

**The Relationship between Systemic Metabolism and
the Structure and Deposition of Human Jaw Bone**

by

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ABSTRACT

The high rate of resorption of the residual ridge in an edentulous jaw may be related to the continuous process of systemic and local bone remodelling in the body throughout life. The aim of this study was to determine the relationship between the histological appearance and deposition of bone in edentulous jaws and the systemic bone metabolism in humans.

A convenient sample of 21 apparently healthy patients scheduled for endosseous oral implants (*a la modum Brånemark*) as they presented for treatment volunteered for the study. Prior to surgery, one gram of tetracycline *per day* was ingested by the patients to label new fronts of bone deposition. The patients were instructed to take the tetracycline for two days starting 22 days before surgery, and again for another two days after a 14 day interval. The two layers of tetracycline accumulation in the bone were used to estimate the daily quantity of bone formation during the 22 day period.

A sample of blood was withdrawn from each patient before surgery and assayed using a radioimmunoassay to detect serum osteocalcin, a non-collagenous bone protein that reflects the metabolic activity of bone formation. Along with the blood, a sample of urine was obtained to measure the concentration of Type I collagen cross-linked N-telopeptide that reflects the metabolic activity of bone resorption. A cylindrical core of bone ($\approx 2 \times 3$ mm) was taken from each of the 54 implant sites with a surgical trephine as the bone was prepared to receive the implants. Static and dynamic histomorphometric parameters of cortical bone were measured using the "point-counting" method.

There was no apparent correlation between systemic metabolism and the

histological appearance or turnover of jaw bone. The histomorphometric parameters measured on each specimen of bone showed substantial variations both between and within patients, while the porosity of the bone decreased from the anterior to the posterior parts of both jaws. Women presented relatively lower values for osteocalcin and higher values for Type I collagen cross-linked N-telopeptide, which supports the view that women are at greater risk of bone loss. Moreover, there was a direct correlation between the values of osteocalcin and Type I collagen cross-linked N-telopeptide, verifying the supportive role of both assays as markers of bone turnover.

It seems that inter- and intra-variation is an intrinsic and widespread characteristic in the structure of jaw bone, and this phenomenon is not necessarily indicative of bone metabolic disorders. We estimated from the variance in bone structure that a sample size of 125 subjects would be necessary in order to offer sufficient statistical power for stochastic contrasts of jaw porosity. Many (45%) of the specimens had woven bone, which suggests that the residual ridges have been subjected to abnormal mechanical stress. Nevertheless, all of the implants that have been exposed intraorally appear to have integrated successfully in the jaws despite the porous structural characteristic of the bone. The widespread variation in normal porosity and the limited number of subjects in the study may be the reason why we could not find any relationship between the jaw bone and systemic bone metabolism. However, the fact that the release of products of bone turnover in the serum and urine did not reflect either the histological appearance or the osteogenic activity of the specimens of bone suggests that the influence of systemic metabolism is less accentuated in the jaws than in other bones (e.g. iliac crest).

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I. Overview

There is a continuous process of bone turnover throughout life (Riggs *et al.*, 1981). After reaching a peak during the second and third decade of life, bone quantity decreases at different rates in various bones. This bone loss seems to affect women more than men, especially during the period from 50 to 65 years of age (Parfitt, 1984), while the highest rate of bone loss corresponds to the period during and after women experience menopause. Bone loss during this later period is so accentuated that half of the decrease seen in the vertebral body occurs within 10 years of menopause (Ribot *et al.*, 1988). When this destructive process exceeds physiological limits, metabolic changes accelerate bone loss which compromises the strength of bones - a condition known as osteoporosis.

Osteoporosis is defined as an absolute decrease in the amount of bone to a level below that required for mechanical support (Riggs, 1991), and the risk of osteoporosis is about four times greater in women than men. It is a known fact that osteoporosis is caused by many factors, such as aging, hormonal imbalance, or low peak bone mass. The loss substantially occurs in the trabecular bone of the hips, vertebrae, and radius (Riggs, 1991). The high rate of bone resorption in edentulous jaws, especially in the mandible, has been attributed by Atwood (1971) to osteoporotic disturbances in systemic bone metabolism. Various studies have attempted to associate the alveolar resorption with osteoporosis (Kribbs *et al.*, 1989; Bras *et al.*, 1982; Bays *et al.*, 1982). However, the evidence is inconclusive due to the statistically insufficient number of subjects, and/or to

the overlap of the results between normal and osteoporotic patients.

Oral implants have been used for many years to stabilize complete dentures (Goldberg & Gershkoff, 1949). They have three basic designs, each relating to their location to or in the jaw bone: subperiosteal; transosteal; and endosseous. Until relatively recently, oral implants were considered unreliable by the dental and medical professions because of their low success rates and unpredictable prognosis (Perry, 1980). In 1981, Brånemark and his associates presented an implant system with an 85% success rate over a 15 year period, and concluded that it was possible to predictably integrate an endosseous implant into the mandible (Adell *et al.*, 1981). Since the first results of this system were reported, the long-term stability of the Brånemark implant design has been reconfirmed by others (Zarb & Schmitt, 1990; Albrektsson, 1988; Adell *et al.*, 1990). Nevertheless, the reason for the failure of between 10 to 15 percent of the implants has not been explained adequately to date.

The dramatic increase in the use of implants has focused interest in the quality of the recipient jaw bone. Jaffin & Berman (1991) and Davis (1990) have suggested that implant failure and the quality of jaw bone are associated. They reported that implants placed into bone with a high rate of resorption were at greater risk of failure than those placed into less resorbed bone. Patients with implants may be at greater risk of osteoporosis because of their age and sex (Zarb & Schmitt, 1990), consequently information on the quality of the supporting bone could be helpful in predicting the success of the endosseous implants.

In any event, there is a renewed interest in the quality of jaw bone, and its

relationship with systemic bone metabolism, as well as its implications for implant survival. Von Wowerm *et al.* (1977, 1978, 1980), in a series of studies using autopsy specimens of bone from the mandible found that quality decreased with age, and that the quality is quite unpredictable and varies markedly between individuals. However, in our opinion, further investigation is necessary for a better understanding of the mechanism of the decrease of jaw bone quality, the factors that cause it, and its repercussions on dental treatment.

A. Objectives & Hypotheses

The objectives of this study are: (1) to characterize histomorphometrically the bone taken from the mandible and maxilla of humans; (2) to assess systemic bone metabolism using bone markers; and (3) to relate the histologic appearance of the jaw bone to measures of systemic metabolism. The hypotheses underlying the study are: (1) imbalances on the systemic bone metabolism influence the conditions of jaw bone; and (2) patients with high systemic metabolism present jaw bone with higher metabolic activity and greater porosity.

II. Bone

Bone is a specialized connective tissue consisting of cells, fibers and ground substance. The fibers and organic components of ground substance form a matrix, where mineral substances are deposited. This deposition of mineral makes bone tissue extremely hard and strong, and distinguishes it from nonmineralized tissues. Bone structure has been divided into trabecular bone and compact cortical bone: both are found in every bone in different amounts and distribution (Parfitt, 1988). Trabecular or cancellous bone develops in a reticular network with cavities that contain red or yellow marrow. Cortical bone is deposited in very dense lamellae, and forms the outer wall of all bone. There are fibrous tissues with ample blood supply that cover the inner (endosteum) and outer (periosteum) surfaces of the bones.

The osteoid - or organic bone matrix - is composed mostly of Type I collagen, and represents about 35% of the total bone content by dry weight. Type I collagen is composed of three polypeptide alpha-chains that are coiled around each other to form a triple-helical conformation (Uitto & Larjava, 1991). Type I collagen molecules aggregate in the extracellular space and form large fibrils that provide the tensile strength of this collagen. The fibers are cemented by a glycosaminoglycan substance into bundles 5 μm thick, that form a structure to support the other elements of the bone. These, in turn, can be divided into acidic (osteocalcin, osteonectin, sialoprotein I, phosphoproteins, proteoglycan, chondroitin sulfate chains) and neutral (bone morphogenetic protein and bone-derived growth factors) elements. The acidic molecules are believed to bind mineral

within the matrix (Termine, 1988); and the principal elements incorporated are calcium, phosphate and carbonate in the form of hydroxyapatite crystals, and amorphous calcium phosphate. The non-collagenous ground substance found in the bone matrix is responsible for stabilizing the crystals that leads to the hardening of the tissue. These minerals are first deposited in gaps located in the collagenous fibers. Other inorganic constituents that are also part of bone mineral are fluoride, sodium, potassium, magnesium and strontium.

Bone tissue consists of three principal cell types: osteoblasts, osteocytes and osteoclasts. Osteoblasts are responsible for the synthesis of organic matrix, and seem to be related to mineralization (Puzas, 1992). Type I collagen is the main product of osteoblasts, but they also produce osteocalcin, osteonectin, and other bone components. Teitelbaum (1992) suggested that osteoblasts help to mineralize bone because of the presence of alkaline phosphatase in the matrix - an enzyme that may break down mineralization inhibitors. During the deposition of the bone matrix, some osteoblasts are surrounded and locked into lacunae before entering a resting phase where they are called osteocytes. Osteocytes "communicate" with superficial bone lining-cells (inactive osteoblasts) through a filamentous network that seems to be involved with the calcium flux between bone surfaces and extracellular fluids (Puzas, 1992). Osteoclasts are large multinucleated cells involved principally in bone resorption. They are attracted to the area to be resorbed when lining-cells are exposed to the bone surface. They adhere to the bone surface, develop a ruffled border and a compartment where intracellular lysosomes fuse to the folded plasma membrane, and release hydrogen ions and enzymes. The

release of hydrogen ions lowers the pH of the area to dissolve and release the mineral component from the matrix into the extracellular fluid. Subsequently, the organic matrix is digested by lysosomal proteases.

A. Bone metabolism

Bone tissue is very active during pre-natal development and the intense growth period that follows. This activity is called bone modelling and refers to the change in size and shape of bones (Baron, 1992). After growth is completed, bone goes throughout life in a constant process of "rejuvenation" - a process called bone remodelling. It occurs when older bone is replaced by new bone to prevent the structure from weakening by fatigue. Some bone with a low mineral density is thereby maintained and used for mineral homeostasis. Low density tissue is used for the rapid release of calcium ions into the blood circulation.

Bone remodelling has six different phases (Figure 1.1): (1) Quiescence is the phase in which there has almost no tissue activity; (2) Activation occurs when the bone surface is exposed and when surface chemicals attracts osteoclasts; (3) Osteoclasts develop the ruffled border and initiate resorption; (4) Reversal is the phase when resorption stops in preparation for new tissue formation; (5) Formation occurs when bone matrix is deposited and its posterior mineralization begins; (6) Finally, after the cycle is finished, the bone returns to a quiescent state.

Bone remodelling happens at the same time in many of the bone structural units

(BSUs). The BSUs correspond to bricks in a wall, that are kept together by a partially collagenous (albeit highly mineralized) substance called the cement line (Parfitt, 1981). BSUs tend to preserve bone mass in the short term because of their approximate quantitative and qualitative balance between resorption and formation (or "coupling") (Peck & Woods, 1988). Therefore, bone resorption and bone formation are coupled when resorption increases actual bone formation is stimulated also.

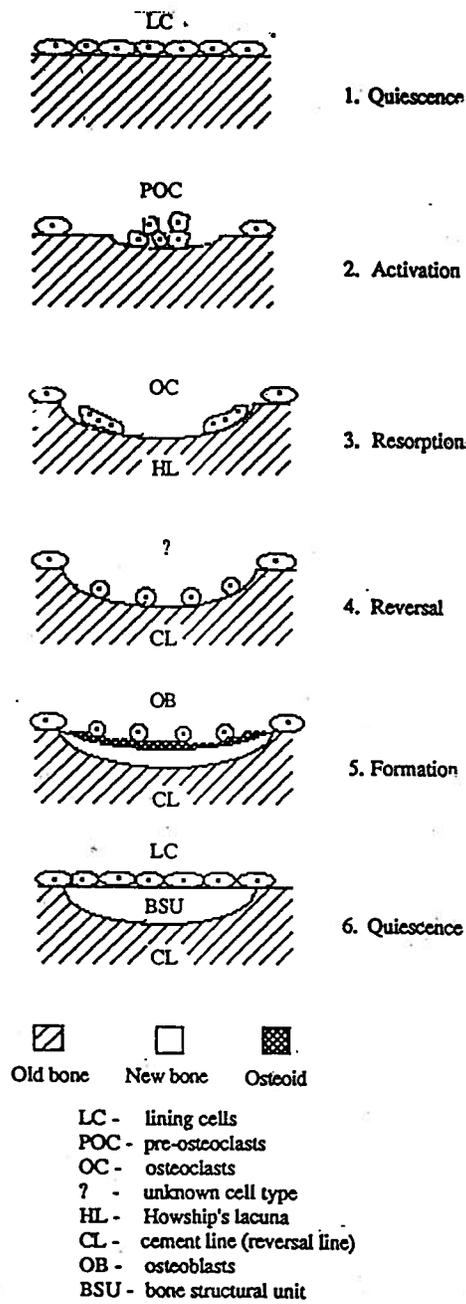


Figure 1.1 - Normal bone remodelling and construction of a single new bone structural unit (From Parffit, 1981).

Bone mass reaches a peak during adult life (Parfitt, 1988). The peak bone mass is about 25 to 30% higher in men than women, and is about 10% higher in Blacks than Caucasians. Individual variations may be related to genetic, hormonal and nutritional factors as well as levels of physical activity. There is an increase in bone resorption which is not compensated by bone formation after the fifth decade of life; therefore, peak bone mass tends to decrease over the years.

Bone remodelling is regulated by hormones, growth factors, and active metabolites that act on bone cells (Raisz & Rodan, 1990). The regulators can be divided into stimulators and inhibitors of bone resorption. The two principal stimulators of bone resorption are parathyroid hormone (PTH) and vitamin (Vit) D. PTH is the principal regulator and determinant of the calcium level in the extracellular fluid, and it increases the concentration of calcium ions. When the calcium level is low, PTH stimulates bone resorption by increasing the number and activity of the osteoclasts at the resorption sites (Gaillard *et al.*, 1979). This increase of bone resorption releases calcium ions into the circulation, reestablishing the serum calcium to a normal level. Vit D has been related to skeletal growth and mineralization (Holick & Adams, 1990). The Vit D active hormone form - 1,25(OH)₂D₃ (1,25 D) - is involved with calcium homeostasis, but in elevated quantities stimulates bone resorption (Maierhofer *et al.*, 1983). Other bone resorption stimulators include thyroid hormone, prostaglandins, interleukins, and epidermal growth factor.

Inhibitors of bone resorption are not as numerous as stimulators. Calcitonin can inhibit osteoclast activity both *in vivo* and *in vitro*, a process that seems to occur through

an alteration in the production of energy within the cell (Holtrop *et al.*, 1974). Even though it has not been demonstrated that sex hormones directly inhibit bone resorption, clinical observations have shown that they play some role in the maintenance of bone mass. Estrogen withdrawal, for example, can reduce bone mass by causing an increase in bone resorption and a small increase in bone formation (Heaney *et al.*, 1978). In healthy conditions, remodelling is a balanced process where resorption is totally countered by formation, in contrast to a condition of metabolic imbalance (*eg.* metabolic bone disease) where resorption exceeds formation. There are many regulators of bone remodelling, and the absence or overabundance of any one of them disturbs the bone.

B. Osteoporosis

Osteoporosis is defined as an decrease in the amount of bone to a level below that required for mechanical support (Riggs, 1991). It is considered a major problem in public health due to the high and increasing number of patients affected. In the United States, there are approximately 1.2 million fractures due to osteoporosis every year (Cummings *et al.*, 1985), with serious physical and social consequences. The principal sites that osteoporotic fractures occur are the vertebrae (650,000 fractures), the hip (250,000 fractures), the distal forearm (Colles' fracture, 200,000). The incidence of osteoporosis is at least four times higher among women than men, and twice greater in white than in black women (Melton, 1988). The hip fracture is particularly serious because of the 12 to 20% reduction in expected survival, and the lifetime risk for this fracture in white women

corresponds to the combined lifetime risk (15%) of breast, endometrial and ovarian cancers.

There are two distinct phases in which bone is lost throughout life (Mazess, 1982): (1) a slow and age-related phase that affects men and women equally, resulting in a similar loss of cortical and trabecular bone; and (2) an accelerated postmenopausal phase due to estrogen deficiency in women, which results in an accentuated loss of trabecular bone. The slow and accelerated phases correspond to two different cellular changes that occur in bone remodelling (Riggs, 1991). During the slow phase osteoblasts fail to completely fill the cavities created by the osteoclasts, which results in the weakening of both the cortical and trabecular bone structure(s). The accelerated phase is caused by the increased number of osteoclasts and their excessive resorption. Osteoclastic mediated bone loss causes the perforation of trabecular bone, increases the size of bone marrow cavities, and changes the structure of cortical bone to a more trabecular-like appearance. These structural changes weaken the bones more than the decrease in the actual amount of bone tissue (Parfitt, 1988).

Osteoporosis is considered to be a multifactorial disorder involving aging, menopause, and local factors regulating bone turnover (Riggs, 1991). As age progresses, there is a decrease in the efficiency of the osteoblast while the decline of oestrogen after menopause reduces the stimulatory effect that this hormone has on bone formation. The regulation of osteoblasts is impaired by an altered production of systemic and local growth factors, whereas the circulating levels of growth hormone and of insulin-like growth factor - which mediate the effect of growth hormone on bone - decreases by

about one half after the fifth decade of life (Bennett *et al.*, 1984). There is also a decrease in calcium absorption from the intestine because of the reduced production of the active Vit D metabolite which regulates intestinal absorption and causes a compensatory hyperparathyroidism that can lead to bone loss (Bullamore *et al.*, 1970).

There are also some risk factors that predispose some people to osteoporosis. An insufficient accumulation and consolidation of bone mass during skeletal growth will favour the occurrence of fractures when age-related bone loss starts. Osteoporosis is more prevalent among Caucasians and Asians, whereas Blacks usually have a higher bone mass compared with other races (Liel *et al.*, 1988). The intake of alcohol, caffeine and cigarette smoking have been linked to the development of osteoporosis, even though the mechanism is not clear (Seeman *et al.*, 1983).

Osteoporosis has been divided clinically into two categories: primary and secondary. Primary osteoporosis is the most frequent, and it in turn is divided into Type I (postmenopausal) and Type II (senile and idiopathic). Type I affects women during a period of 15 years after menopause, and it is characterized by an accentuated loss of trabecular bone. Senile osteoporosis is a result of bone loss caused by aging, whereas idiopathic osteoporosis can affect both men and women at any age and for no apparent reason (Riggs, 1991). Secondary osteoporosis is a direct result of diseases or agents that cause bone loss, such as malignant disease, parathyroid disease, corticosteroid, and inflammatory disease.

Examinations used to diagnose osteoporosis may be divided into two groups: (1) those that measure bone density, such as photon absorptiometry and computed

tomography; and (2) those that measure bone metabolism, such as biochemical markers and bone histomorphometry. The measurement of bone density in the vertebrae and radius confirms and estimates the severity of bone loss, and determines the responsiveness to the treatment (Cohn, 1990). Photon absorptiometry consists of measuring the attenuation of a narrow beam of photons as it passes through the bone, while computed tomography measures bone density by constructing a two-dimensional map of the bone in a particular area.

Assessment of bone remodelling is helpful in establishing the rate that bone loss is occurring. There are assays that measure specific circulating metabolic bone markers released into the blood and urine during the processes of bone resorption and formation (Delmas, 1990). The rate of release reflects the speed of the bone remodelling. Bone metabolism can also be assessed by histomorphometric studies on bone biopsies. This examination consists of measuring parameters like the quantity of organic bone in the biopsy specimen and estimating the rate of bone resorption and formation (Meunier, 1988).

Treatment of osteoporosis consists of drugs that inhibit bone resorption and drugs that stimulate bone formation. Antiresorptive drugs are best used to maintain the existing bone mass, although the prolonged use of them not only decreases bone resorption but also disturbs bone formation because of the coupled relationship of the two processes (Riggs, 1991). Antiresorptive drugs are calcium, oestrogen, calcitonin, and Vit D. Sodium fluoride, low intermittent doses of PTH, and a combination of calcitonin and phosphate stimulate bone formation, thereby increasing bone mass and diminishing the risk of

fractures. From all the drugs mentioned above (with the exception of calcium), only oestrogen and calcitonin have been approved by the United States Food and Drug Administration - the others are still classified as experimental drugs.

C. The process of bone resorption in the jaws

Usually the loss of natural teeth can be managed by a complete denture placed on the residual alveolar bone. Unfortunately, this prosthetic treatment does not always have a satisfactory result, due in large part to the continuous resorption of the residual ridge (Langer *et al.*, 1961; Kapur *et al.*, 1964). Atwood (1971) considered this process of resorption as a "non-recognized" disease that has an irreversibly deleterious effect on the jaws. Resorption of alveolar bone in the mandible occurs along the external layer of cortical bone. The rate and pattern of resorption varies both within and between individuals (Tallgren, 1971). Atwood (1971), citing studies in Australia (Johnson, 1963) and Scandinavia (Bergman *et al.*, 1964), stated that this condition occurs without reference to race or geography. Possible factors, suggested by Atwood (1971), related to this phenomenon are tooth loss, aging, hormonal imbalance, size and shape of the ridge, metabolic imbalances, and physical forces directed to the jaw bone.

Findings from previous studies support Atwood's statements about the unpredictable process of resorption that affects jaw bone. Atwood (1963), using microradiographs, described post-extraction changes in alveolar ridge shape as a result of resorption of the external cortical bone. He based his diagnosis of osteoporosis on

the microscopic appearance of the osteon or Haversian canal - the structural unit of bone - by measuring increased variation in osteon density, increased number of incomplete osteons, and increased porosity. By applying these criteria Atwood found that one-half of the biopsy specimens had osteoporosis. Atwood's study lacked information about the history of the subjects, so it was not possible to explain the shape and structure of the bone solely on faulty metabolism. There may well have been surgical reasons for the loss of alveolar bone.

A longitudinal study that measured the variation in alveolar bone height over 25 years of denture-use showed an average of a 9-10 mm reduction in the lower jaw, and a 2.5 to 3 mm reduction in the upper jaw (Tallgren, 1971). The reduction rate of the alveolar bone varied in time from person to person, and no identifiable pattern of reduction was found. Tallgren also observed that more pronounced resorption occurred in mandibles with large beta-angles and small gonial angles (Figure 1.2).

Atkinson & Woodhead (1968) estimated the density of *post-mortem* bone specimens by measuring the amount of water displaced by them. They found that the porosity increased with age, and that it was more prevalent in the alveolar bone compared to the basal cortex. Edentulous and dentulous ridges lost similar quantities of bone with advancing age, with an allowance for a greater decrease in the height of the ridges. An increase in porosity coincided with mandibular areas of intense growth activity in children, an observation that the investigators suggested may result from predisposition to higher metabolism in these areas.

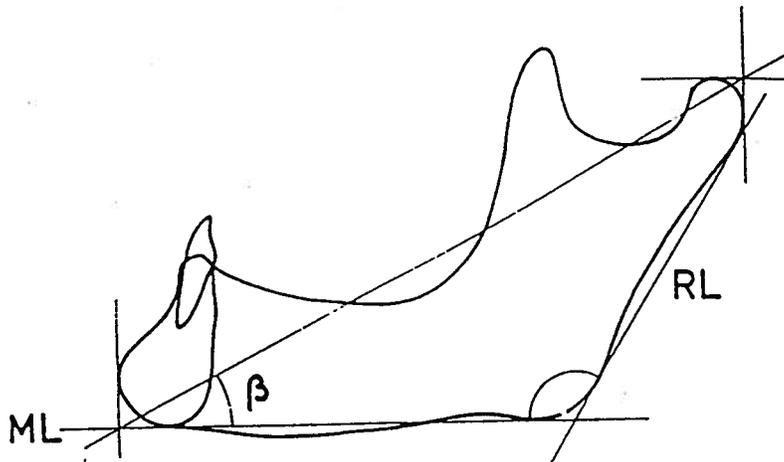


Figure 1.2 - The shape of the mandibular base in terms of the beta-angle and the gonial angle (RL-ML) (From Tallgren, 1971).

Von Wowern & Stoltze (1980) analyzed the pattern of age-related bone loss in mandibles, using microradiograms of a specific site in the buccal cortex. They examined the percentage of bone mass, the mean cortical width and the percentage of resorption on osteons. They reported that the cortical porosity and the percentage of resorption on osteons increased with age, especially after the age of 50 years; but neither porosity nor resorption seemed to be related to gender. Although men had greater bone mass than

women, their pattern of bone loss was similar. There was also a marked individual variation in bone mass; nevertheless, the authors concluded that mandibular bone loss is as dependent on the individual as it is on age. It seems that, because there is no gender difference in the pattern of bone loss, jaw bone may not be so susceptible to the hormonal imbalance after menopause.

a. Studies relating jaw bone resorption and systemic metabolism

Morphometric analysis has been used to compare cortical porosity and mean cortical width between the mandible and the iliac crest (Von Wower, 1979). There was no correlation between the results of both bones: histomorphometric measurements of iliac bone do not predict the amount of bone mass in the mandible (and vice-versa). Von Wower (1985) measured bone density of mandible and distal radius using dual-photon absorptiometry, and reported that the results did not correlate. Bone density of the mandible also did not correlate with that of the forearm and lumbar spine (Von Wower *et al.*, 1988). Therefore, it seems that the evaluation of bone mass in the mandible must be done by analyzing the mandible itself.

Systemic conditions may influence the metabolism and resorption of alveolar bone. Kribbs *et al.* (1989) measured the bone density between the roots of lower first and second premolars in a group of normal and osteoporotic subjects. They tried to use periapical radiographs to diagnose systemic osteoporosis by determining bone density against the standardized density of an aluminium step-wedge; reported that the

osteoporotic patients, when compared to normal subjects, had relatively porous mandibles. However, density values in both groups overlapped, so it was not possible to distinguish clearly between the bone appearance of normal and osteoporotic subjects. Moreover, the radiographic method they used may have been inaccurate. A close inspection of their methods suggests that the step-wedge may have had structural problems that upset the standardization of bone density (this will be discussed later).

Bras *et al.* (1982) used the thickness of the mandibular angular cortex to diagnose metabolic bone loss, and concluded that postmenopausal women had thinner cortices. But, they made no allowance for the attachments of the masseter and medial pterygoid muscles, nor for the variance in the resorption of the mandible (Atkinson *et al.*, 1968). Bays *et al.* (1982) used photon absorptiometry and bone histomorphometry to diagnose metabolic bone disease in patients with severe mandibular atrophy, and they found that almost half of the subjects had bone metabolic disorders. However, they did not perform any close examination of the mandibles in order to rule out any other cause of the atrophy. A similar study was done by Habets *et al.* (1987), who reported a higher degree of resorption in mandibles of subjects with bone disorders; but they used the mandibular angular cortex [as proposed initially by Bras *et al.* (1982)] to establish a relationship between mandibular atrophy and the presence of metabolic bone diseases. In summary, it is still unclear as to whether or not the process of resorption in jaw bones is related to systemic metabolic conditions.

III. Oral implants

A lot of effort has been directed towards developing an implant that provides denture support while compensating for the loss of alveolar bone. Essentially, there are three categories of implant design all based on the location of the implant to the jaw: subperiosteal, transosteal, and endosseous. The subperiosteal implant consists of a metallic frame placed under the periosteum overlying the cortical bone, with vertical posts that traverse the oral mucosa, and to which the denture is fixed (Goldberg & Gershkoff, 1949; Weinberg, 1950). The transosteal implant is composed of two posts that pass through the lower border of the mandible - through the alveolar ridge - and holds the denture in the mouth (Cranin, 1970). Between these posts there are another five endosseous posts supporting a plate that fits the inferior border of the mandible. The endosseous implant can be shaped like the root of a tooth or like a blade (Linkow, 1966). The implant is inserted directly into the jaw bone, and protrudes through the mucosa with an attachment for the denture.

In the United States, the National Institute of Health (NIH)-Harvard Conference reviewed the scientific evidence supporting the use of oral implants, and established criteria for judging implant success (Perry, 1980). None of the implant designs were suitable for unrestricted use in humans because of the inadequate data on the outcome of the treatment after five years. However, the Conference failed to consider the results of the clinical trials performed in Sweden on an endosseous cylindrical, threaded implant designed by Brånemark *et al.* (1977). The Swedish trials obtained a 89-100% success

rate after 10 years, thereby fulfilling the criteria of the NIH-Harvard Conference for unrestricted use in humans.

The Swedish group began their investigation of implants following a study on the repair and regeneration of bone and bone marrow (Brånemark *et al.*, 1985). The initial research focused on the induction of bone and bone marrow using an optical titanium chamber that was placed in the fibula of rabbits. They discovered that the chamber could not be removed without breaking the bone surrounding it. Apparently, the bone had grown along the microirregularities on the surface of the titanium chamber. Following this observation, they experimented with commercially pure titanium cylinders in the edentulous mandible to serve as support for dentures (Brånemark *et al.*, 1977).

The Swedish implant is placed and activated as a two-stage surgical procedure (Brånemark, 1985). Initially, a hole is drilled in the jaw bone and the implant is tapped into place and covered by mucosa for a healing period of three to six months. At the second stage, the top of the implant is exposed and a titanium cylinder which protrudes through the mucosa is attached. The cylinder is used to attach the implant to the denture (Figure 1.3).

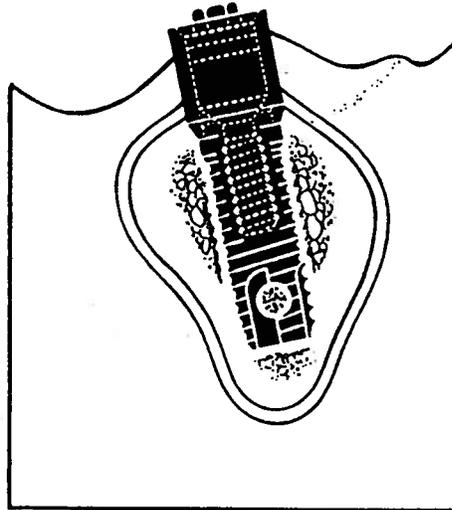


Figure 1.3 - Implant and abutment placed. From Adell *et al.* (1985) In: Tissue-Integrated Prostheses, Osseointegration in Clinical Dentistry, eds. Brånemark, Zarb, Albrektsson, pp.231.

The encouraging results of this technique promoted the interest of the dental community, and then attempts to replicate the clinical trial were initiated. Zarb & Schmitt (1990), using exactly the same technique, reported a success rate of almost 90%, while a multicentre study, involving 11 teams and spread over nine countries, obtained similar success (Albrektsson, 1988). A recent study by Adell *et al.* (1990) analyzed 4,636 implants placed within a 20 year period, and the results demonstrated the same long term stability. Therefore, it seems that the Swedish implant offers a satisfactory way to

compensate for denture instability.

A. Criteria for the Brånemark implant system

Before the NIH-Harvard conference, the reports on oral implants lacked criteria for an effective evaluation of clinical conditions or outcomes; thus, it was difficult to compare data from different research centres. The criteria for successful implants established at the NIH-Harvard conference were: (1) the implant should not move more than 1 mm in any direction; (2) bone loss around the implant no greater than one-third of its vertical height; (3) a favorable prognosis for five years, but there was no minimal success rate percentage offered. Albrektsson *et al.* (1986) elaborated on the criteria above by suggesting five criteria that an implant must meet: (1) implant immobility; (2) no radiographic evidence of peri-implant radiolucency; (3) 0.2 mm allowable bone loss annually after the first year of load; (4) absence of signs and symptoms of pain and infection; and (5) a minimal success rate of 85% at the end of a five-year observation period, and 80% at the end of a ten-year period.

It seems that the bone in some areas of the jaws is not as favourable as others for the integration of implants (Albrektsson, 1992). In an attempt to discriminate between favourable and unfavourable bone, Albrektsson & Zarb (Albrektsson, 1992) divided the jaws into two zones: Zone 1 - the anterior region of the mandible and maxilla; and Zone 2 - the posterior region of the mandible and maxilla. They suggested that the criteria for the success rates in Zone 1 should be 90% after 5 years and 85% after 10 years, whereas

in Zone 2 a success rate of 85% and 80% was offered as an acceptable criteria. However, no evaluation of the clinical trials has been performed to confirm that these zones correspond to more or less favourable bone.

The diversity of jaw bone has been classified by Lekholm & Zarb (1985), using panoramic radiographs, into: (1) the bone shape reflecting the contour of the residual alveolar ridge; and (2) the bone quality reflecting the proportion of cortical to trabecular bone. In addition, they described five different residual jaw shapes: (A) minimal/no alveolar ridge resorption; (B) moderate resorption; (C) advanced resorption with all of the basal bone remaining; (D) some basal bone resorption; and (E) extreme basal bone resorption. The four categories of bone quality are: (I) jaw composed of almost all compact bone; (II) a thick layer of cortical bone surrounding trabecular bone; (III) a thin layer of cortical bone surrounding trabecular bone; and (IV) basically low density trabeculae bone. The classification of bone shape is similar to the classification already proposed by Atwood (1971) for the residual alveolar ridge. However, because the classification is based on panoramic radiographs that lack subtle detail, it is difficult to use the observations clinically to predict the quality of bone.

B. Bone formation and the tissue-metal interface

New bone formation around oral implants is dependent upon the structure and chemical properties of the implant surface and upon the first layer of molecules absorbed by the implant (Albrektsson *et al.*, 1983). A study comparing different metals

demonstrated that titanium is more biocompatible and corrosive resistant than most other metals (Lundskog, 1972). The formation of an oxide layer on its surface within seconds after being exposed to air prevents direct contact between the bone and the pure metal (Clark, 1968). This layer is approximately 10nm ($1\text{nm} = 10\text{\AA}$) thick, and probably reduces the corrosion of the titanium and diffusion of metallic ions into the surrounding tissues (Albrektsson, 1981). It also attracts calcium and phosphate ions to form calcium phosphate - a highly biocompatible ceramic (Havawa, 1991).

After the implant site is prepared by sequential drills and the implant is tapped into place, a process of repair of the surrounding tissue starts. The blood clot, necrotic cells and the remaining damaged bone matrix are removed by phagocytes (Weinlaender, 1991). The periosteum and endosteum begin an intense cell proliferation which forms a cellular tissue - the connective tissue callus - around the implant. After a week, the cells of the callus differentiate into osteoblasts and begin to produce the osteoid (organic bone). Finally, the osteoid is mineralized by the deposition of calcium and phosphate ions.

Albrektsson *et al.* (1981) described the bone-metal interface using electronmicroscopy on specimens of bone obtained from implants that failed because of reasons other than a loss of integration. They believed that a direct contact between the metal oxide coated implant and bone tissue is a prerequisite to long term survival of the implant, and they described the interface as a zone in which it is difficult to determine the transition of inorganic to organic components. Linder *et al.* (1983) developed a method to address the inherent difficulties of preparing thin sections of a hard metal implant for

electromicroscopy by using a titanium-coated epoxy implant that can be sectioned without significant harm to the specimen. The interface described for this titanium coated implant was similar to the interface found with the bulky titanium implant used *in vivo*, even though the magnification of the electron microscope used in the studies by Albrektsson *et al.* (1981) was lower than the magnification used by Linder *et al.* (1983).

A summary of the biological mechanism by which a titanium implant is integrated with bone has been constructed by Albrektsson (1983); however, the description is controversial. Metallic ions (Na^+ , K^+ and Cl^-) and small molecules of blood attach themselves to the titanium and change the chemical properties of the implant surface. Proteins and lipids, with high free-energy, attach to the titanium surface, but the plasma lipids may form complexes with other molecules which may restrict their binding ability (Hayhoe & Quaghino, 1980). Proteoglycans and glucosaminoglycans form a layer approximately 20nm thick on the implant surface, and then interact with the cells of the surrounding tissues. Collagen filaments are observed from 20nm to 1 μm around the implant, and are arranged in a three-dimensional lattice. Going further in the direction of the surrounding bone, the network of filaments is replaced by bundles of collagenous fibrils and fibers. Osteocytic processes also approach the oxide layer, but are separated from the metal by the protein layer. Calcium deposits are found very close to the oxide surface in sections of the bone-implant interface.

In contrast to these observations by Albrektsson (1983), the protein layer between bone and titanium oxide has not been found in some recent studies. Chehroudi *et al.* (1992), studying the effect of implant surface topography on bone formation, found a

direct contact between bone and metal without an interposed protein layer. Listgarten *et al.* (1992), in their study on the relationship of the titanium surface to the surrounding tissues, also described a direct bone contact with collagen fibers contacting the implant surface in some areas. These findings indicate that it may be necessary to look more closely at the bone-implant interface.

Albrektsson *et al.* (1981,1983) reported that there was no soft tissue between bone and metal. Using the Donath method of light microscopy, a technique that was not clearly described, Albrektsson *et al.* (1990) measured the "quantity" of bone-metal contact, and indicated that there was an average of 90% of direct bone contact with the implant at the cortical passage after 1 year of implantation. However, some sections had thin white lines of 1 to 10 μm between bone tissue and metal. No tissue or staining was found on these lines and they were regarded as indicative of bone anchorage. The authors, however, had no explanation for the white lines, so it is possible that the bone is not in direct contact with the entire surface of the implant; the lines could also be an artifact of the histologic technique. In any event, there is no doubt that a clear description for the bone-metal interface does not exist. Moreover, there is no conclusive data affirming that the *in vitro* and *in vivo* situations are similar. The composition of the interface may vary in different areas of the implant. Unfortunately, there is no technique to prepare histological sections without incorporating a source of error. So far, it is not known if the composition of the interface at a molecular level is really critical for the implant's long-term success because the implants have been successful, despite the inconsistency of the

findings of Albrektsson *et al.* (1981,1983), Chehroudi *et al.* (1992) and Listgarten *et al.* (1992).

C. Failure of dental implants

Longitudinal studies on the Brånemark implant system have shown a failure rate of 10-15% (Zarb *et al.*, 1990; Albrektsson, 1988). There is only a small number of studies in the literature about implant failure. Usually, the discovery that the implant has not integrated with the bone is made at the second surgical stage, or during the first year under load (Albrektsson *et al.*, 1989).

A few studies showed that there might be some association between implant failure and the quality of the jaw bone. Jaffin *et al.* (1991) using panoramic radiographs described a higher failure rate in Type IV bone (as classified by Lekholm & Zarb, 1985), therefore suggesting some relation between the bone quality and the implant prognosis. The number of unsuccessful implants found by Davis (1990) suggests that bone quantity and quality, also classified using panoramic radiographs, are more important in the maxilla than in the mandible. Unfortunately, panoramic radiographs present serious limitations to this analysis due to erratic magnification, distortion, the overlapping of structures and ghost images (Hamada, 1989). Consequently we cannot draw any firm conclusions about the actual quality of the bone associated with failed implants, but it is reasonable to assume that the porosity and chemical composition of the bone will influence the

integration or attachment of implants.

IV. Bone histomorphometry

Bone histomorphometry is used largely for diagnosis, treatment and management of osteoporosis and other metabolic bone diseases (Menieur, 1988). Usually, a core of bone is taken with an 8mm inner diameter trephine 2cm behind the anterior and superior iliac spine and 2cm above the iliac crest. The specimen is considered to be high quality when it has two layers of cortical bone interposed by intact trabeculae bone. It is fixed without decalcification and embedded in methylmetacrylate resin. Nonconsecutive serial sections, 4 to 20 μ m thick, are cut with specially designed microtomes. Thinner sections (10 μ m or less) are stained to identify bone cells and to distinguish between calcified bone and osteoid tissue, while sections more than 10 μ m are not stained and are examined by fluorescent light microscopy to identify tetracycline labels that may have been used to label new fronts of bone formation. The sections are measured using a manual point-method to determine area, perimeter, and the distance between bone elements (Recker, 1982). The manual point-counting method superimposes a grid on the microscopic image to help measure static and dynamic parameters relating to the structure of the bone, and to the rate of new bone formation.

A. Tetracycline labelling

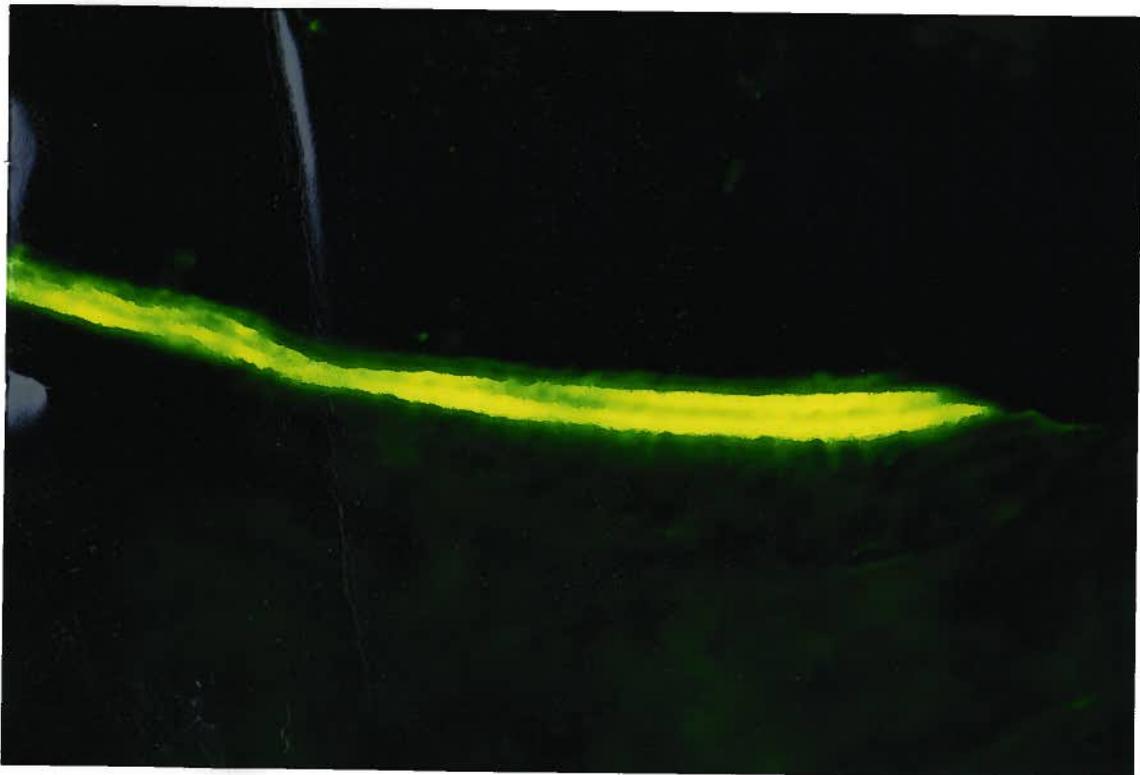
The antibiotic tetracycline chelates calcium and is deposited at sites of bone formation that can then be identified by fluorescent light (Milch *et al.*, 1957). When the level of tetracycline in blood is raised, the antibiotic binds to all bone surfaces that are accessible, and tetracycline is incorporated permanently into sites of active mineralization (Parfitt, 1983). Tetracycline binds preferentially to recently formed mineral because the crystal is small, and has a large surface area. The fact that newly formed bone has a low density and high water content favours the diffusion of tetracycline into the tissues and forms instantaneous labels.

When bone is deposited intermittently in the presence of tetracycline, the distance between the layers of tetracycline offers a measure of the rate of deposition that is seen under a fluorescent microscopy at 360-nm wavelength (Photo 1.1). In order to correctly label the forming bone and accurately estimate the quantity of bone deposited, it is necessary to follow a rigid drug-intake schedule. The schedule starts 22 days before the biopsy when the patients take one gram of tetracycline orally for two days, and after 14 days repeats the dose. The biopsy should not be performed for at least 2 days after intake to allow the tetracycline to "cement" in the tissue. When labelling is done properly, the slide shows two well-defined and easy to measure layers. The edges of each layer locate the bone-forming surface at two points in time. The center of the layer is the measuring point because it can be localized often as much as 20 times more accurately

than its edges (Pødenphant, 1990).

Photo 1.1 - Tetracycline labels under fluorescent microscope at 360-nm wavelength.

(Photo provided by Dr. M. Anderson from Calgary General Hospital, Calgary, Alta)



Tetracycline does not alter the function of osteoblasts and other bone cells: it is stable when incorporated into forming bone (Parfitt, 1983). Tetracycline remains *in situ* as long as the crystal remains intact both *in vivo* and *in vitro*. There are two characteristics of bone formation at the bone structural unit (BSU) that govern the choice of an optimum schedule for dispensing the tetracycline: the rate of new bone deposition, and the length of time taken to complete the formation phase of a typical BSU. There are, however, factors that may contribute to labelling errors. Long intervals between the intake of tetracycline may increase the number of BSUs with a single label. On the other hand, the mineralization of the newly formed bone starts usually after five to 10 days of maturation; therefore, some BSU could "escape" labelling at the beginning or at the end of the formation cycle. In addition, an individual BSU can have its appositional rate increased or retarded during formation, and a "resting" osteoid can still accept labelling (Recker, 1982).

B. Bone histomorphometric measurement techniques

Histomorphometric measurements can also be made using two other methods: a semi-automatic one and a fully automatic method. A semi-automatic method consists of three essential components; an electromagnetic sensitive x-y plate, a cursor and a computer for calculations (Vigoritta, 1984). The slide image is projected by a projecting microscope onto the x-y plate and traced manually by a cursor, and the coordinates are

electronically sent to a computer programmed to generate the appropriate data. The automatic method consists of two successive procedures: the acquisition and the processing of data (Juvin *et al.*, 1990). Images of the slides are sent by a photomultiplier to a computer and stored. The analysis is performed through the reflection of different colours in the same sample. Shape filtering of the images produced is performed by the computer and the surface area and length of the images are estimated.

It is not clear which of the three different techniques for measuring histomorphometric parameters is most precise, accurate and efficient. Chavassiex *et al.* (1985) compared the three methods and found that none was notably outstanding for measuring all of the parameters. They concluded that the automatic method was preferable for measuring bone volume, but that the manual method offered better results for measuring the osteoid volume; however, the semiautomatic method was most suitable for measuring the osteoid surface. Therefore, a combination of all three methods would insure a superior analysis of the specimen.

It is usual to obtain several biopsies for diagnosis and evaluation of therapy during treatment of metabolic bone diseases at different times. Vernejoul *et al.* (1981) did not find a variation in the mean value of the parameters of bone specimens taken from left and right iliac bone in a group of normal patients; however, there was a significant variation in specimens from the same individual. For example, bone volume measurements showed a significant variation between the values from left and right iliac bone, but there was no variation between the mean of the values of each side. Thus, the

authors concluded that bone volume is not reliable for comparing examinations from a single individual, although it is a good parameter to compare the results of groups of patients. Another study by Chavassieux *et al.* (1985a) concluded that each metabolic bone disease causes different degrees of intersample variation. In conclusion, bone histomorphometry is more reliable for inter-group analyses when various samples are to be compared.

Other limitations of bone histomorphometry are the considerable inter-observer and some intra-observer variations reported by Compston *et al.* (1986). In addition, both Ballanti *et al.* (1989) and Pødenphant (1990) agree that bone histomorphometry is accurate for diagnosing individuals with advanced and severe disease, and also when comparing groups. They suggested combining bone histomorphometry with other metabolic bone disease diagnostic exams, such as circulating bone markers and bone density, because of its limited sensitivity when applied to an individual.

Bone histomorphometry offers a measure of morphologic and dynamic bone changes; however, it is best suited for studying groups of patients, and it should be combined with other methods in order to accurately diagnose metabolic bone diseases in individual patients.

V. Serum Osteocalcin

Osteocalcin (Oc), also called bone gamma-carboxyglutamic acid containing protein

(BGP), is the most abundant noncollagenous protein of mammalian bone (Price *et al.*, 1980). It has a molecular weight of 5,800 and contains three residues of gamma-carboxyglutamic acid, a vitamin K-dependent amino acid. It is synthesized by osteoblasts and incorporated into the extracellular matrix of bone. Oc has been described as the most satisfactory marker of bone turnover, which in turn reflects bone formation (Delmas, 1991). Its role in bone metabolism has not been defined fully, but osteocalcin binds to calcium and especially to hydroxyapatite (due to the presence of gamma-carboxyglutamic acid in its molecular constitution). Because of this property, Oc may play an important part in the mineralization of bone.

There may also be a relationship between calcium and the regulation of Oc (Gundberg *et al.*, 1991). Induced hypocalcemia in humans (by administering citrate) causes an expected increase of parathyroid hormone (PTH) - the hormone responsible for calcium homeostasis, and stimulates bone resorption to increase calcium blood level. The hypocalcemia also causes an increase of Oc as acute and rapid as the PTH increase, and conflicts with the fact that the Oc found in serum is newly synthesised (and has not been stored). Oc should not rise rapidly unless it had been stored in the osteoblasts, although no evidence of storage has been found. It is also unlikely that bone formation would be increased (an increase of Oc is related to an increase of bone formation), at the initiation of bone resorption, leading to reestablishment of calcium equilibrium. These intriguing results suggest that further investigation is necessary in order to verify the actual origin of serum Oc.

Oc was first measured from serum using a radioimmunoassay (Price, 1980). This assay consists of the substance (X) to be measured, an antibody to the substance X, a "tracer" - a preparation of substance X marked by radioactive isotopes - and a "standard" preparation with known quantity of substance X. The principle of the assay is as follows: (1) the tracer and antibody are added to the sample with an unknown quantity of substance X, and a competition for the antibody begins between X and the tracer; (2) the more X there is, the less the radioactive tracer will bind to the antibody; (3) the bound and unbound substances are separated, and the rate of radioactive free substance is measured; and (4) when the standard is added to the sample, more tracer will be displaced from the antibody and the rate will be lower (Thorell & Larson, 1978). Gundberg *et al.* (1985) compared a very well-defined radioimmunoassay with two commercial kits (Immuno Nuclear Kit and Seragen Kit) to measure Oc, and concluded that there was a good correlation between the three assays. They also advised the use of only one assay with a research project because of the differences between the assays.

Oc is present specifically in bone, and the serum concentration of Oc reflects changes in bone metabolism. The serum concentration may reflect the portion of newly synthesized Oc in that, instead of being adsorbed, it is released into the circulation. A wide range of values from normal subjects has been reported, which makes it difficult to compare data (Azria, 1989). Power and Fottrell (1991) reviewed the various aspects of the Oc radioimmunoassay that could contribute to these differences, and they suggested that some of the structural features of Oc, such as the peptides from the break-down of

Oc in liver and plasma, may influence Oc immunoreactivity. The peptides, reacting with antibodies raised against intact Oc, may explain the wide-range of Oc values published. Another factor that contributes to the range of values is the multiple components of the radioimmunoassay and the variety of methods by which these components can be obtained, since their specificity to react with Oc may vary.

Oc serum concentration varies throughout life. Duda *et al.* (1988) measured serum Oc from patients with an age distribution from the second to the eighth decade. The values decreased as age increased, and reached the lowest point between age 30-50 years. From the lowest point at the fifth decade, the values increased with age. Women had lower values than men. In this same study the investigators also measured bone alkaline phosphatase from normal subjects and patients with metabolic disease. The expectation was that alkaline phosphatase and Oc values would correlate and better reflect bone metabolism. However, even though bone alkaline phosphatase and osteocalcin presented the same pattern of decrease and increase related to aging, the values did not correlate in four of the nine bone diseases. This fact is not surprising because bone formation is such a complex process that biochemical markers reflect different aspects of osteoblast function, and vary from disease to disease.

A longitudinal study in normal men agreed with the study by Duda *et al.* (1988), also describing an increase of serum Oc with age (Orwool *et al.*, 1990). Another study comparing the age-related changes of serum Oc between men and women also reported that men presented higher values than women (Vanderschueren *et al.*, 1990). However,

the authors described an increase of Oc only in women in the sixth decade of life. Trying to correlate low bone density and Oc, Sherman *et al.* (1992) found that older women with lower density in the spine and the radius had higher serum osteocalcin values. It seems the Oc does reflect bone metabolism. It is known that women are subjected to an increase of bone metabolism during and after the period of menopause, and that this increase usually results in a permanent loss of bone tissue.

VI. Type I collagen cross-linked N-telopeptide in urine

This section will discuss the methods that have been used to monitor bone resorption rates in humans, with a particular emphasis on the method described by Hanson *et al.* (1992) that identifies a cross-linked fragment of Type I collagen.

The metabolic changes caused by osteoporosis and other metabolic bone diseases may be assessed by quantifying the activity of a prominent enzyme or the release of bone matrix components into the circulation. Both hydroxyproline and collagen pyridinium crosslinks are excreted into the urine and reflect the rate of bone resorption. Hydroxyproline may be useful only when a significant increase of resorption happens, as in Paget's disease or hyperparathyroidism (Singer *et al.*, 1978). Bone resorption is not the only source; diet, the turnover of connective soft tissues, complement fractions also contribute to urinary hydroxyproline. In addition, the fact that the liver metabolizes much of the hydroxyproline from collagen degradation make this marker unreliable (Delmas,

1991).

Pyridinoline (Pyr) and deoxypyridinoline (D-Pyr) are two non-reducible pyridinium crosslinks present in collagen (Eyre *et al.*, 1984). Their concentrations in connective tissues are very low and vary with tissue type. Pyr is present in Type I collagen of bone and Type II collagen of cartilage, whereas D-Pyr is found in large quantities only in bone Type I collagen. Pyr and D-Pyr are excreted in urine, both freely and bound to a peptide. Uebelhart *et al.* (1991) used a high performance liquid chromatography assay to measure Pyr and D-Pyr concentrations in pre and post menopausal women, and found that there was an increase of 62% in Pyr and of 85% in D-Pyr after menopause. Furthermore, the postmenopausal women who received hormone therapy returned to premenopausal levels, in contrast to unchanged levels of the placebo group.

Beardsworth *et al.* (1990) described age changes with urinary excretion of Pyr and D-Pyr in normal adults and children. Pyr and D-Pyr levels were higher in children than in adults, which reflects the intense bone metabolic activity during growth. However, there was no evidence of sex or age variation within mature adults, except in the 20-30 year age group. This fact is surprising since bone metabolism changes with age (Parfitt *et al.*, 1984). Eastell *et al.* (1992) found a positive correlation between Oc and D-Pyr measurements in pubertal girls, and in pre and postmenopausal women as expected, because bone formation and bone resorption are coupled processes. Because the correlation was significant in postmenopausal women, the authors suggested that this relationship could be used as an indicator to identify candidates for bone loss: subjects

with increased D-Pyr and with no correspondent (decreased or stable) Oc are likely to lose bone.

Even with satisfactory results, the Pyr and D-Pyr assay is inconveniently complex (Delmas, 1991). Pyridinoline cross-linking residues are excreted primarily in a peptide-bound form, so Hanson *et al.* (1992) developed a less complex, specific immunoassay with a monoclonal antibody to detect a cross-linked fragment of Type I collagen secreted in urine. They looked for a cross-link excreted as a reproducible fraction of total bone-derived pyridinolines, and used it as a quantitative measure of the systemic rate of bone resorption. The antibody was raised against the pyridinoline-containing fragments of N-telopeptide cross-linking domain of Type I collagen. The results obtained from normal subjects of various ages, pre and postmenopausal women, and patients with Paget's disease support the hypothesis that the excretion of the cross-linked N-telopeptide provides a direct and reproducible measure of bone resorption.

VII. Bone density

Bone density is defined by the ratio of the intensity of incident light from the radiograph by the intensity of light transmitted by the densitometer.

$$\text{Bone Density} = \frac{\text{intensity of incident light}}{\text{intensity of transmitted light}}$$

Under controlled exposure factors and processing, a radiograph is darker or lighter depending on the amount of bone tissue that serves as an obstacle to the X-rays. When there is a decrease of bone tissue, a larger quantity of X-rays interacts with the film, causing it to be darker. To measure bone density, the radiograph is placed on a densitometer that emits a calibrated light beam through the radiograph. The dark radiograph permits less light to come through it, and the bone is described as having low density. Measurements of bone density are used to: (1) identify patients who are losing bone abnormally; (2) monitor the progress of this loss; (3) and to evaluate the treatment to slow down bone loss so as to promote bone formation.

There are several techniques to evaluate the amount of bone tissue the skeleton has lost. The choice of technique depends on various factors, such as the radiation dose, the site of measurement, the cortical or/and trabecular bone to be assessed, and the cost. Moreover, the technique selected must be assessed for accuracy, precision and sensitivity. Accuracy is the capacity to measure the exact quantity of bone present at a specific site. Precision is the reproducibility or reliability of the technique when independent measurements are made at the same site. Sensitivity is the ability to detect small variations in the quantity of bone present. The five techniques available are:

- A. Radiogrammetry**
- B. Radiographic photodensitometry**
- C. Single photon absorptiometry (SPA)**
- D. Dual photon absorptiometry (DPA)**

E. Quantitative computed tomography (QCT)

A. Radiogrammetry

Radiogrammetry involves a morphometric measurement of cortical bone using a micrometer on radiographs and is related to bone dimension instead of bone porosity. It is the simplest and least expensive method to quantify bone tissue loss (Tothill, 1989). Measurements are made on the appendicular skeleton, such as radius, humerus, femur, and the midshaft of a metacarpal. It is not feasible to use this technique in the upper and lower jaws. The limitations of this technique are: (1) the difficulty in placing the limb in the exactly same position for several radiographic examinations (the same positioning allows the alignment of the edges of bone on the radiograph image when comparing radiographs); (2) observer variation caused by inexperience or fatigue; (3) a 30-60% change of bone tissue quantity is necessary to show clearly on radiographs; and (4) the technique gives information only about cortical bone, and it is not applicable to bone containing many trabeculae (such as the vertebrae and the distal radius).

B. Radiographic photodensitometry

The optical density (i.e. the amount of light that comes through the film) in radiographic films is measured using a photodensitometer. As explained above, when

bone loss occurs the overall mineral content of the skeleton decreases, and a larger quantity of X-rays interacts with the film to produce a darker image. Because of variations in exposure, film characteristics, processing and soft body tissue thickness, a step-wedge is attached to the film prior to exposure to serve as a reference. The photodensitometer scans the radiograph to form a projection curve of bone density in specific areas of interest, and this is compared to the curve obtained from the step-wedge.

In an ideal situation the step-wedge would have the same atomic number and specific gravity as the minerals in the bone. Various materials, such as solutions of calcium, potassium, suspensions of similar materials in plastic, bone, ivory and metal have been used to fabricate step-wedges. Aluminum is the most commonly used material in wedges, particularly when alloyed with zinc because it has radiolucent characteristics similar to bone mineral (Colbert & Bachtell, 1981). The unit of measurement is called an "aluminum equivalent", and is expressed in g/cm.

Photodensitometry has been used mostly on radiographs of upper limb where the cortical and trabecular layers are quite distinct. It is ill-suited, however, to small bones or for detecting small, early changes in trabecular bone (Tothill, 1989).

C. Single photon absorptiometry (SPA)

SPA also utilizes measurements of beam attenuations to quantify bone density. It uses a radioactive source, usually Iodine 125 (average energy = 28keV) or Americium

²⁴¹ (energy = 60 keV), that emits a monoenergetic gamma ray that passes through the site of interest. A gamma camera captures and measures the beam attenuation caused by the object. The attenuation corresponds to the mineral content of the bone and it is expressed also in g/cm. The soft-tissue thickness may interfere with the results, so to avoid this problem the limb to be scanned is immersed in a water bath. The scanning can be made in a single pass across the bone or in a rectilinear manner to analyze a larger area. As in the photodensitometry, the SPA does not distinguish between cortical and trabecular bone, thereby decreasing its sensitivity, nor is it suitable for the jaws because of the need for a relatively constant thickness of soft tissues to obtain accurate measurements (Cameron & Sorensen, 1963).

D. Dual photon absorptiometry (DPA)

DPA is similar to SPA, but it uses two photon energies instead of one to provide bone scans not possible with the SPA. The need for a constant thickness of soft-tissue around the bone is not so critical for accurate measurements because of the dual photon beams. The radioactive sources are Gadolinium 153 (44 keV, 100 keV), or a combination of Iodine 125 with Americium 241 (29 keV and 60 keV respectively). Because of the two sources of energy, DPA can be applied to other bones such as the spine and the femur. Using especially developed equipment, Von Wowern (1985) used this technique to measure bone density of mandibles. Like SPA, this technique does not differentiate

between cortical and trabecular bone, making the detection of small changes in the quantity of bone tissue difficult.

E. Quantitative computed tomography (QCT)

QCT also follows the principle of beam attenuation caused by the object. This technique provides images of parts of the body as well as measuring the content of bone mineral from the image or image data set. It produces a cross-sectional image from any part of the body. Through mathematical reconstruction from the attenuated outline, a two-dimensional map of the beam attenuation coefficients in a cross section can be produced. Tissue density can be obtained from any point on the image, so it is possible to measure trabecular bone without interference from the surrounding cortical bone. A bone equivalent (i.e. K_2HPO_4) standard reference is used for calibrating the scanner and determining the bone density, which is expressed as K_2HPO_4 equivalent (mg).

There are two types of QCT: a whole-body scanner, and a special purpose scanner used with only appendicular skeleton. The whole-body QCT uses x-rays in single or dual-energy, and it is able to measure the trabecular bone of the vertebral body core. It has been used on the jaws in order to obtain cross-sectional images of the residual alveolar ridges which are of particular interest when evaluating the shape of the bone for implants (Hamada, 1989). However, the measurement of bone density in a specific site of the jaws is difficult because the pixels that make up the image, and from which bone

density is calculated, are too large for the reduced resolution usually required for implants. The special purpose QCT uses gamma-rays photons instead, and it is also able to distinguish between trabeculae and cortical bone; however, its use is limited to the appendicular skeleton.

MATERIALS AND METHODS

I. The patients

The patients that participated in this study were selected from the Implant Clinic at the Faculty of Dentistry at UBC, and from private dental practices in Vancouver in the order in which they requested treatment with implants. Patients requiring implants were chosen because of the convenience for obtaining bone specimens. Twenty one patients - 11 women and 10 men - ranging in age from 44 to 78 years agreed to participate in the study (Table 2.1). The age distribution was similar in a study by Parfitt *et al.* (1984) who demonstrated that bone changes are greater between 50-65 years of age.

Table 2.1 - Age and sex distribution of implant patients.

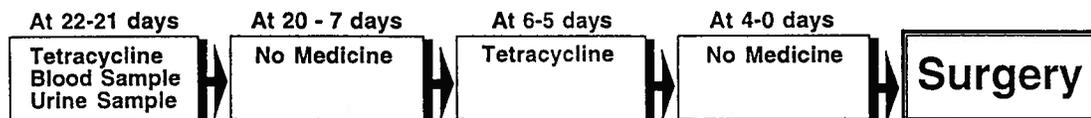
	Age group		
	<u>65 ys & under</u>	<u>Above 66 ys</u>	<u>Total</u>
Women	8	3	11
Men	7	3	10
Total	15	6	21

II. Patient preparation

All of the patients consented to participate in the study as required by The University of British Columbia Behavioural Sciences Screening Committee (Appendix 1). They were asked to fast over-night until the following morning when 10ml of blood were withdrawn using a standard procedure. The blood was centrifuged immediately to separate the serum, which was removed and frozen for storage at - 70°C. The subjects provided a urine sample at the same time of the day on three consecutive days. The three samples were used to obtain a mean value for Type I collagen cross-linked N-telopeptide concentration in urine. In addition, they were asked to take tetracycline to label the mineralizing front of new bone. Twenty two days before the implant surgery, the patients took 150mg of Demeclocycline four times a day for two days, and after a 14 day interval, 250 mg of Tetracycline four times a day for another two days. The implant

surgery was performed four days later.

Intake of Tetracycline Preoperatively



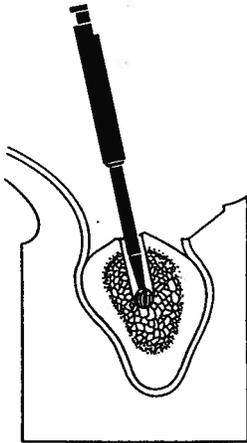
III. Bone biopsies from implant sites

A core of bone was taken during the preparation of the implant site with a surgical trephine that was smaller than the trephine recommended by Menieur (1988) for biopsy of iliac bone. The trephine (Nobelpharma Inc, Canada) had a 3 mm diameter, and was modified with a longitudinal slot approximately 10 mm above the cutting edge to allow removal of the core of bone (Photo 2.1). Circumferential marks at 5, 7 and 9 mm from the cutting edge were made also to orient the length of the cut during surgery. The effects of the modified trephine on the implant site were tested during a pilot-study on a pig's mandible, following the same procedures for placing an implant (Figure 2.1). The trephine was used after a 2mm pilot drill marked the exact location the implant was to be placed. After the sample was taken, the site was prepared with the 3mm twist drill

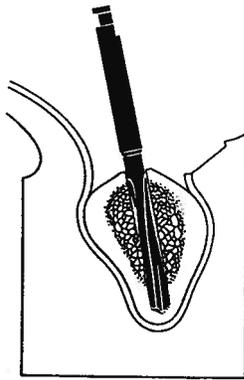
followed by the others sequential for standard preparation of the site. The threads made in bone by the twist drill were well defined, so the trephine had no obvious ill-effects on the bone left around the implant site. After the implant was placed in the bone, it made a high-pitched metallic sound when tapped with a metal instrument, indicating close adaptation between bone and implant (Adell *et al.*, 1985).

The bone specimens were cylindrical, 2mm wide and usually 3mm long (Photo 2.2). Occasionally the cut by the 2mm pilot drill removed some of the cortical bone from the top of the specimen, consequently, it was not possible to obtain an intact layer of cortical bone. Fifty nine implants were placed in the 21 patients, ranging from one to eight implants *per* patient. A core of bone could not be obtained from 11 sites, because of large marrow spaces; one man did not yield any specimen for this reason, so no histomorphometric information was available data from any of the four implant sites in his mouth. The 48 specimens of bone were obtained from different areas within the jaws and were identified by the tooth* normally located in the area (Table 2.II).

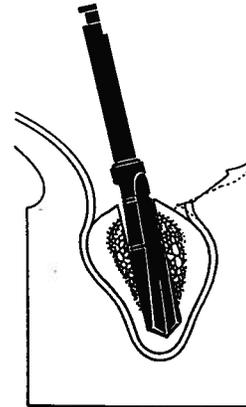
*Teeth are identified by numbers as established by the Federation Dentaire Internationale (Torres & Ehrlich, 1990).



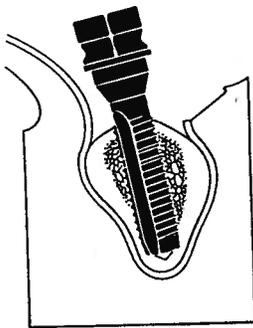
Pilot Drill



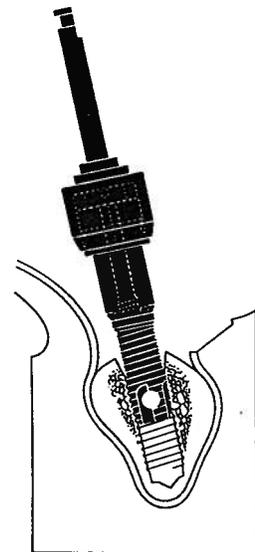
2mm Drill



3mm Drill



Screw Tap Preparation



Implant Placement

Figure 2.1 - Steps of implant placement. From Adell et al. (1985) In *Tissue-Integrated Prostheses, Osseointegration in Clinical Dentistry*, eds. Brånemark, Zarb, Albrektsson, Chapter 13.

Photo 2.1 - Trepine used to obtain specimens of bone from implant sites demonstrating the longitudinal slot used to remove the specimen and the circular marks used as a depth gauge during surgery.

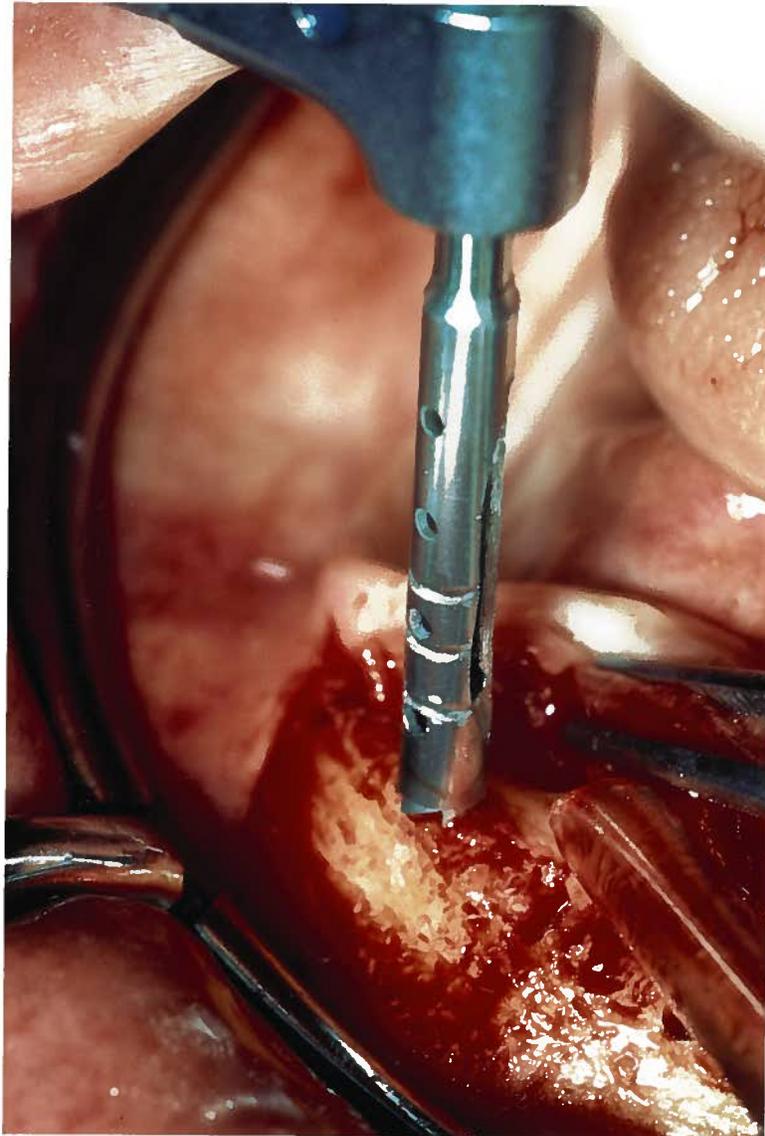


Photo 2.2 - Specimen of bone taken from implant site.

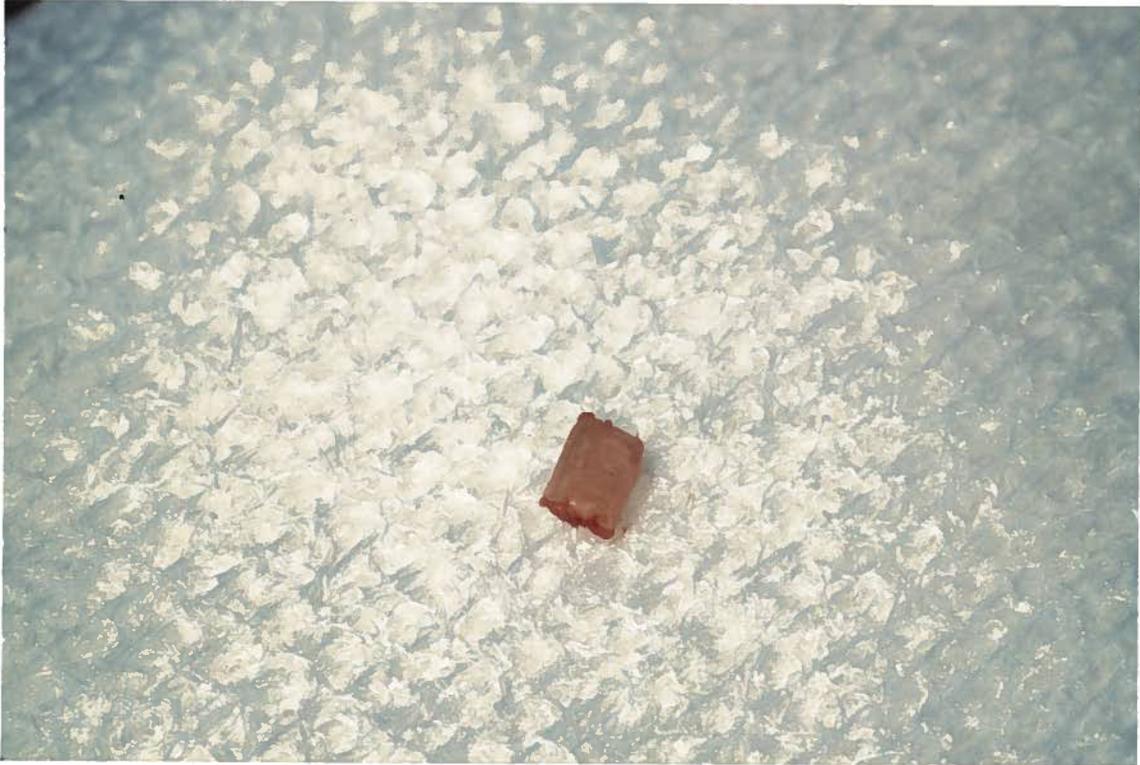


Table 2.II - The location of 48 specimens of bone taken from the implant sites in 20 patients.

		<u>Tooth number corresponding to implant site</u>															
		<u>Right</u>								<u>Left</u>							
<u>Upper jaw</u>		18	17	16	15	14	13	12	11	21	22	23	24	25	26	27	28
		-	-	1	-	-	2	3	3	3	2	1	1	1	1	-	1
		-	1	1	1	3	7	1	1	1	2	6	2	-	2	1	-
<u>Lower jaw</u>		48	47	46	45	44	43	42	41	31	32	33	34	35	36	37	38

IV. Preparation and staining of thin sections of undecalcified bone tissue

The specimens of bone were sent to the Department of Laboratory Medicine at the Calgary General Hospital for histomorphometric analysis under the direction of Dr. M.A. Anderson¹ as follows:

A. Preparation of bone specimen for embedding

Each bone specimen was placed immediately in Millonig's fixative (100 ml 37%

¹Dr. M.A.Anderson MD
841 Centre Avenue East, Calgary, AB, T2E 0A1

formaldehyde solution, 900 ml tap water, 18.6g NaH_2PO_4 , 4.2g NaOH, 5g sucrose) for at least 24 hours to preserve the tissues and the tetracycline markers. The fixative denatured and preserved the bone cells (Faugere & Malluche, 1983). They were stored in 70% ethanol until time for embedding. The specimens were dehydrated in a sequence of solutions as follows, before embedding in methyl methacrylate:

1. 50% acetone-water mixture for 24 h.
2. 100% acetone, changed every 24 h for three days.
3. 50% acetone-uninhibited methyl methacrylate monomer for 24 h.
4. absolute uninhibited methyl methacrylate monomer for 24 h.

B. Preparation of the embedding medium.

Hydroquinone was added to the medium to inhibit premature polymerization, and was extracted under a fume hood before the specimens were embedded. To extract hydroquinone, one liter of medium was mixed with one liter of 5% NaOH in a two liter separatory funnel. NaOH was separated and drained through the stopcock three times. The medium was washed three times with equal volumes of distilled water, and filtered twice through 75 - 100 g of 4 - 20 mesh calcium chloride pellets.

One gram of dried benzoyl peroxide was used for each 100 ml of medium to initiate polymerization, and the mixture heated under a fume hood. The mixture (medium

plus catalyst) was heated in a water bath stirred continuously to 75-80°C. The mixture was then placed in a bath of ice until it reached 35-40°C (lower temperatures resulted in a consistency that was too hard for embedding). The water bath followed by the ice bath was repeated until the mixture had a consistency of honey.

C. Embedding

The mixture was poured into a glass scintillation vial for embedding, and the specimen was placed into the vial with the surface to be sectioned turned towards the bottom of the vial. A paper label was placed on the upper surface of the bone, the vial cap was replaced and the specimen was placed under a fume hood at room temperature until the embedding medium hardened. When the plastic surface could be indented slightly, it was placed in a 45°C oven to complete the polymerization. Finally, the warm vial was placed in an ice bath to freeze and crack the vial, so that the excess of embedding medium could be trimmed.

D. Sectioning

The trimmed block was placed with its cutting surface upon the block holder of a Jung Model K Sledge Microtome (Nussloch, Germany). The sectioning speed was set from slow to moderate, and the block and blade were flooded with 70% alcohol. A steel

trimming blade was used to cut a 10 μm section of each specimen, and the microtome carriage was adjusted so that the block was close to the surface of the blade. After the block was trimmed one-third to one-half way into the bone, the trimming blade was replaced with a carbide-tungsten tipped cutting blade. The microtome was set to produce a 5 μm thick section, the carriage was adjusted so that the block did not touch the blade, and subsequently the 5 μm section was stained.

The 10 μm sections were examined under fluorescent light microscopy. The non-gelatinized face of the slide was placed down on the wet section to transfer the section to the slide, and cotton-tipped applicator dipped in chloroform was waved over the section several times to tease out wrinkles. Haupt's adhesive (1 gm of gelatin dissolved at 100 ml distilled water at 50°C, 15 ml glycerol and 2 gm phenol crystals) was dropped onto the section to bond it to the slide, and a 2% formalin solution was dropped onto the slide before it was flattened and dried between filter paper. The slide was placed in a Copland jar filled with warm xylene, and put into a 45°C oven for two hours to remove the plastic leaving the section fixed to the slide.

E. Staining

The xylene saturated slides were dried in air to allow the stain to penetrate the bone. The tetracycline labels were visible in the 10 μm sections without stain when coverslipped with Permount (Fisher). The 5 μm section was stained in Weigert's

hematoxilin for 12-15 minutes and rinsed in distilled water to identify bone cells and osteoid (Goldner, 1938), Appendix 2.

The sections were mordanted in 5% phosphomolybdic acid solution (5.0 gm phosphotungstic acid, 100.0 ml distilled water) for 3 - 5 minutes to help the stain penetrate the specimen. The sections were rinsed in acetic acid water for a few minutes to eliminate the phosphotungstic acid and to differentiate the colour tones. Each section was stained in light green solution 0.1% (0.1 gm light green, 100.0 ml acetic acid water 0.2%) for 12 - 15 minutes. The slides were treated with acetic acid water for 5 minutes. The 5 μ m sections were dehydrated through a series of alcohol changes from 70% to 100% alcohol, then cleared through xylene baths, mounted and coverslipped with Permount after staining.

V. Measurements of histomorphometric parameters

The histomorphometric measurements were made by superimposing a grid built into the eyepiece of the microscope. The area, perimeter and distance of the features of bone tissue were measured to calculate the histomorphometric parameters of each specimen. The area occupied by bone tissue is proportional to the number of regularly arranged grid points overlying it. Each point represents the area lying in a square whose centre coincides with the point and whose sides are of length (d) - the distance between the points (Figure 2.2). The perimeter of woven bone is proportional to the number of

intersections of equidistant parallel lines with the projected perimeter of woven bone (Figure 2.3). Each intersection represents the distance (d) that surrounds each line for a distance of $d/2$, where d is the distance between the lines. The distance between the two tetracycline labels is proportional to the number of scaled unit intervals between them (Fig 2.4). A scale unit represents the distance (d) that is calibrated by the micrometer in the eyepiece.

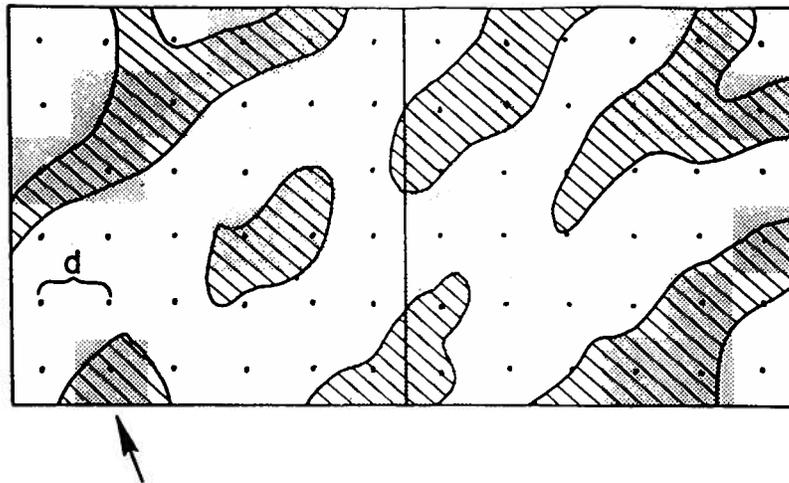


Figure 2.2 - An illustration of two adjacent fields of 36 test points each as they might appear in a microscope overlying a bone section. Twenty-five of the 72 points overlie the cross-hatched feature. Each point actually represents a shaded square (arrow) and d is the distance between the points. (In Bone histomorphometry: techniques and interpretation, ed. RR Recker, 1982)

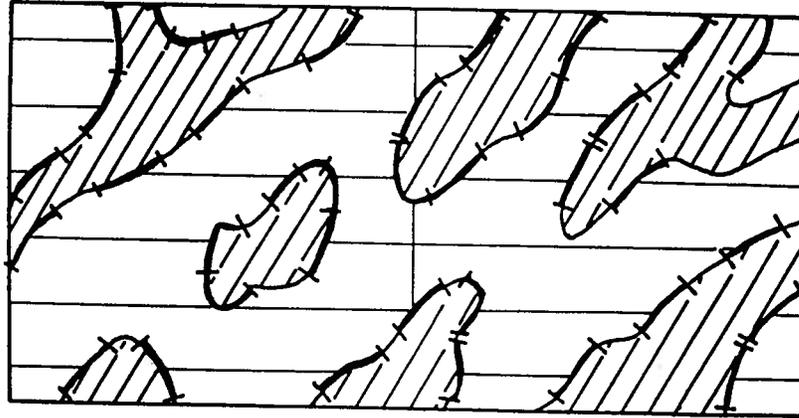


Figure 2.3 - An illustration of two adjacent fields with six parallel test lines each. There are 37 intersecting test lines with the outline of the cross-hatched feature. Each intersection actually represents the distance (d) between the lines, located for $d/2$ either side of the intersection. (In Bone histomorphometry: techniques and interpretation, ed. RR Recker, 1982)

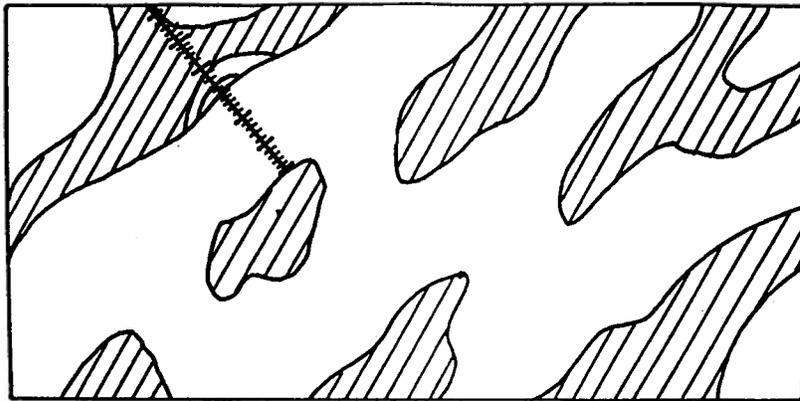


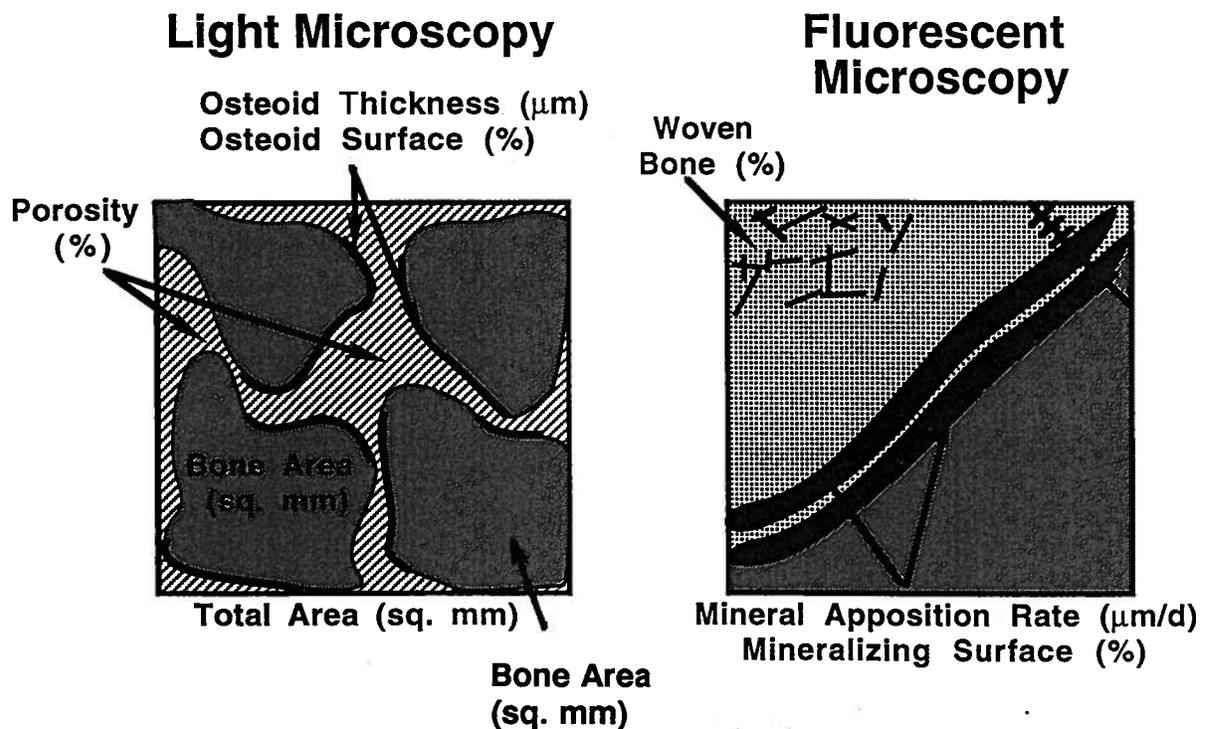
Figure 2.4 - An illustration of a graduated scale of 30 units in length superimposed over the section as it might appear in a microscope overlying a bone section. The distance between the "tetracycline labels" is about 2 units. (In Bone histomorphometry: techniques and interpretation, ed. RR Recker, 1982)

Eight bone histomorphometric parameters, six static or structural and two dynamic, were measured in each specimen. The static parameters were:

- (1) Total area (Tt.Ar) representing the extension (size) of the specimen;
- (2) Bone area (B.Ar) is the area constituted only by bone tissue, no bone marrow;
- (3) Porosity (Po) is the percentage of porosity in bone tissue;
- (4) Osteoid thickness (O.Th) is the average thickness of the osteoid;
- (5) Osteoid surface (OS/BS) is the percentage of bone surface covered by osteoid;
- (6) Woven bone (Wo.B) refers to the percentage of woven bone in the specimen.

The dynamic parameters measured with the Tetracycline labels were:

- (7) Mineral apposition rate (MAR) i.e. the daily amount of mineral deposited in the specimen during Tetracycline intake;
- (8) Mineralizing osteoid surface (MS/OS) i.e. the percentage of mineralized osteoid.



Total area, bone area and porosity were measured in all 48 specimens. The thickness and surface of the osteoid were measured from 27 specimens, woven bone was seen in 22 specimens, and mineral apposition rate and mineralizing surface were measured from 16 specimens.

The formulae used to calculate each of the histomorphometric parameters is presented in the Appendix 3.

VI. Radioimmunoassay for serum osteocalcin

The serum samples were sent to the Mineral Metabolism Laboratory (Chicago, Illinois) where the assays were performed under the direction of Dr. M DeMuzio².

Human osteocalcin cross-reacts with the antibody raised against purified bovine osteocalcin. For this reason the assay is performed with rabbit antiserum to bovine osteocalcin. The assay buffer consists of 0.01 M phosphate pH 7.4, 0.1225 M NaCl, 0.025 M EDTA, 0.1% BSA, and 0.1% Tween 20. All assays included:

- 100 μ l of unknown serum;
- 10,000 cpm [¹²⁵I] osteocalcin (SA, 6-10 μ C/ng) prepared by the solid lactoperoxidase method;
- 100 μ l of a 1:750 dilution of antiserum (final dilution, 1:4500);

²Dr.M. DeMuzio
962 Sherman Road, Northbrook, IL 60062

- assay buffer, to a final volume of 600 μ l.

Standard curves were generated by replacing unknown serum with 0.1-500 ng/ml bovine osteocalcin diluted in clarified human serum.

The assays were incubated for 20 h at 25° C and terminated by adding 300 μ l of 1.5% charcoal-dextran or precipitation with goat antirabbit immunoglobulin G. Nonspecific binding was determined on each sample by omitting anti-serum. All samples were assayed in duplicate, and standard curves were constructed using B/Bo vs. nanograms *per* ml osteocalcin standard. B is defined as counts *per* minute in supernatant ([¹²⁵I] osteocalcin bound to antibody) minus NSB. Bo was the average of six separate determinations of [¹²⁵I] osteocalcin bound to antibody in the absence of unlabelled osteocalcin.

VII. Immunoassay for detection of Type I collagen cross-linked N-telopeptide in urine

The urine samples were analyzed by Dr. A-M Bollen³ at the University of Washington where the assay was developed (Hanson et al., 1992). Each well in the microtiter plate was coated with 100 μ l of urinary peptide-BSA conjugate diluted in 0.05 M carbonate and bicarbonate [pH 9.6]. This coating retains the N-telopeptide on the

³Dr A-M Bollen

Department of Orthodontics, SM-46; University of WA; Seattle; 98195.

bottom of the well to facilitate posterior antibody binding. The plate was sealed and incubated overnight at 4°C. Next day, the plate was washed twice with ELISA wash buffer [0.15 M NaCl containing 0.05% vol/vol Tween 20], patted dry, and 100 µl of urine and 100 µl of standards were placed in each well. Standard is a solution with known concentration of Type I collagen cross-linked N-telopeptide that is used as reference. The samples were diluted twice by adding 100 µl double-strength assay buffer [0.29 M NaCl, 0.01 M KCl, 0.162M Na₂HPO₄, 3 mM KH₂PO₄, 0.002% wt/vol thimerosal, and 0.1% vol/vol Tween 20, pH 7.4], and serial dilutions were made by transferring 100 µl of the initial well into 100 µl assay buffer [1:1 dilution of double strength buffer in water] in the next well, etc. After the dilutions were complete, 100 µl of the sample were left in each well and 100 µl of 1H11 ascites antibody were added quickly. The plate was incubated for one hour at room temperature, washed three times and patted dry before 100 µl of goat antimouse secondary antibody conjugated to horseradish peroxidase was added. The plate was incubated again at room temperature for one hour, washed five times, patted dry and the colour was developed by adding 100 µl per well of 0.1 mg/ml of tetramethylbenzidine in 0.1 M Na acetate, pH 5.0 and 0.01%wt/vol H₂O₂. The reaction was stopped with 100 µl per well of 1.5 N H₂SO₄ and the absorbance was read at 450nm using a Titertek Multiskan-plus plate reader.

A. Calculation of Bone Collagen Equivalent (BCE)

The immunoassay measurements (picomoles type I collagen *per* micromole creatinine) were calibrated using a standard of bacterial collagenase-digested human bone collagen and were expressed as equivalent moles of bone type I collagen (bone collagen equivalent - BCE) normalized to creatinine. The steps to calculate BCE are follows:

1. P1(μg)

P1 is the term used to indicate the collagen crosslink that was measured with the assay. On each of the ELISA plates a standard (and several dilutions of this standard) of known concentration was included. The absorptions for each of the standard concentrations were plotted on semi-logarithmic scale, with P1 concentration on the X-axis and the absorption on the Y-axis. The results were plotted in a sigmoidal curve. For each of the urine samples the measured absorption was located on the standard curve and the corresponding P1 concentration located on the X-axis.

2. Creatinine (mg/dl)

The creatinine measurement (Sigma kit) is based on the yellow/orange colour that forms when the metabolite is treated with alkaline picrate. The creatine-picrate colour faded rapidly under acid conditions. This method was used to prevent other chromogens

from interfering with the creatinine results, and it measures the difference in colour intensity before and after acidification. This difference is proportional to creatinine concentration.

a. Measurements

1. measured absorbance [A] of sample = INITIAL [A]test
2. measured absorbance [A] of creatinine standard,
catalog no. 925-3 = INITIAL [A]standard
3. added acid reagent
4. measured absorbance [A] of sample = FINAL [A]test
5. measured absorbance [A] of standard = FINAL [A]standard

b. Calculations

$$\text{Creatinine [mg/dL]} = \frac{\text{INITIAL [A]test} - \text{FINAL [A]test}}{\text{INITIAL [A]stand} - \text{FINAL [A]stand}} \cdot .3^*$$

* Concentration [mg/dL] of creatinine standard, catalog no. 925-3

3. P1/creat ($\mu\text{g}/\text{mg}$)

The P1 value is divided by creatinine value.

4. BCE/creat ($\text{pmol}/\mu\text{mol}$)

BCE (Bone Collagen Equivalent) refers to the quantity of bone collagen (in picomoles) that has to be resorbed in bone to result in the amount of P1 in the urine. One microgram of P1 is derived from 380 picomoles of BCE. To convert microgram P1/mg creatinine to picomoles BCE/micromoles creatinine, the P1 value was multiplied by 42.94, taking in account the creatinine molecular weight.

VIII. Measurement of bone density using Radiographic photodensitometry

This study planned to include the measurement of bone density at the sites in the jaws where implants were. Radiographic photodensitometry was chosen because it had been used with apparent success to measure bone density in the mandible (Kribbs *et al.*, 1983) and there was no justification for submitting the subjects to more costly examinations. Kribbs *et al.* (1983) used periapical films with a small-sized step-wedge attached. They reported that there was a considerable amount of overlap of bone density values from normal and osteoporotic patients. Nevertheless, we used these data as a

reference for our study and we obtained from Dr. Kribbs the same step-wedge.

The objectives of this part of the study were: (1) to compare the results of bone density and bone histomorphometry from each implant site; and (2) to compare the results obtained with implant patients with reported by Kribbs *et al.* (1983).

The technique was tested performed on radiographs of a dried mandible. Fifteen periapical radiographs were taken of the same site between the roots of lower left pre-molars on the same dried human mandible. The step-wedge was attached to a film positioner in such a way that the wedge position would be equivalent to the top part of the film. The step-wedge was made of type 6061 aluminum alloy and was machined in eight 0.5 mm incremental steps 1.5 mm wide (range 4.5 to 8 mm). The film positioner was fixed to a table so that every film was exposed at the same angulation.

The radiographs were divided into five groups. Each group received a different exposure: 18, 21, 24, 31, and 36 impulses, in order to verify that the bone and step-wedge density would increase or decrease corresponding to the degree of exposure (the higher the exposure, the higher the density). There were three films in each group to confirm the reproducibility of the density measurements. Each radiograph was processed using an automatic processor (Air Techniques Inc, Hicksville NY), and scanned by Ultrosan XL Enhanced Laser Densitometer (LKB Bromma, Uppsala, Sweden). Each film was scanned three times, again to verify reproducibility of the technique, between the first and second pre-molar from the alveolar crest to the bottom edge of the film. A template was used to reproduce the same film position in each scanning to certify that the same

area of the film was being scanned each time. The films were scanned in a random order, and for each scanning every film was removed from the Ultrascan and then repositioned for the next scanning.

RESULTS

This chapter is divided into six parts. The first four parts present the results of the bone histomorphometry, the quantity of serum osteocalcin and the Type I collagen cross-links bounded to n-telopeptide in urine, and the density of bone measured in a dry mandible. The fifth part considers the relationship between the osteocalcin and the Type I collagen cross-linked N-telopeptide results; and the final part compares the values from the systemic bone metabolism with the histomorphometric parameters.

I. Bone histomorphometry

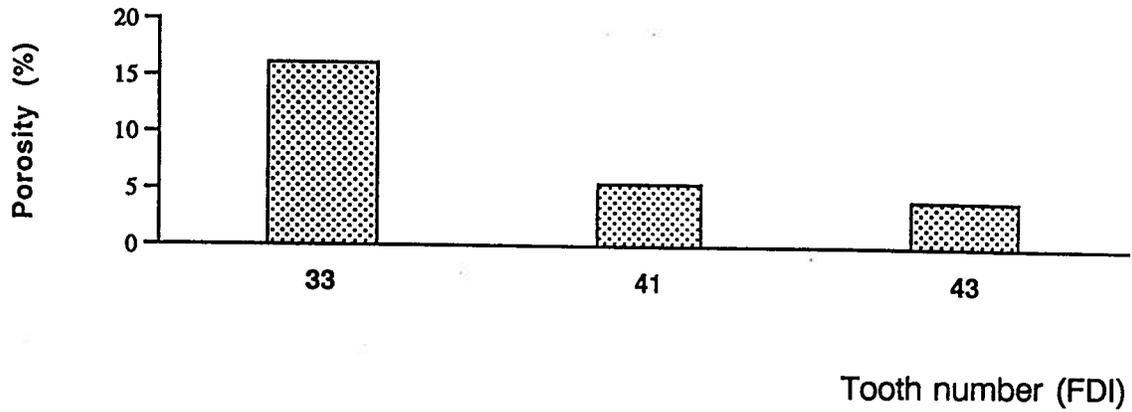
The histomorphometric parameters measured on each specimen of bone showed substantial variations both between (Table 3.1) and within patients (Figure 3.1). The variation was widespread and did not differ with sex or age. There was no evidence, therefore, that women and older patients had more active or porous jaw bone than men or younger patients. There was no difference either in the porosity of cortical bone in the maxilla or in the mandible; even though the percentage of cortical bone in the upper jaw was less than in the lower jaw. There was a wide range of values when specimens were compared from identical locations in different patients. For example, the porosity of the bone taken from the area of the maxillary right canine varied from 4.4% on patient #9 to 26.7% on patient #13. However, the porosity of the bone decreased from the anterior

to the posterior parts of both jaws (Figure 3.1).

Table 3.1 - The distribution of histomorphometric parameters measured on specimens of bone from the maxilla and the mandible.

Parameters	Range	Mean	Sd
<u>Static</u>			
Total area (sq.mm)	0.43 - 6.65	2.58	1.42
Bone area (sq.mm)	0.42 - 5.73	2.16	1.22
Porosity (%)	0.0 - 40.8	13.2	11.3
Osteoid thickness (μm)	1.0 - 18.6	8.8	4.2
Osteoid surface (%)	1.9 - 47.2	17.0	14.1
Woven bone (%)	1.0 - 88.0	12.3	20.6
<u>Dynamic</u>			
Mineral apposition rate ($\mu\text{m}/\text{day}$)	0.29 - 1.06	0.77	0.25
Mineralizing surface (%)	20.1 - 133.0	67.3	32.2

Patient #3



Patient #9

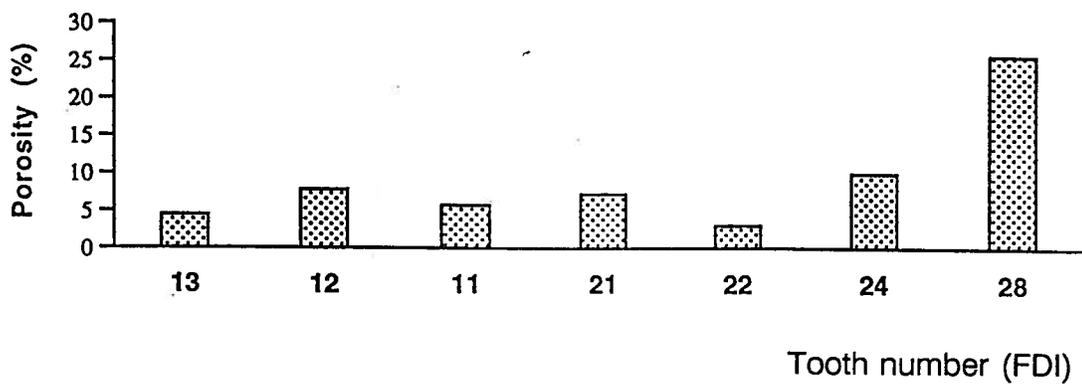


Figure 3.1 - Porosity values associated with specific tooth sites in patients #3 and 9.

II. Serum osteocalcin (Oc)

The normal limits for serum osteocalcin provided by the Mineral Metabolism Laboratory (Chicago, Illinois) is 2-8 ng/ml. From the 21 patients, three patients had values below (0.9, 1.2, 1.5), and one had values above (8.1) the limits. The mean value for serum osteocalcin was 3.1 ng/ml(SD 1.4) with a range 0.9 - 5.3 for women; and 4.0 ng/ml(SD 1.7) with a range 1.5 -8.1 for men.

III. Type I collagen cross-linked N-telopeptide in urine

The women presented higher mean value of cross-linked N-telopeptide in urine, expressed in bone collagen equivalent (BCE, pmol/ μ mol) (Table 3.II). This was not surprising because of the fact women lose more bone than men throughout life (Riggs, 1991). This assay is relatively new, therefore, the normal range has not been established.

Table 3.II - Comparison of the Type I collagen cross-linked n-telopeptide concentrations in the urine of women and men.

	<u>Concentrations (pmol/μmol)</u>		
	Range	Mean	Sd
Women	35-243	102.7	64.2
Men	26-137	54.8	32.8

IV. Bone density in a dry mandible

The results of the various scannings of the step-wedge in every film provided irregular curves that did not correspond to the steps of the wedge. It was impossible, therefore, to establish the aluminium-equivalent unit to measure bone density. These unexpected results hint at errors in either the Gel-Scan, or the step-wedge, or possibly in both.

To verify the reliability of the Gel-Scan, a radiograph taken of a different step-wedge was scanned and the result was a curve that corresponded to the step-wedge design. This radiograph was also scanned by a calibrated densitometer (Macbeth TD502, New York) and the result was similar to the one obtained by the Gel-Scan. Consequently, we concluded that there was no apparent problem with the Gel-Scan. The absorption measured by the Gel-Scan and the density measured by densitometer were plotted and

correlated, showing that absorption and density were the same (Figure 3.2).

The results indicated, therefore, that there was a structural problem with the step-wedge used by Kribbs et al. This fact raises some questions about this bone density measurement technique and also about the conclusions made by Kribbs et al. (1983). Monajery & Arbor (1992) also used radiographic photodensitometry to verify differences in jaw bone density between normal and osteoporotic patients, and found that it was impossible to distinguish one group from the other. However, the authors stated that the densitometer used was not as sensitive as the one used by Kribbs et al. (1983). We could not proceed with this part of the study nor with the plan to compare jaw bone density values in our group of 21 patients with the results published by Kribbs et al. (1983) - the original step wedge was used also by Kribbs et al., so our observation questioned the reliability of their data. The use of another step-wedge was not possible because we could not verify the value of radiograph photodensitometry as a measure of bone density, verification of this technique was beyond the scope of the study.

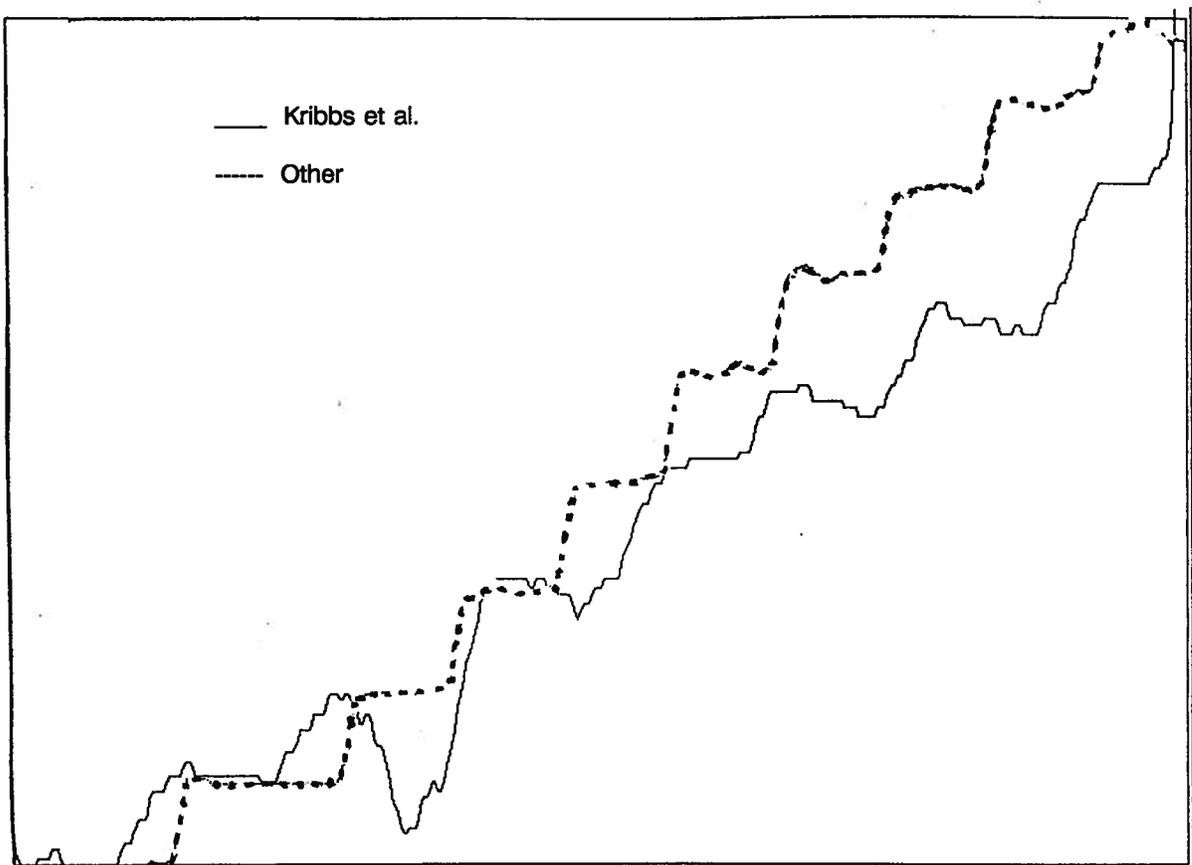


Figure 3.2 -Comparison between the scans of the step-wedge used by Kribbs et al. and another step-wedge.

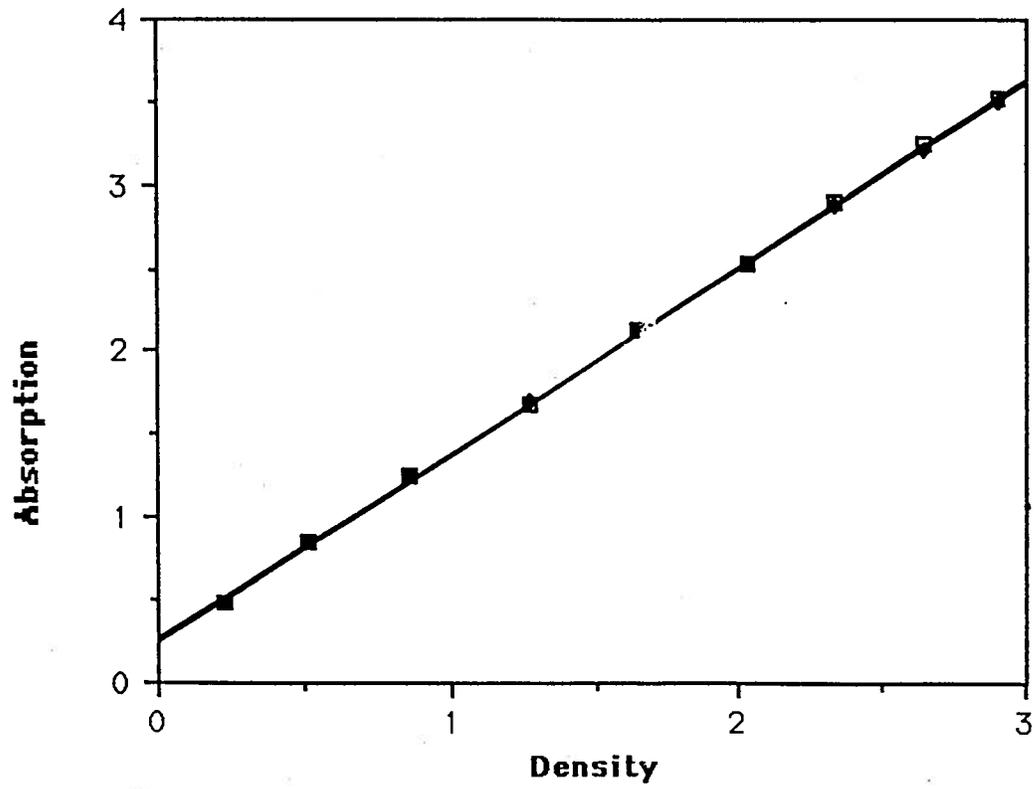


Figure 3.3 - Absortometry values by densitometry values for the second step-wedge.

V. The relationship between the osteocalcin and Type I collagen cross-linked N-telopeptide results

The objective of measuring serum osteocalcin and Type I collagen cross-linked N-telopeptide in urine was to assess the systemic bone metabolism of the patients. Because the N-telopeptide assay is relatively new there is no published information on its association with the Oc assay. Eastell et al. (1992) found a positive correlation between Oc and deoxypyridinoline (D-Pyr), especially in postmenopausal women. This relationship is not surprising since bone formation and bone resorption are coupled processes. Therefore, there was reason to believe that Oc and N-telopeptide would correlate because of the relationship demonstrated previously between pyridinolines and N-telopeptide (Hanson et al., 1991). The results from men and women were plotted separately (Figure 3.4 A & B). There was a higher correlation among men than women; confirming that women tend to lose bone tissue because of the imbalance between bone resorption and bone formation.

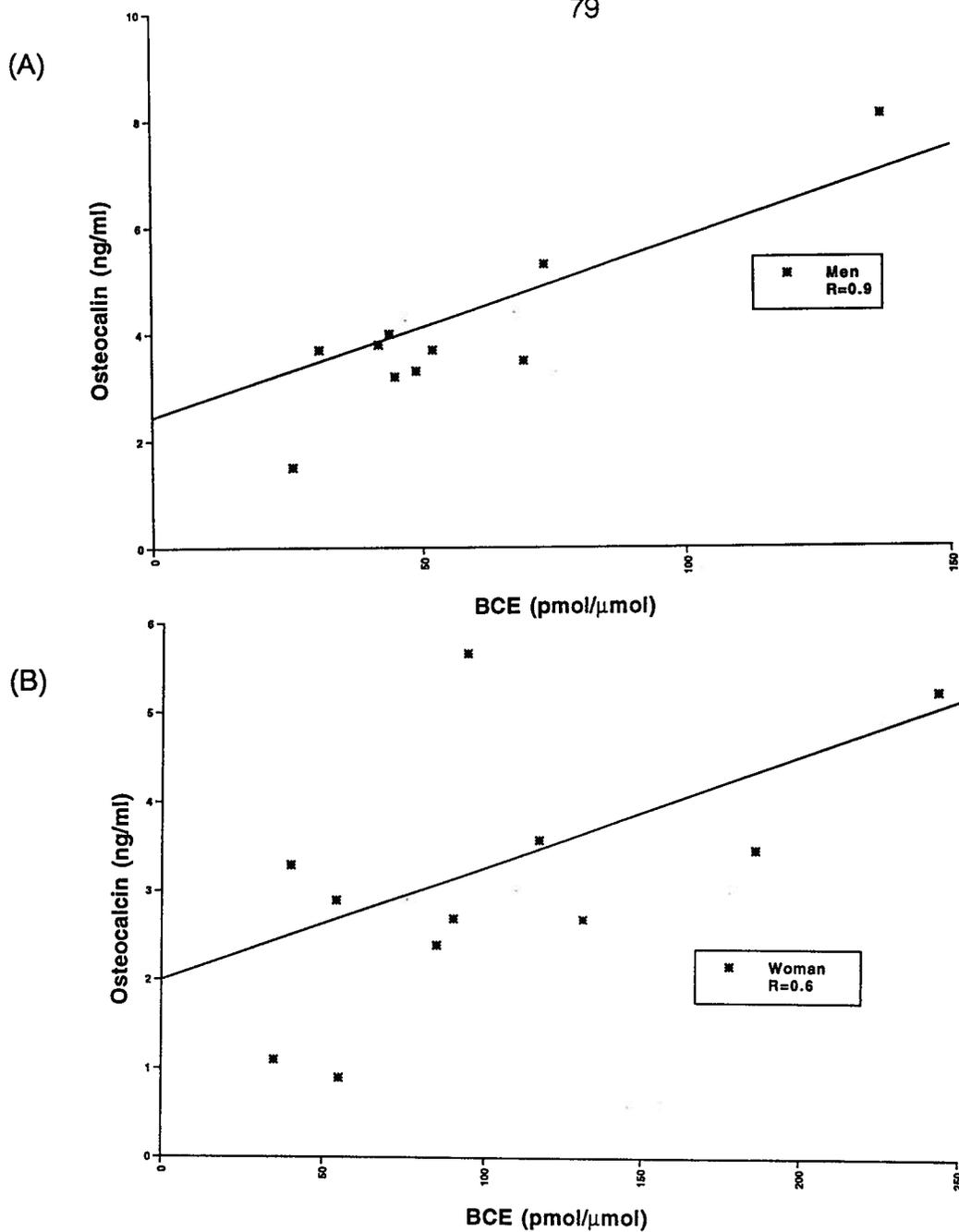


Figure 3.4 Correlation, expressed as correlation coefficient (r), between serum Osteocalcin and Type I collagen cross-linked N-telopeptide (BCE) in men (A, $r=0.9$) and women (B, $r=0.6$).

VI. The relationship between the values from systemic bone metabolism and the histomorphometric parameter porosity

The last objective of this study was to analyse the relationship between systemic metabolism and the histological appearance of jaw bone, on the assumption that patients with high bone remodelling rates would have unusually porous cortical bone. The mean value of the porosity from each patient was calculated and plotted against the osteocalcin and Type I collagen cross-linked N-Telopeptide results (Figure 3.5 and 3.6). The osteocalcin levels in the serum and the N-telopeptide levels in urine did not appear to be correlated with the porosity of the bone specimens. This suggests that systemic bone metabolism may not have a substantial influence on the structure of jaw bone. However, when the patients were analyzed individually, one woman (patient #6) and two men (patients #8 & 13) had very high systemic metabolic values suggesting that the bone remodelling rate was high; whereas patients #8 and 13 had high values for porosity (40.8,17.0,28.8,30.7,26.7%), but patient #6 had low values for porosity (6.3,4.6%). The two women patients that apparently were candidates for bone loss also had conflicting porosity results (patient #2: 5.5% and patient #12: 10.2,23.7%). The man who presented with extreme porosity - so much so that it was not possible to obtain any specimen of bone from his jaws - had the third highest value for bone formation among the men and a low value for bone resorption. The patients with the lowest values for systemic metabolism had low values for bone porosity, however, patient #9 had values as different

as 4.7 and 25.8% and patient #18: 0.9 and 13.7%. Our observations suggest that the histological appearance of jaw bone may not be as influenced by systemic metabolism as it is in other bones (eg. iliac bone).

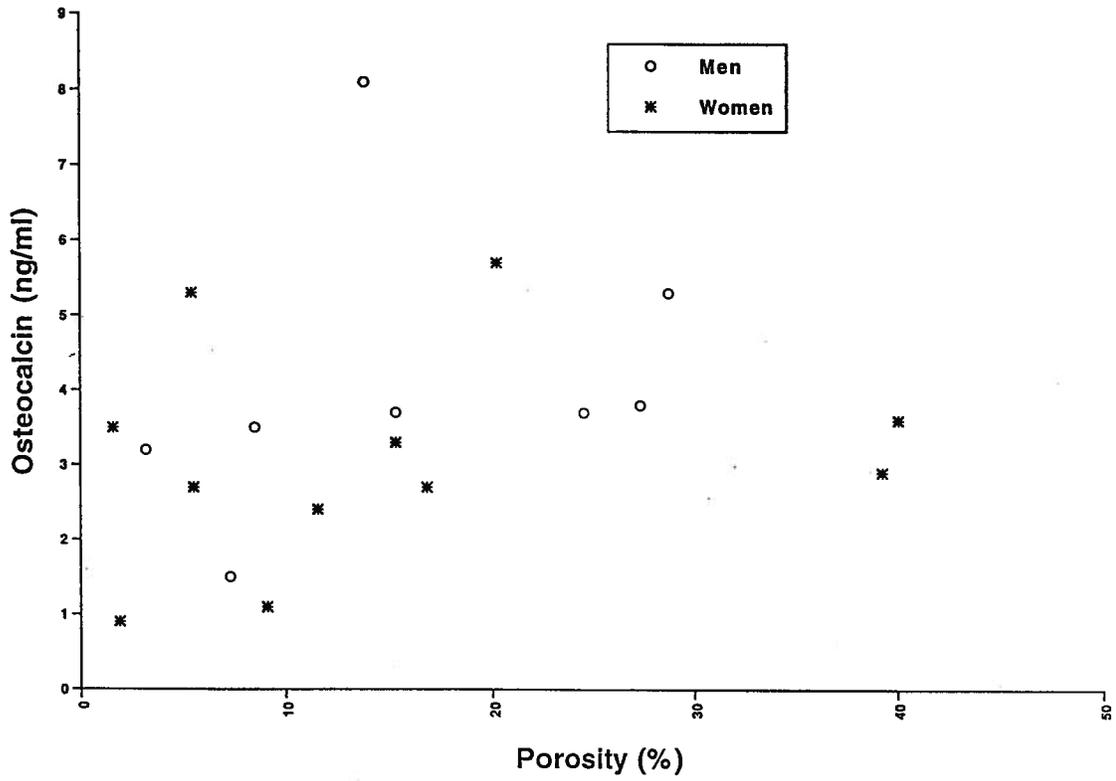


Figure 3.5 - Porosity values plotted against osteocalcin values in men and women.

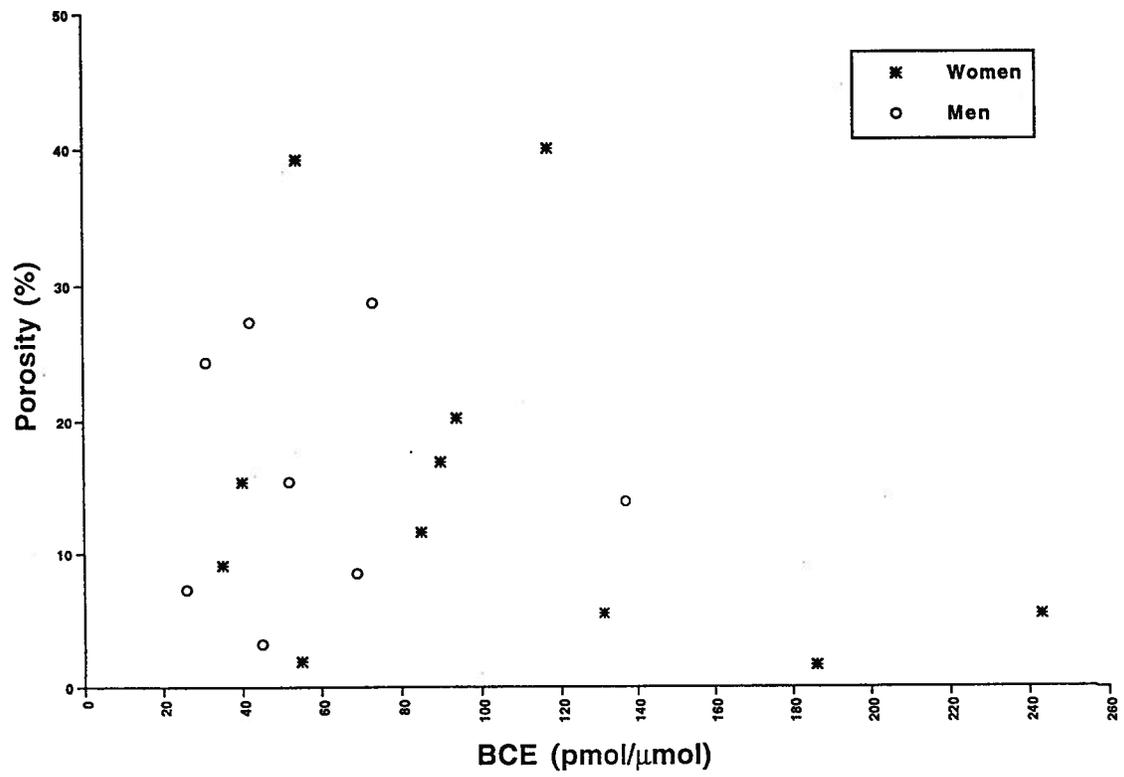


Figure 3.6- Porosity values plotted against Type i collagen cross-linked N-telopeptide (BCE) values.

DISCUSSION

I. Jaw bone analysis

According to the static and dynamic histomorphometric parameters measured in the specimens of bone, both bone quantity and metabolic activity in the jaws presented a considerable variation between and within patients. The porosity of the cortical bone ranged from 0.0 to 40.8%, and, in a few patients, the porosity increased towards the posterior part of the jaws with the implication being that there are different amounts of bone from region to region around the jaws. Others (Von Wowern, 1977; Atkinson & Woodhead, 1968), also reported the variability of cortical porosity in jaw bone. However, these previous studies were limited to human autopsy specimens. The present study confirmed - in vivo - those previous findings. Von Wowern (1977) measured bone porosity in specimens of cortical bone from the incisal, premolar and molar areas, and also found that there was a gradual increase of bone porosity from the incisive to molar regions. This variability is not an exclusive characteristic of jaw bone, but is present throughout the skeleton (Nordin, 1977). Variations exist in porosity and bone activity not only between different bones, but also in different parts of the same bone, because both have been described as factors influenced largely by functional stresses.

The porosity of bone taken from close to the mental foramen increased with age. Von Wowern & Stoltze (1980) measured histomorphometric parameters in a standard site

in the mandible from subjects without bone disorders, and found that the porosity of cortical bone increased with advancing age and that it was unrelated to gender. They had verified previously that the buccal cortex of the mandible at the mental foramen was subjected to the least structural variation, probably due to minimal influence of masticatory muscles. Consequently, they obtained their specimens exclusively from this region (Von Wowern & Stoltze, 1978). They found that porosity varied between subjects, so their results were more suitable to inter-group comparisons.

Tetracycline labels were seen in only 16 of the 48 specimens, and dynamic parameters were measured from some sites and not from others in the same patient. It is possible, therefore, that bone was not deposited evenly throughout the jaw during the period of drug-intake. The histomorphometric studies of iliac bone conducted by others (Recker et al., 1988; Vedi et al., 1988) obtained only one specimen of bone, and no previous reports of this observation have been found.

Woven bone was present in 23 specimens taken from 12 patients, however, the significance of woven bone in the jaws is still unclear. Woven bone indicates rapid, uncontrolled bone formation and high bone turnover, and is found commonly in a postfracture bone union (Meunier, 1988). Apparently, it is less susceptible than lamellar bone to endocrine and other bone regulators (Frost, 1983), and it is found also in some pathological conditions, such as Paget's disease (Meunier, 1988). From the 23 specimens with woven bone, 70% of the anterior specimens had less woven bone than the posterior specimens. As woven bone has been associated with accentuated strain

by Burr et al. (1989), this finding suggests higher mechanical stress on posterior part of the alveolar ridge.

Even though it seems that the bone supporting implants is characterized by a wide range of porosity, there may be other reasons that contribute to the histomorphometric variations. The pilot drill used to mark the implant site did remove some of the cortical bone, and in the process it may have altered the structure of the specimen taken by the trephine. In fact, the pilot drill in one patient removed all of the cortical bone from the only specimen obtained. Specimens taken from ten patients had no tetracycline labelling, for reasons that are not clear. It is possible that the tetracycline was not ingested as directed, even though every patient was given a written schedule for the marker and reminded by phone to take the drug the preceeding evening. Therefore, it seems advisable (in future studies) to eliminate the use of the pilot drill when obtaining the specimens of bone, and to try to establish a more efficient way of verifying patient compliance.

II. Osteocalcin and N-telopeptide

It was not surprising that the Type I collagen cross-linked N-telopeptide and the osteocalcin measurements had a higher correlation among men than women because of the imbalance between bone formation and bone resorption that usually affects women after the fifth decade of life. Eastell et al. (1992) have shown a significant correlation

between serum osteocalcin and urinary pyridinolines (a marker for bone resorption), and, in addition, Hanson et al. (1991) demonstrated a significant correlation between urinary pyridinolines and N-telopeptide. The measurements of serum osteocalcin and N-telopeptide are used to assess the level of bone turnover. Nevertheless, the use of these two examinations alone does not offer sufficient information to diagnose osteoporosis or any other metabolic bone disease (Riggs, 1991). There were three patients (two women and one man) with osteocalcin values below the limits usually found in healthy patients (0.9, 1.2, 1.5 ng/ml), and one man with a high osteocalcin value (8.1 ng/ml). We can only conclude from this that the three patients had a very low turnover rate at the time of the study.

Vanderschueren et al. (1990) also reported that women presented lower Oc values than men, and that women in the sixth decade and men in the eight decade had a significant increase in Oc values. They measured osteocalcin in specimens of iliac bone and found that the age-related pattern of serum Oc reflects changes in bone Oc. They concluded that serum osteocalcin is a valid indicator of bone metabolism. In our study, however, the older patients presented higher Oc values than younger patients. A decrease with age was probably not seen because all of our patients were over 50 years of age, when osteocalcin concentrations normally show an increase (Vanderscheren et al., 1990). These lower Oc values, when compared to the finding of Vanderscheren et al. (1990), probably reflect differences in the radioimmunoassays relating to antibody specificity and sensitivity. In radioimmunoassays, antibodies are raised against bovine

osteocalcin to cross-react with human osteocalcin. Antibodies can be raised differently, and, they recognize different parts of the Oc molecule and the Oc fragments, which can result in different readings for serum Oc.

The Type I collagen cross-linked N-telopeptides concentration in the urine was similar to the concentration reported by Hanson et al. (1992). They measured Type I collagen cross-linked N-telopeptides from urine samples of more than 1000 healthy men and women of all ages, and found that women had relatively higher values than men. The difference in values found between men and women is probably due to the deficiency of oestrogen after menopause, which usually causes an increase in bone resorption (Holtrop et al., 1974).

III. Jaw bone and systemic metabolism

From all the histomorphometric parameters that were measured, porosity (Po) was the one most likely to reflect systemic metabolism. The percentage of cortical bone porosity has been used to demonstrate the pattern of age related bone loss in mandibles (Von Wowern & Stoltze, 1980), and to demonstrate the relationship between the mandible and the iliac bone (Von Wowern & Melsen, 1979). There was no evidence, however, that porosity was associated with osteocalcin or N-telopeptide values, which suggests that the systemic bone metabolism may not substantially influence the amount of porosity in jaw bones. When the results were analyzed individually, more evidence that bone metabolic

markers are not predictable indicators for bone status in the jaw was found. Two of the three patients who had the most active systemic metabolism had very porous bone, whereas the third patient had very dense bone. The patients with the least active systemic metabolism did not have particularly dense bone. Two women, whose bone marker values suggested that they were under a process of bone loss, had both low and high values of porosity. The presence of extreme porosity in bone made it very difficult, and in some situations impossible, to obtain a specimen of bone with the surgical trephine. In one male patient the marker values suggested a stable systemic metabolism, yet because of accentuated porosity it was impossible to obtain a single specimen from the five sites that were drilled.

These confusing observations from the jaws do not correspond with reports about the iliac bone. Garcia-Carrasco et al. (1988) found a direct and strong correlation between the results of serum osteocalcin and the histomorphometric parameters of the iliac bone of adults without bone diseases. Hodsman et al. (1993) reported that, during treatment, osteoporotic patients had an increase in both serum osteocalcin and the histomorphometric parameters of iliac bone, showing a positive correlation between the examinations. Patients with rheumatoid arthritis had a moderate correlation between serum osteocalcin and bone histomorphometry of the iliac bone (Kroger et al., 1993). It seems that the jaws are not as susceptible as the iliac bone to the changes and patterns of systemic bone metabolism. Perhaps a better approach to establish this relationship would be to analyze porosity only in specimens of bone from the standard site so as to

avoid the intrinsic porosity variation of the jaws. This new procedure could not be attempted in this study because it would have involved additional bone removal from an area not usually required during implant surgery.

IV. Jaw bone and the implants

Despite the intrinsic and widespread variation in bone structure, the short-term outcome of the endosseous implants was not affected. All of the implants that have been exposed were successfully integrated. Jaffin & Berman (1991) and Davis (1990) reported that implants placed in porous bone presented a higher failure rate than those placed in more dense bone. They used panoramic radiographs, and the clinical appearance and "feel" of the bone while drilling, to identify quality of bone. These are not very accurate methods to evaluate bone porosity because panoramic radiographs present serious limitations due to erratic magnification, distortion, the overlapping of structures and ghost images (Hamada, 1989). These studies, therefore, do not offer conclusive results. The formation of bone around implants depends upon the periosteal and endosteal cells that proliferate and differentiate into osteoblasts and eventually produce osteoid (Weinlaender, 1991). The new bone formation around implants is related therefore to the process of bone repair. Osteoporotic bones do not have a defective repair capacity, and they appear to heal normally after fracture (Parfitt, 1988). This indicates that porous bone is capable of forming bone satisfactorily. The failure of implants is probably caused by

reasons other than bone porosity or the local bone turnover rate. The optimistic results described by Langer et al. (1993) from wide implants placed in the sites of previous failed implants supports this view.

V. Future directions

The surgical technique developed to obtain specimens of bone from implant sites without jeopardizing the implant's placement offers the opportunity to analyze the jaw bone in vivo. The results from this study were similar to previous studies, confirming the intra- and inter- variation of jaw bone and its pattern of age-related bone loss. However, even though it seems that jaw bone is affected by aging much like the rest of the skeleton, there is no information on subjects with systemic metabolic bone diseases. It is true that from the measurements of bone markers it was concluded that jaw bone is not affected by the systemic metabolism as much as other bones; however, this group of patients did not appear to have metabolic disorders. It is still unknown as to what extent jaw bone is affected by metabolic bone diseases.

It is necessary to investigate the jaw bone of healthy and osteoporotic patients histomorphometrically. Because of the accentuated variation of jaw bone in normal subjects a larger sample size is needed for sufficient statistical power. Kimmel et al. (1990) reported that a difference of 35% in bone volume is the smallest difference that could be used to distinguish between osteoporotic and normal patients. Therefore, we

estimated that a sample of 125 subjects would be required to compensate for the variation in bone when comparing the responses from osteoporotic and normal subjects (Appendix 4). We believe that the ideal study should have the same number of osteoporotic patients with matching sex and age.

CONCLUSIONS

From the experiments used in this thesis, the conclusions can be summarized as follows:

- (1) The histomorphometric values of the specimens of bone obtained from implant sites presented a large variation both between and within patients.
- (2) The variation of the histomorphometric values from the implant sites suggest that the variation is an intrinsic characteristic of jaw bone, and was similar to the results of previous studies (Von Wowern, 1977; and Von Wowern & Stoltze, 1980)
- (3) No difference was found between the specimens of bone from the maxillae and the mandible, nor between men and women, in different age groups.
- (4) The extent of the porosity in jaw bone did not influence the short-term outcome of endosseous implants in this group.
- (5) Serum Osteocalcin and N-telopeptide cross-links assays were higher correlated among men than women.
- (6) Conflicting results between bone markers and bone histomorphometry did not allow any definite conclusions. Further investigation is still necessary to establish this relationship.
- (7) The radiographic measurements of bone density had to be abandoned because of structural difficulties with the step-wedge used to standardize bone density.

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Appendix 1**Informed consent**

Title: A biochemical, and histomorphometric study of bone associated with oral implants in humans.

Investigators: Drs. M MacEntee, B Wu, D McDonnell, A Esteves.

Address : Faculty of Dentistry

Phone Number: (604) 822 5064

I understand that I will participate in a study conducted by members of the Faculty of Dentistry of The University of British Columbia. The following information has been provided so that I can make an informed decision about my willingness to participate:

1. The study is designed to evaluate the bone around dental (oral) implants, with the aim of providing a better understanding of the factors influencing the success of implants.
2. I will provide a sample of urine and blood. There will be no charge for these tests, but I understand that I am responsible for the cost of other tests prescribed by my surgeon and not used in the study.
3. I will be given a supply of two antibiotics (tetracycline) at no charge, and asked to take 150mg of *democlocycline* four times a day for two days starting exactly twenty two days before the implants are placed in the bone, followed by fourteen days without the bone marker, and again for two days I will take 250mg of *tetracycline* four times a day, ending the intake of the marker four days before surgery. The antibiotics are used to mark changes in the bone, and not for therapeutic reasons.

INTAKE OF BONE MARKING ANTIBIOTICS PREOPERATIVELY

DAY

22-----21	20-----7	6-----5	4-----0(Surgery)
Democlocycline	No Marker	Tetracycline	No Marker

4. I understand that tetracycline is an antibiotic that is not usually taken before implant surgery, and I am not aware that I am allergic to it.
5. I aware that pregnant women should not participate in the study.

6. The study will require no extra time for my treatment.
7. My participation will be strictly confidential, all records will be secure and made available only to the investigators, and my identity will remain confidential in any reports from the study.
8. I understand also that I am free to withdraw from the study at any time, without jeopardizing the treatment or care I receive now or in the future.
9. All questions about the study have been answered satisfactorily, and I know that I can contact Dr MacEntee or his assistant for further information at the address above.

I acknowledge receipt of a copy of this consent form.

(signature of participant)

(signature of witness)

Appendix 2**Goldner Trichrome Stain**

Constituents of Weigert's hematoxylin

solution A: 1gm haematoxylin

100 cc absolute alcohol

solution B: 4 cc 29% ferric chloride

95 cc distilled water

1 cc hydrochloric acid (concentrated)

working solution: equal parts of A and B

The section was stained in the filtered working solution of fuchsin-ponceau-orange G for 30 minutes and rinsed in acetic acid water to remove excess of stain.

Constituents of Masson Fuchsin-Ponceau-Orange stock solution

2.0 gm Ponceau de xyldine [ponceau 2r]

1.0gm acid fuchsin

2.0 gm Orange G

300.0 ml acetic acid water 0.2%

Masson Fuchsin-Ponceau-Orange G working solution

10.0ml stock Fuchsin-Ponceau-Orange G solution

90.0ml acetic acid water 0.2%

Acetic Acid Water 0.2%

2.0 ml glacial acetic acid

1000.0ml distilled water

Appendix 3

Formulae for calculation of the histomorphometric parameters

A. Static parameters

1. Total Area (Tt.Ar) is defined as the extent of the specimen examined and is expressed in square millimeters.

$$Tt . Ar = hTb . T \times K_G^2$$

hTb.T - Points superimposing cortical bone, including marrow.

KG - Grid calibration constant at correct magnification.

2. Bone Area (B.Ar) is defined as the extent of the bone tissue and is expressed in square millimeters.

$$B . Ar = hB \times K_G^2$$

hB - Points superimposing bone tissue.

KG - Grid calibration constant at correct magnification.

3. Porosity (Po) represents the percentage of porous present in the cortical bone.

$$Po = \frac{\sum iPo \cdot Pm \times 100}{\sum i \cdot Pm}$$

iPo.Pm - Intersections on porosity perimeter.

$\sum i.Pm$ - Summation of the intersections on perimeter.

4. Osteoid Thickness (O.Th) refers to the mean thickness of osteoid present in the cortical bone and is expressed in μm .

$$O.Th = \frac{\sum O.Wi \times K_{MC} \times \pi / 4}{n O.Wi}$$

$\sum O.Wi$ - Summation of the micrometer-based width measurements taken of osteoid.

Kmc - Micrometer calibration constant at correct magnification.

n O.Wi - number of sampling units.

5. Osteoid Surface (OS/BS) represents the percentage of osteoid in the cortical bone.

$$OS / BS = \frac{iO \cdot P_m \times 100}{\Sigma i \cdot P_m}$$

iO.Pm - Intersections on osteoid perimeter.

Σ i.Pm - Summation of the intersections on perimeter.

6. Woven Bone (Wo.B) represents the percentage of woven bone present in the sample.

$$Wo \cdot B = \frac{iWo \cdot P_m \times 100}{\Sigma i \cdot P_m}$$

iWo.Pm - Intersections on woven bone perimeter.

Σ i.Pm - Summation of the intersections on perimeter.

B. Dynamic parameters

7. Mineral Apposition Rate (MAR) is the amount of mineral deposited in 24 hours and is expressed in $\mu\text{m}/\text{day}$.

$$MAR = \frac{\Sigma L . W_i \times K_{mc} \times \frac{\pi}{4}}{n L . W_i \times t}$$

$\Sigma L.W_i$ - Summation of the micrometer-based width measurements taken of tetracycline labels.

K_{mc} - Micrometer calibration constant at correct magnification.

$n L.W_i$ - number of sampling units.

8. Mineralizing Osteoid Surface (MS.OS) represents the percentage of osteoid mineralized in the cortical bone.

$$MS / OS = \frac{MS / BS \times 100}{OS / BS}$$

MS/BS - Mineralizing surface.

OS/BS - Osteoid surface.

Appendix 4Calculation of sample size

$$n = \frac{Z_{\alpha} + Z_{\beta}^2 \times 2 \delta^2}{\Delta^2}$$

δ = standard deviation of response variable = 11.3

Δ = smallest difference of bone volume between osteoporotic
and normal subjects* = 4.6

$(Z_{\alpha} + Z_{\beta})$ = multiplier which depends on the level of significance α
and power $1 - \beta = 10.4$

$n = 125$

* Kimmell et al. (1990)