume of a sample. The second method determines bone density as a volume percentage by applying Delesse's principle (7), which states that an area fraction measurement of bone can be taken as an estimate of the volume fraction of bone (8). Later on, in the 17th century, Cavalieri showed that the volume of any object may be estimated from parallel sections separated by a known distance *t*, by summing up the areas of all cross sections of the object and multiplying this figure by *t* (9). The approach of Delesse is commonly used to analyze bone biopsy sections taken with a trephine (10). The bone specimen is first dehydrated, defatted, and embedded in methylmetacrylate. The piece of bone is then cut into serial sections, which are either stained or microradiographed. Finally, morphometry to determine the bone area fraction (and, consequently, volumetric density) can be done manually by point counting (8) or planimetry (5) or automatically by using a densitometric scanning device for stained sections (11) and computer-assisted videodensitometry for microradiographs (12).

Turnover of bone tissue can be evaluated by measuring the extent of the bone surface area under resorp-

tion or formation. Findings from a study of steroid treatment in patients with multiple myeloma (13) indicated that surface values (= values of areas under resorption or formation) are only meaningful when they are accompanied by values for concomitant changes in bone volume.

Robb & Jowsey (12) described a method for reproducible and rapid quantitative measurements of fractional bone volume in sections of a bone biopsy specimen by the aid of microradiographic images of bone sections. The presence or absence of bone was determined by high-speed digitization and computer analysis of video scans of microradiographic images of bone sections. The bone specimens were taken from normal persons in various age groups and from two groups of patients with multiple myeloma, before and after therapy, respectively. The data indicated the potential of that technique for accurate and reproducible measurements of fractional bone volume in investigative and clinical diagnostic studies of age-dependent and disease-dependent processes.

The computer-assisted image processing used in this study offers the possibility of assessing fractional bone volume by measuring directly on histologic sections in the microscope.

Aim

The purpose of the study was to evaluate a computer-assisted image-processing method for quantitative measurements of fractional bone area in sections of rabbit calvarial bone by testing the reproducibility of the method, by testing different factors affecting image processing of microscopic images, and by comparing this automatic method with a manual method, performed with the aid of a Merz grid but automatically calculated.

A second aim was to apply this automatic method in an experimental study of rat skull bone and compare the results with the manual method.

Materials and methods

Sections

Part I. Stained paraffin sections of rabbit skull bone, originally prepared for a different study, were examined. The material originated from a project in which initially four trephine calvarial defects were produced in young rabbits. The surgical procedure has been described in detail (14–16). The calvarial specimens were fixed in buffered formalin, demineralized in formic acid, trimmed, and embedded in paraffin wax. Serial sections, 7 μm thick, were cut in the middle part of the lesion and stained with hematoxylin and eosin (15). For the testing of the reproducibility of the method in this study, only the parts of the sections which present the non-surgically involved bone or the bone distant from the defect (that is, 'normal' bone) were used in the present investigation.

Part II. Stained paraffin sections of rat skull bone, originally prepared for a different study, were examined. The material originated from a project in which one group of rats was gastrectomized (experimental group) and the other group had a sham operation (control group). In our laboratory we have shown that gastrectomy causes a dramatic change in the structure of skull bone (B. Klinge et al. Unpublished observations). The surgical procedure has been described in detail (17).

The calvarial specimens were in vivo fixed in buffered Stefanini solution, decalcified in formic acid, trimmed, and embedded in paraffin wax. Serial sections, 7 μm thick, were cut transversally in the middle part of the skull and then stained with hematoxylin, eosin, and van Gieson.

Ten biopsy specimens from the control group (sham operation) and 10 specimens of the experimental group (killed 6 to 16 weeks after gastrectomy) were analyzed in this study. For every specimen, two to three sections were measured with the automatic and manual method, respectively.

The sections were placed on the microscope, and the middle suture was used as a reference. The measurements were taken on the part of the sections distant from the middle suture, with the suture as borderline.

Image processing using the automatic method

The histologic sections were placed on a microscope (Leitz, Aristoplan). A video camera (Hamamatsu, CCD C3077) was coupled to the microscope and transferred the image to a display monitor (Mitsubishi, colour monitor, model FT3420 ETKL), connected with a PC (Victor, V386T) with dedicated software, which performed the measurements. The computer received information in analog form, and the signals therefore had to go through an analog-to-digital (A/D) conversion. The reverse process (D/A) must occur before a digitally stored image can appear on a gray-scale monitor for subjective interpretation and measurements. The conversion of the analog to the digital signal was done by measuring the light intensity in the analog image and expressing it as a digital figure of corresponding value.

PC-Image (Foster Findlay Associates Ltd., Newcastle upon Tyne, UK) is an application program for image processing and measurements. It includes the following functions: image processing, display and control, image analysis and transformation, geometric and arithmetic operations on images, and image file management.

The video image of each section of bone was viewed on the display monitor, and a desired part of the image could then be selectively outlined with a cursor and subsequently digitized. This area is the so-called region of interest (ROI).

The image analyzer converts the image of the selected

area to digital gray values (0–256), and subsequently these are correlated to the number of pixels. A pixel is the smallest unit of resolution of the measuring field.

Features are identified on the basis of their gray level in image analysis. Features of interest in the image can be partially masked by a variation in shading across the image (due to inappropriate staining or light circumstances). To reduce random noise, caused by video camera recording and digitization, an integration function was applied. Each pixel is measured eight times, and this function assigns for each pixel the pixel middle value. In this manner the noise is reduced in the image. Shade correction was used to correct systematic errors in image brightness, usually caused by uneven illumination of the sample. This is done by correcting the image under investigation with a 'shade' image. The shade image is normally an image captured under the same illumination conditions but without the sample (= empty glass). This shade image is captured and saved in a file. When the processing function 'shade correction' is chosen, the shade image is subtracted from the currently displayed image. Then, a median filter was used. If a pixel is accidentally given an extreme value, it is eliminated from the image and replaced by a reasonable value—the median value—in the neighborhood. This means that noise (spuriously high- or low-valued pixels) will be replaced by a suitable neighboring value. Thus, the filter has a smoothening effect, which makes it easier to set up a correct threshold.

The features of interest (= bone) were identified by their gray level and were selected by means of the gray level thresholding technique (18). In this technique the operator defines a range of brightness values in the ROI, thus defining the pixels within that range as belonging to the foreground and referring all the other pixels to the background. Such an image is then displayed as a binary or two-level image, using black and white to distinguish the regions. In some sections of the second part of the study—that is, sections from the experimental group—it was necessary to exclude some areas of soft tissue which had the same gray level as bone tissue. This was done by marking these areas with a cursor. These areas were then automatically excluded from the calculations of fractional bone volume.

Dark points in each image of the bone section, which represent bone, correspond to digitized points of low gray value. Points that do not represent bone in the bone section image have high gray values and are distinguished from the points representing bone by the threshold technique. The points of high value are then assigned the value zero. The number of low-intensity points (= non-zero points) measured in a defined region is thus proportional to the amount of bone in that region. To calculate the fractional bone value (FBV), or percentage of bone, the number of non-zero points (= NZP) within an area is divided by the total number of pixel points (TNP) digitized within the area and multiplied by 100—that is: FBV (%) = (NZP/TNP) × 100. The value of FBV was computed and displayed by the computer for each bone section placed in front of the video camera. The results of the automatic method were compared with the results obtained with a 'manual' method, using a Merz grid.

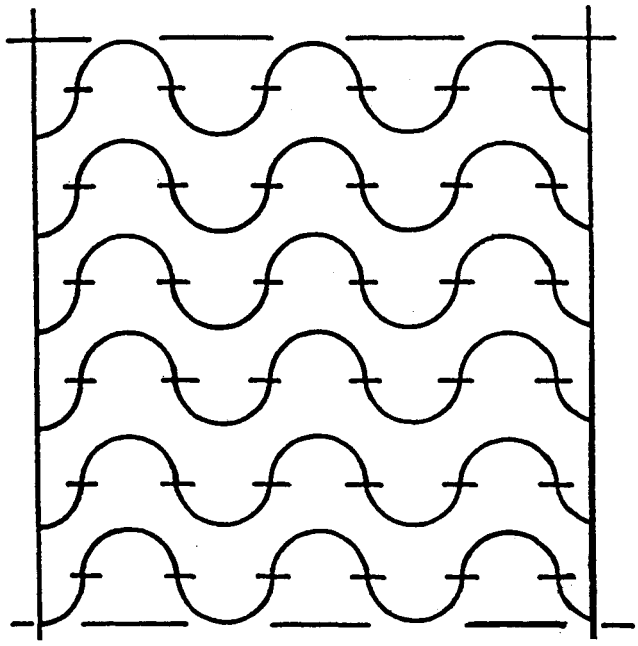

Fig. 1. The Merz grid, using semicircular parallel lines plus 36 regularly arranged test points.

The manual method

The area occupied by a particular component is proportional to the number of regularly arranged test points overlying it. Each point is considered to be representative of the area lying in a square whose center coincides with a test point and whose sides are of length d, the distance between the points (Fig. 1). Various grids are available for use on integrating eyepiece reticules.

Merz improved the more conventional grids by retaining the 36 test point arrangement but using semicircular parallel lines instead of straight parallel lines to compensate for the non-randomly oriented structures (Fig. 1).

In this study the Merz grid was placed over the same area as the area measured by the automatic method. The Merz grid was used because this grid was the only one available that covered exactly the same area as the automatic method when adjusting the magnification. The same area could then be seen on the image analysis monitor as in the microscope. The measurements with the Merz grid were automatically calculated by means of a Leitz Microvid connected between the microscope and a computer. The Microvid display is attached to the microscope and contains images of several grids.

These grids can be superimposed directly over the section in the microscope. The Merz grid was thus displayed over the section in the microscope. The test points overlying bone could then be selectively pointed out with a cursor, using the mouse of the computer, which is connected to the Microvid. By counting the test points, the percentage of fractional bone volume on that area could be calculated by the computer.

In the first part of the study, the measurements were performed 10 times on the same area, with the automatic and the manual method (Merz grid), respectively. The machines were shut off and on, and the same number of measurements were performed, using both methods. The paired *t* test was used to calculate the difference between the means of the manual and automatic measurements.

Reproducibility of the automatic method

The reproducibility of the video digitizing system was tested by digitizing the same outline (= region of interest) in a section 13 times on the same day; this on 3 different sections of a bone biopsy specimen. This determined the reproducibility of the operator skill to visually set the right gray level threshold, which in turn controls which picture points will be included in the image.

The variance in bone mass in the sections from bone specimens was analyzed by studying several sections from two specimens. Ten and 11 sections were measured from the two specimens, respectively, and the mean and standard deviations of fractional bone value were calculated to indicate the variance in the sections from the same bone biopsy specimen.

The variance in processing the same image at different times was calculated to indicate the day-to-day reproducibility of the method by analyzing the same picture once a day for 10 days.

Factors affecting image analysis of microscopic images

Computer-assisted image analysis of microscopic images can be affected by several factors, such as specimen preparation, uneven or inappropriate staining of the sections, non-uniform illumination, and the light intensity in the microscope.

The influence of inappropriate specimen preparation and staining was avoided by selecting only the sections of highest quality, without any cracks or other artefacts in the section.

The non-reproducibility of the illuminating conditions on the specimen might represent a problem. During the day the illumination is totally different from that in the evening. The image-processing facility, called 'shade correction', can be used to solve this problem.

The influence of external illumination was evaluated by measuring the same area several times at different times (= different illumination). The variance of processing the same picture at different times has been calculated by analyzing the same picture 12 times in the morning (1100 h) and 12 times in the evening (1730 h) of the same day during the winter. The procedure was started each time with a subtraction of the 'shade' image, taken at that time.

The influence of the light intensity in the microscope has been tested by measuring the same area 12 times at light intensity 5 (= dark condition), followed by 12 measurements of the same area at light intensity 6 (= light condition). The procedure was started each time with a subtraction of the shade image at the light and dark conditions, respectively.

Table 1. Precision of the automatic method (digitization of the same outline on the same day)

Section	No. of trials	Mean percentage of bone	CV*
K90A1	13	99.7	0.0006
K90A1a	13	60.6	0.0013
K90A1b	13	39.5	0.0013

* CV = coefficient of variation.

Results

Part I

Reproducibility of the automatic method. The variance in digitization of the same outline in three different sections was very low (Table 1). The coefficient of variation (CV = SD/mean) varied in accordance with the mean percentage of FBV. The CV was a little higher when the mean FBV was lower but stabilized at the lowest mean of FBV.

The variance of the automatic measurements in different sections from two specimens was also low (Table 2). There was little variance (average = 0.3) between different sections of the same specimen.

The day-to-day reproducibility, obtained by analyzing the same image once each day during 10 days, was good. The FBV varied between 96.2% and 97.8%. The CV was only 0.0066.

Manual versus automatic method. The CV of the auto-

Table 2. The variance of the automatic measurements in different sections from two specimens

Biopsy	No. of sections	Mean percentage of bone	CV*
K90	10	99.42	0.003
K91	11	99.43	0.003

* CV = coefficient of variation.

Table 3. Means of fractional bone volume of 10 control specimens and 10 experimental specimens measured by the manual and automatic method. Results expressed as percentage of bone

	Controls ($n = 10$)	Experimental ($n = 10$)
Manual method		
Mean	87.5	27.3
CV*	0.066	0.272
Automatic method		
Mean	90.2	31.8
CV*	0.043	0.141

* CV = coefficient of variation.

matic method was 100 times lower than that of the manual method in measuring the same area. An F test to compare the variances of the two methods showed that the automatic method was significantly ($p < 0.001$) more precise than the manual method.

The differences between the means of the manual and automatic measurements were statistically significant ($p = 5.4$ e-6) according to the paired t test. However, there was a positive correlation between the two methods (Pearson $r = 0.79$).

The shutting off and on of all the machines had a minor influence on the mean fractional bone volume in both methods (3% difference in the manual method and 0.5% in the automatic method).

The difference between the automatic and the manual measurements was less than 6% in 60% of the total number of measurements.

Factors affecting image processing. Image processing was hardly affected by external illumination (daylight). The results indicate a high reproducibility independent of the time of measurement. The difference between the means at different times was only 0.06% and was not statistically different according to the paired t test ($p = 0.07$).

The results indicate that the light intensity in the microscope did not affect the reproducibility of the measurements. The difference between the means at different light intensities was only 0.09% and was not statistically significant according to the paired t test ($p = 0.19$).

Part II

Table 3 shows the means of the FBV of the 10 control specimens and the 10 experimental specimens, measured by the manual and the automatic method. The means of the FBV of the control group and experimental group differed statistically significantly according to the unpaired t test ($p < 0.05$), for both the automatically ($p = 3.3$ e-16) and the manually ($p = 8.1$ e-14) measured values. The specimens of the experimental group showed 60% less fractional bone volume than the control group, both for the automatic and for the manual method.

The differences between the means of the manual and automatic measurements were statistically significant according to the paired t test ($p < 0.05$), both in the control ($p = 0.01$) and in the experimental group ($p = 0.03$). There was a positive correlation, however, between the two methods ($r = 0.91$ in the control group and $r = 0.66$ in the experimental group).

The CV of the measurements was higher with the manual method than with the automatic method, both in the control and in the experimental group. The CV of the measurements in the experimental group was higher with both the manual and the automatic method. There was no significant difference in variance between the two methods in the control group according to an F test. However, the automatic method was more precise in the experimental group, according to an F test ($p = 0.07$).

The difference between the automatic and manual method was less than 9% for 90% of all measurements.

Discussion

The purpose of this study was to evaluate a computer-assisted image processing method for quantitative measurements of fractional bone area in sections of a bone biopsy specimen (part I). A second purpose was to apply this method in an experimental study to determine bone loss by measuring fractional bone area in sections of a bone specimen (part II).

Reproducibility of the method

The low CV in measurements of the same area in the same section on the same day reflect the high reproducibility of the method. This also determines the reproducibility of the operator skill to set the right gray level threshold visually.

The day-to-day reproducibility, obtained by analyzing the same image once each day during 10 days, was good. The CV was only 0.006.

The bone sections were quite consistent in terms of bone mass as obtained by this method by measuring different sections from two specimens.

Manual versus automatic method

The computer-assisted image analysis method was compared with a manual method, performed with the aid of a Merz grid but automatically calculated (parts I and II). The differences between the means of manual and automatic measurements were statistically significant ($p < 0.05$) according to the paired t test. However, there was a positive correlation between the two methods (parts I and II). The variations between the two methods were less than 6% for 60% of all the measurements in part I and less than 9% for 90% of all the measurements in part II.

Part I. The manual method gave a 100-fold larger standard deviation in 20 measurements of the same area: 10 measurements before and 10 measurements after shutting off the machines, respectively. An F test to compare the variances of the two methods showed that the automatic method was significantly ($p < 0.001$) more precise than the manual method. This greater variation is to be expected because missing one test point has a greater impact on the total percentage of FBV than missing some gray levels in thresholding in the automatic method.

Part II. In the second part the automatic method was applied in an experimental study on rat skull bone to measure bone loss. The means of FBV of the control group and the experimental group differed significantly according to the unpaired t test ($p < 0.05$), both automatically and manually measured. The specimens of the experimental group showed 60% less FBV than those of the control group, both for the automatic and for the manual method. These results confirm our previous findings that gastrectomy causes a drastic change in skull bone of the rat (B. Klinge et al. Unpublished observations). These results support the gastrin-gastrocalcin hypothesis, explaining the importance of the stomach in maintaining calcium homeostasis in the rat (19–21). However, more measurements in different and larger areas of rat skulls are needed to confirm this hypothesis. Two to three sections were measured per specimen with the manual and automatic methods, respectively. Systematic random sampling of 5 to 10 sections is generally advocated (22). In addition, it is generally recommended that 100–200 points are counted per specimen (22). The selection of the sections of the highest quality caused the loss of many sections. Therefore, only two or three sections per specimen were analyzed (about 100 points per specimen were counted). The selections may have introduced a systematic error.

There was no significant difference in variance between the two methods in the control group according to an F test. However, the automatic method was significantly more precise ($p < 0.1$) in the experimental group, according to an F test.

The CV of the measurements of the same area with the manual method was higher than with the automatic method, both in the control and in the experimental group. The mean FBV in most manual measurements is lower than the mean FBV of the automatic measurements, both in controls and in experimental groups. This was also seen in the first part of the study. This may be due to the limitation of the manual method. If 1 or 2 of the 36 test points of the grid are accidentally lying over a little bone lacuna, this has a greater impact on the total percentage of measured FBV than missing some gray levels in thresholding with the automatic method.

In the second part, however, the CV for both methods is much higher than in the first part of the study. This may be due to the quality of the bone sections. Although the highest-quality specimens were selected, the rat sections were of lower quality than the sections of the first part of the study, probably owing to different fixation methods.

The CV in the experimental group is higher in both methods. This difference is greater in the manual method. This may be due to the difficulties of defining bone in the experimental sections. There is a marked increase of vasculature and number of corpuscular blood elements. The bone appeared to be less compact, and this may give a higher CV in both methods. The higher CV in the automatic method can also be due to exclusion of areas of soft tissue, which had the same gray level as bone tissue. It can be concluded that a greater methodologic error may be expected because it is more difficult to define bone in sections with less bone.

Factors affecting image processing

Non-uniform illumination is a problem in imaging. Even elaborate arrangements of lights and ring lights can only approximate uniform illumination of an area. The results of this study indicate that factors like non-uniform illumination and different light intensities of the microscope have a minor influence on the reproducibility of the measurements. The reason for this is that these factors can be corrected for by operator interaction by using several image-processing functions.

Factors like inappropriate staining and preparation of the specimen, which influence image analysis of microscopic images, were avoided as much as possible by selecting the specimens of the highest quality. In preparing specimens for manual analysis, the conventional histologic care and precautions should be taken. However, in preparing specimens for image analysis, extra precautions are necessary. The reason for this is that the automatic method will readily include measurements of artefacts that have the same gray level contrast as the histologic specimens. Although a completely automatic scanner is the goal of image analysis, at present its use is restricted to that of analyses of the highest-quality specimens. Even in these cases, man/instrument interaction is often required to define or exclude areas to be measured in the specimen.

The application of automatic image processing methods to the complex microscopic patterns of bone is not new. Many studies described new computerized methods for measuring variables of bone histomorphometry and indicated the potential of these techniques for rapid, reproducible, and accurate measurements (12, 23–28). For each variable in bone histomorphometry the best method would be the less time-consuming one and the one that gives the values closest to the true values. The rapidity in performing measurements was another argument in favor of automatic analyzers in earlier studies. In this study the measurements with the Merz grid were calculated automatically by means of a Leitz Microvid connected between the

microscope and a computer. This reduced the time necessary for performing the measurements with the manual method. Thus, there was no significant difference in time between the two methods. But, on the other hand, the automatic method gives a much better reproducibility and the possibility to save images in a file. However, it should be taken into consideration that the automatic method is much more expensive than the manual method.

It is generally recommended that quantitation of bone and bone cells are performed by the use of three-dimensional stereologic methods (9, 29). These methods require two parallel sections, which are often, but not always, of known thickness and specified orientation (29). The new stereologic tools, such as a nucleator, fractionator, and dissector, were a breakthrough in stereology because they are truly three-dimensional and because they are the only stereologic methods whereby particle number and sizes can be estimated in an unbiased manner. However, in studies characterized by pronounced differences between the groups and by very large interindividual variation, two-dimensional methods can be used, provided systematic random procedures are included in all steps of the quantitation procedure and unbiased counting methods are used (22).

The methods described in this study deal with basic stereologic principles for two-dimensional quantitation and are tools for obtaining efficient and unbiased quantitative estimates of three-dimensional structures. These methods require only a single, uniformly positioned thin section. The data of this study indicate that the automatic method is an efficient tool to determine bone loss by measuring fractional bone volume in sections of a bone specimen. The automatic method was compared with the manual method but gave higher reproducibility. This technique is simple to operate and can be applied to less sophisticated image analysis systems.

However, it should be kept in mind that the quality and the standardized preparations of the sections are of paramount importance for precise measurements. To get higher reproducibility, the influence of the quality of the histologic sections should be carefully considered. The expense of the automatic method should also be taken in account.

Image processing and analysis can be of particular use in bone research—for example, in the study of bone around osseointegrated implants, healing processes after different kinds of synthetic bone implants, and in evaluating tissue regeneration procedures.

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