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UNIVERSITY OF ALBERTA

LONGITUDINAL CHANGES IN BONE MINERAL DENSITY IN PATIENTS FOLLOWING TOTAL HIP REPLACEMENT

BY

SAFWAT GIRGIS

A thesis

submitted to the FACULTY OF GRADUATE STUDIES AND RESEARCH

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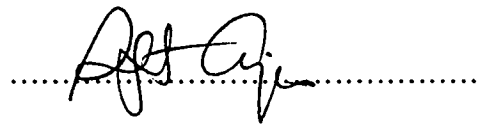

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled LONGITUDINAL CHANGES IN BONE MINERAL DENSITY IN PATIENTS FOLLOWING TOTAL HIP REPLACEMENT submitted by S. GIRGIS in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE IN EXPERIMENTAL MEDICINE.

.....
Dr. T. R. Overton (Supervisor)

.....
Dr. C.L. Chik (Co-supervisor)

.....
Dr. G.K. Nguyen

Date: .....

This thesis is dedicated to my wife Rose, my son Daniel, and my parents Isis and Abdel Malak

Abstract

This thesis presents data on a one-year study of the relative effects of total hip replacement surgery on the bone mineral density (BMD) in ten osteoarthritic patients (three males + seven females), aged 49 to 81 years.

Bone mineral density (BMD) in the lumbar spine (L_1 to L_4), and in the proximal femur (neck, trochanter, intertrochanteric region, and Ward's triangle) was measured using dual energy x-ray absorptiometry (DEXA). BMD measurements were made for each subject at baseline (one month before surgery), and at 4 and 12 months after surgery.

Bone biopsies from the intertrochanteric region of the femur were obtained intraoperatively and processed for the evaluation of histomorphometric variables. Biochemical data collected at baseline included: 25-hydroxy vitamin D, parathyroid hormone, total proteins, serum ionized calcium, and serum alkaline phosphatase.

At baseline, BMD measurements and bone histomorphometric variables did not show a significant correlation, but a strong correlation was observed between BMD measurements and biochemical data .

For all subjects, BMD in the spine and proximal femur at 4 and 12 months were not significantly different from baseline. In 4 of 10 patients, however, BMD in the spine was significantly different between baseline and 4-months, and also between 4-months and 12-months.

These data suggest that the new biomechanical status following total hip replacement may have a positive effect on BMD in the lumbar spine.

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List of Abbreviations

25 (OH) D	25-hydroxy vitamin D
1,25 $(OH)_2$ D_3	1,25 dihydroxy vitamin D 3
ANOVA	Analysis of variance
BGP	Serum bone Gla protein
BMC	Bone mineral content
BMD	Bone mineral density
BMU	Bone multicellular unit
BRU	Bone remodelling unit
BSU	Bone structural unit
DER	Dual energy radiography
DEXA	Dual-energy x-ray absorptiometry
DPA	Dual-photon absorptiometry
HP	Hydroxylysylpyridinoline
LP	Lysylpyridinoline
OA	Osteoarthritis
PTH	Parathyroid hormone
QDR	Quantitative digital radiography (same as DER)
QCT	Quantitative computed tomography
RA	Rheumatoid Arthritis
RMD	Reversible mineral deficit
ROI	Region of interest
SPA	Single-photon absorptiometry
TBD	Trabecular bone density
TRAP	Tartrate-resistant acid phosphatase

Chapter 1: Introduction and Literature Review

1.1 Introduction

Osteoporosis is a disorder characterized by decreased bone mass and by increased susceptibility to fractures (Cummings *et al.*, 1985). Bone mass tends to decrease after the fourth decade of life and hence, osteoporosis could be considered as a phenomenon of aging. The definition of osteoporosis was a controversial issue until recently. Collins (1966) defined osteoporosis as a generalized form of atrophy of bone and reserved the term "bone atrophy" for a more localized process such as might occur in a paralyzed limb. "Localized osteoporosis" is, however, a term also frequently used to describe the changes in immobilized limbs or around severely arthritic joints (Urovitz *et al.*, 1977). Sissons (1955) defined osteoporosis as a morphological change in bone in which the quantity of supporting tissue is reduced while remaining highly mineralized. Osteoporosis has also been described as increased bone porosity by McLean and Urist (1961). The current, general definition of osteoporosis is that there is a decrease in bone mass of otherwise normal bone [no detectable differences from normal bones in the relative properties of mineralized and non-mineralized matrix (Faugere, 1985)].

The term osteopenia, often used to describe osteoporosis, is misleading because while both terms may mean the same thing (reduced bone mass of otherwise normal bone), many researchers reserve the term osteoporosis for those cases of osteopenia in which there is an actual or potential mechanical failure of bone (Malluche *et al.*, 1986). Remagen *et al.* (1989) defined "clinical osteoporosis" as bone atrophy in the presence of at least one fracture, most commonly a vertebral compression fracture. In summary, while the definition of osteoporosis given by Faugere (1985) cited above is generally useful, the related terms of bone atrophy, localized osteoporosis, clinical osteoporosis, and osteopenia

must be borne in mind when consulting the literature on osteoporosis.

Both men and women lose bone gradually, starting in the fourth decade of life at a rate between 0.25 and 1% per year; women usually experience accelerated bone loss (2 to 3% per year) at menopause which persists for about 3 to 7 years (Avioli, 1984). Not surprisingly, early menopause increases the probability of women developing osteoporosis (Peck *et al.*, 1987). It is generally believed that the reason for the increased bone loss at menopause is due to the decrease of estrogen secretion with decreased ovarian function. On average, women at age 70 years, have lost 50% of their skeletal mass; men of the same age have a 20 to 30% loss (Peck *et al.*, 1987). While age-related bone loss occurs in both sexes and in all ethnic groups studied (Cummings *et al.*, 1985), most investigators have found that the most rapid rate of bone loss occurs in white females in the few years following surgical or natural menopause (Cann *et al.*, 1980; Mazess, 1982). Hui *et al.* (1982) have reported for very old women (age 90 years), that loss of cortical bone mass ceases as the rate of endosteal bone loss falls below the rate of periosteal bone gain and the cortical thickness starts to increase very slowly.

Demographic factors are important in determining individual susceptibility to osteoporosis and its associated fractures. In the U.S., bone mass was found to be greater and the rates of vertebral and hip fractures to be lower in blacks than in whites. A genetic difference in the rate of parathyroid hormone (PTH) secretion has been hypothesized as a reason for this difference (Mangaroo *et al.*, 1985). Geographic location has also been implicated as a factor in hip fracture rate. Lewinnek *et al.* (1980) reported higher rates of hip fracture in the U.S. and Western Europe compared with Asia, Eastern Europe and Africa. Variations in diet, physical activity, and exposure to sunlight have been suggested as factors for these variations.

Although the whole skeleton is at risk in osteoporosis, certain bones have an increased tendency to fracture. The lower thoracic and upper lumbar vertebral bodies, the proximal femur (subcapital, intertrochanteric, subtrochanteric), the proximal humerus, and distal radius (Colles' fracture) are the most common sites for osteoporotic fractures (Melton, 1983). The incidence of fractures increases steeply for both men and women after age 65 years. It has been estimated that 13% of all Canadian women over 65 years of age will sustain an osteoporotic fracture of the proximal femur (and Spassof, 1986). Vertebral fractures usually occur spontaneously when the spine is subjected to the normal daily stresses of lifting and bending while forearm and hip fractures usually follow minimal trauma such as falls (Gryfe, 1977). Vertebral crush fractures vary in both severity and strength and some lead to permanent vertebral deformities. Permanent Vertebral Deforming Events (PVDE) usually start as biconcavities of the vertebral body with a loss of vertebral height; wedging follows with subsequent complete crushing of vertebral bodies, and their collapse. Back pain usually accompanies vertebral fractures, but this subsides fairly rapidly. Secondary postural pains become dominant as the patient loses height and kyphosis (Dowager's hump) increases (Wolff and Dixon, 1988). A "toggle neck" will develop later when the neck muscles (splenius capitis and other cervical extensors) weaken. Intubation for surgical procedures may become more difficult, and as the spinal column shrinks and shortens, the space in the chest and abdomen decreases; lung capacity is thus reduced and the potential is higher for respiratory problems. The decreased space in the abdominal cavity can also lead to the development of hiatus hernia (Kleerkoper *et al.*, 1984).

Fracture of the long bones (proximal femur) is about 2.5 times as common in women as in men, and occurs, on average, about 15 years later than the vertebral compression fractures (Nagent de Deuxchaisnes, 1983). The later onset of hip fractures in both men and women might be related to the fact that the femoral neck contains mostly

cortical bone (cortical/trabecular ratio of 75/25) in contrast to the vertebral body which is mostly trabecular bone (cortical/trabecualr ratio of 40/60) (Wolff and Dixon, 1988).

The socio-economic impact of osteoporosis and its associated fractures on public health is high especially in North America where women live, on average, about 40% of their lives after menopause. In Canada, life expectancy for females is 80 years (Statistics Canada, 1987) and currently, 6.5% of the female population is aged 65 years and older; this proportion is estimated to be 9.2% for the year 2011 (Statistics Canada, 1985). Proximal femur fractures are by far the most serious health problem in this aging female population. Melton *et al.* (1983) have reported that 3.3% of U.S. women 85 years and older had sustained a fracture of the proximal femur. It has been estimated for the U.S. that 237,000 hip fractures occur every year at an estimated cost of U.S. 7 billion dollars/year (Holbrook *et al.*, 1984). In addition to the direct cost of the acute treatment, an equivalent amount of money is lost indirectly (e.g. lost earnings). In addition to the large cost, the mortality in this group is increased significantly and is estimated to be between 12 to 20% (Cummings *et al.*, 1985). Morbidity is also very significant; many patients develop chronic complications such as bed sores after surgery (Versluysen, 1986). Half of those able to walk before sustaining a hip fracture, cannot walk adequately afterwards (Miller, 1978), and independence is greatly reduced. One-third of patients will become totally dependent on society after a proximal femur fracture (Jensen *et al.*, 1982). Immobility of patients will further aggravate the rate of bone loss (i.e. disuse bone loss) and increase the potential risk for other fractures. The extent of the proximal femur fracture problem is increasing, even with the development of new technologies and therapies, and is expected to increase over the next decade in parallel with the increase in the size of the aging population (Melton, 1988).

The problem of bone mass loss and subsequent fracture has encouraged a lot of researchers to investigate this process. Certain clinical disorders increase the rate of bone

mass loss beyond the normal rate alluded above. In osteoarthritis (OA), proximal femur fractures do not usually occur spontaneously unless the osteoarthritic changes are accompanied by a significant decrease in bone mass (Malluche *et al.*, 1986). However, patients with severe osteoarthritic changes in the hip joints are often scheduled for elective total hip replacement surgery. In such patients, it might be expected that bone mass in the affected limb would be lower than age-matched control subjects due to the lack of usual mechanical stresses on bone, as a result of severe pain which accompanies osteoarthritic changes in the hip and limits mobility. Patients with OA who were scheduled for total hip replacement surgery were selected for our study in which bone mineral density (BMD) in the proximal femur and the lumbar vertebrae were measured before, and during 12 months after the hip replacement. It was expected that the new biomechanical status of these individuals, with physiotherapy and re-ambulation, would cause observable changes in bone mass due to the greater general mobility facilitated by the hip replacement. The information obtained in this study could be of value for clinical decisions concerning the type of rehabilitation program to be undertaken by particular individuals. The findings might indicate, for example, whether an aggressive approach to re-ambulation is dangerous, and could perhaps result in fractures in other bones which have not yet adapted to the new skeletal biomechanics. Alternatively, a more aggressive approach to therapy may speed patient's recovery by increasing the rate of increase of bone mass, and may improve the likelihood of a successful return to an independent lifestyle. Additionally, correlation of the (BMD) measures with histomorphometric data for samples from the same bone obtained intraoperatively might provide important information concerning the similarities and differences between these two methods for quantitative investigation of bone. Two hypotheses are to be tested in this study: that there will be a measurable increase in BMD post-operatively in patients undergoing hip replacement with re-ambulation; that BMD in these patients will be positively correlated with bone histomorphometric indices.

1.2 History of osteoporosis

Although recognized for more than a century (Cooper, 1826), osteoporosis was only described in 1847 by an Irish physician (Smith), known for Smith's fracture (of the distal radius). For a female patient, Smith observed "she is diminishing away in stature until she was reduced to about two-thirds of her former height" (Karpas *et al.*, 1987). Although many of our current concepts about osteoporosis were formulated over forty years ago by Fuller Albright (1940), only recently has it been possible to test many of his hypotheses using modern technological methods. Albright and Reifenstein (1948) noted that in osteoporosis "the decrease of bone tissue is due to the fact that the osteoblasts lay down too little bone matrix; that matrix which is laid down is normally calcified; osteoporosis is a disorder of tissue metabolism, not of calcium or phosphorous metabolism".

A conference sponsored by the U.S. National Institutes of Health in 1984, publicized the prevalence and seriousness of osteoporosis. The roles of estrogen therapy, calcium, exercise, general nutrition, and other strategies in reducing bone loss were evaluated and important new areas for research investigation were suggested. These findings were supported and extended in a 1987 conference co-sponsored by the same institution.

Diagnostic methods for osteoporosis were introduced early in this century. In the 1930's and 1940's, routine x-rays were used to detect osteoporosis (Davies and Saha, 1985). Radiographs, however, are insensitive indicators of bone loss; the bone mass has to be decreased by at least 30% to be detected (Lashmann, 1955). Other radiographic information may also be useful in the diagnosis of osteoporosis, including the measurement of cortical width for long bones and the use of trabecular indices, such as the Singh or Calcaneal indices, that classify the degree of bone loss according to the trabecular pattern

shown in an x-ray (Singh, 1970). Single-photon absorptiometry (SPA) was first described by Cameron and Sorenson (1963), and the basic configuration for the commercial model available today was first introduced in 1972 (Mazess and Cameron, 1972). SPA provided more accurate and more precise assessments of bone mass than radiographic methods, but they lack the ability to distinguish between cortical and trabecular bone and are limited to the appendicular skeleton. Dual-photon absorptiometry (DPA) was originally reported by Reed (1960), and the first equipment used was also described by Reed (1966). Roos *et al.* (1970) described the methodology of lumbar spine DPA. The main advantage of DPA over SPA and radiographic methods is the elimination of soft tissue effects in the photon transmission measurement (Huddeleston, 1988). Computed tomography (CT) was first applied to the quantification of bone by Rüegsegger *et al.* (1974). Hangartner *et al.* (1985), subsequently developed a special-purpose CT scanner with a radioisotope (^{125}I) source. These techniques have facilitated precise, quantitative measurements of the mineral content of bone, and have made it easier to monitor changes in bone mass in osteoporosis. Currently, dual-energy x-ray absorptiometry (DEXA), a technology that uses two alternating x-ray energies for transmission measurements is considered the best available technique for BMD measurement since negligible radiation dose levels are involved and the method has high reproducibility (Wahner, 1988). A precise determination of BMD is important because of the strong relationship between BMD and fracture risk. Attributable risk (excess risk/population risk) associated with BMD in fractures is 73% for the spine and 83% for the femur; these may be compared to a 21% attributable risk of cholesterol for heart disease in males or a 78% attributable risk of smoking for lung cancer (Browner, 1986).

The therapeutic approach to osteoporosis has included the following agents: estrogen, progestin, fluoride, calcium, calcitonin, vitamin D, parathyroid hormone (PTH), bisphosphonates, and coherence treatment (Activation, Depression, Free, Repeat-ADFR).

However, no therapeutic measures to prevent bone loss appear to have been undertaken prior to 1925 (Frost, 1981). Estrogen was isolated and purified in 1923, and by 1938 ethinylestradiol and stilbestrol had been synthesized (Albright, 1940). Studies in the 1950's showed that long-term estrogen replacement reduced bone loss (Lindsay, 1982). In the mid-1970's, there were reports that hormone replacement therapy increased the risk of endometrial carcinoma (Shapiro *et al.*, 1980). Progestogen, when added to the estrogen treatment, was shown to protect against uterine carcinoma (Mack and Ross, 1989); estrogen, used as a single agent, had been implicated as a probable causative factor in endometrial carcinoma. The role of calcium in osteoporosis was first investigated by Nordin (1960) who reviewed studies linking calcium deficiency with osteoporosis in animals. Jowsey and Raisz (1968) reported, in animal studies, linkage between calcium and PTH in osteoporosis and noted the role of PTH in controlling serum calcium levels. Solomon (1968) was unable to establish any relationship between calcium intake and cortical bone mass. However, Markovic *et al.* (1979) studied two Yugoslav populations with two different calcium intakes, and found higher bone mass in the population with higher calcium intake. Many studies (Horsman *et al.*, 1977; Recker *et al.*, 1977; Nilas *et al.*, 1984) have shown that calcium supplementation in post-menopausal women has a small effect in preventing cortical bone mass loss, but Riis *et al.* (1987) found no effect of calcium in preventing trabecular bone loss.

Vitamin D supplements have also been used in the treatment of osteoporosis, since a significant number of osteoporotic patients were found to suffer from calcium malabsorption (Gallagher, 1973). It was also found that such patients had reduced levels of $1,25(OH)_2$-vit D_3 (the most potent metabolite of vitamin D and is essential for active absorption of calcium from the gut) (Bishop *et al.*, 1980; Lawoyin *et al*, 1980; Caniggia *et al.*, 1984). Gallagher *et al* (1979) have shown that ostoporotics with impaired calcium absorption had a variable response to vitamin D; some patients responded to low doses of

vitamin D (1000 U/day), while others required higher doses (up to 40,000 U/day). Increased calcium absorption in these patients was reflected by increased cortical bone mass. Vitamin D metabolites have also been used in the treatment of osteoporosis. Caniggia *et al.* (1985) reported a 6% increase in bone mass in patients given 1 μg/day $1,25(OH)_2$-vit D_3.

The effects of fluoride on bone were first described by Moller (1932); fluoride excess leads to increased radiodensity of the axial skeleton. Rich and Ensinck (1961) were the first to report the use of fluoride to treat osteoporosis. Wallache *et al.* (1977) were the first to report the use of salmon calcitonin to treat osteoporosis: they found that serum calcium levels were increased in their subjects. Chesnut *et al.* (1983) reported a study in which calcitonin was administered in addition to 1200 mg of calcium and 400 IU of vitamin D per day; a significant increase in total body calcium was found. Gruber *et al.* (1984) reported a significant increase over 18 months in total bone mass in a group of post-menopausal women who received 100 MRC units of calcitonin per day along with supplemental calcium and vitamin D.

Reeve *et al.* (1980) described the use of PTH in the treatment of a group of osteoporotic post-menopausal women; trabecular bone volume was increased with the formation of new bone on the surface of existing trabeculae. Bisphosphonates have also been used in the treatment of osteoporosis although earlier clinical studies with high doses of etidronate were disappointing due to the development of osteomalacia (Canfield *et al.*, 1977; Boyce *et al.*, 1984). However, recent studies using lower doses of etidronate in a cyclical protocol (Watts *et al.*, 1990) did not find osteomalacia in their subjects. Chesnut (1985) reported an increase in total body calcium in patients who received 1200-1600 mg of clodronate intermittently over an eleven month period.

Coherence treatment of osteoporosis (ADFR) was first suggested by Frost (1981),

and involved the use of a combination of drugs applied at different phases of the bone remodelling cycle, to first activate (A) bone remodelling units (BRUs) (described below) with phosphate or PTH; second, to depress (D) the subsequent resorptive phase of the bone remodelling cycle using bisphosphonates or calcitonin; third, to let the final phase of bone formation to proceed without any agent (F); finally, to repeat (R) the whole process again. Using the "right" drug and selecting the "right" timing is very critical for the success of this kind of treatment. Anderson (1984) reported encouraging results using 1500 mg of oral phosphate daily for 3 days as an activator followed by 5 mg/kg etidronate per day for 15 days, and then 10 weeks of no treatment. The whole cycle was repeated at 3-month interval for a total of 8 cycles. The results indicated a significant increase in trabecular bone volume. Watts *et al.* (1990) have also reported a significant increase in spinal bone density (between 4.2% and 5.2% over two years) in subjects receiving intermittent cyclical treatment with etidronate, compared to placebo controls. To date, the optimal therapy for ostoeporosis has not been elucidated. Estrogen therapy is effective and is the mainstay of treatment. Fluoride and the use of bisphosphonates as single agents have not been shown to be effective and the role and type of ADFR remains to be established.

1.3 Bone Biology

On a functional level the skeleton can be considered to have two distinct parts: first, the axial skeleton, consists of the skull, spine, and pelvis; second, the arms and legs make up the appendicular skeleton. The skeleton is made of bone, and bone is a composite material with organic and inorganic components. The organic components are primarily a matrix of collagen and non-collagenous glycoproteins, phosphoproteins, proteolipids, and mucopolysaccharides, which together are referred to as "osteoid". Type I collagen is the most abundant structural protein of osteoid, accounting for more than 95% of the total volume (Katz and Li, 1973a&b). The inorganic or mineral components of bone consist of hydroxyapatite which is an insoluble macrocrystalline mineral [$Ca_{10}(PO_4)(OH)_2$]. This

mineral is initially deposited on the organic matrix as calcium phosphate salt and is later transformed into apatite crystals. Variable quantities of other ions, such as carbonate, magnesium, sodium, calcium, and fluoride are also found in bone, and these may play important structural and metabolic roles (Asling *et al.*, 1963).

The osteon is the smallest individual functional unit of mature bone. Each osteon consists of a central Haversian canal containing blood vessels and connective tissue. At the surface of the Haversian canal, osteoclasts (bone-resorbing cells) and osteoblasts (bone-forming cells) may be seen, indicating bone modelling or remodelling activity (Frost, 1963b).

Osteoclasts, which are the principal cells for breaking down bone matrix and bone mineral (Raisz, 1983), are mono- or multi-nucleated cells characteristically seen in the lacunae of resorbed bone. Osteoclasts react positively to stains for acid phosphatase and may vary in histological appearance. The study of Gaillard (1959) has demonstrated that osteoclasts are highly mobile within the bone envelopes (closed bone surfaces on which the metabolic activity takes place), and go through resorbing and resting cycles. Parathyroid hormone, an 84-amino acid polypeptide secreted by the parathyroid gland, regulates the extracellular calcium in the kidney, the intestine, and in bone (Fischer *et al.*, 1982). In bone, there is little evidence that osteoclasts have receptors either for PTH or 1,25 $(OH)_2$-vit D_3 (the most potent metabolite of vitamin D that has a role in the active absorption of calcium in the intestine), or respond to these hormones directly. The observation of normal osteoclastic activity in parathyroidectomized newborn rats (Krukowski and Khan, 1980) and the lack of PTH involvement in the osteoclastic effect associated with osteoporosis (Rodan *et al.*, 1978) supports the notion that osteoclasts do not respond directly to PTH. Malluche *et al.* (1985) observed that marginal or no correlation exists between circulatory levels of PTH and the number of osteoclasts, and that a good correlation occurs between serum PTH and number of osteoclasts in bones of patients with end-stage renal failure.

Faugere *et al.* (1984a) supported the hypothesis advanced by Rodan and Martin (1981) that PTH acts upon osteoblasts to cause bone resorption. According to this hypothesis, osteoblasts protect bone matrix against osteoclastic resorption and the effect of PTH is to reduce this protective effect. Lian *et al.* (1984) have further reported that osteoclast-deficient bone was poorly resorbed, both *in vivo* and *in vitro*, suggesting that osteocalcin (an enzyme secreted by osteoblasts and used as a biochemical marker to detect an increase in osteoblastic activity) might be an essential component of bone matrix and that osteocalcin enhances the recruitment of osteoclasts. Osteoclasts are believed to be involved in calcium homeostasis and, possibly, the induction of bone remodelling (Krempien, 1981). Holtroop (1977) found that the increase in serum calcium levels after a PTH injection, was not coincidental with an increase in the number of osteoclasts demonstrating that there are mechanisms other than the increase in the number of osteoclasts that are responsible for the short-term transfer of calcium from bone. Malluche *et al.*(1982) have reported that an increase in the number of osteoclasts does not necessarily result in an increase in total osteoclastic activity.

Osteoblasts are mono-nuclear cells with a basophilic cytoplasm, and are the principle cells involved in osteoid formation. Osteoblasts are usually arranged in a pallisade-like manner and bone is formed by osteoblasts in two successive stages: matrix or osteoid formation followed by mineralization. Matrix formation occurs through the biosynthesis of collagen and ground substance of bone consisting of proteoglycans, glycoproteins and other components (Hancox, 1972). Mineralization denotes deposition of hydroxyapatite crystals.

Approximately 10 to 20% of the trabecular surface exhibits osteoid which can be histologically demonstrated if undecalcified bone samples are processed by mineralized bone histology. Some 30 to 40 percent of osteoid is covered by active osteoblasts which

look polyhedral in shape (Schenk, 1976a; Malluche *et al.*, 1982c); 60 to 100% of osteoid seams (the surface of the newly formed bone) exhibit a zone with characteristic staining properties that allow them to be identified with Toludine blue, and this zone is called the mineralization or calcification front (Malluche, 1986). At active bone formation sites where osteoid seams are covered by active osteoblasts, matrix biosynthesis and mineralization proceed at the same rate resulting in a constant osteoid-seam thickness. Osteoid thickness decreases at sites of "maturing seams" (Parfitt *et al.*, 1977) where osteoblasts are progressively less active, causing depression of mineralization preceded by a decrease in matrix synthesis. At the tissue level, matrix and mineral apposition are considered to be balanced processes.

Osteoblast function has been found to decrease with age. Bennett *et al.* (1984) showed that serum levels of both growth hormone and insulin-like growth factor-I (IGF-I, somatomedin C, which mediates the effect of growth hormone on bone and cartilage), decline with age, which means that the bone formation rate is decreased. It is more likely, however, that the decrease in osteoblast function results from impaired production of growth factors by bone cells. At least 12 local regulators of growth, produced by bone, cartilage, or marrow cells, have been identified (Centrella *et al.*, 1985). The most important of these appear to be IGF-I and IGF-II (known to be identical to skeletal growth factor (Mohan *et al.*, 1988). When osteoblasts have completed their bone matrix deposition, approximately 10% of the these become trapped by the advancing edge of bone and are surrounded, at first partly and then entirely, by the matrix thus becoming osteocytes. Osteocytes are situated in lacunae and have cellular processes which traverse the matrix in canaliculi, connecting the osteocytes with each other and forming a syncytium with each other and with the osteoblasts of the trabecular surface.

Histologists have long recognized that the skeleton is constructed of many small elements of bone made at different times (Parfitt, 1983); these are referred to as bone

structural units (BSUs) (Figure 1.1). The BSUs are held together by a highly mineralized connective tissue which is visible in bone sections as the cement line (in a two-dimensional bone section). The strength of bone is increased by this method of construction, but slippage at the cement plane (a three-dimensional structure) can be produced by prolonged mechanical stress (Prockop *et al.*, 1979).

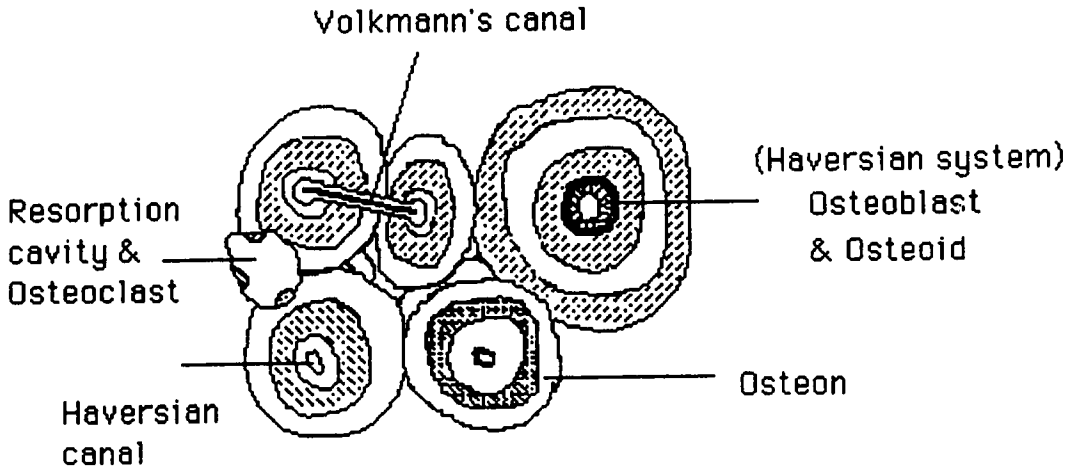

Figure 1.1 Schematic diagram of the mature cortical bone section showing bone structural units (BSUs) as the Haversian system and its components

The characteristic BSU of adult cortical bone is the secondary osteon or Haversian system, a cylinder about 300 to 700 μm in diameter roughly parallel to the long axis of the bone, with a central canal about 40 to 50 μm in diameter. The canals are connected to each other by transverse Volkmann's canals and periodically divide and reunite to form a branching network. Within the canal runs blood vessels, lymphatics, nerves and loose connective tissue, all of which are continuous with those of the bone marrow and the periosteum. Osteons constitute approximately two-thirds of cortical bone by volume, a proportion which decreases with age, the remainder being interstitial bone (Parfitt, 1983).

In trabecular bone, the BSUs are flattened, and lie roughly parallel to the plane of the trabecular plates; they are sometimes referred to as trabecular osteons. The BSUs have a thin crescent shape about 600 μm long and about 60 μm in depth at the center, tapering at each end. In the three-dimensional reality they are much larger and have a more complicated shape, with prolongations in different directions that interlock with adjacent BSU. In cortical bone (or compacta) the porosity or soft tissue content, is usually less than 10% by volume; in trabecular bone, porosity is more than 75% by volume. In the aging skeleton, however, all degrees of porosity may be found. The ratio of surface area to volume of bone is about four times higher in trabecular than in cortical bone, but because of the difference in porosity, the ratio of surface area to volume of bone tissue overall is nearly the same (Parfitt, 1983).

Cortical bone provides about 80% of the total skeletal mass and constitutes the outer surface of all bones, but most cortical bone is found in the shafts of long bones in the appendicular skeleton. Trabecular bone (or spongiosa) constitutes the remaining 20% of skeletal mass and is a rigid matrix of mineralized tissue which forms the greater part of the vertebral bodies, the epiphysis of the long bones and is abundant at other sites such as the ribs and pelvis. Trabecular bone has a large surface area (about two thirds of the total bone surface), and is the most metabolically active part of the skeleton, with a high rate of bone turnover, and a blood supply that is much greater than that of cortical bone. Trabecular bone is composed of trabeculae or struts which are usually oriented to resist deformational stresses, and their number, size, and distribution are related to biomechanical factors.

The structural difference between cortical and trabecular bone is visible with the naked eye, but lamellar bone (the main type of bone in a mature skeleton) and woven bone (fetal or fibrous bone) are distinguished only by the microscopic orientation of collagen fibre bundles (Hancox, 1972). The difference in structural organization between lamellar

and woven bone arises from differences in methods of formation. Lamellar bone is formed only through apposition on an existing surface; each osteoblast is coordinated with its neighbors and together they make a continuous layer of bone laid down on one side of the cell (osteoblast). Such spatial orientation and polarized extrusion of protocollagen strands toward the bone matrix is a prerequisite for normal collagen production. The deposited collagen exhibits a lamellar pattern with circular layers of collagen alternating with longitudinal layers (Boyde, 1972). Woven bone is formed directly through the condensation of fibrous tissue without the necessity for an adjacent free bone surface, by the rapid and uncoordinated action of individual osteoblasts. Each osteoblast forms an island of new matrix, and adjacent islands fuse together into nodules (Parfitt, 1983). Mineralization occurs rapidly and simultaneously in each nodule (in the absence of proper seams) and without relation to collagen fibers or blood vessels. This can be observed as a diffuse rather than a focal uptake of tetracycline (an antibiotic drug that when administered orally or parenterally, adsorbs on bone surfaces that are actively mineralizing at the time of administration). This process somewhat resembles the mineralization of cartilage rather than mature lamellar bone (Boyde, 1972; Courpron *et al.*, 1976).

The differences between lamellar and woven bone are relevant to osteoporosis for several reasons. First, only woven bone can be formed in cell culture; this places certain limits on the applicability of cell culture experiments to the study of the pathogenesis and treatment of osteoporosis. Second, woven bone formation is less susceptible to endocrine and other regulation than is lamellar bone formation (Frost, 1985); this is why fracture healing may be quite normal in osteoporotic patients with subnormal rates of bone formation and impaired osteoblast function. Third, the first bone formed in response to sodium fluoride administration, although deposited in apposition to an existing bone surface, has several of the features of woven bone (Baylink, 1967) and is not as strong as an equivalent volume of lamellar bone. Finally, the controlled production of woven bone

in specific locations may turn out to be an important strategy for the restoration of normal bone structure in severe osteoporosis .

Each bone has four bone surfaces: periosteal, intracortical (including both Haversian and Volkmann's canals), endocortical (Figure 1.5) and trabecular. Bone envelopes are defined as closed and unbounded surfaces that divide space into an outside and inside (Parfitt, 1983). The periosteal envelope encloses all the hard and soft tissues of a single bone. The endosteal envelope (trabecular, endocortical and intracortical) encloses all the soft tissue within the bone. This classification of bone envelopes is very important to the understanding of bone modelling and remodelling activities.

Bone modelling describes the process of bone growth. There are two types of bone growth, longitudinal and appositional growth (i.e. growth of bone in length and width). Longitudinal growth occurs by enchondral ossification, a process which creates new trabeculae until the epiphyseal growth plate fuses. Appositional growth takes place by periosteal apposition of new bone and resorption of old bone. The term bone remodelling is used to describe the dynamic processes in the adult skeleton after closure of the epiphyseal growth plates (Parfitt, 1983). Bone tissue is continuously renewed in order to repair the tissue damage caused by normal mechanical usage. A certain number of osteons enter a "remodelling cycle", resulting in their partial or complete removal and replacement by new osteons. Osteons undergoing such remodelling are called active bone remodelling units (BRUs) (Frost, 1961b). Bone remodelling represents the physiologic basis of bone turnover and renewal in mature skeleton. The remodeling process can be generalized into three parts (Figure 1.2). First, on the bone surface, the local remodelling cycle begins with "activation", a change in the local milieu that attracts bone remodeling units (BRU). This is a coordinated sequence of cellular events, where osteoclasts resorb bone by the release of hydrolytic enzymes over a 1 to 2 week period. Second, once resorption is

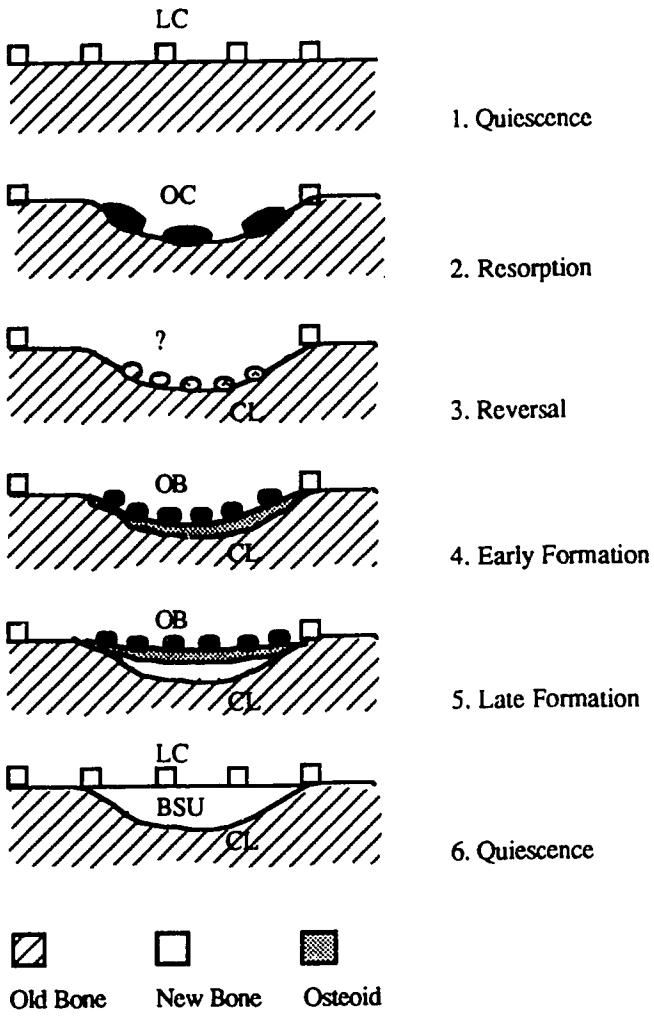

Figure 1.2 Normal bone remodelling of a single and construction of a single new BSU. [Adapted from Parfitt, (1984)].

LC = flat lining cells, OC = osteoclasts, CL = cement line, OB = osteoblasts, BRU = bone remodeling unit, BSU = bone structural unit.

completed, the osteoclasts disappear and lining cells deposit a cement substance, the reversal line. Third, osteoblasts then appear and refill the cavity, first with a collagenous substance (i.e. osteoid), over a 3 to 4 months period. Mineral is deposited in this osteoid matrix over several months beginning about two weeks after formation. Some of the osteoblasts become enclosed within the newly formed matrix forming the osteocytes.

Histological cross-sections of compact bone show osteoclastic activity near the center of a future osteon. The diameter of the new osteon is the composite result of activity and duration of the osteoclast resorptive phase. In trabecular bone, the mean wall thickness (distance between cement lines) is the composite result of osteoclastic activity, duration of osteoclastic resorption, and activity of osteoblasts. Approximately 10 days after deposition of bone matrix by osteoblasts (Figure 1.3), mineralization starts at a distinct mineralization front, and when the newly deposited bone has been completely mineralized, the new remodelling cycle is complete. In the adult skeleton, the time span between activation of resorbing cells and completion of the BSU (Frost, 1963b), is 4 to 6 months in young healthy individuals.

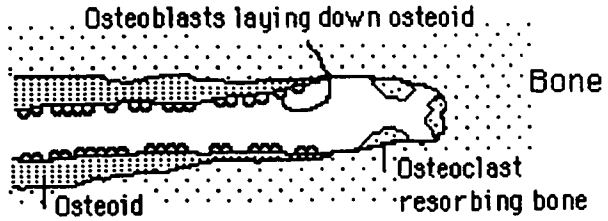

Figure 1.3 Diagram of a bone remodelling unit (BRU). Osteoclasts are eroding from left to right a longitudinal cavity, which is being refilled by osteoblasts lining an osteoid seam which subsequently will be mineralized

The overall rate of bone resorption and formation is determined by (a) the amount of matrix resorbed and formed per cell, and per unit tissue cell-level activity, (b) the number of cells per resorption and apposition (formation) site, and the lifetime of the individual resorption and apposition sites-osteon level activity, and (c) the number of resorption and apposition sites per unit volume bone-tissue level activity. In uremia for example, overall bone resorption and formation, (i.e tissue level activity) may be increased, whereas cell-level resorption and formation may be depressed (Malluche *et al.*, unpublished

observations). Therapy of uremic patients with 1,25 $(OH)_2$-vit D_3 may depress tissue-level resorption and formation activities while enhancing cell-level activity.

The rate of bone remodelling is often assumed to depend on the working speed of the bone cells (osteoblasts and osteoclasts) with cells working more quickly and increasing the bone remodelling rate, or working more slowly and decreasing the bone remodelling rate. However, the relative consistency of the mean wall thickness indicates that the overall bone remodelling rate is largely independent of the rate of individual cell activity which vary over a wide range (Landeros and Frost, 1964). This conclusion is very important to the understanding of the kinetic and biomechanical indices of bone remodelling as a consistent remodelling rate reflects the bone remodelling activities of the entire skeleton and is not affected by sampling problems when examined by histological techniques (i.e not site specific). Thus, indices of bone remodelling (formation or resorption) are influenced mainly by changes in the whole body rate of remodelling activity.

Changes in bone cell activity might be transient reflecting a stimulus which initiated the events for bone remodelling. For instance, if the number of osteons entering a remodelling cycle (activation frequency) is temporarily increased, as seen with increased levels of thyroxine (Meunier *et al.*, 1972) or after administration of PTH (Malluche *et al.*, 1982d), numerous osteoclasts and a negative bone balance might be observed; subsequently, numerous osteoblasts, and a positive bone balance might be seen before the surface cell density and the resultant bone balance will return to baseline levels.

Every BRU is associated with a temporary deficit of bone mineral which has three components. The first component is the volume of bone that has been removed and has to be replaced. The second is the volume of the new osteoid that will eventually be mineralized. The third is the lower density of newly formed bone which needs an extended time for complete mineralization. The sum of the these components is the total turnover-

related reversible mineral deficit (RMD). The deficit (Figure 1.4) is reversible because replacement of each component is inevitable as the BRU completes its evolution and the new bone reaches maturity.

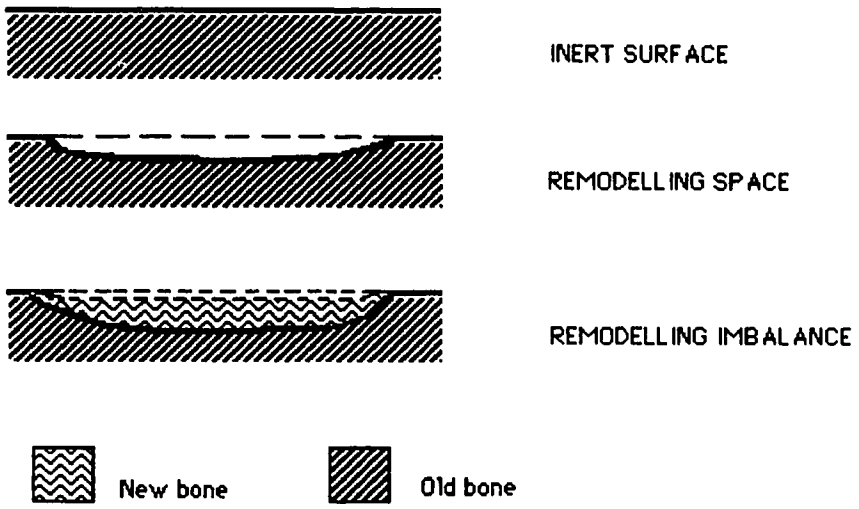

Figure 1.4 Diagrammatic representation of mineral deficit during bone remodelling

1.4 Age-related Changes in bone mass in men and women

Changes in bone mass during human life can be classified into three periods. First, from conception until the epiphyseal closure there is a progressive increase in both cortical and trabecular bone volumes. Second, after cessation of longitudinal growth, there is a period of consolidation during which cortical porosity (still high in late adolescence) continues to decrease and bone tissue density increases to a peak (Kleerekoper *et al.*, 1981). The cortices become thicker because of net apposition on endosteal as well as on periosteal bone surfaces (Garn, 1981). For trabecular bone, some researchers reported no increase in the amount of trabecular bone after epiphyseal closure (Courpron *et al.*, 1976), but in other studies (Malluche *et al*, 1982c) both the total amount of trabecular bone, and

the trabecular thickness increased after closure of the epiphyses. During this period, peak adult bone mass representing the sum of contributions of growth (90 to 95%) and consolidation (5 to 10%) is reached between the age of 35 to 40 years for cortical bone and probably also for trabecular bone. Peak adult bone mass is about 25 to 30% higher in males than in females and about 10% higher in blacks than whites. These differences are partly under genetic control but must also reflect a variety of hormonal, nutritional and socioeconomic factors. Third, a few years after peak adult bone mass is attained, age-related bone loss begins which is a universal phenomenon of human biology that occurs regardless of sex, race, occupation, habitual physical activity, or dietary habits (Garn, 1970; Kleerkoper *et al.*, 1981; Melton *et al.*, 1981; Meunier *et al.*, 1981). Bone is lost from nearly every site that is amenable to measurement, but only from the endosteal surfaces in contact with the bone marrow, since the periosteal surface continues to gain small amounts of bone due to its direct exposure to mechanical strains. In males, the average cortical bone loss is about 0.3% of peak adult bone mass per year and trabecular bone loss is slightly faster. In females, the average loss is about 1% of peak adult bone mass per year for both cortical and trabecular bone, with an accelerated rate during the 4 to 8 years after menopause and a slower rate at earlier and later times (Krølner *et al.*, 1982; Riggs *et al.*, 1986). The absolute reduction in thickness is about 50 µm/year (Parfitt, 1980). After about age 90 years, the rate of endosteal loss may fall below the rate of periosteal gain, so that cortical thickness, having declined progressively for 40 to 50 years, very slowly increases (Hui *et al.*, 1982).

Age-related bone loss is a complicated process and to understand it, we have to look separately at cortical and trabecular losses.

1.4.1 Cortical bone loss

Cortical bone loss (Figure 1.5) occurs primarily at the cortical endosteal surface (inner cortical surface) which makes it easily detectable radiographically. Bone could also

be lost from within the cortex, and to a lesser extent from the periosteum. The loss from all of these bone envelopes will lead to an increase in bone porosity with a variable severity from the outer to inner surfaces (Mathews *et al*, 1981). In the outer half, the increase is about 1 to 2% and is mostly accounted for by the rise in the number of Haversian canals that follow the continued formation of new osteons (Martin *et al.*, 1980). In the inner half, the increase in bone porosity is about 5 to 10%, and is mainly due to an increase in the size

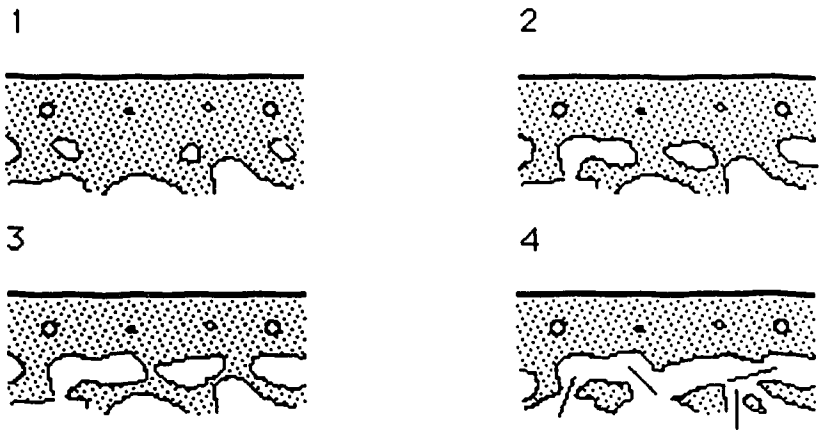

Figure 1.5 Diagram of microscopical evolution of cortical bone loss. Successive stages in osteoclast-dependent thinning of cortical bone. 1- Normal adult cortex with larger Haversian canals closer to the inner cortical surface. 2- Enlargement of the endosteal spaces and communication with the marrow cavity. 3- Further enlargement and conversion of the inner third of the cortex to a structure that topologically resembles trabecular bone, with expansion of the marrow cavity. 4- Perforation and disconnection of the new trabecular structure. Adapted from Parfitt (1984).

of the spaces rather than in their number. The differences in type and extent of porosity between the outer and inner regions of the cortex are related to the mechanisms of cortical thinning.

1.4.2 Trabecular bone loss

Trabecular bone loss (Figure 1.6) is more important to assess than cortical bone

loss, due to the fact that the trabecular bone is metabolically more active than the cortical bone. There are two important values that have to be calculated to be able to assess the amount of trabecular bone loss; first, trabecular density which is the number of trabecular plates per mm; second, the calculated separation between trabeculae as an index of the

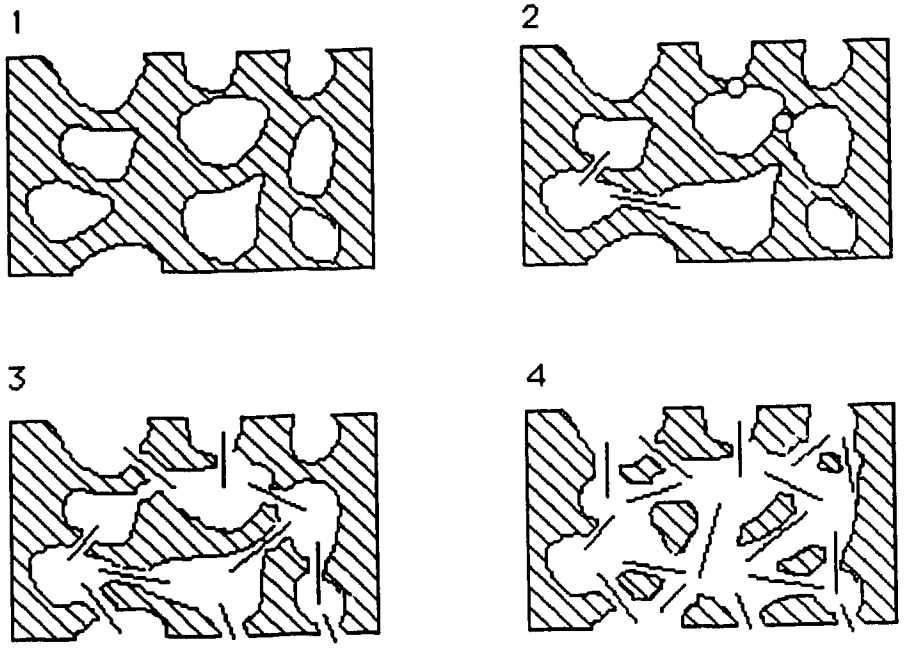

Figure 1.6 Diagram illustrating microscopical evolution of trabecular bone loss. Successive stages in the conversion of the continuous trabecular network present at skeletal maturity to the discontinuous network seen in the elderly. 1- Fragments are isolated trabecular profiles seen in the two-dimensional section and, in fact, are connected in the third dimension rather than lying free in the marrow cavity. 2- Perforation (focal break in continuity) starts. 3- Perforation increases and fragmentation of trabeculae starts to appear. 4- More perforations as well as more fragmentation that bring adjacent marrow cavities into communication. Adapted from Parfitt (1984).

average diameter of the marrow cavities between the trabeculae. A fall in trabecular plate density and an increase in trabecular plate separation both indicate that some structural elements have been lost and that those remaining are less connected (Kleerekoper *et al.*, 1985). Applying this model to post-menopausal women, it has been found that the trabecular bone loss occurs predominantly by a reduction in the number of trabeculae,

rather than in the trabecular thickness (Parfitt, 1983; Villaneuva *et al.*, 1983). More precisely, there is a loss of the central regions of the plates, a process that begins with focal perforation and continues by enlargement of the perforation.

Age-related bone loss begins at about the fourth decade and continues throughout life, or at least into old age. As noted above, for men, the rate of loss is about 0.3% /year and is slightly greater in trabecular than cortical bone. In women, estrogen deficiency at menopause accelerates bone loss for about 4 to 8 years; some 15 to 20% of trabecular bone and about 10% of cortical bone are lost during this period (Riggs *et al.*, 1986). This large menopause-related loss is superimposed on the slow, age-related bone loss (1%/year) and coupled with the smaller peak bone mass in women accounts for the greater risk of osteoporosis in women.

The rate of bone turnover, the amount of old bone replaced by new bone/unit time, is determined by the number of BRUs in the skeleton at any given time. The elderly were previously believed to have a decrease in bone turnover (Melsen *et al.*, 1978); however, more recent evidence supports the notion that the elderly may have an increase in bone turnover. Delmas *et al.* (1983) and Duda *et al.* (1988) have found age-related increases in bone Gla protein (BGP or osteocalcin) and serum alkaline phosphatase both of which indicate a high rate of osteoblastic activity. Fogelman *et al.*, 1980, found that whole body retention of diphosphonates (index of bone turnover) has also increased with age. Similarly, early histomorphometric studies have shown that older females have decreased rates of bone remodelling; however, more recent studies suggest that remodelling is increased (Eastell *et al.*, 1988; Dahl *et al.*, 1988).

Despite the increase in the number of BRUs with age, there is evidence that the formative part of the remodelling cycle is impaired. The age-related decrease in wall thickness of trabeculae (evidence of decreased bone function at the BRU level) was found

in a histomorphometric study by Meunier *et al.* (1980). A remodelling imbalance in the elderly occurs when osteoblasts fail to replace completely the bone resorbed by the osteoclasts. In the presence of this imbalance, the higher the bone turnover, the greater the number of BRUs, and the greater the rate of bone loss. Increased secretion of PTH with aging is probably one reason for the increase of bone turnover, and because of the co-existance of age-related remodelling imbalance, bone loss will be increased. In summary, the slow phase of bone loss, although age-related, probably results from the summation of several age-related processes.

In addition to the age-related bone loss in the normal elderly population, bone loss in the elderly population could also be aggravated due to the existence of other clinical conditions. Chronic inflammatory diseases may exacerbate bone loss; rheumatoid arthritis (RA) is one example (Bjelle *et al.*, 1970). Rheumatoid arthritis is a chronic inflammation of the joints and may lead to deformity and loss of the joint function. It is more common in women than in men and virtually can affect any joint in the body. The symptoms of RA include swelling, pain and stiffness of the joints which severely limits the movement of patients (Bjelle, 1970). The limited physical movement in these patients, in addition to the effect of corticosteroid therapy on the bone, will result a net loss of bone mass and the development of osteoporosis and its associated fractures (Saville and Kharmosh, 1967). Corticosteroids, when used on regular basis for the treatment of RA, produce a direct inhibition of bone formation and an indirect enhancement of resorption and these two effects lead to a negative bone balance (Peck *et al.*, 1984). Calcium malabsorption also occurs when corticosteroids are given in high doses (Hahn *et al.*, 1981), but may only occur in those patients with low levels of 1,25 $(OH)_2$-vit D_3. Surprisingly, Crilly *et al.* (1981) have reported increased serum levels of 1,25 $(OH)_2$-vit D_3 in short-term treatment of corticosteroids. Gennari *et al.* (1983) have suggested that high doses of corticosteroids will decrease the sensitivity of the gut wall for the effect of 1,25 $(OH)_2$-vit D_3. Krokor *et*

al. (1985) have shown that glucocorticoids interfere with 1,25 $(OH)_2$-vit D_3 receptors in the intestine of dogs. Glucocorticoids also induce a state of secondary hyperparathyroidism due to the fall in serum calcium levels in addition to directly releasing PTH from the parathyroid gland (Fucik *et al.*, 1976). The secretion of calcitonin (a hormone secreted by C-cells of the thyroid gland and a potent inhibitor of osteoclastic activity), is also decreased in response to calcium infusion in the presence of high levels of corticosteroids (Gennari *et al.*, 1986). The synthesis of osteocalcin which reflects osteoblastic activity was found to be inhibited in corticosteroid-treated patients patients (Reid *et al.*, 1986; Delmas *et al.*, 1987; Lukert *et al.*, 1986). Also, glucocorticoids were found to acutely decrease testosterone levels in men (MacAdams *et al.*, 1985) and also in women (Nordin *et al.*, 1985). It has been suggested that suppression of ACTH production by high levels of corticosteroids causes the low levels of testosterone (Grilley *et al*, 1979). All the above factors will be reflected in an increase of bone resorption and a decrease of bone formation that results in a 4 to 35% loss of bone mass depending on the site, dose, and the duration of corticosteroid treatment. Hahn *et al.* (1978) have reported a 17.4% decrease in bone mass of the proximal femur for a group of 33 RA patients who received 14.7 mg/day of prednisone for an average of 9.4 years. Sambrook *et al.* (1986) have reported a 9.6% decrease in bone mass of the lumbar spine in comparison to 12.2% decrease in the femoral neck for a group of 44 RA patients who received 8 mg/day of prednisolone for an average period of 7.5 years. Als *et al.* (1984) have reported a 27% decrease in the bone mass of the distal forearm in a group of 24 RA patients who received lower doses of prednisone between 2.5 to 10 mg/day for a period of 6.1 years.

The precise quantification of bone mass is the key factor in the success of any research involving bone mass changes during the course of bone or joint disease and/or during therapy. While the x-ray remains a valuable tool for the detection of qualitative changes in bone, it cannot provide a quantitative assessment of the mineral content of bone

(bone mass, g/cm). The need for more sophisticated methods for the quantitative evaluation of bone have resulted in the development of several bone densitometry techniques that have facilitated the measurement of bone mineral content (BMC, g/cm) and bone mineral density (BMD g/cm^2).

1.5 Bone densitometry

The term "bone densitometry" describes several radiological methods for measuring the quantity of bone tissue. Bone densitometry has two main uses: first, to detect low bone mass (osteopenia); and second, to monitor bone changes over time with good precision. A controversy still exists concerning the significance of bone density screening in the detection of osteoporosis (Cummings and Black, 1986). Clinical indications for bone densitometry include male hypogonadism, menopause, radiographic osteoporosis, and immobilization (Mazess, 1989). Bone densitometry usually measures bone mineral density (BMD as g/cm^2) or bone mineral content (BMC as g/cm). Erickson *et al.*, (1989) have shown that BMD determined by dual-photon absorptiometry (DPA) correlates strongly with bone strength. In the lumbar vertebrae, factors other than BMD or BMC could contribute to the decrease of bone strength (e.g. microfractures and cortical porosity), but it is unlikely that the the decrease in bone strength due to these factors is greater than that due to the gradual bone loss (Vernon and Pirie, 1973). Epidemiological studies have also shown that individuals with low BMD have the greatest risk for fractures (Hui *et al.*, 1988; Riggs *et al.*, 1986; Melton *et al.*, 1988). For example, the relative risk of spinal fractures increases by about 1.5 times for every standard deviation decrease in spinal BMD, and the relative risk for femur fracture increases by about 2.6 times for every standard deviation decrease in femur BMD (Mazess, 1989). These particular findings suggest a relation between hip fracture and both spinal and femur BMD; however, other studies have indicated that spinal BMD is not reduced in hip fracture (Firooznia *et al.*, 1986; Mazess *et al.*, 1988). More data are required to resolve these discrepancies.

Precision and accuracy, are two important considerations in bone mass measurement techniques. Precision is the reproducibility of the technique, accuracy is the ability to truly measure the absolute amount of bone present. Methodological considerations such as subject positioning are very important in repeated measurements. In longitudinal studies, good precision is necessary to monitor small changes over time (average rate of bone loss in women is about 1% per year). The accuracy of the technique is important in cross-sectional studies, and especially so when the measurement is used for diagnostic purposes.

A variety of techniques is available for measuring the appendicular and/or the axial skeleton. These are: single-photon absorptiometry (SPA), dual-photon absorptiometry (DPA), dual-x-ray absorptiometry (DEXA), computed tomography (CT), and *in vivo* neutron activation analysis (IVNAA).

1.5.1 Single-photon absorptiometry (SPA)

SPA was first described twenty-eight years ago by Sorenson and Cameron (1963). SPA uses a sealed radioactive source (^{125} I) with an average energy of 27.4 keV which is appropriate for transmission measurement through the human forearm. The radioactive source is coupled mechanically to a NaI (Tl) scintillation counter. SPA has a small diameter photon source (1 to 3 mm) and is best used in sites where anatomical uniformity exists (i.e. distal third or mid shaft of radius). The precision of SPA in the forearm is between 1 and 2% (Vogel *et al.*, 1988). During the past decade, SPA has been used with rectilinear scanning to measure anatomically variable regions such as the os calcis. Clinical precision at these sites is between 2 and 4% with an accuracy of better than 3%. One of the limitations of SPA is that the soft tissue thickness around the bone must be constant (Wahner *et al.*, 1984); thus the limb must be covered by a uniform thickness of tissue-equivalent material (water bath, water bag or tissue-equivalent gel). Scanning time is about

10 minutes for a distal radius measurement using SPA with a radiation dose of less than 0.05 mSv. The inability of SPA to measure the axial skeleton limits its clinical usefulness; the limitation of SPA to mainly cortical bone measurements is also a problem since monitoring of trabecular bone is necessary in studying rapid skeletal changes.

1.5.2 Dual-photon absorptiometry (DPA)

DPA was developed in the early 1970's, but commercial machines did not become available until the late 1970's due to the unavailability of suitable "dual-photon" radioisotope sources. DPA can be used to measure bone mass in the axial and the appendicular skeleton without the requirement for constant tissue thickness at the measurement site. Commercial DPA uses a radioisotope source ^{153}Gd which has two principal γ-ray energies (44 and 100 KeV) (Ross, 1974). Most DPA machines use rectilinear scanning covering an area of 15 x 20 cm. DPA has a precision of between 1 and 2% with an accuracy of better than 3% in the lumbar spine. Scanning time is about 20 minutes for a spine measurement and the radiation dose is less than 0.02 mSv (Mazess *et al.*, 1989). The newer DPA scanners have multiple scintillation detectors which reduce the time needed to complete a scan by about half (10 minutes). DPA has a spatial resolution of 3 to 4 mm which is adequate for scans of the femur or the spine in normal subjects. However, spinal artifacts (osteophytes, calcified aorta and spinal fractures) which occur in about 15 to 20% of patients over 65 years of age, cause problems for quantification of bone mass.

1.5.3 Dual-energy x-ray absorptiometry (DEXA)

DEXA is identical in principle to DPA; however, an x-ray tube rather than an isotope source provides the two photon energies (40 and 70 KeV). This method offers several advantages over DPA. Spatial resolution is improved, precision is improved (1% vs 2%) with accuracy of better than 3%, scanning time is greatly reduced (4 minutes for the spine and 3 minutes for the hip), and a lower radiation dose (0.01 mSv) is given. Long-

term stability of DEXA machines is significantly better than for DPA. The improved spatial resolution of DEXA helps not only to recognize artifacts (such as calcified ligaments and compression fractures) and eliminating them from the analysis, but also allows better location of regions of interest (ROI) reducing errors in longitudinal studies (Kelly *et al.*, 1988). Several dual-energy x-ray absorptiometers are commercially available. A system developed by Lunar Inc. uses a cerium filter and a constant high voltage tube operated at 120 KeV, to provide energies of 40 and 70 KeV. Norland Inc.(XR26) uses a samarium filter with a similar x-ray tube that gives beam energies of 45 and 80 KeV. Using K-edge filters (e.g. cerium and samarium) provides a lower patient dose at any given precision level (0.01 vs 0.03 mSv) than the switched energy approach (i.e. Hologic QDR-1000) Sorenson *et al.* (1989). The Hologic densitometer has an x-ray tube that provides alternating pulses at 70 Kvp and 140 Kvp, generating effective dual-energy photons at 43 and 110 KeV, respectively, in the unattenuated beam. A calibration disk containing several x-ray absorbing materials (bone and soft tissue equivalents) provides pixel by pixel calibration data and serves as an internal reference system.

1.5.4 Computed tomography (CT)

Two types of CT systems are used for bone mass measurement (i) commercial whole body CT systems which are used to measure axial skeleton, and (ii) special purpose CT systems used to measure the appendicular skeleton.

(i) Commercial whole body CT systems:

A conventional x-ray CT scanner is used with a bone phantom and specialized scanning and analysis software to produce cross-sectional images of bone. Both single and dual-energy methods have been developed (Cann, 1981). Accuracy is better than 15% and precision is about 1 to 3% for the lumbar spine. In order to achieve this precision, two different approaches to scanning and analysis have been developed. The first uses multiple contiguous transverse slices from each of several vertebrae in order to construct a large

volume (ROI) (Banks *et al.*, 1986; Cann *et al.*, 1987); the second is a clinical approach that uses only a small ROI (3 cm^3 or < 5% of vertebral mass) from a single slice of each of 2 to 4 vertebrae. The latter approach is dependent on (a) selection of the ROI, (b) angulation of each vertebrae, and (c) congruence of the lateral scout view and the tomographic slice. The first approach (reformatting) provides the better sensitivity and precision but is costly because of the computational and interactive analysis requirements. Scanning time for vertebrae is about 20 minutes with a radiation dose between 2.0 and 5.0 mSv

(ii) Special purpose peripheral CT (pCT):

Special purpose peripheral CT is used to measure appendicular sites (proximal tibia, distal radius, and distal femur). Two pCT machines have been developed; the first uses the γ-ray spectrum of energy ^{125}I; the second uses x-ray tube instead of the ^{125}I source. The γ-ray pCT has a precision (SD) of better than ±0.5% (Overton *et al.*, 1987). The accuracy of the method is reported to range between 0.6 and 3.4 % (Hangartner *et al.*, 1987). Scanning time for the distal radius is 90 seconds per slice. Six to eight contiguous slices are measured at each subject visit with a radiation dose of less than 0.1 mSv.

1.5.5 In vivo neutron activation analysis (IVNAA)

IVNAA involves the irradiation of a natural isotope present in the human body (^{48}Ca) to produce a radioactive form (^{49}Ca) by neutron activation. Neutrons are generated in a reactor, a linear accelerator, or in a radioisotope neutron source (Pu - Be). Since ^{48}Ca is a high-energy γ-ray emitter, with a short half-life (8.8 minutes), subjects must be measured immediately after the irradiation ends; high efficiency scintillation detectors must also be used in order to achieve the best precision for the lowest radiation dose. IVNAA can be carried out for the total body to measure total body calcium (TBCa), or on limited regions such as hand (Nilas *et al.*, 1985), or spine (Leblanc *et al.*, 1980). The normal radiation dose is between 3 and 20 mSv with a typical precision of 4 to 5%.

1.6 Bone Histomorphometry

Bone histomorphometry is a histological method for the quantitative evaluation of the skeletal constituents; the method provides data concerning the amount, structure and quality of bone (Meunier *et al.*, 1981). Bone histomorphometry is carried out in many different research centers around the world and a variety of equipment and techniques are used. While the methods for processing the bone tissue are fairly well standardized, methods for quantitative histological analysis vary from simple manual procedures to sophisticated, computer-based automated processes. Due to the quantities of data resulting from histological methods, this field has developed in-step with that of computer technology. However, costs for such new technologies are high and this limits their availability to major histomorphometry research centers. A bone biopsy, taken with a minimal physical force to avoid fracture of bone trabeculae or cortices, hemorrhage, compression of spongy bone, or possibly cellular damage from excessive heat during drilling, is the starting point for histomorphometric procedures (Malluche *et al.*, 1986). The most common type of bone biopsy in humans is taken horizontally through the superior iliac crest (transiliac biopsies). A trephine needle with an internal diameter between 5 and 10 mm is used to provide a bone sample about 2 cm long. The transiliac bone biopsy is performed under local anaesthesia with valium pre-sedation, or under general anaesthesia, and usually takes between 30 to 60 minutes to complete. Transiliac biopsies contain both cortical (external and internal), and trabecular bone of adequate quantity and of good quality. Repetition of the biopsy whenever a follow-up is needed should be performed on the contra-lateral side. Subsequent biopsies are taken from alternating sides, although they may be of doubtful value due to the local effects induced on bone remodelling by the previous biopsy. Rib biopsies are also used, but these must be obtained intra-operatively; usually the right 11th rib is a preferred site, being a floating rib, and almost completely below the pleural reflection. Bone samples pass through many

processing steps before analysis including fixation, dehydration, sectioning, staining, mounting, and evaluation. The biopsy should be fixed immediately to preserve it from cellular destruction. Fixatives are divided into two major groups: precipitant fixatives that precipitate the cytoplasmic proteins as a coagulum and include ethanol, mercuric chloride, picric and chromic acids; non-precipitant fixatives that fix proteins by denaturing them and include formalin, osmium tetroxide and potassium dichromate. The bone specimens are then dehydrated, and embedded in plastic monomer (methylmethacrylate or Spurr's) until it hardens (Spurr, 1969). The plastic block containing the bone biopsy is then sectioned using a heavy duty microtome (Jung) to obtain thin sections (10 to 20 μm). These sections are processed stained (for the evaluation of static variables), or unstained (for the evaluation of dynamic variables). The final stage in the preparation of the bone sections is mounting on microscopic slides, and analysis by light microscopy (stained sections) or fluorscent microscopy (unstained sections).

The introduction of tetracycline double labelling (Misch *et al.*, 1958; Frost 1963a & 1969) as a means to advance from static bone histology to the evaluation of the dynamics of bone formation and resorption, have made bone histomorphometry a valuable tool for diagnosis and research into metabolic bone diseases. Tetracycline, when administered to the patient either orally or parenterally, is picked up by all the surfaces of bone but retained only by those that are metabolically active at the time of administration (Frost, 1963a). Tetracycline compounds are usually given in two separate dosing schedules separated by several days so that bone is deposited over the first label; four to five days follow the second label before the taking of the biopsy. The tetracycline dose is adjusted according to kidney function. It should be noted that phosphate binders or antacids also bind tetracycline, therefore, these drugs should not be given on the days of tetracycline labelling. Except for nausea and vomiting, tetracyclines are considered to be safe drugs. In rare cases, photosensitivity and allergic reactions might be observed. It is recommended to use

two different derivatives of tetracycline as each will give a different color fluorescence which make it easier to differentiate between the two labels.

The manual analysis of bone samples is usually done using optical integration grids projected over the histologic structure. The method is based on the principle of geometrical probability (Delesse, 1847). The Merz and Shenk (1970) grid (Figure 1.7) utilizes these principles, and is most widely employed for manual, quantitative bone histology.

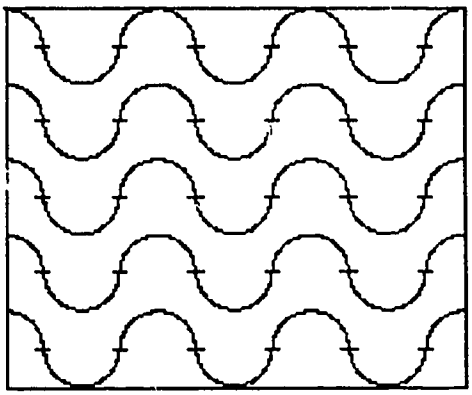

Figure 1.7 Diagram illustrating Merz grid for manual evaluation of bone sections

Volumetric fractions are obtained by point-counting procedures where the fraction of grid points overlying a structure provides an unbiased estimate of the volume fraction. Surface measurements are made by counting random intersections between sampling lines and histological features; distances are measured directly using a stage micrometer with an eyepiece micrometer which calibrates the measuring unit according to the magnification used. The probability of sampling a given structure in a random plane, i.e. the plane of a section, depends on the position of the structure. The probability is zero if they are parallel and is one if they are perpendicular. According to Buffon (1977), the count must be corrected mathematically by multiplying the measured value for the surface density by the factor $\pi/4$. Trabecular bone represents an isotropic non-random structure with preferential

spatial orientation of trabeculae which follow the strain patterns produced by mechanical forces. Therefore, the integrating grid of Merz and Shenk with its semicircular sampling lines, is preferable over straight-line grids. The probability of an intersection between the semicircular grid sampling lines of Merz and Shenk, and the trabecular structure is largely independent of the trabecular orientation relative to the grid. When straight-line grids are employed, these errors are avoided by counting twice, with the grid in two orthogonal positions. This manual technique is very slow and lacks precision in the measurement of bone histomorphometric variables. Reproducibility of the manual method has been reported in several studies where significant differences for all variables between intra- or inter-observer measurements were observed (De Vernejoul *et al.*, 1981; Compston *et al.*, 1986). With the development of digital imaging techniques, it has become easier and faster to analyze the bone sections with higher precision (Malluche, 1982). The primary data (area of the bone structure; perimeter of the bone structure; distances such as the mean wall thickness; cell counting) are collected either manually by selecting the desired region or by automated ROI definition. Malluche *et al.* (1986) have evaluated the difference between the manual and the semiautomatic techniques by analyzing 200 optical fields by the manual technique, and 50 fields by the semi-automatic technique at the same magnification. They found a significant difference in mean trabecular width between the two techniques, which they attributed to the fact that the semi-automatic method measured actual surfaces while the manual method obtained random hits and intersections for area and surface measurements. They also found that there was remarkably little inter-observer variation in the structural variables of bone using the semi-automatic method, even when three different observers evaluated the slides at different times. This finding demonstrated the value of the semiautomatic method.

Bone histomorphometry techniques are best suited to the characterization of basic mechanisms of bone diseases. The technical error with the usual histomorphometric

variables of trabecular wall thickness, osteoid thickness, and mineral apposition rate, is about the same as other techniques of research (e.g. radio-immunoassay), between 5 and 15% (Eriksen *et al.*, 1986). For some secondary derived indices such as formation period and erosion rates, the method error is about 20 to 30% but this has to be taken in the context of changes due to metabolic bone diseases. The technical error in bone histomorphometry of about 30%, is significantly better than the error in calcium balance studies where ±70% is not unusual (Melsen *et al.*, 1989). In individual subjects, the relatively high intra-bone variance (i.e. the variance of bone histomorphometric indices between different compartments of bone) does not provide a basis for a firm diagnosis. However, bone histomorphometry is very valuable as an adjunctival method for the investigation of metabolic bone diseases.

In 1987, a new system of terminology for quantitative bone histomorphometry was adopted. The need for standardization for bone histomorphometry had been recognized for many years, and a committee chaired by Parfitt (1987) proposed the new unit system after wide consultation in this field. The new unit system has made it easier for other scientists to understand bone histomorphometry, and the new terminology is self-explanatory and descriptive. The committee recommended that the symbols should only consist of abbreviations with each symbol having only one meaning, and finally, the system should be sufficiently flexible to be applied to all surfaces and all types of bone either cortical or trabecular. All the results presented in this thesis conform to the new system standards.

*1.7 **Biochemistry of bone turnover***

Measuring bone formation and resorption by non-invasive methods is relatively easy in diseases characterized by a rapid change in the rate of bone turnover, such as Paget's disease and renal osteodystrophy (Delmas, 1984). In osteoporosis where bone mass is lost over a long period of time, non-invasive methods are less precise and most

conventional markers of bone turnover (such as serum alkaline phosphatase or osteocalcin) are usually normal. Bone is comprised of inorganic (hydroxyapatite) and organic (bone matrix) components. The organic matrix constitutes about 35% of the total weight and is composed mainly of type I collagen. The non-collagenous compartment of the bone matrix contains several proteins such as osteocalcin, osteosialoprotein, and proteoglycan (Price, 1983; Termine *et al.*, 1983). The rate of formation or degradation of bone matrix could be determined by measuring enzymatic activities such as alkaline or acid phosphatase of osteoblasts or osteoclasts, or by measuring bone matrix components released into the circulation during formation or resorption as indicated in Table 1.1.

Bone Formation	Bone Resorption
Serum:	Plasma:
- Total and bone specific alkaline phosphatase	- Tartrate-resistant acid phosphatase
- Bone Gla protein (BGP, osteocalcin)	- Free gamma carboxyglutamic acid
- Procollagen I extension peptides	- Fragments of non-collagenous protein
- Other collagenous bone proteins	
Urine:	Urine:
- Non-dialyzable hydroxyproline	- Total and dialyzable hydroxproline
	- Hydroxyproline
	- Pyridinoline crosslinks

Table 1.1 Biochemical markers of bone turnover. Adapted from Delmas (1983).

Serum alkaline phosphatase activity is the most commonly used marker of bone formation but the test for it lacks sensitivity and specificity (Delmas, 1988), and the activity increases with aging. In osteoporosis, alkaline phosphatase levels are either normal or slightly elevated and poorly correlated with bone formation rates determined by bone histomorphometry (Brown *et al.*, 1984; Podenphant *et al.*, 1987). A mineralization defect in elderly patients may be reflected in higher serum alkaline phosphatase levels.

Techniques have been developed to differentiate between liver and bone isoenzymes of alkaline phosphatase (Duda, 1988). These techniques rely on the use of differentially effective activators and inhibitors (heat, urea, and phenylalanine) and separation by electrophoresis and specific antibodies. These assays have improved the sensitivity of this marker but most of these techniques are indirect or technically difficult.

Serum bone Gla protein (BGP) is synthesized predominantly by osteoblasts and incorporated into the extracellular matrix of bone but a fraction of newly synthesized BGP is released into the circulation where it can be measured by radioimmunoassay (Lian *et al.*, 1988). BGP is recognized by antibodies directed against it, and depends on the epitope recognized by the antibody. In a high bone turnover state, some antisera may see fragments of BGP released during bone resorption in addition to the intact molecule (Gundberg *et al.*, 1986). Delmas *et al.* (1985) and Bataille *et al.* (1987) have reported that most polyclonal antibodies raised against the intact molecule do not recognize significant amounts of BGP fragments in serum. This implies that such assays are specific markers of bone formation whenever formation and resorption are uncoupled. BGP is gradually increased in the serum of females from the fourth to the tenth decade; the increased release of BGP is also accompanied by an increase in serum alkaline phophatase and urinary hydroxyproline (Delmas, 1983) that reflects an increase in bone turnover. Eastell *et al.* (1988) have compared bone histomorphometric data from young normal females to that from a group of healthy elderly women who had passed their menopause an average of 17 years previously; they found a significant increase in serum BGP in the older of the two groups. High serum BGP in post-menopausal women could be reduced to normal levels by estrogen therapy. Two independent studies (Johonson *et al.*, 1988; Selemenda *et al.*, 1987) have shown that in untreated post-menopausal females followed for 2 to 4 years, serum BGP was the best single biochemical marker to correlate highly with the spontaneous loss of BMD in the radius and lumbar spine. In vertebral osteoporosis, for

those patients with low osteoblastic activity, BGP was in the lower range of normals, but for those patients with high bone turnover, BGP was increased significantly in about one-third of the patients. Serum BGP correlated significantly with bone histomorphometric variables reflecting bone formation but not with those variables reflecting bone resorption, in both the trabecular and cortical endosteal envelops (Brown *et al.*, 1987). For patients treated with fluoride, the increase in serum BGP correlated with the increase in BMD (Pak *et al.*, 1989). Patients who used corticosteriods chronically for long times, had subnormal levels of BGP (Reid *et al.*, 1986). In another study (Garrel *et al.*, 1988), it was found that the decrease of serum BGP was dependent on the daily dose of prednisone, and could be observed after only a short time of treatment.

Procollagen I extension peptides circulate in the blood and might be useful markers for bone formation, as collagen is by far the most abundant organic component of bone matrix. Simon *et al.* (1983) have shown that a single dose of 30 mg of prednisone suppressed serum pcoll-I-C (carboxy terminal of procollagen I) without decreasing urinary hydroxyproline suggesting that circulating pcoll-I-C reflects bone formation.

Osteonectin is another bone protein that circulates in the blood (Fischer, 1987). Unfortunately, platelets which contain significant amounts of osteonectin, are secreted during thrombin stimulation which alters the sensitivity of this marker.

Tartrate-resistant acid phosphatase (TRAP) is abundant in osteoclasts and is released into the circulation. Plasma TRAP is increased in a variety of metabolic bone disorders with increased bone turnover (Stephen, 1983). TRAP is a labile enzyme which can be decreased in stored frozen plasma samples, and can originate from blood cells as it corresponds to plasma isoenzyme 5. These limitations have to be considered when using this enzyme as a bone marker.

Free hydroxyproline released during degradation of collagen cannot be reutilized in collagen synthesis. Thus, hydroxyproline presence in urine reflects the resorption of bone matrix which contains approximately half of the human collagen. Urinary hydroxyproline increases with aging in normal women when corrected for glomerular filtration rate (Delmas *et al.*, 1983). Hydroxyproline is poorly correlated with bone resorption assessed by calcium kinetics or by bone histomorphometry. On the other hand, the amount of hydroxyproline glycosides varies in bone and soft tissue and this suggests that the urinary excretion of these compounds might be a more sensitive marker of bone resorption.

Hydroxylysylpyridinoline (HP) and lysylpyridinoline (LP), are the two non-reducible pyridinium crosslinks present in the mature form of collagen. HP is widely distributed in type I collagen of bone and in type II collagen of cartilage. LP has only been found in type I collagen of bone (Black *et al.*, 1988). HP and LP can be measured by reverse phase HPLC (high pressure liquid chromatography) of a cellulose-bound extract of hydrolyzed urine. Uebelhart *et al.* (1989) have shown that urinary HP and LP represent more sensitive markers of bone resorption than hydroxyproline in Paget's disease of bone and in primary hyperparathyroidism.

One major difference between circulating bone markers and histomorphometry is that circulating bone markers reflect the cellular activity of the whole skeleton, including cortical and trabecular bone and depend on both the number and activity of bone cells. Histomorphometry, on the other hand, is limited to a small volume of bone (trabecular or cortical) and detects a specific defect at the cellular level. These differences are very important as there is growing evidence that bone mass and bone turnover in osteoporotic patients, before and during treatment, varies with skeletal site (appendicular vs axial) and type of bone (cortical vs trabecular) (Delmas,1988).

1.7.1 Inter-relationship between bone histomorphometric variables and biochemical markers of bone turnover

It is well established that histomorphometric variables of bone reflect changes in a variety of clinical states with abnormal bone and calcium metabolism. On the other hand, circulating biochemical markers have been shown to be sensitive indices of cellular activity in patients with metabolic bone diseases. However, it has been difficult to establish a significant relation between the two methods of investigation in well-defined clinical situations.

Serum alkaline phosphatase correlates well with variables of osteoblastic activity, such as the number of osteoblasts/unit trabecular boundary length, mineral apposition rate, peritrabecular fibrosis, fraction of trabecular surface covered by osteoid and cancellous bone volume (Malluche *et al.*, 1986). Serum levels of 1,25 $(OH)_2$-vit D_3 correlated well with cancellous bone volume in a group of patients with renal failure; however, this correlation was not established in normal population. Faugere *et al.* (1985) found that administration of 1,25 $(OH)_2$-vit D_3 to normal rats, and to rats with pathologic losses of bone after oophorectomy, increased trabecular bone volume.

Serum Gla protein (BGP) correlated inversely with the volume and surface of lamellar osteoid and directly with volume and surface of woven osteoid. Correlations between resorption variables (such as bone osteoclast interface and osteoclastic index) and BGP were weaker than those between osteoblastic variables and BGP (Malluche, 1984). These data suggest that BGP is an index of osteoblastic activity. The negative correlation between BGP and both volume and surface of lamellar osteoid might support that notion. The lack of correlation between BGP and variables of mineralization, such as fractional labelling of osteoid and mineralization lag time, indicates that BGP mainly affects bone formation activity and not bone mineralization. These results suggest that osteoblasts are

the most likely site of BGP production, which agrees with the hypothesis that plasma BGP arises from synthesis of BGP in bone and not from the release of BGP through bone resorption (Price *et al.*, 1981). The good correlation between BGP and histomorphometric variables of bone formation suggest a promising role for BGP as a non-invasive tool for the assessment of bone formation in patients with renal osteodystrophy and osteoporosis (Malluche *et al.*, 1986).

*1.8 **Bone and Mineral Metabolism***

Osteoporosis, like coronary artery disease, is a multifactorial disease. Nutritional as well as hormonal factors are very important in determining loss of bone mass. Calcium, in addition to other minerals, is very important to maintain a healthy skeleton. On the other hand, hormonal factors such as PTH and calcitonin are also important in regulating the mineral balance in humans.

Calcium homeostasis is established when the rate of entry of calcium into the extracellular fluid is equal to the rate of loss of calcium from it. Calcium enters the extracellular fluid by three routes: gastrointestinal absorption, mineral release from bone, and reabsorption by the kidney (Nordin, 1976). Gastrointestinal absorption contributes quantitatively to the available calcium pool but it is not involved in the short-term correction of extracellular calcium. Calcium is lost into the gastrointestinal tract through the digestive juices, into bone by mineralization and into urine. PTH and 1,25 $(OH)_2$-vit D_3 are the major humoral mediators for maintaining the extracellular calcium homeostasis; however, genetic differences have an important role in determining the end organ response to PTH and 1,25 $(OH)_2$-vit D_3. Blacks in the US, while known to have greater bone mass than whites throughout life, have recently been reported to have greater resistance to the bone resorption effect of PTH (Bell *et al.*, 1985). Blacks appear to protect their skeletons by making better use of dietary calcium.

Nutrient interaction is another important factor in determining calcium balance. Increased protein intake to twice normal, has been shown to lead to a 50% increase in urinary calcium (Heaney *et al.*, 1982), in contrast to a 30 to 35% increase in calcium levels by doubling the calcium intake. The increase in urinary calcium as a result of increased protein intake is not accompanied by a change in intestinal absorption of calcium so the end result is a net loss of calcium. Sodium intake also leads to increased urinary calcium excretion through a complicated relationship which depends on PTH, glomerular filtration rate, and degree of hydration (Robertson, 1976). In general, an increase in urinary sodium will lead to an increase in urinary calcium. Aluminum-containing antacids, by reducing phosphate absorption from the diet, decrease plasma phosphate level and thereby increase urinary calcium levels (Spencer *et al.*, 1979). Caffeine also slightly increases calcium excretion in urine (Heaney *et al.*, 1982), but a substantial intake of caffeine will lead to a faster depletion of calcium reserves. Even though urinary calcium increases linearly after acute infusion of calcium in individuals with normal kidney function (Nordin, 1976), changes in urinary calcium cannot fully account for the maintenance of extracellular calcium homeostasis.

Calcium absorption deficiency in the elderly is one of the most interesting aspects of bone research. It has been found that calcium absorption decreases with age in both men and women after age 65 (Gallagher *et al.*, 1979). The decrease in calcium absorption has been determined by calcium balance studies (Nordin, 1976), by jejunal perfusion studies (Ireland, 1973) and by radiocalcium absorption tests (Gallagher, 1979). The decrease in calcium absorption has been linked to a defect in the active calcium transport mechanism regulated mainly by the physiologically active vitamin D metabolite 1,25 $(OH)_2$-vit D_3. Impaired calcium absorption appears to be the result of two defects: increased resistance of the intestine to 1,25 $(OH)_2$-vit D_3 activity and, later in life, impaired conversion of the major circulating form of vitamin D, 25 (OH)-vit D, to 1,25 $(OH)_2$-vit D_3 (Eastell, 1988).

Plasma levels of 25 (OH)-vit D decrease with age by more than 50% (Chapuy *et al.*, 1983; Omdahl *et al.*, 1985; Tsai *et al.*, 1987). This decrease could be attributed to decreased dietary intake of vitamin D, decreased absorption of vitamin D, decreased hepatic hydroxylation to the 25-hydroxy metabolite, increased metabolic clearance, decreased epidermal synthesis, and decreased vitamin D binding protein. A study by Epstein *et al.* (1986) indicated that serum levels of 1,25 $(OH)_2$-vit D_3 increase up to age 65 and then stabilize or decrease. Because 1,25 $(OH)_2$-vit D_3 increases while active calcium absorption is decreasing, this finding suggests that resistance of the intestine to the action 1,25 $(OH)_2$-vit D_3 is increased. Armbrecht *et al.* (1980) have shown that intestinal 1,25 $(OH)_2$-vit D_3 receptors are decreased in aged rats. Data regarding malabsorption of vitamin D in the elderly are conflicting. In a study by Barraguy *et al.* (1978) tritium-labelled vitamin D absorption was found to be decreased in an elderly group of patients compared to young persons. Clemens *et al.* (1986) found no difference between elderly and younger subjects with regard to vitamin D absorption. Plasma 25 (OH)-vit D was increased in the elderly subjects after exposure to ultra-violet light (Snell *et al.*, 1978). A decrease in 7-dehydrocholesterol in skin biopsies of elderly subjects was reported by MacLaughlin *et al.* (1985). Serum 1,25 $(OH)_2$-vit D_3 decreases in the elderly, and is affected by a decrease in renal function (glomerular filtration rate), and decreased intake of calcium (Gallagher *et al.*, 1979). The most likely cause for the decrease in serum 1,25 $(OH)_2$-vit D_3 with aging is decreased renal 1 α-hydroxylase activity. Tsai *et al.* (1987) found that serum levels of 1,25 $(OH)_2$-vit D_3 in the elderly were slightly increased after 24 hours infusion with PTH. The idiopathic increase in PTH with aging is not large enough to maintain 1,25 $(OH)_2$-vit D_3 level while the decrease in 1,25 $(OH)_2$-vit D_3 will lead to a decrease in intestinal calcium absorption (Nordin *et al.*, 1979). A distinction has to be made between severe vitamin D deficiency, defined as 25 (OH)-vit D levels less than 5 ng/mL which is associated with clinical and histological osteomalacia, and less severe deficiency in the range between 5 and 30 ng/mL, associated with calcium malabsorption, but not with histologic abnormality of

bone (Parfitt, 1983; Heaney *et al.*, 1986). According to this standard, most elderly persons are vitamin D-deficient. The fall in 1,25 $(OH)_2$-vit D_3 levels with age is further complicated by an age-related decline in renal glomerular filtration rate and a decrease in the response of renal 1 α-hydroxylase to PTH. In osteoporotic subjects, Francis *et al.* (1984) found evidence that the intestinal mucosa itself becomes more resistant to the action of 1,25 $(OH)_2$-vit D_3, and this change will account for an increase in vitamin D requirements in the elderly (at or above 25 to 30 ng/mL).

The long-term effect of PTH on bone is to increase the rate of bone remodelling (Reeve, 1986). On the proximal renal tubule, PTH increases the production of 1,25 $(OH)_2$-vit D_3 and decreases phosphate reabsorption; on the distal renal tubule, PTH increases calcium reabsorption. Serum PTH increases with age. Gallagher *et al.* (1980) found a 50% increase by a "mid-region" assay, and a 30% increase with an "intact assay". Delmas *et al.*(1983) found that urinary cyclic AMP excretion was also increased with age and Marcus *et al.* (1984) have reported that nephrogenic cyclic AMP excretion was also increased. Both of these compounds are measures of the biological activity of PTH in the kidney. A more precise method for detecting small increases of PTH was described by Forero *et al.* (1987). These researchers used a renal membrane assay for adenylate cyclase after immunoextraction of serum to show that bioactive PTH was higher in the elderly. Serum intact PTH as determined by either a NH_2-terminal-specific radioimmunoassay (Young *et al.*, 1987), or by the two-site immunoradiometric assay (Eastell *et al.*, 1989) was increased by about 50% in men and women over age 65 years of age. A decrease in glomerular filtration rate, which in turn decreases plasma 1,25 $(OH)_2$-vit D_3, may stimulate PTH secretion directly by an action of the parathyroid gland or indirectly by decreasing plasma calcium. The increase in serum PTH is associated with a decrease in bone mass in a group of young persons with adenoma of the parathyroid gland, to about 10% below age-matched normals (Eastell *et al.*, 1986; Martin *et al.*, 1986); this loss is partially reversed by

the removal of the adenoma. PTH levels are increased by about 10% in post-menopausal women with vertebral fractures (Joly *et al.*, 1980). Riggs *et al.* (1981) have also reported decreased PTH levels in post-menopausal women; this could be explained by the increase in bone resorption due to decreased estrogen levels. The effect of PTH on the number of osteoclasts is not likely to account for the long-term setting of plasma calcium, since the amount of bone mineral removed by osteoclasts is balanced by an equal amount of mineral deposited into bone by osteoblasts due to the tight coupling between osteoclasts and osteoblasts (Farley and Baylink, 1982). Therefore, the mechanisms that induce calcium translocation across the bone membrane, and maintain a bone/extracellular fluid equilibrium, must be dissociated from bone turnover or bone remodelling. This implies that at least two independently regulated systems exist, one regulating bone remodelling, the other regulating the contribution of bone to extracellular calcium homeostasis. This notion is in agreement with the clinical observation that plasma calcium levels are not directly affected by the rate of bone turnover; for example, plasma calcium may be normal despite prevalence of low or high bone turnover as seen in medullary carcinoma of the thyroid (Melvin *et al.*, 1973). A similar dissociation exists in uremic patients who might present with hypocalcemia despite high bone turnover or with hypercalcemia associated with low or high bone turnover.

Extracellular fluid is saturated with respect to bone mineral and while the bone fluid compartment must be in equilibrium with the bone mineral, there is growing acceptance of the idea that a cellular envelope effectively isolates the fluid compartment of bone from that of the general extracellular fluid (Neuman *et al.*, 1977). However, there is no agreement as to the cellular mechanism by which the gradient in ionic composition is maintained across such a cellular envelope. There is evidence that PTH affects the fluxes across this bone membrane, but it is uncertain to what extent these PTH effects are mediated by alterations in serum phosphate or stimulation of 1 α-hydroxylase activity in the kidney. It is possible

that PTH and 1,25 $(OH)_2$-vit D_3 act synergistically on the translocation of calcium across the bone membrane or that the action of PTH is mediated by vitamin D metabolites. Recent findings suggest that both hormones, PTH and 1,25 $(OH)_2$-vit D_3, might be needed for maintenance of normal bone and mineral accretion rates, at least in a uremic dog model (Malluche *et al.*, 1982e)

Calcitonin works as an antiresorbant in the remodelling cycle. It has been found that patients who have had a total thyroidectomy (calcitonin deficient), have lower bone density values than normals (McDermott *et al.*, 1983). Women were found to have lower plasma levels of calcitonin than men at all ages (Deftos *et al.*, 1980). Using a highly sensitive assay method for monomeric calcitonin, (Body and Heath, 1983) could not confirm that calcitonin levels decrease with age; however, they confirmed lower values of calcitonin for women.

Phosphorus is one of the bulk phase components of bone mineral; normally there are about 2 moles of phosphate for every 3 moles of calcium (for a phosphorus:calcium ratio of 0.6) (Heaney, 1988). Phosphate deficiency limits the amounts of bone mineral that can be deposited in the mineralization process. The combination of systemic hypophosphatemia and severe local deficiency (extracellular fluid that surrounds the osteoblasts) will result in poor osteoblast function. Osteoblasts withdraw minerals (calcium + phosphorus) from the surrounding extracellular fluid and thus are subjected to low calcium/phosphorus concentrations. While osteoblasts have their own internal supply of calcium, the lack of phosphate will render them more sensitive to low phosphate levels (Lotz *et al.*, 1968). The extracellular fluid should have a molar ratio of phosphorus/calcium about 3 times higher than that found in bone (i.e. 1.8 to 2.5) to provide a sufficient supply of phosphate during bone mineralization. A phosphate/calcium ratio in the extracellular fluid of about 0.65, which is considered normal in the adult human and very close to the ratio in bone, will subject the osteoblasts to a low phosphate environment during

mineralization. Harris *et al.* (1976) have found that adult dogs which received phosphate supplements, have increased bone remodelling.

While calcium and phosphorous comprise a significant portion of the bulk of bone minerals, other minerals such as copper have shown to possess essential roles in bone metabolism by being vital components of metalloenzymes that are responsible for many cellular functions, including formation and resorption of bone (Opsahl *et al.*, 1982). Copper has an important metabolic function with its role in cross-linkage of collagen. Bone and tendon formed under conditions of copper deficiency are structurally weak. Zinc deficiency results in stunted growth and inadequate collagen formation (Heaney, 1988). Zinc is also a component of many enzymes (e.g. serum alkaline phosphatase, carbonic anhydrase) that are linked to bone formation. Manganese is essential in being a co-factor for many enzymes involved in carbohydrate metabolism (Robertson, 1976); deficiency is apparent during growth as bone matrix synthesis becomes impaired.

To summarize, several methods have been developed to investigate the quality and quantity of bone. These include densitometry, histomorphometry, biochemical markers, hormonal and mineral assays. The problem of bone mass loss in general, and osteoporosis in particular, is attracting a lot of research interest due to the recent recognition of the enormous socio-economic costs associated with these conditions. The work presented in this thesis focuses on one population of patients (total hip replacement), in whom changes in bone mass at the lumbar spine and proximal femurs, before and after the surgery are monitored by using a dual-energy x-ray absorptiometry (DEXA). Bone histomorphometry was performed on bone biopsies obtained during the hip surgery and biochemical data were also collected. We anticipated that the new, post-surgical biomechanical status in such patients would have a positive effect on the bone mass.

Chapter 2 : Experimental Design and Methodology

2.1 Overview

This study was designed to evaluate bone density changes in patients undergoing hip replacement surgery. The new biomechanical status following implantation of the hip prosthesis was expected to lead to an increase in bone mineral density (BMD) in the lumbar spine and contralateral femur over the 12-month period of study. Patients scheduled for hip replacement surgery were recruited with the collaboration of four local orthopedic surgeons; 15 patients were entered into the study protocol (Figure 2.1). Patients received two oral tetracycline bone labels before surgery in order to evaluate the dynamics of bone remodelling in a bone biopsy taken intraoperatively. BMD in lumbar spine and both femurs was measured at baseline using dual-energy x-ray bone absorptiometry (Hologic QDR-1000). Following surgery, two additional BMD measurements were made at about 4 and 12 months. Blood samples for biochemical tests were taken 1 or 2 days before surgery.

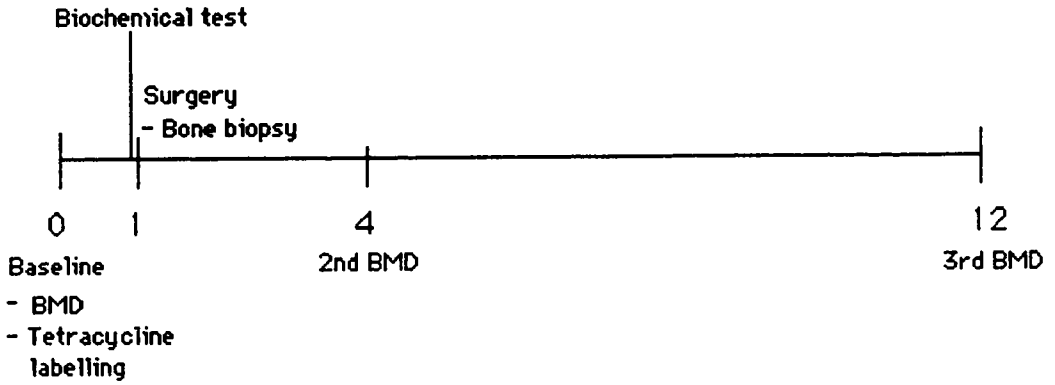

Figure 2.1 Study design. The horizontal scale depicts in months; BMD= bone mineral density

2.2 *Study design*

The study design involved one group of patients who had already been scheduled for total hip replacement. No specific control group was included in the design of this longitudinal study since each patient was considered to be his/her own control. At baseline, patients received the two tetracycline labels, had BMD measurements and upon their admission to the hospital, had blood collected for routine and special chemistry assays (PTH, 25 (OH)-vit D, and serum ionized calcium). The hip replacement surgery was performed about 1 month after the baseline measurements, and a bone biopsy was taken intraoperatively and processed for quantitative bone histomorphometric evaluation. Four months after the baseline measurements a second set of BMD measurements was made; after 12 months, the third and final BMD measurement was made.

2.3 *Sample size*

The sample size estimation for this study was based upon the changes expected in BMD in the lumbar spine and the proximal femur during a 12-month period. Previous data suggested mean changes of 3% per year in BMD for both the lumbar spine and the proximal femur (Riggs *et al.*, 1981; 1983). The standard deviation (σ) of the changes in bone mass was estimated at $\pm$ 3.0%. The sample size estimate was calculated as follows (Sokal and Rohlf, 1969):

$$n \geq 2\,(\sigma/\delta)^2\{t_{\alpha(\nu)} + t_{2(1-P)(\nu)}\}^2$$

n = sample size

δ = the smallest true difference in bone density that is desired to be detected in our study sample (estimated at 4%)

σ = true standard deviation of bone density changes of $\pm$3%

α = significance level (set at 0.05)

P = desired power that a difference will be found to be significant (if it is as small as δ) set at 0.8

ν = degrees of freedom of the sample standard deviation

Substituting the estimates into the equation, and using a 2-tailed test, a minimum sample size of 10 subjects was indicated in order to observe a clinically significant result over the one-year study period. Allowing for a 10% drop-out rate, the sample size was set at 11 subjects.

2.4 Subjects

Fifteen Caucasians (6 males [illegible] aged 48 to 80 years were entered into the study; all subjects were from Edmon[illegible]nding areas. All subjects were patients scheduled for primary total hip [illegible] surgery; all had a diagnosis of osteoarthritis (OA) with a long history of painful and limited movement of the affected hip joint. All patients were mobile at baseline, but six of ten patients completing the protocol were using canes at baseline. None of the patients had used any drug that might have an effect on the rate of bone mass loss (such as corticosteroid, thyroid hormones, etc).

2.5 Screening

Patients were contacted through lists provided by the collaborating orthopedic surgeons; those individuals who showed an interest in participating in the study were given an information sheet (Appendix A) outlining the purpose of the study, and an informed consent form (Appendix B) describing in detail the different stages of the study. Appointments for participating patients were scheduled about four weeks prior to surgery. At that time, a medical history was taken and baseline BMD measurements for the spine and both proximal femurs were made using the Hologic QDR-1000 bone densiometer. Tetracycline was provided (18 capsules of 250 mg of Oxytetracycline hydrochloride and 9 tablets of 300 mg of Declomycin; see appendix H), along with a dosing schedule for bone labelling (Appendix C). Upon admission to the hospital for surgery, blood samples were taken for PTH, 25 (OH)-vit D, and serum ionized calcium measurements, in addition to

routine serum and urine profiles.

2.6 Bone histomorphometry

The use of tetracyclines as markers of bone formation has been improved by giving them in an appropriate sequential manner. Tetracycline is taken up by all the bone surfaces following its administration but only those areas of bone matrix under active mineralization at the time of administration will retain the label. Different types of tetracycline are usually used as each compound fluoresces differently when viewed with ultraviolet light. For example: Declomycin has a typical golden-yellowish fluorescence and tetracycline hydrochloride fluoresces greenish-yellow (Malluche *et al.*, 1986). These color differences help to discriminate a single label from two merging labels resulting from low bone formation. In this study the tetracyclines were administered orally and patients were instructed about the side effects of the drug, and given a detailed explanation of the labelling procedure (Appendix E).

The following protocol was employed in this study:
The first label, given on day 1 and continued until day 3 inclusive, was Oxytetracycline hydrochloride (two 250 mg capsules, t.i.d). This dosage was followed by 11 days without tetracycline; the second label, given on days 15, 16 and 17, was Declomycin (one 300 mg tablet, t.i.d). The labelling protocol was completed 3 days prior to surgery.

Bone biopsies were taken intraoperatively. A trabecular bone core (0.8 mm diameter and 2 cm long) was taken from the intertrochanteric region using a Mayo trephine after osteotomy of the femoral neck (Figure 2.2). Initially, an electric drill biopsy needle was used to take the bone sample but this method produced too much sample damage for quantitative bone histomorphometry. Bone biopsies were immediately placed in 70% ethanol to await additional preparation steps.

2.6.1 *Fixation and dehydration of bone biopsy*

The purpose of fixation is to preserve the bone tissue constituents and bone cells in a condition as close to *in vivo* status as possible. Bone biopsy specimens were fixed in 70% ethanol for 24 h; ethanol is a precipitant fixative that precipitates the cytoplasmic

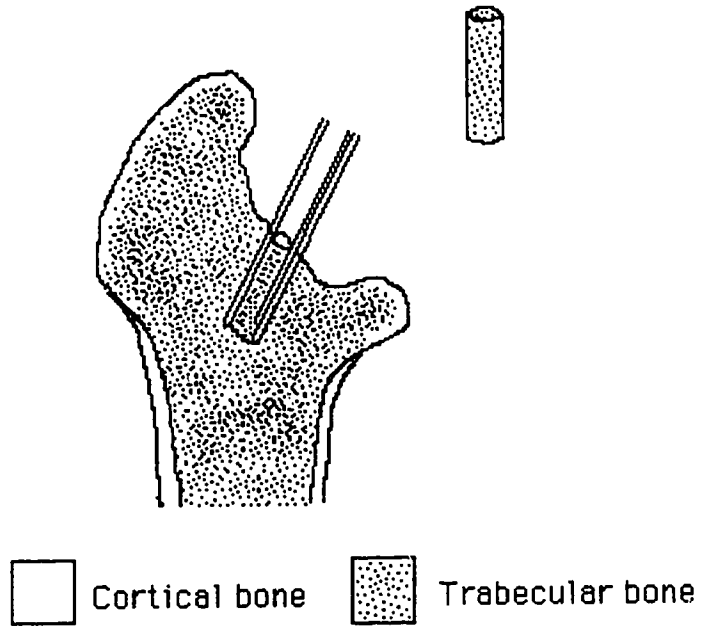

Figure 2.2 A diagram of the technique used in an intertrochanteric bone biopsy

proteins. Ethanol was also chosen as it does not wash out the tetracycline labels. Prior to embedding, the bone biopsies were dehydrated thoroughly because the plastic monomers used for subsequent embedding are not miscible with water. The bone biopsies were kept in a 50% acetone/water solution for 24 h, then in a 70% acetone/water for 24 h, and finally in 100% acetone for 24 hours.

2.6.2 *Embedding*

The major objective of embedding the bone tissue samples was to have a medium in

which the final degree of hardness approximated the hardness of the bone. A nonflammable and nontoxic embedding substance was sought to penetrate the bone specimen without causing significant artifacts such as bubbles. Methylmethacrylate is commonly used as an embedding medium (Baron *et al.*, 1983) and this has the advantage of being able to accept a variety of stains (Goldner trichome and von Kossa, etc). However, due to a variety of technical difficulties in our laboratory, Spurr's embedding solution (Spurr, 1969) was used in the present study, and this medium limited the choice of stains to either Toludine blue or Giemsa.

Spurr's embedding solution is made by mixing the following agents :

Vinylcyclohexene dioxide (epoxy resin) ERL 4206	18.4 mL
Diglycidyl ether polpropyleneglycol (flexinilizer) DER 736	9.2 mL
Nonenyl succinic anhydride (hardener) NSA	51.0 mL
Diemethylaminoethanol DMAE (accelerator) S-1	0.6 mL

The more DER that is used, the softer the block. The fixed, dehydrated bone biopsy was transferred from the 100% acetone solution to a clean glass bottle containing a 50/50 solution of Spurr's/acetone for 24 hours. The specimen was then transferred to a 70/30 Spurr's/acetone for another 24 hours and finally, to a 100% Spurr's for 24 hours. Bone biopsies were then placed in Peel-A-Way plastic moulds and a 100% Spurr's was added to totally cover the bone, and the samples were placed in an oven. Slow, stepwise polymerization was required and was achieved by gradually increasing the temperature by 15 °C to 20 °C every 24 hours to a maximum of 60 °C until the block was fully hardened. The time needed for the polymerization process varied according to the amount of DER used. On average, 3 days of polymerization was needed to obtain a fully hardened block clear of air bubbles. Before removing the bone block from the plastic mould, an identification label was affixed using few drops of Spurr's, and the block was placed in the oven for 24 hours.

2.6.3 Sectioning

Special microtomes, equipped with diamond or carbide-edged knives, are required for sectioning embedded, undecalcified bone samples. A sledge Jung microtome model K was used for this study. During sectioning, the block was kept moist with ethanol. Cutting bone sections required skill to get consistently good results. Ten-micron thick sections were cut for staining with Toludine blue and 20-micron thick sections were cut but not stained. Sections were kept floating in 70% ethanol until staining.

2.6.4 Staining

Numerous staining procedures are available for bone (Baron *et al.*, 1983), however, this discussion is limited to the stain used in this work (Toludine blue). Toludine blue provides an excellent view of cement lines, osteoid seams and the calcification front. Ten-micron bone biopsy sections were put through one change of distilled water for a few minutes, stained in filtered 1% Toludine blue in veronal acetate buffer (pH 7.0) for 45 minutes, and then rinsed in 3 changes of distilled water.

Stock Veronal Acetate Buffer:

Na Acetate	1.943 g
Na diethylbarbiturate	2.943 g
Distilled water	100 mL

For pH 7.0

stock veronal acetate buffer	5.0 mL
0.1 N Hydrochloric acid	6.0 mL
Distilled water	14 mL

then check and bring pH to 7.0

The expected results of staining with Toludine blue (Baron *et al.*, 1983) are as follows:

Osteoid seams	light blue

Cement lines	bright purple
Calcification front	dark, granular purple
Cell nuclei	dark purple
Cell cytoplasm	light blue

2.6.5 Mounting

When staining was complete, sections were floated onto glass slides from the last rinse of distilled water. The slide was kept upright and the section allowed to dry. One drop of Permount was placed on the slide, another drop on the section, and the cover slip pressed on top to allow the Permount to spread and air bubbles to be removed. The slide was then wrapped with polyurethane, sandwiched between two blank slides, and clamped over the area of the bone section to ensure that all the air bubbles were eliminated. Slides were then placed in a 60 °C oven for 2 to 3 days to dry, after which they were rinsed in xylene to remove the extra Permount, and marked with an identification number.

2.6.6 Evaluation

Three approaches are commonly used for histomorphometric evaluation of bone: manual (point counting techniques); semi-automatic computerized techniques; fully automatic computerized image analysis. In our study, we used both manual and semi-automatic techniques.

a) Manual technique

The manual technique used in our study was limited to the measurements of distances between the two tetracycline labels in the unstained bone sections as viewed by a fluorescent microscopy. A stage micrometer was used to calibrate the eye piece micrometer under 100 x magnification. Inter-label distance was calculated by averaging the inner (i) and outer (o) inter-label distances (Figure 2.3) at 4 different, equally distant locations perpendicular to the tetracycline labels.

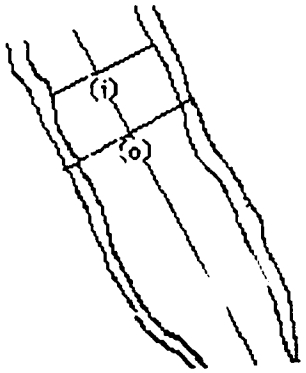

Figure 2.3 Technique for measuring the inter-label distance between the two tetracyclines labelling fronts in bone (i) Inner-label distance (o) Outer-label distance

b) Semiautomatic technique:

The semi-automatic approach to histomorphometric analysis was pioneered by Malluche (1982). The method uses a microscope equipped with a drawing tube through which the image-digitizing platen, positioned underneath the drawing tube, is projected over the optical field. The digitizing platen is connected to a computer for data acquisition and analysis. For this study, we assembled a semi-automatic image analysis system (Figure 2.4) using a microscope equipped with a TV camera which was interfaced (Data Translation Inc, MA) to a Macintosh IIcx computer. A second color monitor was attached directly to the camera to facilitate image inspection before capture. The image size was 640 X 480 pixels and after digitization images were stored in disk files. Image analysis software (IMAGE 1.22), was then used for image evaluation. All the structures to be measured were selected by a moving cursor; measurement of area, perimeter, distances, and cell counting were easily performed. Computer measurements are usually in pixels unless calibrated against other measuring units. To calibrate our system, a slide grid was digitized under 10 x magnification, and the slide image was used to calibrate the image system as follows; 0.1 mm was equivalent to 810 pixels, the size of each field was 0.468 mm^2, and the pixel size was 1.523 micron. Primary data were kept in files and transferred

later to another software program (Appendix F) which was written by the author and runs on the Macintosh IIcx computer (see sample run, Appendix G). The program transferred all the primary data to the histomorphometric variables required. Images were stored on floppy disks or printed when permanent records were required.

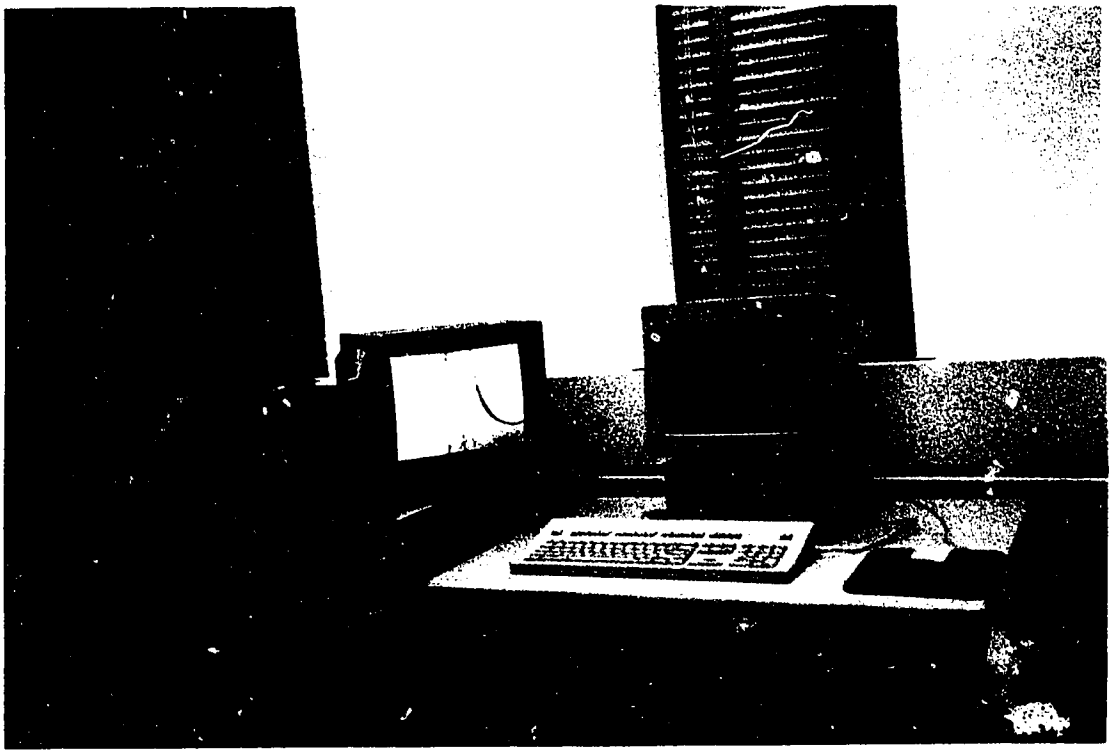

Figure 2.4 The semi-automatic system used in the evaluation of the bone sections

2.6.7 Histomorphometric variables

For each biopsy three sections were stained with Toludine blue for evaluating static remodelling variables, and one section was processed unstained for evaluating the dynamic variables. For the static variables, 10 fields per section were selected in random order, corresponding to a total evaluated surface area of 4.68 mm^2 per section and 14.04 mm^2 per biopsy (total for the 3 sections).

The variables evaluated were either static or dynamic:

Static variables

1) Bone volume (Cn-BV%TV), cancellous-bone volume % tissue volume,was expressed as a percentage of total bone tissue volume occupied by the trabeculae. In a two-dimensional analysis, the corresponding measurement, BAr/TAr (bone area/tissue area), was measured under 10 x magnification by selecting the two areas using the electronic cursor.

2) Osteoid volume (Cn-OV%BV), cancellous-osteoid volume % bone volume, was expressed as a percentage of total cancellous bone volume occupied by osteoid. In a two-dimensional analysis, the corresponding measurement, OAr/BAr (osteoid area/bone area), was measured under 10 x magnification by selecting the two areas using the electronic cursor.

3) Osteoid surface (Cn-OS%BS), cancellous-osteoid surface % bone surface, was expressed as a percentage of total cancellous bone surface covered by osteoid. In a two-dimensional analysis, the corresponding measurement, OPm/BPm (osteoid perimeter /bone perimeter), was measured under 10 x magnification by selecting the two areas and measuring the perimeter.

4) Trabecular thickness (Cn-TbTh), cancellous-trabecular thickness, was expressed in microns and corresponds to trabecular width (Cn-TbWi) in a two-dimensional analysis. A correction factor of $\pi/4$ was applied to the mean value to correct for obliquity. This variable was measured directly under 10 x magnification at four equally distant locations perpendicular to an oriented middle direction in the trabeculae and the results were averaged.

Dynamic variables

Mineral apposition rate (MAR) was expressed in microns per day, and is the rate of progression of the tetracycline labelled calcification front. A correction factor of $\pi/4$ was applied to the mean value to correct for obliquity. This parameter was measured by calculating the inner and outer distance between the two labels at four equally distant locations and averaging the results. Only sections with distinct labels were evaluated.

2.7 Bone Mass Measurement

The capability of precisely measuring bone mineral density (BMD), was the key element in this study design. Quantitative Computed Tomography (QCT) and Dual-Photon Absorptiometry (DPA) have been widely used for clinical bone mass measurements but they have several disadvantages. QCT involves a patient radiation exposure of at least 250 to 300 mrem (Cann, 1981), and a scanning time of about 30 minutes which causes problems for elderly patients. DPA is limited by its inability to provide a selective measurement of vertebral body BMD due to the low resolution of its image, and also by the relatively low specific activity and short half-life of its radioactive source (^{135}Gd), factors which decrease long-term precision of this technique and increase operating costs. Dual-energy x-ray absorptiometry (DEXA) is similar in many aspects to DPA, however, an x-ray source rather than a radioisotope source, facilitates precise and accurate BMD measurements in the spine that can be completed in less than 10 minutes with negligible radiation exposure (Sartoris *et al.*, 1989).

A Hologic QDR-1000 (Hologic Inc., Waltham, MA) bone densitometry system, depicted in Figure 2.5, was used in this study to measure BMD in the lumbar spine and proximal femur. The technique is based upon the detection of a collimated x-ray beam after transmission through the patient. An x-ray tube provides alternating pulses at 70 Kvp and 140 Kvp (generating effective dual-energy photons at 43 and 110 KeV respectively).

These beams pass through a calibration disk containing several x-ray absorbing materials (epoxy-resin-based equivalents to bone and soft tissue), prior to passing through the patient. The calibration disk provides a pixel by pixel calibration and serves as an internal

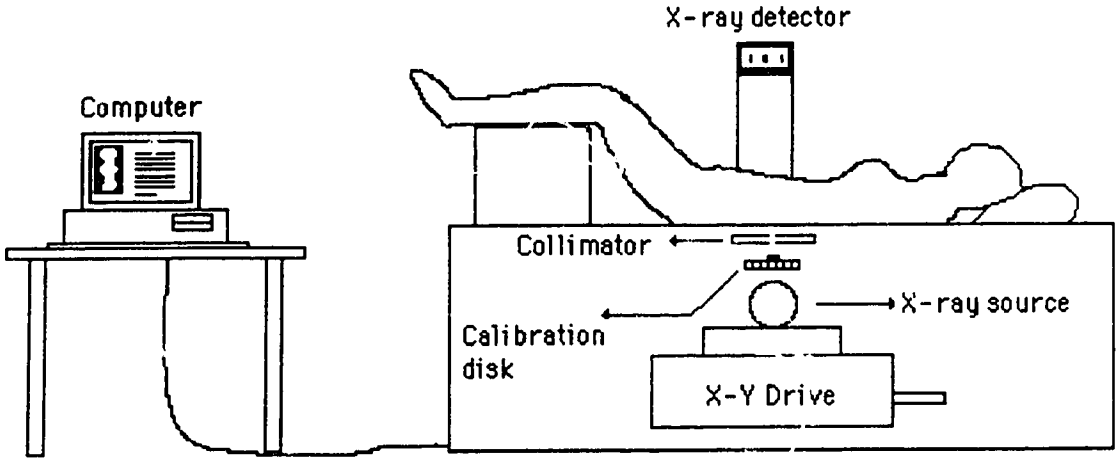

Figure 2.5 Diagram of Hologic QDR-1000

reference system. Comparison of attenuation by the patient with that of the calibration disk and patient then yields a measurement of patient BMD. This continuous self-calibration and internal reference compensates for any fluctuation in energy or flux of the x-ray tube output and for any detector variations, and helps to compensate for beam hardening (shift to higher average energy of the x-ray beam as it travels through the object). Energy discrimination is not used in the detector because only one energy is used at any one time. The object is scanned by the source and detector moving together in a rectilinear pattern. A high resolution image of the object is displayed on the computer monitor, and the region of interest (ROI) selected by electronic cursors. The total mineral content is then divided by the area of interest and the results expressed as g/cm^2 (Pacifici *et al.*, 1988). The technique is capable of measuring different body regions (vertebrae, proximal femur, wrist etc). In our study, we also used the "metal removal" version of the computer software which

allowed us to exclude the region of the hip prosthesis from the calculation of bone mineral density.

DEXA measurements correlate highly (r= 0.895) with DPA but DPA yields consistently higher (6.8%) values (Pacifici *et al.*, 1988). Cullem *et al.* (1989) reported DEXA BMD measurements *in vivo* with a precision of better than 1% in normal volunteers. The *in vitro* precision of DEXA is reported at 0.41%, and the short-term *in vivo* precision is 1% (Pacifici *et al.*, 1988). Wahner *et al.* (1989) reported *in vivo* precision of 1.3% for the lumbar spine and 2.2% for the femur neck region. Solsman *et al.*, (1989) reported *in vivo* precision of 1% for the lumbar spine and 1.8% for the femur neck. In a cadaver study by Charles *et al.* (1990), a high correlation (r= 0.96, p<0.0001) was found between vertebral ash weight and bone mineral content (BMC) determined by DEXA. A key comparison was also the high correlation (r= 0.85, p<0.0001) between measured vertebral volume in cm^3 and frontal projected area in cm^2 determined by DEXA.

2.7.1 Regions of interest

In our study, regions of interest (ROI) were as follows:

i) Four lumbar vertebrae (L_1 - L_4).

ii) In the proximal femur (Figure 2.6).

a- Trochanteric region.

b- Inter-trochanteric region.

c- Ward's triangle.

d- Neck of femur.

e- Greater trochanter.

*2.8 **Biochemical tests***

A blood profile was obtained for all the patients upon admission for surgery. In addition to the routine biochemical tests, PTH, 25 (OH)-vit D, and serum ionized calcium,

were also obtained. For the PTH assay, the method used varied between the two hospitals at which surgery was performed; 4/10 patients had a (carboxy) C-terminal assay and for

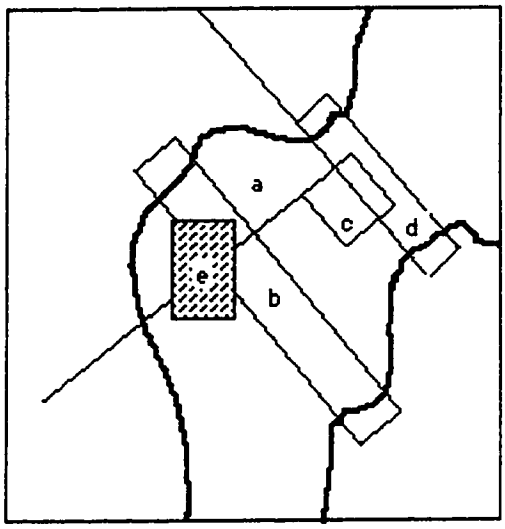

Figure 2.6 Proximal femur regions for DEXA measurements
(Symbols are described in the text)

6/10 patients, the (amino) N-terminal assays were used. Unfortunately, these differences in PTH assays were not identified until later in the study when PTH results were received and thus four PTH values were excluded from the analysis of data. Assays for 25(OH)-vit D, however, were performed in one laboratory and these data are included in this report. Biochemical data were later correlated to both the bone histomorphometric variables and the BMD measurements in the analysis.

*2.9 **Data editing***

All data points were used in the analysis except those BMD values that were obtained using the metal removal version of the software. An explanation is provided, where appropriate, concerning deletion of data points.

2.10 ***Data analysis***

All data were organized in a spread sheet (Microsoft Excel 2.2) on a Macintosh computer. These data files were later exported to a statistical analysis program (Stat View SE + Graphics). Data analysis included both descriptive and inferential statistics; drop outs were not included in the analysis. The level of significance was $\alpha = 0.5$ with power ($P=1-\beta$) of 0.80. Analysis of variance (ANOVA), Student's t-test, and Pearson correlation were employed in the study evaluation. The difference in bone mass at different sites over 3 longitudinal BMD measurements was tested using a repeated-measures ANOVA and Student's t-test. The bone mass change between baseline and the subsequent two measurements, was also calculated as a percentage difference, and a 2-tailed, paired Student's t-test was used to test for significant difference. The significance of the percentage differences between the 3 longitudinal BMD measurements was also tested for a difference from zero ($\mu=0$) using a 2-tailed one-sample t-test. Bone histomorphometric variables were correlated with BMD measurements at approximately the same site and displayed in a Pearson correlation matrix. Biochemical data were also correlated in this way to both BMD measurements and bone histomorphometric variables.

Chapter 3: Results

Twenty-one patients were initially recruited to this 12-month longitudinal study of bone density changes in the lumbar spine and proximal femur following total hip replacement. Six patients withdrew from the study after the pre-operative baseline measurement. Reasons for withdrawal included: post-operative complications (1), cancellation of surgery (1), leaving country post-operatively (1), and personal reasons unrelated to the protocol (3). Fifteen patients had post-operative measurements, but only 10 (7 women + 3 men) completed the 12-month protocol. Anthropometric characteristics of all patients at baseline, and of the patients completing the protocol are given in Table 3.1.

Table 3.1 **Anthropometric characteristics of study subjects**

	Patients at baseline (n=21)		Patients completing protocol (n=10)	
	Male	**Female**	**Male**	**Female**
n =	8	13	3	7
Age (yrs)	61.0 ± 13.0 *	71.0 ± 8.0	62.0 ± 5.0	69.0 ± 10.0
Height (cm)	172.0 ± 4.0	162.0 ± 5.0	175.0 ± 3.0	162.0 ± 6.0
Weight (Kg)	81.0 ± 13.0	68.0 ± 9.0	84.0 ± 10.5	67.0 ± 9.0

* values represent mean ± SD

There were no significant differences in anthropometric characteristics between individuals completing the protocol and those withdrawing. Patient recruitment occurred over a nine-month period. Bone mass measurement intervals varied somewhat between patients and depended upon the availability and mobility of each patient. Three bone mass

measurements (BMD) were made: at baseline, and subsequently at average intervals of 4.3 ± 0.8 months and 12.2 ± 0.7 months after surgery. Measurements were made in each subject for the lumbar spine (L_1 to L_4), the proximal femur (neck, trochanteric, intertrochanteric, and Ward's triangle), and the contralateral proximal femur (i.e the non-operated side). After surgery, BMD measurements in the lumbar spine and contralateral femur were made as before, but for the operative side, BMD in the greater trochanter only was measured by selecting a region of interest (ROI) and using the metal removal version of the analysis software.

In addition to the BMD measurements, bone histomorphometry was also performed on bone specimens obtained intraoperatively. Bone histomorphometric primary data (areas, distances, and perimeters) were collected for each patient first as averages of 10 fields per bone section and then as averages of 3 sections per specimen. These primary data were then used to calculate the bone histomorphometric variables (BV/TV, OV/BV, OS/BS, TbTh, and MAR).

*3.1 **Baseline data***

BMD measurements

Baseline BMD values (g/cm^2) for the lumbar spine and proximal femurs are shown in Table 3.2. For the contralateral femur (non-operated side), two cases were deleted as those patients had a contralateral hip replacement during the course of this study.

Bone histomorphometry

Mean ± standard deviation of bone histomorphometric variables for the 10 patients completing the protocol are shown in Table 3.3. For mineral apposition rate (MAR), five cases were excluded due to the difficulty of distinguishing between the two tetracycline labels, otherwise, complete data was available for the 10 patients completing the protocol.

Table 3.2 **BMD (g/cm^2) for the lumbar spine and proximal femur in 10 patients at baseline**

Lumbar Spine		Proximal femur (n=10)			Contralateral femur *
			Right	Left	
L1	0.96 ± 0.21**	Neck	0.78 ± 0.21	0.80 ± 0.19	0.71 ± 0.11
L2	1.01 ± 0.20	Troch	0.60 ± 0.19	0.62 ± 0.16	0.58 ± 0.17
L3	1.10 ± 0.19	Inter	1.00 ± 0.32	1.02 ± 0.25	0.93 ± 0.23
L4	1.14 ± 0.18	Ward's	0.56 ± 0.25	0.59 ± 0.19	0.48 ± 0.14

* (n = 8)
** values represent mean ± SD

Table 3.3 **Selected bone histomorphometric variables for 10 patients at baseline**

Parameter (unit)	values**
BV/TV (%)	25.0 ± 10.0
OV/BV (%)	2.6 ± 1.1
OS/BS (%)	38.0 ± 10.0
TbTh (microns)	157.0 ± 50.0
MAR (microns/day)*	1.0 ± 0.2

* (n = 5)
** values represent mean ± SD
BV= Bone Volume, TV= Tissue Volume, OV= Osteoid Volume, OS= Osteoid Surface, BS= Trabecular Bone Surface, TbTh= Trabecular thickness, and MAR= Mineral Apposition Rate

Biochemical Data

Measurements were made for serum alkaline phosphatase, 25(OH)-vit D, serum ionized calcium, and total serum protein, and these variables were included in the analysis. PTH was measured in all 10 patients but 4/10 data points were excluded since a different portion of the PTH molecule was measured in the radioimmunoassay. Descriptive statistics for the biochemical variables are given in Table 3.4.

Table 3.4 **Measured values for selected biochemical variables**

	values	Range	Normal range
Alkaline Phosphatase	69.10 ± 22.01*	39-111	30-100 U/L
25(OH)-vit D	75.50 ± 24.82	44-136	40-192 nmol/L
Serum ionized Calcium	2.33 ± 0.13	2.00-2.45	2.00-2.60 mmol/L
Total Proteins	70.70 ± 12.67	48-98	60-80 g/L
PTH **	21.23 ± 5.03	15.6-29.8	10-65 ng/L

* values represent mean ± SD
** (n = 6) since a different assay was used in the measurement for the other 4 patients

3.2 Analysis of baseline data

Correlations between the bone histomorphometric variables and the BMD values, at approximately the same site at baseline, are shown in Table 3.5. All of the bone histomorphometric variables were included in the correlation analysis and no significant correlation was found.

Table 3.5 **Correlation coefficient* between bone histomorphometric and BMD variables at baseline in 10 patients completing the protocol**

	BV/TV	OV/BV	OS/BS	TbTh	MAR*
Neck BMD	0.21	-0.34	-0.21	0.25	-0.18
Troch BMD	0.22	-0.29	-0.09	0.29	-0.29
Inter BMD	0.28	-0.22	-0.01	0.34	-0.23
Ward's BMD	0.11	-0.42	-0.29	0.17	-0.25

* Pearson correlation coefficient
** (n=5) since MAR could not be determined in the other five patients

Bone histomorphometric variables were also compared with the biochemical data (Table 3.6) and again, no significant correlations were revealed in this analysis of data.

Table 3.6 **Correlation coefficient* between bone histomorphometric and biochemical variables at baseline in 10 patients completing the protocol**

	BV/TV	OV/BV	OS/BS	TbTh	MAR**
Alkaline Phosphatase	-0.28	0.16	-0.11	-0.28	0.30
25(OH)-vit D	0.27	-0.23	-0.22	0.27	-0.12
Serum Calcium	0.18	0.01	0.16	0.20	-0.[illegible]2
Total Proteins	-0.13	-0.04	0.09	-0.12	-0.29
PTH ***	-0.3	-0.28	-0.3	-0.23	†

* Pearson correlation coefficient
** (n=5)
*** (n = 6)
† (n =1, and correlation could not be established)

BMD measurements in the proximal femur regions (neck, trochanter, intertrochanteric region, and Ward's triangle) were also compared with the biochemical data (Table 3.7) and several significant correlations were found.

Table 3.7 **Correlation coefficient* between BMD and biochemical variables at baseline in 10 patients completing the protocol**

	Neck BMD	Troch BMD	Inter BMD	Ward's BMD
Alkaline Phosphatase	-0.18	-0.61	-0.60	-0.24
25(OH)-vit D	0.70	0.59	0.59	0.75
Serum Calcium	0.32	0.51	0.59	0.51
Total Proteins	0.08	-0.06	0.11	0.21
PTH **	0.30	0.17	0.33	0.56

* Pearson correlation coefficient
** (n=6)
*** ($r=0.63$, $p< 0.05$)

BMD changes over time

Longitudinal changes in spinal BMD for the 10 patients completing the protocol are shown in Figures 3.1 and 3.2. Four of 10 patients showed a trend of increasing BMD after the second measurement at 4 months (statistically significant, $p< 0.01$) (Figure 3.1).

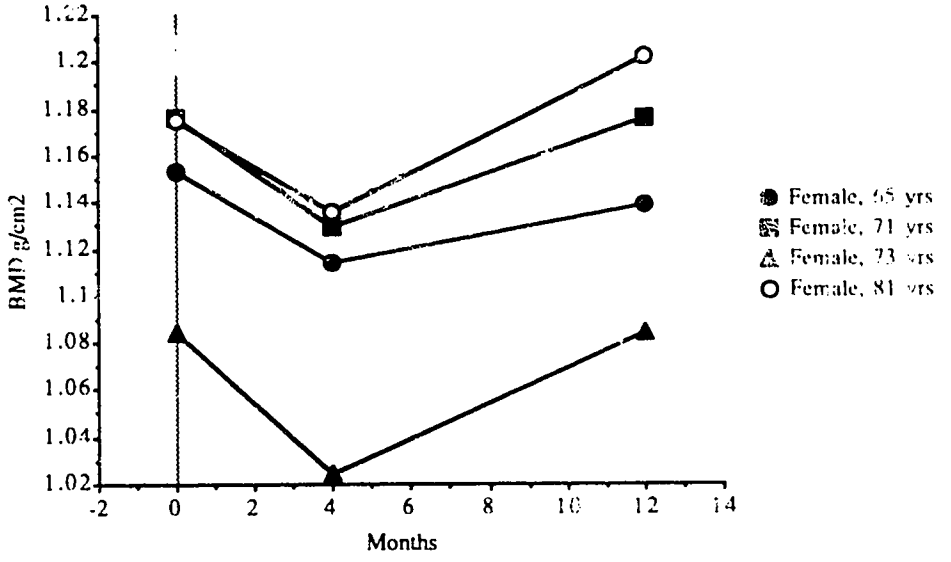

Figure 3.1 Spinal BMD changes over the 12 months follow-up in 4/10 patients

Longitudinal spinal BMD changes in the other 6 patients were not significant (Figure 3.2).

3.4 Analysis of the BMD changes

The main objective of the statistical analysis for this part of the study was to determine if the observed BMD changes were statistically significant. BMD values at baseline and at 4 and 12 months were compared using a paired, 2-tailed Student's t-test. For the lumbar spine, BMD values for all patients (n=10) were included in the analysis; however, for the proximal femur (right or left side regardless of surgery), six cases were

excluded because of surgical procedures which extended into the bone measurement region

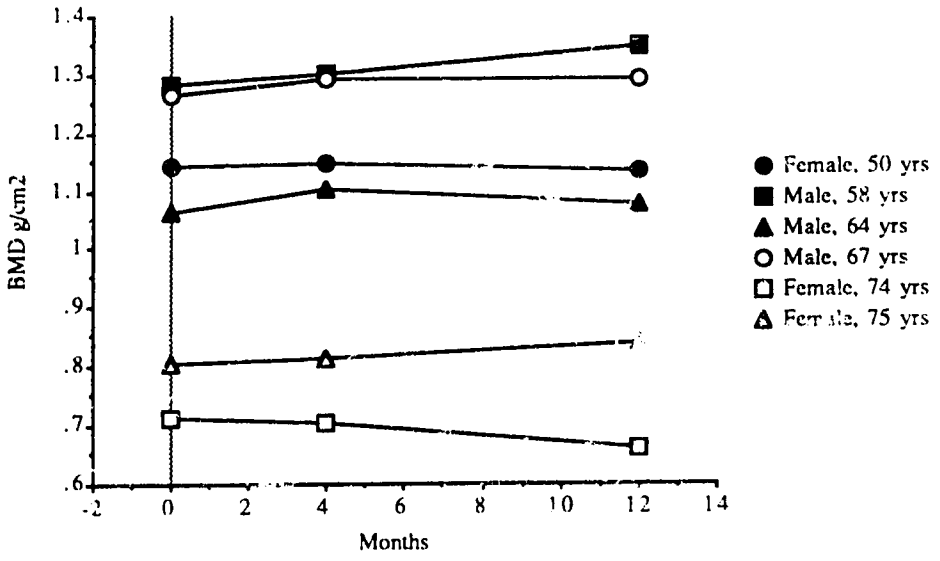

Figure 3.2 Spinal BMD changes over the 12 months follow-up in 6/10 patients

and thus distorted the analysis. For the contralateral femur (non-operative side), two cases were deleted because of contralateral hip replacement during the course of this study. Post-operatively, BMD measurements in the greater trochanter on the operative side were made using the metal removal version of the analysis software.

For the lumbar spine and the contralateral femur, most BMD measurements made at 4 and 12 months were not significantly different from baseline. However, for the contralateral femur (Ward's triangle), a significant change between baseline and 4 months was detected ($p < 0.02$). For the greater trochanter (operative side), BMD changes were not significantly different between 4 and 12 months measurements.

Percentage changes in BMD between baseline, 4 months, and 12 months were also calculated (Table 3.8) and compared using a 2-tailed, paired Student's t-test. For the

lumbar spine, the percentage differences were not significantly different over the 12-month study period. For the the left intertrochanteric region, however, the percentage change in BMD between baseline and 4 months, and baseline and 12 months was significantly different ($p < 0.0001$); the percentage change between baseline and 4 months and between 4 and 12 months of the same region was also significantly different ($p < 0.0001$).

Table 3.8 **Percentage change in BMD for the lumbar spine and several regions in both femurs**

Region	Baseline to 4 months	4 to 12 months	Baseline to 12 months
L_1	-0.5 ± 5.0	1.0 ± 12.0	1.0 ± 13.0
L_2	-0.2 ± 4.0	2.0 ± 12.0	0.9 ± 9.5
L_3	-1.0 ± 3.0	2.0 ± 10.0	1.0 ± 10.0
L_4	-0.3 ± 5.0	2.0 ± 7.0	2.0 ± 8.0
Rt Neck	-4.0 ± 6.0	2.4 ± 8.3	-1.0 ± 5.0
Rt Trochanteric	-0.3 ± 2.0	-1.7 ± 3.1	-1.0 ± 4.0
Rt Intertrochanteric	-3.0 ± 5.0	2.2 ± 3.6	-1.0 ± 5.0
Rt Ward's triangle	6.5 ± 4.0	-0.1 ± 12.3	6.7 ± 11.5
Lt Neck	-3.7 ± 5.0	-1.0 ± 3.0	-4.8 ± 4.7
Lt Trochanteric	1.0 ± 3.0	-3.8 ± 6.0	-3.0 ± 3.4
Lt Intertrochanteric	6.1 ± 5.9	-45.0 ± 4.0*	-41.5 ± 7.0*
Lt Ward's triangle	7.0 ± 8.0	-3.4 ± 2.7	3.0 ± 8.0
Contra Neck	-3.4 ± 5.0	0.7 ± 6.0	-3.0 ± 5.0
Contra Troch	0.6 ± 2.6	-2.8 ± 4.6	-2.2 ± 3.3
Contra Inter	1.0 ± 7.0	-1.2 ± 5.3	0.04 ± 6.85
Contra Ward's	7.0 ± 6.3	-2.0 ± 8.0	5.0 ± 9.0

* Percentage change for this time period is significantly different from the change for the baseline to the 4-month time period (Student's paired t-test, $p<0.0001$)

Percentage changes between the 3 BMD measurements were also compared to zero change in a one-sample, 2-tailed Student's t-test. For the lumbar spine, BMD changes were not significantly different from zero; in contrast, for the proximal femur, some percentage changes were significantly different from zero. In the left intertrochanteric region, the difference between baseline and 12 months, and between 4 months and 12 months was significantly different from zero ($p < 0.001$). In the right Ward's triangle, the difference between baseline and 4 months was significantly different ($p < 0.02$). For the contralateral femur, a significant difference from zero was detected between baseline and 4 months ($p < 0.02$).

Chapter 4: Discussion

The loss of bone mass in the elderly is a multifactorial phenomenon. For example, estrogen deficiency in post-menopausal women is a factor known to be related to an increased rate of bone loss. Immobilization or limited usage of certain limbs due to joint problems is also a contributing factor to the bone loss. While mechanical usage of bone helps to prevent bone loss, bone is influenced negatively by disuse which might occur as a result of fracture, fixation, and implants or prosthetic joint replacement (Brown, 1985). Inactivity, and absence of strain transmitted to bone or even the absence of tension applied to the bones through muscular movements, are also thought to produce disuse osteopenia (Wolff, 1982). Hence, exercise has been recommended to reverse bone loss in post-menopausal women. Dalsky (1988) have reported an increase of 5.2% in spinal bone mineral content (BMC) in a group of post-menopausal women who were enrolled in a nine-month exercise program, in comparison to a 1.4% loss of BMC in the control group. In our study, 12 months after total hip replacement surgery, there was no significant change in spinal bone mineral density (BMD). As for the proximal femur, few significant changes in BMD were seen; however, deletion of cases with missing values greatly reduced the power of the analysis and we could not draw reliable conclusions from these limited data.

Correlation coefficients between bone histomorphometric and BMD variables at approximately the same site were not significant in our study. Fox *et al.* (1989) reported no significant correlation between BMD and bone histomorphometric variables. In the Fox *et al.* study the sample size was larger and they used a transiliac biopsy to obtain the bone histomorphometric variables and correlated those variables to BMD measured in the lumbar spine and the proximal femur. However, the correlation coefficients obtained in our study were similar to those obtained by Fox *et al.* leading us to draw the same conclusion that the

two methods of investigation (bone density measurement and bone histomorphometry) are measuring two different aspects of bone behavior and that is why we cannot get significant correlation between them.

Correlations between bone histomorphometric and biochemical data reported in our results were different from the findings of Malluche *et al.*(1986). While Malluche showed significant correlations, our correlations were weaker and not significant. For example, the correlation between mineral apposition rate (MAR) and serum alkaline phosphatase in our study was r=0.3 (p< 0.63), while Malluche *et al.* reported r=0.72 (p< 0.001). In our work, a weak correlation (r=0.27, p< 0.456) was found between 25(OH)-vit D level and trabecular bone volume. Faugere *et al.* (1985) have reported a correlation (r=0.73, p< 0.0[illegible]) between 1,25$(OH)_2$-vit D_3 and trabecular bone volume. For serum ionized ca[illegible] findings were also inconsistent with the data of Malluche *et al.*(1986) since they found a negative relationship between serum ionized calcium and all the osteoblastic indices, while our data indicated a positive relationship. A possible reason for the differences between our data and those of Malluche *et al.* may relate to the fact that his patient population were in renal failure, in whom serum calcium levels fall, and increasing amounts of osteoid accumulate in the skeleton.

Correlations between proximal femur BMD and biochemical data in our study were significant; 25(OH)-vit D correlated strongly with both Ward's triangle BMD (r=0.75, p< 0.01) and neck BMD (r=0.7, p< 0.05). These findings are different from the findings of Tsai *et al.* (1987) that found insignificant correlation between 25(OH)-vit D and both spinal and distal radius BMD. In our study, an insignificant correlation was also found between spinal BMD and 25(OH)-vit D, however, for the proximal femur BMD, a strong correlation was seen. These findings are not unexpected given the fact that calcium and vitamin D are very important requirements for maintaining the bone mass (Heaney, 1988). However, we have to be cautious in the interpretation of these results since the biochemical

data reflect the activity of the whole skeleton while the BMD measurements are site-specific. The same argument could be made concerning the correlation between bone histomorphometry and biochemical markers of bone activity. Also, we have to take into account the fact that the changes in bone mass in osteoporosis occur over a long period of time in comparison with other metabolic bone diseases where dramatic changes of bone mass can occur over a short period of time (e.g. in Paget's disease). A much larger study is therefore needed before definitive conclusions can be drawn from a study of this nature.

4.1 Changes in bone mass

Physical exercise is an essential factor in maintaining bone mass. Pocock (1989) has reported that muscle strength, physical activity, and weight, but not age, can predict femoral neck bone mass. Eisman (1990), however, was doubtful about that conclusion due to the fact that the femoral neck has a higher proportion of cortical bone compared with the predominantly trabecular bone of the lumbar spine. Eisman (1990) suggested an alternative explanation: the older subjects in his study were unable to exercise enough to modify BMD at the femoral neck. Eisman's findings are supported by Nilsson (1971) who found no significant correlation between quadriceps strength and distal femur BMD in athletes. On the other hand, Eisman (1990) reported that BMD changes in the lumbar spine correlated significantly with changes in physical fitness and biceps strength; femoral neck BMD changes did not correlate with the changes in these variables. Quadriceps strength did not correlate with BMD changes in either site. In summary, BMD of the femoral neck did not correlate with any parameter of physical fitness.

In our study, BMD changes in the lumbar spine 12 months after total hip replacement were not significantly different from the baseline or the 4-month measurement. Over this period, patients completed a rehabilitation program at a local hospital, including a variety of physical exercises (e.g. leg, back). It was anticipated that these patients would

have regained bone mass at least to a level to compensate for their bone mass loss during the short immobilization period after surgery. Although bone mass did not increase significantly, there was an increasing trend in spinal BMD between 4 and 12 months in 4 of 10 patients and no significant difference of BMD in the remaining six patients. In the 4 of 10 patients, a decrease in BMD was seen at the 4-month measurement in comparison with the baseline measurement (an average of -4.01%) followed by a recovery and a small increase at the 12-month measurement (an average of +4.54%). The overall change from the baseline was +0.3%. We anticipated that BMD would be increased significantly at both sites (lumbar spine, proximal femur), following the hip replacement. Our failure to detect such changes could be due to a variety of reasons; in particular the sensitivity and precision of the BMD measuring technique could be inappropriate. The ability of the DEXA to detect small changes in bone mass may be a limiting factor in this study (*in vivo* precision of 1 to 1.3% for lumbar spine Pacifici *et al.*, 1988; Wahner *et al.*, 1989, and *in vivo* precision of 1.8 to 2.2% for femoral neck Wahner *et al.*, 1989; Solsman *et al.*, 1989). Any changes smaller than these limits are not detectable using the DEXA unless we have more subjects in cross-sectional studies. In some BMD measurements, we found that a change in measurement area was not reflected in a change in the calculated BMD which suggests that the method lacks sensitivity. Tracing the bone borders using DEXA is automatic and without operator interaction. In some cases, this has proven to be a problem as artifacts in the field (e.g. sclerotic tissue) lead to a lack of precision in the definition of the bone contours. It is also possible that this technical difficulty might have added to the problem of precisely quantifying the bone mass in some study subjects. An additional technical problem was that the metal removal version of the QDR-1000 software (version 4.26) could not be used to calculate BMD after the implantation of the prosthesis. This problem was not identified until results were obtained by measuring the lumbar spine with both the regular software version and the metal removal version when we noted a 20% increase in BMD values by using the metal removal version. This discrepancy is due to the design of

the metal removal software in which the operator must enter threshold values for air, bone, fat, and soft tissue. In the regular version of the software, these values are preset and the operator input is not required. Because of these analysis difficulties, BMD measured in the proximal femur using the metal removal software were excluded from the analysis. However, BMD data obtained using the metal removal version of the software were compared to similar BMD data (obtained by the metal removal version of the software) at a subsequent measurement time. The limited usefulness of the metal removal software was quite unfortunate, as one of the objectives of this study was to assess BMD changes in the greater trochanter before and after the implantation of the hip prosthesis and thereby, establish a technique for the orthopedic surgeon to monitor long-term local effects of the implant.

The relatively short immobilization period following the surgery could be another factor accounting for the lack of detection of significant changes in BMD in this group of patients. Patients started their rehabilitation program 3 to 5 days after the surgery, and mobilization (mechanical usage) reduced the rate of bone loss to a minimum in the absence of post-operative complications (e.g. infection, thrombosis, shortening of one limb etc). Full mobility was usually restored about 5 to 6 weeks after surgery. The second BMD measurement in our study occurred between 4 and 6 weeks after the end of the rehabilitation program. The timing of the second BMD measurement might explain the lack of detection of a change in BMD between baseline and the 4 months. Indeed these patients were only partially immobilized compared to the traumatic hip fracture patients who would be totally immobilized until the hip was repaired. The small number of patients in our sample undoubtedly had an effect in the failure to detect significant differences in bone mass. A minimal sample size of 10 subjects was indicated for this study; however, a larger sample would have added to the power of the statistical analysis. A larger sample size is therefore needed to increase the confidence level of any statistically significant change in

bone mineral density over the one-year period of the study.

4.2 Study Compliance and sample size

Patient compliance in the study was generally good given the age and physical condition of the patients; over the 12-month period, 6 of 21 patients dropped out of the study. The number of cases available was quite unpredictable and recruitment proved to be very difficult. Out of 369 primary total hip replacements performed in Edmonton in 1990 (Hospital Health Records, 1990), only 21 patients were recruited to this study. Many problems had to be overcome to achieve the purpose of the study. One of the major problems that had to be overcome during the study was the hospital waiting-list system. Hospitals usually notify the patient only 1 to 2 days in advance of the surgery date. However, for this study, the patient had to be informed at least 4 weeks in advance to allow time for baseline DEXA measurements and for tetracycline labelling. This change was very difficult to implement as it added a lot of work for the hospital staff. Additional problems encountered included the cancellation and rescheduling of surgery which was totally beyond our control.

4.3 Significance of this work

This thesis presents the first comparison between BMD measurements and histomorphometric variables at approximately the same bone site. Although the longitudinal BMD changes were not statistically significant, the increasing trend in BMD observed in 4 of 10 patients supported our hypothesis. Correlation between biochemical data (25(OH)-vit D) and proximal femur BMD showed a strong relationship between these two variables which was expected as the level of 25(OH)-vit D represents the stores of vitamin D in the human body, however, previous studies (Tsai *et al*, 1987) could not prove this finding by comparing 25(OH)-vit D to spinal BMD.

4.4 Future application

DEXA measurements in the proximal femur before hip replacement surgery might be of value in selecting patients for the surgery since the long-term prognosis for the implant in patients with very low initial bone mass is questionable. Such DEXA measurements might also help decide which kind of prosthesis might be optimal for that patient. Follow-up after surgery with DEXA, at least once a year, could be helpful to monitor the biomechanical effects of the prosthesis on the bone mass in the spine and the proximal femur.

4.5 Conclusion

The study did not show any significant changes in BMD measurements over 12 months. However, a significant increase in BMD was found in 4 of 10 patients and in the remaining 6 subjects, there was no significant change in BMD. In this age group an expected rate of bone loss could be about 1% per year. The one-year study period was too short to detect statistically significant changes in bone mass in this group of patients. We suggest follow-up for another year (i.e. BMD to be measured at 24 months) which may yield additional information. We also suggest, if the study is to be repeated, a larger sample size should be used, serum biochemical tests should be performed with every BMD measurement, the inclusion of BMD and biochemical measurements from an age-matched control group, and physical activity parameters should be measured before and after the hip replacement surgery in correspondence with every BMD measurement. Additionally, a more successful way has to be found to recruit patients for studies of this nature by encouraging more orthopedic surgeons to collaborate. It was quite evident that the orthopedic surgeon's perception of the study was definitely a determining factor for his patients to participate.

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Appendix A

UNIVERSITY OF ALBERTA
DEPARTMENT OF APPLIED SCIENCES IN MEDICINE AND THE DEPARTMENT OF MEDICINE

INFORMATION SHEET

TITLE: Bone Mineral Density and Morphological Features in the Lumbar Spine and Proximal Femur

INVESTIGATORS: DR. TR Overton, DR. CL Chik and, DR. S Girgis

The purpose of this 12-month research project is to monitor changes in trabecular bone density (TBD) in the lumbar spine and proximal femur, in patients following surgery for hip repair or replacement. We expect that the new biomechanical status of the lower limbs, with re-ambulation, will cause observable changes in bone mass at all sites. The information provided by direct bone mass measurement might be of value for clinical decisions concerning the type of rehabilitation program to be undertaken for particular individuals, and to monitor the rate of progress.

We will use the Dual-Energy Radiography (DER) for bone mass measurement; this instrumentation provides the best available precision with radiation exposure less than 0.15 mGy per measurement.

Three measurements will be made (1 initial baseline measurement before surgery followed by 2 measurements after surgery over a one-year period beginning as soon as mobilization is achieved after surgery).

The risks of participating in this study are outlined in the " Consent to Participate " form; drug risks, including even the remotest side effects, have been described.

Your participation in this study will be greatly appreciated. However, you should be aware that you may choose to withdraw at any time without explanation or prejudice to future follow-up for skeletal investigation. We ask you to sign the "Consent to Participate" form. We urge you to ask about any part of the study which you do not understand, at any time during your participation. Costs of public transportation or parking fees incurred in attending the study related visits will be reimbursed; any medication required for the study will be provided to you free of charge. Information obtained from your participation in this study may be published in medical reports but your identity will be kept confidential.

Persons who can be contacted about this research:

Dr. S. Girgis	tel: 492-6780	office
	478-0237	residence
Dr. TR. Overton	tel: 492-6344	

Appendix B

UNIVERSITY OF ALBERTA
DEPARTMENT OF APPLIED SCIENCES IN MEDICINE AND THE DEPARTMENT OF MEDICINE

CONSENT TO PARTICIPATE IN A STUDY

TITLE: Bone Mineral Density and Morphological Features in the Lumbar Spine and Proximal Femur

INVESTIGATORS: DR TR Overton, DR CL Chik and DR S Girgis

1- I agree to participate in a 12-month research project to monitor changes in trabecular bone density (TBD) in the lumbar spine and proximal femur, following surgery for hip replacement or repair.
2- I understand that I will receive two, 3-day courses of tetracycline 10 days apart during the month before surgery for the purpose of bone analysis.
3- I understand that I will give an extra 5 ml of blood during the usual intravenous procedure for the purpose of some additional lab tests needed for the study.
4- I understand that one, 8 mm diameter bone biopsy will be taken from the operative site and that this will be analyzed by the bone research unit.
5- I authorize Dr. Chik / Girgis and / or assistants as may be selected by them to perform the following procedures on myself:
   a- Medical history and physical examination.
   b- Any screening / diagnostic procedures that are in their opinion, necessary and desirable in determining the status of my skeletal health.
   c- Three sets of bone density measurement to be made for the proximal femur and the lumbar vertebrae of the spine.
6- Dr. Overton / Chik / Girgis or assistants has explained the purpose of the study to me, and I understand the necessity for the several study visits and the procedures outlined in this consent form.
7- It is possible that one or more of the following risks or discomforts may occur during some of the procedures:
   a- The measurement of bone density involves a total radiation exposure of less than 0.15 mGy to the lumbar spine and both right and left hip joints .
   b- Side effects of tetracycline may include nausea, heartburn, vomiting or diarrhea.
8- I understand that there will be no cost to me for study related visits, or doctor's fees, or any study-related medication.
9- I understand that I will be informed of any significant findings which may develop during the research that may affect my willingness to continue participating in the study.
10- I understand that the information obtained from my participation in this study may be published in medical reports, but that my identity will be kept confidential.
11- I understand that copies of the study information sheet and the signed consent form will be given to me.
12- The study described here has been explained to me and I understand the inherent risks and benefits, and I voluntarily consent to participate in it. I have read this form and I have had the opportunity to ask questions which have been answered to my satisfaction. I understand that I may refuse to participate, or may withdraw from the study at anytime without explanation or prejudice to my future medical care.

Subject name (print)	Subject signature and date
Witness name (print)	Witness signature and date
Investigator name (print)	Investigator signature and date

Appendix C

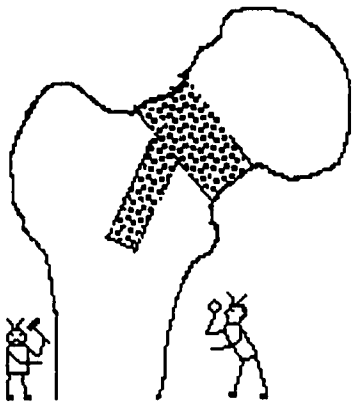
Dosing schedule for taking the tetracycline

Mr / Ms: _____

Labelling day	Take pills on these days	Times taken			Comments
Yellow Capsules: 1* Oxytetracycline 250 mg (2 caps three times daily)		6:30 am	2:30 pm	10:30 pm	
2 *		6:30 am	2:30 pm	10:30 pm	
3 *		6:30 am	2:30 pm	10:30 pm	
4					
5					
6					
7					
8					
9					
10					
11					
12					
13					
14					
Red Tablets: 15 * Declomycin 300 mg (1 tab three times daily)		6:30 am	2:30 pm	10:30 pm	
16 *		6:30 am	2:30 pm	10:30 pm	
17 *		6:30 am	2:30 pm	10:30 pm	
18					
19					
20					
21					Surgery Date

Appendix D

OPERATING ROOM REGISTRATION FORM FOR PARTICIPANTS IN THE BONE DENSITY STUDY



PATIENT'S NAME	DATE OF SURGERY	SURGEON
	15 May 1990	Dr. Johnston
	29 May 1990	Dr. Johnston

Thanks for your cooperation, it is very well appreciated,

Sincerely yours,

Dr.S.Girgis

Appendix E

INSTRUCTIONS FOR BONE BIOPSY LABELLING PROCEDURES

FOR: _____

1- Please keep to the following schedule as closely as possible.

2- As far as possible the first set of labelling medication should be taken on days 1, 2, and 3 at _____
and the second set of labelling medication on days 15, 16, and 17 at _____
_____as indicated on the record sheet by the starred dates. This schedule has been chosen to cause minimum interference with normal food habits. Ordinarily only breakfast will be affected. If you are unable to follow this schedule please contact your doctor or the nurse coordinator.

3- It is important to take the medication on an empty stomach or 1 hour before, or 2 hours after a meal with water, juice, pop, or clear tea or coffee.

4- You should not take any milk, cheese, ice-cream, butter, yogurt or other dairy products or antacids containing aluminum (e.g. Amphojel, Gelusil, Basaljel, Rolaids, Riopan, Maalox, Mylanta, Gaviscon, Di-Gel, Univol) 1 hour before or 2 hours after taking the labelling medication.

5- Please record exactly the times and dates that you take the labelling medication. If you miss taking a dose, please indicate it . Please bring the record sheet with you when you come for the surgery.

6- The most common side effects of the tetracycline-based drugs used for bone labelling are gastro intestinal upsets such as heartburn, loss of appetite, nausea and/or vomiting, diarrhea, and skin sensitivity to sunlight (avoid sunburn).

Appendix F

A FORTRAN program for the bone histomorphometric parameters

```
      REAL  TbAr, OAr, TAr, TbPm, OPm, RsiPm, RsaPm, dLPm, RsiAr, RsaAr,
     2        MOsWd, MTWd, Ldist, Dint, M, Rr, SIGf, SIGr, TBV, VOS, VOSf,
     3        Sf, Sr, Sra, Sfa, Sfo, Sv, MV, MOsc, Fs, Mf, MLT, Fv, SIG, VS, TTi,
     4        Ra, Mra
      character*20 fnme, lnme
      write (*,*)' WELCOME TO THE BONE HISTOMORPHOMETRY PROGRAM '
      write (*,*)' PLEASE PRINT YOUR NAME and DATE '
      write (*,*)'
      accept 10, fnme
10    format (A)
     write (*,*)'
     write (*,*)' Enter Trabecular Bone Area '
      read *, TbAr
      write (*,*)' Enter Osteoid Bone Area '
      read *, OAr
      write(*,*)' Enter Tissue Area '
      read *, TAr
      write(*,*)' Enter Trabecular Perimeter in μ '
      read *, TbPm
      write(*,*)' Enter Osteoid Perimeter in μ '
      read*, OPm
      write(*,*)' Enter Mean Osteoid Width dir in μ '
      read *, MOsWd
      write (*,*)' Enter Mean Trabecular thickness dir in μ '
      read *, MTWd
      write (*,*)' Enter Inter-label distance in μ '
      read *, Ldist
      write (*,*)' Enter Tetracycline Dose interval in days '
     read *, Dint
      TBV  = TbAr/TAr *100
      VOS  = OAr/TbAr *100
      VOSf = OAr/(OAr + TbAr) *100
      Sf   = OPm/TbPm *100
      M    = Ldist*.7853/Dint
      write (*,*)
      write (*,*)'************************************************************'
      write (*,*)'THANK YOU, NOW PLEASE ENTER PATIENT NAME & NUMBER'
      write (*,*)
      accept 20, lnme
20    format (A)
      write (*,*)' BONE MEASUREMENTS ','***********************************'
      write (*,*)
      write (*,*)' TRABECULAR BONE VOLUME (TBV) %                =', TBV
      write (*,*)' OSTEOID BONE VOLUME    (VOS) %                =', VOS
      write (*,*)' FRACTIONAL OSTEOID VOLUME (VOSf) %            =', VOSf
      write (*,*)' FRACTIONAL FORMATION SURFACE (Sf)%            =', Sf
      write (*,*)' APPOSITIONAL RATE   (M) μ/day                 =', M
      write (*,*)' MEAN TRABECULAR THICKNESS(μ)                  =', MTWd
      write (*,*)' MEAN OSTEOID WIDTH(μ)                         =', MOsWd
      End
```

Appendix G

Sample run for the bone histomorphometry program

```
WELCOME TO THE BONE HISTOMORPHOMETRY PROGRAM
 PLEASE PRINT YOUR NAME and DATE

Safwat GIRGIS  03 Nov 1990

 Enter Trabecular Bone Area
.203
 Enter Osteoid Bone Area
.004
 Enter Tissue Area
.468
 Enter Trabecular Perimeter in µ
1575
 Enter Osteoid Perimeter in µ
980
 Enter Mean Osteoid Width dir in µ
4
 Enter Mean Trabecular thickness dir in µ
279
 Enter Inter-label distance in µ
26
 Enter Tetracycline Dose interval in days
11

**********************************************************
THANK YOU, NOW PLEASE ENTER PATIENT NAME & NUMBER

xxxxxxxxxx 1L1
 BONE MEASUREMENTS ***********************************

 TRABECULAR BONE VOLUME (TBV) %          =43.37
 OSTEOID BONE VOLUME   (VOS) %           =1.97
 FRACTIONAL OSTEOID VOLUME (VOSf) %      =1.93
 FRACTIONAL FORMATION SURFACE (Sf)%      =62.22
 APPOSITIONAL RATE   (M) µ/day           =1.86
 MEAN TRABECULAR THICKNESS(µ)            =279
 MEAN OSTEOID WIDTH(µ)                   =4
```

Appendix H

Drugs, chemicals, and materials

Oxytetracycline Hydrochloride 250 mg capsules (Rugby Laboratories Inc., New York, NY)

Declomycin 300 mg tablets (Cyanamid Canada Inc., Markham, Ont)

Spurr's (Embedding medium) (Ernest Fullam Inc., Latham, NY)

Toludine Blue 0-certified (Sigma Chemical co., St Louis, MO)

Hydrochloric Acid 0.1 N (Sigma Chemical co., St Louis, MO)

Peel-A-way plastic moulds (Peel-A-way Scientific, South El monte, CA)

Jung microtome model K (Nussloch, Heidelberg, Germany)

Na Acetate (Fisher Scientific, Edmonton, AB)

Na diethylbarbiturate (BDH Inc., Edmonton, AB)

Permount (Fisher Scientific, Edmonton, AB)

Xylene (Fisher Scientific, Edmonton, AB)

Image analysis software (IMAGE 1.22, Wayne Rasband, National Institute of Health, Research Services Branch, NIH, Bethesda, MD).

Hologic QDR-1000 x-ray bone densitometer (Hologic Inc., Waltham, MA)

Microsoft Excel 2.2 (Microsoft, Redmond, WA)

Stat View TM SE + Graphics, 1988 (Abacus Concepts Inc., Berkley, CA)