Methotrexate and bone formation and turnover in rheumatoid arthritis

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METHOTREXATE AND BONE FORMATION AND TURNOVER IN RHEUMATOID ARTHRITIS

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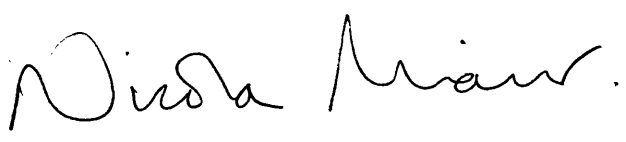

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ABSTRACT

This study was designed to explore the effects of the anti-folate drug methotrexate (MTX) on bone formation and turnover in rheumatoid arthritis (RA), using 116 RA subjects, bone histomorphometry and bone cell culture.

Low dose MTX is a disease-modifying drug (DMARD) used in RA treatment. Evidence exists that high dose MTX causes osteoporosis. As RA is a risk factor for osteoporosis, the study was performed to determine whether therapy with MTX results in bone loss.

There were four groups of subjects: those who had taken MTX continuously for the past five years; those on another DMARD for the same time; those starting MTX; and those starting sulphasalazine (another DMARD). Data collected included: annual bone mineral density (BMD) measurements at the spine, hip and forearm; serum and urinary markers of bone turnover; measurement of RA disease activity and joint damage. Paired bone biopsies from four subjects before and after one year's MTX treatment were analysed by histomorphometry. MTX was added to cultures of normal human marrow-derived stromal cells and trabecular-derived osteoblast-like cells.

Analysis of the clinical data revealed that MTX was associated with significantly reduced BMD of the forearm which remained significant at the mid-portion forearm site after adjustment for confounding factors. No significant adverse effect of MTX on bone formation or resorption was found in the markers of bone turnover or by histomorphometry of the cancellous envelope. In cultures of normal human bone cells MTX affected proliferation and colony formation in a dose-dependent way. Differentiation was also affected, resulting in population shifts on flow cytometry.

MTX therapy was associated with reduced bone mass of the forearm, most of which could be explained by confounding factors. However, a significant adverse treatment effect persisted at the forearm mid-portion site which is predominately cortical and not prone to fracture. A preferential effect on cortical bone has been predicted from case reports and may explain the lack of effect seen on histomorphometry of the cancellous bone. In vitro, the anti-folate effect of MTX dominated, impairing proliferation of normal bone cells. These findings are reassuring for clinicians and patients using low-dose MTX in the treatment of RA.

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1. INTRODUCTION

1.1 Bone Biology

1.1.1 Functions of Bone

Vertebrates are unique in possessing an internal skeleton. The 206 bones of the human skeleton are a specialised form of connective tissue and perform many functions including the following three vital ones. These are: (1) the protection of vulnerable internal organs including the bone marrow, (2) the mechanical provision of levers for the striated muscles to act on thereby permitting locomotion, and (3) the metabolic reservoir of calcium, phosphate and bicarbonate ions available from bone and essential for homeostasis (Baron, 1996).

There are two forms of bone with distinct features at the macroscopic and microscopic levels which result in different functions.

1.1.2 Cortical Bone

Cortical, or compact, bone comprises 80% of the adult skeletal mass and is found in the long bones and the outer aspects of the flat bones (the ilium and skull) and vertebral bodies. Cortical bone is dense and strong and 85% by volume is calcified. Only 10% is soft tissue and thus cortical bone provides most of the protective and mechanical functions of bone (Monier-Faugere et al, 1998).

1.1.3 Trabecular Bone

Trabecular, cancellous or spongy bone is much more porous than cortical bone, consisting of a meshwork of connecting struts with cells of the bone marrow, blood vessels and connective tissue filling the spaces. Trabecular bone in the adult is found mainly in the vertebral bodies, iliac crest, calvariae and at the ends of the long bones (metaphysis and epiphysis) (Baron, 1996). It is only 20% calcified, with bone

marrow making up more than 75% of tissue volume. Although trabecular bone only contributes 20% towards the total skeletal mass, it is four times more metabolically active than cortical bone and so the metabolic function is divided equally between the two bone types (Monier-Faugere et al, 1998).

1.1.4 Bone Matrix

In normal or lamellar bone, collagen fibres are aligned in a preferential direction whereas in woven bone the collagen fibres are laid down in random bundles. The pattern of collagen fibres is seen best under polarised light or by electron microscopy. Primitive woven bone occurs in various pathological states such as fracture healing, Paget's disease of bone, osteogenesis imperfecta and in primary and secondary hyperparathyroidism (Ng et al, 1997).

Type 1 collagen fibres account for 90% of the protein in bone. Calcification of the collagen results in the formation of spindle-shaped or plate-shaped crystals of hydroxyapatite [$3Ca_3(PO_4)_2 \bullet (OH)_2$] on and within the collagen fibres and within the ground substance between the fibres (Glimcher, 1998). Woven bone is less able to undergo calcification and is therefore weaker than lamellar bone. Collagen synthesis is described in Section 1.1.10 below. The bone matrix contains many growth factors, proteolytic enzymes and their inhibitors, all of which contribute to the modelling and remodelling of bone as described in Section 1.1.12 (Ng et al, 1997).

1.1.5 Osteoblasts

The primary function of the osteoblast is bone formation. Osteoblasts are small cuboidal cells which differentiate from bone marrow stromal stem cells initially into pre-osteoblasts, then osteoblasts (Beresford, 1989). Fully differentiated osteoblasts

express alkaline phosphatase on their cell surface and synthesize osteocalcin (also called bone gla-protein), which can be used as a marker of bone formation (Section 1.1.12) (Raisz et al, 1998).

In the normal adult skeleton, bone formation only occurs on a bone surface which has previously been eroded (Monier-Faugere et al, 1998). Mature osteoblasts are found in a cluster along a bone surface where they synthesise osteoid at a bone forming site (Raisz et al, 1998). Osteoid is uncalcified bone tissue which can undergo calcification by the association of hydroxyapatite (section 1.1.4) to form mature bone. It is thought that each osteoblast can produce a pre-determined quantity of osteoid in its functional life-span which has been estimated as between three days and three months (Parfitt, 1995).

1.1.6 Osteocytes

Osteocytes are cells found deeper within the bone matrix. Originally osteoblasts which have become trapped within the matrix they produced, osteocytes are thought to have a regulatory role in bone turnover. They have long processes which extend through small canals or canaliculi in the bone and are in contact by gap junctions with the cell processes from other osteocytes as well as with osteoblasts and lining cells (Puzas, 1996). Recent work has suggested that cortical bone osteocytes undergo apoptosis in response to mechanical stimuli, affecting bone formation and resorption on a bone surface not adjacent to the osteocyte location (Noble et al, 1997).

1.1.7 Osteoclasts

Osteoclasts are bone resorbing cells which develop from the fusion of several cells derived from a haematopoietic stem cell of different lineage from osteoblasts and chondrocytes (Mundy, 1996). They can have between four and twenty nuclei and are found on the surface of bone which is being resorbed, usually only one to four such fused cells for each resorption area. The osteoclast makes a resorption pit by using integrins on the cell membrane to attach to the bone surface, creating a sealed region (Ng et al, 1997). The cell membrane adjacent to the bone surface is deeply folded and the osteoclast can secrete various compounds across this ruffled border which result in bone resorption. Because the space is sealed off from the rest of the extracellular environment, concentrations of enzymes such as collagenase can become high enough to enable efficient bone resorption to occur and the osteoclast makes the pH of the fluid in the space optimal by secreting protons (Mundy, 1996).

1.1.8 Chondrocytes

Chondrocytes synthesise cartilage and are located in the growth plates at the ends of the long bones. Like osteoblasts, they are derived from bone marrow stromal stem cells (Beresford, 1989). Chondrocytes are arranged in columns in the growth plate and as they mature they undergo hypertrophy (Baron, 1996). Endochondral ossification involves chondrocytes (Section 1.1.9).

1.1.9 Intramembranous and Endochondral Ossification

There are two ways in which bones can develop in the fetus. Flat bones such as the calvariae, scapulae and ilia form by intramembranous ossification while the long bones of the limbs form by endochondral ossification (Baron, 1996).

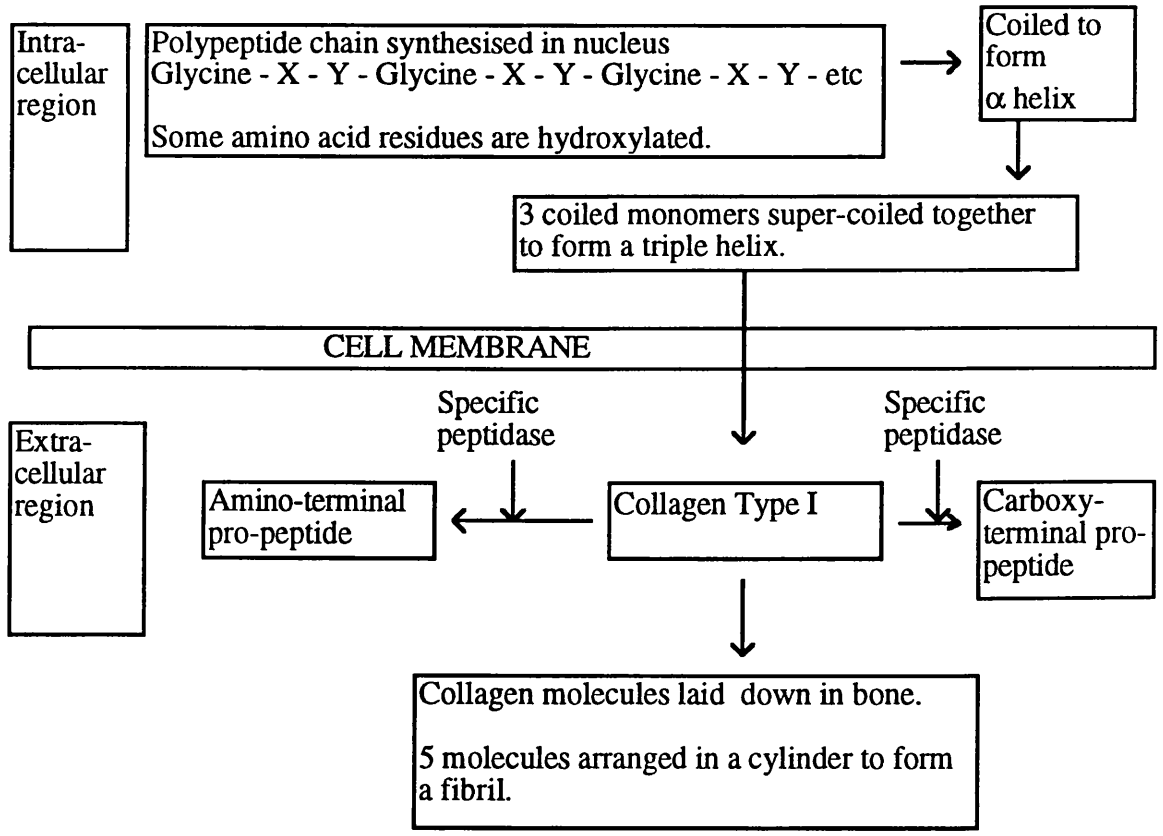
During intramembranous ossification, mesenchymal stem cells within the embryonic connective tissue proliferate and then differentiate into pre-osteoblasts and subsequently osteoblasts. These cells produce a bone matrix of woven bone with the embryonic bone marrow within it. The woven bone is gradually remodelled (Section 1.1.12) to form mature lamellar bone (Baron, 1996).

During endochondral ossification, the mesenchymal stem cells divide and differentiate into pre-chondroblasts and then chondroblasts. The chondroblasts synthesise cartilage which in the embryo is avascular. Across the growth plate of a developing long bone different zones have been identified. The zone in which the chondrocytes initially proliferate and synthesise the cartilage matrix is termed the proliferative zone. The cells hypertrophy before undergoing apoptosis in the hypertrophic zone. The cartilage then calcifies partially (zone of provisional calcification) and is partially resorbed by osteoclasts. The portion of the long bone which is destined to become the mid-shaft is a ring of woven bone formed by intramembranous ossification. As this woven bone becomes calcified, blood vessels which will form the bone marrow invade it and also extend into the cartilage (zone of invasion). Osteoblasts are now present in the growth plate and lay down woven bone on the sites of resorbed cartilage. This primitive trabecular bone is called the primary spongiosum and is later remodelled to form mature lamellar trabecular bone, or the secondary spongiosum (Baron, 1996).

1.1.10 Collagen Synthesis

There are at least nineteen genetically distinct collagenous proteins (Rowe et al, 1998). Cartilage contains mainly Type II, and basement membranes Type IV. Bone collagen is almost exclusively Type I, the most abundant collagen type in the body. It is present in many connective tissues including tendons, skin, ligaments, dentine and sclerae and, to a lesser extent, in blood vessels, lungs and other visceral supporting tissues (Rowe et al, 1998). Type I collagen is a complex molecule, consisting of three polypeptide chains, two of α 1(I) collagen and one of α 2(I) collagen (Monier-Faugere et al, 1998). The synthesis of collagen is illustrated in Figure 1.1.

Figure 1.1 The synthesis of type I collagen



The type I collagen molecule consists of multiple glycine-X-Y repeats, where X is often proline or hydroxyproline. Glycine has no side chain, which is important in the conformation of the protein as it allows tight coiling of the polypeptide chain to form the α -helix.

The three coiled monomers (two α 1 and one α 2) are super-coiled together, creating a rope-like structure.

Type I collagen undergoes several post-translational modifications. Intra-cellularly, before secretion from the osteoblast, some of the proline and lysine residues are hydroxylated. Hydroxyproline provides conformational stability to the helix at physiological temperatures (Rowe et al, 1998). The three α chains of procollagen spontaneously adopt a triple helical conformation within the cell and the helix is secreted from the cell. Cleavage of the amino-terminal and carboxy-terminal pro-peptides is done by specific peptidases. These released pro-peptides have been utilised as serum markers of bone formation (Section 1.2.6). In the matrix of the bone, collagen forms fibrils consisting of five collagen molecules arranged in a three-dimensional cylinder with staggering of the molecules. The regular overlapping pattern can be seen on electron microscopy, when cross-striations are separated by 640 Å (Rowe et al, 1998). The collagen molecules complex with non-collagenous proteins such as proteoglycans (Ng et al, 1997) and the covalent bond associated may also confer strength. However, the main reason for collagen's durability are the collagen crosslinks (Section 1.1.11).

1.1.11 Collagen Crosslink Formation

The final maturation stage of a collagen fibril is the extra-cellular formation of intra- and inter-molecular covalent pyridinium crosslinks, which are unique to mature bone (Termine et al, 1996). Multiple cross-linking sites can combine to form pyridinium ring structures, binding several collagen monomers together. Lysyl oxidase acts on the collagen lysine and hydroxylysine residues, forming deoxypyridinoline (Dpd) or pyridinoline (Pyd) respectively (Knott et al, 1998). These cross-links are non-reducible and insoluble and are only released when bone is resorbed. Pyd occurs mainly in collagen types I and II, while Dpd is found mainly in collagen type I (Knott et al, 1998). They thus have different tissue distributions with Pyd having its highest concentration in cartilage although it is also present in bone. Dpd has its highest concentration in bone where the ratio Pyd: Dpd is approximately 4:1 (Knott et al, 1998). Dpd is therefore the most bone-specific resorption marker available at present (Robins, 1995). Dpd and Pyd are excreted in the urine where two-thirds are small peptides and the remainder are free amino acids. As they naturally fluoresce, Pyd and Dpd can be measured in urine by high pressure liquid chromatography (HPLC) after hydrolysis and pre-fractionation over cellulose, using an ion-paired reversed-phase method developed by Black (Black et al, 1988) as discussed in Section 2.9.1. Alternatively, the cross-links can be measured by an enzyme-linked immunosorbent assay (ELISA) utilising a monoclonal antibody assay (Robins et al, 1994) described in Section 2.9.2. Urinary excretion of cross-links has been shown to be increased in conditions with increased collagen breakdown such as rheumatoid arthritis (Black et al, 1989) and Paget's disease of bone (Uebelhart et al, 1990).

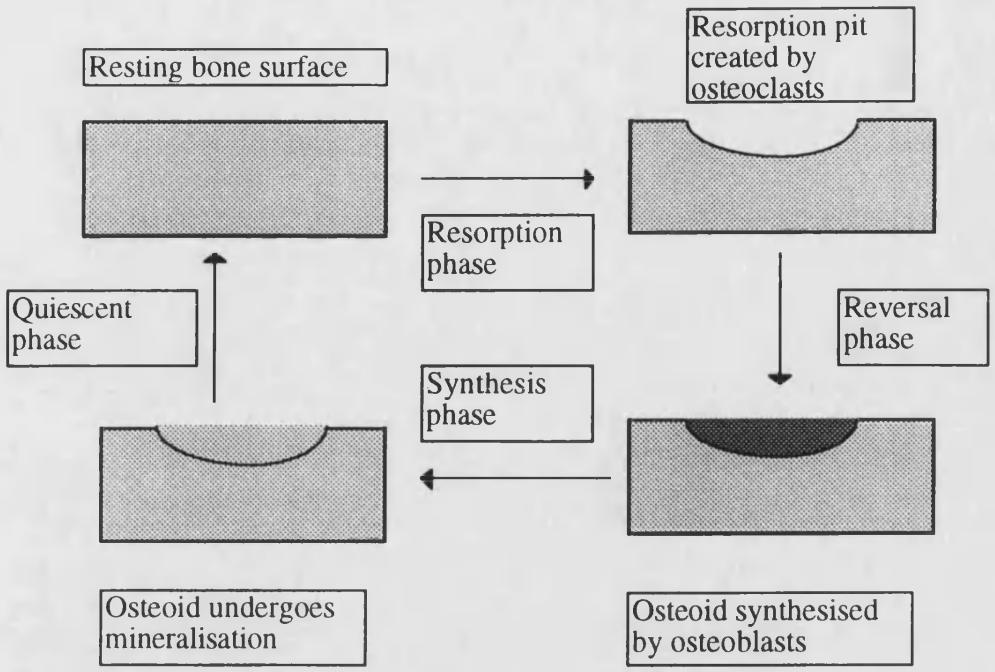
1.1.12 Bone Modelling and Remodelling

During growth, the bones need to increase in size and change in shape. This is achieved by modelling, where resorption and formation of bone happen at different sites in the skeleton. This results in a redistribution of bone in response to changing biomechanical demands. The proliferation and differentiation of cartilage cells at the growth plates results in the longitudinal modelling or growth of the long bones. The bones increase in width by appositional modelling when bone is resorbed from the inner (endosteal) surface of the long bone and new bone is laid down on the outer (periosteal) surface (Ng et al, 1997).

After growth has ceased the bone tissue is not static as small quanta of bone are resorbed and replaced with new bone. In this remodelling cycle, the skeleton renews itself without any change in the macroscopic appearance of the bones (Monier-Faugere et al, 1998). Remodelling differs from the modelling which occurs in growth as there is coupling, or linkage, of the resorption and formation both anatomically and quantitatively (Canalis, 1996). The functions of bone remodelling are thought to be predominately mechanical in cortical, load-bearing bone and metabolic in trabecular bone.

The remodelling cycle consists of sequential events involving different cell types and is illustrated in Figure 1.2. The team of cells which undertake the remodelling of a quantum of bone is referred to as a bone multicellular unit (BMU).

Figure 1.2 The remodelling cycle in bone



The first event in the remodelling cycle is activation of the bone surface with the arrival of mononucleated osteoclast precursors and the retraction of the bone lining cell layer, exposing the bone matrix. The signalling factors for activation are not fully understood but are thought to include both systemic hormones and locally produced polypeptide growth factors such as members of the transforming growth factor-β family (Canalis, 1996).

After the osteoclast precursors have fused into mature, multinucleated osteoclasts, resorption of the bone can proceed. The osteoclasts remove two-thirds of the bone in the resorption cavity over a period of about one week. The remaining resorption is carried out by mononuclear cells over a longer period of about thirty-six days.

After resorption ceases, there is a one to two week reversal phase during which the reversal or cement line is laid down. Osteoblast precursors are attracted to the eroded surface and, after differentiation into mature osteoblasts, they lay down osteoid (uncalcified matrix protein) in the bone formation phase of the remodelling cycle. Formation is a slower process than resorption. The osteoid seam is mineralised, initially at a rate of 1 to 2μm per day but then more slowly. The newly-synthesised bone enters a phase of quiescence. During the first three to six months of this phase the young bone matures to contain larger hydroxyapatite crystals with a corresponding increase in bone mineral density. In normal adult bone 80% of the bone surface is in the quiescent phase at any time.

If normal skeletal metabolism is occurring, the complete remodelling cycle takes approximately 100 days in cortical bone and 200 days in trabecular bone (Eriksen et al, 1994).

Thus, in health, new bone is formed to completely replace the bone resorbed by osteoclasts, resulting in loss of neither bone mass nor strength. If coupling of the resorption and formation processes is lost, either net bone loss or gain will occur.

1.2 Osteoporosis

1.2.1 Definition

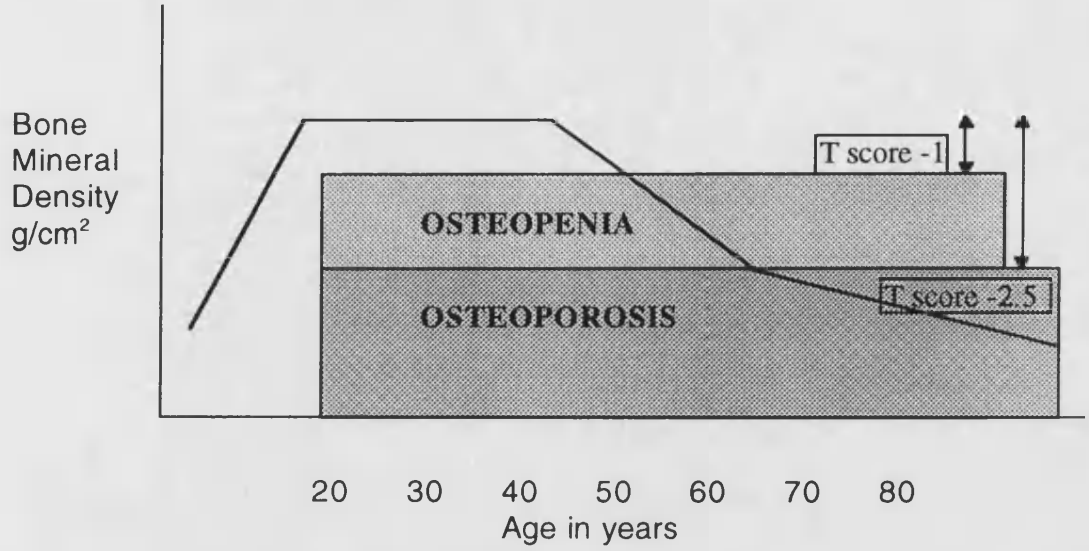
The 1993 Consensus Development Conference statement defined osteoporosis as 'a disease characterised by low bone mass and microarchitectural deterioration of bone tissue, leading to enhanced bone fragility and a consequence increase in fracture risk' (Christiansen et al, 1993). Subsequently, a working group of the World Health Organisation proposed a classification of white women as osteopenic or

osteoporotic on the basis of their bone mineral density (BMD) measurement (Section 1.2.5) (Kanis et al, 1994). The threshold BMD values are shown in Table 1.1 and are illustrated in Figure 1.3. Severe osteoporosis was defined by a BMD more than 2.5 standard deviations (SD) below the young adult mean in the presence of at least one non-traumatic (fragility) fracture.

Table 1.1 WHO criteria for osteoporosis and low bone mass

Diagnosis	**T score: BMD expressed in standard deviations (SD) relative to mean for young adults**
normal bone mass	no more than 1.0 SD below young adult mean
low bone mass (osteopenia)	between 1.0 and 2.5 SD below young adult mean
osteoporosis	more than 2.5 SD below mean
severe osteoporosis	more than 2.5 SD below mean and fragility fractures

Figure 1.3 Schematic diagram of WHO criteria for the diagnosis of osteoporosis and osteopenia.



The mean bone mineral density for a female population is shown.

1.2.2 Epidemiology

Women have lower peak bone mass than men and have rapid bone loss related to the reduction in circulating oestrogen at the time of the menopause (Heaney, 1996). In addition, there is age-related bone loss of approximately 1 to 2% per year in both sexes after the age of 50 years (Wasnich, 1996). Studies have shown that Caucasians have lower bone mass than do Black or Asian populations (Wasnich, 1996). As a result, the group most at risk of osteoporosis are white postmenopausal women, and fracture rates confirm this (Center et al, 1997). However, osteoporosis in other groups, including men, is being increasingly recognised (Jackson, 1996).

It has been estimated that more than 50% of postmenopausal women in the United States have low bone mass and another 20 to 30% fulfil the criteria for osteoporosis (Center et al, 1997). The prevalence of osteoporosis rises steeply with age, so that while less than 15% of women aged 50 to 59 have BMD measurements consistent with osteoporosis, this increases to 70% of women over the age of 80 years (Center et al, 1997). For England and Wales, Kanis has estimated that 22.5% of women and 5.8% of men over the age of 50 have osteoporosis (Kanis et al, 1994).

Fracture rates vary with the type of fracture, the population studied and the method of data collection. In general, fracture rates for all types of osteoporotic fractures are increasing. This is due to demographic changes, with increasing numbers of people aged eighty years and older. Lifetime risks of fracture have been estimated and for a 50 year old white women were 16% to 19% for a hip fracture in her remaining life, and approximately 5% for a man of the same age (Center et al, 1997). Prevalence rates for vertebral fractures are harder to determine as only about one third come to clinical attention, but, as for hip fracture, rates rise exponentially with age in women. Below the age of 50 higher prevalence rates in men than women have been found. It has been suggested that this is due to the more physical nature of work undertaken by many men (O'Neill et al, 1996).

1.2.3 Clinical Features

Low bone mass without fracture causes no symptoms and osteoporosis has been called the silent epidemic. The clinical consequences of osteoporosis are all related to the fractures which may occur. The three most common fractures are a Colles' fracture of the distal forearm, hip fracture and vertebral body fracture. Apart from the pain of the fracture itself, considerable morbidity and even mortality may result.

Osteoporotic bone does not take longer to heal after fracture than normal bone, but the surgeon may have difficulty in finding enough bone stock for successful instrumentation (for example, pin and plate fixing of a fractured hip) and bone grafts are sometimes necessary. Forearm fractures cause least problems and do not necessarily result in admission to hospital. However, if the person lives alone, they may need help with activities of daily living.

Vertebral fractures cause pain, loss of height and change in body shape; the so-called Dowager's hump. If multiple vertebral fractures have occurred, the lowest ribs may rest on the iliac crest, resulting in abdominal protrusion which may be mistaken for obesity. Apart from the discomfort this causes, respiration may be impaired and the abdominal contents compressed. Vertebral fractures may be silent (painless) or simply attributed to 'back ache'. It has been estimated that only one third come to medical attention (Cooper et al, 1992). In those which are recognised, the patient often describes a sudden severe pain over the fracture site which may radiate around the thorax to the midline, anteriorly. This pain is worse on taking a deep breath or moving, and usually settles after four to six weeks. It is important to consider underlying causes of vertebral fracture, such as multiple myeloma and metastatic disease. Only 10 to 15% of vertebral fractures occur on falling. The remainder are related to lifting or other loading of the spine and may seem to have occurred spontaneously (Cooper et al, 1992).

The most serious fragility fracture is that of the hip. These are also the most accurately recorded because medical attention is always sought. There is significant mortality associated with hip fractures. Several studies have demonstrated an excess mortality of between 10 and 40% over the subsequent

year (Center et al, 1997). The majority of deaths occur in the first six months after the fracture, and it has been suggested that at least some of these deaths may be due to co-existing serious diseases which may have contributed to the fall. Estimates of deaths directly due to the fracture vary from 15 to 40%. Among the survivors of hip fracture, many have serious continuing disability and require long-term nursing care. Other fractures may occur in osteoporosis and fractures of the humerus, tibia and pelvis are not unusual.

1.2.4 Secondary Osteoporosis

Osteoporosis may be age-related, postmenopausal, or secondary to a variety of medical conditions and drugs. Common causes of secondary osteoporosis are shown in Table 1.2 and include the inflammatory joint disease rheumatoid arthritis (Section 1.3).

Table 1.2 Secondary causes of osteoporosis

Underlying Cause	Examples
Endocrine	Cushing's syndrome, thyrotoxicosis, hypogonadism
Associated with inflammatory diseases	rheumatoid arthritis, inflammatory bowel disease, ankylosing spondylitis
Drug-related	corticosteroids, heparin, anticonvulsants
Haematopoietic	multiple myeloma, lymphoma, mastocytosis
Gastrointestinal disease	Coeliac disease, inflammatory bowel disease
Hepatic disease	Primary biliary cirrhosis
Non-weight-bearing	Bed-bound, astronauts
Pregnancy	Transient osteoporosis of pregnancy

1.2.5 Bone Mineral Density Measurement

Osteoporosis cannot be diagnosed from plain radiographs unless a typical fracture is seen, but the radiologist may comment that the bones are osteopenic, with apparent rarefaction of the bony substance and fewer characteristic markings than normal. The mainstay of detection is measurement of the BMD (Kanis et al, 1994) which is also used to monitor treatment (Kanis et al, 1996). This is usually derived from a measure of bone mineral content (BMC) which can be estimated by a variety of techniques. BMD is the most important factor in determining bone strength and has been shown to be associated strongly with future fracture risk. Each standard deviation reduction in BMD is associated with a 1.5 to 3-fold increase in risk of future fracture (Melton et al, 1993) and BMD accounts for 75-85% of the variance in bone strength. There are several ways to measure BMD with good accuracy and excellent precision. Dual-energy x-ray absorptiometry (DXA) is widely used to measure BMD as it needs only a low radiation dose, has high reproducibility and can measure BMD at both axial (spine and hip) and appendicular (forearm) sites. Thus the sites most prone to osteoporotic fracture can be studied directly. This is important as Cummings showed that the best predictor of future hip fracture was the hip BMD (Cummings et al, 1993).

DXA measures bone mineral content (BMC, g or g/cm), which is then converted into an areal (two dimensional) density (BMD, g/cm^2) using the size of the bone measured.

In assessment of the lumbar spine BMD, degenerative changes (osteophytosis) can falsely elevate the BMD without reducing fracture risk (Jones et al, 1996). Lateral lumbar spine BMD measurements which exclude the posterior vertebral elements

are now possible but have been associated with less precision than the traditional antero-posterior view due to positioning difficulties (Bjarnason et al, 1996). In practice, lumbar spine BMD values in patients with degenerative changes of the lumbar vertebrae should be disregarded and treatment decisions made on the basis of measurements at other sites.

1.2.6 Clinical Markers of Bone Turnover

It is possible to assess the rate of bone turnover by invasive (bone biopsy) or non-invasive methods. Biochemical markers of bone turnover refer to the measurement of compounds in either serum or urine which are related to bone resorption and formation. Markers of bone turnover are shown in Table 1.3. As osteoporosis occurs in the setting of an imbalance (uncoupling) of formation and resorption, these markers may have clinical usefulness. Convenient specific immunoassays are being developed for these compounds. An advantage of markers over bone density measurements is that they change in response to therapy much more quickly.

For a measured change to be significant, it must be at least three times the coefficient of variation of the measurement. Depending on the site, typical coefficients of variation in BMD are 1 to 2% and there is therefore no advantage in repeating BMD estimation before at least a year when looking for a response to treatment. However, significant changes in markers of bone turnover can be seen within weeks, so in theory, non-responders or those with poor compliance could be identified early.

Markers of both formation and resorption are increased in postmenopausal women (Lufkin et al, 1992). A prospective study of elderly French women over two years found that two urinary markers of bone resorption, carboxyterminal-telopeptide and

free deoxypyridinoline, were predictive of hip fracture risk, independent of baseline BMD (Garnero et al, 1996). There has been difficulty in translating results from population studies to the individual patient. The hope is that a combination of baseline BMD measurement together with markers of bone turnover will enable treatment of osteoporosis to be better targeted to these at greatest risk of future fracture.

1.2.7 Bone Histology

Despite advances in non-invasive assessments of bone such as BMD by DXA and serum and urine markers of bone turnover (Section 1.2.6), bone biopsy remains the most informative means of assessing the effect of therapies at the tissue level (Monier-Faugere et al, 1998). Histological data is required in the development of new treatments for osteoporosis and other metabolic bone diseases. Bone biopsy is useful in the diagnosis of osteomalacia, especially in the elderly where there may be co-existing osteoporosis. In renal bone disease, bone biopsy reveals the extent of aluminium accumulation in patients undergoing dialysis. The techniques of bone biopsy and histomorphometry are described fully in Chapter 6.

Table 1.3 Markers of bone turnover

	Tissue of origin	Method	Specificity	Measured in this study
Formation Markers				
Bone specific alkaline phosphatase	Bone	Colorimetric Electrophoretic Precipitation IRMA ELISA	Cross-reactivity with the liver isoenzyme may occur	Yes
Osteocalcin	Bone	RIA ELISA	Osteoblast specific	Yes
PICP	Bone (soft tissue & skin)	RIA ELISA	Osteoblast & fibroblast	No
PINP	Bone (soft tissue & skin)	RIA ELISA	Osteoblast & fibroblast	No
Resorption Markers				
Hydroxyproline	Bone, cartilage, soft tissue, blood, skin	Colorimetric HPLC	All collagenous proteins	No
Pyridinoline	Bone, cartilage, tendon, blood vessels	HPLC ELISA RIA	Mature collagen in cartilage & bone	Yes
Deoxy-pyridinoline	Bone, dentine	HPLC ELISA RIA	Mature collagen in bone	Yes
ICTP	Bone, skin	RIA	Newly synthesised Collagen type I	No
INTP	Bone, skin	ELISA	Newly synthesised Collagen type I	No
Bone sialoprotein	Bone, dentine, calcifying cartilage, platelets	RIA	Osteoblast product, specificity unclear	No
Tartrate-resistant acid phosphatase	Bone, blood	Colorimetric	Unstable isoforms in osteoclasts, platelets and erythrocytes	No

PICP Carboxy-terminal Propeptide of Type I Procollagen; PINP Amino-terminal Propeptide of Type I Procollagen; ICTP Carboxy-terminal Cross-linked Telopeptide of Type I Collagen; INTP Amino-terminal Cross-linked Telopeptide of Type I Collagen; IRMA Immunoradiometric assay; ELISA Enzyme-linked immunosorbent assay; RIA Radio-immuno assay; HPLC High Pressure Liquid Chromatography

1.3 Rheumatoid Arthritis

1.3.1 Introduction and Definition

Rheumatoid arthritis (RA) is the most common inflammatory joint disease in the Western world, with a prevalence estimated at 1 to 2% (Alarcón, 1995). The American College of Rheumatology 1987 revised diagnostic criteria (Arnett et al, 1988) are shown in Table 1.4. Of note is that the joint swelling must have been present for six weeks but rheumatoid factor does not have to be present in the serum. The cause of RA is unknown but the trigger is assumed to be environmental, possibly a virus, in a genetically-susceptible person.

Table 1.4 American Rheumatism Association revised diagnostic criteria for rheumatoid arthritis, 1987

Early morning stiffness lasting at least one hour
Synovial swelling of at least 3 joints observed by a physician
Synovial swelling of the proximal interphalangeal, metacarpophalangeal or wrist joints
Symmetrical synovial swelling
Rheumatoid nodules
Rheumatoid factor positive
Erosions and/or periarticular osteopenia in hand and/or wrist joints on radiographs

Criteria 1 to 4 must have been present for at least 6 weeks. Rheumatoid arthritis is defined by four or more criteria.

1.3.2 Clinical Features

RA is characterised by an inflammatory infiltrate in the lining of synovial joints, termed synovitis. The symptoms are joint swelling, pain and stiffness, and the joints first affected are often the small joints of the hands and feet; the metacarpo-phlangeal, proximal inter-phlangeal and metatarso-phlangeal joints. An explosive onset is not uncommon, with nearly every synovial joint affected, often on waking one morning. Rheumatoid disease is a systemic illness and almost every system can be affected. Extra-articular features include nodules, pericarditis, pulmonary fibrosis and vasculitis. The result of the joint inflammation is usually erosive damage as shown in Figure 1.4, a Larsen standard film.

1.3.3 Therapy of Rheumatoid Arthritis

Prior to referral to a rheumatologist, most patients will have been treated by their general practitioner with analgesics and non-steroidal anti-inflammatory drugs (NSAIDs). In the past, the introduction of a drug such as intra-muscular gold was delayed until there were definite signs of joint damage (marginal erosions) on radiographs. Over the last twenty years, however, with the publication of double-blind, randomised, placebo-controlled trials of such "second-line" agents, confirming that they can delay the progression of RA (Felson et al, 1990), there has been a trend to start the drugs as soon as the diagnosis is made. The terminology has changed so that the drugs are now referred to as disease-modifying anti-rheumatic drugs (DMARDs) or disease-controlling anti-rheumatic drugs (DCARTs). Table 1.5 lists the DMARDs in clinical use in Britain today. Increasingly, combinations of different DMARDs are being used to try and achieve better suppression of the inflammatory process (Tugwell et al, 1995; O'Dell et al, 1996).

Figure 1.4 Rheumatoid erosions in a wrist joint

Standard reference films for Larsen score of radiographic damage in rheumatoid arthritis (Larsen, 1997)

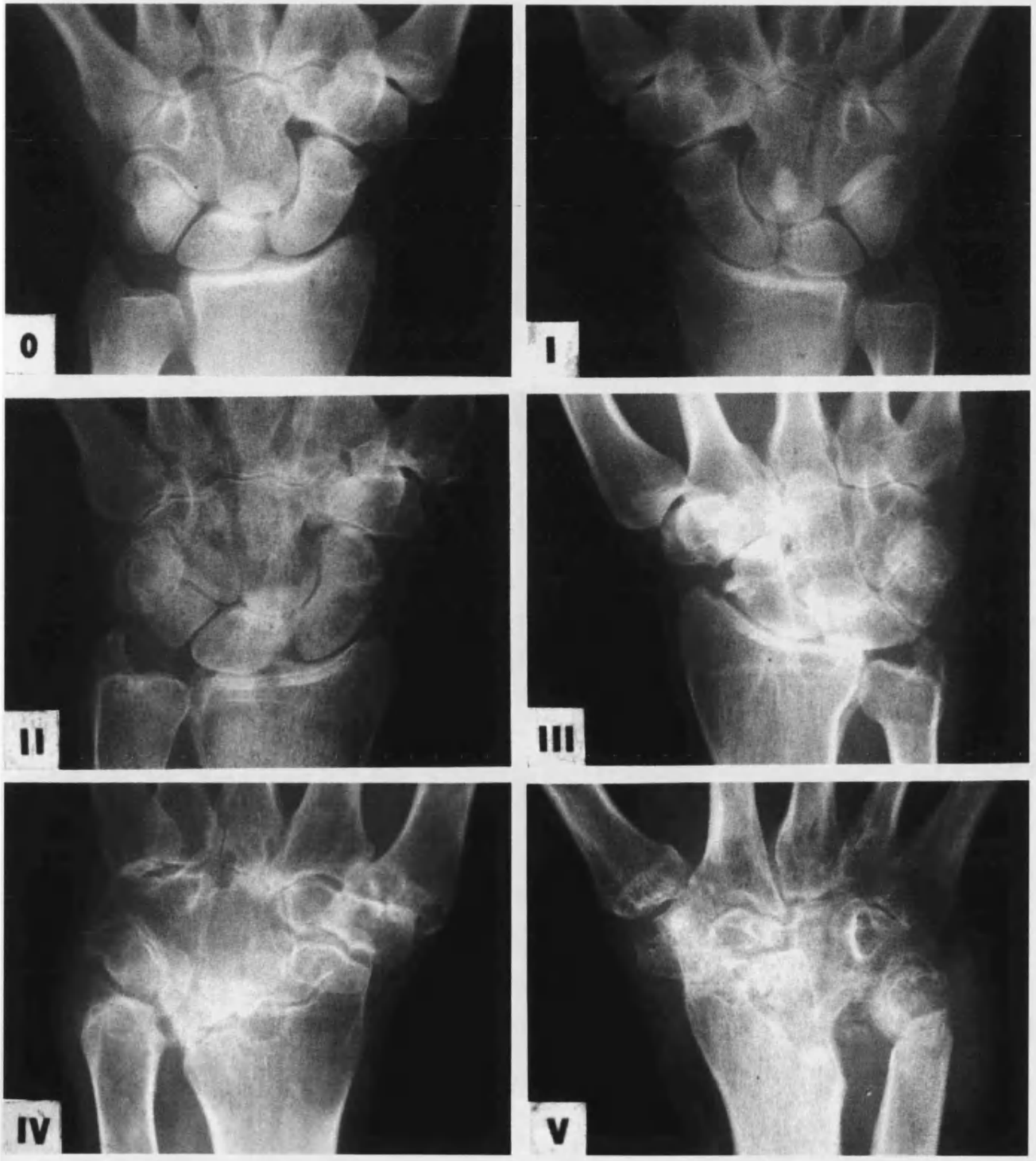


Table 1.5 Disease modifying drugs for rheumatoid arthritis (DMARDs)

Methotrexate
Sulphasalazine
Intramuscular gold
Oral gold
Azathioprine
D-Penicillamine
Hydroxychloroquine
Cyclosporin-A
Corticosteroids

1.3.4 The Concept of Disease Activity in Rheumatoid Arthritis

Rheumatoid arthritis is said to be active when synovitis or extra-articular features are present, necessitating a change in treatment with the aim of suppressing the disease process. This change could be the introduction of a DMARD in a previously untreated patient, an increase in the dose of their current DMARD or a change to a different DMARD if the maximum dose of the drug has not given sufficient benefit. In combination therapy the new DMARD is added to the current regime, with continuation of the other drug.

In the clinic, the rheumatologist considers many pieces of evidence before deciding to change a patient's treatment. The patient may request a change in treatment if they have symptoms of joint pain, swelling and stiffness leading to difficulties in everyday activities including work. The rheumatologist may decide after examining the joints that the level of synovitis detectable clinically merits a change in treatment, even if the patient is uncomplaining.

Laboratory investigations may suggest that the disease is inadequately treated. Blood tests confirm an inflammatory response when the liver produces excess of

certain proteins such as C-reactive protein (CRP). The additional proteins present in the plasma result in increased plasma viscosity (PV) and higher erythrocyte sedimentation rate (ESR). The CRP, PV and ESR are all used in clinical practice to inform treatment decisions. In addition, the patient with inflammation due to any cause may have an anaemia of chronic disease, with low haemoglobin (Hb) and an elevated platelet count. The anaemia improves or resolves with adequate suppression of the inflammatory process.

The importance of identifying and treating active rheumatoid disease is two-fold. In the first place, suppressing inflammation will lead to improvement in the patient's symptoms and joint function in the short-term. Perhaps more importantly synovitis is associated with joint erosion and damage. The long-term outcome of the disease (Section 1.3.7) may be improved by adequate treatment of synovitis in the early stages (Emery et al, 1995). Annual radiographs of the hands and feet are used to monitor the development of erosions in the affected joints. Joint erosions are strictly an outcome measure (Section 1.3.7) but disease progression demonstrated by such radiographic changes may be another reason to change medication.

1.3.5 Assessment of Disease Activity in Rheumatoid Arthritis

Rheumatoid disease activity may be assessed in the clinic in many different ways, according to physician preference and local practice. In the research setting, it is desirable for investigators to use the same parameters to assess disease activity so that results from different studies may be compared.

The European League Against Rheumatism (EULAR) has developed a core set of measurements of disease activity recommended for use in trials of early RA patients

(van Gestel et al, 1996). The suggested measurements are listed in Table 1.6 and include patient-based, physician-based and laboratory assessments.

Table 1.6 EULAR core data set recommended for assessing disease activity in rheumatoid arthritis

Number of swollen joints	Richie or 28 joint count
Number of tender joints	Richie or 28 joint count
Joint pain	on a 10cm visual analogue scale
Patient's global assessment of disease activity	on a 10cm visual analogue scale
Assessor's global assessment of disease activity	on 5 point scale
Acute phase response	ESR or C-reactive protein
Functional assessment	HAQ
Radiological assessment	Larsen score of hands and feet

Although not all of the subjects in this study had early RA, it was decided to use the EULAR core data set with a 28-joint count (Fuchs et al, 1989) so that all subjects had the same measurements made at each visit. This maximised data collection efficiency and made analysis between the different groups easier. From the measurements of disease activity a modified disease activity score (Section 1.3.6) was derived for each patient encounter.

1.3.6 Modified Disease Activity Score

The different measures of disease activity, such as tender joint count or ESR, can be studied separately or can be combined into composite scores. A disease activity score (DAS) has been developed by the investigators who developed the EULAR core data set (van Gestel et al, 1996). A modified version of

the score is used if the joint count is of 28 joints (Prevoo et al, 1995) rather than all synovial joints.

The modified DAS is given by the formula:

$DAS = 0.555\sqrt{(TJC)} + 0.284\sqrt{(SJC)} + 0.7\ln(ESR) + 0.0142(VAS\ PG)$,

where TJC is the tender joint count (28 joints), SJC is the swollen joint count (28 joints), ln is the natural logarithm, ESR is the erythrocyte sedimentation rate and VAS PG is the patient global activity score marked on a 100mm visual analogue scale (VAS) (van Gestel et al, 1996).

The DAS has been validated in 227 patients with RA of duration less than one year (van Gestel et al, 1996) and such patients can be divided into those with low disease activity (DAS $\leq$ 2.4), moderate disease activity (DAS $>$ 2.4 to $\leq$ 3.7) and high disease activity (DAS $>$3.7). Response criteria based on the DAS have been developed and validated and are shown in Table 1.7 (van Riel et al, 1996). The classification of the improvement in DAS depends on the DAS at baseline. A subject with a high baseline DAS of more than 3.7 needs to have a reduction in DAS of more than 1.2 units for it to be classed as a moderate response. This change in someone with low DAS of less than 2.4 at baseline would be termed a good response. The precision of the DAS is 0.6 so a change of more than 0.6 units is unlikely to have occurred by chance (Stucki, 1996).

Table 1.7 Response criteria based on the EULAR disease activity score

DAS at time of comparison with baseline	Improvement of > 1.2 from baseline DAS	Improvement of ≤ 1.2 and > 0.6 from baseline DAS	Improvement of ≤ 0.6 from baseline DAS
DAS ≤ 2.4	Good response	Moderate response	No response
2.4 < DAS ≤ 3.7	Moderate response	Moderate response	No response
DAS > 3.7	Moderate response	No response	No response

1.3.7 Outcome Measurements in Rheumatoid Arthritis

The Health Assessment Questionnaire (HAQ) was developed by Fries (Fries et al, 1980) to evaluate the long-term outcome of RA patients. Kirwan modified the questions relating to disability for use in British patients (Kirwan et al, 1986). The modified HAQ consists of three questions for each of eight types of activities of daily living, such as dressing, washing, reaching and getting out of bed or a chair. The respondent indicates for each question whether they can do the relevant activity with no difficulty (score 0), some difficulty (score 1), a lot of difficulty (score 2), or find it impossible to do (score 3). If aids such as adapted taps or jar opener are usually used, or if help from another person is required, this raised the score for the item to 2, but no higher. The score is added up and can be a maximum of 24 as the highest score for each of the eight stem questions was taken. This is then divided by eight, giving a score of 0 to 3, with 0 indicating no impairment.

1.3.8 Bone Loss Related to Rheumatoid Arthritis

RA is associated with three sorts of bone disease: erosions, juxta-articular osteopenia, and generalised axial and appendicular osteoporosis (Goldring, 1996).

The bone close to a joint with active RA demonstrates increased bone turnover and blood flow, and rapid trabecular bone loss from the distal radius has been shown in early RA (Sambrook et al, 1990). Erosions and juxta-articular osteopenia are included in the diagnostic criteria for RA (Arnett et al, 1988), but the generalised bone loss associated with the disease has only recently been recognised. The site most often reported to have reduced BMD in RA is the hip.

In a controlled, cross-sectional study, Shenstone et al found significantly reduced femoral neck BMD in premenopausal women with RA of less than five years duration (Shenstone et al, 1994). Another cross-sectional study of postmenopausal women found reduced hip BMD compared with controls (Hall et al, 1993). The reduction was greater and also present at the spine if their therapy included corticosteroids. A Dutch study found the mean ESR over the six months prior to BMD measurement was negatively associated with hip BMD (Laan et al, 1993). A twin study of British and Australian twins where at least one had RA found that the BMD was reduced at most skeletal sites in the twin with RA (Sambrook et al, 1995). The greatest difference was again at the femoral neck where the mean reduction was 9.7%. Gough followed 148 patients with early RA over two years and compared them with 730 normal controls (Gough et al, 1994). Some were treated with corticosteroids which may have contributed to the observed bone mass losses of up to 10%. For subjects with active RA despite treatment (CRP greater than 20 mg/dl), the greatest loss in BMD over either one or two years was at the hip, with losses double those seen at the spine. In a group of 120 postmenopausal women with RA, bone mass measured at the hip and distal forearm was significantly reduced at both sites compared to age-matched controls (Lane et al, 1995). Although the reduction

was greatest in the subgroup who were current users of corticosteroids, it was also significant in those who had never used steroids.

It has been suggested that the measurement of hand bone mineral content may be useful in early RA as it correlates with disease activity and BMD at other sites (Peel et al, 1994b). Deodhar et al found that hand bone mineral content (BMC) was inversely correlated in female RA patients with age, disease duration, and two radiological assessments of damage (Larsen's and modified Sharp's) (Deodhar et al, 1994). In this cross-sectional study, disease activity or functional status was not correlated with BMC. However, a longitudinal study by the same group, with repeat hand BMC estimation after one year in eighty-one patients, found that the rate of loss of BMC correlated with baseline disease activity as assessed by C-reactive protein levels (Deodhar et al, 1995).

The biochemical assessment of bone turnover in RA patients may be affected by the disease process. The ratio of pyridinium collagen cross-links (Section 1.1.10) in synovial tissue is 10:1 (Pyd: Dpd) compared with approximately 50:1 in articular cartilage and 4:1 in bone (Pearce et al, 1995). In active RA with proliferation of synovial tissue, bone resorption could be over-estimated if Pyd alone or the ratio were measured. The deoxypyridinoline (Dpd) cross-link is more specific for bone, and thus in RA subjects Dpd alone rather than the ratio should be used (Black et al, 1989).

1.4 Methotrexate

1.4.1 Introduction

Methotrexate (MTX) is the anti-metabolite drug, 4-amino-N10-methylpteroyl-L-glutamic acid, whose structure is close to that of tetrahydrofolate as shown in Figure 1.6 (Jolivet et al, 1983). It is a weak bicarboxylic organic acid with a molecular weight of 454, is negatively charged at neutral pH and has limited lipid solubility. The main action of MTX is the competitive inhibition of dihydrofolate reductase (DHR) shown in Figure 1.7. This enzyme reduces dietary folate (which is metabolically inactive) first to dihydrofolate and then tetrahydrofolate which is essential in the synthesis of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). MTX can thus effectively halt protein synthesis if given in high doses (Jolivet et al, 1983) and this action is utilised in chemotherapy. MTX also inhibits other enzymes such as thymidylate synthetase, glycinamide ribotide transformylase and 5-aminoimidazole-4-carboxamide ribotide transformylase, all of which affect protein synthesis (Jolivet et al, 1983). In addition, MTX has anti-inflammatory and cytokine-modulating effects described in Section 1.4.5 below. MTX enters cells through the active transport system used by folate and leucovorin (folinic acid) and is converted to polyglutamated forms by the enzyme folyl-γ-polyglutamate synthase. The MTX polyglutamates are retained intracellularly for a long time and are probably even more active than the parent compound (Chabner et al, 1985). The importance of polyglutamation was demonstrated in studies of a human breast cancer cell line when reduced polyglutamation was associated with resistance to MTX (Jolivet et al, 1983).

Figure 1.5 The structure of tetrahydrofolate and methotrexate

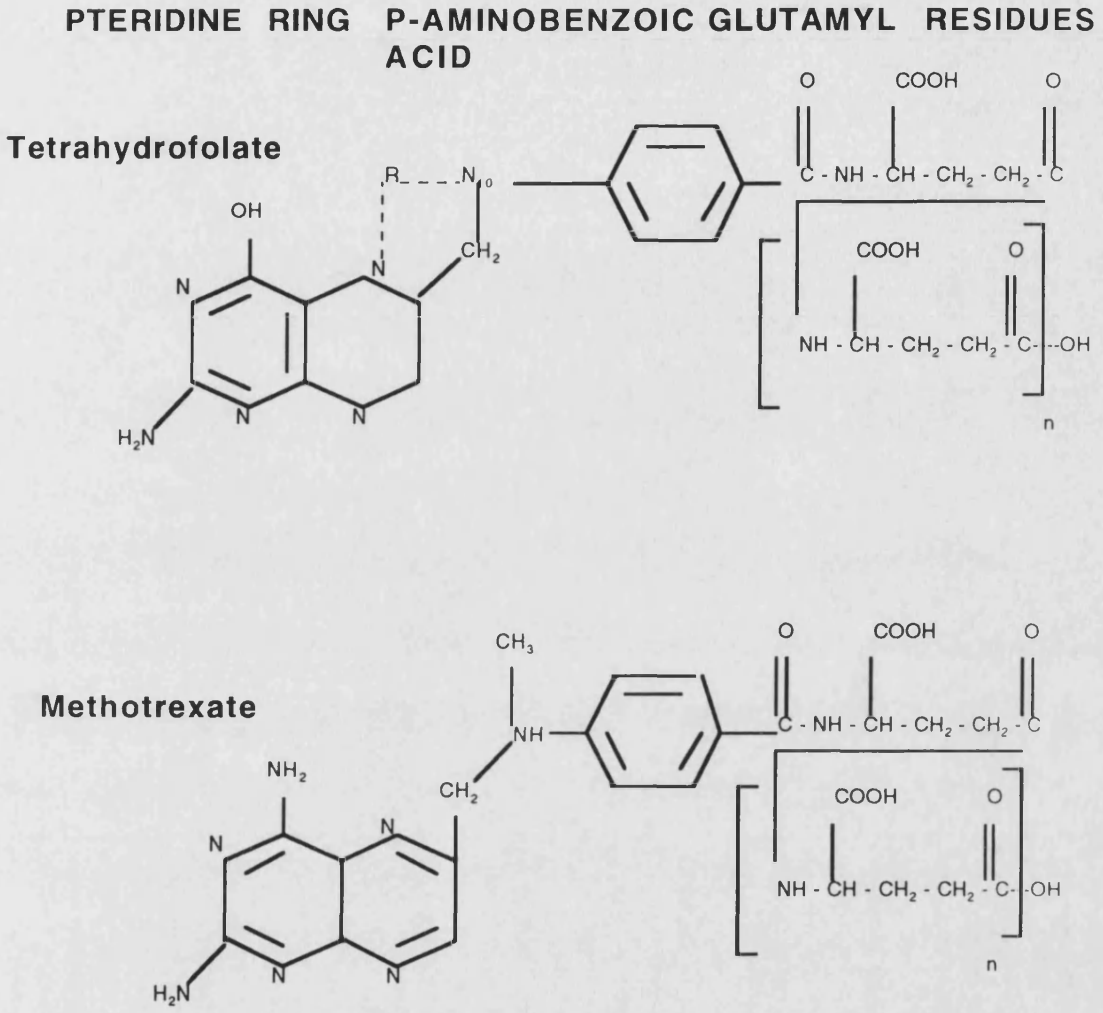
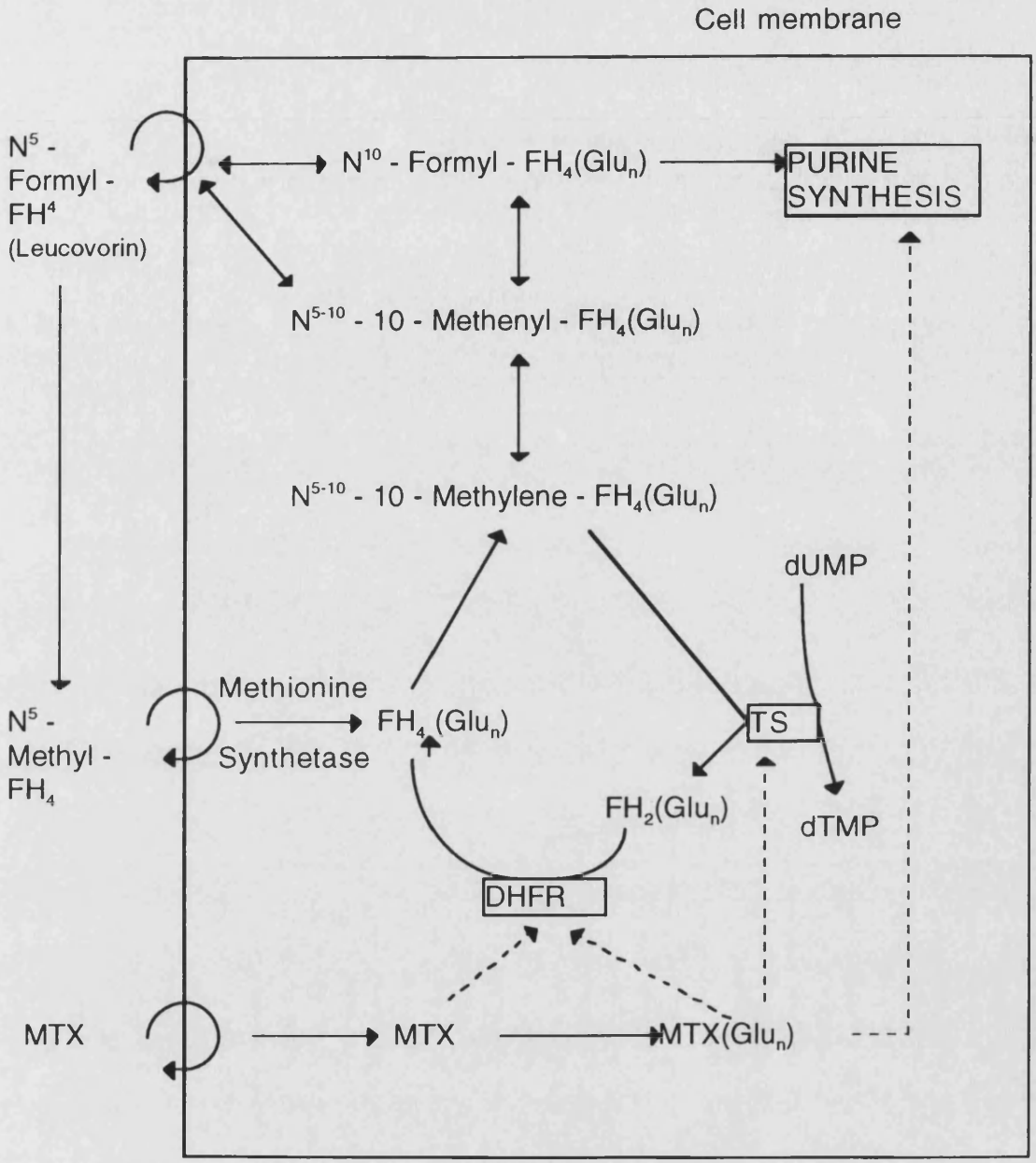


Figure 1.6 Main actions of methotrexate



MTX denotes methotrexate, DHFR dihydrofolate reductase, TS thymidylate synthetase, FH_4 tetrahydrofolate, FH_2 dihydrofolate, Glu glutamyl, dTMP thymidylate, and dUMP dioxyuridylate. Broken lines indicate enzyme inhibition.

1.4.2 Indications for Methotrexate

MTX at the high dose of 100 to 1000mg/m^2 body surface area per cycle with leucovorin rescue is used in combination with other anti-neoplastic drugs to treat many solid and haematological malignancies (Jolivet et al, 1983). Tumours sensitive to MTX include breast and ovarian carcinomas, acute lymphocytic and non-lymphocytic leukaemias and osteosarcoma.

At the lower dose of 2.5 to 25mg per week, MTX is used to treat a variety of chronic conditions (Weinblatt, 1995) including rheumatoid arthritis (Section 1.4.3). MTX has also been used as a steroid-sparing agent in polymyalgia rheumatica and giant cell arteritis (Krall et al, 1989), severe asthma (Yntema et al, 1993) and Crohn's disease (Weinblatt, 1995). It has been used since the 1960's as a disease-modifying treatment in psoriasis (Tung et al, 1990) and improves both the skin and joint symptoms. However, radiological damage may progress despite this (Abu-Shakra et al, 1995).

The teratogenic effects of MTX have led to its use, in conjunction with the prostaglandin E_1 analogue misoprostol, as an abortifacient (Hausknecht, 1995).

1.4.3 Low Dose Methotrexate in Rheumatoid Arthritis

The first report of the use of MTX in RA was in 1951 by Gubner and colleagues (Gubner et al, 1951). They gave daily MTX in a dose of 1 to 2mg to seven cases of inflammatory arthritis. Of these, six probably had RA and one had psoriatic arthritis. There was a definite improvement in symptoms after only two to six days but side effects limited therapy. Most subjects had an increase in inflammatory joint symptoms on stopping MTX.

The first placebo-controlled trials of MTX in RA were published in 1985 (Williams et al, 1985; Weinblatt et al, 1985). Williams found a significant improvement in clinical variables in the MTX group compared to placebo after eighteen weeks of therapy, but one third of the MTX treated patients withdrew from the trial because of side-effects (Williams et al, 1985). Weinblatt used a double-blind, placebo-controlled, cross-over design and found significant differences in joint counts, morning stiffness and disease activity assessment according to the physician at the end of the twelve weeks treatment in the group which had received MTX (Weinblatt et al, 1985). Similar improvements were seen in the group originally treated with placebo, after cross-over.

A double-blind, randomised trial of twenty-six weeks' treatment with either intra-muscular gold sodium thiomalate or MTX (either oral or intra-muscular) demonstrated a useful clinical improvement in both groups, with no statistically significant difference between the drugs (Morassut et al, 1989). However, the numbers studied were small with only fifteen out of the eighteen patients randomised to MTX, and twelve out of the seventeen randomised to gold completing the trial. A larger study comparing intramuscular MTX with intramuscular gold (IMG) had a total of 126 subjects completing one year of follow-up, seventy-three on MTX and fifty-three on IMG, with greater than 50% mean improvement in clinical variables with no significant differences between groups (Rau et al, 1997). There were more adverse drug effects in the IMG group (83.9%) than in the MTX group (66.7%), but most were mild. A total of thirty-eight subjects withdrew because of side effects, six in the MTX and thirty-two in the IMG group. However, more subjects on IMG achieved remission, 25% compared with 14% in the MTX group.

Double-blind trials of MTX against azathioprine have failed to show a significant difference in effectiveness between the two agents (Hamdy et al, 1987; Willkens et al, 1992). However, a meta-analysis published in 1992 of radiographic progression in DMARD-treated RA found that MTX was superior to azathioprine and equally effective as gold salts in reducing disease progression as assessed by new erosions (Alarcón et al, 1992). Further evidence that MTX may be classified as a DMARD came from a study by Rau who prospectively evaluated the radiographs of twenty-four RA patients commencing MTX (Rau et al, 1991). All the subjects had failed intramuscular gold treatment previously and had radiographs of hands and feet taken every one or two years. The follow-up period varied from one to five years. There was a significant reduction in the percentage of joints developing erosions in the patients once they had been started on MTX compared to when they were on gold. However, there have also been studies which found MTX to have no effect on radiographic progression (Bologna et al, 1997), leading some commentators to suggest the beneficial effect on symptoms is due solely to its anti-inflammatory actions (Section 1.4.5).

There have been open observational studies of MTX therapy in RA published with follow-up for up to seven years (Weinblatt et al, 1988; Fehlauer et al, 1989; Weinblatt et al, 1994; Kremer et al, 1992; Weinblatt et al, 1992; Bologna et al, 1997). Drug survival rates have been estimated as 50% at five years (Alarcón et al, 1989), and 46% at seven years (Weinblatt et al, 1992), although this included all dropouts, including administrative ones. Some authors have reported more than 70% of subjects remaining on MTX after a five year retrospective study (Bologna et al, 1997) or a prospective study for a mean of seven

and a half years (Kremer et al, 1992). As the experience of prescribing MTX increased, the main reason for discontinuation was adverse events (60%), rather than lack of efficacy (7.7%) (Alarcón et al, 1989). Many adverse events are minor and do not require cessation of MTX, but some are potentially life-threatening (Section 1.4.6).

Low dose, weekly MTX is now the DMARD most likely to be prescribed first for RA in the United States (Weinblatt, 1996). In the United Kingdom, MTX vies with sulphasalazine (SPZ) as the first-line DMARD of choice in RA as it is better tolerated, has a longer drug survival and faster onset of action, but does need careful monitoring (Anonymous, 1995). Recently, MTX has been used in combination with other DMARDs with reportedly superior disease suppression and no increase in side effects (O'Dell et al, 1996; Tugwell et al, 1995).

1.4.4 Pharmacology of Low-Dose, Pulse Oral Methotrexate

When MTX is taken in low-dose by mouth, the resulting serum levels of the drug vary greatly from subject to subject. One study comparing the bioavailability of intra-muscular or sub-cutaneous routes with the oral route, found a range of 0.54 to 0.99 (mean 0.85) of the parental dose (Jundt et al, 1993). Peak serum levels after oral tablet treatment occurred at approximately three hours post dose (0.06 ± 0.02 μmol/l) and MTX was undetectable (<0.01μmol/l) between sixteen and twenty-four hours post-dose (Jundt et al, 1993). In contrast, Sinnett et al could still detect MTX in the serum at twenty-four hours in all ten RA patients they studied despite having a less sensitive detection system (minimum 0.03μmol/l) (Sinnett et al, 1989). They also found rapid absorption after a single oral dose, with peak serum concentrations at one and a quarter hours. MTX elimination (either by renal excretion of the

unmetabolised drug, or by cellular uptake) was consistent between subjects, with a mean serum half life of nearly five hours (Sinnett et al, 1989). Differing bioavailability is probably due to factors such as age, renal and hepatic function, and gastrointestinal features such as differing ability of gastrointestinal flora to metabolise MTX (Jundt et al, 1993). Food has not been shown to affect absorption or bioavailability (Oguey et al, 1992).

A study of tissue levels of the drug following intra-venous MTX in RA patients found that at twenty-four hours after administration, synovial fluid levels were greater than serum, but levels were undetectable in both compartments by seventy-two hours (Tishler et al, 1989). Bologna and colleagues assayed the concentrations of MTX in the synovial membrane, and in cortical and trabecular bone of ten RA patients treated with the drug for a mean of twenty-six months (Bologna et al, 1994). They were able to do this as the patients came to surgery; five were having wrist synovectomy, two each having shoulder and knee arthroplasty and one having a hip arthroplasty. The day prior to surgery, each patient had an intra-muscular injection of 10mg MTX. They were found to have similar high mean levels of MTX in the synovial membrane (0.285 nmoles/g) and in both trabecular (0.292 nmoles/g) and cortical (0.286 nmoles/g) bone whilst the plasma concentration was ten-fold less (0.0252 nmoles/ml).

1.4.5 Actions of Methotrexate in Rheumatoid Arthritis

MTX has been shown to have multiple anti-inflammatory and cytokine modulating effects at low dose and they are summarised in the text below and in Table 1.8. The precise action of MTX, as for all DMARDs, remains unclear, but some authors have proposed it is primarily an anti-inflammatory agent, rather than an

immunosuppressive, at the doses used in RA (Bologna et al, 1997; Furst, 1997; Klippel et al, 1985).

In the clinic, MTX reduces symptoms in the RA patient much more rapidly than do other DMARDs, in keeping with an anti-inflammatory effect. Within one week of an intra-venous injection of MTX in eighteen RA patients, there was a significant fall in two markers of the acute phase response, the erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) (Segal et al, 1989a). Cronstein and colleagues used a murine model to study the effect of MTX on inflammation (Cronstein et al, 1993). They found that, through the intracellular accumulation of 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR), MTX could increase adenosine release and inhibit leukocyte accumulation at sites of cell injury. Previously, an Australian group had shown a specific inhibition of polymorphonuclear chemotaxis *in vitro* when RA patients had received MTX (O'Callaghan et al, 1988). Other *in vitro* studies have concentrated on the metabolism of arachidonic acid because many pro-inflammatory molecules are generated from this precursor. Both acute (twenty-four hours after the first ever dose of MTX) and chronic (after six to eight weeks of treatment) suppression of leukotriene-B4 synthesis by neutrophils from RA patients was found (Sperling et al, 1992), suggesting reduced activity of the 5-liopoxygenase enzyme. Further evidence of an anti-inflammatory effect comes from studies of polymorphonuclear leukocytes from patients with RA who are treated with MTX, which have reduced production of superoxide anions (Laurindo et al, 1995). Also, in a rat model, MTX reduced adherence of leukocytes in mesenteric venules (Asako et al, 1993).

Many of the cytokines which are deranged in RA have been studied with regard to MTX. MTX has inhibitory actions on IgM-rheumatoid factor synthesis (Olsen et al, 1987), macrophage activation (Johnson et al, 1988; Hu et al, 1988), actions of interleukin-1 (IL-1) (Segal et al, 1989b) and production of interleukin-1β (IL-1β) by peripheral blood mononuclear cells (Barrera et al, 1994) and by synovial fluid mononuclear cells (Thomas et al, 1993). The production of interleukin-6 (IL-6), interleukin-8 (IL-8), interleukin-1-receptor-antagonist (IL-1Ra), tumour necrosis factor-α (TNFα) and soluble tumour necrosis factor receptor (sTNFR) have all been shown to be affected by MTX (Kremer, 1994). Soluble interleukin-2 receptor (sIL-2R) and p55 levels are reduced by MTX in RA (Barrera et al, 1993). MTX therapy increases the expression of the anti-inflammatory cytokines interleukin-4 (IL-4) and interleukin-10 (IL-10) and decreases the expression of the pro-inflammatory cytokines interleukin-2 (IL-2) and interferon-γ (IFNγ) (Constantin et al, 1998).

It has been suggested that the pre-treatment ratio of serum IL-1Ra to interleukin-1β (IL-1β) predicts the clinical response to MTX (Seitz et al, 1995). However, serum IL-2R levels did not predict a response to treatment with MTX in refractory RA (Polisson et al, 1994). A study following subjects for thirty-six months after starting MTX measured interleukin-2 (IL-2), IL-6, TNF-α and IL-1Ra serially (Straub et al, 1997). The best predictor for the outcome at three years was baseline IL-6. Specifically, a decrease in IL-6 during the first six months of treatment with either intramuscular MTX or intramuscular gold was predictive of a good outcome (Straub et al, 1997).

Table 1.8 Actions of methotrexate in rheumatoid arthritis

Type of action	Cell type or chemokine acted on	Effect
Anti-inflammatory effects	**Acute phase response**	Reduced CRP and ESR
	Neutrophils	Inhibition of chemotaxis
		Inhibition of neutrophil adherence due to increased adenosine release secondary to intracellular accumulation of AICAR
		Suppression of LT-B4 synthesis
		Reduced production of superoxide anions
	Mononuclear lymphocyte effects	Increased T cells and T helper cells
		Decreased T suppresser cells
		Decreased absolute lymphocyte counts
		Reduced IgM Rheumatoid factor
Cytokine effects	**Macrophages**	Inhibition of activation
	Pro-inflammatory cytokines	Inhibition of IL-1 production from macrophages
		Inhibition of IL-1 activity
		Inhibition of synthesis of IL-2, IFN-γ, TNF-α, sTNF-αR and SIL-2R
		Inhibition of IL-6, IL-8
	Anti-inflammatory cytokines	Increased expression of IL-4 and IL-10
Other effects	**Collagenolytic proteases**	Inhibition
	Angiogenesis	Inhibition

1.4.6 Side Effects of Methotrexate

Table 1.9 shows the main adverse effects associated with low dose, weekly MTX therapy (Furst, 1997). The most common reason for discontinuation of MTX is toxicity rather than lack of effect (Weinblatt et al, 1994) and the gastrointestinal system is most often adversely affected, with mouth ulcers, nausea and vomiting (Weinblatt, 1996). Mouth ulcers occurred in up to 30% of patients in one survey (Anonymous, 1995), but can often be avoided or alleviated by the concurrent administration of low dose folic acid (Section 1.4.7).

Bone marrow suppression with pancytopenia can be an idiosyncratic reaction and may occur at any time during treatment so regular full blood count monitoring every four to six weeks is mandatory (Gutierrez-Ureña et al, 1996). More often though, a predisposing cause for the pancytopenia exists such as untreated folate deficiency, hypoalbuminaemia, impaired renal function, concurrent infection, or treatment with either probencid, which impairs the renal excretion of MTX, or the anti-folate antibiotics trimethoprim or sulphamethoxazole (Weinblatt, 1996; Steuer et al, 1998). Impaired renal function has been shown to increase the risk of serious side effects by approximately four times (Rheumatoid arthritis clinical trial archive group, 1995). Long-term MTX therapy is associated with hepatic fibrosis and cirrhosis when used in psoriasis patients, but the risk of hepatotoxicity appears less in RA (Schnabel et al, 1994). Up to 70% of RA patients may develop elevated transaminases during five years of MTX therapy (Scully et al, 1991) but these are not reflected by abnormalities on liver biopsy and the blood tests usually return to within normal levels if the MTX is withdrawn for a few weeks. It has been suggested that the

changes seen on liver biopsy are due to the rheumatoid process rather than MTX (Rau et al, 1989).

Pulmonary toxicity in the form of acute interstitial pneumonitis is a rare but potentially fatal complication of treatment with MTX. In one series, the fatality rate was 17.2% (Kremer et al, 1997). Lung function tests do not enable identification prior to the clinical symptoms of pneumonitis (unproductive cough and dyspnoea) and many asymptomatic RA patients have slightly abnormal pulmonary function tests (Cottin et al, 1996). As pre-existing lung disease may be a predisposing factor for the development of this side effect, some authors have recommended that a chest radiograph be taken prior to commencing treatment, which may also be useful for comparison if symptoms develop (Kremer et al, 1997).

MTX is teratogenic (Bermas et al, 1995) and should not be prescribed to premenopausal women unless adequate contraception is practised. In both sexes impaired fertility has been reported and, in women, menstrual dysfunction and amennorrhoea may occur (Anonymous, 1994).

Concern that MTX may increase the risk of post-operative infections and delay wound healing leads approximately 20% of British orthopaedic surgeons to stop MTX for two weeks before and two weeks after an arthroplasty (Steuer et al, 1997), although the manufacturers (Lederle, UK) do not recommend this.

Although MTX is thought to have low oncogenic potential, there are case reports of Epstein-Barr associated non-Hodgkin's lymphoma developing in MTX-treated RA patients (Bachman et al, 1996). The malignancies resolved completely on stopping the MTX and no chemotherapy was necessary.

Opportunistic infections such as *Herpes zoster* encephalomyelitis (Lyon et al, 1997) and *Pneumocystis carinii* pneumonia (Wallis et al, 1989) have been reported in patients on low dose MTX for RA or psoriatic arthritis.

Other unwanted effects secondary to MTX treatment include hair loss, accelerated nodulosis, cardiac nodules, photosensitivity and dermatitis (Anonymous, 1995; Scully et al, 1991).

Table 1.9 Adverse effects associated with methotrexate

System involved	Adverse effect	% affected
Gastrointestinal	Nausea, vomiting	19-65
	Stomatitis	2-55
Central nervous system	Headache, fatigue, malaise	13-35
Haematological	Anaemia	1-2
	Leucopenia	2-21
	Thrombocytopenia	1-5
Immune	Opportunistic infection	Rare
Skin	Alopecia	1-6
	Rash	2-15
Liver	Cirrhosis	Rare
	Elevated transaminases	8-30
Osteopathy	Bone pain, fracture	Case reports only
Pulmonary	Pneumonitis	1-7
Pseudolymphoma		Case reports only
Teratogenicity		Definite

1.4.7 Use of Folic Acid and Folinic Acid in Methotrexate Therapy

Following high dose MTX as a chemotherapeutic treatment, folinic acid (leucovorin) rescue must be given within forty-eight hours to prevent necrosis of the bone

marrow and gut epithelium (Rosen et al, 1974). If this is not done, severe toxic reactions occur with cytopenia and diarrhoea.

Both folic acid (1 to 27.5mg per week) and folinic acid (a synthetic tetrahydrofolate, 1 to 20mg per week) have been used to try and reduce the incidence of side effects from low-dose MTX (Anonymous, 1995).

A systematic review was published in 1997 of the seven double-blind, controlled trials of folate or folinic acid supplementation in RA patients treated with low dose MTX (20mg per week or less) (Ortiz et al, 1998). It showed that folic acid (either low or high dose) reduced mucosal and gastrointestinal side effects by 79% which was statistically significant, but folinic acid only reduced such side effects by 42% (not significant). At higher doses, folinic acid was associated with higher tender and swollen joint counts, suggesting antagonism of the treatment effect of MTX. This was not seen with folic acid. It was concluded that low dose folic acid (5mg per week) was the most effective and cheapest way to reduce gastrointestinal side effects from MTX.

1.4.8 Evidence that High Dose Methotrexate Causes Osteoporosis

MTX osteopathy has been described in children receiving high dose MTX as chemotherapy for haematological malignancies and osteosarcomas. The syndrome of bone pain, radiographic osteopenia and fractures has been reported in both short term (Ecklund et al, 1997) and long term use up to five years (Schwartz et al, 1984). A recent retrospective survey found that 9% of children receiving high dose MTX ($12g/m^2$ for an average of nine cycles) as therapy for osteosarcoma had developed radiographic changes consistent with osteopathy (Ecklund et al, 1997).

1.4.9 Animal Studies of the Effect of Methotrexate on Bone

The effects of MTX are different depending on whether high or low dose MTX is used and whether or not the animals have experimental arthritis.

In a rat heterotopic bone model using high doses, the timing of the dose of MTX affected the result (Nilsson et al, 1987). Thus, if MTX was administered at the same time as the heterotopic bone (from another animal) was implanted, new bone formation in the implant was reduced by 40%. When MTX was given ten days after implantation, it had less effect as heterotopic bone formation was reduced by only 22%. However, if the MTX was given ten days before the bone was transplanted, it was associated with increased bone formation of 33% (Nilsson et al, 1987). In a subsequent paper, the same group reported the findings from studies of MTX and another chemotherapeutic drug, adriamycin (ADR), on the formation of both heterotopic and orthotopic (native) bone in rats (Nilsson et al, 1990). MTX and ADR are often used in combination to treat bone malignancies. Both drugs inhibited new (heterotopic) bone formation but had relatively little effect on orthotopic bone. The drugs did not seem to affect bone turnover as much as new bone formation. Unlike ADR, MTX inhibited the overall growth of these young rats.

May and co-workers administered low-dose MTX (corresponding to doses used in RA in humans) to rats over a sixteen week period (May et al, 1994). Histomorphometry of the long bones demonstrated suppressed osteoblast activity and increased osteoclastic recruitment in the treated animals compared with controls.

In a study of the short and long-term effects of MTX on the rat skeleton, Wheeler and colleagues gave MTX daily for five days on two occasions and then harvested

the bones after 30, 80 or 170 days (Wheeler et al, 1995). They performed histomorphometric and biomechanical studies and found the MTX-treated groups had significantly lower trabecular bone volume at the 80 and 170 day time points. The mineral appositional rate was only reduced at 170 days but the trabecular mineralising surface and longitudinal bone growth was significantly depressed in all three MTX-treated groups. In contrast to the suppressed bone formation indices, bone resorption was enhanced as trabecular osteoclast surface was increased at all time points compared with controls. The cortical cross-sectional area and periosteal mineral appositional rate were lower in the femur and the tibia at all time points but the periosteal mineralising surface was lower in the MTX treated group only in the femur. MTX had minimal effects on the biomechanical properties of the bones studied. In summary, in normal rat bone, MTX decreased bone volume, bone formation and osteoblast activity and increased osteoclast activity and these effects were still detectable long after the treatment period (Wheeler et al, 1995).

In a study of rats with adjuvant-induced arthritis, low-dose MTX maintained bone mass and prevented the bone loss seen in untreated arthritic animals (Segawa et al, 1997). This suggested that the suppression of the arthritis by MTX was more important than any adverse effect of the drug on bone formation or turnover.

1.4.10 In Vitro Studies of the Effect of Methotrexate on Bone

A group from Utrecht have studied the effect of MTX in cultures of human osteoblasts (Scheven et al, 1995) and articular cartilage (van der Veen et al, 1996). They found that MTX inhibited proliferation of human trabecular-derived osteoblast-like cells in a dose-dependent fashion over the range 1 to 100nM, with little additional suppression occurring above this dose (Scheven et al, 1995). The

addition of $1,25(OH)_2$ Vitamin D_3 caused further suppression of proliferation but enabled differentiation to a mature osteoblast-like phenotype, with associated alkaline phosphatase and osteocalcin expression. In a second paper MTX had no effect on proteoglycan synthesis in cultures of human articular cartilage (van der Veen et al, 1996). As before, MTX inhibited proliferation of osteoblasts but did not affect either the number of cells expressing alkaline phosphatase nor the activity of this enzyme.

May and colleagues studied the effect of MTX on mouse bone cells *in vitro* (May et al, 1996). Neonatal mouse calvariae-derived bone cells were cultured with either 10% (for osteoblast-like cells) or 2% (for osteoclast-like cells) fetal calf serum. MTX at a range of doses from 0.5nM to 600nM was added after one week in culture. No effect on osteoblast or osteoclast cell number was observed, but MTX did cause a reduction in the level of osteocalcin in the supernatant and in the calcification of the matrix produced by the cells. This suggested, in contrast to the human studies of Scheven et al described above, that in the mouse, MTX affected cell differentiation rather than proliferation.

In a study comparing the two anti-rheumatic drugs MTX and sulphasalazine (SPZ), a dose-dependent toxic effect of MTX on the rat osteosarcoma cell line UMR 106 at doses greater than 10nM after only twenty-four hours was found (Preston et al, 1997). These concentrations are similar to those found in the serum of RA patients during the first twenty-four hours after taking their weekly dose (Section 1.4.4). MTX did not inhibit proliferation or viability of human umbilical endothelial cells and only caused reduced viability to 34% after four days in culture of peripheral blood mononuclear cells. The bone cell line thus seemed more sensitive than the other

cell lines tested to the toxic effects of MTX. Interestingly, SPZ at a dose of 100μg/ml inhibited UMR 106 proliferation significantly after forty-eight hours or longer in culture. SPZ is not thought to have any affect on bone in humans, although this has not been studied.

1.4.11 Reports of the Effect of Low Dose, Weekly Methotrexate on Bone in Humans

The first case report of osteoporosis associated with low dose MTX use was in 1993 when Preston reported two patients (one with psoriatic arthritis and one with RA), each of whom had stress fractures of tibia after five and six years respectively of MTX therapy (Preston et al, 1993). Neither had ever taken oral corticosteroids. Bone biopsies were performed which excluded osteomalacia but showed reduced osteoid surfaces and osteoid thickness and a low bone formation rate.

A further case report in 1995 was of a 75 year old man with a fifteen year history of RA who had been taking MTX 7.5mg/day for five years (Schapira et al, 1995). He developed an insufficiency fracture of the tibia but had been taking prednisolone 10mg/day for at least five years and may thus have had corticosteroid-associated osteoporosis.

MTX-associated fragility fractures were reported in two postmenopausal women with RA in 1996 (Maenaut et al, 1996). The first had previously been treated with oral corticosteroids (mean dose 15mg/day for 1.5 years) prior to starting MTX, and had already sustained a fragility fracture of the malleolus in the past. She had taken MTX for eight months when multiple metatarsal fractures occurred. The second had never been treated with corticosteroids and after three years of MTX treatment suffered two stress fractures of the distal tibia, separated by six months. In each

case, BMD measurements were available for before and after MTX therapy. Both women had lost bone mass at the radius whilst the lumbar bone mass was stable. The difference in the Z score expressed as percentage of age-matched control values for BMD of the distal 8cm of the radius was 35% and 29% respectively.

It was reported in an abstract in 1992 that the serum osteocalcin was reduced in nine patients after starting MTX for RA (Ward et al, 1992). Samples had been taken before and at up to eighty-one months after starting MTX.

During the first year of this study, several abstracts were presented and they are summarised in Table 1.10. All found no effect of MTX on the bone density or fracture rates in RA (West et al, 1995; Buckley et al, 1995; Ide et al, 1995; Stainbrook et al, 1995).

Table 1.10 Abstracts published in 1995: MTX and bone in RA

Authors	Number of subjects on MTX	Control group	Steroid co-therapy	Calcium co-therapy	Vitamin D co-therapy	Outcome measure
West,S.G.et al	34	no	yes	yes	yes	BMD: LS, FN, mid-radius
Buckley,L. et al	63	yes	not stated	yes	yes	BMD: LS and FN
Stainbrook, D. et al	343	yes	yes	no	no	fragility fracture
Ide,M. et al	51 in total	yes	yes	no	no	BMD: LS and radius. Serum osteocalcin

In 1996, a group which had presented their findings the previous year (Ide et al, 1995) reported follow-up BMD data on their cohort. Serial scans of the forearm suggested that MTX may protect against appendicular bone loss in RA as the loss of

bone in the distal forearm was less in the group treated with MTX alone compared to those treated either with MTX and corticosteroids, or other DMARDs (Ide et al, 1996).

Also in 1996, a group from Mount Sinai, New York reported a significant reduction in the BMD of the proximal third of the forearm in eleven postmenopausal women receiving the drug for primary biliary cirrhosis (Blum et al, 1996). They were compared with eleven patients who had received MTX for less than one month. Bone turnover was assessed by serum osteocalcin, serum bone-specific alkaline phosphatase and urinary N-telopeptide and was not significantly different between the two groups. However, both osteocalcin and alkaline phosphatase were correlated negatively with bone mass and contributed to the impact of MTX on forearm BMD.

A three year follow-up study of the subjects presented after two years as an abstract in 1995 (Buckley et al, 1995) was published in 1997 (Buckley et al, 1997). At three years, low dose MTX was not associated with lower BMD of the femoral neck or lumbar spine compared with subjects on low dose prednisolone in a group of ninety-five subjects who completed the study. However, the combination of MTX and prednisolone did result in greater bone loss from the lumber spine.

A two year prospective but uncontrolled study of nineteen non-steroid treated RA patients from Angiers, France found no difference in BMD of the lumbar spine, hip, femoral neck, radial ultra-distal or proximal third region (Pascaretti et al, 1997). The only marker of bone turnover assessed was osteocalcin. There was no difference between the groups. The lack of a control group, failure to measure bone resorption

or assess RA disease activity are criticisms of this study, as is the small number of subjects.

The only full paper published on the effect of MTX on BMD in RA was in 1997 (Buckley et al, 1995). RA subjects who were taking part in a randomised, placebo-controlled study of calcium and vitamin D_3 supplementation on BMD of the lumbar spine and femoral neck were included. After three years, lumbar spine and femoral neck BMD was not affected by low dose MTX but those patients who were on prednisolone greater than 5mg per day in addition to MTX had more loss in the lumbar spine than those who were on MTX alone. Forearm BMD measurements were not made.

1.4.12 Summary of the Evidence of an Effect of Methotrexate on Bone

MTX impairs bone formation *in vitro*. The adverse effect is less in animal models of inflammatory arthritis than in normal animals. Methotrexate osteopathy is not uncommon in children treated with high dose MTX for malignancies and maintained on it to prevent remission. However, in adults with inflammatory conditions, osteopathy is rare in patients who are not taking long-term oral corticosteroids. Cortical bone in the forearm was adversely affected in MTX-treated primary biliary cirrhosis (Blum et al, 1996). In the few case-controlled studies of long-term, low-dose MTX therapy for RA in adults, it does not appear to cause a deleterious effect on bone mineral density or bone turnover. However, there have been no studies where an attempt has been made to control for factors which may affect bone mineral density such as disease duration, activity and joint damage.

2. METHODS: CLINICAL STUDY

2.1 Introduction

The aim of the clinical study was to investigate the effect of methotrexate (MTX, Section 1.4) on bone density and bone turnover when used to treat rheumatoid arthritis (RA, Section 1.3). The study was designed to test the hypothesis that MTX had an adverse effect on bone turnover, based on evidence from animal and *in vitro* studies, chemotherapy experience and case reports (sections 1.4.8 to 1.4.12). The study included patients who had been exposed to MTX for both a short and long time, as adverse drug effects may become apparent only with prolonged use, especially in a tissue such as bone with a slow turnover.

As bone mineral density is currently the best available method for predicting future fracture risk (Melton et al, 1993), the primary outcome measure was the bone mineral density measured by dual x-ray absorptiometry of the lumbar spine, hip and forearm.

Bone turnover was measured by formation markers in the serum (bone specific alkaline phosphatase and osteocalcin) and by resorption markers in the urine (pyridinoline and deoxypyridinoline collagen cross-links). Together these measurements constituted the secondary outcome measures.

In addition, histomorphometric analysis of dual-labelled iliac crest bone biopsies was performed on a subgroup of subjects to study the effects of MTX at the tissue level *in vivo*.

2.2 Study Design

The study was a pragmatic, open and non-randomised study of RA patients. Those treated with MTX were compared with those who had not received MTX.

It was planned to recruit patients into the four groups described below. No group included normal subjects; all had RA.

Starting MTX Group (Starting MTX)

Patients who had developed RA within the previous two years, who had not been treated with a disease-modifying drug (DMARD) before (section 1.3.3), who had active RA (section 1.3.4) and were starting MTX for the first time.

Control Group for Group Starting MTX (Starting SPZ)

Patients who had developed RA within the previous two years, who had not been treated with a disease-modifying drug before, who had active RA and were starting sulphasalazine (SPZ) for the first time. As they had never had a DMARD before, by definition they had not had MTX in the past.

Established on MTX Group (Continuing MTX)

RA patients who had been taking low dose weekly MTX continuously for at least five years.

Control Group for Group Continuing MTX (Continuing Other DMARD)

RA patients who had been taking a disease-modifying anti-rheumatic drug (DMARD) other than MTX continuously for at least five years, and who had never taken MTX.

The conditions of entry to the study are set out in Table 2.1.

Table 2.1 Entry criteria to study

Group	Disease duration	Drug therapy
Start MTX	Preferably less than 2 yrs	No previous treatment with MTX and needed to start or change to MTX
Start SPZ	Preferably less than 2 yrs	No previous treatment with MTX and to start SPZ
Continue MTX	At least 5 years	MTX for at least 5 yrs. No limit on previous DMARDs
Continue Other DMARD	At least 5 years	Present DMARD for at least 5 yrs, and no MTX ever. No limit on previous DMARDs

MTX, Methotrexate; SPZ, Sulphasalazine; DMARD, disease-modifying anti-rheumatic drug.

Men and women between the ages of 18 and 80 who fulfilled the American College of Rheumatology 1987 revised diagnostic criteria for RA (Table 1.3) (Arnett et al, 1988) were eligible for inclusion. Ethical approval for the study was granted by the local ethics committee and each participant gave signed, informed consent.

Exclusion criteria are shown in Table 2.2. Postmenopausal women using hormone replacement therapy (HRT) were not excluded but a record was kept of the use of such drugs throughout the study. It had been hoped to avoid recruiting women who were perimenopausal as they are in a state of increased bone turnover (Lufkin et al, 1992), but many women presenting with RA fell into this category and the exclusion was relaxed.

Table 2.2 Exclusion criteria for recruitment to study

Current treatment with corticosteroids, apart from inhaled steroids for airways disease
Previous maintenance treatment with corticosteroids for any disease, apart from short reducing courses for reversible airways disease (not permitted within the previous 6 months)
Intra-venous pulse treatment with corticosteroids for flare of RA within the previous 3 months
Previous or current treatment with bisphosphonates, calcitonin or Cyclosporin A
Previous or current illness known to effect bone mass such as hyperparathyroidism, multiple myeloma, Cushing's syndrome
Current untreated hyperthyroidism. Replacement therapy with thyroxine following previous hyperthyroidism was permitted provided the dose of thyroxine had been stable for at least 6 months
Hypogonadal men receiving testosterone replacement therapy
Serious illness such as malignancy, heart failure or vasculitis
Unable or unwilling to give informed consent

2.2.1 Subject Recruitment

The study subjects were patients attending the Royal National Hospital for Rheumatic Diseases NHS Trust, Bath.

Subjects were recruited during the first year of the study. The medical notes of all out-patients attending the hospital were scrutinised and potential subjects identified. The doctor who saw the individual in clinic was asked to seek the patient's permission for an initial telephone contact by the author and two hundred and thirty-one patients agreed to be telephoned. The telephone conversation enabled

subjects to be further screened and the study protocol was discussed briefly. At this point one hundred and thirty-nine patients were excluded, most commonly due to refusal to attend study visits. The reason most often given was unwillingness to have additional hospital visits, particularly when others were relied on for transport. Those who did agree to be seen were sent an appointment card, a container to bring the urine sample in and instructions on how to collect the fasting, second void morning urine sample. Additional patients were seen in person during their clinic visits and study appointments arranged then.

By these means, one hundred and thirty-eight subjects attended for first interview, when twenty-two were excluded, leaving one hundred and sixteen recruited to the study (Figure 2.1). The pattern of recruitment throughout the year is shown in Table 2.3.

Figure 2.1 Recruitment to the study

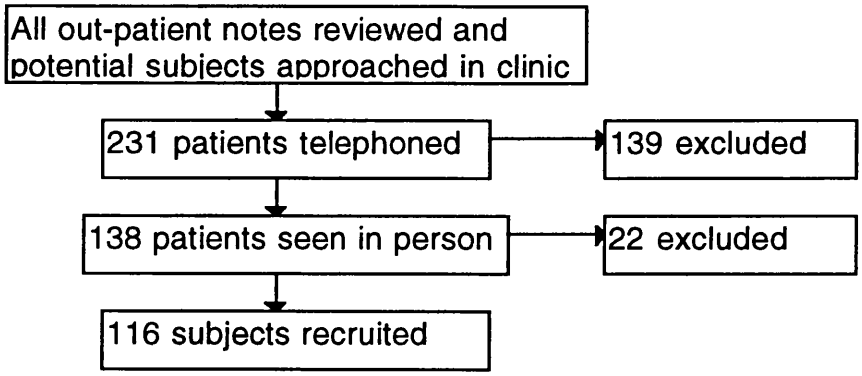


Table 2.3 Recruitment of subjects

Month	Potential subjects seen	Number recruited	Cumulative number recruited
May 95	13	9	9
June 95	13	12	21
July 95	19	16	37
August 95	18	17	54
September 95	17	15	69
October 95	9	9	78
November 95	16	11	89
December 95	7	6	95
January 96	11	8	103
February 96	11	9	112
March 96	4	4	116
Total	**138**	**116**	**116**

There were several reasons for exclusion at the first visit. One patient had long-standing RA treated with D-Penicillamine who was in a flare of RA requiring a pulse of steroids. Another had been placed on oral prednisolone by the General Practitioner, again for a flare. Two patients had already started taking the study DMARD (MTX and SPZ) in error. Several patients with mild disease had decided not to start taking the recommended DMARD and several decided they were not willing to wait the two weeks until the second baseline assessment before starting therapy. One patient had abnormal baseline liver function tests and could not start MTX or SPZ. Another had secondary fibromyalgia and all the joints were tender despite his RA being under good control. Both physician and patient assessment of disease activity could have been inaccurate and he was therefore excluded.

2.2.2 Study Visits

The main determinants of bone mineral density at any site are age and, in women, menopausal age (years since the menopause). In patients with RA, disease activity and duration of RA may also affect bone density and are potential confounders. Study visits were therefore planned to detect changes in both bone turnover and RA disease activity. It was assumed that the rheumatoid disease activity and bone turnover would change rapidly in the groups starting either MTX or SPZ as their active RA was treated. Therefore these subjects were seen more frequently than those on stable RA therapy in whom the disease could be expected to be under better control.

Subjects Starting MTX or SPZ

These patients were seen five times over one year. As the day-to-day variation in crosslink excretion has been shown to be as high as 26% (Colwell et al, 1993), two baseline visits were performed approximately two weeks apart to obtain two sets of baseline data for bone turnover and disease activity. The subjects were then seen at three months, six months and a year after starting the DMARD (either MTX or SPZ). The subgroup with RA of duration less than two years at study entry were invited to attend a sixth visit two years after starting the DMARD.

Continuing MTX or Other DMARD

These patients were seen three times over one year. They were seen on two occasions, a fortnight apart at study entry to provide baseline data (similar to the prospective groups). The third and final visit was a year later.

The timing and number of visits is shown for each group in Table 2.4.

Table 2.4 Number of study visits for the different groups

Group	Time 0	2 weeks	3 months	6 months	12 months	24 months
Start MTX	Yes	Yes	Yes	Yes	Yes	Yes (if early RA)*
Start SPZ	Yes	Yes	Yes	Yes	Yes	Yes (if early RA)*
Continue MTX	Yes	Yes	No	No	Yes	No
Continue Other DMARD	Yes	Yes	No	No	Yes	No

* Early RA was arbitrarily defined as RA duration of less than 2 years at study entry.

A morning research clinic was set up. Serum osteocalcin has a diurnal rhythm, with the peak between 04.00 and 08.00 and the nadir between noon and 14.00 (Gundberg et al, 1985). It is therefore recommended that blood samples for osteocalcin estimation are taken at the same time and preferably before 10.00. However, patients with active RA do not like attending early morning clinics due to their morning stiffness and some patients had a long way to travel (from South Wales, for example). The latest clinic appointment time was 12.30, with blood samples taken at 13.00. This was not ideal, but was necessary to maximise recruitment because many patients who were used to afternoon appointments refused to come if they could not be seen at 12.00 or 12.30. The appointment time for a subject was the same throughout the study, so that if they came at 11.00 the first time, all subsequent visits would be at 11.00. This meant that the diurnal variation in osteocalcin was less important when percentage change in osteocalcin in a single subject was considered but it has implications for mean group values. Although the clinic was on a Thursday, during the first year potential subjects were seen on any suitable morning to maximise the number recruited.

The study visits were used to collect baseline demographic data (first visit) and information about the bone turnover and rheumatoid activity (all visits). The measurements obtained at the different study visits are shown in Table 2.5. The same data was collected in all groups of subjects to enable four-way statistical analysis. All the joint assessments were by one observer (the author) which eliminated inter-observer variation.

Intra-articular corticosteroid injections were performed as clinically necessary, but not at the first visit as it has been shown that the serum osteocalcin level is reduced one day after such an injection and takes two weeks to return to normal (Emkey et al, 1996). The osteocalcin could still have been suppressed at the second baseline visit two weeks later.

Table 2.5 Measurements at study visits

Visit	At study entry	2 weeks	3 months	6 months	1 year	2 years
Baseline data	Yes					
BMD	Yes				Yes	Yes
Bone turnover	Yes	Yes	Yes	Yes	Yes	Yes
RA activity	Yes	Yes	Yes	Yes	Yes	Yes
HAQ	Yes	Yes	Yes	Yes	Yes	Yes
Radiographs	Yes				Yes	Yes

The First Visit

At the first visit, for which an hour was available, a detailed patient information sheet was read through with the patient and any questions about the study protocol answered. If the patient agreed to take part in the study, they were asked to sign a consent form. All female patients with early RA who were starting MTX were asked if they would agree to have a transiliac crest bone biopsy before starting MTX, with the biopsy repeated after one year. If the patient was commencing a DMARD (MTX or SPZ), the implications and possible side effects of the drug were discussed.

A proforma was completed which was designed to identify osteoporotic risk factors and, in women, menopausal status. A full medical history and examination were performed. The history included details of previous DMARD use. If a thyroid stimulating hormone had not been estimated in the last year, this was done. Serum calcium and Vitamin D measurements were not performed.

The assessments of RA activity and bone turnover were completed (see sections 2.5 and 2.4).

Subsequent Visits

For the second and later visits, half an hour of clinic time was available. At each visit a proforma was completed and the assessments of RA activity and bone turnover were made.

2.3 Measurement of Bone Mineral Density

The bone mineral density (BMD) of the lumbar spine, hip and forearm was measured by dual x-ray absorptiometry (DXA) on a Hologic QDR 4500 machine (Hologic, USA) in the Clinical Measurements Department at the RNHRD. The BMD was done at entry to the study and then after one year. All the scans were done on

the same device which is calibrated every day using a lumbar vertebra phantom. The coefficient of variation of the phantom measurement throughout the study period was 0.51%. Typical precision errors for the sites scanned have been reported as 1.0% for the lumbar spine, 1.6% for the femoral neck (Laskey et al, 1991) and 1.0% for the forearm (Hologic Inc datasheet).

The areas scanned and measurements made are shown in Tables 2.6 and 2.7 and an example of the printout is shown in Figures 2.2 to 2.4.

Table 2.6 Bone mineral density measurements

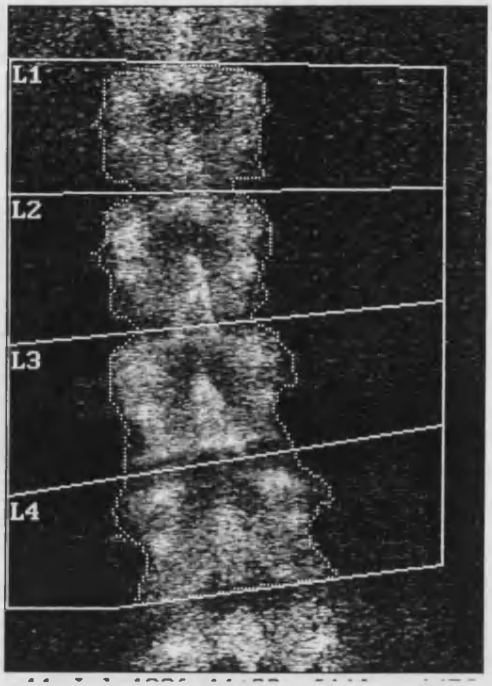
Anatomical Area	Regions scanned
Lumbar spine	lumbar vertebrae 1 to 4 and result averaged
Hip	neck of femur
	intertrochanteric
	trochanteric
	Ward's area
	total hip
Forearm - Radius and Ulna	proximal third
	mid-portion
	ultra-distal
	total forearm
Forearm - Radius alone	proximal third
	mid-portion
	ultra-distal
	total forearm

Table 2.7 Bone mineral measurements given for each area scanned

Region	Bone mineral content, BMC (g)	Bone mineral density, BMD (g/cm2)	T score	T%	Z score	Z%	Annual change in BMD (absolute and %)
Lumbar Spine	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Neck of Femur	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Inter-trochant-eric	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Trochant-eric	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Ward's area	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Total Hip	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Radius & Ulna:							
Proximal Third	Yes	Yes	Yes	Yes	Yes	Yes	No
Mid-portion	Yes	Yes	Yes	Yes	Yes	Yes	No
Ultra-Distal	Yes	Yes	Yes	Yes	Yes	Yes	No
Total Forearm	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Radius Alone:							
Proximal Third	No	Yes	Yes	Yes	Yes	Yes	No
Mid-portion	No	Yes	Yes	Yes	Yes	Yes	No
Ultra-Distal	No	Yes	Yes	Yes	Yes	Yes	No
Total Forearm	No	Yes	Yes	Yes	Yes	Yes	Yes

Figure 2.2 Printout of bone mineral density measurement estimation of lumbar spine

k = 1.129 d0 = 42.9(1.000H) 8.449



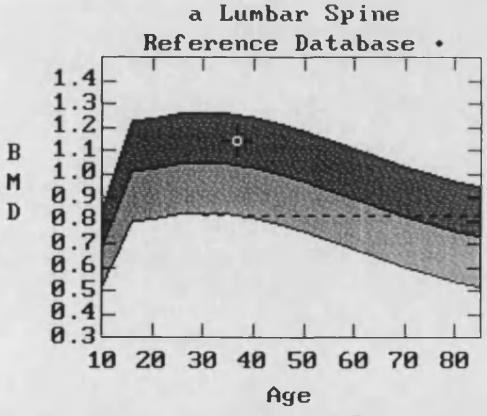
B0728950C Fri 28.Jul.1995 11:23
Name: OBM
Comment: R.A. R26
I.D.: Sex: F
S.S.#: - - Ethnic: W
ZIPCode: Height: 164.50 cm
Operator: JBS Weight: 88.70 kg
BirthDate: 14.Aug.58 Age: 36
Physician: DR BHALLA
Image not for diagnostic use

TOTAL BMD CV FOR L1 - L4 1.0%

C.F. 1.022 0.992 1.000

Region	Est.Area (cm^2)	Est.BMC (grams)	BMD (gms/cm^2)
L1	13.91	13.83	0.994
L2	14.67	16.89	1.151
L3	16.38	20.04	1.223
L4	17.65	20.15	1.142
TOTAL	62.61	70.91	1.133

Clinical Measurement, RNHRD, Bath.



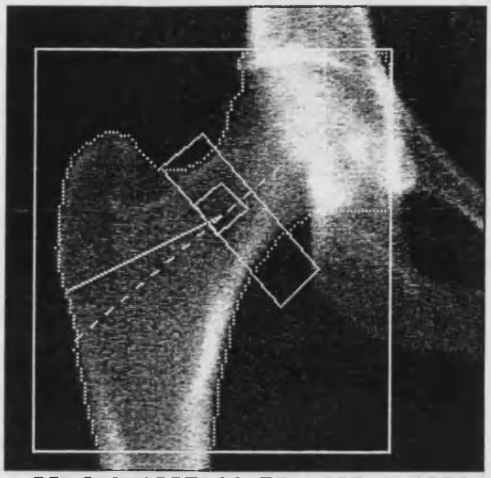
B0728950C Fri 28.Jul.1995 11:23
Name: OBM
Comment: R.A. R26
I.D.: Sex: F
S.S.#: - - Ethnic: W
ZIPCode: Height: 164.50 cm
Operator: JBS Weight: 88.70 kg
BirthDate: 14.Aug.58 Age: 36
Physician: DR BHALLA

BMD(L1-L4) = 1.133 g/cm^2

Region	BMD	T(30.0)	Z
L1	0.994	+0.63 107%	+0.73 109%
L2	1.151	+1.12 112%	+1.23 113%
L3	1.223	+1.27 113%	+1.39 114%
L4	1.142	+0.24 102%	+0.35 104%
L1-L4	1.133	+0.78 108%	+0.89 109%

Figure 2.3 Printout of bone mineral density measurement estimation of hip

k = 1.131 d0 = 46.4(1.000H) 6.675



·28.Jul.1995 11:59 [95 x 108]
Hologic QDR-4500A (S/N 45023)
Right Hip V8.10a:3

I.D.: Sex: F
S.S.#: - - Ethnic: W
ZIPCode: Height: 164.50 cm
Operator: JBS Weight: 88.70 kg
BirthDate: 14.Aug.58 Age: 36
Physician: DR BHALLA
Image not for diagnostic use

TOTAL BMD CV 1.0%

C.F.	1.022	0.992	1.000

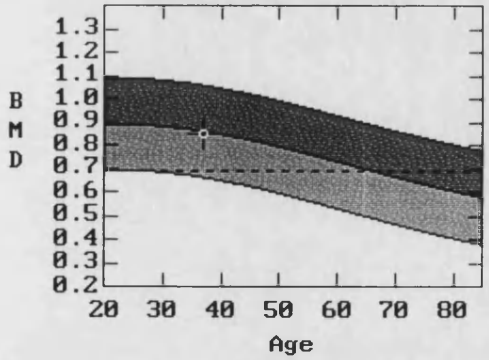
Region	Est.Area (cm^2)	Est.BMC (grams)	BMD (gms/cm^2)
Neck	4.74	3.99	0.842
Troch	11.08	7.73	0.697
Inter	20.47	22.44	1.096
TOTAL	36.30	34.16	0.941
Ward's	1.11	0.74	0.671

Midline (98,124)-(22, 58)
Neck 49 x 15 at [-20, 11]
Troch -12 x 44 at [0, 0]
Ward's 11 x 11 at [-7, 7]

HOLOGIC

Clinical Measurement, RNHRD, Bath.

a Right Hip
Reference Database •

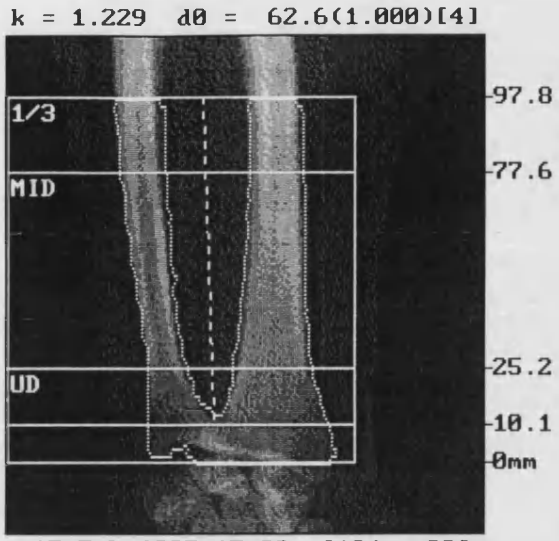


BMD(Neck[R]) = 0.842 g/cm^2

Region	BMD	T	Z
Neck	0.842	-0.53 94% (22.0)	-0.21 98%
Troch	0.697	-0.27 97% (30.0)	-0.19 98%
Inter	1.096	-0.37 96% (29.0)	-0.29 96%
TOTAL	0.941	-0.28 97% (28.0)	-0.18 98%
Ward's	0.671	-1.14 84% (20.0)	-0.39 94%

B07289500 Fri 28.Jul.1995 11:30
Name: OBM
Comment: R.A. R26
I.D.: Sex: F
S.S.#: - - Ethnic: W
ZIPCode: Height: 164.50 cm
Operator: JBS Weight: 88.70 kg
BirthDate: 14.Aug.58 Age: 36
Physician: DR BHALLA

Figure 2.4 Printout of bone mineral density measurement estimation of forearm

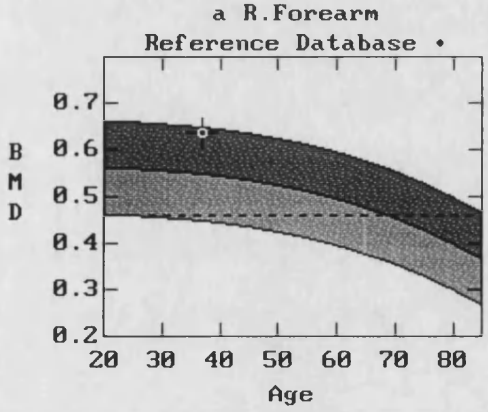


I.D.: Sex: F
S.S.#: - - Ethnic: W
ZIPCode: Height: 164.50 cm
Operator: JBS Weight: 88.70 kg
BirthDate: 14.Aug.58 Age: 36
Physician: DR BHALLA
Forearm Length: 26.7 cm
Image not for diagnostic use

TOTAL BMD CV IS LESS THAN 1.0%
C.F. 1.022 0.992 1.000

RADIUS + ULNA	Area (cm2)	BMC (grams)	BMD (gms/cm2)
1/3	4.87	3.64	0.748
MID	12.17	8.15	0.669
UD	5.43	2.48	0.457
TOTAL	22.47	14.27	0.635

Clinical Measurement, RNHRD, Bath.



B0728950E Fri 28.Jul.1995 11:37
Name: OBM
Comment: R.A. R26
I.D.: Sex: F
S.S.#: - - Ethnic: W
ZIPCode: Height: 164.50 cm
Operator: JBS Weight: 88.70 kg
BirthDate: 14.Aug.58 Age: 36
Physician: DR BHALLA

BMD(Radius+Ulna[R] TOTAL) = 0.635 g/cm^2

Region	BMD	T	Z
1/3	0.748	+1.10 109% (20.0)	+1.38 112%
MID	0.669	+1.54 114% (20.0)	+1.83 117%
UD	0.457	+0.89 111% (20.0)	+1.09 114%
TOTAL	0.635	+1.39 113% (20.0)	+1.68 116%

Bone mineral content (BMC), from which the BMD was derived, was recorded for the following: lumbar spine, neck of femur, trochanteric, intertrochanteric, total hip, Ward's region, radius and ulna proximal third, mid-portion, ultra-distal and total forearm.

Estimates of BMD were available for all the regions where the BMC was measured and for the proximal third, mid-portion, ultra-distal and total forearm of the radius alone as well as the radius and ulna combined.

For subjects with early RA (arbitrarily defined as less than two years' duration), they were invited to have a third BMD measurement of the lumbar spine, hip and forearm at two years. If they consented, a lateral lumbar spine BMD was also obtained as the software became available during the course of the study. A total of twenty-nine subjects with early RA had this measurement at twelve and twenty-four months after entry to the study.

The annual change in BMD was recorded for each region and expressed both as absolute change and percentage. For those areas where the change in BMD was not provided from the printout, annual changes were calculated from the BMD values.

The lumbar spine BMDs were reviewed at the end of the first year by Dr A.K. Bhalla, Consultant Rheumatologist, who was not aware of the clinical details of the subjects. He identified any vertebrae which had morphological changes, which would lead to inaccurate BMD values. The scans were re-analysed excluding such vertebrae.

2.4 Measurement of Bone Turnover

At each visit, samples were collected for markers of bone formation (serum) and resorption (urine).

2.4.1 Markers of Bone Formation

Serum was stored at -70°C in four aliquots for later estimation of osteocalcin and bone-specific alkaline phosphatase and only thawed on the day of analysis.

2.4.2 Markers of Bone Resorption

Fasting, second void urine samples were brought to each visit in two containers. Samples were collected according to instructions previously sent to the subject. One urine sample was sent to the Biochemistry Department at the Royal United Hospital NHS Trust, Bath for urinary creatinine measurement by automatic analyser (Roche Cobas Mira and Integra), whilst the other was stored at -20°C in two aliquots for later estimation of the collagen cross-links, pyridinoline and deoxypyridinoline. Samples were only thawed on the day of analysis.

2.5 Measurement of Rheumatoid Arthritis Disease Activity

The following measures of disease activity in RA (section 1.3.5) were made at each visit:

length of early morning stiffness of the joints in minutes;

a twenty-eight joint count was performed in which swelling and tenderness were scored separately (Fuchs et al, 1989);

the subject indicated on two separate 100mm visual analogue scales the level of the pain they were experiencing and a measure of overall disease activity;

blood was taken for haemoglobin (Hb), C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR) estimation.

From these measures, a modified Disease Activity Score (DAS, Section 1.3.6) (van Gestel et al, 1996) was derived.

2.6 Measurement of Rheumatoid Arthritis Disease Impact

Each subject completed the modified Health Assessment Questionnaire (HAQ, Section 1.3.7) (Kirwan et al, 1986) at each visit.

Radiographs of hands and wrists were taken on entry and then annually. The radiographs were scored using Larsen standard plates (Larsen et al, 1977) at the end of the study by Dr Gordon Evison, Consultant Radiologist. He had no knowledge of the patient clinical details, other than the diagnosis of RA. In subjects who agreed, elbow radiographs were taken at one year follow-up. These were taken because preliminary analysis during the first year of the study indicated that the forearm BMD of the long-term MTX group was significantly lower than in their control subjects (those continuing DMARDs other than MTX). The elbow films were also scored according to the Larsen plates.

2.7 Other Data Collected

During the course of the study, any changes in therapy were noted. Changes in treatment were initiated by the General Practitioner, at other RNHRD clinics or by the patient.

Appropriate monitoring and baseline blood tests for the DMARD were performed as for a usual outpatient appointment and the General Practitioner asked to continue monitoring according to hospital guidelines.

2.7.1 Physical Activity

Weight-bearing exercise is essential for bone health and completely bed-bound individuals may loose as much as 40% of their bone mass over a year (Marcus, 1996). Peak bone mass attained is partly determined by the length of time spent walking outdoors (Cooper et al, 1995). Patients with RA often have reduced physical activity due to lower limb joint disease. The EVOS study used a validated questionnaire and spinal radiographs to determine the impact of factors such as physical activity and diet on the prevalence of vertebral osteoporosis in nineteen European countries (O'Neill et al, 1995; Silman et al, 1997). Part of the EVOS questionnaire was administered to the study patients. The subject filled in the questionnaire alone but the responses were checked to ensure comprehension. The questions related to physical activity, both current and previous, and also to diet (Section 2.7.2). In the analysis, a score derived from the length of time spent walking outdoors at the present time was used and is shown in Table 2.8.

Table 2.8 Score for outdoor walking from questionnaire

Time spent walking out of doors each day	Score
none	1
some, but less than half an hour	2
between half an hour and one hour	3
more than one hour	4

2.7.2 Calcium Intake

The dietary components of the questionnaire asked about current and previous milk intake, current intake of various calcium-containing foods and intake of tea, coffee and alcohol. Dietary calcium during childhood affects the peak bone mass attained (Cadogan et al, 1997). In postmenopausal women there is reduced calcium absorption from the gut and calcium supplements (with or without Vitamin D_3) may reduce bone loss and fractures (Dawson-Hughes et al, 1990; Reid et al, 1993; Chapuy et al, 1992). A score shown in Table 2.9 was developed for the current calcium intake and used in the analysis.

Table 2.9 Score for calcium intake from questionnaire

Foodstuff	**Score is the average numbers of days per week each foodstuff is eaten**
Hard cheese	0 to 7
Soft cheese	0 to 7
Yoghurt	0 to 7
Milk	0 to 7
Other e.g. ice cream	0 to 7
Total	**0 to 35**

2.8 Analysis of Bone Formation Markers

2.8.1 Osteocalcin

Osteocalcin was estimated by a competitive immunoassay (Novocalcin, Metra Biosystems, UK) using osteocalcin-coated strips in a ninety-six well plate. Standard, control or sample (25μl) were added to wells in duplicate and incubated with an anti-osteocalcin antibody at room temperature for two hours. After three washes, an anti-mouse alkaline phosphatase conjugate was incubated for another hour at room temperature before another three washes. Finally, a p-Nitrophenyl Phosphate (p-NPP) substrate was added for thirty minutes at room temperature before the reaction was stopped by 50μl of 3M NaOH. The resulting optical densities were read at 405nm. In addition, six standards and a high and low control were supplied, allowing forty patient samples to be processed per assay as each was done in duplicate with a standard curve performed with each assay. A four-parameter calibration curve fitting equation was used to construct a standard curve and to determine the concentrations of the controls and samples. The software to calculate this was provided by Metra Biosystems. Interassay variation in this study was 6.9%.

2.8.2 Bone-Specific Alkaline Phosphatase

This was estimated by an immunoassay (Alkphase-B, Metra Biosystems) using monoclonal anti-bone-specific alkaline phosphatase (BALP) antibody-coated strips which capture serum BALP. Initially, 125μl of assay buffer were added to each well, then 20μl of standard, control or patient sample in duplicate. The plate was incubated at room temperature for three hours. After four washes, incubation for thirty minutes with p-NPP was done which detects the enzyme activity of the

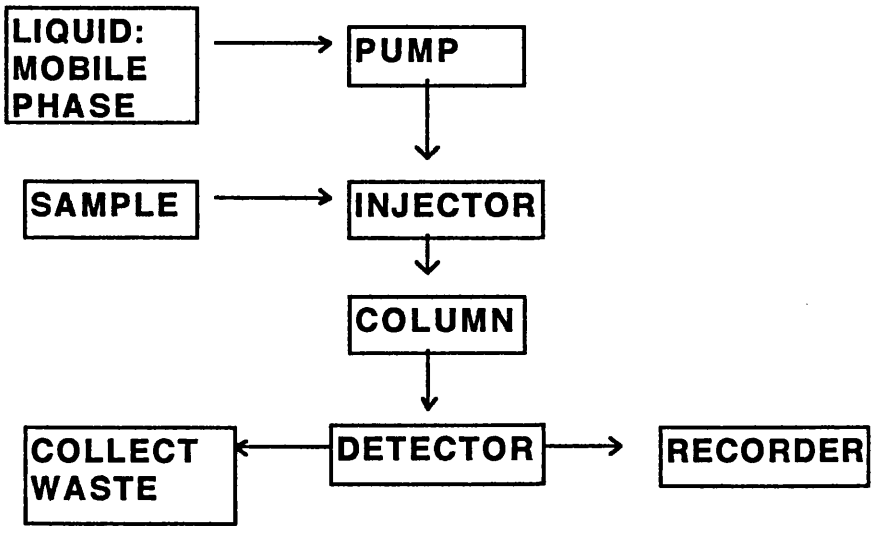
BALP and the reaction stopped with 100μl of 3M NaOH. A quadratic calibration curve was used to construct the standard curve and then generate BALP serum values from the optical densities obtained at 405 nm, again using software supplied by Metra Biosystems. Interassay variation in this study was 4.8%.

2.9 Analysis of Bone Resorption Markers

2.9.1 High Pressure Liquid Chromatography Method

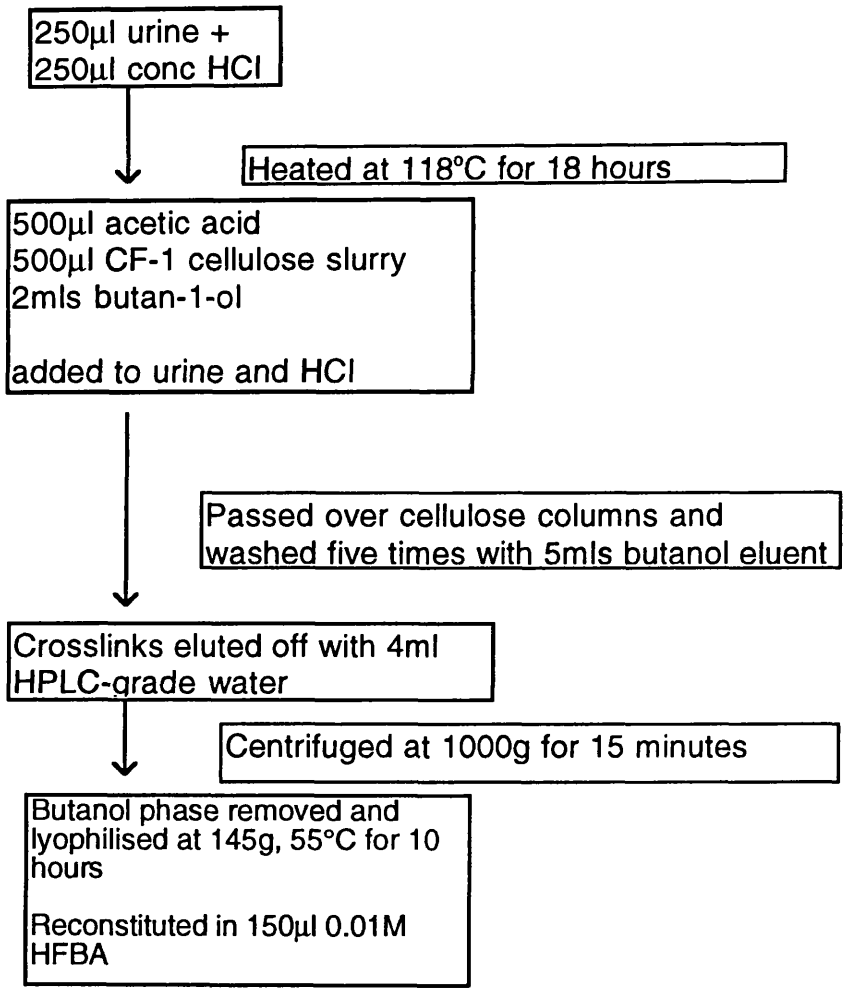
High Pressure Liquid Chromatography (HPLC) is a powerful technique for separating the different components in a chemical mixture (Schram, 1980). The compounds of interest (solutes) are dissolved in a liquid solvent (the mobile phase) and then passed at high pressure through a chromatographic column (the stationary phase). The principle of the HPLC technique is shown schematically in Figure 2.5.

Figure 2.5 Flow diagram of the principle of separation of compounds by HPLC



By altering the solvent composition and flow rate and column characteristics, the compounds can be made to come off the column at different times. The collagen cross links, Pyd and Dpd (Section 1.1.10), naturally fluoresce and can be quantified in a urine sample which has been passed over a HPLC column by a fluorescence detector set to the appropriate excitation (295nm) and emission (395nm) wavelengths (Colwell et al, 1993). An analysing computer integrates the area under the curve for each peak and by comparison with a standard, the concentration of Pyd and Dpd can be determined. The usual ratio of urinary Pyd to Dpd is 4:1 (Robins et al, 1991), although this may be increased in RA, when turnover of tissues such as cartilage and synovium have a larger contribution of Pyd than normal (Pearce et al, 1995). However, there are many contaminating compounds in urine and the samples need to be purified by fractionation over cellulose columns before analysis. In addition, the collagen cross links are hydrolysed by heating for eighteen hours with concentrated hydrochloric acid prior to fractionation. The technique for preparing and purifying the urine samples before passing them over the column is illustrated in Figure 2.6.

Figure 2.6 Flow diagram of the method for preparing urine samples for collagen cross-link estimation



All chemicals used in the estimation of Pyd and Dpd by HPLC were of HPLC-grade and were obtained from BDH Laboratory Supplies, Poole, Dorset, unless otherwise stated.

Preparation of urine samples for collagen crosslink estimation (Figure 2.5)

Overnight hydrolysis with hydrochloric acid

In the fume hood, 250µl of thawed urine was placed with 250µl of concentrated hydrochloric acid in a 100mm long pyrex tube with a teflon-lined screw cap (Aldrich Chemical Company Inc., Milwaukee, Wisconsin, USA) and heated at 118°C in a heating block (Stuart Scientific, UK) overnight for eighteen hours. The heating block held twenty-four tubes (twelve samples in duplicate) and all samples from a single patient (three or five visits) underwent prefractionation in parallel on the same day to reduce variability.

Pre-fractionation

The pre-fractionation was done over cellulose columns which were washed with a butan-1-ol eluent. In the fume hood, the butanolic eluent was prepared from butan-1-ol, glacial acetic acid and HPLC-grade water in the ratio 4:1:1 by volume. The 5% CF1 cellulose slurry was made using 25g fibrous cellulose powder CF1 (Whatman Laboratory Division, Maidstone, Kent, UK) suspended in 500mls butanolic eluent. The cellulose was kept suspended in solution by magnetic stirring. In the fume hood, the hydrolysed urine samples had 500µl glacial acetic acid, 500µl 5% CF1 cellulose slurry and then 2mls butan-1-ol added to each tube. During this stage the cross-links bound to the cellulose. Working in the fume hood, the cellulose columns were made by pipetting 5mls of the cellulose slurry into polyprep columns (Biorad Labs Inc., Hemel Hempstead, UK) suspended over a container to catch the butanol eluent washes. Each column was washed once with 5mls of butanol eluent prior to the urine sample being poured onto the relevant column. To ensure maximum retrieval of the cellulose (and thus cross-links) from the pyrex tubes, the tubes were

washed three times with 5mls butanolic eluent and shaken vigorously prior to tipping the contents onto the correct column. Finally, the columns were washed twice with 5mls butanolic eluent.

Elution of the crosslinks and lyophilization

The Biorad columns were then transferred to a rack where correspondingly labelled LP4 tubes (Luckham, UK) were suspended underneath. The crosslinks were eluted off the columns by 4mls of HPLC grade water. The samples were then centrifuged at 1000g for fifteen minutes to separate the aqueous from the butanolic phase. The butanol phase was aspirated off and the tubes transferred to the centrifuge evaporator (RC10, Jouan Ltd, UK) with a cold trap (RCT90, Jouan Ltd, UK) cooled to -90°C to collect the evaporated solvent for lyophilization overnight. Spinning at 145g for ten hours with heating to 55°C and a pulsed vacuum was sufficient to dry forty-eight tubes completely.

Reconstitution

The following day, the residue was reconstituted with 150μl of 0.01M HFBA, placed in a 200μl conical insert (02-MTV, Chromacol Ltd, London, UK) and then in a 2ml HPLC vial (2-CV, Chromacol Ltd, London, UK), with the top crimped. Prepared samples were stored in the dark at 4-8°C until they were run on the HPLC column.

Separation of crosslinks by reverse-phase, ion-paired HPLC

The method followed was that described by Colwell et al (Colwell et al, 1993) and is shown schematically in Figure 2.5. This is an isocratic method: that is, the same ratio of mobile phase components is maintained throughout the run.

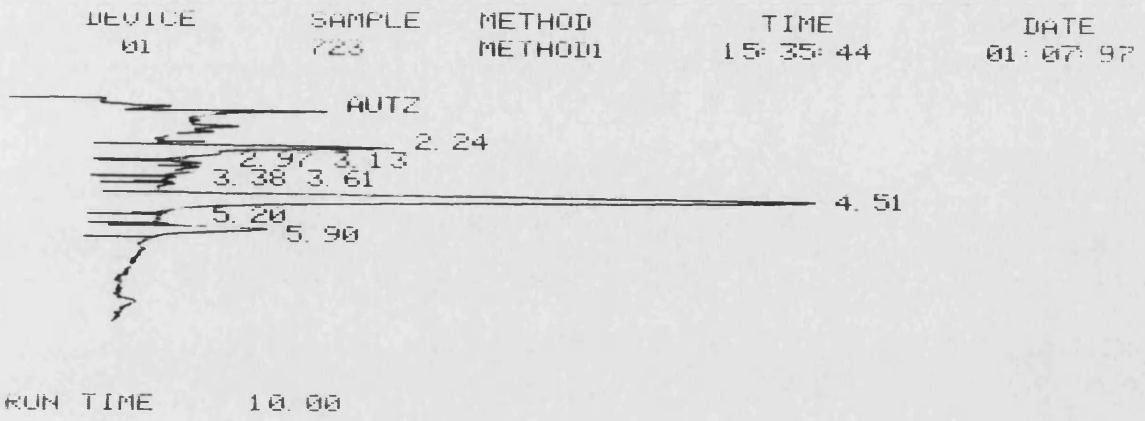
The column was 33 x 4.6mm long, packed with 3µm octadecyldimethylsilane (ODS) (LC-18-DB, Supelco Inc., Bellefonte, PA, USA), used in conjunction with a sampler (Marathon, Spark Holland), flurometer (JASCO 820-FP Intelligent Spectrofluorometer, Japan Spectroscopic Co. Ltd, Japan), solvent delivery system (Milton Roy CM400 multiple solvent delivery system, LDC Analytical, Thermo Instrument Systems, Stone, Staffs, UK), LDC Analytical CI-4100 data acquisition system (LDC Analytical, Stone Staffs, UK) and pump (Bambi Air, UK).

The mobile phase was 90% solution A and 10% solution B, where solution A was 10mM HFBA (2.64mls in 1 litre HPLC-grade water), pH adjusted to 2.5 with 10M NaOH, made fresh every day. Solution B was 75% acetonitrile (HPLC grade, BDH Laboratory Supplies, Poole, Dorset, UK), 25% solution A by volume.

A 20µl loop was used to inject the sample onto the column and fluorescence was measured with excitation at 295nm and emission at 395nm.

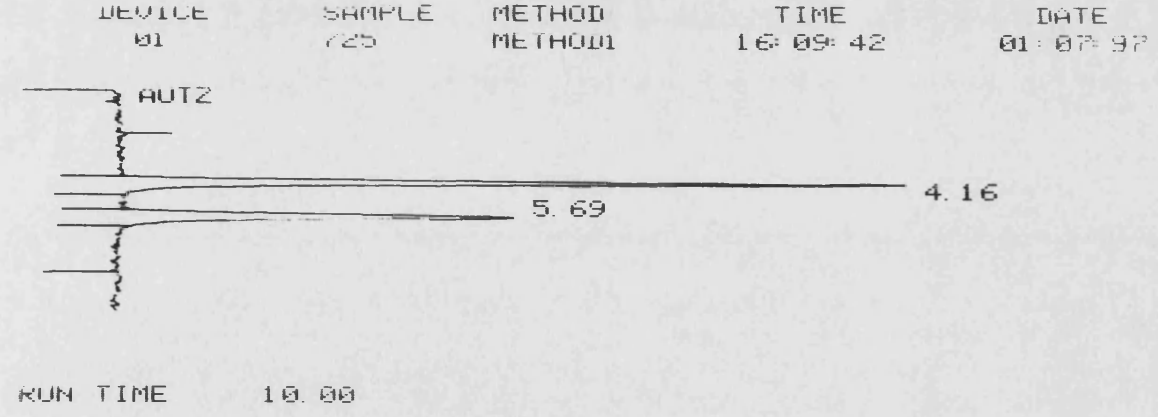
The Pyd usually came off the column at four to five minutes and the Dpd at seven to eight minutes, with good separation of the peaks. The analysis took ten minutes, with two minutes for calculation. An example of the printout obtained is shown in Figure 2.7 with the peaks of interest labelled. An internal standard was not used. External standards from Dr D Black (Glaxo Ltd, Greenford, UK) were run every twenty samples to ensure stability of the system and to allow calculation of the conversion factors for Dpd and Pyd. A printout from the standards is shown in Figure 2.8.

Figure 2.7 HPLC output of collagen cross-links



Pyridinoline peak at 4.51 minutes; deoxypyridinoline peak at 5.90 minutes.

Figure 2.8 HPLC output of the deoxypyridinoline and pyridinoline standards



Pyridinoline peak at 4.16 minutes; deoxypyridinoline peak at 5.69 minutes.

From the area under the peaks, the concentrations of Dpd and Pyd for each sample were calculated using the formulae:

Dpd (nmol/ml) = area(Dpd) x (150/250) x 1000 x rf(Dpd) and

Pyd (nmol/ml) = area(Pyd) x (150/250) x 1000 x rf(Dpd),

where area (Dpd) and area (Pyd) are the areas under the two peaks printed out (integrated automatically);

150/250 is the correction factor for the starting volume of urine (250µl) and the final volume of the purified crosslinks (150µl);

1000 converts the concentration from nmol/l to nmol/ml;

rf is the response factor, established from the external controls. For Dpd the concentration of the external control was 0.518 and for Pyd it was 0.76. The response factor for each was given by the following formulae:

rf(Dpd) = 0.518 / (area of Dpd external control peak)

rf(Pyd) = 0.76 / (area of Pyd external control peak).

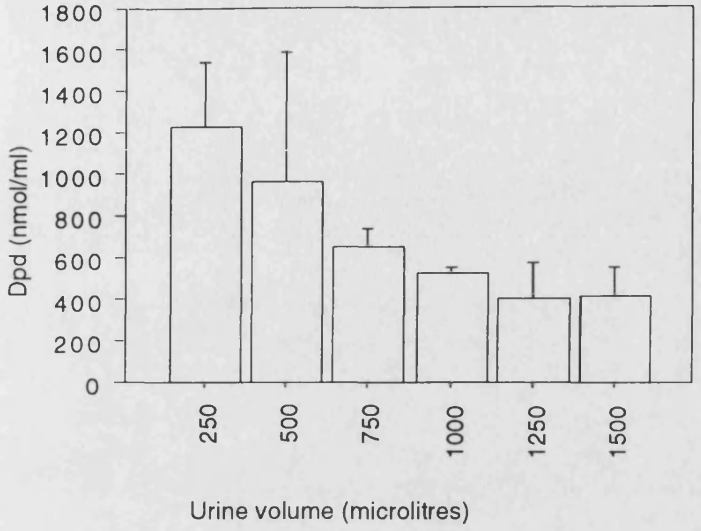
The drug sulphasalazine has a metabolite which is excreted renally and which interferes with the quantification of the LP and HLP peaks by HPLC. The interfering peak can be delayed on the column by changing the proportion of the mobile phase from 90(A):10(B) to 96(A):4(B) (Peel et al, 1994a). The samples from patients on sulphasalazine were processed at the same time as ones from other patients, but they were stored in the dark at 4-8°C and would have been run together at the end.

HPLC was initially used for analysis of the urine samples in this study and the in-house protocol was followed. The ion-paired, reversed-phase method of Black (Black et al, 1988) had been modified by previous investigators so that the starting volume of urine had been increased from 250µl to 1ml. This was on the (incorrect) assumption that a greater starting volume would result in greater recovery of the crosslinks of interest: pyridinoline (Pyd) and deoxypyridinoline (Dpd). Although it had been planned that two aliquots of urine of at least 2mls were to be stored from each sample, a smaller volume had often been saved. As samples were prepared in duplicate, frequently there was not enough urine to process two 1ml volumes and thus two different volumes of 1ml and 500µl or even 1ml and 250µl were used, with appropriate correction of the final results for the starting volume. When the first results were obtained, it became apparent that the smaller starting volume (250µl) yielded a greater final concentration of Dpd and Pyd than did 500µl or 1ml of urine, inversely proportional to the starting volume.

An experiment was done using urine from a Pagetic patient with varying starting volumes of urine and varying volumes of cellulose slurry packing the pre-fractionation columns. The results are shown in Figure 2.9 and it can be seen that the optimum starting volume of urine is 250µl, with 5ml cellulose slurry in the columns, as in the method of Colwell et al (Colwell et al, 1993).

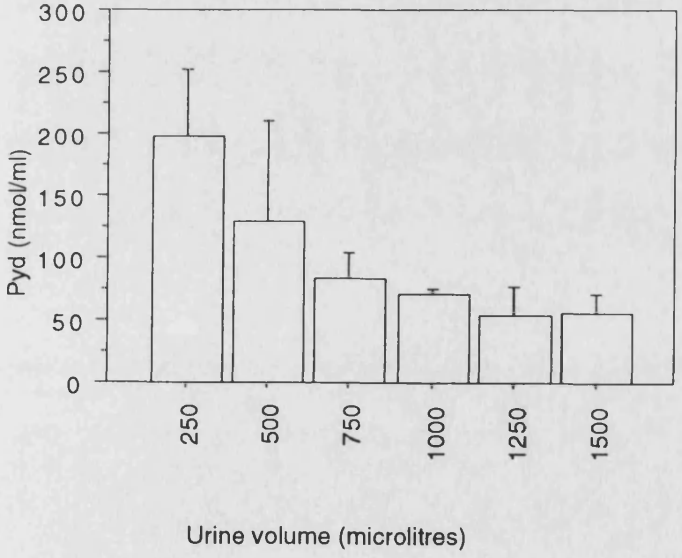
Figure 2.9 Recovery of deoxypyridinoline and pyridinoline from Pagetic urine using different starting volumes of urine

(A) Deoxypyridinoline (Dpd)



Results shown as the mean and the 95% confidence level, n = 4

(B) Pyridinoline (Pyd)



Results shown as the mean and the 95% confidence level, n = 4

The urine samples were prepared again in duplicate, using 250μl of urine. However, recurrent technical difficulties with the HPLC sampler and pump meant that after eight months' work and processing of 624 samples in duplicate, results for Dpd were only available on 163 samples and only 17 out of the 116 subjects had complete Dpd and Pyd results for all samples. At this time, it was decided to process all the samples for free Dpd alone by ELISA (Pyrilinks-D, Metra, UK). This kit was chosen as Dpd is more specific than Pyd for bone (Section 1.3.8) and is the only ELISA commercially available which measures the cross-linked collagen rather than the C- or N-terminal peptides. All the remaining urine samples were then analysed again by ELISA.

2.9.2 ELISA Method

This was a competitive enzyme immuno-assay using monoclonal anti-Dpd antibody coated on the well (Pyrilinks-D, Metra Biosystems, UK). The Dpd in the sample competed with conjugated Dpd-alkaline phosphatase for the antibody and the reaction was detected with a p-NPP substrate. The samples, standards and control solutions were diluted 1 in 10 with assay buffer in a microtitre plate and 50μl of the diluted samples added to the anti-Dpd coated wells in duplicate. Enzyme conjugate (100μl) was then added to each well and the plate incubated in the dark at 2-8°C for two hours. The plate was then washed three times with wash buffer and 150μl of the working substrate (p-NPP) added to the wells prior to a one hour incubation at room temperature. The reaction was stopped with 100μl of 1M NaOH and the optical densities read at 405nm on a plate reader. A 4-parameter curve-fitting equation was used to construct the standard curve and determine the concentrations of the controls and samples. Metra provided the software for use with the ELISA. Inter-assay variation in this study was 5.5%.

3. RESULTS: CLINICAL STUDY

3.1 Introduction

In this chapter the results from the clinical study are presented. First, the methods of analysis are discussed and the characteristics of the subjects are detailed. The adverse events, drop outs and protocol violations are addressed. The results of the primary outcome measure (bone mineral density) and the secondary outcome measures (markers of bone turnover) are described for all subjects. Then the subgroups of postmenopausal women, subjects with early rheumatoid arthritis (RA), and men are discussed in turn.

3.2 Analysis of Results

The analyses were performed by the author using the Statview 4.5 for Macintosh statistical package. For the four-way comparisons between groups, analysis of variance (ANOVA) with Fisher's Protected Least Significant Difference (PLSD) post hoc testing was performed. Confidence levels were set at 95% and the p values for significant differences determined. For two-way comparisons between groups either a paired or an unpaired t-test was used as appropriate. All possible inter-group comparisons were performed but for clarity only those where differences were significant are illustrated.

The regression analysis on the subgroup of postmenopausal women (Section 3.6) was performed by Dr Daphne Kounali, School of Mathematical Sciences, Bath University. She studied the whole data set and gave advice on the appropriate statistical tests to apply. Due to the small number of subjects and the strong influence of menopausal status on bone mineral density, regression and discriminant analyses were limited to the subgroup of postmenopausal women. The

statistical models she developed and applied to the postmenopausal data set are discussed in Section 3.6.

3.3 Characteristics of Subjects Included in the Study

There were one hundred and sixteen subjects recruited to the study by the methods described in Chapter 2. All were Caucasian. The number of subjects recruited into each study group, their sex distribution, age, body mass index, rheumatoid factor status and rheumatoid arthritis (RA) duration in months or years are shown in Table 3.1.

Table 3.1 Subjects recruited to the study

Group	Number	Male: Female	Age in years, mean ± st dev (range)	RA* duration mean ± st dev (range)	Body mass index (kg/m^2) mean ± st dev	Rheumatoid Factor status, number (%) Rheumatoid Factor +ve
Starting MTX*	36	8: 28	54.8 ± 14.1 (21-77)	69.3 months ± 85 (3-360)	24.25 ± 3.83	24 (67%)
Starting SPZ*	23	5: 18	55.8 ± 16.6 (23-78)	19.9 months ± 29.6 (3-144)	23.31 ± 2.65	14 (61%)
Continue MTX*	28	6: 22	62.6 ± 8.9 (46-79)	15.8 years ± 6.6 (6-34)	25.86 ± 3.96	26 (93%)
Continue Other DMARD *	29	7: 22	60.1 ± 10.9 (29-72)	15.4 years ± 6.9 (6-30)	24.64 ± 3.75	26 (90%)

*MTX, Methotrexate; SPZ, Sulphasalazine; DMARD, disease-modifying anti-rheumatic drug; RA, rheumatoid arthritis.

The group continuing Methotrexate (MTX) was well-matched with the group continuing other disease-modifying anti-rheumatic drugs (DMARDs) for age, body mass index and duration of RA. There were no differences between the two groups for these three parameters by unpaired t-testing.

Subjects starting either MTX or Sulphasalazine (SPZ) were less well-matched for disease duration and there was a significant difference between them by unpaired t-test ($p = 0.0102$, Table 3.1). This was due to the twenty-one subjects included who had failed a previous DMARD, sixteen subjects in the group starting MTX, but only five subjects in the group starting SPZ. These subjects did not fulfil the entry criteria as defined in Section 2.2 but were recruited to increase numbers.

Overall, twenty subjects in the group starting MTX and eighteen in the group starting SPZ had early RA (arbitrarily defined as RA duration less than twenty-four months) and the results for this subgroup are presented in Section 3.7.

3.3.1 Osteoporotic Risk Factors

The distribution of various risk factors for osteoporosis by group is shown in Table 3.2. Except for smoking-related risk factors, the number of subjects affected in each group was five or less and the distribution between groups was approximately equal.

No subjects had taken oral corticosteroids for any condition within eight years of the study apart from those with chest disease where short reducing courses more than six months prior to study entry were permitted. No subjects with early RA had been treated with intra-venous or intra-muscular steroids. Oral steroids for up to eighteen months had been taken by three subjects, between eight and thirteen years prior to study entry.

Pack years is a useful way of expressing both the duration and intensity of smoking cigarettes in an individual. A person who had smoked twenty cigarettes a day (one pack) for five years was scored as five pack years, as was someone who had smoked five cigarettes a day for twenty years, or ten a day for ten years. In the groups continuing on their therapy, those on MTX had a higher mean smoking pack years score than those on other DMARDs. The number of subjects still smoking in each of these groups however was the same.

The group starting SPZ had higher mean weekly alcohol intake than the group starting MTX. This was due to one individual who had a heavy alcohol intake.

3.3.2 Menopausal Status

The menopausal status and hormone replacement therapy (HRT) use for all the women recruited is shown in Table 3.3. Postmenopausal women were the majority in each group but only a minority were taking HRT, although more had tried it in the past. Overall, HRT had been discussed with 40% of postmenopausal subjects by either their General Practitioner or Hospital Consultant.

Table 3.2 Risk factors for osteoporosis among subjects

Risk factor / Group	Starting MTX n = 36	Starting SPZ n = 23	Continue MTX n = 28	Continue Other DMARD n = 29
Hyperthyroid	0	0	2 taking T_4[a]	0
Steroid therapy[b]	4 IV pulse 1[c] (see below)	0	6 IV/IM pulse 4 chest disease 2[d] (see below)	1 IV pulse 2 chest disease
Family history osteoporosis[e]	4	2	4	1
Previous fracture[f]	4	1	3	2
Current smoker	7	9	4	4
Pack years, mean	7.0	10.8	15.1	10.5
Alcohol u/wk, median	0	1.5	0	0
Late menarche[g]	2	0	3	3
Early menopause[h]	0	2	5 (1 surgical)	2
Nulliparous[i]	4	4	5	2

[a] Dose of thyroxine replacement was appropriate as checked by serum thyroid stimulating hormone (TSH) levels.

[b] Intra-venous (IV) or intra-muscular (IM) pulses of steroid were for RA flare and had been given at least six months before study entry. Steroids given for chest disease (chest dis), either asthma or chronic obstructive pulmonary disease, were short, rapidly reducing courses of oral steroids given infrequently, and at least six months before study entry.

[c] This patient had less than 10mg prednisolone daily for one year for sarcoidosis, thirteen years prior to study entry.

[d] One subject had oral prednisolone between 5 and 10mg daily for RA for one year and the other had between 5 to 15mg prednisolone daily for eighteen months for pemphigus, twelve and eight years prior to study entry respectively.

[e] Family history of osteoporosis was taken to be present if there was a clear history of osteoporotic fractures in a first or second degree relative.

[f] Previous fracture refers to previous fragility fractures, not traumatic ones.

[g] Late menarche was taken as older than fifteen years of age.

[h] Early menopause was taken as younger than forty-five years of age, whether natural or surgical.

[i] Nulliparity refers to not having given birth to any live children.

Table 3.3 Menopausal status of the female subjects

Group / number of subjects (%)	Number of women	Pre-meno-pausal	Peri-meno-pausal	Post-meno-pausal (PMW)	Current HRT* use (% of PMW)	HRT ever used (% of PMW)	HRT ever discussed (% of PMW)
Starting MTX	28	8 (28.6)	5 (17.8)	15 (53.6)	2 (13.3)	4 (26.7)	5 (33.3)
Starting SPZ	18	4 (22.2)	4 (22.2)	10 (55.6)	2 (20)	3 (30)	3 (30)
Continue MTX	22	2 (9.1)	1 (4.5)	19 (86.4)	3 (15.8)	7 (36.8)	7 (36.8)
Continue Other DMARD	22	4 (18.2)	3 (13.6)	15 (68.2)	4 (26.7)	7 (46.7)	9 (60)

3.3.3 Rheumatoid Arthritis Therapy

The mean length of time subjects had been using their current DMARD for each group and the mean number of different DMARDs taken by each subject prior to their current drug is shown in Table 3.4. None of the group continuing MTX had been prescribed it as their first DMARD. Prior to 1991 when these patients started MTX, it was reserved for those who had failed other DMARDs. Of note are the seventeen subjects in the group continuing other DMARDs who were still using their first ever prescribed DMARD. This group were taking a variety of drugs and this is illustrated in Table 3.5. None of these drugs is thought to have a direct effect on bone.

Table 3.4 DMARD therapy of subjects

Group	Mean time on current DMARD (months)	Mean number of previous DMARDs	Range of number of previous DMARDs	Number with no previous DMARD therapy (total number in group)
Starting MTX	26.0	0.5	0 - 3	25 (36)
Starting SPZ	7.8	0.1	0 - 1	21 (23)
Continue MTX	83.6	2.1	1 - 6	0 (28)
Continue Other DMARD	121.7	0.6	0 - 2	17 (29)

Table 3.5 Current DMARD therapy in the group continuing DMARDs other than Methotrexate

DMARD / number of subjects	Men	Pre-menopausal women	Peri-menopausal women	Post-menopausal women	Total
IM Gold	2	1	2	3	8
D-Penicill-amine	3	1	0	4	8
Sulpha-salazine	1	1	1	1	4
Oral Gold	1	0	0	3	4
Hydroxy-chloro-quine	0	1	0	3	4
Azathio-prine	0	0	0	1	1
Total	7	4	3	15	29

3.3.4 Rheumatoid Arthritis Disease Activity

The assessment of disease activity in RA is discussed in Section 1.3.5 and the disease activity score (DAS) which was used in this study in Section 1.3.6.

The mean number of swollen and tender joints, mean erythrocyte sedimentation rate (ESR) and mean DAS for each group at baseline is shown in Table 3.6. The number of subjects in each group who had a normal C-reactive protein (CRP, less than 0.001 units/ml) and the mean CRP value for each group are also shown. Although the group starting SPZ contained a greater percentage of individuals with normal CRP (59%) than did any of the other groups, the combined DAS for this group was the highest. The DAS was calculated from the ESR, the tender and swollen 28-joint counts and the patient's global assessment of disease activity. The number of subjects in each group with a low ($\leq$ 2.4), moderate (2.4 to $\leq$ 3.7) or high ($>$ 3.7) DAS at baseline and at one year is shown in Table 3.7. The majority of subjects had moderate or high DAS throughout the study.

Table 3.6 Rheumatoid arthritis disease activity at baseline of the entire study group

Group / mean ± standard deviation	Tender joint count (out of 28)	Swollen joint count (out of 28)	ESR* (mm/hr)	Mean CRP ** (units/ml)	Number (%) with CRP < 0.001 units/ml	Disease activity score (DAS)
Starting MTX n = 36	8.93 ± 6.47	12.65 ± 3.96	48.76 ± 24.31	0.034 ± 0.046	9 (25)	5.691 ± 1.003
Starting SPZ n = 22	13.48 ± 6.25	13.57 ± 4.83	41.20 ± 23.43	0.025 ± 0.048	13 (59)	5.973 ± 0.978
Continue MTX n = 28	7.64 ± 5.67	11.61 ± 4.18	42.02 ± 24.89	0.022 ± 0.30	12 (43)	5.267 ± 1.006
Continue Other DMARD n = 29	8.17 ± 5.28	10.10 ± 4.77	38.12 ± 19.53	0.014 ± 0.016	14 (48)	5.314 ± 0.889

The measurements from the two baseline visits were averaged.

* ESR: erythrocyte sedimentation rate by Westergren's method (mm per hour). Normal values for up to 50 years old: male less than 10, female less than 12; for age 51 to 60: male less than 12, female, less than 19; for over 60 years old: male less than 14, female less than 20.

** CRP: C-reactive protein. A CRP of < 0.001 was taken as zero to enable a mean value to be calculated.

Table 3.7 Disease Activity Score (DAS) at baseline and at one year

Group, time point / number of subjects	Low DAS (≤ 2.4)	Moderate DAS (2.4 to ≤ 3.7)	High DAS (> 3.7)
Starting MTX Baseline	0	0	36
Starting MTX One year	0	7	28
Starting SPZ Baseline	0	1	21
Starting SPZ One year	1	8	8
Continue MTX Baseline	0	2	27
Continue MTX One year	0	3	26
Continue other DMARD Baseline	0	2	25
Continue other DMARD One year	0	3	25

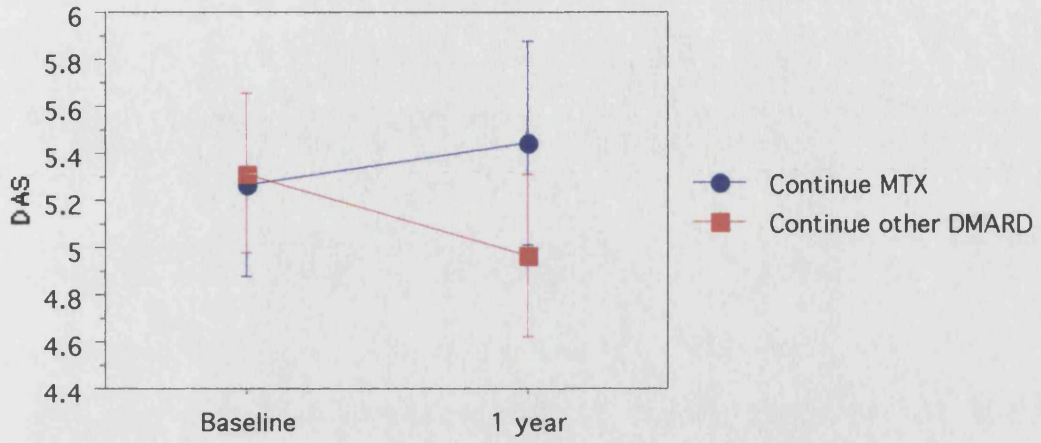
There are differences in numbers of subjects at baseline and one year by group due to dropouts and failure to complete DAS in some cases

Analysis of variance of the four groups showed that there was no difference in the measures of disease activity at baseline between the two groups continuing on their DMARD. However, those starting a DMARD had significantly higher DAS than those continuing on their therapy (Table 3.6). This is unsurprising as the decision to change or start a DMARD was related to disease activity.

When the two groups starting a DMARD were compared, only the tender joint count was significantly higher in those starting SPZ than in those starting MTX (Table 3.6).

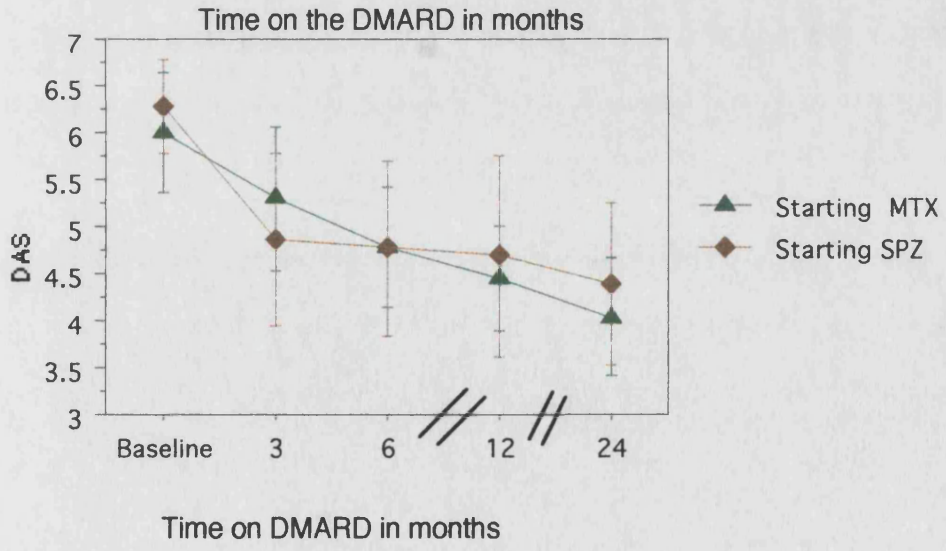
The changes in disease activity (DAS) over the twelve months of the study are shown in Figure 3.1 for those continuing their DMARD. The mean change in DAS was an increase of 0.175 units for those continuing MTX and a reduction of 0.351 units for those continuing another DMARD. These changes were not significantly different from baseline (by paired t-test), nor were the differences between the groups (by unpaired t-test). As a difference in DAS of less than 0.6 units may have occurred by chance alone (Stucki, 1996), these groups were stable with regard to disease activity over the course of the study.

Figure 3.1 Disease activity score (DAS) in subjects continuing on a DMARD



Results are shown as the mean ± 95% confidence interval

Figure 3.2 Disease activity score (DAS) in subjects starting a DMARD



Results shown as mean ± 95% confidence interval. There was no difference between the two groups when compared by repeated measures ANOVA. There was a reduction in disease activity score at later time points compared to baseline ($p < 0.0001$).

Figure 3.2 shows the change in disease activity over two years for those starting MTX or SPZ. In both groups there was a significant reduction in the DAS compared to baseline values at each later time point (by repeated measures ANOVA). The mean reduction in the DAS was 1.046 for the group starting MTX and 1.469 units for those starting SPZ. However, there was no significant difference at any time point between the two groups point (by repeated measures ANOVA).

3.3.5 Functional Status

The mean Health Assessment Questionnaire (HAQ) score (Section 1.3.7) and the mean outdoor walking score (Section 2.7.1) for the four groups at study entry are shown in Table 3.8. The outdoor walking score was defined according to the length of time spent walking outside on an average day. The responses: more than one hour, half to one hour, less than one hour and none, scored 4, 3, 2 and 1 respectively. There were no significant differences between the four groups by ANOVA testing for either functional measurement at baseline (Table 3.8).

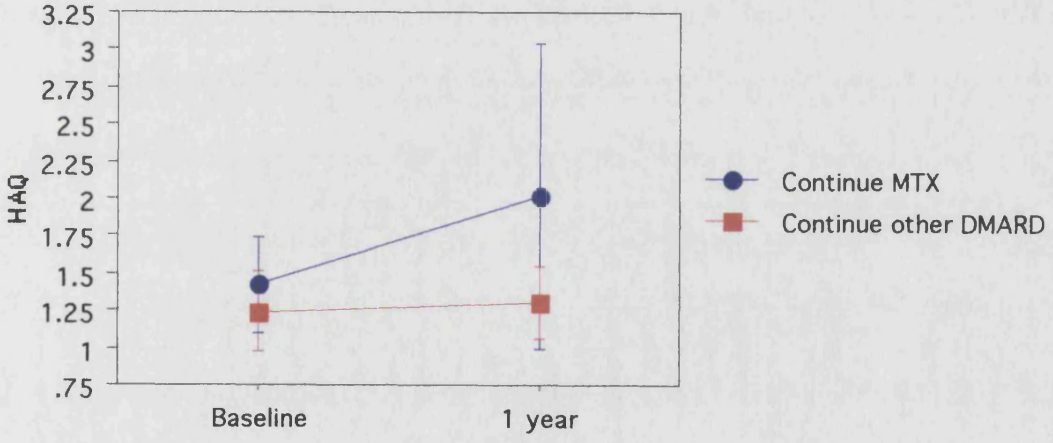
Table 3.8 Functional status at baseline by group

Group / mean ± standard deviation	HAQ* (0 to 3)	Outdoor walking score** (1 to 4)
Starting MTX	1.156 ± 0.753	2.806 ± 0.786
Starting SPZ	1.105 ± 0.557	2.714 ± 0.784
Continue MTX	1.420 ± 0.829	2.857 ± 0.848
Continue Other DMARD	1.244 ± 0.704	2.714 ± 0.937

* Health Assessment Questionnaire score (Kirwan, 1986, Section 1.3.7)
** Derived from EVOS Questionnaire (O'Neill, 1995, Section 2.7.1)

The changes in HAQ over one year are shown in Figure 3.3 for those continuing their DMARD and there was a mean increase in HAQ score of 0.602 in the group continuing MTX and 0.055 in the group continuing other DMARDs. These changes were not significant compared to baseline (by paired t-test) and there was no significant difference between the two groups at one year (by unpaired t-test).

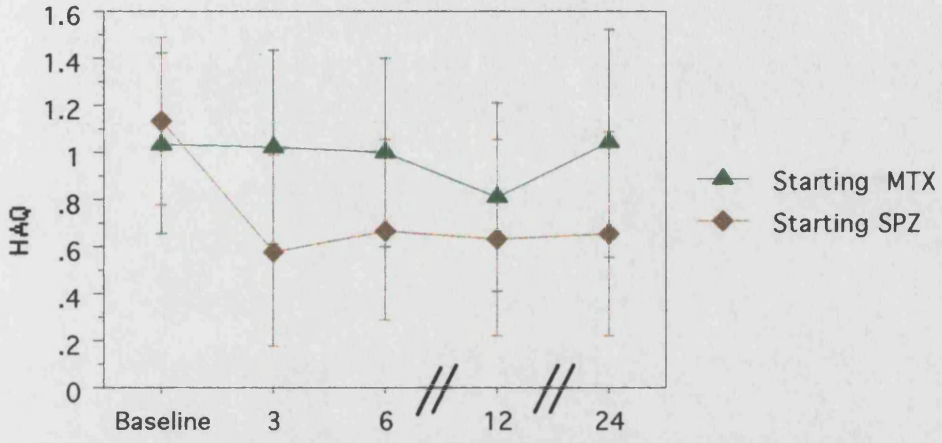
Figure 3.3 Health assessment questionnaire (HAQ) in subjects continuing on a DMARD



Results shown as mean ± 95% confidence interval

The serial HAQ scores over the two years for those for those starting either MTX or SPZ is shown in Figure 3.4 (mean reduction of 0.166 and 0.397 respectively). There was a significant difference between the two groups when they were compared by repeated measures ANOVA ($p = 0.0165$).

Figure 3.4 Health assessment questionnaire (HAQ) in subjects starting a DMARD



Results shown as mean ± 95% confidence interval. There was a significant difference between the two groups by repeated measures ANOVA, p = 0.0165.

Radiographic Score of Damage

The mean Larsen scores of joint damage of the hands and wrists for the different groups at baseline and one year, and for elbows at one year is shown in Table 3.9. Significant differences by analysis of variance for radiographic damage at baseline are shown in Table 3.10. As expected, there were significantly more erosions in the hands and wrists of subjects with long-standing RA (those continuing MTX or other DMARDs) when compared with the propesctive subjects. In addition, there were significantly more hand and elbow erosions in

the group continuing MTX compared to those continuing other DMARDs. This could reflect the fact that the MTX group had been on more DMARDs in the past and had all failed at least one drug, whereas the continuing other DMARD group were more likely to have continued on their first ever DMARD (Table 3.4). Alternatively, MTX may be less effective at suppressing disease progression. However, Larsen scores of wrist radiographs at baseline were similar in the two groups continuing their DMARD.

The Larsen scores for wrist and hand radiographs at one year are also shown in Table 3.9 and the analysis of the change over one year is shown in Table 3.11. The group starting MTX had a significant increase in Larsen score of the wrists compared with each of the other three groups, whilst the Larsen score of the hands significantly increased compared with the two long-term groups, but not those starting SPZ. Thus, MTX was less effective than SPZ in this cohort of subjects in preventing disease progression as measured by radiographic erosions of the wrist. The two groups continuing either MTX or another DMARD did not have a significant difference in change of Larsen score over the year of the study.

Table 3.9 Radiographic damage of hands and wrist joints at baseline and of hands, wrists and elbows at one year of the entire study group

Group / mean ± std dev	Baseline Hands	Baseline Wrists	1 year Hands	1 year Wrists	1 year Elbows
Starting MTX	1.65 ±1.45	2.03 ±1.56	2.11 ± 1.45	2.66 ± 1.47	1.00 ± 1.26
Starting SPZ	1.29 ±1.31	1.43 ±1.40	1.39 ± 1.20	1.50 ± 1.20	0.56 ± 0.51
Continue MTX	4.36 ±1.03	3.96 ±1.11	4.39 ± 1.03	4.07 ± 1.09	4.39 ± 1.03
Continue Other DMARD	3.35 ±1.67	3.31 ±1.56	3.57 ± 1.64	3.50 ± 1.53	3.57 ± 1.64

Table 3.10 Statistical analysis of the variance of radiographic damage (Larsen score) of the entire study group at baseline between the four groups

P values at the 95% confidence level	Continue MTX versus Continue Other DMARD	Continue MTX versus Start MTX	Continue MTX versus Start SPZ	Continue Other DMARD versus Start MTX	Continue Other DMARD versus Start SPZ
Larsen score hands	0.0073	< 0.0001	< 0.0001	< 0.0001	< 0.0001
Larsen score wrists	NS*	< 0.0001	< 0.0001	0.0005	< 0.0001
Larsen score elbows	0.0143	< 0.0001	< 0.0001	< 0.0001	< 0.0001

*NS is no significant difference by analysis of variance with Fisher's Protected Least Significant Difference Post Hoc Testing

Table 3.11 Statistical analysis of the variance of the change in Larsen Score over one year of the entire study group

P value at the 95% confidence level	Continue MTX versus Start MTX	Continue Other DMARD versus Start MTX	Start MTX versus Start SPZ
Change Larsen score of hands over first year	0.0006	0.0204	NS*
Change in Larsen score of wrists over first year	0.0004	0.0022	0.0057

*NS is no significant difference by analysis of variance with Fisher's Protected Least Significant Difference Post Hoc Testing

The change in mean Larsen score of the hands and wrists and the analysis between the groups starting either MTX or SPZ by unpaired t-test is shown in Table 3.12. There was a mean increase in Larsen score for both groups at both sites each year. The greatest change was at the wrists during the first year of treatment in the group starting MTX (0.842) which was significantly higher than the corresponding change in the group starting SPZ (0.200, p = 0.0215). By the second year, however, the mean annual increase in the Larsen score of the wrists had fallen to 0.200 in the MTX group, whilst it was 0.231 in the SPZ group. The overall change from baseline to two years was less in the MTX group than in the group starting SPZ (0.308 compared with 0.500, p = 0.0097). This may represent regression to the mean.

Table 3.12 Change in radiographic score during the study in subjects starting a DMARD

Mean Larsen score ± standard deviation	Starting MTX (number of subjects)	Starting SPZ (number of subjects)	P value at the 95% confidence level by unpaired t-test
Hands: change from baseline to 1 year	+0.579 ± 0.769 (19)	+0.267 ± 0.458 (15)	0.1745
Hands: change from 1 year to 2 years	+0.467 ± 0.640 (15)	+0.231 ± 0.439 (13)	0.2732
Hands: change from baseline to 2 years	+1.200 ± 1.207 (15)	+0.500 ± 0.674 (12)	0.0851
Wrists: change from baseline to 1 year	+0.842 ± 0.958 (19)	+0.200 ± 0.414 (15)	0.0215
Wrists: change from 1 year to 2 years	+0.200 ± 0.561 (15)	+0.231 ± 0.439 (13)	0.8742
Wrists: change from baseline to 2 years	+0.308 ± 0.630 (15)	+0.500 ± 0.798 (12)	0.0097

Results shown as mean change in Larsen score ± standard deviation (n).

3.3.7 Summary of Demographic and Rheumatoid Arthritis Characteristics of the Subjects

The groups of patients starting either MTX or SPZ were well-matched, especially when only those with RA of less than two years' duration were considered.

The subjects continuing on MTX were different from those continuing on other DMARDs as in general they had RA which had been more difficult to treat, reflected by the greater number of erosions in the hands and elbows (though not the wrists) and the number of different DMARDs they had been on prior to MTX. The difference in erosions at the elbow was important as significant differences in some measurements of forearm BMD were found in the two groups continuing DMARDs (Section 3.6).

MTX and SPZ had similar, rapid beneficial effects on rheumatoid disease activity which were sustained over the period of follow-up. The reduction in DAS was significant compared to baseline at all subsequent time points, although the majority of subjects in all four groups had high DAS throughout the study period. SPZ was more effective than MTX in improving the functional status (HAQ) over the first year of treatment, but this advantage was lost by two years.

The changes in radiographic scores at the wrist and hands over one year were not significantly different between the two groups on long-term treatment. The group starting MTX did have a significant increase in Larsen score of the wrist over the first year of the study compared with the group starting SPZ, suggesting MTX was less effective in retarding disease progression at this site. However, over two years, the group starting MTX had significantly less progression at the wrist than the group starting SPZ.

3.4 Adverse Events During the Study

3.4.1 Incident Fractures During the Study

There were two incident osteoporotic or fragility fractures during the study period, both of the humerus.

One woman who had taken MTX for five years, fractured the surgical neck of the humerus after a simple fall at home. She was aged sixty-nine, had suffered from RA for thirty-four years and was on no treatment for osteoporosis despite having fractured her hip in the past.

The second subject was in the group starting MTX. There was no fracture history and she slipped on a wet bathroom floor and fractured the distal humerus. She was aged fifty-six, was diagnosed with RA six years ago and had previously been treated with SPZ. She was taking hormone-replacement therapy having first had menopausal symptoms four years earlier.

3.4.2 Adverse Drug-Related Events

There were twenty-six adverse events during the study. The fourteen events which were recognised side effects of MTX, SPZ or other DMARDs are shown in Table 3.13, together with the numbers in each group who stopped their DMARD.

3.4.3 Adverse Events Not Related to Drugs

The twelve other adverse events which were probably not related to the DMARD therapy are shown in Table 3.14. The only death which occurred during the study was a seventy-eight year old woman who had a myocardial infarction within three months of starting SPZ.

Table 3.1 3 Adverse events related to medication

Group	Type of adverse event (number of subjects)	Number of subjects who stopped DMARD due to side effects
Starting MTX	mouth ulcers (3) nausea (2) abnormal LFTs* (1) hair loss (2) rash (1)	0
Starting SPZ	abnormal LFTs* (1) rash (2) shingles (1)	2
Continue MTX	mouth ulcers (1)	0
Continue Other DMARD	none	0

* abnormal LFTs were elevated serum liver function tests (transaminases) which resolved after one month off treatment

Table 3.1 4 Adverse events probably unrelated to medication

Group	Type of adverse event (number of subjects)
Starting MTX	carpal tunnel syndrome (2) ruptured Achilles tendon (1) ruptured flexor pollicis longus tendon (1) Achilles tendinitis (1) fibromyalgia (2) tinnitus (1) haematuria (2) sciatica (1)
Starting SPZ	fatal myocardial infarction (1)
Continue MTX	none
Continue Other DMARD	none

3.4.4 Protocol Violations

During the period of the study subjects were followed up in their usual clinic where a change in medication was sometimes felt necessary on clinical grounds. A total of nine protocol violations occurred and are shown in Table 3.15. The most common change in medication was from another DMARD to methotrexate.

Table 3.15 Protocol violations during the first year

Group	Type of violation (number of subjects)
Starting MTX	intramuscular steroid for flare of RA (1)
Starting SPZ	stopped SPZ (3) changed to MTX(2)
Continue MTX	none
Continue Other DMARD	changed to MTX (2) intravenous steroid for flare of RA (1)

3.4.5 Changes in Therapy Which Could Affect Bone

Patients and General Practitioners also made changes in medication which may have had an effect on bone. These are shown in Table 3.16. The subject who took a Chinese medication was unable to discover the content other than "herbs" but was told that it did not contain corticosteroids. She had symptomatic, rapid relief from the preparation. There have been reports of Chinese medication containing undeclared non-steroidal anti-inflammatory drugs and benzodiazipines (Gertner et al, 1995).

Table 3.1 6 Change in medication during study which may affect bone

Group	Medication (number of subjects)
Starting MTX	took Chinese medicine for 2 months (1)
Starting SPZ	started hormone replacement therapy (1)
Continue MTX	started thiazide for hypertension (2)
Continue Other DMARD	none

3.4.6 Dropouts and Failure to Attend Study Visits

There were five dropouts (4.4%) from the study after the baseline visit. Four were in the group starting SPZ. A woman died from a myocardial infarct between the first two baseline visits and the three month visit. A man failed to attend after the three month visit and could not be traced despite phone calls, letters and contacting his GP. He had probably moved back to Ireland. Another woman decided that she did not want to participate further in the study after the baseline visits, although she was happy to continue on SPZ. A woman who had failed to tolerate SPZ due to side effects of nausea and vomiting, failed MTX after one month with the same symptoms and was treated subsequently with intra-muscular gold did not attend the one year visit.

A woman in the group continuing a DMARD other than MTX was unable to attend the appointment at one year due to chronic obstructive pulmonary disease requiring continuous oxygen therapy.

Finally, a woman in the group starting MTX failed to attend the three month appointment but was seen at six months and at one year.

Data where available on those subjects who dropped out was included in the analysis.

3.4.7 Missing Data

A total of sixteen serum samples could not be found to analyse and sixteen urine samples were all used up in the HPLC preparation and could not therefore be analysed by ELISA.

Three urine samples were not brought for study visits and one patient left before having his blood taken.

The radiograph packets for two subjects were lost and thus could not be scored.

Hip BMD measurements could not be obtained in one subject in the continuing MTX group as she had had bilateral hip replacements.

It was not possible to position the forearm for bone densitometry in some patients with severe rheumatoid elbow or shoulder disease. Forearm BMDs could not be obtained in one subject in the continuing MTX group and two subjects in the continuing Other DMARD group.

3.5 Results of the Whole Cohort

3.5.1 Bone Mineral Content, Bone Mineral Density and Bone Mineral Density Z Score of All Subjects at Baseline

The mean bone mineral content (BMC, g) of the four groups for each of the regions scanned is shown in Table 3.17. The bone mineral density (bone mineral content expressed as areal density, BMD, g/cm^2) and BMD expressed as standard

deviations from the age- and sex-matched normal reference data (Z scores) are shown in Tables 3.18 and 3.19 respectively.

Table 3.17 Bone mineral content (BMC) of the entire study group at baseline

BMC (g) Mean ± standard deviation	Start MTX n = 36	Start SPZ n = 22	Continue MTX n = 28 #	Continue Other DMARD n = 29 #
Lumbar spine	63.47* (a) ± 15.83	61.31 ± 13.74	54.85* (a) ± 16.81	60.45 ± 15.65
Neck of femur	4.01* (b) ± 0.89	3.91 ± 0.77	3.53* (b) ± 1.12	3.77 ± 0.75
Trochanteric	7.97 ± 2.46	7.85 ± 2.68	7.14 ± 2.10	7.84 ± 2.49
Inter-trochanteric	20.16 ± 6.27	20.29 ± 5.68	19.49 ± 8.95	20.34 ± 6.45
Ward's region	0.71** (c) ± 0.23	0.72** (d) ± 0.19	0.55** (c, d) ± 0.24	0.63 ± 0.19
Total hip	32.14 ± 9.31	32.04 ± 8.77	30.17 ± 11.09	31.94 ± 9.29
Proximal third forearm	3.50* (e) ± 0.83	3.54* (f) ± 0.89	2.97* (e, f) ± 1.26	3.48 ± 1.02
Mid-portion forearm	6.72 ± 2.10	7.18* (g) ± 2.61	5.51* (g) ± 2.97	6.88 ± 2.70
Ultra-distal forearm	2.24 ± 0.68	2.28 ± 0.65	1.93 ± 0.79	2.23 ± 0.69
Total forearm	12.46 ± 3.54	13.01* (h) ± 4.07	10.40* (h) ± 4.94	12.59 ± 4.33

Analysis by ANOVA with Fisher's Protected Least Significant Difference post hoc testing
* $p < 0.05$, ** $p < 0.01$, a, b, c etc. identify the groups with significant differences.
# There was no hip or forearm scan available for one subject in the continue MTX group and two subjects in the group continuing other DMARDs group (Section 3.8.7).

There were no significant differences in BMC at any site between the two groups continuing on their DMARD (either MTX or other DMARD, Table 3.17) or the two groups starting either MTX or SPZ (result not shown). However, the BMC was

significantly lower in the group continuing MTX than in those starting MTX at the lumbar spine, neck of femur, Ward's area of the hip and the proximal third of the forearm (radius and ulna combined). The BMC of the long-term MTX group was also lower than the group starting SPZ at Ward's area of the hip, and the proximal third, mid-portion and total forearm measurements (radius and ulna combined).

Table 3.1 8 Bone mineral density (BMD) of the entire study group at baseline.

BMD (g/cm^2) Mean ± standard deviation	Start MTX	Start SPZ	Continue MTX	Continue Other DMARD
Lumbar spine	1.002* (a) ± 0.183	0.995* (b) ± 0.139	0.892* (a,b) ± 0.197	0.963 ± 0.148
Neck of femur	0.770* (c) ± 0.144	0.754 ± 0.100	0.681* (c) ± 0.197	0.710 ± 0.125
Trochanteric	0.686* (d) ± 0.159	0.675 ± 0.115	0.613* (d) ± 0.155	0.650 ± 0.133
Inter-trochanteric	1.066** (e) ± 0.206	1.032 ± 0.151	0.939** (e) ± 0.199	0.985 ± 0.189
Ward's region	0.625** (f) ± 0.189	0.625* (g) ± 0.146	0.491* (g) / ** (f) ± 0.217	0.548 ± 0.156
Total hip	0.898* (h) ± 0.178	0.879 ± 0.126	0.796* (h) ± 0.180	0.841 ± 0.157
Proximal third forearm	0.655*** (i) ± 0.110	0.660*** (j) ± 0.113	0.527** (k) / *** (i,j) ± 0.166	0.639** (k) ± 0.130
Mid-portion forearm	0.541** (l) ± 0.099	0.555** (m) ± 0.103	0.445* (n) / ** (l,m) ± 0.145	0.515* (n) ± 0.114
Ultra-distal forearm	0.375** (o) ± 0.091	0.384** (p) ± 0.081	0.353* (q) / ** (o,p) ± 0.126	0.375* (q) ± 0.077
Total forearm	0.525** (r) ± 0.098	0.536** (s) ± 0.097	0.441* (t) / ** (r,s) ± 0.136	0.507* (t) ± 0.105
Proximal third radius	0.658*** (u) ± 0.106	0.670*** (v) ± 0.109	0.533**(w) / ***(u,v) ± 0.172	0.650** (w) ± 0.128
Mid-portion radius	0.558** (x) ± 0.094	0.575** (y) ± 0.094	0.473* (z) / ** (x,y) ± 0.149	0.538* (z) ± 0.108
Ultra-distal radius	0.401 ± 0.098	0.416 ± 0.087	0.375 ± 0.130	0.398 ± 0.089
Total radius	0.535** (aa) ± 0.096	0.551** (ab) ± 0.093	0.458* (ac) / ** (aa,ab) ± 0.139	0.522* (ac) ± 0.103

Analysis by ANOVA with Fisher's PLSD post hoc testing.

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; a, b, c etc. identify the groups with significant differences.

Table 3.19 Bone mineral density expressed as the Z score of the entire study group at baseline

Z score Mean ± standard deviation	Start MTX	Start SPZ	Continue MTX	Continue Other DMARD
Lumbar spine	0.624 ± 1.491	0.542 ± 1.282	-0.022 ± 1.378	0.473 ± 1.143
Neck of femur	-0.022 ± 1.265	-0.141 ± 0.837	-0.476 ± 1.603	-0.310 ± 1.013
Trochanteric	0.216 ± 1.448	0.182 ± 0.965	-0.0267 ± 1.320	-0.041 ± 1.043
Inter-trochanteric	0.117* (a) ± 1.256	-0.049 ± 0.856	-0.469* (a) ± 1.112	-0.310 ± 1.088
Ward's region	0.419 ± 1.443	0.444 ± 0.951	-0.244 ± 1.600	0.070 ± 1.199
Total hip	0.077 ± 1.244	-0.005 ± 0.802	-0.446 ± 1.173	-0.251 ± 0.997
Proximal third forearm	0.076# (b) ± 1.181	0.218# (c) ± 1.221	-1.746# (b,c,d) ± 1.943	-0.121# (d) ± 1.383
Mid-portion forearm	-0.200** (e) ± 1.212	0.138# (g) ± 1.262	-1.621** (e,f) / # (g) ± 1.713	-0.615** (f) ± 1.399
Ultra-distal forearm	-0.253 ± 1.247	-0.043 ± 1.167	-0.417 ± 1.799	-0.182 ± 1.054
Total forearm	-0.152*** (h) ± 1.211	0.129*** (i) ± 1.225	-0.410** (j) /***(h,i) ± 1.674	-0.403** (j) ± 1.260
Proximal third radius	0.021# (k) ± 1.183	0.268# (m) ± 1.140	-1.715# (k,m,n) ± 2.038	0.009# (n) ± 1.415
Mid-portion radius	-0.281** (o) ± 1.227	0.089*** (p) ± 1.101	-1.453* (q)/** (o)/*** (p) ± 1.785	-0.557* (q) ± 1.327
Ultra-distal radius	-0.261 ± 1.252	0.021 ± 1.130	-0.439 ± 1.653	-0.232 ± 1.112
Total radius	-0.233** (r) ± 1.222	0.118*** (s) ± 1.125	-1.297* (t)/** (r)/*** (s) ± 1.678	-0.365* (t) ± 1.237

Analysis by ANOVA with Fisher's PLSD post hoc testing

Z score is the bone mineral density expressed as standard deviations above (positive values) or below (negative values) the mean value of an age- and sex-matched reference population (Section 1.2.5).

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, # $p < 0.0001$. a, b, c etc. identify the groups with significant differences.

BMD estimates for the group continuing MTX were significantly lower than the group starting MTX at all sites except the ultra-distal portion of the forearm (for

the radius and ulna combined, and the radius alone). The values in the long-term MTX group were also significantly lower than in the group starting SPZ for many sites (Table 3.18). This may reflect the longer duration of RA and the older age of the cohort on long-term MTX compared to subjects starting a DMARD. However, there were also significant differences between the group continuing MTX when compared to those subjects who were continuing a different DMARD from MTX although these groups were well-matched for age and disease duration (Section 3.3). The BMD of the forearm (except for the ultra-distal portion) was significantly lower in the group on MTX compared to those taking other DMARDs and the differences became highly significant ($p < 0.0001$) in the proximal third portion when BMD was corrected for age and sex (Z score, Table 3.19). These differences may be partially explained by the increased Larsen scores of the elbows in the group continuing MTX (Section 3.3.6), but was an unexpected finding.

Many of the significant differences in BMD measurements between the long-term MTX group and those starting MTX or SPZ were no longer significant when the Z score was considered, confirming that they were due to age differences between the groups (Table 3.19).

3.5.2 Change in Bone Mineral Density of All Subjects Over One Year

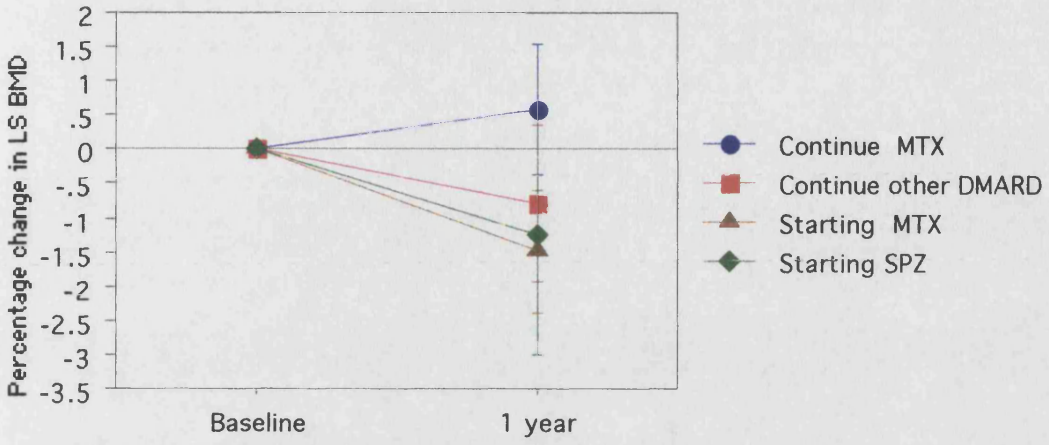
The percentage changes in bone mineral density (BMD) over the first year of the study are shown in Table 3.20. The percentage changes in BMD are shown in Figures 3.5 to Figures 3.10 for the regions where the differences by group reached significance at the 95% confidence level.

Table 3.20 Percentage change in bone mineral density of all subjects over one year.

% change in BMD Mean ± standard deviation	Start MTX	Start SPZ	Continue MTX	Continue Other DMARD
Lumbar spine	-1.490** (a) ±2.684	-1.259* (b) ±3.537	+0.583* (b)/ ** (a) ± 2.435	-0.783 ±2.936
Neck of femur	-1.887* (c,d) ±3.960	-0.161 ±4.250	+0.437*(c) ±4.566	+0.354*(d) ±4.017
Trochanteric	-2.561 ±7.050	-1.144 ±5.024	-0.740 ±3.253	-2.008 ±3.979
Inter-trochanteric	-2.085 ±4.598	-0.587 ±4.607	-1.358 ±3.935	-2.141 ±3.840
Ward's region	-1.353*(e) / **(f) ± 6.871	+0.732 ±5.121	+3.639** (f) ±6.707	+2.827* (e) ±7.929
Total hip	-2.174 ±4.221	-0.728 ±4.523	-0.750 ±2.897	-1.669 ±3.262
Proximal third forearm	-1.913* (g,h) ±3.966	+0.302*(g) ±2.791	+0.095*(h) ±4.335	-1.518 ±2.875
Mid-portion forearm	-2.472 ±4.284	-1.112 ±1.843	-0.767 ±4.043	-1.826 ±3.232
Ultra-distal forearm	-2.903* (i) ±4.398	-2.608 ±4.048	-0.376* (i) ±6.918	-1.761 ±3.387
Total forearm	-2.347* (j) ±3.766	-1.024 ±1.984	-0.491* (j) ±4.117	-1.767 ±2.698
Proximal third radius	-1.037 ± 3.954	-0.212 ±3.077	-0.016 ±5.205	-1.682 ±3.603
Mid-portion radius	-1.772 ±3.965	-1.116 ±2.601	-0.883 ±4.996	-2.160 ±3.670
Ultra-distal radius	-2.610 ±5.214	-3.077 ±4.689	-0.887 ±7.827	-2.469 ±3.660
Total radius	-1.715 ±3.592	-1.400 ±2.811	-0.646 ±5.185	-2.113 ±3.110

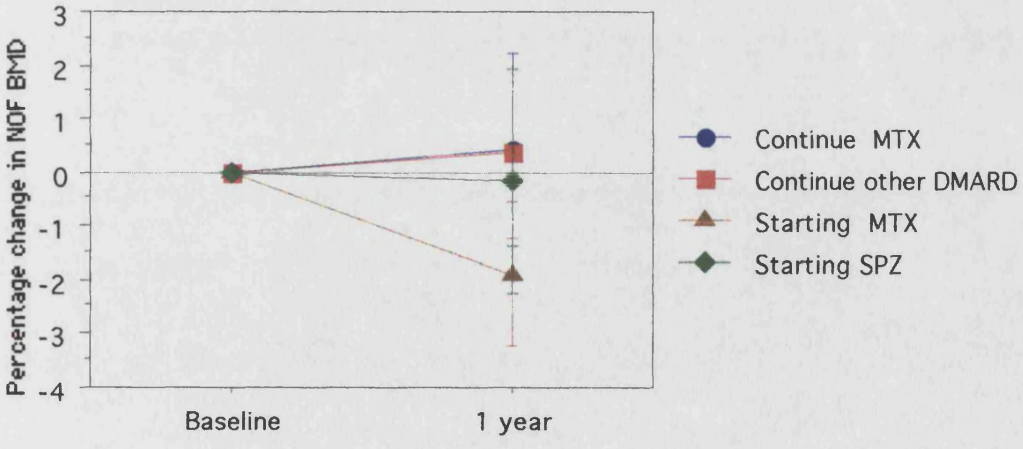
Analysis by ANOVA with Fisher's Protected Least Significant Difference post hoc testing
* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, a, b and c etc. identify the groups with significant differences

Figure 3.5 Change in lumbar spine bone mineral density over one year



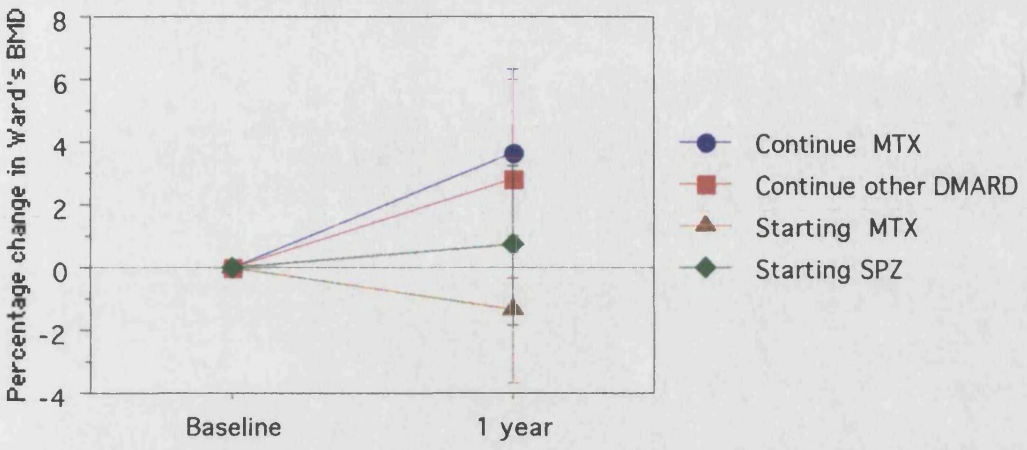
Results shown as mean percentage change ± 95% confidence interval

Figure 3.6 Change in neck of femur bone mineral density over one year



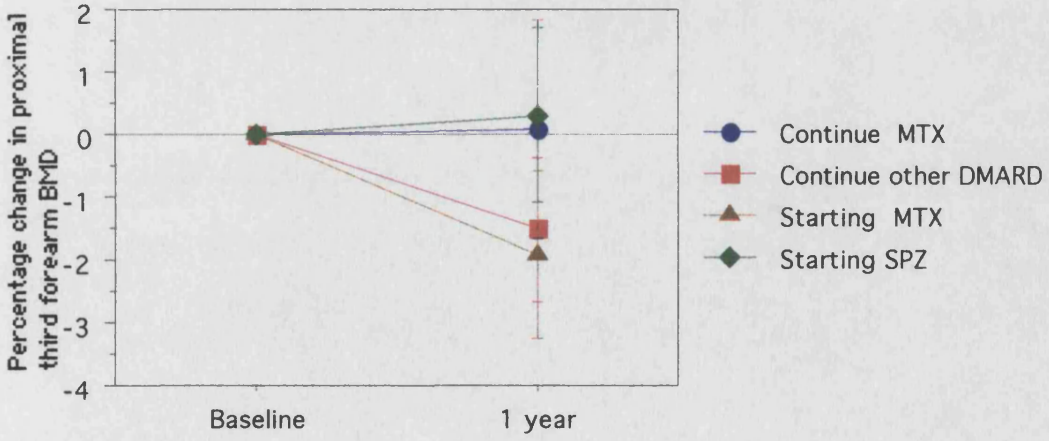
Results shown as mean percentage change ± 95% confidence interval

Figure 3.7 Change in Ward's area bone mineral density over one year



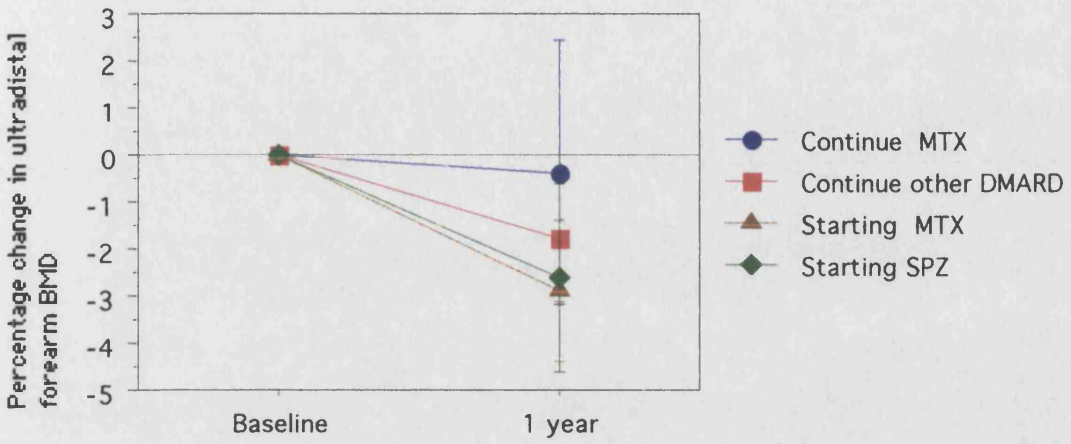
Results shown as mean percentage change ± 95% confidence interval

Figure 3.8 Change in proximal third forearm bone mineral density over one year



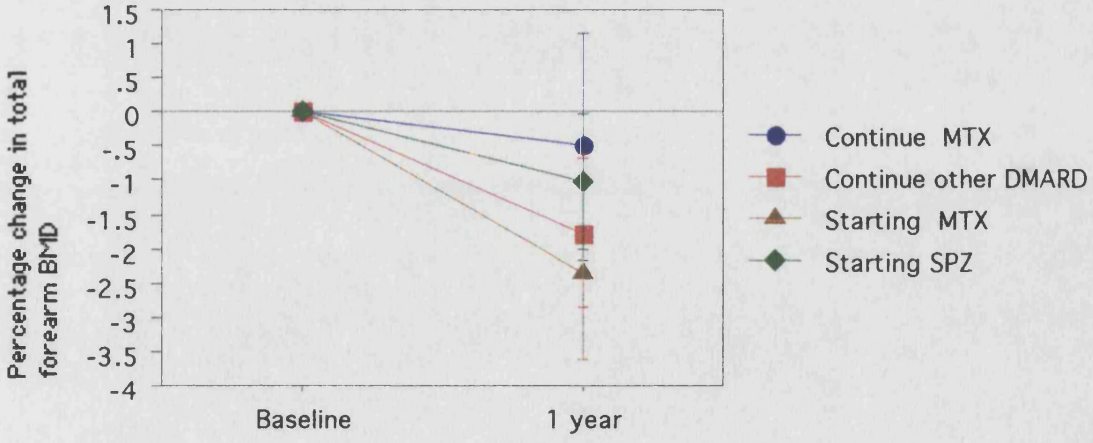
Results shown as mean percentage change ± 95% confidence interval

Figure 3.9 Change in ultra-distal forearm bone mineral density over one year



Results shown as mean percentage change ± 95% confidence interval

Figure 3.10 Change in total forearm bone mineral density over one year



Results shown as mean percentage change ± 95% confidence interval

The mean bone mineral density of the lumbar spine increased in the group continuing on MTX whilst it decreased in the other three groups (Table 3.20). The greatest loss was in the group starting MTX where the mean loss was 1.5%. There were significant differences between the group continuing MTX and each of the groups starting a DMARD but not between the two groups continuing on their DMARD (Table 3.20 and Figure 3.5). The loss of bone mass in the lumbar spine in early RA has been described previously (Shenstone et al, 1994; Gough et al, 1994). The increase in lumbar spine BMD seen in the group continuing MTX may be due to degenerative changes.

There were no significant inter-group differences in the rate of annual change of BMD for the trochanteric, inter-trochanteric or total hip measurements (Table 3.20). The group starting MTX had significantly more bone loss at the neck of femur site than each of the two groups continuing on their DMARD and the mean BMD actually increased in both these groups. However there was no difference in the rate of change between the group starting MTX and the group starting SPZ (Table 3.20 and Figure 3.6). Similarly, the group starting MTX had more bone loss from Ward's area and this reached significance only when compared with the two long-term groups (Table 3.20 and Figure 3.7).

The only significant difference in bone loss between the group starting MTX and the group starting SPZ was found in the proximal third of the forearm (for the radius and ulna combined, Table 3.20 and Figure 3.8). This is the same site where highly significant differences had been found at baseline between the two groups which had been on long-term DMARD treatment. In addition, compared to the group continuing MTX, the group starting MTX had sustained significantly more bone loss

from the ultra-distal (Figure 3.9) and total forearm (Figure 3.10) regions, again for the ulna and radius combined.

These results, taken together with the baseline Z score analysis (Table 3.19), suggested that MTX therapy was associated with increased bone loss from the forearm, particularly the proximal third region. The increased loss occurred early rather than later in treatment with MTX, as subjects continuing on MTX no longer had excess bone loss from this site.

3.5.3 Markers of Bone Turnover of All Subjects at Baseline

The mean baseline levels of serum osteocalcin (OC), bone specific alkaline phosphatase (AP) and urinary deoxypyridinoline corrected for urinary creatinine (Dpd) for the four groups are shown in Table 3.21. The mean results from the two baseline visits a fortnight apart are given. There were significantly higher mean values of the markers of bone formation (OC and AP) in the group which had been on MTX for at least five years when compared with the other three groups. There were no significant differences between groups for the marker of resorption (Dpd).

Table 3.21 Markers of bone formation and resorption of all subjects at baseline

Mean ± standard deviation of the mean	Start MTX n = 36	Start SPZ n = 22	Continue MTX n= 28	Continue Other DMARD n = 29	Normal range female ; male
Serum OC (ng/ml)	9.496*** (a) ± 2.570	9.587*** (b) ± 2.291	12.921*(c) / ***(a,b) ± 4.412	10.818* (c) ± 3.873	3.7-10.0 ; 3.4-9.1
Serum AP (U/litre)	24.101* (d) ± 10.808	22.171* (e) ± 9.300	30.062* (d,e) / **(f) ± 14.809	20.099** (f) ± 8.124	10-22 ; 12-23
Urinary Dpd / urinary creatinine (nM/mM)	9.333 ± 4.006	7.281 ± 3.725	8.474 ± 3.134	7.904 ± 3.932	3.0-7.4 ; 2.3-5.4

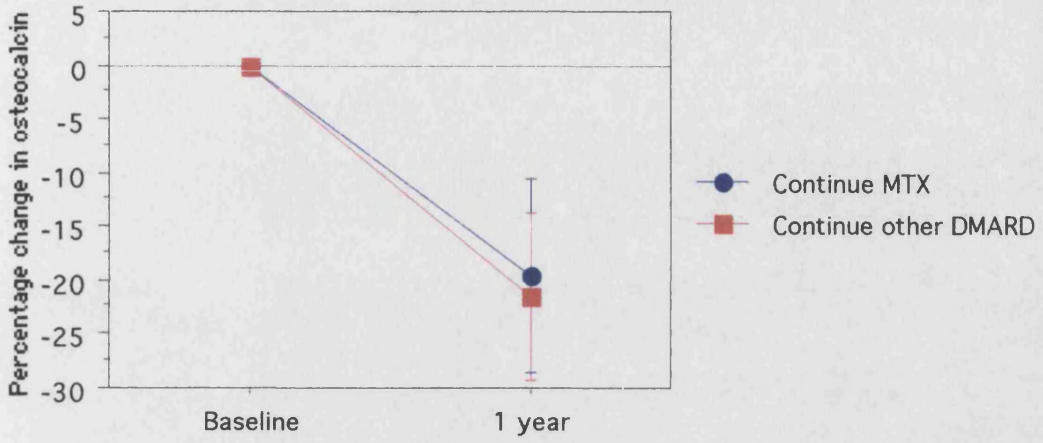
Analysis by ANOVA with Fisher's PLSD post hoc testing

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, a, b, c etc. identify the groups with significant differences.

3.5.4 Change in Markers of Bone Turnover of All Subjects Over One Year

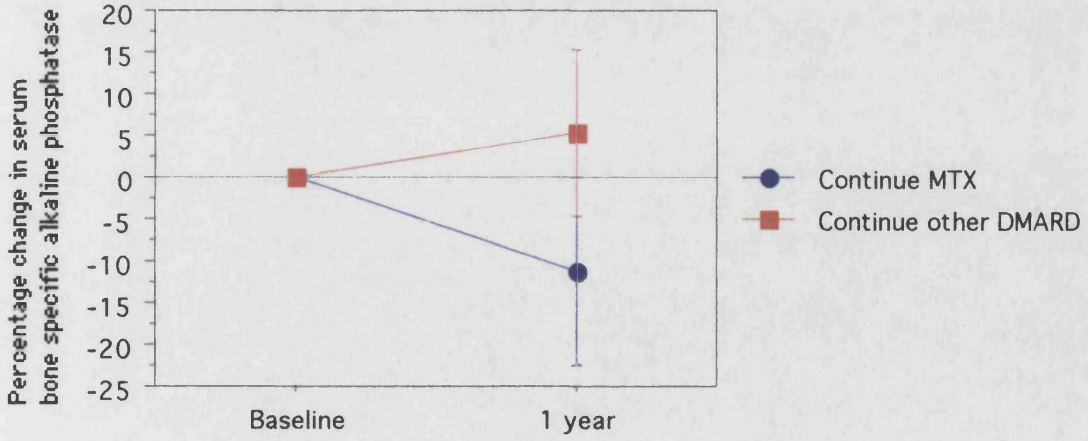
The percentage change in the markers of bone turnover from mean baseline values over the year of the study for the subjects continuing their DMARD are shown in Figure 3.11 for OC, Figure 3.12 for AP and Figure 3.13 for Dpd. The changes were compared by unpaired t-test and the only significant difference was in mean AP. The group continuing MTX mean AP value decreased by 11% whilst it increased by 5% in the group continuing other DMARDs ($p = 0.0198$).

Figure 3.11 Osteocalcin in subjects continuing a DMARD



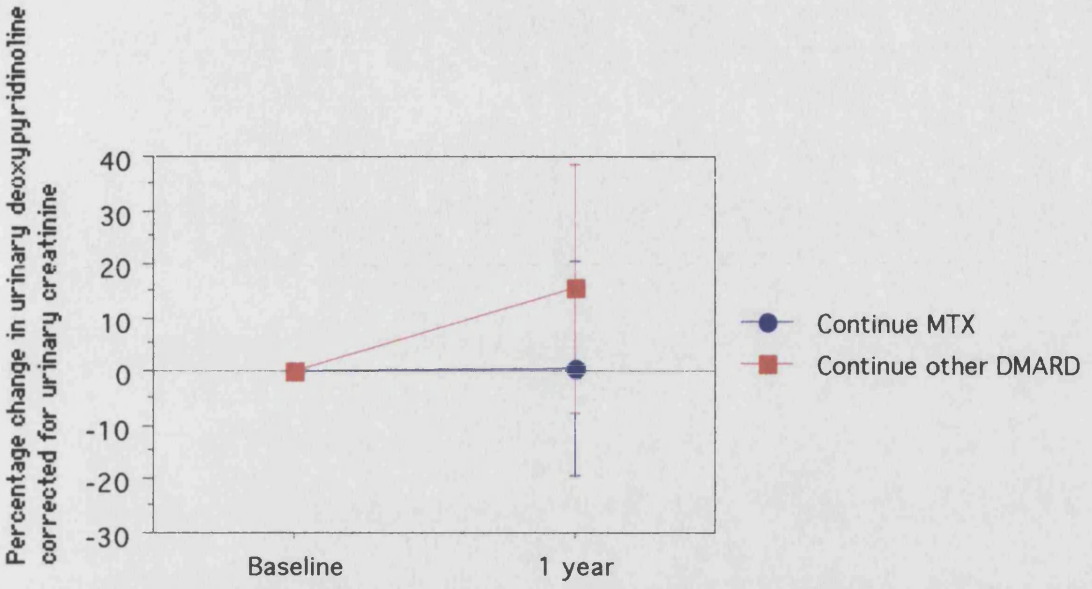
Results shown as mean percentage change from baseline ± 95% confidence interval

Figure 3.12 Bone-specific alkaline phosphatase in subjects continuing a DMARD



Results shown as mean percentage change from baseline ± 95% confidence interval

Figure 3.13 Urinary deoxypyridinoline in subjects continuing a DMARD

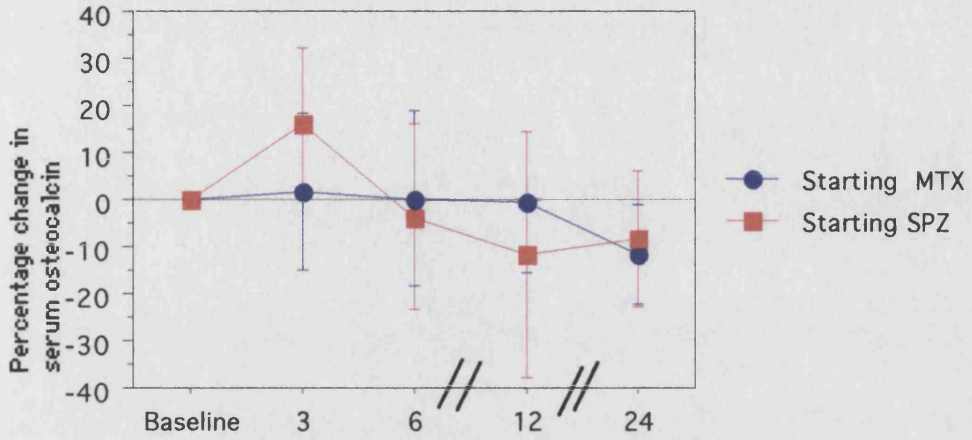


Results shown as mean percentage change from baseline ± 95% confidence interval

For the subjects starting MTX or SPZ, the percentage change from baseline over the first two years of treatment are shown in Figure 3.14 for OC, Figure 3.15 for AP and Figure 3.16 for Dpd.

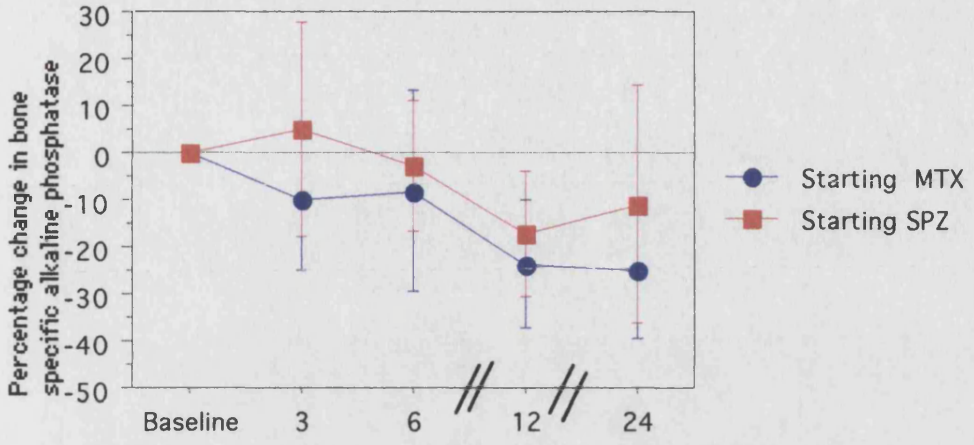
The two groups were compared by repeated measures ANOVA, with the different time point measures included as a compact variable. There were no significant differences between the two groups by this analysis for any of the bone turnover measures, suggesting MTX has no effect on OC, AP or Dpd in these subjects.

Figure 3.14 Serum osteocalcin in subjects starting a DMARD



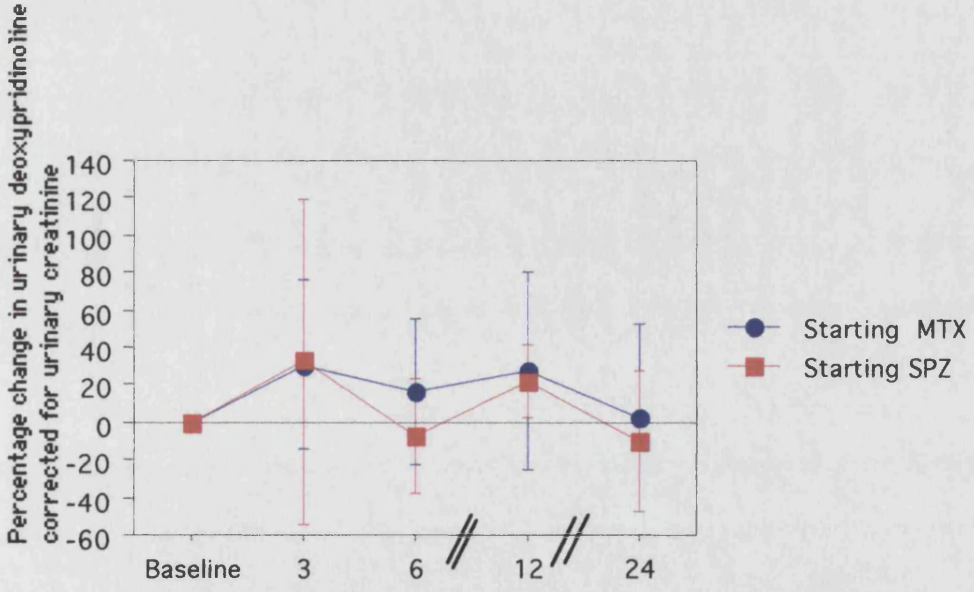
Results are shown as mean percentage change from baseline ± 95% confidence interval. The groups were compared by repeated measures ANOVA.

Figure 3.15 Serum bone specific alkaline phosphatase in subjects starting a DMARD



Time on treatment in months. Groups were compared by repeated measures ANOVA.

Figure 3.16 Urinary deoxypyridinoline in subjects starting a DMARD



Time on treatment in months. Groups were compared by repeated measures ANOVA.

3.6 Results of Postmenopausal Women

Postmenopausal women are at greatest risk of osteoporosis and so, if MTX has an adverse effect on bone, this is the group in which it could be expected to be seen. The BMD at any given time and site is influenced by many factors which are often not truly independent. In RA, disease duration and disease activity interact to produce damage (erosive changes on radiographs) but the nature of the relationship between these factors is not known. In a postmenopausal woman, the BMD will depend on both how much bone mass was lost in the immediate postmenopausal period but also on what peak bone mass she had attained in her youth. The peak bone mass is influenced by genetic and environmental factors, such as diet and exercise.

Due to the numbers in each group (only nine subjects starting SPZ), it was not possible (or desirable) to enter every variable on which data had been collected into the model. A much larger, multi-centre study design would be required for such an analysis. The factors which are known to have the greatest impact on BMD, both from population studies and a survey of the data in this study, were chosen and included in the multiple regression model.

Dr Daphne Kounali (School of Maths, University of Bath) undertook the statistical analysis of this group. The BMD for the different regions was expressed as the Z score (standard deviations from the age-appropriate mean) and the change in BMD as percentage change in Z score which removed the requirement for age as an independent variable. In the analysis of the markers of bone turnover, however, age was a covariate.

3.6.1 Statistical Methods Applied to the Dataset of Postmenopausal Women

The many different variables on which data had been collected were studied and plotted out for the different groups. There were no differences found between the groups in many of the life-style variables such as diet, exercise, smoking and other features such as family history. Trends were noted such as smokers tended to have lower BMD and subjects who were more physically active, both now and in the past, tended to have higher BMDs. The variables which were studied and not included in the regression analysis are shown in Table 3.22.

Table 3.22 Variables studied and not included in the regression analysis

Family history of osteoporosis
Previous osteoporotic fracture
Smoking
Parity
Oral contraceptive use
Breast feeding
Use of thiazide diuretics
Use of non-steroidal anti-inflammatory drugs
Calcium intake
Walking score

The variables found to strongly influence BMD were: the duration of rheumatoid arthritis (disease duration), the menopausal age (number of years since the onset of the menopause), the body mass index (BMI), and the disease activity at baseline (DAS, Section 1.3.6). The Larsen x-ray score of the hand, wrist and elbow and the changes in hand and wrist x-ray scores over the year were also used in the model as covariates. Due to the small number of subjects, the Larsen scores were dichotomised into 'low' and 'high' scores, where 'high' was any score greater than zero. This enabled the Larsen score to be used as a nominal variable. Similarly, the change in Larsen score over the year was coded as '0' for no change (increase) and as '1' for any increase. In practice, no subject had an increase of more than one in Larsen score at any site, and no subjects had an improvement (reduction) in Larsen score.

The confounding factors which were used as the covariates in the analysis are shown in Table 3.23.

Table 3.23 Confounding factors (covariates) used in the analysis

Duration of RA (months)
Disease activity (DAS)
Body mass index (kg/m^2)
Menopausal age (years)
Baseline hand x-ray score
Change in hand x-ray score
Baseline wrist x-ray score
Change in wrist x-ray score
Baseline elbow x-ray score

Factorial analysis of variance (ANOVA) and repeated measures analysis of variance was performed. Duncan's multiple range test was used to examine multiple comparisons between the four groups (Barnett, 1982). When the assumptions needed for analysis of variance were violated, such as normality and homogeneity of variance, either Friedman's non-parametric test was used, or the data were log-transformed prior to ANOVA (Conover, 1980).

Multiple regression analysis was used to examine apparent effects of MTX on BMD (Section 3.6.4). The potentially confounding covariates above in Table 3.23 were entered into the model (McCullagh et al, 1984).

To test the strength of association between nominal variables Mantel-Haenszel chi-square test was used and Fisher's exact test was employed when relatively small cell sizes were found (Everitt, 1984).

Finally, discriminant analysis was used to identify which measurements were important for distinguishing among treatment groups using a multivariate approach (Tatsuoka, 1971).

3.6.2 Unadjusted Results of Postmenopausal Women

The primary outcome measures were the BMD Z score and the change in BMD Z score over one year and the results of the analysis of these unadjusted measurements by ANOVA are shown in Table 3.24 and Table 3.25.

Table 3.24 Baseline unadjusted BMD Z score

	Starting MTX	Starting SPZ	Continuing MTX	Continuing Other DMARD
Baseline Lumbar Spine BMD Z score	0.66 ± 1.56 [-0.20 1.52] (15)	*(a) 1.04 ± 1.30 [0.04 2.04] (9)	*(a) -0.43 ± 1.24 [-1.10 0.19] (18)	0.38 ± 1.01 [-0.17 0.94] (15)
Baseline Neck of Femur BMD Z score	*(b) -0.019 ± 0.97 [-0.56 0.52] (15)	0.09 ± 0.61 [-0.37 0.56] (9)	*(b) -0.95 ± 1.15 [-1.5 -0.38] (18)	-0.35 ± 1.13 [-0.98 0.28] (15)
Baseline Total Hip BMD Z score	-0.04 ± 1.18 [-0.69 0.62] (15)	0.18 ± 0.9 [-0.52 0.88] (9)	-0.80 ± 0.88 [-1.2 -0.36] (18)	-0.49 ± 1.18 [-1.14 0.16] (15)
Baseline Total Forearm BMD Z score	*(c) -0.32 ± 1.26 [-1.02 0.38] (15)	*(d) 0.17 ± 1.08 [-0.66 0.99] (9)	*(c,d) -1.94 ± 1.48 [-2.68 -1.21] (18)	-0.80 ± 1.44 [-1.63 0.029] (14)
Baseline Ultra-distal Forearm BMD Z score	-0.59 ± 1.13 [-1.23 0.027] (15)	0.03 ± 1.01 [-0.75 0.82] (9)	-0.81 ± 1.22 [-1.41 -0.20] (18)	-0.47 ± 1.03 [-1.07 0.13] (14)
Baseline Mid-portion Forearm BMD Z score	*(e) -0.28 ± 1.26 [-0.98 0.41] (15)	0.143 ± 1.17 [-0.76 1.04] (9)	*(e) -2.19 ± 1.45 [-2.9 -1.46] (18)	-1.02 ± 1.6 [-1.9 -0.09] (14)
Baseline Proximal 1/3 Forearm BMD Z score	*(f) -0.02 ± 1.35 [-0.76 0.73] (15)	0.28 ± 1.19 [-0.6 1.2] (9)	*(f) -2.3 ± 1.5 [-3.1 -1.5] (18)	-0.5 ± 1.6 [-1.4 0.41] (14)

Results are shown as the mean ± standard deviation of the mean, [95% confidence interval for the mean], (n). *Significant differences between the groups, $p < 0.05$. a, b, c etc. identify the groups with significant differences.

Of note, although the mean values of BMD at all sites were lower in the group continuing MTX compared to the group on other DMARDs, these differences were not significant. This is despite the significant differences observed in the data for all subjects (Table 3.19). There were no differences between the two groups starting a DMARD. The group continuing MTX did have significantly lower Z scores than one

or other, or both, the groups starting a DMARD for each region except the total hip and ultra-distal forearm regions.

The unadjusted percentage change in BMD expressed as Z score over the first year of the study is shown in Table 3.25.

Table 3.25 Unadjusted percentage change in BMD Z score of postmenopausal women

	Starting MTX	Starting SPZ	Continuing MTX	Continuing Other DMARD
% Change in Lumbar Spine BMD Z score	-3.86 ± 59.18 [-36.64 28.91] (15)	22.76 ± 42.86 [-22.21 67.74] (6)	10.27 ± 50.9 [-15.04 35.59] (18)	-21.27 ± 49.08 [-48.46 5.9] (15)
% Change in Neck of Femur BMD Z score	8.6 ± 139.5 [-68.58 85.9] (15)	33.6 ± 168.9 [-143.5 210.9] (6)	-1.02 ± 56.2 [-28.9 26.9] (18)	-28.8 ± 73.4 [-69.5 11.8] (15)
% Change Total Hip BMD Z score	3.3 ± 45.4 [-21.84 28.46] (15)	-64.7 ± 141.4 [-213.08 83.6] (6)	16.98 ± 64.9 [-15.3 49.3] (18)	-25.7 ± 136.6 [-101.4 49.9] (15)
% Change Total Forearm BMD Z score	*(a) 100.49 ± 278.39 [-53.68 254.66] (15)	*(b) -16.39 ± 34.89 [-53.02 20.33] (6)	*(a,b) -23.39 ± 119.94 [-85.06 38.27] (17)	-184.82 ± 464.25 [-452.87 83.23] (14)
% Change Ultra-distal Forearm BMD Z score	-7.77 ± 92.68 [-59.09 43.55] (15)	20.85 ± 27.49 [-8.01 49.7] (6)	-38.77 ± 117.1 [-98.9 21.44] (17)	172.16 ± 722.7 [-245.1 589.45] (14)
% Change Mid-portion Forearm BMD Z score	-3.02 ± 367.87 [-206.74 200.7] (15)	-18.2 ± 28.2 [-47.8 11.38] (6)	110.42 ± 460.28 [-126.24 347.07] (17)	-170.47 ± 559.32 [-493.42 152.46] (14)
% Change Proximal 1/3 Forearm BMD Z score	-882.61 ± 3422.1 [-2278.25 to 1013.04] (15)	-17.9 ± 40.4 [-60.3 24.4] (6)	-15.4 ± 60.7 [-46.6 15.7] (17)	-11.9 ± 88.6 [-63.1 39.2] (14)

Results are shown as the mean ± the standard deviation of the mean, [95% confidence interval for the mean], (n). * Significant differences between the groups, $p < 0.05$ (*). (a) and (b) identify groups with significant differences.

There were significant inter-group differences identified only for the change in total forearm BMD Z score (Table 3.25). Those continuing MTX therapy had significantly greater bone loss at this site (23%) than each of the groups starting treatment. The mean percentage loss was greatest in those continuing other DMARDs (a reduction of 184.82% in Z score), but the confidence interval was very wide reflecting heterogeneity in the group.

The results of the markers of bone turnover (secondary outcome measures) by group are presented in Table 3.26.

Table 3.26 Unadjusted markers of bone turnover

	Starting MTX	Starting SPZ	Continuing MTX	Continuing Other DMARD
Baseline Osteocalcin	10.07 / 2.91 [8.46 11.69] (15)	10.36 / 2.62 [8.35 12.37] (9)	14.31 / 4.37 [12.21 16.42] (19)	12.13/ 4.38 [9.71 14.55] (15)
Osteocalcin at one year	9.95 ± 3.7 [7.82 12.09] (14)	10.36 ± 2.62 [8.35 12.37] (9)	11.57 ± 4.15 [9.43 13.71] (17)	9.2 ± 2.98 [7.48 10.92] (14)
Baseline Bone Specific Alkaline Phosphatase	23.92 ± 9.45 [18.68 29.16] (15)	24.97 ± 13.37 [14.69 35.26] (9)	33.38 ± 15.4 [25.96 40.81] (19)	23.5 ± 9.19 [18.41 28.59] (15)
Bone Specific Alkaline Phosphatase at one year	21.74 ± 4.73 [19 24.48] (14)	18.26 ± 6.71 [1.59 34.9] (3)	26.11 ± 9.77 [21.23 30.99] (16)	21.43 ± 7.24 [17.24 25.61] (14)
Baseline Deoxypyridinoline/Creatinine	10.07 ± 3.11 [8.34 11.79] (15)	6.86 ± 3.92 [3.85 9.87] (9)	9.39 ± 2.98 [7.91 10.88] (18)	9.97 ± 4.33 [7.58 12.37] (15)
Deoxypyridinoline/Creatinine at one year	8.77 ± 6.21 [5.33 12.21] (15)	7.67 ± 3.16 [4.36 10.99] (6)	8.86 ± 5.02 [6.28 11.45] (17)	10.17 ± 4.96 [7.03 13.33] (12)

Results are shown as the mean ± standard deviation of the mean, [95% confidence interval for the mean], (n). Analysis by ANOVA. Significant differences are described in the text below.

There were significant decreases in all groups between the mean baseline and one year values for both markers of bone formation, serum osteocalcin and bone specific alkaline phosphatase. The group continuing MTX had significantly higher mean values of the bone formation markers than either the group starting SPZ or the group continuing on other DMARDs.

3.6.3 Association of Confounding Factors Between Groups

Due to the study design, the groups differed in some factors such as disease duration and the distribution of the confounding factors between the groups is shown in Table 3.27.

Table 3.27 The difference between groups with regard to confounding factors

	Starting MTX	Starting SPZ	Continuing MTX	Continuing Other DMARD
*Duration of RA (years)	6.18 ± 5.8	1.37 ± 1.14	15.3 ± 5.89	17.07 ± 6.85
*Disease activity (DAS)	5.7 ± 0.9	5.9± 0.8	5.5 ± 0.77	5.7 ± 0.73
*Body mass index (kg/m^2)	24.56 ± 5.48	23.92 ± 3.57	25.3 ± 4.18	23.89 ± 3.89
*Menopausal age (years)	14.83 ± 9.57	21.77 ± 11.5	19 ± 10.2	13.7 ± 6.01
^Baseline hand x-ray score	1 / 14	1 / 7	0 / 19	0 / 15
#Change in hand x-ray score	10 / 5	5 / 1	18 / 1	12 / 2
^Baseline wrist x-ray score	2 / 13	0 / 19	0 / 19	0 / 15
#Change in wrist x-ray score	7 / 8	5 / 1	18 / 1	12 / 2
^Baseline elbow x-ray score	4 / 8	1 / 5	3 / 16	1 / 13

*Mean ± standard deviation of the mean shown. ^The number with low / high (> 0) Larsen scores are given for the baseline x-ray scores, and # the number with 'no change in x-ray score' / 'increase in x-ray score' are given.

The relationship between the two confounding factors 'disease duration' and 'x-ray score' was examined and the results shown in Table 3.28. Unsurprisingly, those subjects who have had RA for a longer time had been treated with a DMARD for longer. However, when the x-ray score of the groups was controlled for, differences emerged in the duration of RA between those subjects who had been treated with MTX or other DMARDs. Disease severity (as judged by x-ray score) interacted with duration of treatment. In the groups continuing on their DMARD (having been treated for a minimum of five years with either MTX or Other DMARDs), if the x-ray score was high, those on MTX had been treated for less time than those on other DMARDs. However, if the x-ray score was low, the MTX group had been treated for longer than those on other DMARDs. This suggests that MTX does indeed have a DMARD rather than purely anti-inflammatory role, as longer treatment was associated with lower x-ray scores. In the groups starting treatment with MTX, they had been treated for a longer time for RA than had those starting SPZ (reflecting the smaller proportion of 'early RA' subjects in the group starting MTX). When subjects with high x-rays scores were studied, they had been treated for a longer time in the group starting MTX than in the group starting SPZ. Thus, in Table 3.28, those on other DMARDs have higher x-ray scores (departure from the mean, 4.39) than those continuing on MTX (departure from the mean, 3.33). However, in subjects starting MTX, they are more likely to have a higher mean x-ray score than those starting SPZ (departure from the mean -4.70 versus -11.41). This is in keeping with the results of Larsen scores and the change in Larsen scores described for the cohort as a whole (Section 3.3.6).

Table 3.28 Interaction of disease duration and disease severity (radiographic score)

Grand Mean = 11.71	Departure from the mean*	Levels of Covariates
Continuing other DMARDs Continuing MTX Starting SPZ Starting MTX Treatment effect p = 0.034	4.39 3.33 -11.41 -4.70	
Baseline hand x-ray score	4.89 -0.20	0 1
Change in hand x-ray score	-0.12 0.76	0 1
Baseline wrist x-ray score	-0.92 0.04	0 1
Change in wrist x-ray score	0.52 -1.89	0 1
Baseline elbow x-ray score	-5.20 1.11	0 1

* due to treatment effect adjusted for disease severity.

3.6.4 Multiple Regression Analysis

This statistical method was used to re-appraise the potential treatment effect of MTX on the presence of the covariates. The differences in the magnitude of the log-transformed baseline BMD Z scores after adjustment for significant covariates is shown in Table 3.29 and the change in BMD Z score presented in the same way in Table 3.30. Significant differences were found after adjustment in the baseline neck of femur BMD Z score (p = 0.0027) and marginally significant differences in the proximal third and mid-portion forearm Z scores (p = 0.06 and 0.07 respectively, Table 3.29). There were also significant differences at these three sites and the total forearm site in the percentage change in Z score over one year (Table 3.30).

Table 3.29 Baseline differences in BMD Z scores accounting for significant covariates

BMD Z score magnitude (log-transformed)	Lumbar spine	Neck of femur	Total hip	Total forearm	Proximal third forearm	Mid-portion forearm	Ultra-distal forearm
Grand mean	-0.5	-0.41	-0.41	-0.05	0.11	-0.12	-0.48
Main effect (departure from the mean)	(Non-significant)	(p = 0.0027)	(Non-significant)	(Non-significant)	(p = 0.06)	(p = 0.07)	(Non-significant)
	0.01	-0.09	-0.33	-0.14	-0.18	0.01	-0.53
Continuing other DMARDs	0.06	0.41	0.07	0.44	0.58	0.90	-0.11
Continuing MTX	-0.02	-0.82	0.08	-0.67	-0.18	-1.22	0.62
Starting SPZ	-0.11	-0.09	0.32	-0.19	-0.72	-0.90	0.56
Starting MTX							
Covariates (Regression coefficients)							
Sign of score	0.16	-0.08	0.19	0.03	*0.094	0.03	0.02
Body mass index	-0.03	-0.03	-0.05	-0.1	-0.02	-0.14	-0.03
Disease duration	-0.01	0.03	0.03	*-0.007	0.02	*-0.06	0.03
Menopausal age	0	-0.01	-0.02	0.04	0.01	0.03	0.03
Disease activity	0.12	-0.03	0	*0.204	1.03	0.16	-0.27
Baseline hand x-ray score	1.15	*0.196	-0.22	-1.24	-0.89	-1.17	0.64
Baseline wrist x-ray score	0.16	2.57	0.45	-0.32	-0.05	-0.48	-0.71
Baseline elbow x-ray score	-0.35	0.7	0.23	-0.38	0.69	-0.15	-0.34

* Significant covariates which contribute to the significant differences

Table 3.30 Change in BMD Z scores accounting for significant covariates

BMD Z score magnitude (log-transformed)	Lumbar spine	Neck of femur	Total hip	Total forearm	Proximal third forearm	Mid-portion forearm	Ultra-distal forearm
Grand mean	1.22	3.14	3.14	3.19	2.86	3.04	3.19
Main effect (departure from the mean)	(Non-sig-nificant)	(p = 0.04)	(Non-sig-nificant)	(p = 0.002)	(p = 0.0001)	(p = 0.001)	(Non-sig-nificant)
Continuing other DMARDs	-0.06 -0.07	-0.11 -0.05	-0.47 -0.21	0.59 -0.24	-0.19 -0.56	-0.45 -0.38	-0.28 0.02
Continuing MTX	0.27	0.27	1.18	-0.88	0.99	1.11	-0.05
Starting SPZ	0.07	0.11	0.47	0.07	0.82	0.81	0.44
Starting MTX							
Covariates (Regression coefficients)							
Sign of score	-0.03	0.23	-0.03	0.35	0.35	-0.28	-0.05
Baseline value	*-0.389	*-0.961	*-0.777	*-1.042	*-1.255	*0.942	*-0.736
Body mass index	0.02	0.05	*0.083	*0.117	*0.091	*0.252	0.08
Menopausal age	*-0.005	*-0.012	*0.002	*-0.030	*0.026	*-0.047	*-0.057
Disease duration	-0.01	-0.06	-0.01	-0.06	-0.03	*0.007	*0.006
Baseline osteocalcin	0.06	*0.06	0.04	*0.059	-0.03	-0.02	0
Baseline alkaline phosphatase	*0.006	0	0.01	*0.017	0.01	-0.02	-0.02
Baseline hand x-ray score	-0.32	-0.77	0.47	1.5	1.84	1.96	-0.66
Baseline elbow x-ray score	0.452	*-1.233	0.36	-0.06	*-1.7588	-0.59	-0.02

* Significant covariates which contribute to the significant differences

Table 3.3 1 Baseline differences in bone turnover markers accounting for significant covariates

BMD Z score magnitude (log-transformed)	Osteocalcin	Alkaline Phosphatase	Deoxypyridinoline / creatinine
Grand mean	12.1	26.96	9.32
Main effect (departure from the mean)	(p = 0.015)	(p = 0.071)	(Non significant)
Continuing other DMARDs	-0.34	-5.82	0.35
Continuing MTX	2.06	6.62	-0.33
Starting SPZ	-2.40	2.88	-2.16
Starting MTX	-2.01	-6.10	1.52
Covariates (Regression coefficients)			
Age	0.16	-0.17	*-0.034
Body mass index	0.06	0.5	0.25
Disease duration	0.05	0.03	*0.052
Menopausal age	0.03	-0.37	0.15
Disease activity	0.08	3.79	0.87
Baseline hand x-ray score	-2.77	17.81	*-7.64
Baseline wrist x-ray score	0.14	-8.75	5.19
Baseline elbow x-ray score	-1.1	-7.31	*4.66

Table 3.3 2 Change in markers of bone turnover accounting for significant covariates

BMD Z score magnitude (log-transformed)	Osteocalcin	Alkaline Phosphatase	Deoxypyridinoline / creatinine
Grand mean	10.3	22.77	9.51
Main effect (departure from the mean)	(Non significant)	(p = 0.014)	(Non significant)
Continuing other DMARDs	-0.50	-0.70	0.56
Continuing MTX	0.55	1.09	-0.81
Starting SPZ	-2.73	3.66	-1.30
Starting MTX	0.79	-2.20	1.47
Covariates (Regression coefficients)			
Baseline value	*0.648	*0.487	*0.089
Age	*0.053	*-0.021	*-0.045
Body mass index	-0.1	-0.41	0.32
Disease duration	-0.07	*0.099	-0.04
Menopausal age	-0.07	-0.3	*0.015
Disease activity	0.11	0.46	*1.368
Baseline hand x-ray score	-0.69	*11.678	*-7.309
Baseline wrist x-ray score	3.35	*4.148	*6.816
Baseline elbow x-ray score	1.47	1.24	*-5.553

The difference in the magnitude of the log-transformed baseline bone turnover markers and change in bone turnover markers after similar adjustment are shown in Tables 3.31 and 3.32 respectively. The baseline OC and percentage change in AP were significantly associated with MTX treatment. For the changes in markers of bone turnover baseline values and age were significant covariates for all three markers.

3.6.5 Discriminant Analysis

In the discriminant analysis, variables were analysed at the same time, not sequentially, as in multiple regression analysis. The relationships between variables could then be included in the model. This was important as the factors contributing to the bone loss associated with RA were not independent. For example, a subject who has had the disease for longer is more likely to have a higher Larsen score. In discriminant analysis, a linear combination of the independent variables is developed, using group membership (in this situation, treatment group) as the dependent factor. The information contained in multiple independent variables is summarised by a single index, by finding a weighted sum of the dependent variables. The weights are estimated so as to result in the 'best' (or furthest) separation of the different treatment groups.

In Table 3.33, the variables were ranked depending on their relative importance in correctly predicting the group membership of any particular Z score. When the model was applied back to the data set, it correctly categorised 97.56% of cases (only one case was incorrectly categorised).

Table 3.34 depicts the correlation between the values of the discriminant functions and the original variables to enable an assessment of the contribution each makes to the discriminant function.

Table 3.33 Standardised canonical discriminant function coefficients (with baseline values)

	Function 1	Function 2	Cumulative percentage of variance explained
Baseline hand x-ray score	0.53	-0.85	
Baseline wrist x-ray score	1.01	0.94	
Baseline prox 1/3 FA BMD	-1.62	-2.01	**77.64%**
Baseline mid-portion FA BMD	0.58	1.89	
Baseline osteocalcin	0.69	0.43	
Disease duration	1.02	-0.36	
Baseline lumbar spine BMD	-0.64	-0.79	
Baseline alkaline phosphatase	0.12	0.05	
Baseline ultra-distal FA BMD	-0.21	-0.34	
Change total FA BMD	0.01	0.81	**94.23%**
Change ultra-distal FA BMD	0.84	0.02	
Change mid-portion FA BMD	0.75	0.46	
Baseline neck of femur BMD	-1.22	-0.37	
Change neck of femur BMD	-0.1	0.07	
Change total hip BMD	0.68	0.61	

Table 3.34 Correlations between discriminating variables and the canonical discriminant functions (with baseline values)

	Function 1	Function 2
Baseline hand x-ray score	0.34	
Baseline wrist x-ray score	0.22	
Baseline prox 1/3 FA BMD	-0.22	
Baseline mid-portion FA BMD	-0.19	
Baseline osteocalcin	0.18	
Disease duration		-0.29
Baseline lumbar spine BMD		-0.23
Baseline alkaline phosphatase		0.19
Baseline ultra-distal FA BMD		-0.18
Change total FA BMD		0.17
Change ultra-distal FA BMD		-0.15
Change mid-portion FA BMD		0.15
Baseline neck of femur BMD		-0.12
Change neck of femur BMD		0.11
Change total hip BMD		0.08

From the discriminant analysis, the factors which explained most of the observed variation were found to be the following: baseline hand and wrist x-ray scores, baseline osteocalcin and baseline mid-portion forearm BMD. Elbow scores were not discriminatory as they were high in most subjects. Subjects on MTX were more likely to have higher hand and wrist x-ray scores, higher baseline osteocalcin and lower baseline mid-portion forearm BMD score. This held for the subjects who were starting MTX as well as those continuing MTX. After duration of treatment with MTX or other DMARDs was controlled for, the baseline proximal third forearm BMD emerged as a discriminant factor, suggesting that long-term MTX therapy is

associated with reduced BMD in this site even after correction for all the confounding factors listed above.

Overall, the statistical analysis showed that the best discriminators between subjects treated with MTX and those treated with other DMARDs were the baseline hand and wrist x-ray scores, the baseline osteocalcin level and baseline BMD, especially of the mid-portion of the forearm (Tables 3.33 and 3.34). Analysis of covariance suggested that differences between groups existed in BMD of the neck of femur and baseline osteocalcin and may be present at the forearm sites of mid-portion and proximal third in addition (Tables 3.29 and 3.31). The changes in BMD or markers of bone turnover were greater in those continuing on MTX than other DMARDs, but less in those starting MTX than SPZ (Tables 3.30 and 3.32).

Many of the changes in BMD at skeletal sites which at first appeared significant, were explained by baseline differences in BMD between the groups. This held for the following sites: lumbar spine, neck of femur, ultra-distal, proximal third and total forearm. Both the proximal third and total forearm changes in BMD were strongly correlated with the elbow x-ray score, suggesting disease activity rather than a toxic effect of MTX is important here.

Thus, the only BMD site where the changes were large enough to overcome the baseline differences between groups was the mid-portion of the forearm, where MTX treatment was associated with significantly greater loss in BMD.

3.7 Results of Subjects with Early Rheumatoid Arthritis

In this section, the results for subjects with rheumatoid arthritis of less than two years' duration are presented. The distribution of the early RA subjects by sex and

menopausal status between the groups starting MTX and SPZ is shown in Table 3.35. By one year, the number in the SPZ group had decreased to sixteen as there had been one death and one subject was lost to follow-up (Section 3.4). All subjects with early RA were invited for a final visit two years after they started their drug and sixteen subjects in the MTX and thirteen in the SPZ group attended. Of these, fifteen subjects in the MTX group and twelve subjects in the SPZ group underwent lateral lumbar spine estimation at both the one and two year time points.

Table 3.35 Subjects with early rheumatoid arthritis

Number of subjects	Male	Premenopausal female	Perimenopausal female	Postmenopausal female	Total
Starting MTX	3	7	4	6	20
Starting SPZ	5	2	4	7	18

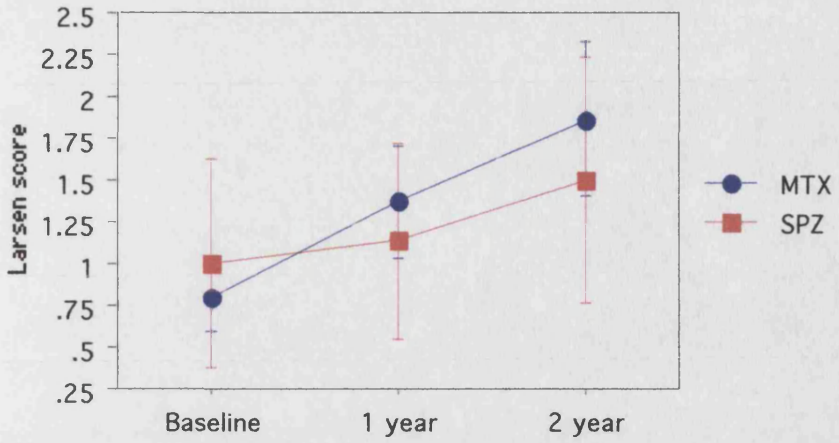
The mean Larsen scores for the hand, wrist and elbow radiographs of the subjects with early RA are shown in Table 3.36 and Figures 3.17 and 3.18 below.

Table 3.36 Larsen scores of subjects with early rheumatoid arthritis

Mean ± standard deviation (n)	Starting MTX	Starting SPZ
Baseline Hand Larsen Score	0.79 ± 0.42 (19)	1.00 ± 1.22 (17)
Baseline Wrist Larsen Score	1.05 ± 0.71 (19)	1.18 ± 1.33 (17)
1 Year Hand Larsen Score	1.37 ± 0.68 (19)	1.13 ± 1.06 (15)
1 Year Wrist Larsen Score	1.89 ± 1.10 (19)	1.20 ± 1.01 (15)
2 Year Hand Larsen Score	1.87 ± 0.83 (15)	1.50 ± 1.17 (12)
2 Year Wrist Larsen Score	1.93 ± 1.22 (15)	1.50 ± 1.17 (12)
Elbow Larsen Score	0.59 ± 0.62 (17)	0.47 ± 0.52 (15)

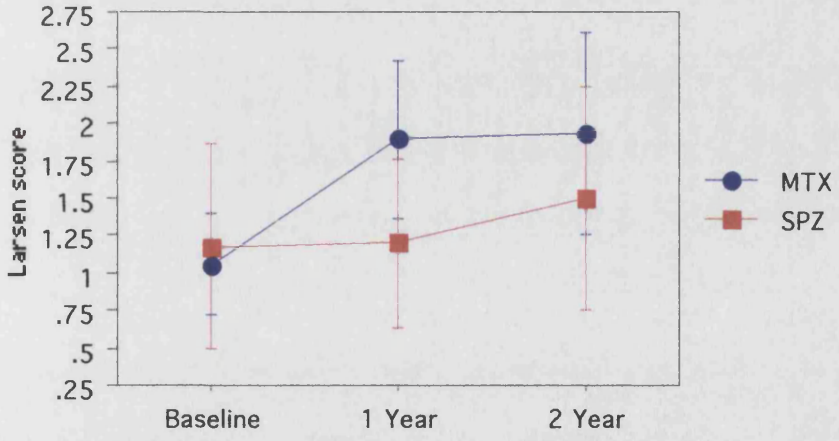
No significant differences between the two groups at any time point by unpaired t-test.

Figure 3.17 Larsen hand scores in early rheumatoid arthritis subjects



Results shown as the mean score ± 95% confidence interval. Analysis by paired t-test: 1 year compared with baseline, p = 0.0041 for MTX, p = 0.0406 for SPZ; 2 years compared with baseline, p = 0.0001 for MTX, p = 0.0261 for SPZ.

Figure 3.18 Larsen wrist scores in early rheumatoid arthritis subjects



Results shown as the mean score ± 95% confidence interval. Analysis by paired t-test: 1 year compared with baseline, p = 0.0012 for MTX, not significant for SPZ; 2 years compared with baseline, p = 0.0104 for MTX, not significant for SPZ.

3.7.1 Bone Mineral Density Z Score of Subjects with Early Rheumatoid Arthritis at Baseline

The bone mineral density Z score of subjects with early RA at entry to the study is shown in Table 3.37. None of the areas scanned was significantly different between groups at baseline by repeated measures ANOVA, confirming that the two groups were well-matched.

Table 3.37 Bone mineral density expressed as the Z score of subjects with early rheumatoid arthritis at baseline

Z score, mean ± standard deviation	Starting MTX n = 20	Starting SPZ n = 18
Lumbar spine	0.337 ± 1.350	0.231 ± 1.067
Neck of femur	-0.012 ± 1.342	-0.139 ± 0.830
Trochanteric	0.307 ± 1.492	0.130 ± 0.976
Inter-trochanteric	0.211 ± 1.357	-0.015 ± 0.691
Ward's region	0.412 ± 1.399	0.396 ± 0.944
Total hip	0.132 ± 1.344	0.013 ± 0.708
Proximal third forearm	0.377 ± 1.231	0.348 ± 1.250
Mid-portion forearm	-0.029 ± 1.319	0.217 ± 1.275
Ultra-distal forearm	-0.064 ± 1.220	-0.077 ± 1.143
Total forearm	0.061 ± 1.287	0.194 ± 1.268
Proximal third radius	0.295 ± 1.243	0.384 ± 1.197
Mid-portion radius	-0.164 ± 1.346	0.157 ± 1.129
Ultra-distal radius	-0.067 ± 1.189	0.034 ± 1.167
Total radius	-0.053 ± 1.291	0.182 ± 1.191

3.7.2 Change In Bone Mineral Density of Subjects with Early Rheumatoid Arthritis

The mean changes in percentage of BMD measurements in the subjects with early rheumatoid are shown in Table 3.38.

During the first year, the group starting MTX lost a greater percentage of BMD at all sites studied than did the group starting SPZ. However, the difference in percentage loss between groups was not significant by repeated measures analysis of variance (ANOVA). The standard deviations for all the sites were large, reflecting the heterogeneity of rates of bone loss within the groups.

The changes in mean percentage of BMD between baseline and two years and between the one and two year time points are also shown in Table 3.38. There were no significant differences in the changes between the two groups at any of the sites measured. Whereas during the first year of treatment the MTX group had mean bone loss at all sites (negative values of the percentage change), during the second year of treatment there was mean increase at all sites except the proximal third (radius and ulna) and all the radius alone measurements. From baseline to the two-year time point, the MTX group had a greater loss of bone than the group starting SPZ at all sites except at Ward's area and the ultra-distal regions of the forearm (radius alone, and radius and ulna combined). Of note, in the proximal third regions (radius alone, and radius and ulna combined), the group starting SPZ had overall gain in BMD whilst the group starting MTX had a loss. These differences did not reach significance by repeated measures ANOVA.

During the first year, the group starting SPZ had suffered less mean bone loss at each site than the group starting MTX. However, over the second year of treatment, the mean percentage loss in the SPZ group was greater than in the

MTX group at the following sites: neck of femur, inter-trochanteric, Ward's area and the total hip, and in the forearm, the ultra-distal and total forearm measurements for the radius and ulna combined. At the proximal third forearm site, a mean loss of 0.26% in BMD occurred in the MTX group, compared with a gain of 0.1% in the SPZ group.

Table 3.38 Mean percentage change of bone mineral density of subjects with early rheumatoid arthritis

Mean percentage change ± standard deviation	Baseline to one year		One to two years		Baseline to two years	
	Starting MTX n = 20	Starting SPZ n = 16	Starting MTX n = 16	Starting SPZ n = 13	Starting MTX n = 16	Starting SPZ n = 13
Lumbar spine	-2.436 ± 2.672	-1.689 ± 3.487	+0.212 ± 2.682	+0.972 ± 6.229	-2.275 ±4.058	-1.164 ± 6.648
Lateral lumbar spine	N/A	N/A	+0.61 ± 6.18	-3.50 ± 11.99	N/A	N/A
Neck of femur	-1.931 ± 4.639	-0.577 ± 4.324	+0.006 ± 3.968	-0.658 ± 4.439	-2.588 ±5.302	-1.550 ± 6.618
Trochanteric	-3.014 ± 4.362	-1.206 ± 5.340	+2.446 ± 4.016	+3.536 ± 15.365	-0.673 ± 4.170	+1.576 17.213
Inter-trochanteric	-2.205 ± 3.421	-0.557 ± 4.898	+2.244 ± 3.651	+0.821 ± 5.855	-0.453 ± 2.557	-0.302 ± 8.865
Ward's region	-2.193 ± 6.998	-0.241 ± 5.218	+1.280 ± 5.835	-2.00 ± 6.157	-1.817 ± 7.881	-2.436 ± 9.007
Total hip	-2.553 ± 3.370	-0.820 ± 4.807	+2.407 ± 3.691	+1.225 ± 7.633	-0.681 ± 2.852	-0.271 ± 10.343
Proximal third	-2.907 ± 4.566	+0.271 ± 2.969	-0.259 ± 3.675	+0.106 ± 8.073	-2.542 ± 6.382	+0.907 ± 8.215
Mid-portion forearm	-3.497 ± 5.106	-1.233 ± 1.863	+0.163 ± 2.400	+0.178 ± 5.529	-2.366 ± 6.036	-1.038 ± 6.432
Ultra-distal forearm	-4.222 ± 3.795	-3.156 ± 3.870	+0.704 ± 4.770	-0.038 ± 4.725	-3.165 ± 6.366	-3.454 ± 5.729
Forearm total	-3.396 ± 4.266	-1.203 ± 2.009	+0.818 ± 2.798	+0.038 ± 5.415	-2.577 ± 5.766	-1.042 ± 6.471
Proximal third radius	-2.026 ± 3.903	-0.231 ± 3.273	-1.447 ± 3.737	+0.917 ± 11.621	-3.114 ± 5.901	+0.445 ± 10.803
Mid-portion radius	-2.201 ± 4.781	-1.292 ± 2.609	-0.822 ± 2.502	-0.624 ± 5.734	-2.101 ± 5.749	-2.006 ± 5.942
Ultra-distal radius	-3.814 ± 3.949	-3.702 ± 4.542	-0.239 ± 4.673	-0.216 ± 5.155	-3.829 ± 6.438	-4.393 ± 7.229
Radius total	-2.424 ± 3.848	-1.649 ± 2.849	-0.763 ± 2.747	-0.087 ± 6.403	-2.644 ± 5.332	-1.922 ± 6.861

There was no significant difference between the groups Starting Methotrexate and Starting Sulphasalazine by repeated measures ANOVA.

3.7.3 Markers of Bone Turnover of Subjects with Early Rheumatoid Arthritis at Baseline

The pre-treatment values of the bone markers osteocalcin (OC), bone specific alkaline phosphatase (AP) and urinary deoxypyridinoline corrected for creatinine (Dpd) are given in Table 3.39. The values of OC were similar in the two groups, the AP in the group starting MTX was higher than in the group starting SPZ, but the difference between the groups was not significant. There was a significantly higher mean Dpd value for the group starting MTX, with p = 0.0044 by unpaired t-test. This suggests that the group starting MTX had more bone resorption than the group starting SPZ, but that formation was similar in the two groups.

Table 3.39 Markers of bone turnover in the subjects with early rheumatoid arthritis at baseline

	Normal range Female: male	Starting MTX mean ± standard deviation n = 20	Starting SPZ mean ± standard deviation n = 18	P value at the 95% confidence level by unpaired t-test
Serum OC (ng/ml)	3.7-10.0: 3.4-9.1	9.620 ± 2.708	9.484 ± 2.195	0.8678
Serum AP (U/L)	10-22: 12-23	27.006 ± 13.277	21.784 ± 9.589	0.1773
Urinary Dpd (nM/mM)	3.0-7.4: 2.3-5.4	10.736 ± 4.117	6.885 ± 3.629	0.0044

3.7.4 Change in Markers of Bone Turnover of Subjects with Early Rheumatoid Arthritis Over One Year and Two Years

The mean percentage change in markers of bone turnover for the first two years of treatment are shown in Table 3.40. There were no differences between the groups for any of the three markers by unpaired t-test.

Table 3.40 Percentage change in markers of bone turnover in the subjects with early rheumatoid arthritis

Mean % change from baseline ± standard deviation	Starting MTX (number of subjects)	Starting SPZ (number of subjects)
Serum osteocalcin at 1 year from baseline	+2.521 ± 32.826 (18)	-14.740 ± 26.898 (11)
Serum osteocalcin at 2 years from baseline	-8.651 ± 20.424 (14)	-4.754 ± 19.252 (11)
Serum AP at 1 year from baseline	-18.107 ± 24.324 (18)	-12.993 ± 17.782 (11)
Serum AP at 2 years from baseline	-23.500 ± 23.842 (14)	-14.918 ± 28.771 (11)
Urinary Dpd at 1 year from baseline	+14.286 ± 85.921 (20)	+20.357 ± 20.469 (12)
Urinary Dpd at 2 years from baseline	+2.732 ± 90.857 (15)	-4.196 ± 44.984 (11)

3.8 Results of Male Subjects

3.8.1 Bone Mineral Density Z Score of Male Subjects at Baseline

The Z scores of the bone mineral density at entry to the study for the male subjects is shown in Table 3.41. Unlike the study group as a whole and the subgroup of postmenopausal women, there were no significant differences between the groups for any of the forearm measurements. The only significant difference between the four groups for age- and sex-adjusted BMD (Z scores) was at the trochanteric region of the hip, where the group continuing MTX had a lower mean Z score than the group starting MTX. There were no significant differences between the two groups continuing on DMARDs.

3.8.2 Change in Bone Mineral Density of Male Subjects Over One Year

These are shown in Table 3.42. In general, compared to the female subjects, the changes seen were much smaller and were often near to zero. The greatest losses in BMD were observed at the ultra-distal portion of the forearm, in all groups except those continuing on MTX.

Table 3.4 1 Z score of the bone mineral density of the male subjects at baseline

Mean ± standard deviation	Start MTX n = 8	Start SPZ n = 6	Continue MTX n = 6	Continue Other DMARD n = 7
Lumbar spine	1.088 ± 1.017	-0.110 ± 1.277	0.127 ± 0.936	0.463 ± 1.158
Neck of femur	0.229 ± 1.136	0.254 ± 1.096	-0.313 ± 1.081	-0.109 ± 0.804
Trochanteric	*0.959 ± 0.920	0.760 ± 1.006	*0.207 ± 1.445	0.647 ± 0.594
Inter-trochanteric	0.927 ± 0.691	0.208 ± 0.618	-0.235 ± 1.271	0.131 ± 0.516
Ward's region	0.492 ± 1.511	0.752 ± 1.592	-0.155 ± 1.091	-0.104 ± 0.961
Total hip	0.774 ± 0.797	0.346 ± 0.709	-0.175 ± 1.294	0.243 ± 0.548
Proximal third forearm	-0.046 ± 0.818	-0.164 ± 1.823	-1.256 ± 2.425	0.252 ± 1.248
Mid-portion forearm	-0.408 ± 0.798	-0.296 ± 1.853	-0.944 ± 1.921	-0.568 ± 0.825
Ultra-distal forearm	-0.268 ± 1.097	-0.252 ± 1.525	0.080 ± 3.127	-0.248 ± 0.777
Total forearm	-0.301 ± 0.863	-0.264 ± 1.831	-0.868 ± 1.749	-0.290 ± 0.829
Proximal third radius	-0.136 ± 0.869	-0.196 ± 1.586	-0.927 ± 2.657	0.434 ± 1.386
Mid-portion radius	-0.643 ± 0.747	-0.379 ± 1.512	-0.675 ± 1.667	-0.587 ± 0.885
Ultra-distal radius	-0.274 ± 1.301	-0.121 ± 1.537	0.145 ± 2.697	-0.154 ± 0.722
Total radius	-0.468 ± 0.906	-0.293 ± 1.663	-0.656 ± 1.551	-0.268 ± 0.927

Analysis by ANOVA with Fisher's PLSD post hoc testing

* Significant difference between the group starting MTX and the group continuing MTX, p = 0.0147

Table 3.4 2 Change in bone mineral density (BMD) over one year of male subjects

Mean percentage change in BMD ± standard deviation	Start MTX n = 8	Start SPZ n = 6	Continue MTX n = 6	Continue Other DMARD n = 7 (n = 6 for hip)
Lumbar spine	+0.984 ± 1.526	-0.743 ± 2.888	+1.848 ± 1.336	+0.926 ± 3.735
Neck of femur	* (a) -2.106 ± 1.859	-0.820 ± 1.374	+0.160 ± 3.486	* (a) +1.498 ± 2.639
Trochanteric	+0.306 ± 2.102	+0.550 ± 1.408	-0.658 ± 2.971	-0.670 ± 2.732
Inter-trochanteric	+0.906 ± 2.557	+1.650 ± 2.240	-0.222 ± 2.626	-0.382 ± 2.737
Ward's region	* (b) -1.315 ± 4.365	+1.415 ± 3.265	+3.082 ± 5.877	*(b) +5.512 ± 3.912
Total hip	+0.631 ± 1.695	+1.107 ± 1.990	-0.253 ± 2.751	-0.352 ± 2.022
Proximal third forearm	-0.574 ± 1.768	-0.564 ± 2.079	-0.409 ± 4.156	-0.195 ± 1.664
Mid-portion forearm	-0.720 ± 2.842	-0.598 ± -0.269	-0.241 ± 4.046	-0.993 ± 1.784
Ultra-distal forearm	-2.623 ± 3.767	-3.559 ± 2.779	+1.101 ± 5.227	-2.642 ± 2.457
Total forearm	-1.064 ± 2.529	-1.205 ± 1.043	-0.057 ± 3.898	-1.061 ± 1.285
Proximal third radius	+0.513 ± 4.092	+0.688 ± 1.694	-1.464 ± 2.956	-0.160 ± 1.847
Mid-portion radius	-0.641 ± 0.140	+3.121 ± 1.057	-0.669 ± 4.622	-1.050 ± 2.108
Ultra-distal radius	-1.570 ± 3.862	-1.976 ± 2.807	+0.526 ± 5.464	-3.149 ± 2.089
Total radius	-0.583 ± 3.266	-0.308 ± 1.312	-0.430 ± 4.244	-1.260 ± 1.448

Analysis by ANOVA with Fisher's PLSD post hoc testing

* Significant difference between the groups starting MTX and continuing other DMARDs: p = 0.0149 for the neck of femur (a) and p = 0.0116 for Ward's area (b)

3.8.3 Markers of Bone Turnover of Male Subjects at Baseline

Subjects on long-term MTX had significantly increased OC at baseline compared with those starting MTX (Table 3.43), but all groups except those starting MTX had

mean OC levels outside the reference value for males. The group continuing MTX also had high AP but it was only just above the normal range and was significantly higher only when compared with the long term other DMARD group. As for the whole study cohort and the postmenopausal women, there was no difference in resorption (Dpd) seen.

Table 3.4 3 Markers of bone turnover at baseline in the male subjects

Mean ± standard deviation	Normal male values	Start MTX n = 8	Start SPZ n = 5	Continue MTX n = 6	Continue other DMARD n = 7
Serum osteocalcin (ng/ml)	3.4-9.1	*(a) 8.293 ± 1.642	9.829 ±2.033	*(a) 10.930 ±3.127	10.351 ±2.326
Serum AP (U/L)	12-23	20.395 ±6.363	19.306 ±3.208	*(b) 24.902 ±10.950	*(b) 15.449 ±4.288
Urinary Dpd (nM/mM)	2.3-5.4	7.085 ±3.568	5.979 ±1.732	6.989 ±3.308	5.441 ±0.929

Analysis by ANOVA with Fisher's protected least significant difference post hoc testing.
* (a and b) Significant differences between groups at the level $p < 0.05$.

3.8.4 Change in Markers of Bone Turnover of Male Subjects Over One Year

The men starting MTX had a mean increase in OC of 11% whilst the other three groups had a reduction of between 25 and 32%. These differences were significant (Table 3.44).

The group continuing DMARDs other than MTX had a large mean increase in AP of 24% whilst it reduced in the other three groups. Again, these differences were significant.

The group continuing MTX had a mean decrease in Dpd of 14% whilst the other three groups had a mean increase, but this did not reach significance.

Table 3.44 Mean percentage change in the markers of bone turnover in the male subjects from baseline to one year

Mean ± standard deviation	Start MTX (n = 7 & 8)	Start SPZ (n = 3 & 4)	Continue MTX (n = 6 & 6)	Continue Other DMARD (n= 7 & 6)
Serum osteocalcin	+11.269* (a,b,c) ± 40.616	-32.413* (a) ± 13.185	-31.481* (b) ± 21.919	-25.947* (c) ± 24.400
Serum AP	-8.830** (d) ± 16.787	-13.560* (e) ± 18.023	-5.085* (f) ± 27.026	+24.056* (e,f) / ** (d) ± 18.004
Urinary Dpd	+20.308 ± 61.943	+16.124 ± 16.136	-13.997 ± 22.181	+22.874 ± 58.194

The numbers are given of subjects results available for formation and resorption markers for each group. AP is serum bone specific alkaline phosphatase; Dpd is urinary deoxypyridinoline corrected for urinary creatinine.
Analysis by ANOVA with Fisher's PLSD post hoc testing. * $p < 0.05$, ** $p < 0.01$. a, b, c etc. identify the groups with significant differences.

3.9 Summary of Results of the Clinical Study and Future Work

When all the subjects were considered together (Section 3.5), the cohort who had been on MTX for more than five years had significantly reduced BMD corrected for age and sex (Z score) compared with the other three groups of subjects in the following areas: forearm proximal third and midportion regions, for both the radius alone and the radius and ulna combined. However, the rate of change in BMD for the proximal third and midportion forearm areas was no different between the two groups continuing their DMARD, suggesting that the loss of bone mass seen in the group continuing MTX had occurred in the past.

There were differences found in the groups starting either MTX or SPZ with regard to annual change in forearm BMD. Those subjects starting MTX had significantly more reduction in BMD in the proximal third region (radius and ulna) than did those starting SPZ.

These results suggest that MTX is associated with early loss of bone from the proximal third of the forearm, but that the rate of loss slows with long-term use. The bone in this part of the forearm is predominately cortical (Schlenker et al, 1976). However, this study does not inform whether the group continuing MTX had lost bone before or after starting the drug.

At baseline, the bone formation markers (OC and AP) were within the normal reference range apart from in the group continuing MTX, where they were increased significantly compared to all the other groups. In the group starting MTX, AP was slightly raised, but this was not significant when compared with the other groups. The mean percentage change in AP over one year in the group continuing MTX (decrease of 11%) was significantly different from the change in the group on other DMARDs (increase of 5%).

Bone resorption markers were elevated above the normal reference range in all four groups, and there was no significant difference between the groups or over the time of the study.

When the subgroup of postmenopausal women were considered (Section 3.6), the discriminant analysis results showed that the factors which explained most of the observed variation between groups were the following values: baseline osteocalcin levels, baseline hand and baseline wrist x-ray scores and the baseline proximal third and mid-portion forearm BMD Z scores. These factors explained much of the

variability between the groups (77.64%). Another 17% of variability was explained by the factors in the bottom half of Tables 3.29 and 3.30. The analysis found that the only site which had a significant negative annual change associated with MTX therapy, after correction for confounding factors, including baseline values of BMD, was the mid-portion of the forearm. Sites where osteoporotic fractures typically occur, such as the ultra-distal forearm site and hip, were not at increased risk of reduction in BMD due to MTX therapy in the model. Other factors, such as baseline BMD and disease duration and activity were more important at these sites. However, it is not known whether other sites would become significant if a larger sample of subjects had been available for analysis.

The results for the subgroup with early RA (less than two years) was presented in Section 3.7. In this small cohort of subjects there was no significant difference between the two groups (Starting MTX and Starting SPZ) when the percentage change in bone mineral density at each site was compared by repeated measures ANOVA (Table 3.38).

The subgroup of males were discussed in Section 3.8. There were no significant differences in baseline BMD or change in BMD between the two groups starting or continuing their DMARDs. However, there were significantly greater losses in BMD occurring over the year of the study at the neck of femur and Ward's area of the hip in those starting MTX when compared with those continuing other DMARDs. As in the group as a whole, MTX treatment was associated with higher osteocalcin levels.

Overall, MTX treatment was not associated with adverse effects by either markers of bone turnover or BMD measurement. The only site where MTX was

associated with increased bone loss after correction for confounding factors in the sub-group of post-menopausal women was the mid-portion of the forearm. This is a predominately cortical bone site and is not prone to insufficiency fractures. These results are reassuring for clinicians prescribing MTX.

4. METHODS: BONE CELL CULTURE

4.1 Introduction

The following experiments were designed to investigate whether or not methotrexate (MTX) had any effect on the behaviour of normal human osteoblast-like bone cells *in vitro*.

Osteoblast-like cells which proliferate from trabecular bone chips and normal human bone marrow cells were studied in a series of experiments and the results are presented in Chapter 5. The culture techniques outlined below (Sections 4.3 and 4.5) have been developed over several years by Dr JN Beresford and others (Gallagher et al, 1996).

All chemicals and reagents were obtained from BDH Ltd, UK or Sigma Ltd, UK and all plastics from Falcon,UK unless otherwise stated.

4.2 Preparation of Marrow Stromal Cells for Culture

Cells were prepared from portions of rib from elective thoracic surgery at Frenchay Hospital NHS Trust, Bristol. The most frequent diagnosis was lung tumour but some patients were undergoing pleurodesis for recurrent pneumothorax. Several samples were from exploratory thoracotomies, where the diagnosis was unknown. Characteristics of patients whose bone was used in this series of experiments are discussed in Chapter 5 (Section 5.2 and Table 5.1).

The sample was placed in serum-free medium until preparation, which was usually the same afternoon following a morning operation.

The portions of rib varied in length from 3cm to 10cm. Working in a laminar flow hood, the connective tissue and periosteum were carefully stripped away from the bone surface using sterile instruments such as locking forceps and scalpel

(Richardsons, UK). This minimised contamination of the cell suspension with fibroblasts. In a clean square petri dish with a small volume of serum-free Dulbecco's Modified Essential Medium (DMEM, Gibco, see Tissue Culture appendix), the rib was cracked open along its length using bone cutters and the marrow washed out by repeated vortexing of rib fragments. The marrow was transferred to a 50ml polypropylene tube with a screw cap with 30ml serum-free DMEM and washed by centrifugation at 350g for five minutes. The remaining rib was reserved for preparation of the trabecular bone (Section 4.4).

Following the wash, many of the adipocytes could be aspirated off with the DMEM as they were at the top of the liquid layer. The cell pellet was resuspended in 25ml serum-free DMEM, layered on top of 20ml Lymphoprep (Nycomed) in a clean 50ml polypropylene tube and centrifuged at 630g for thirty minutes, with the brake off. This separated the mononuclear cells from erythrocytes and other marrow components. The mononuclear cells, which included the marrow stromal cells of interest, were removed by carefully drawing up the interface into a 25ml pipette and transferring it to a clean 50ml polypropylene tube. Following the addition of 20ml serum-free DMEM, the cells were centrifuged at 980g for ten minutes to separate the Lymphoprep. The cell pellet was resuspended in 15ml serum-free DMEM and filtered through a 70μm cell filter (Becton Dickinson, UK) before counting. Cells were counted by either Coulter counter (Section 4.6.4) or haematocytometer (Section 4.6.5) with Trypan Blue staining to identify viable cells. Total cell counts were typically 60 to 120 million cells per sample, depending on the length of rib and age of the patient.

4.3 Primary Culture of Marrow Cells

The prepared mononuclear cells were established in primary culture in petri dishes for colony formation experiments (Section 4.7), or in $25cm^2$ or $75cm^2$ flasks. The cells were plated out at a density of 2×10^4 cells/cm^2. The medium was DMEM (with the supplements listed in the Tissue Culture appendix) supplemented additionally with 100μM L-Ascorbate-2-phosphate magnesium salt n-hydrate (Asc-2-P, Wako Pure Chemical Industries Ltd, Japan) and heat-inactivated fetal calf serum (FCS, Sigma-Aldrich Co Ltd, Poole, UK), either 10% or 15% by volume. During the course of this work, the preferred concentration for growing marrow-derived stromal cells changed from 10% to 15% FCS, based on work by other members of the Bath Bone Research Group. Similarly, the length of time to the first change of medium and wash with PBS, was reduced from seven to three or four days, as this was found to accelerate growth (reduce time to confluence) and increase the yield of colonies. In contrast, trabecular-derived bone cells were cultured throughout in DMEM with 10% FCS by volume and fed first after seven days with no wash step (Section 4.5).
Cultures were routinely set up with and without 10nM Dexamethasone (DX). Cells were fed twice a week and cultured at 37°C in an humidified atmosphere of 95% air and 5% CO_2.

4.4 Preparation of Trabecular Bone Chips

Trabecular (cancellous) bone cells were grown in flasks as outgrowths from chips of bone after the method of Beresford et al (Gallagher et al, 1996). The source of the bone was either rib from Frenchay Hospital, Bristol or bone fragments from orthopaedic operations at the Royal United Hospital NHS Trust, Bath. The origin of

the bone was usually the femur (reamings from the shaft to make space for the femoral stem of a total hip replacement) but samples from the iliac crest (donor site of bone graft for spinal fusion surgery) or tibia (total knee replacement) were also received.

The trabecular bone was carefully separated from the cortical bone and any connective tissue or cartilage elements were discarded. Trabecular bone was distinguished as it was white and could be cut into small fragments with a sharp pair of scissors. In contrast, the cortical bone was yellow, tended to splinter and could only be cut with bone cutters. The preparation was done in the hood, using a small amount of serum-free DMEM to keep the bone moist. The trabecular chips were suspended in calcium- and magnesium-free phosphate buffered saline (PBS) in a 50ml polypropylene tube and washed by vortexing three times, allowing settling each time. The PBS was aspirated off, the bone chips were resuspended in clean PBS and the wash step repeated twice more. The chips were then placed in a clean square petri dish and any remaining haematopoietic elements (red tissue) were discarded. Further wash steps were performed as necessary to obtain clean, white trabecular chips.

4.5 Primary Culture of Trabecular Bone

The trabecular chips were placed in 25cm^2 or 75cm^2 flasks, with DMEM supplemented with 10% FCS, 100μM Asc-2-P and with and without 10nM DX. The cultures were left to settle for seven days and thereafter were fed twice a week and, as for the marrow cultures, grown at 37°C in an humidified atmosphere of 95% air

and 5% CO_2. No wash step was performed at the time of the first feed, unlike the marrow cultures.

4.6 Redistribution and Passaging of Marrow-Derived and Trabecular Bone Cells

The technique is the same for both types of cell cultures and consists of treatment with collagenase followed by trypsin EDTA (Gallagher et al, 1996).

Redistribution of the cells within the flask was carried out at four weeks of culture. By disaggregating the cells, contact inhibition of proliferation was reduced and the cells reached confluence more quickly.

Passaging was done when the cell layer was confluent over the bottom of the flask. The cells were counted and further experiments done in secondary culture.

4.6.1 Collagenase Incubation

The medium was discarded and the cell layer washed gently with serum-free DMEM or PBS twice. The cells were incubated at 37°C for 60 to 90 minutes with 4ml (for a 75cm^2 flask) of collagenase VII solution containing 2mM $CaCl_2$ (1 in 500 dilution of sterile 1M stock) and 25 units/ml collagenase VII in serum-free DMEM. The $CaCl_2$ is needed for optimal activity of the enzyme .

4.6.2 Trypsin Incubation

At the end of the first incubation, the cells could be seen rounding up under the microscope. The medium was aspirated off and the cell layer washed gently twice with PBS. Trypsin EDTA (Gibco, UK), 4ml for a 75 cm^2 flask, was added to the cell layer and further incubation at 37°C for fifteen minutes carried out. As the trypsin

acted, the cell layer could be seen lifting off the bottom of the flask in a sheet. The trypsin activity was halted by the addition of 0.5ml FCS and the cells pipetted up and down vigorously, to dislodge the cells from the dense matrix. The cell suspension was transferred to a 50ml polypropylene tube and the flask washed out with 10ml serum-free DMEM to obtain the maximum yield of cells.

4.6.3 DNA-ase Step

The cell suspension was centrifuged at 350g for five minutes with the brake off. The cell pellet was resuspended in 1ml of serum-free DMEM containing 10μg/ml DNA-ase and pipetted vigorously to further disaggregate the cells. The cell suspension was filtered through a 70μm cell strainer (Becton Dickinson, UK), spun again for five minutes at 350g and resuspended in 1 or 2ml serum-free DMEM. The cells were then counted and plated out for experiments in secondary culture.

4.6.4 Cell Counts by Coulter counter

A Coulter Electronics Multisizer Counter (Coulter, UK) was used and a known volume of cell suspension (usually 100μl) was placed in a counting cup with a known volume of filtered isoton (usually 8ml). The cell count for two 500μl aliquots were obtained and summed, giving a count per ml. The cell concentration was then given by the following equation:

(count x isoton volume/ sample volume) x 10 = cell count per ml

4.6.5 Cell Counts by Haematocytometer

A 50µl aliquot of the cell suspension was mixed with 50µl of Trypan Blue. A small volume (approximately 20µl) of the mixture was introduced into one half of the haematocytometer. Under high power magnification (x 400, Wilovert microscope) viable cells which had not taken up the Trypan Blue were counted in at least four squares of the grid to give a count of at least one hundred. The suspension was diluted if necessary, if the cells were too dense to count, or clumped. Non-viable cells, identified by a blue halo, were then counted. The cell concentration was given by the following equation:

(count/number of squares counted in) x 100/50 x 10^4 = cell count per ml

The percentage of viable cells was given by:

$$\text{Cell viability (\%)} = \frac{\text{Viable cell count}}{\text{(Viable + non-viable cell counts)}} \times 100\%$$

4.7 The Effect of Methotrexate on Colony Forming Units-Fibroblastic Formation from Normal Human Bone Marrow Stromal Cells.

Bone marrow stromal cells include clonogenic adherent precursors which under appropriate conditions can form colonies and which have been termed colony-forming units fibroblastic (CFU-F) (Friedenstein et al, 1970; Friedenstein et al, 1987). Although they have the appearance of fibroblasts, the cells from a proportion of the colonies can form bone and cartilage if transplanted into diffusion chambers in allogeneic recipient animals demonstrating that they retain their osteogenic

potential (Friedenstein et al, 1987). In addition they express alkaline phosphatase (Section 4.7.1), suggesting that they have acquired osteoblast-like characteristics.

A series of experiments was performed to investigate the effect of methotrexate on the formation of colonies and on their differentiation into osteoblast-like cells, and the results are presented in Chapter 5 (Section 5.3). All these experiments were done in 60mm diameter ($28cm^2$) petri dishes, with four replicates for each condition. Freshly prepared marrow cells were plated out at $4x10^5$ cells per dish (1.5×10^4 cells per cm^2) and washed with PBS before the first feed. The medium used was DMEM supplemented with 100μM Asc-2-P and either 10% or 15% FCS. Following the first feed, cells were fed every three or four days. If used, methotrexate (MTX, Wyeth-Lederle, Gosport, Hants, UK), folate and folinic acid was added to the medium from the outset in all experiments.

After eighteen to twenty-one days in culture, the dishes were washed once with PBS and the colonies fixed with methanol for five minutes. The methanol was aspirated off and the cultures air dried. The colonies were then stained with Fast Red (Section 4.7.1) for alkaline phosphatase-positive colonies containing osteoblast-like cells. These were counted under low power microscopy (Olympus, magnification x 5).

The same dishes were then restained with 1% methylene blue (Section 4.7.2) in 10mM Borate buffer (appendix bone cell culture) and the colonies counted. This stained all colonies, whether fibroblastic or osteogenic.

4.7.1 Fast Red Stain for Alkaline Phosphatase-Positive Osteogenic Colonies

To make 30ml of stain, 6mg of Naphthol AS-MX was dissolved in 600µl dimethyl formamide in a glass container. This was diluted to 30ml with 0.1M Tris buffer, pH 9.2. Just before use, 30mg Fast Red TR salt was dissolved in the solution and 2ml placed in each petri dish of fixed cells. After ten minutes at room temperature, the stain was washed off with tap water and the dishes air dried. Colonies containing cells expressing alkaline phosphatase had been stained red and could be counted at low magnification (x 5). A colony was counted if it contained at least fifty cells: clusters of fewer than fifty cells were not included.

4.7.2 Methylene Blue Stain for All Colonies

The stain for total colony counts used was 1% Methylene Blue in 10mM Borate buffer and 2mls was placed in each petri dish and left for thirty minutes at room temperature. The stain was washed off with tap water and the dishes air dried. Colonies were stained blue and could be counted at low magnification, as above. Clusters of less than fifty cells were not counted.

4.8 Experiments with Passaged Bone Cells

Following collagenase and trypsin passaging, the cells were plated out in twenty-four well plates at 1 x 10^4 cells per cm^2, unless stated otherwise.

4.9 Protein Assay

The Bio-Rad DC Protein Assay is a calorimetric assay for protein concentration following detergent solubilisation and is similar to the well-documented Lowry

Assay (Hartree, 1972). The protein to be quantified reacts with an alkaline copper tartrate solution and Folin reagent. There are two steps which lead to the blue colour development: the reaction between protein and copper in an alkaline medium, and the subsequent reduction of Folin reagent by the copper-treated protein.

Reagents used
Reagent A an alkaline copper tartrate solution
Reagent B a dilute Folin reagent
Reagent S SDS (detergent)

Protein standards were made from 4mg/ml stock bovine serum albumin (BSA) by doubling dilutions to the following concentrations:

0, 62.5, 125, 250, 500, 1000, 2000 and 4000μg/ml

Method

The protein was dissolved by the addition of 4M NaOH at 50°C prior to the assay.

Reagent A' was prepared by adding 20μl of Reagent S to every 1ml of Reagent A to be used.

In a 96 well plate, 5μl of samples and standards were added in triplicate. 25μl of Reagent A' was added followed by 200μl of Reagent B. The plate was gently agitated to mix solutions. After fifteen minutes at room temperature the plate was read at 750nm on a plate reader (Dynatech MR5000).

4.10 Alkaline Phosphatase Assay

The mature osteoblast expresses the enzyme alkaline phosphatase (AP), which has an essential role in matrix mineralisation, both on the cell surface and in the cytoplasm (Raisz et al, 1998).

Principle of the Assay

Colourless p-Nitrophenyl Phosphate (pNPP) was used as a substrate for alkaline phosphatase and formed yellow p-Nitrophenol on hydrolysis by the enzyme. The assay was done in a ninety-six well plate which was read at 410nm.

Method

Reaction Buffer was made from adding 0.5% Triton-X to 221 Alkaline Phosphatase Buffer (2-amino-2-methyl-1-propanol, 1.5M, pH 10.3). The Triton-X lysed the cells, enabling quantification of total AP, both cytoplasmic and cell surface.

Standards were made from stock p-Nitrophenol (p-NP 10µM) diluted in Reaction Buffer as in Table 4.1.

Table 4.1 Preparation of standards for alkaline phosphatase assay

Standard Number	p-NP (µl)	Reaction Buffer (µl)	[p-NP] (pg/ml)
1	2.5	2497.5	10
2	12.5	2487.5	50
3	25	2475	100
4	50	2450	200
5	20	480	400
6	40	460	800
7	50	450	1000
8	60	440	1200
9	70	430	1400
10	90	410	1800
11	100	400	2000

Aliquots of 50µl of the standards were placed in a ninety-six microtitre well plate in triplicate. The plate was stored at -20°C until use.

Assay

150µl of Reaction Buffer was added to each standard well to make the volume up to 200µl.

Controls

The control solution was the Substrate Buffer which was made by dissolving 4.6mg of p-NPP in 10ml Reaction Buffer and 200µl was placed in the nine control wells.

Samples

The samples were thawed, vortexed and 50µl placed in the sample wells in duplicate. Substrate Buffer (150µl) was then added to each sample well and the plate was incubated at 37°C for ten minutes. At this time, the colour production was checked and if necessary, the plate was incubated for a further ten minutes before reading at 410nm on a plate reader. The duration of the incubation was noted as the enzyme activity is expressed as unit per cell per minute i.e. [n-NPP produced (pg/ml)] / 1 x 10^5 cells / minute.

4.11 Flow Cytometry of Human Bone Marrow Stromal Cells

4.11.1 Principle of Flow Cytometry

Flow cytometry (also known as fluorescent activated cell sorting, FACS) is a powerful way of studying the expression of cell surface markers, such as receptors or cell-associated molecules (Carter et al, 1990). Other applications of flow cytometry include the study of cell kinetics, membrane potential and chromosomal analysis (Ormerod et al, 1990).

In flow cytometry, rapid measurements are made on cells (or particles) as a fluid stream containing the cells passes a sensor. Laser-based flow cytometers measure

the scattered and fluorescent light generated by cells as they pass through the illuminating laser beam. The cells are labelled with fluorochromes attached to antibodies bound to the molecule of interest (Ormerod, 1990). These compounds release energy when they are excited by the laser beam and the photons are collected by photodetectors and converted into electronic signals. The signals are computed into a graphical display of the events. A powerful feature of the technique is that each measurement, or event, is made on a single cell rather than pooled (Carter et al, 1990).

Suitable antibodies against the molecules of interest must be available and be appropriately specific. Monoclonal antibodies are therefore preferable. In the experiments described below, indirect staining by a secondary, directly conjugated goat anti-mouse antibody against the primary (mouse) antibody was used. An advantage of using secondary staining is that more than one secondary antibody can bind to the primary antibody, increasing the fluorescence intensity of the cell.

Single, dual or triple labelling with one, two or three different primary antibodies is possible, resulting in two, four or eight subpopulations. The cells are sorted into populations depending on the binding of the antibodies and results are given as the percentage of the total cells in each population. Population shifts according to different experimental conditions can be studied. Sorted subpopulations of cells can be further cultured but this was not undertaken in this project.

4.11.2 Monoclonal Antibodies Used in Flow Cytometry of Bone Cells

The monoclonal antibody B4-78 recognises the bone/liver/kidney isoform of alkaline phosphatase (Lawson et al, 1985).

Osteoblasts develop from multipotential bone marrow stromal cells and the characteristics of the intermediary cell types is unknown. Simmons and Torok Strob have developed a monoclonal antibody against a subpopulation of bone marrow stromal cells which they have called Stro-1 (Simmons et al, 1991). It has been shown that this antibody identifies all the cells which can produce colonies (CFU-F) *in vitro*, and it is thought that Stro-1 may be a marker of the stem cell for osteoblasts (Gronthos et al, 1994). The expression of Stro-1 is lost as the cell becomes more mature and committed to the osteoblast phenotype.

Hybridomas for the monoclonal mouse anti-human antibodies Stro-1 and AP (B4-78) were supplied by the Development Studies Hybridoma Bank, University of Iowa. The B4-78 antibody was purified by Dr Carolyn Jefferis by passage over a protein G column and Dr Susan Walsh prepared the Stro-1 antibody from the hybridoma supernatant.

4.11.3 Model of Osteoblast Differentiation

Osteoblasts differentiate from multipotential stem cells which give rise to other mesenchymal lineages such as chondrocytes and adipocytes. Dr JN Beresford and colleagues have developed a model of osteoblast differentiation based on the four populations of cells obtained by FACS analysis of cells labelled with B4-78 and Stro-1 (Stewart et al, 1996). The model is shown in Figure 4.1 and Table 4.2.

It is possible that the least well-characterised (double negative) population includes both early and late stage cells of the osteoblast lineage, osteoblasts or osteocytes.

Figure 4.1 Model of osteoblast differentiation using Stro-1 and alkaline phosphatase (AP) expression determined by flow cytometry

	Stro-1-negative	**Stro-1-positive**
AP-positive	Stro-1 negative and AP positive cells: mature osteoblasts	Stro-1 and AP positive cells: pre-osteoblasts
AP-negative	Stro-1 and AP negative cells: uncommitted osteoprogenitor cells and post-mature osteoblasts (osteocytes)	Stro-1 positive and AP negative cells: committed osteoprogenitor cells

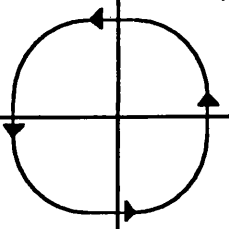


Table 4.2 Cell populations by flow cytometry after dual labelling

	Stro-1	**AP (B4-78)**
Least differentiated	negative	negative
Progenitor osteoblast	positive	negative
Committed osteoblast	positive	positive
Mature osteoblast	negative	positive

4.11.4 Experimental Design

Bone marrow-derived cells were grown under standard culture conditions in DMEM (with additions, see appendix) with 100μM Ascorbate-2-phosphate and 15% fetal calf serum by volume (Section 4.3). Additional conditions were Methotrexate (0, 1, 10, 100 or 1000nM MTX) and Dexamethasone 0 or 10nM. At the end of primary culture, cells were passaged, dual-labelled with the monoclonal antibodies B4-78

and Stro-1 and subjected to fluorescence-activated cell sorting. The number of days the cells were in culture is shown in Table 4.3.

Table 4.3 Culture time for marrow-derived osteoblast-like cells prior to flow cytometry

Results shown in:	Days in primary culture before redistribution	Days in culture after redistribution
Figures 5.19 and 5.20	23	48
Figures 5.21 and 5.22	44	24
Figures 5.23 and 5.24	44	29

4.11.5 Dual-Labelling of Human Bone Marrow Derived Cells

Cells were passaged as described above (Section 4.6), and counted.

In brief, the cells were blocked, incubated with the primary monoclonal antibodies (B4-78 and Stro-1, or controls), washed and incubated with the secondary (fluorescent) antibodies. After two further washes, the cells were fixed in paraformaldehyde and stored overnight at 4°C before flow cytometry the following day.

Protocol in detail

For each experimental condition, the following FACS tubes were prepared:

B4-78 monoclonal antibody only (O/A)

Stro-1 monoclonal antibody only (S/I)

B4-78 & Stro-1 monoclonal antibodies (S/A) (2 tubes),

where O was a non-specific monoclonal antibody (OBL) developed in-house by Dr Susan Walsh , A was B4-78, S was Stro-1 and I was mouse monoclonal Ig G_1.

OBL was used as the IgM isotype control for Stro-1 and I was used as the IgG isotype control for B4-78. In addition, cells which were blocked and fixed, but not incubated with any antibody, were used to align the FACS laser beam (negative cells). Negative control tubes were: cells incubated with the secondary (fluorescent) antibody only (2nd ab only), and cells incubated with the two non-specific antibodies, OBL and I (O/I).

Cells were blocked by placing on ice with blocking buffer for 20 minutes, where blocking buffer was Hanks Buffered Saline Solution (HBSS, Gibco, UK) with 10mM HEPES and 5% FCS and 10% human AB serum by volume. The serum blocked non-specific binding in human cells.

After blocking, 10^5 cells were placed in each tube, and all tubes, except the negative cells, were centrifuged at 350g for five minutes at 4°C. The block was aspirated off and the primary antibody was added to each tube, except the 2nd ab only cells, which had 500µl of wash buffer instead.

Primary antibodies

Freshly thawed O,I,S and A antibodies were used. The volumes needed for each tube were:

O and S	500µl (hybridoma supernatant)
A	3µl (15µg/ml)
I	10µl (15µg/ml)

Thus for an experiment with four conditions, the following solutions of antibodies were prepared:

S/A 500µl / 3µl for eight tubes = 4 mls (S) + 24µl (A)

S/I 500µl / 10µl for two tubes = 1 ml (S) + 20µl (I)

O/A 500µl / 3µl for two tubes = 1 mls (O) + 6µl (A)

O/I 500µl / 10µl for one tube = 500µl (O) + 10µl (I)

The appropriate volume (503µl or 510µl) was added to each tube, the pellet was resuspended by gentle shaking and they were incubated on ice for one hour. Following this, each tube (including 2nd ab only tube) was filled to the top with cold washing buffer and spun at 350g for five minutes at 4°C with a low brake. The wash was aspirated off, and the second antibody added.

Secondary antibody

These were two fluorescent antibodies which bound to the primary antibody and allowed identification of differentially-labelled cells. The goat anti-mouse human-adsorbed secondary conjugates anti-IgG_1-rhodamine-phycoerythrin (R-PE) and anti-IgM-fluorescein isothiocyanate (FITC) were supplied by Southern Biotechnology Associates. R-PE absorbs light at 488nm and emits light in the orange spectrum whilst FITC absorbs light at 495nm and emits a green light (Ormerod, 1990). One laser can excite both compounds and thus differential detection can be made.

A 1.5ml secondary antibody suspension was made using the following volumes:

150µl human AB serum

1.35 mls HBSS with 5% FCS

7.5 µl R-PE

60 µl FITC,

and 100µl of the suspension was added to each tube. The cell pellet was resuspended and incubated on ice for forty-five minutes, in the dark to prevent fading of the fluorescent labels.

Fixing step

Following the second incubation, the cells were washed using cold washing buffer and centrifuged as before. After the wash was aspirated, 500µl of cold PBS with 1% paraformaldehyde was added to each tube and the pellet resuspended. Lids were placed on the tubes and they were kept at 4°C overnight.

4.11.6 Flow Cytometry Analysis of Dual-Labelled Passaged Human Bone Marrow-Derived Cells

Analysis of the dual-labelled cells was done on a Becton Dickinson FACstar Plus flow cytometer with a 488nm Argon laser. The machine was calibrated daily using standard beads provided by the manufacturer. The FACS laser beam was aligned using the negative control sample and the lower threshold set for the recording of events. The single positive controls were used to set the machine compensation for the spectral overlap of FITC and RPE, and the four cell populations defined. The positive controls also confirmed the effectiveness of the secondary antibody binding. The double-labelled samples were then analysed and distributions in the four quadrants recorded for each sample. Results are presented in Section 5.7 and Figures 5.18 to 5.24.

4.12 Appendix For Bone Cell Culture

Stock solutions for tissue culture were obtained from Gibco BRL, UK unless otherwise stated.

Dulbecco's Modified Essential Medium (DMEM) (with NEAA, without glutamine, pyruvate and bicarbonate), with the following additions:

Sodium Bicarbonate (7.5%)	11.4ml/l
HEPES buffer (1M)	20ml/l
Glutamine (200mM)	10ml/l
Sodium pyruvate (100mM)	10ml/l
Penicillin/Streptomycin (25 IU)	2.5ml/l
Amino acids	Alanine (35.6mg/l), Asparagine (60.0mg/l),

Aspartic acid (53.0mg/l), Glutamic acid (59.0mg/l), Proline (46.0mg/l)

This was made up to 1 litre with Milli-Q water, adjusted to pH 7.2 with sodium hydroxide and filter-sterilised. It was stored at 4°C in 500ml aliquots.

Folate-free DMEM

To make 500ml:

Milli-Q water	438ml
Na Bicarbonate 7.5%	5.7ml
Amino acids (as above)	5ml
Penicillin/Streptomycin (as above)	1.25ml
HEPES	2.38g
Glutamine	15ml
Folate-free DMEM 10x stock	50ml

Prepared in hood, buffered with 10 M NaOH to pH 7.2 and filter sterilised.

Phosphate buffered saline (PBS)

10 PBS tablets were dissolved in 1 litre of Milli-Q water and autoclaved. It was allowed to cool to room temperature before use.

Collagenase IV

This was made up in sterile water or serum-free medium and stored in 1ml aliquots at -20°C. It was used at 25 U/ml final concentration.

Deoxyribonuclease I (Dnase I)

This was made up in 150mM sodium chloride to 2000 units/ml. It was used at 20 units/ml final concentration.

1 α, 25 - dihydroxyvitamin D_3 (calcitriol)

This was made up in absolute ethanol and stored in sealed evacuated tubes under gaseous nitrogen in 250 μl aliquots of 3.44×10^{4} M at -20°C.

Ascorbate - 2- phosphate (asc-2-P)

This was supplied as a desiccate and stored at -20°C in a box containing desiccant. It was made up in serum-free DMEM to a stock strength of 10mM and filter-sterilised before being stored at -20°C as 5 ml aliquots. It was used at a final concentration of 100 μM.

Dexamethasone (DX)

This was made up in serum-free DMEM to a stock concentration of 5×10^{-5} M and filter-sterilised before being stored at -20°C as 1ml aliquots. It was used at a final concentration of 10^{-8} M.

Methotrexate

This was obtained from Wyeth-Lederle in 2ml vials of 25mg/ml. The molecular weight of Methotrexate in the hydrous form is 454.5. Therefore 1000nM Methotrexate is 0.45mg in 1ml. As 1ml contained 25mg, 18μl contained 0.45mg and concentrations of 100nM to 1nM were made by serial dilutions.

Folic acid

Normal DMEM contains 4mg/l. Folic acid and normal human serum levels are 4 to 18μg/l. Folic acid is concentrated in red blood corpuscles at a concentration of 160 to 180μg/l. Folic acid was added back to folate-free medium in some experiments. The stock was kept sterile and 19.4mg was measured out in the hood, weighed in a sealed container and dissolved in 1.94ml PBS or folate-free DMEM to give 10mg/ml. A dilution was made by adding 10μl of this to 10ml PBS or folate-free DMEM gave 10μg/ml. This was used in experiments either 1 in 100 or 1 in 1000 to give final concentrations of 10μg/l or 100μg/l.

Folinic acid

Folinic acid stock was kept sterile. The molecular weight is 511.5, thus 1M is 511.5g/l and 1mM is 511.5mg/l. A working stock solution of concentration 1mM was made by diluting 14.5mg in 28.35ml PBS or folate-free DMEM and was used at 1 in 1000 in experiments, at a final concentration of 1μM.

5. BONE CELL CULTURE RESULTS

5.1 Introduction

The results from the series of experiments performed on human bone cells in culture to explore any direct effect of the drug methotrexate (MTX) are presented in this chapter. The experimental methods were described in Chapter 4.

5.2 Characteristics of Donors

The available features of donors are shown in Table 5.1. They include age, sex, diagnosis and drug treatment where known.

Table 5.1 Characteristics of donors of bone

Sex	Age (years)	Diagnosis	Medication
Male	19	Pneumothorax	None
Male	83	Diaphragmatic hemia	Omeprazole
Female	57	Lung cancer	HRT
Male	62	Lung cancer	None
Female	72	Squamous cell lung carcinoma	Thyroxine Dothiepin
Female	75	Oesophageal carcinoma	None

5.3 Analysis of Results

When possible, experiments were set up in quadriplicates to enable analysis of variance to be performed. An exception was the flow cytometry experiments where only duplicates were available. Results were analysed by the author using Statview 4.5 for Macintosh. Statistical tests used included paired t-test and analysis of variance (ANOVA) with post-hoc testing with Fisher's Protected Least Significant

Difference (PLSD). Levels of significance were set at 95% and standard errors or standard deviation of the means are illustrated.

5.4 Effect of Methotrexate on the Proliferation and Migration of Cells from Trabecular Bone Chips in Vitro

Trabecular bone chips were prepared as described in Section 4.4 and cultured in a twenty-four well plate with 1ml of medium per well. The medium was Dulbecco's Modified Essential Medium (DMEM) with the supplements listed in the appendix to Chapter 4 and additionally 100μM L-ascorbate-2-phosphate and fetal calf serum (FCS) 10% by volume. Experimental conditions were with or without 10nM Dexamethasone (DX) and 0, 1, 10, 100 or 1000nM Methotrexate (MTX, Wyeth-Lederle, Gosport, Hants, UK). The wells were assessed for proliferation of cells on the surface of the bone chips and migration of the cells out onto the surface of the plastic by light microscopy on the ninth, fourteenth and eighteenth day of culture. An arbitrary scoring system of '1' for cell proliferation (as seen in Figure 5.1) and '2' for migration (as seen in Figure 5.2) was used. The mean scores at the three time points are shown in Table 5.2. The results demonstrated the permissive effect of DX on bone cell proliferation and migration, with the highest scores on day eighteen being in the group treated with 10nM DX but no MTX. MTX delayed rather than abolished proliferation and migration, as shown by the scores on day nine. By day eighteen, there was evidence of proliferation even at the highest dose of MTX (1000nM), both in the presence and absence of DX. On day twenty-five, the well contents were stained with Fast Red (Section 4.7.1) to demonstrate the expression of alkaline phosphatase. Staining was heaviest over the chips, but was also present

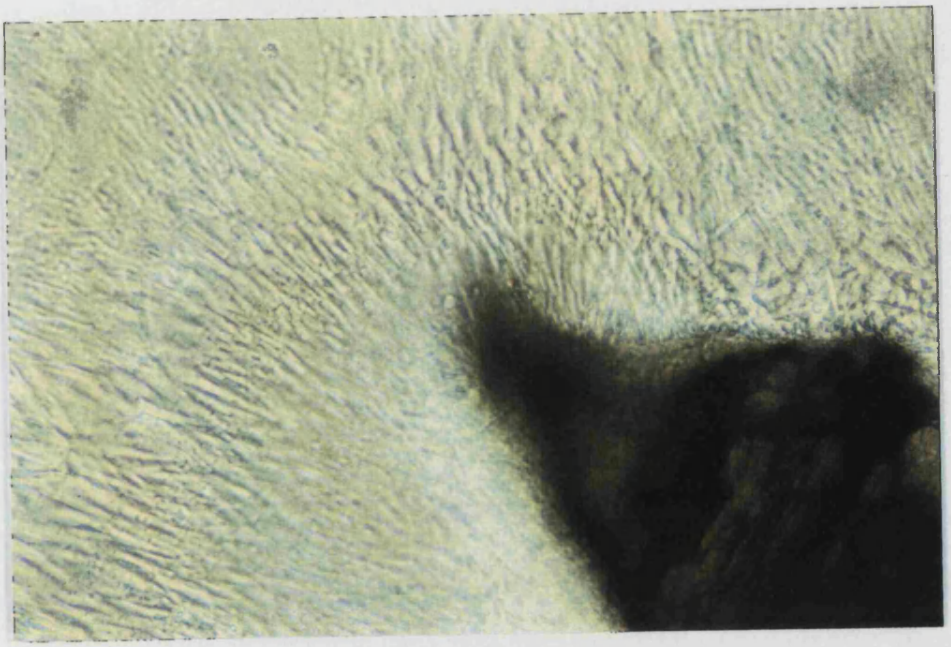
over the cell layer which had migrated onto the well surface, confirming an osteoblast-like phenotype.

In summary, MTX inhibited and delayed, but did not abolish, the proliferation and migration of osteoblast-like cells from normal human trabecular bone.

Figure 5.1 Proliferation of cells on the surface of a trabecular bone chip in culture

Magnification x 200

Figure 5.2 Migration of cells from a trabecular bone chip out onto the surface of the culture flask



Magnification x 200

Table 5.2 Proliferation and migration of bone cells from trabecular chips

[MTX] nM (n)	-DX Day 9	-DX Day 14	-DX Day 18	+DX Day 9	+DX Day 14	+DX Day 18
0 (3)	1.67	1.67	1.33	1.67	1.67	2
1 (2)	0	0	0.5	0	2	2
10 (2)	0	1	2	0	0.5	1
100 (2)	0	0	1	0	0	0.5
1000 (3)	0	0.67	0.67	0	0	1.33

Results are shown as mean per well. Proliferation on cells on the surface of the bone chips was scored as '1', migration out onto the plastic was scored as '2'. MTX, methotrexate; -DX, no added dexamethasone; +DX, dexamethasone 10nM.

5.5 Effect of Methotrexate on Colony Formation of Normal Human Bone Marrow Stromal Cells

5.5.1 Dose Response Experiments

The method for these experiments is given in Section 4.7. A 28cm^2 plate stained with Fast Red for alkaline phosphatase (AP) positive colonies is shown in Figure 5.3, and one stained with both Fast Red and Methylene Blue (for all colonies) is shown in Figure 5.4.

Figure 5.3 Colonies expressing alkaline phosphatase stained by Fast Red stain

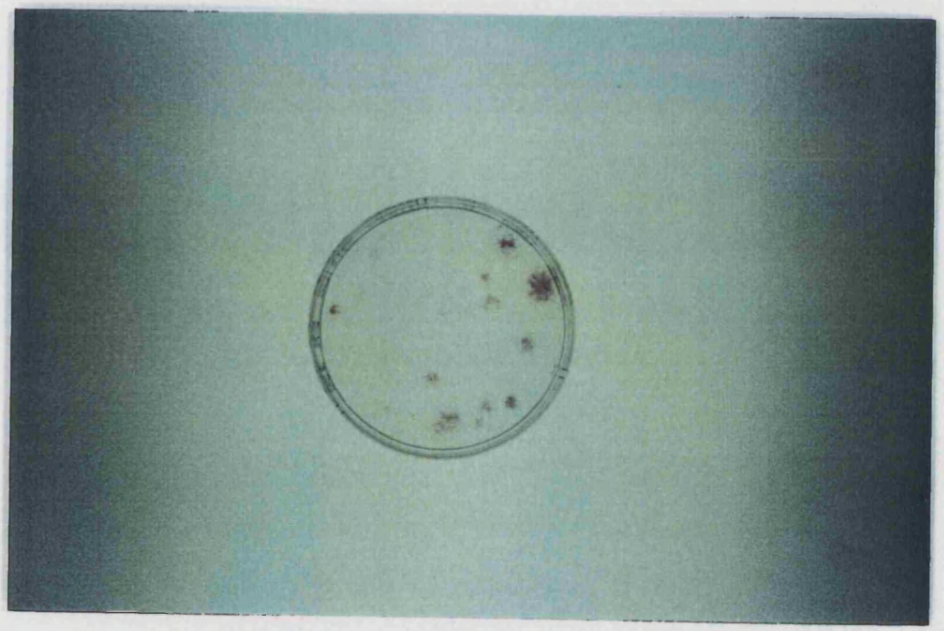


Figure 5.4 All colonies stained by Fast Red and then Methylene Blue

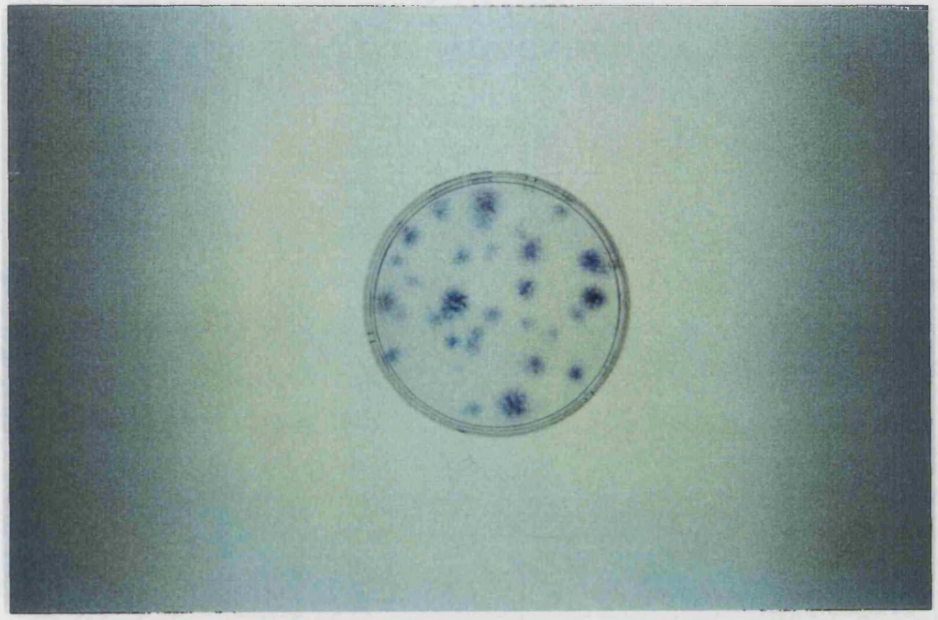
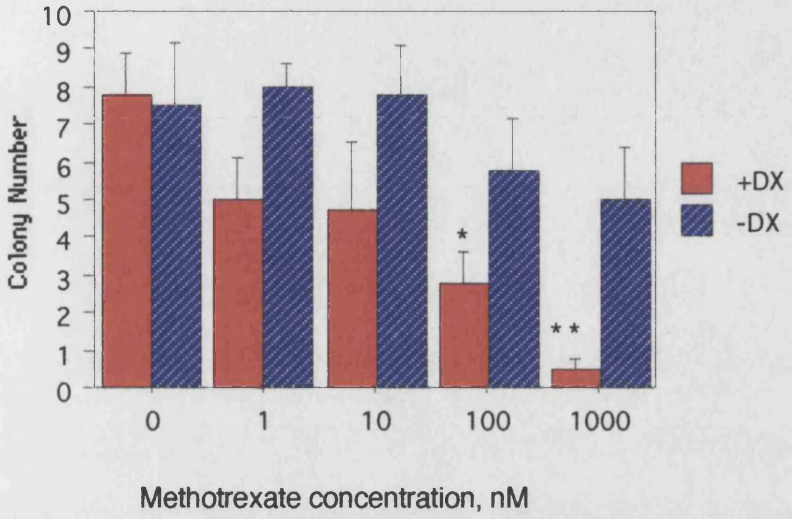


Figure 5.5 shows the effect of Methotrexate (MTX) 1 to 1000nM on the numbers of colonies formed after twenty-one days in primary culture. There were very few AP-positive colonies, only five colonies in the forty plates (result not shown). In the presence of 10nM DX, MTX had a dose-dependent inhibitory effect on total colony formation, treatment with 100nM and 1000nM MTX being associated with significantly fewer colonies than controls.

The colonies which developed in the absence of DX were also fewer in the presence of 100nM and 1000nM MTX, but the difference from controls did not reach significance (Figure 5.5).

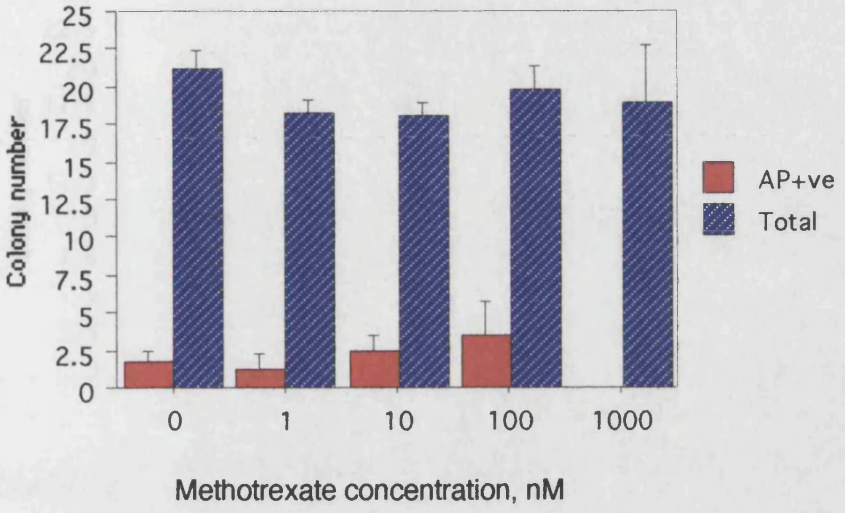
Figure 5.5 Total colony formation in the presence of methotrexate, with and without 10nM dexamethasone



Colonies stained with Methylene Blue after 21 days in culture.
Results shown as mean colony number ± standard error. Analysis by ANOVA with Fisher's Protected Least Significant Difference, n = 4.
* significant difference from 0nM MTX, p = 0.006
** significant difference from 0nM MTX, p = < 0.0001, and from 1nM MTX, p = 0.006, and from 10nM MTX, p = 0.0089

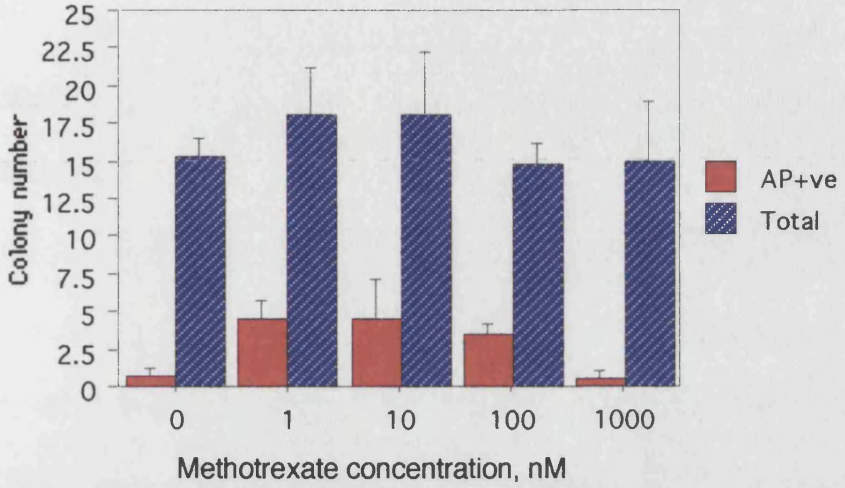
Figures 5.6 and 5.7 show the total colonies and alkaline phosphatase-positive (AP +ve) colonies which grew from a different donor in the absence (Figure 5.6) and presence (Figure 5.7) of 10nM DX respectively. There was no significant difference between control cultures (no MTX) and any of the doses of MTX for either AP positive or total colony numbers in either DX condition.

Figure 5.6 Total and AP-positive colony formation in the presence of methotrexate and no dexamethasone (a)



Colonies stained with Fast Red and then Methylene Blue after 18 days in culture.
Results shown as mean colony number ± standard error. Analysis by ANOVA with Fisher's Protected Least Significant Difference post hoc testing, n = 4.

Figure 5.7 Total and AP-positive colony formation in the presence of methotrexate and 10nM dexamethasone (a)

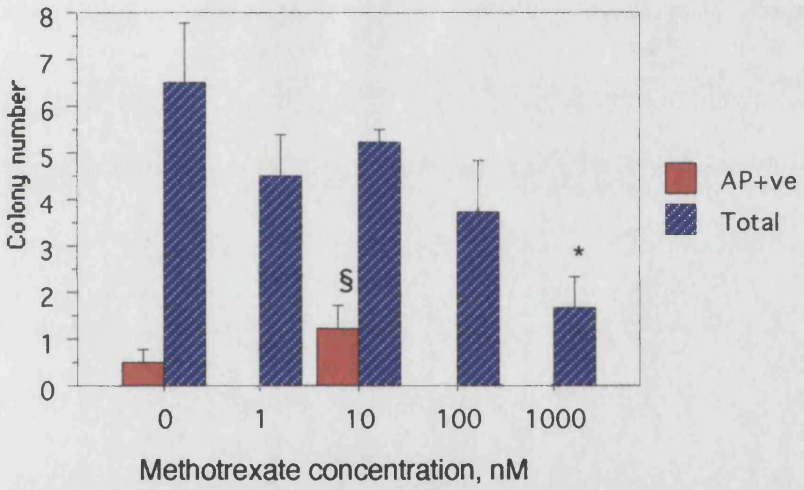


Colonies stained with Fast Red and Methylene Blue after 18 days in culture.
Results shown as mean colony number ± standard error. Analysis by ANOVA with Fisher's Protected Least Significant Difference post hoc testing, n = 4.

Figures 5.8 and 5.9 show the total colonies and alkaline phosphatase (AP) positive colonies which grew from a third donor. There were few AP positive colonies even in the presence of DX (Figure 5.9). Glucocorticoids at doses which correspond to physiological levels (such as those used here) have been shown to lead to the development of an osteoblast-like phenotype in human bone derived cells in culture (Gallagher et al, 1996). The total colony number was suppressed in the absence of DX in a dose-dependent manner, but only reaching significance at the highest concentration of MTX (Figure 5.8). In the presence of DX, in contrast to both the results above, MTX at the dose 100nM appeared to have a permissive effect on

colony formation, although the standard error was large (Figure 5.9). Again, the highest dose of MTX, 1000nM was associated with significant reduction in colony number.

Figure 5.8 Total and AP-positive colony formation in the presence of methotrexate and no dexamethasone (b)



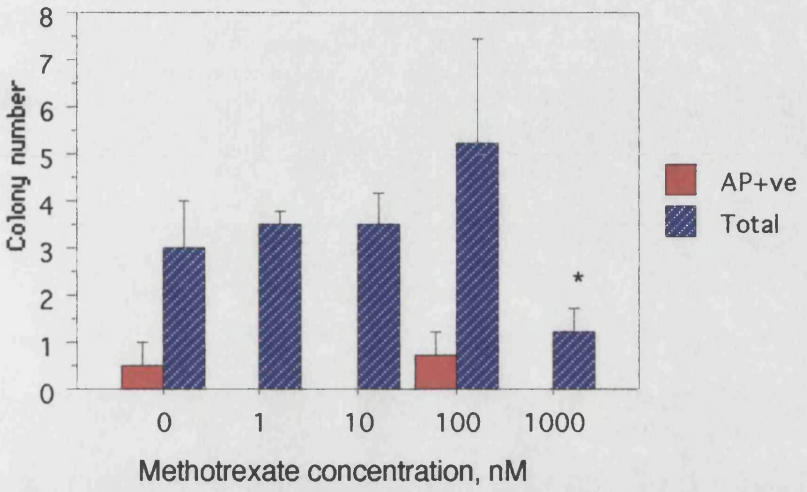
Colonies stained with Fast Red and Methylene Blue after 20 days in culture.

Results shown as mean colony number ± standard error. Analysis by ANOVA with Fisher's Protected Least Significant Difference post hoc testing, n = 4.

* Significant difference from 0nM MTX, p = 0.0036 and from 10nM MTX, p = 0.0217

$ Significant difference from 1nM MTX, p = 0.003, and from 100nM MTX, p = 0.003, and from 1000nM MTX, p = 0.003

Figure 5.9 Total and AP-positive colony formation in the presence of methotrexate and 10nM dexamethasone (b)

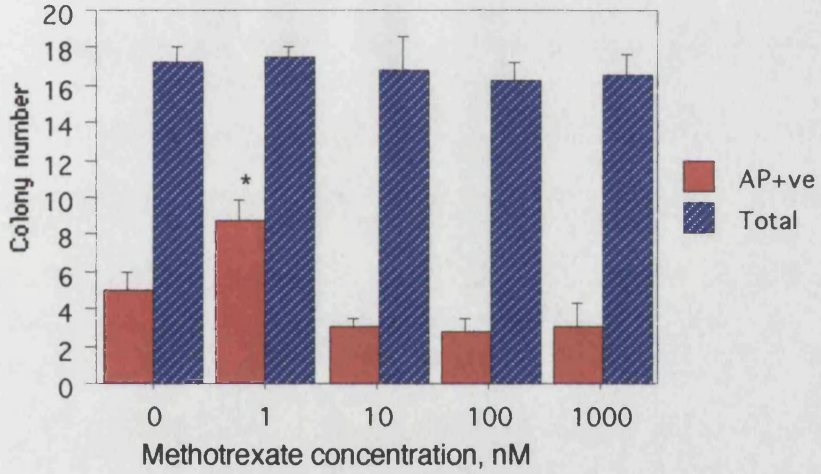


Colonies stained with Fast Red and Methylene Blue after 20 days in culture.
Results shown as mean colony number ± standard error. Analysis by ANOVA with Fisher's Protected Least Significant Difference post hoc testing, n = 4.
* Significant difference from 100nM MTX, p = 0.0253

Figure 5.10 shows colony formation in the absence of DX from another donor and illustrates both the total colony numbers and those which were osteoblast-like (AP-positive). The corresponding counts in the presence of DX are shown in Figure 5.11. In this experiment, the effect of very low doses of MTX was explored (1 to 1000 picomolar, equivalent to 1 nanomolar), diluting by a thousand-fold the doses used previously. The number of colonies which developed, both AP-positive and total, was much greater than in the previous experiments. In the absence of DX (Figure 5.10), these small doses of MTX had no effect on total colony number. Figure 5.11 shows that the lowest dose of MTX, 1pM, significantly suppressed both AP positive

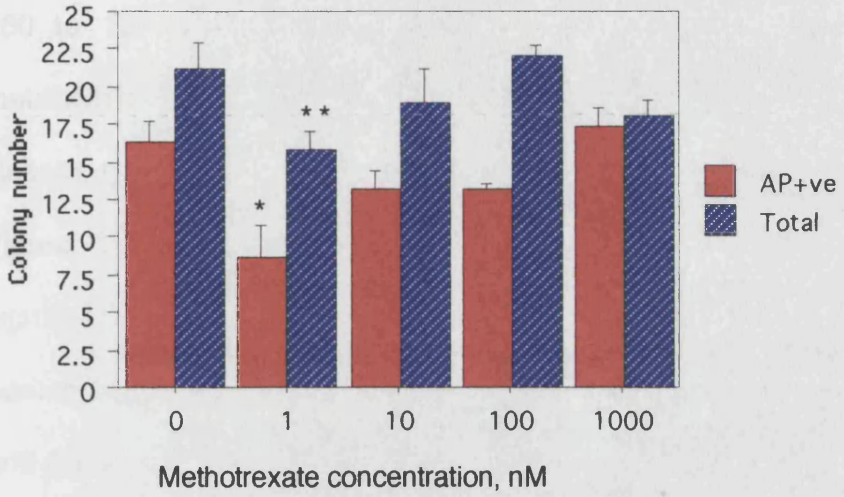
and total colony formation compared to control, whilst in Figure 5.10 the number of AP positive colonies was increased in the presence of 1pM MTX and no DX.

Figure 5.10 Total colony and AP-positive formation in the presence of very low dose methotrexate and with no dexamethasone



Colonies stained with Fast Red and Methylene Blue after 18 days in culture.
Results shown as mean colony number ± standard error. Analysis by ANOVA with Fisher's Protected Least Significant Difference post hoc testing, n = 4.
* Significant difference from 10pM MTX, p = 0.0042, and from 100pM MTX, p = 0.0016, and from 1000pM (= 1nM) MTX, p = 0.0031.

Figure 5.11 Total and AP-positive colony formation in the presence of very low dose methotrexate and with 10nM dexamethasone



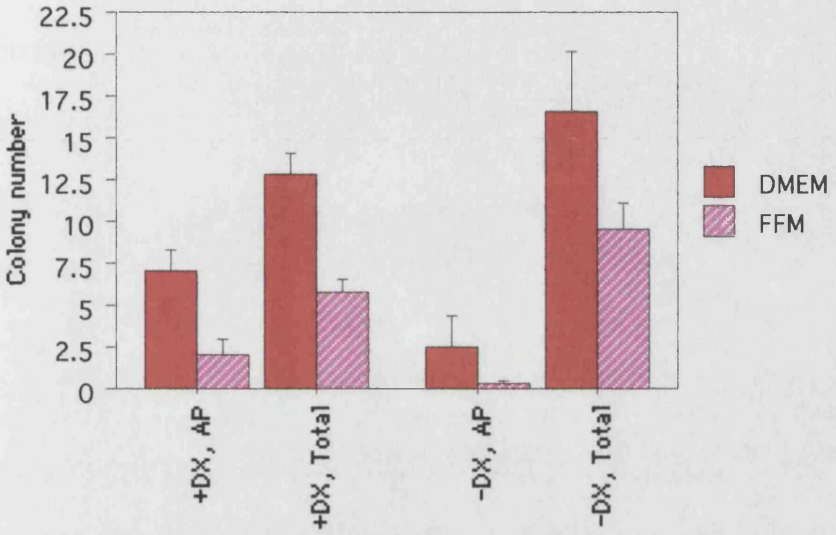
Colonies stained with Fast Red and Methylene Blue after 18 days in culture.
Results shown as mean colony number ± standard error. Analysis by ANOVA with Fisher's Protected Least Significant Difference post hoc testing, n = 4.
* Significant difference from 0pM MTX, p = 0.0017, from 10pM MTX, p = 0.0362, from 100pM MTX, p = 0.0362, and from 1000pM (= 1nM) MTX, p = 0.0011.
** Significant difference from 0pM MTX, p = 0.0202, and from 100pM MTX, p = 0.01.

In summary, colony formation was inhibited in the presence of 100 and 1000nM MTX but the results were not consistent between donors. There was considerable variation in the number of colonies (both AP positive and total) in the control, untreated groups between experiments. Possible reasons for this are discussed in Section 5.5.4 below.

5.5.2 Colony Formation with Folate-Free Medium

The medium used routinely for bone cell culture was Dulbecco's Modified Essential Medium (DMEM, Sigma, UK) with additions (see Tissue Culture appendix). DMEM contains 4mg/l folate whilst in humans the normal serum folate level is 1.8 to 14.0μg/l. Folate is concentrated in the red blood cell where the normal levels are 160 to 180μg/l. As one of the many actions of MTX is interference in folate metabolism (section 1.4.1), colony formation in folate-free medium was investigated. Results from the pilot experiment utilising folate-free medium (FFM) is shown in Figure 5.12 and no MTX was included in the design. Colony formation was significantly reduced with FFM, whether or not DX was present. The mean percentage reductions in AP-positive colonies were 90% and 71% in the absence and presence of DX respectively, and for total colony formation were 42% and 55% in the absence and presence of DX respectively.

Figure 5.12 Total and AP-positive colony formation in folate-free medium

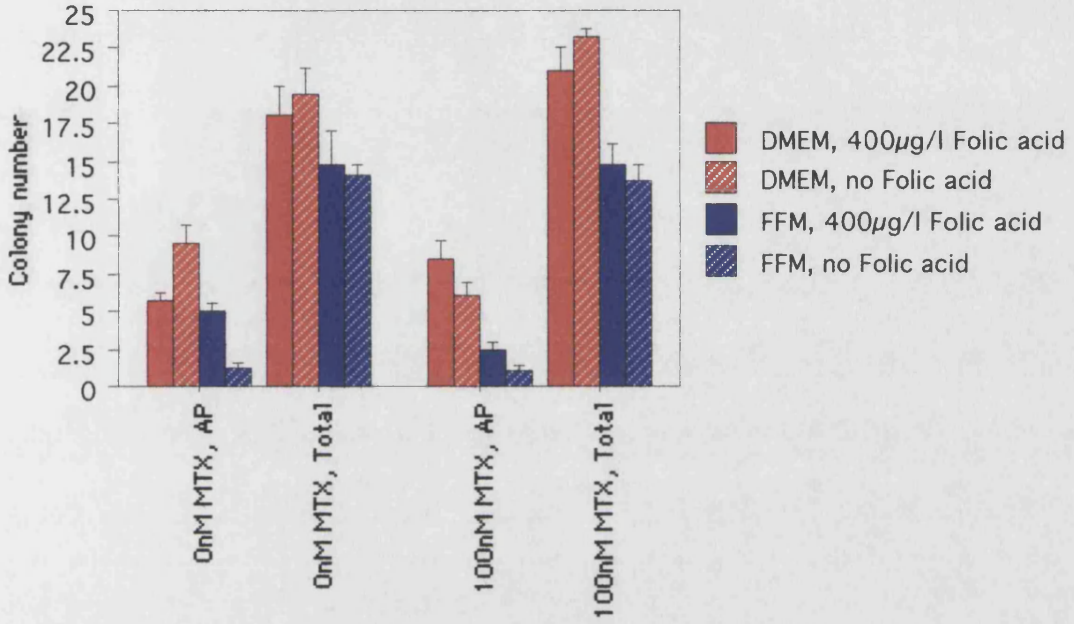


Colonies stained with Fast Red and Methylene Blue after 18 days in culture.
Results shown as mean colony number ± standard error. Analysis by ANOVA with Fisher's Protected Least Significant Difference post hoc testing, n = 4.
For each condition (with and without DX) and for both total and AP-positive colonies, formation was significantly reduced in the folate-free medium, $p < 0.001$.

5.5.3 Effect of Methotrexate with Folate-Free Medium on Colony Formation from Normal Human Bone Marrow Stromal Cells

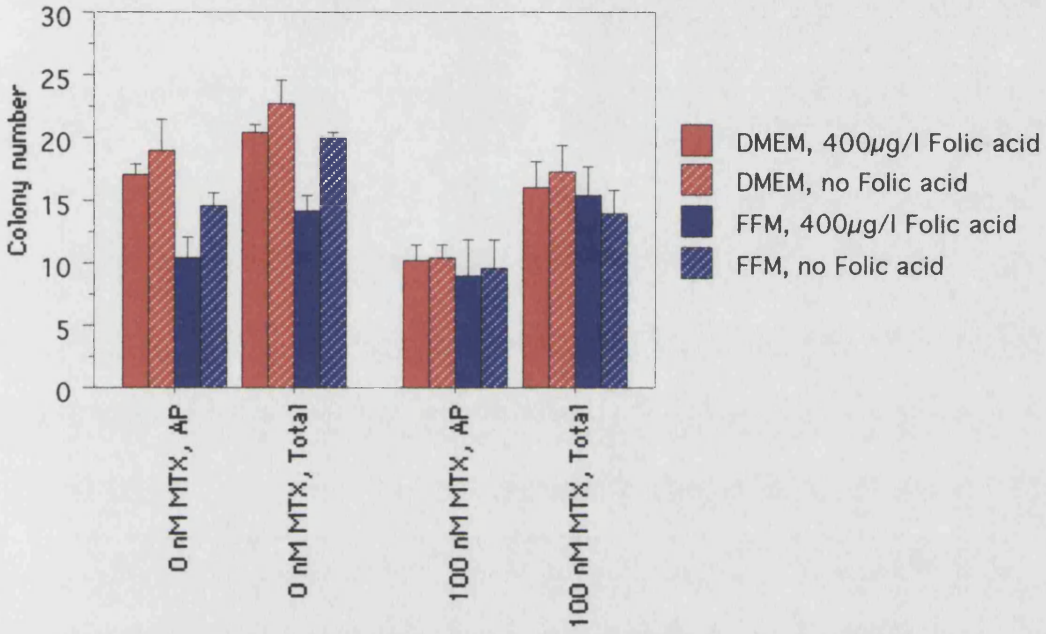
Subsequently, the effect of adding back 400μg/l folic acid to the FFM was explored and the results shown in Figures 5.13 and 5.14 with mean colony numbers shown in Table 5.3. This dose of folic acid did not overcome the inhibitory effects of FFM on the development of colonies. In fact, the number of colonies was greater in the FFM without folic acid supplement in the presence of DX (Figure 5.14), except for total colony formation in the presence of 100nM MTX. The number of colonies formed was generally lower for those cultures grown in FFM. In the presence of 10nM DX (Figure 5.14), the number of AP-positive colonies was remarkably similar in all four media conditions when 100nM MTX was present. This contrasts greatly with the outcome in the absence of DX (Figure 5.13), when AP-positive colony formation was suppressed in the FFM groups, especially when no folic acid had been added back. The addition of 100nM MTX did not suppress further the osteoblast-like (AP-positive) differentiation in these DX-free culture conditions when, in the absence of folate, AP-positive colonies were already very suppressed.

Figure 5.13 Total and AP-positive colony formation in folate-free medium or DMEM and with or without folic acid and with no dexamethasone



Colonies stained with Fast Red and Methylene Blue after 18 days in culture.
Results shown as mean colony number ± standard error. Analysis by ANOVA with Fisher's Protected Least Significant Difference post hoc testing, n = 4.

Figure 5.14 Total and AP-positive colony formation in folate-free medium or DMEM and with or without folic acid and with 10nM dexamethasone



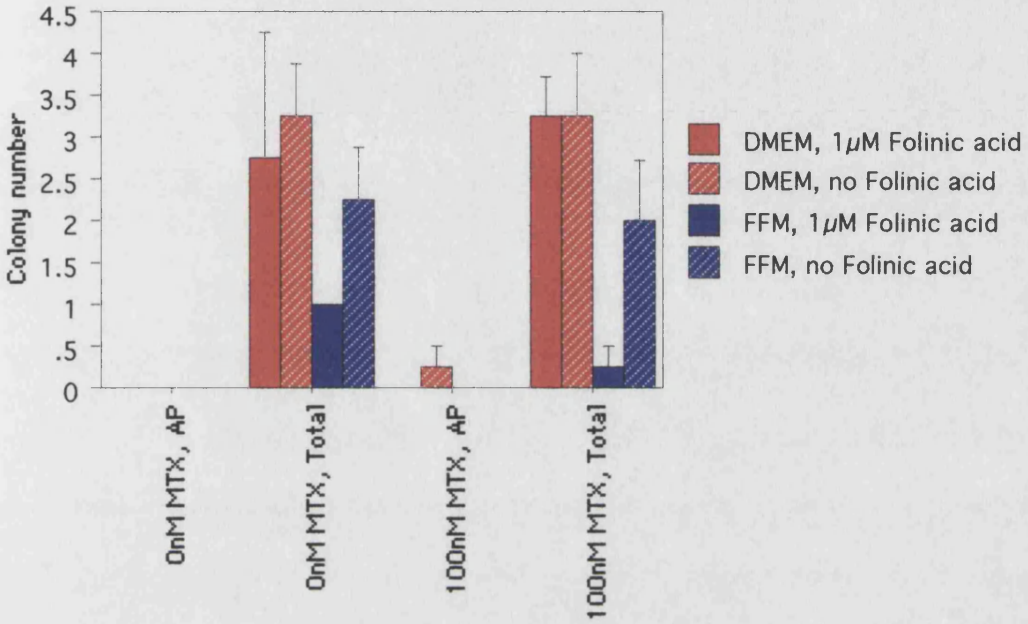
Colonies stained with Fast Red and Methylene Blue after 18 days in culture.
Results shown as mean colony number ± standard error. Analysis by ANOVA with Fisher's Protected Least Significant Difference post hoc testing, n = 4.

Table 5.3 Mean colony number for experiment shown in Figures 5.13 and 5.14

0 or 10nM DX	0 or 100nM MTX	Total/ AP colonies	DMEM + 400μg/l Folic acid	DMEM	FFM + 400μg/l Folic acid	FFM
0	0	Total	18	19.5	14.75	14
0	100	Total	21	23.25	14.75	13.75
0	0	AP	5.75	9.5	5	1.25
0	100	AP	8.5	6	2.5	1
10	0	Total	20.5	22.75	14.25	20
10	100	Total	16	17.25	15.5	14
10	0	AP	17	19	10.5	14.5
10	100	AP	10.25	8	9	9.5

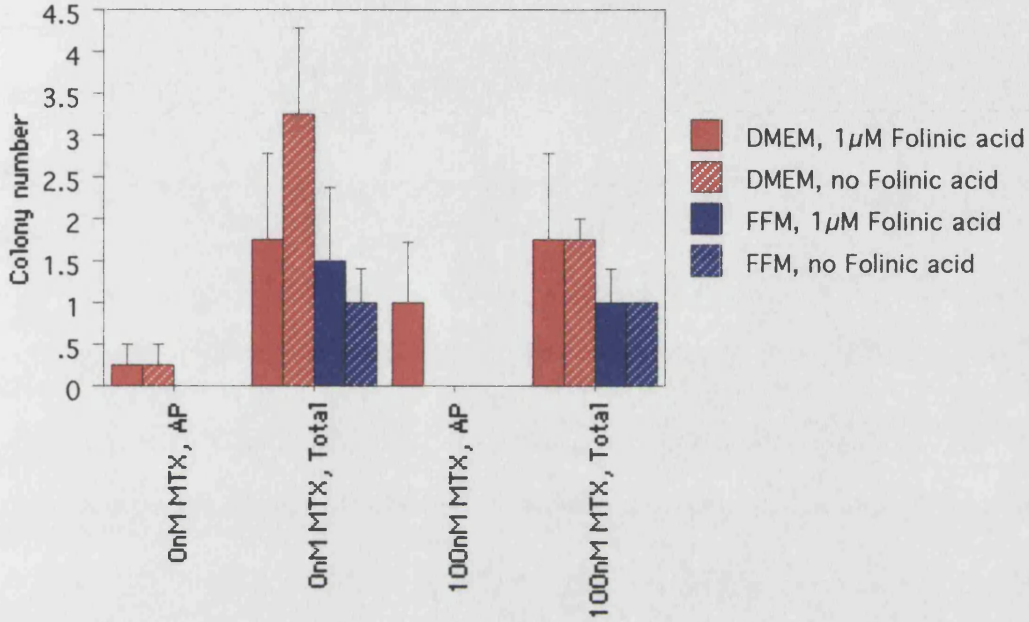
The final colony formation experiment was done using folinic acid 1μM as a supplement to the media instead of folic acid. Otherwise the experimental design was the same as previously and the results are illustrated in Figure 5.15 for cultures in the absence of DX and Figure 5.16 for colony development in the presence of 10nM DX. Mean colony number for the different conditions is shown in Table 5.4. There were few AP-positive colonies with none developing at all in the FFM in the presence of DX. When the total colonies were counted, it was found that the addition of 1μM folinic acid had in fact inhibited their formation in the FFM groups in the absence of DX (Figure 5.13). This result is interesting as folinic acid is the compound used to rescue cells from the effects of MTX toxicity as it is down-stream from folic acid in folate metabolism. The effect of adding folinic acid at this concentration did not have a significant effect on colony formation, either AP-positive or total, in the presence or absence of DX.

Figure 5.15 Total and AP-positive colony formation in folate-free medium and with or without folinic acid with no dexamethasone



Colonies stained with Fast Red and Methylene Blue after 18 days in culture.
Results shown as mean colony number ± standard error. Analysis by ANOVA with Fisher's Protected Least Significant Difference post hoc testing, n = 4.

Figure 5.16 Total and AP-positive colony formation in folate-free medium and with or without folinic acid and with 10nM dexamethasone



Colonies stained with Fast Red and Methylene Blue after 18 days in culture.
Results shown as mean colony number ± standard error. Analysis by ANOVA with Fisher's Protected Least Significant Difference post hoc testing, n =4.

Table 5.4 Mean colony number for experiment shown in Figures 5.15 and 5.16

0 or 10nM DX	0 or 100nM MTX	Total/ AP colonies	DMEM + 1μM Folinic acid	DMEM	FFM + 1μM Folinic acid	FFM
0	0	Total	2.75	3.25	1	2.25
0	100	Total	3.25	3.25	0.25	2
0	0	AP	0	0	0	0
0	100	AP	0	0.25	0	0
10	0	Total	1.75	3.25	1.5	1
10	100	Total	1.75	1.75	1	1
10	0	AP	0.25	0.25	0	0
10	100	AP	1	0	0	0

5.5.4 Summary of Colony Formation Experiments and Future Work

Compared to other workers in this field, generally low colony numbers were observed in this series of experiments. This may be related to the source of bone or plating density. It has been reported that in lung cancer there is reduced CFU-F formation (Chasseing et al, 1997). Several lung cancers, particularly squamous cell carcinomas, have associated non-metastatic features which are endocrine. Parathormone-related peptide (PTH-rP) was first described in patients with lung cancer but many other hormones with effects on bone may be synthesised by malignant cells. Other groups use higher plating densities with the development of higher colony numbers (Scutt et al, 1995). The low colony counts, particularly of AP positive colonies even in the control cultures makes it difficult to draw conclusions about the effect of MTX in this system. Additionally, MTX had differing effects between donors with an effect seen predominately either on total colony numbers or on osteoblast-like differentiation. Folate was shown to have an important role in colony formation as its removal lead to a dramatic reduction in colony number. Partial replacement of folate with either folate or folinic acid did not restore colony numbers to those seen with DMEM and paradoxically had an inhibitory effect under some conditions.

The size of individual colonies was not measured in this series of experiments. MTX may have no consistent effect on colony number (which reflects the transition from a non-adherent to an adherent state) but may effect colony size, which reflects proliferative potential.

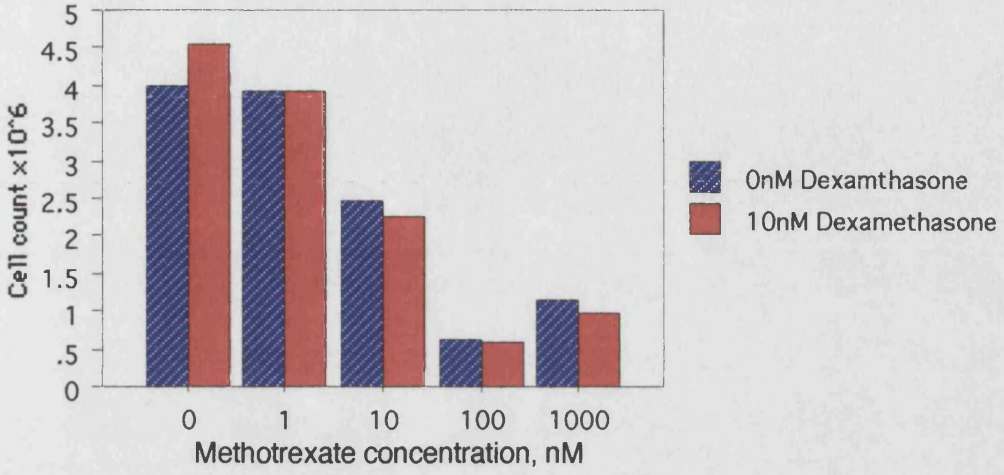
In conclusion, MTX affected colony formation but not in a consistent way in marrow from these donors. Future investigations would aim to confirm and extend the above

findings, using bone marrow from donors without malignancy, using different plating densities, assessing colony size and performing a larger number of experiments to try and overcome the problem of donor variation in this system.

5.6 Effect of Methotrexate on the Proliferation of Normal Human Marrow Stromal Cells

Primary human marrow-derived bone cells were cultured in DMEM with additions as in Chapter 4 appendix, 100μM Asc-2-P, 15% FCS and with or without 10nM DX. After one week in culture, the cell layer was washed gently once with PBS and the media replaced with varying concentrations of MTX (0, 1, 10, 100 or 1000nM).
The cells were cultured to confluence (forty-four or sixty-eight days in culture) and were passaged for labelling prior to flow cytometry (section 5.7 below).
Proliferation was assessed by cell number determined by Coulter counter. Cells from each experimental condition were pooled and counted twice. The results are shown in Figure 5.17, with cell counts corrected for one $75cm^2$ flask. Doses of MTX of 10nM and above inhibited proliferation with maximum inhibition occurring at 100nM. There was a trend towards greater inhibition in the cells cultured in the presence of 10nM DX.

Figure 5.17 Cell counts of normal human bone marrow cells cultured in the presence of methotrexate



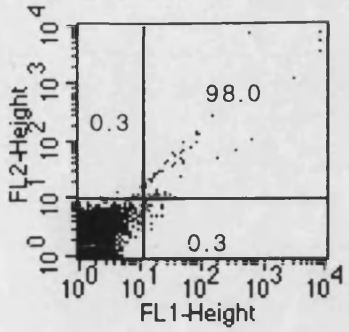
Cells were pooled from each condition prior to counting by Coulter counter.
There were no significant differences between cells grown in the presence or absence of 10nM Dexamethasone by paired t-test, n = 2.

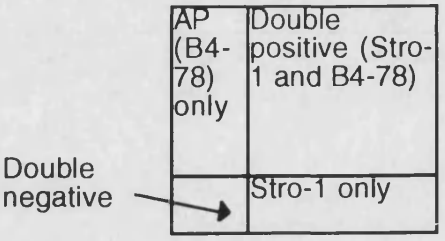
5.7 Effect of Methotrexate on the Surface Expression of Stro-1 and Alkaline Phosphatase in Normal Human Marrow-Derived Bone Cells Assessed by Flow Cytometry

The full method for this series of three experiments is given in Section 4.11. Cells were dual-labelled with the monoclonal antibodies B4-78 and Stro-1 at the end of primary or secondary culture. B4-78 recognises bone, liver and kidney isoform of alkaline phosphatase and is therefore a marker for committed osteoblasts (Lawson et al, 1985). Stro-1 is a marker of less well differentiated cells and is thought to identify marrow stromal cells which have the potential to become osteoblasts (Gronthos et al, 1994). After labelling, the cells were fixed and analysed by flow cytometry the following day. They were sorted into four populations, depending on their labelling. The distribution of the cells between the four populations varied according to the culture conditions and representative dot plots are shown in Figure 5.18.

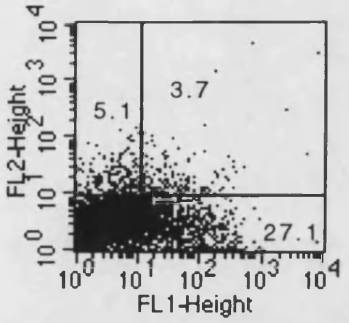
Figure 5.18 Representative dot plots from flow cytometry

(A) Control. Cells incubated with the irrelevant second antibody only.

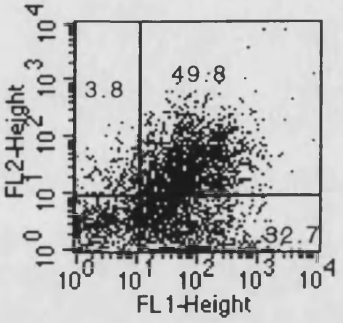




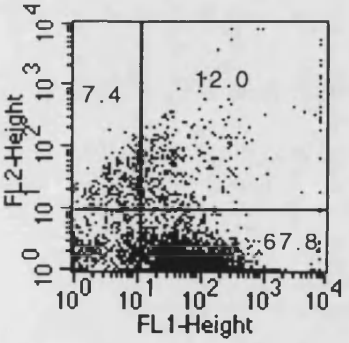
(B) Dual-labelled cells: no MTX, no DX.



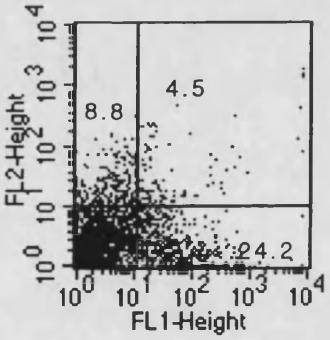
(C) Dual-labelled cells: 100nM MTX, no DX.



(D) Dual-labelled cells: no MTX, 10nM DX.



(E) Dual-labelled cells: 100nM MTX, 10nM DX.



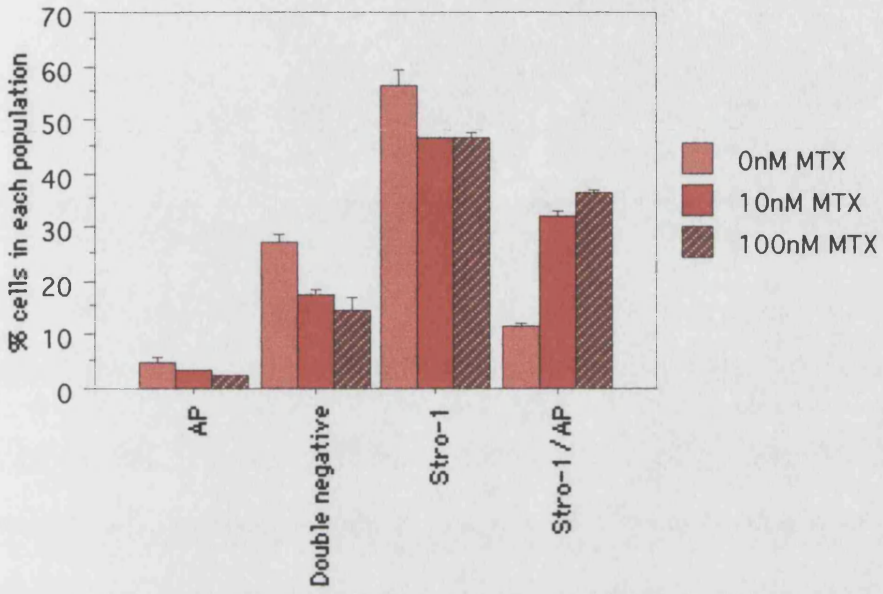
Cells were dual-labelled with B4-78 and Stro-1 monoclonal antibodies and sorted by flow cytometry into four populations. The percentage of cell events in each population is indicated in the quadrants of the figures. DX is Dexamethasone.

Population shifts were observed according to the experimental conditions. The presence of DX was associated with a larger percentage of cells having a more mature osteoblastic phenotype, that is, more cells in the AP-positive (upper left quadrant in Figure 5.18) and dual-positive (AP- and Stro-1-positive, upper right quadrant in Figure 5.18) populations, as previously described (Stewart et al, 1996). Results from the three different experiments are shown in Figures 5.19 to 5.24. Each result is the mean and standard deviation of two aliquots of cells which were labelled separately.

Figures 5.19 and 5.20 show the results from the first experiment with no DX and 10nM DX respectively. MTX at doses of 10nM and 100nM was used. MTX at 10nM in the absence of DX (Figure 5.19) increased the proportion of cells expressing both Stro-1 and AP, whilst decreasing the other three populations. The same dose in the presence of DX (Figure 5.20) also increased the dual-labelled population (AP- and Stro-1 positive) whilst having little effect on Stro-1-positive cells.

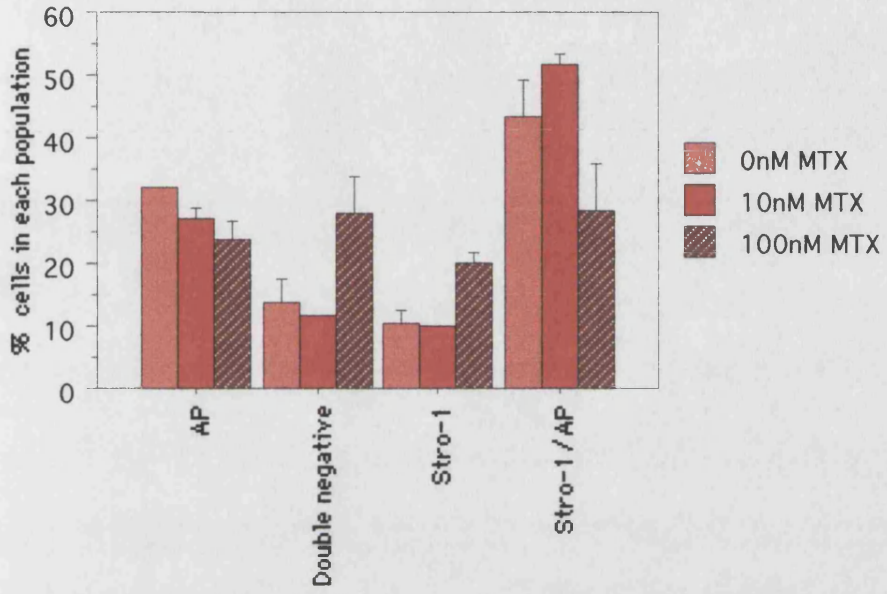
The effect of 100nM MTX was not noticeably different from that of 10nM MTX in the absence of DX, whilst in the presence of 10nM DX, it increased the proportion of double negative (neither AP nor Stro-1 labelled) cells and reduced the proportion of dual-labelled (Stro-1 and AP) cells.

Figure 5.19 Flow cytometry of dual-labelled human bone marrow cells cultured in the presence of methotrexate and no dexamethasone (a)



Cells were labelled with B4-78 (AP) and Stro-1 monoclonal antibodies. Results are shown as the mean percentage and standard deviation of two samples of cells for each sorted population.

Figure 5.20 Flow cytometry of dual-labelled human bone marrow cells cultured in the presence of methotrexate and 10nM dexamethasone (a)



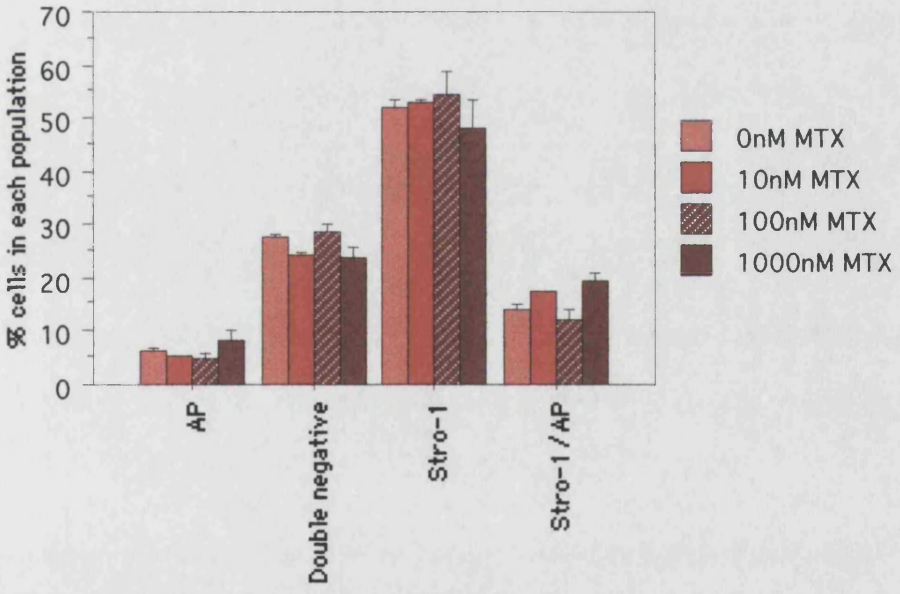
Cells were labelled with B4-78 (AP) and Stro-1 monoclonal antibodies. Results are shown as the mean percentage and standard deviation of two samples of cells for each sorted population.

Figures 5.21 and 5.22 show the results from a second experiment, in the absence of DX in Figure 5.21 and with 10nM DX in Figure 5.22. The experiment was planned to include MTX at the dose 1nM and 1000nM in addition to the 10nM and 100nM previously used. Unfortunately, the 1nM MTX with no DX cultures became infected but all the other conditions were available for analysis.

In the cultures grown in the absence of DX (Figure 5.21), 1000nM MTX increased the proportion of cells in the committed osteoblast populations (AP-positive and

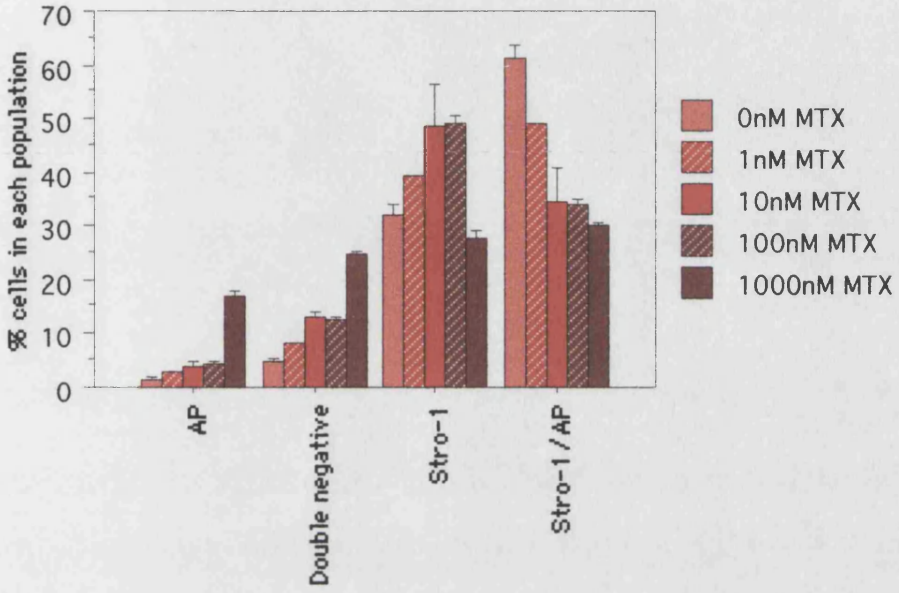
dual-positive cells). In the presence of DX (Figure 5.22), 1000nM MTX increased the proportion of cells in the AP-positive and double negative populations. The dual-positive (AP- and Stro-1-positive) cells were decreased in a dose-dependent way by MTX. In both experiments, 1000nM MTX had similar effects on both the dual positive and dual-negative cell populations (Figures 5.21 and 5.22).

Figure 5.21 Flow cytometry of dual-labelled human bone marrow cells cultured in the presence of methotrexate and no dexamethasone (b)



Cells were labelled with B4-78 (AP) and Stro-1 monoclonal antibodies. Results are shown as the mean percentage and standard deviation of two samples of cells for each sorted population.

Figure 5.22 Flow cytometry of human bone marrow cells cultured in the presence of methotrexate and 10nM dexamethasone (b)

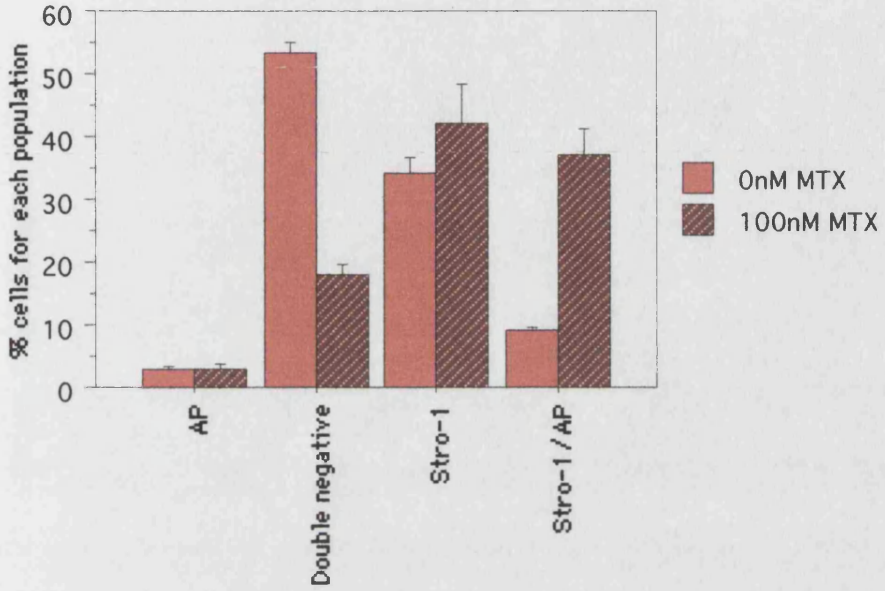


Cells were labelled with B4-78 (AP) and Stro-1 monoclonal antibodies. Results are shown as the mean percentage and standard deviation of two samples of cells for each sorted population.

The final flow cytometry experiment used a single dose of MTX, 100nM. The results without DX are shown in Figure 5.24 and those with 10nM DX in Figure 5.25.

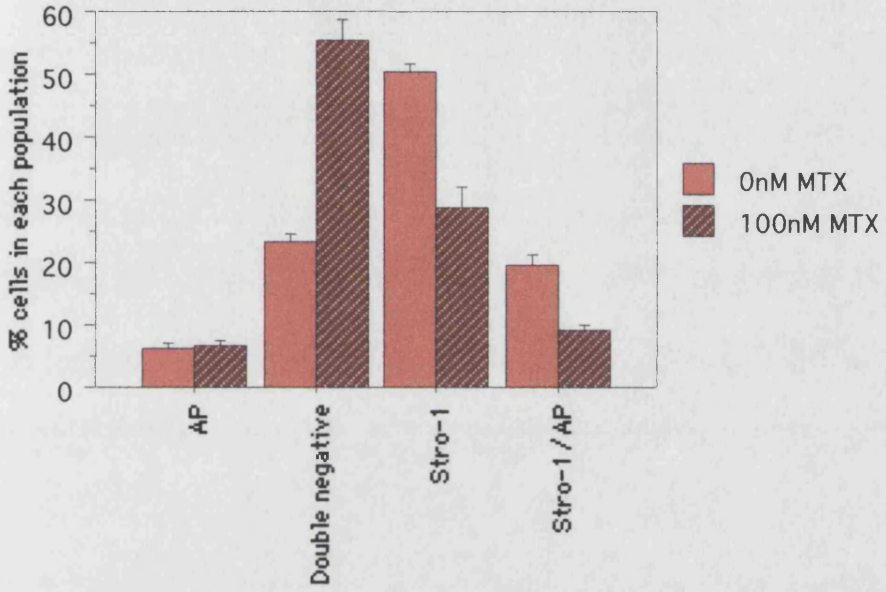
The results were similar to those obtained in previous experiments as 100nM MTX in the absence of DX (Figure 5.24) decreased the double-negative population whilst increasing the double-positive population of cells. In the presence of DX, the opposite effect was seen and additionally, the proportion of Stro-1 positive cells was also reduced.

Figure 5.23 Flow cytometry of human bone marrow cells cultured in the presence of 100nM methotrexate and no dexamethasone



Cells were labelled with B4-78 (AP) and Stro-1 monoclonal antibodies. Results are shown as the mean percentage and standard deviation of two samples of cells for each sorted population.

Figure 5.24 Flow cytometry of dual-labelled human bone marrow cells cultured in the presence of 100nM methotrexate and 10nM dexamethasone



Cells were labelled with B4-78 (AP) and Stro-1 monoclonal antibodies. Results are shown as the mean percentage and standard deviation of two samples of cells for each sorted population.

5.7.1 Summary of Flow Cytometry Experiments and Further Work.

These experiments demonstrated that MTX pushed osteoblast-like cells further down the path of osteoblast commitment, or increased their degree of differentiation. The effect is greater in the presence of DX, which is a recognized enhancer of osteoblast differentiation. This may be an effect secondary to the inhibitory effect on cell proliferation demonstrated in the experiments above (Sections 5.5 and 5.6).

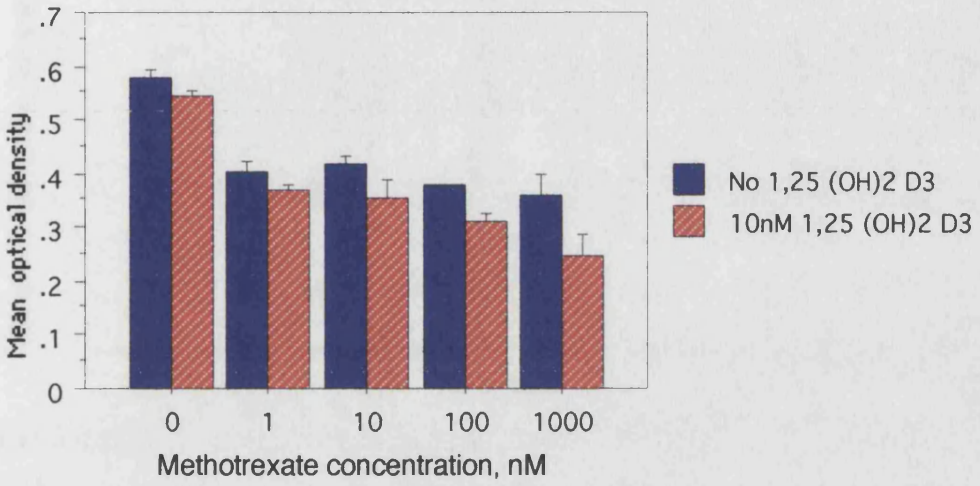
(Sections 5.5 and 5.6). Further work would confirm and extend the above results. The sorted populations of cells could be cultured and subjected to further flow cytometry to investigate whether the phenotypes are stable.

5.8 Effect of Methotrexate on the Proliferation and Differentiation of Passaged Trabecular-derived Osteoblast-like Bone Cells and the Influence of Dexamethasone and 1,25 $(OH)_2$ Vitamin D3

In this experiment, osteoblast-like bone cells were cultured from chips of trabecular bone as described in Section 4.5. When confluent, the cells were passaged, counted and established in secondary culture, in twenty-four well plates in a variety of experimental conditions. If cells had been cultured in the presence or absence of 10nM DX during primary culture, this was continued. MTX in a range of doses (1 to 1000nM) was added to the culture medium during the week of secondary culture. In some experiments, 1, 25 $(OH)_2$ Vitamin D_3 (calcitriol) was added to the cultures for the final forty-eight hours. Calcitriol, like DX, tends to enhance the differentiation of osteoblasts at the expense of proliferation. Proliferation was assessed by elution of Methylene Blue and spectometry at 650nm. This has been shown to correlate well with proliferation assessed by different means. Thymidine uptake was not used as this would be unreliable in the presence of MTX (Section 5.9).

Figure 5.25 shows the dose-dependent inhibition of proliferation by MTX and the additive effect of calcitriol.

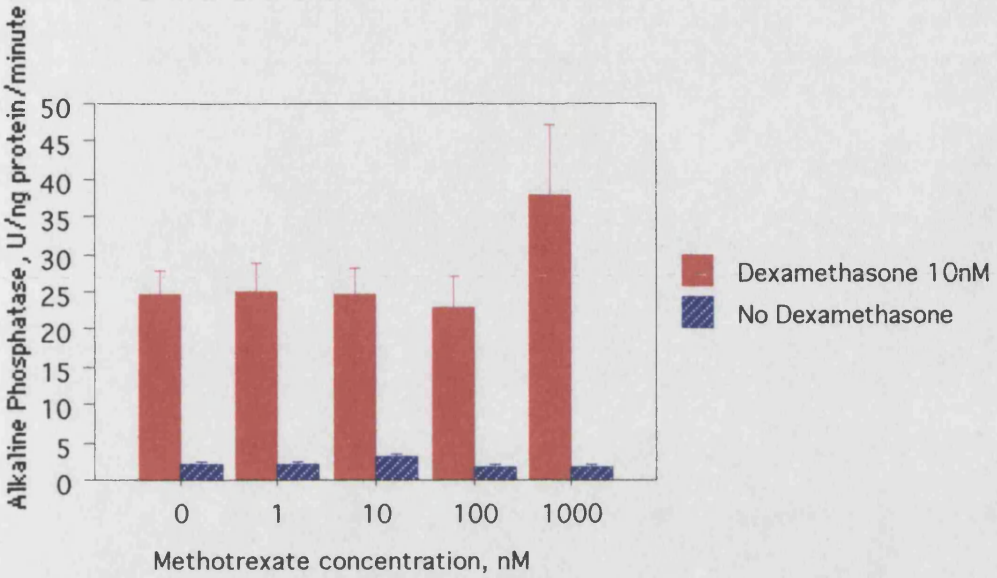
Figure 5.25 Proliferation of trabecular-derived osteoblast-like bone cells in secondary culture in the presence of methotrexate and vitamin D_3



After 7 days in secondary culture, the cell layer was stained with Methylene Blue and the elute read at 650nm. Results are shown as the mean optical density ± standard error (n = 4).

Alkaline phosphatase activity in the cell layer and supernatant was measured using a Sigma assay (Section 4.10). Figure 5.26 shows that the level of AP activity was much greater in the presence of DX and was greatest when 1000nM MTX was present. This result was in keeping with the action of DX as a promoter of differentiation to the osteoblast phenotype.

Figure 5.26 Alkaline phosphatase activity of trabecular-derived osteoblast-like cells in secondary culture



Alkaline phosphatase activity was determined on day 8 of secondary culture. MTX was added on day 3 and calcitriol on day 6. Results are shown as the mean ± standard error, n = 4.

5.8.1 Summary of Secondary Trabecular Experiments and Further Work

Further work would aim to confirm and extend the above findings. It would be informative to analyse the supernatant for osteoblast specific products such as osteocalcin and to study the synthesis of collagen in the presence and absence of MTX.

5.9 Summary of the Bone Cell Culture Experiments

The series of experiments described in this chapter were designed to investigate the effect of MTX on cells of the osteoblast lineage in culture. The techniques of

bone cell culture developed by Dr JN Beresford's group were utilised. Thus, marrow-derived cells were cultured in conditions to promote the development of osteoblast-like cells (expressing alkaline phosphatase), and the cells which grew out from trabecular bone chips were studied in secondary culture. In the time available it was not possible to study in depth the possible mechanisms of the observed effects, by for example, expression of mRNA (messenger ribonucleic acid) of osteoblast products. Rather, the differences in expression of alkaline phosphatase (AP) and the Stro-1 surface marker were explored in different culture conditions. MTX had a generally inhibitory effect of proliferation, as assessed by colony number (Section 5.5) and cell counts (Section 5.6). Proliferation was not estimated by incorporation of radiolabelled nucleotides or precursors such as tritiated thymidine, as one of the main actions of MTX is inhibition of thymidylate synthetase and counts would have been unreliable. MTX also affected differentiation, as the colony-formation and flow cytometry experiments (Section 5.7) demonstrated. However, there were large differences in proliferation and colony-formation rates observed between donors. These differences were sometimes greater than the treatment effects of MTX. Overall, whilst MTX consistently inhibits proliferation irrespective of the cells' origin (marrow or bone surface), there were no consistent effects on colony formation (Section 5.5) or differentiation (Section 5.8) and MTX had inhibitory rather than toxic effects on the cells cultured using these systems.

6. BONE HISTOMORPHOMETRY OF METHOTREXATE-TREATED RHEUMATOID ARTHRITIS

6.1 Introduction

6.1.1 Bone Histomorphometry

Histology is the study of a tissue by microscopic examination of thin sections which have been mounted on slides. The sections can be stained to highlight features of interest and provide qualitative information. Histomorphometry is a histological technique in which numerical values are assigned to various elements of a tissue. This enables quantitative differences between treatment groups or disease states to be measured. Inferences about underlying pathophysiological processes may then be drawn. Over the last twenty years histomorphometric techniques have been developed and applied to bone (Compston et al, 1991).

The first site to be studied was rib (Frost, 1969) but the optimum site for biopsy is the iliac crest, just behind the anterior superior iliac spine. This site has several advantages (Recker, 1994). It is easily accessible and the procedure is safe and relatively easy to perform with little patient morbidity. A full thickness biopsy can be obtained with both the inner and outer cortex and the tissue of interest, the cancellous bone, is present in abundance.

Examination of undecalcified bone sections which have been appropriately stained (Section 6.4) allows measurement of structures such as cancellous area, percentage osteoid extent and resorption cavities (Eriksen et al, 1994). These measurements are termed static and are listed together with normal values obtained from British subjects in Table 6.1 (Recker, 1994; Vedi, 1986; Vedi et al, 1982; Garrahan et al, 1990).

Table 6.1 Static measurements

Measurement	Units	Values in Normal Subjects
Cancellous bone area (B.Ar)	% (of tissue area)	*27.7; 29.6; 29.6
Osteoid perimeter (OP)	% (of bone perimeter)	*17.7; 14.5; 21.2
Osteoid width (OW)	μm	**7.70
Mean wall width (W.Wi)	μm	***62.0; 50.4; 40.0
Resorption cavity maximum depth	μm	^28.9
Resorption cavity mean depth	μm	^15.6
Resorption cavity area	μm^2	^4752
Reconstructed cavity length	μm	^229.5

* Values from Vedi, S. et al 1982. Age groups: 31 to 40 years (n = 6); 41 to 50 years (n = 3); 51 to 60 years (n = 6).

** Value from Vedi, S. 1986 (Thesis). Value from 57 normal subjects, 24 males and 33 females aged 19 to 80 years old.

*** Values from Croucher, P.I. et al 1989. Age groups: 19 to 40 years (n = 11); 41 to 60 years (n = 13); 61 to 81 years (n = 4).

^ Values from Garrahan, N.J. et al 1990 (13 normal subjects; 8 female and 5 male).

Additional information can be obtained if the subject has taken a compound which labels bone prior to the biopsy. The property of tetracycline antibiotics to bind avidly to bone surfaces undergoing mineralisation can be exploited to enable dynamic measurements to be made (Eriksen et al, 1994). In this study, two time-spaced doses of demethylchlortetracycline were taken by the subjects prior to the biopsies (Section 6.3). When unstained sections are examined under ultra-violet light, tetracyclines fluoresce and identify actively mineralising surfaces. The dynamic measurements of mineral apposition rate and mineralising perimeter were

measured and normal values obtained from British subjects are shown in Table 6.2 (Vedi et al, 1982; Compston et al, 1994).

Table 6.2 Dynamic measurements

Measurement	Units	Values in Normal Subjects
Mineral apposition rate (MAR)	μm/day	*0.60; 0.61; 0.61
Mineralising perimeter (MP)	% (of bone perimeter)	**10.8

* Values from Vedi, S. et al 1982. Female values only used. Age groups: 31 to 40 years (n = 6); 41 to 50 years (n = 3); 51 to 60 years (n = 6).

** Values from Compston, J.E. et al 1994. Mean value from 40 healthy controls, 17 men aged 38 to 74 and 23 women aged 32 to 80 years old.

6.1.2 Histomorphometric Considerations of the Remodelling Cycle

The bone remodelling cycle has been described in section 1.1.11. The realisation that bone cells exist in teams and co-ordinate their activities of resorption or synthesis came from Frost's study of tetracycline incorporation into bone (Frost, 1969). Histomorphometry allows study of these remodelling events at the bone multicellular unit (BMU) level. Resorption cavity, osteoid seam and mineralisation parameters can all be studied independently, or measures can be combined into derived indices of bone formation (Section 6.7.3).

6.1.3 Histomorphometry in Rheumatoid Arthritis

Studies by Compston's group in non-steroid treated RA subjects have shown that male and females less than fifty years old have reduced trabecular plate thickness

compared with controls consistent with premature bone loss (Mellish et al, 1987). The mean wall thickness of completed packets of trabecular bone was significantly reduced compared with controls, again in both sexes (Compston et al, 1989). These results are consistent with reduced bone formation.

A further study found low bone turnover as shown by reduced eroded perimeter, tissue based bone formation rate and activation frequency. Mean and maximum eroded depth and cavity area were also reduced when compared with controls (Compston et al, 1994), suggesting a reduction in osteoclast activity. It was concluded that the reduced bone mass in RA is due to a negative remodelling balance.

6.2 Subjects Who Underwent Bone Biopsy

All the women recruited to the study (Section 2.2.1) with recent onset rheumatoid arthritis (within two years) who were commencing Methotrexate (MTX) were asked if they would be willing to undergo bone biopsy but most refused. Reasons for refusal included unwillingness to stay in hospital overnight and work or child-care commitments. However, eight did agree to have the biopsy in the first year, but two of these were unsuccessful (no bone was obtained) and they did not undergo a second biopsy one year later. All six women attended for a second biopsy and one of these was unsuccessful. Thus, five paired biopsies were available for analysis. However, on sectioning one of the biopsies it was found to contain only fat and no bone. Furthermore, one of the biopsies fractured during the biopsy procedure and so structural analysis (Section 6.5.9) was not possible. Structural analysis was also not possible on a biopsy which was very oblique with a semi-circular rim of cortical

bone. The characteristics of the subjects who provided the four paired biopsies which were available for analysis are given in Table 6.3.

Table 6.3 Characteristics of the donors

Subject	Sex	Age (years)	Menopausal status	Previous fragility fracture	Duration of rheumatoid arthritis (in months)	Dose of MTX at the time of the second biopsy (mg per week)
1	Female	38	Pre-menopausal	No	3	10
2	Female	51	Peri-menopausal	Wrist fracture 3 years ago	3	12.5
3	Female	48	Peri-menopausal	No	11	7.5
4	Female	40	Pre-menopausal	No	8	15

MTX is Methotrexate

6.3 Biopsy Technique and Preparation

The subjects were given information sheets about the bone biopsy procedure and the tetracycline administration. They gave informed consent for each biopsy and the study was approved by the local ethics committee. Prior to each biopsy, the bone was labelled with demethylchlortetracycline, 300mg twice daily in the following schedule: the label was taken on days one, two, thirteen and fourteen with the biopsy obtained three to five days after the last dose. Subjects were asked to avoid dairy products for two hours before and two hours after the tetracycline doses to optimise absorption. Full thickness transiliac crest bone biopsies were taken with a

Bordier trephine (8mm French Gauge internal diameter) from two centimetres below and behind the anterior superior iliac spine. The first biopsy was from the right iliac crest and the second from the left side after a year of MTX therapy. The procedure was done under local anaesthetic with mild intravenous sedation with midazolam. The patients were observed in hospital overnight following the biopsy. The biopsies were placed in 70% alcohol for at least two days and then dehydrated by using 80%, 90% and finally 100% alcohol in turn, with a minimum period of forty-eight hours at each concentration. During dehydration the biopsies were gently agitated. The biopsies were sent to Dr Juliet Compston's Laboratory in Addenbrooke's Hospital, Cambridge, where they were embedded in LR White Resin (London Resin Company, UK) by Dr Shobna Vedi and stored in the dark until processing. Sections of 8μm or 15μm thickness were cut on a microtome (Bright Instrument Company Ltd, Huntingdon, UK).

6.4 Slide Preparation for Histomorphometric Analysis

6.4.1 Toluidine Blue Stain

The stain was prepared from 20 mg Toluidine Blue in 10 ml McIlvan Buffer.

McIlvan Buffer was 25 mls Solution A and 75 mls Solution B, pH 4.2, where Solution A was 28.3g disodium hydrogen orthophosphate in 1 litre distilled water and Solution B was 21g Citric acid in 1 litre distilled water.

Sections of the bone biopsy cut at 8μm were free-floated in filtered Toluidine Blue stain for ninety seconds. The sections were then washed twice for five minutes in distilled water before placing on slides (SuperFrost, BDH Laboratory Supplies, Poole, Dorset, UK) and blotted dry. Stained sections were mounted using Inhibisol

(TAAB Laboratories Equipment Ltd, Reading, Berks., UK) and DPX mounting medium (AGAR Scientific Ltd, Stansted, Essex, UK).

6.4.2 Von Kossa Stain

Sections cut at 8μm were free-floated in 1.25% Silver Nitrate for two to three hours in bright light until they went black. They were washed twice in distilled water and then fixed in 10% mono-sodium thiosulphate for two minutes. They were washed twice again in distilled water before being counter-stained with filtered van Gieson stain (20ml Acid Fucin in 80ml Picric acid) for twenty minutes. After two more washes in distilled water, the sections were mounted on slides using Inhibisol and DPX.

6.4.3 Tetracycline Label

Unstained 15μm sections of bone were mounted on slides using Inhibisol and Xam neutral medium (BDH Laboratory Supplies, Poole, Dorset, UK).

6.5 Histomorphometric Measurements

6.5.1 Introduction

The measurements were made in Dr Juliet Compston's laboratory at Addenbrooke's Hospital, Cambridge. All measurements apart from the structural analysis (Section 6.5.9) were made using a "Digicad" digitizing tablet and cursor with an LED point light source (Kontron Limited) and an Olympus BHS-BH2 binocular light microscope with a BH2-DA drawing attachment (Olympus Optical Company, London, UK).

6.5.2 Cancellous Bone Area

This was derived from measurement of the bone area and bone perimeter and expressed as a percentage of total area of cancellous bone. Using a semi-automated technique, three non-contiguous Von Kossa stained sections per biopsy were analysed at an objective magnification of x4 (final magnification of x100, Figure 6.1). The eyepiece was mapped and the lens calibrated at the start of each measuring session. The cancellous surface of the bone was traced using a computer mouse on a viewing pad. Then any holes present in the cancellous structure were defined. Finally the length of any osteoid seam was recorded before moving onto the next field. The number of fields in a section depended on the size of the biopsy and all the cancellous bone was measured. Care was taken not to include any cortical bone by starting measurements at a fixed distance away from the cortical edge, utilising the markings on the eyepiece. Similarly, the extreme edges of the biopsy, where there was usually some bone dust, were not included in measurements.

The bone area for a given section was given by the formula:

Total field area = a x n

True bone area = b - h

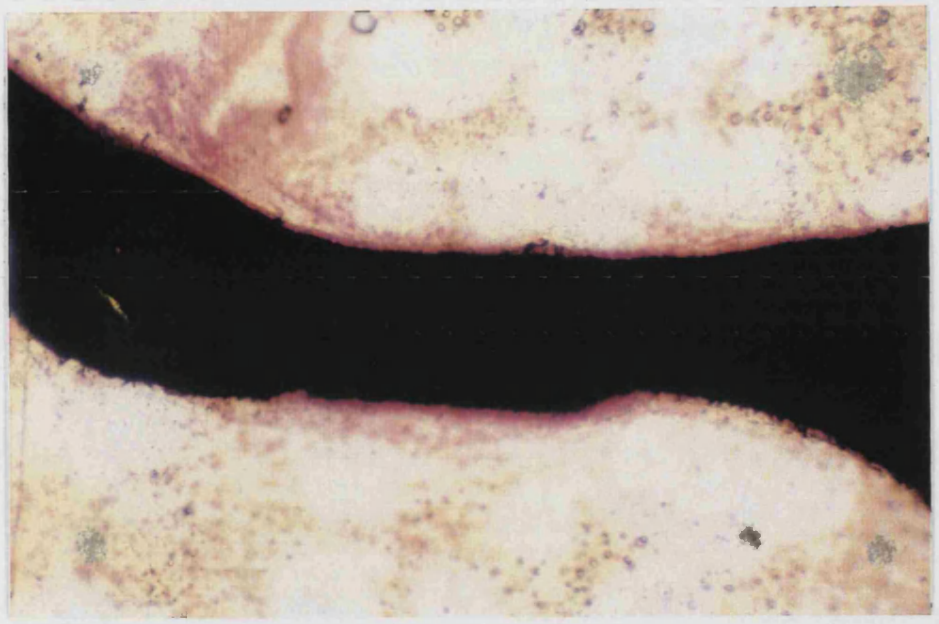
Mineralised bone area (%) = (true bone area) / (total field area) x 100%

$$= (b - h) / (a \times n) \times 100\%,$$

where a was the field area, b the bone area, h the hole area and n was the number of fields.

The mean cancellous bone area of the biopsy was calculated by averaging the results from the three sections measured.

Figure 6.1 Section of cancellous bone demonstrating cancellous bone area and osteoid perimeter



Von Kossa stain with van Gieson counterstain, magnification x 100

6.5.3 Osteoid Perimeter

This was measured at the same time as the cancellous bone area above (Section 6.5.2) and reflects bone turnover. The osteoid perimeter (OP) was expressed as a percentage of the total cancellous mineralised bone perimeter and was given by the formula:

OP (%) = (ol) / (bl+hl) x 100%,

where ol was the osteoid length traced out, bl was the length of the cancellous bone perimeter and hl was the length of the holes. The mean osteoid perimeter was calculated by averaging the results from the three sections measured.

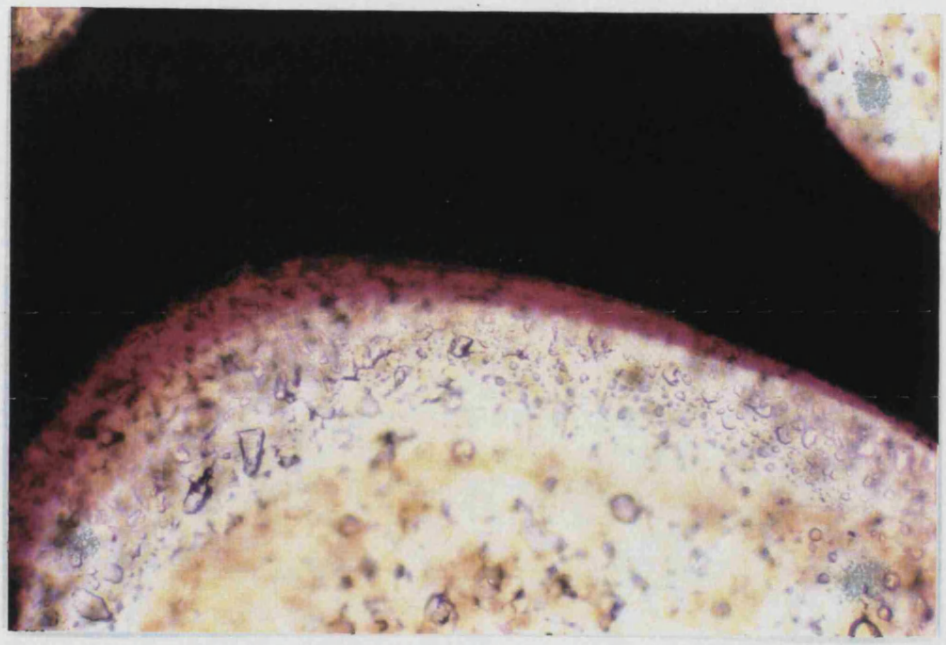
6.5.4 Osteoid Width

This measurement was also done on Von Kossa stained sections, but at objective magnification x20 (final magnification x500, Figure 6.2). The lens was appropriately calibrated and the eyepiece mapped. Osteoid seams were identified by scanning the section with the x10 objective lens, moving to the x20 for measurements. Points on opposing margins of the osteoid seam were marked, at four equidistant points. If a seam was less than 3μm wide it was not included as it was considered to be endosteal membrane rather than osteoid. Every osteoid seam in three sections from each biopsy was measured. If a seam was very long or very short, the number of measurements made was increased or decreased accordingly. The osteoid width (OW) for a section was given by the formula:

OW (μm) = (Σ distance recorded) / (Σ number of measurements made).

The mean osteoid width for the biopsy was calculated from the average for the three sections.

Figure 6.2 Section of cancellous bone demonstrating an osteoid seam



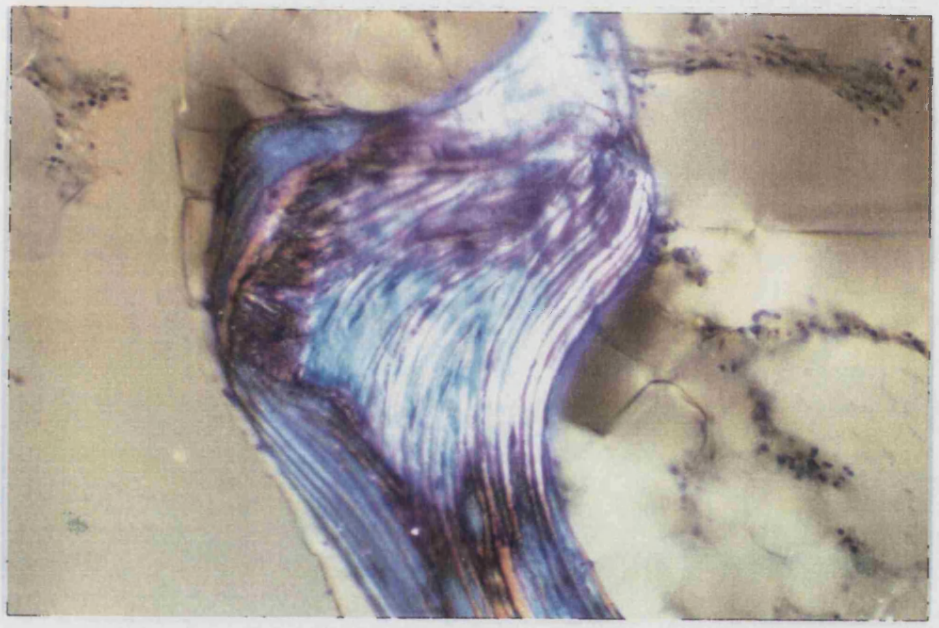
Von Kossa stain with van Gieson counterstain, magnification x 500

6.5.5 Mean Wall Width

Completed cancellous wall (or packet) width was measured using Toluidine Blue stained sections, a polarising lens and the x10 objective magnification (final magnification x250). The lens was appropriately calibrated and the eyepiece mapped. Completed packets were identified by the alignment of collagen fibres within the cancellous bone under polarised view (Figure 6.3). Non-polarised light was used to ensure that active formation was not continuing on the outside of the packet and to identify the cement line on the inner surface of the packet (Figure 6.4). The endosteal surface and then the cement line were traced using the computer mouse. The packet width (the distance between the two lines drawn) was automatically measured in μm at four equidistant points along the packet. For each

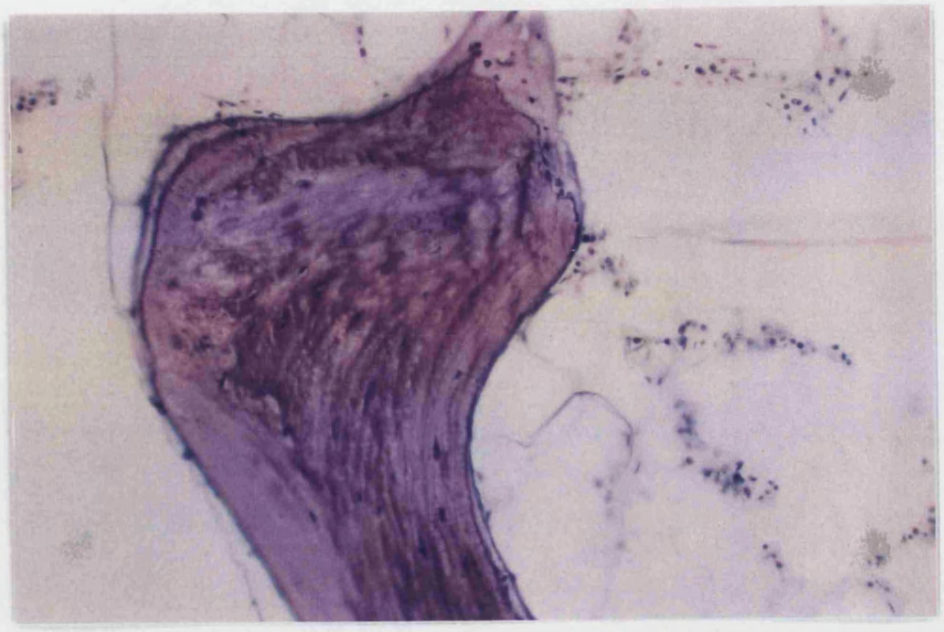
biopsy, three sections were examined and at least ten packets for each section. The mean packet width for each biopsy was calculated.

Figure 6.3 Cancellous bone section demonstrating a completed bone packet viewed under polarised light



Toluidine Blue stain, polarised light, magnification x 250

Figure 6.4 Cancellous bone section demonstrating a completed bone packet viewed under transmitted light



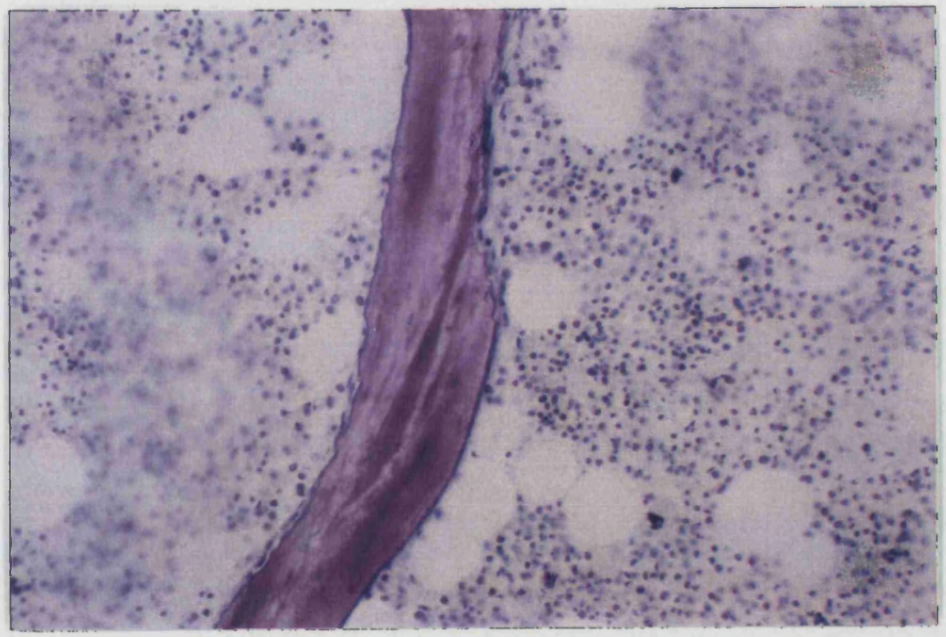
Toluidine Blue stain, normal transmitted light, magnification x 250

6.5.6 Resorption Cavities

Resorption cavities were measured using Toluidine Blue stained sections, a polarising lens and magnification of x10 or x20 (objective). The computerised technique used to measure resorption cavities was developed by Dr Compston's group (Garrahan et al, 1990). The section was viewed with unpolarised light to identify a suitable resorption cavity for measurement (Figure 6.5). It was ensured that the resorption cavity was not undergoing formation (no osteoid present) and under polarised light that ragged ends of resorbed collagen fibres were visible (Figure 6.6). The endosteal surface which included the resorption cavity was traced and the margins of the cavity identified. The endosteal surface on the opposite side

of the trabecular strut from the resorption cavity of interest was also recorded. The computer programme then fitted a cubic spline curve to the cavity and calculated the cavity depth (mean and maximum), cavity area and reconstructed the endosteal surface over the cavity (reconstructed length).

Figure 6.5 Cancellous bone section demonstrating a resorption cavity viewed under transmitted light



Toluidine Blue stain, transmitted light, magnification x 250

Figure 6.6 Cancellous bone section demonstrating a resorption cavity viewed under polarised light

Toluidine Blue stain, polarised light, magnification x 250

6.5.7 Mineral Apposition Rate

This dynamic measurement was done on unstained sections. An ultra-violet light source was used and the room was darkened to protect the fluorescent labels from deterioration and to enable their visualisation. Labels were identified using the x10 objective magnification but the measurements were done using the x20 objective. As before, the eyepiece was mapped and the lens calibrated prior to a measuring session.

A section was methodically scanned using the medium power lens (x10 objective) until a label was seen. At x20 objective magnification it was ascertained whether the

label was a double or single label as the measurement could only be done on double labels. Furthermore, it was decided whether the label related to the labelling period of interest. This was determined by the position of the label in the bone. If the label was in the centre of a trabecular strut rather than at the edge it was due to previous tetracycline exposure, either given deliberately to label the bone or therapeutically. If a label was judged to have been laid down in the past, it was not measured.

At x20 objective magnification, the inter-label distance was measured (Figure 6.7). Measurements were taken from the middle of the first label to the middle of the second label, perpendicularly opposite. If the label was blurred so that it was difficult to identify the middle of the label (due to the orientation of the section), no measurement was made. As for the osteoid seam width measurement (Section 6.5.4), four equidistant measurements were made for each label along its length (or more or fewer depending on double label length). All the double labels in a section were measured and the mean inter-label distance (ILD, μm) for the section was given by the following formula:

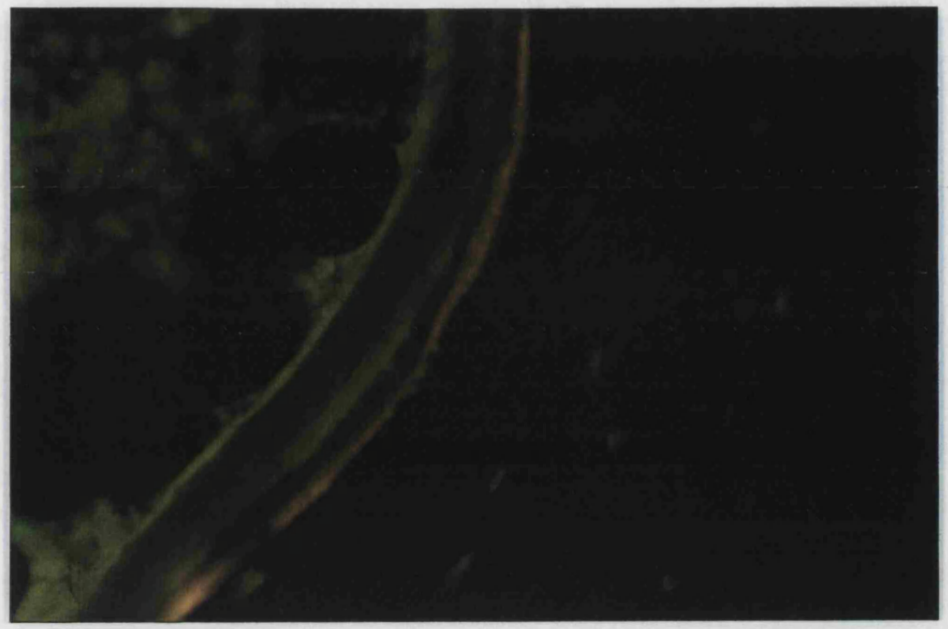
ILD = (Σ inter-label measurements) / (number of measurements).

From this the mineral apposition rate (MAR, μm/day) was calculated by dividing the mean inter-label distance by the number of days between the labels (twelve).

MAR = ILD / 12.

The mean MAR for the biopsy was calculated from at least three sections.

Figure 6.7 Cancellous bone section demonstrating a double tetracycline label viewed under ultraviolet light



Unstained 15μm section, ultraviolet light, magnification x 250

6.5.8 Mineralising Perimeter

This dynamic measurement assesses the percentage of the total cancellous bone perimeter undergoing active mineralisation during the labelling period. It is also known as the surface extent of tetracycline labelling.

The interpretation of tetracycline labelling can be difficult. Although two, time-spaced labels were given, often only a single label was present on a given trabecular strut. Occasionally, no label was visible, as occurred in the first biopsy

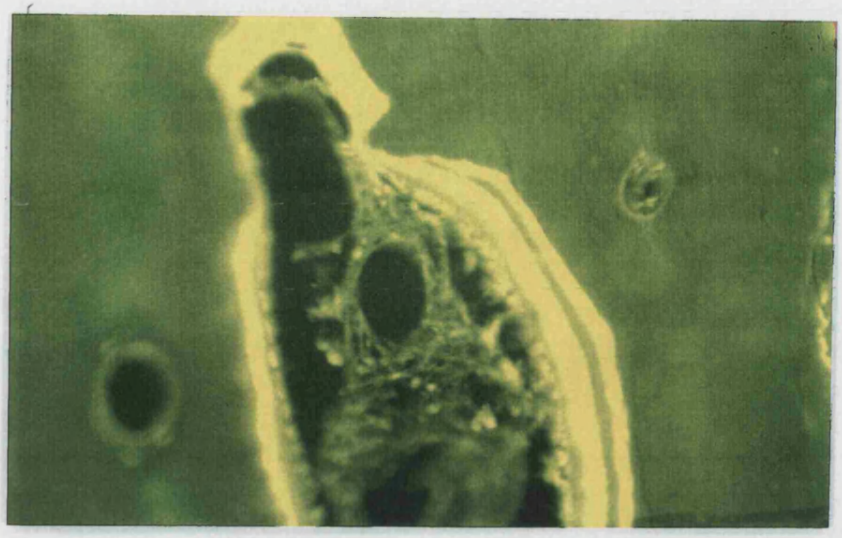
from subject 2 in this study (Section 6.7.2). In addition, fluorescence from previous tetracycline exposure was sometimes present (Section 6.5.7).

A single label may result from one of two possible mechanisms. Frost has described 'label escape' which occurs if formation is either initiated or terminated during the inter-label period (Frost, 1969). The alternative cause of a single label is if formation continues, but mineralisation ceases (or starts) during the inter-label period. When measuring the surface extent of tetracycline labelling, the single (sL) and double labels (dL) are recorded separately. The mineralising perimeter can be calculated in four different ways, all expressed as a percentage of the cancellous bone perimeter.

sL + dL	the sum of the single and double labelled surfaces
dL	the sum of the double labelled surface only
sL	the sum of the single labelled surface only
dL + (1/2 sL)	the sum of the double labelled and half of the single labelled surfaces

This last, dL + (1/2 sL) is the most commonly used and was used in this study. It recognises that of the single labelled surfaces, some will have started and some stopped undergoing mineralisation during the inter-label period. Single and double labels are illustrated in Figure 6.8.

Figure 6.8 Cancellous bone section demonstrating single and double tetracycline labels



Unstained 15μm section, ultraviolet light, magnification x 250

The measurements of the surface extent of tetracycline labelling were made on unstained sections, as for the mineral apposition rate (Section 6.5.7). The light source was ultra-violet light and the sections were not exposed to natural or artificial light. The x10 objective lens was calibrated and the eyepiece mapped as before. In each field, the bone perimeter (BP) was traced round, and then any double (dL) and single (sL) labels traced individually. The x20 lens was used to determine whether a label was single or double before it was measured. For each section, the mineralising perimeter (MP) was given by the following formula:

MP (%) = (dL + (1/2 sL)) / BP.

The values from the sections were averaged to give a measurement for each biopsy.

6.5.9 Strut (Pattern) Analysis

The structural or strut analysis measurements were made on a separate measuring system using a camera mounted on a light box. Toluidine Blue stained slides were used and an image of the entire section was captured on a compatible computer. The image was segmented into a binary image. This could then be edited by removing features such as bone dust or heavily stained resin which did not correspond to trabecular struts. After editing, the image was skeletonised automatically and trabecular junctions (nodes) and the ends of trabeculae (termini) identified. Analysis of three sections from each biopsy was done and the mean number of nodes, termini and the node to terminus ratio and other measures of interest were calculated for each biopsy.

6.6 Validation of Measurements

Before the study biopsies were measured, inter- and intra-observer variability were calculated for each measurement. Three sections from six different biopsies previously prepared and measured by an experienced histomorphometrist (Dr Vedi) were used and were measured twice for all parameters except the mineralising perimeter and strut analysis. The first values were used to compare with results obtained by Dr Vedi to give an inter-observer variation rate (Table 6.4). The same sections were re-measured after at least a week and compared with the results obtained previously. From this, an estimate of intra-observer variability was obtained (Table 6.5).

The observer variance (S) of the difference was given by the equation

$$S = (\Sigma d^2) / (2n),$$

where d is the difference between the first and the second measurement and n is the number of biopsies studied.

The standard deviation (SD) of the difference is the square root of the observer variance of the difference.

$$SD = \sqrt{(S)}$$

The standard error (SE) of the difference is given by the formula:

$SE = SD / \sqrt{n}$, where SD is the standard deviation of the difference.

The coefficient of variation (CV) for the two series of measurements is given by the formula:

$CV (\%) = 100\% \times \sqrt{(variance)} / \mu = 100\% \times SD/\mu$, where μ is the mean of the measurements.

6.6.1 Inter-Observer Variation

The coefficients of variation for the inter-observer variation for the different measurements are shown in Table 6.4 and the range is from 5.58% for the mineral apposition rate to 48.67% for the mineralising perimeter. The author had consistently lower values than Dr Vedi for the measurements of osteoid extent and the dynamic measurements (mineral apposition rate and mineralising perimeter). In the case of the osteoid perimeter, this measurement, along with the cancellous bone area were done first whilst training was occurring. In the case of the dynamic measurements, the fluourescent labels may have faded with time. However, the large variation in measurements obtained on the same sections and equipment but

by two different observers emphasises the importance of inter-observer variation. All measurements of the study biopsies were done by one observer, the author, thus eliminating inter-observer variation.

Table 6.4 Inter-observer variation

Feature	Number of biopsies (n)	Mean (μ)	Mean differ-ence (d)	Sum of d squared	Variance (S)	Standard deviation (SD, $\sqrt{S}$)	Co-efficient of variation (%)
Cancellous bone area (%)	6	20.96	2.25	105.91	8.83	2.97	14.17
Osteoid perimeter (%)	6	11.12	1.93	128.33	10.69	3.27	29.41
Osteoid width (μm)	6	7.41	1.27	16.72	1.39	1.18	15.93
Wall width (μm)	6	40.36	2.81	126.54	10.55	3.25	8.05
Mineral apposition rate (μm/day)	6	0.65	0.03	0.02	0.002	0.04	5.58
Mineralising perimeter (%)	5	4.659	2.738	51.426	5.143	2.268	48.67

6.6.2 Intra-Observer Variation

The coefficients of variation for the intra-observer variation are shown in Table 6.5. The mineralising perimeter measurements were not repeated. The coefficients of variation range from 1.76% for wall width to 31.74% for osteoid perimeter. The coefficient of variation for all the measurements except for osteoid perimeter was less than 10% confirming lower variability than in the case of inter-observer measures.

Table 6.5 Intra-observer variation

Feature	Number of biopsies (n)	Mean (μ)	Mean differ-ence (d)	Sum of d squared	Variance (S)	Standard deviation (SD, √ S)	Co-efficient of variation (%)
Cancellous bone area (%)	6	19.50	0.68	17.66	1.47	1.21	6.22
Osteoid perimeter (%)	6	11.65	0.88	158.93	13.24	3.64	31.24
Osteoid width (μm)	6	8.03	0.02	0.69	0.06	0.24	2.99
Wall width (μm)	6	39.23	0.56	5.69	0.47	0.69	1.76
Mineral apposition rate (μm/day)	7	0.77	0.05	0.03	0.002	0.05	6.06

6.7 Results

All results are presented as the unadjusted two-dimensional values, with no correction to three-dimensional values. This is justified as comparison between the pre- and post-treatment values is of interest and correction would affect all values equally.

6.7.1 Static Measurements

The results for these measurements as mean values and standard deviations are given in Table 6.6. The mean cancellous bone area as a percentage of marrow area for the four subjects before and after treatment with MTX is shown in Figure 6.9. There was an increase in mean bone area in each subject after MTX therapy compared with the baseline measurements.

The mean osteoid perimeter as a percentage of cancellous bone perimeter is shown in Figure 6.10. In three out of the four subjects, there was an increase in osteoid perimeter over the year of treatment with MTX.

The mean osteoid seam width for the four subjects is shown in Figure 6.11. In two out of the four subjects there was an increase in mean osteoid width over the study period. There was a decrease in the remaining two subjects.

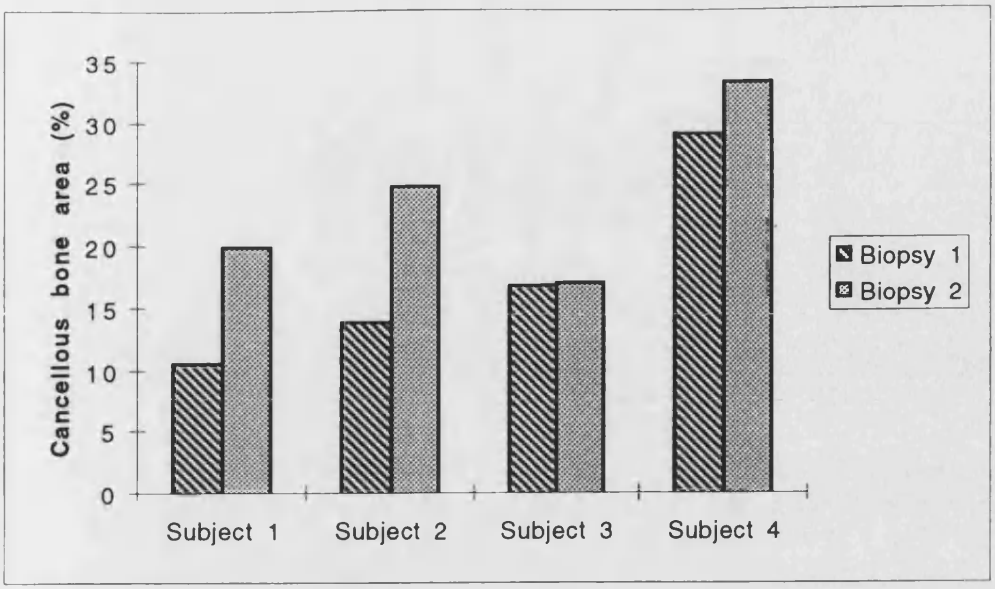
The mean completed wall width of the four subjects is shown in Figure 6.12. As for the cancellous bone area, there was an increase in all four subjects.

Table 6.6 Results for static measurements

	Cancellous bone area (%)	Osteoid perimeter (%)	Osteoid seam width (μm)	Wall width (μm)
Subject 1 1st biopsy	10.63 ±0.66	4.38 ±0.54	7.07 ±0.52	40.7 ±1.32
Subject 1 2nd biopsy	19.99 ±5.01	5.43 ±0.95	8.96 ±0.93	44.36 ±3.07
Subject 2 1st biopsy	13.86 ±1.83	10.47 ±4.74	10.5 ±1.64	39.74 ±2.08
Subject 2 2nd biopsy	24.94 ±11.23	13.14 ±6.58	10.83 ±4.40	40.97 ±2.49
Subject 3 1st biopsy	16.84 ±1.90	2.88 ±1.71	10.77 ±1.41	36.36 ±0.57
Subject 3 2nd biopsy	17.1 ±2.85	6.53 ±0.87	7.15 ±0.24	41.31 ±2.08
Subject 4 1st biopsy	29.09 ±2.08	3.25 ±1.07	10.33 ±0.67	39.57 ±2.24
Subject 4 2nd biopsy	33.22 ±4.11	2.17 ±2.16	7.04 ±0.62	40.84 ±4.09

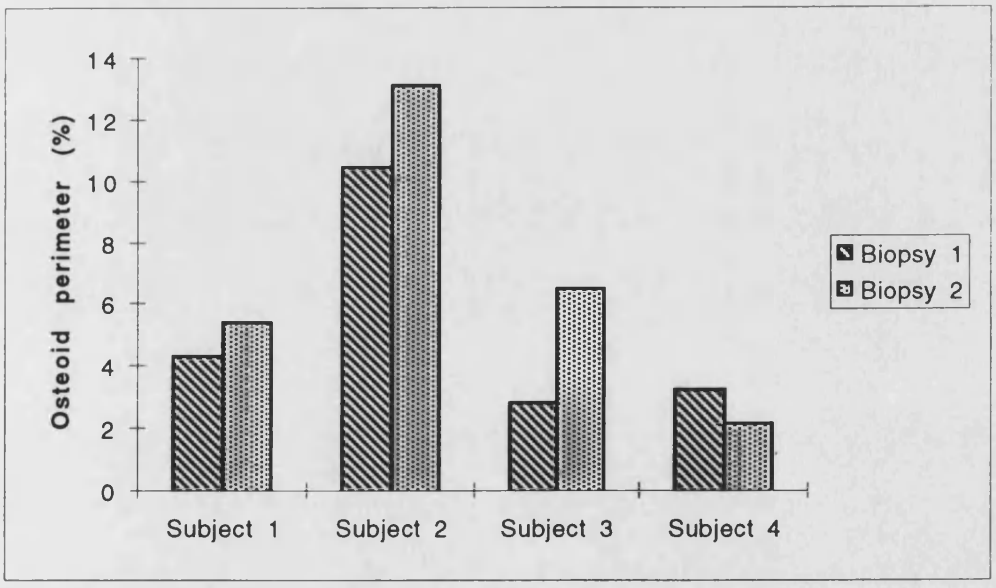
Results shown as mean ± standard deviation of the mean for at least three sections from each biopsy

Figure 6.9 Mean cancellous bone area



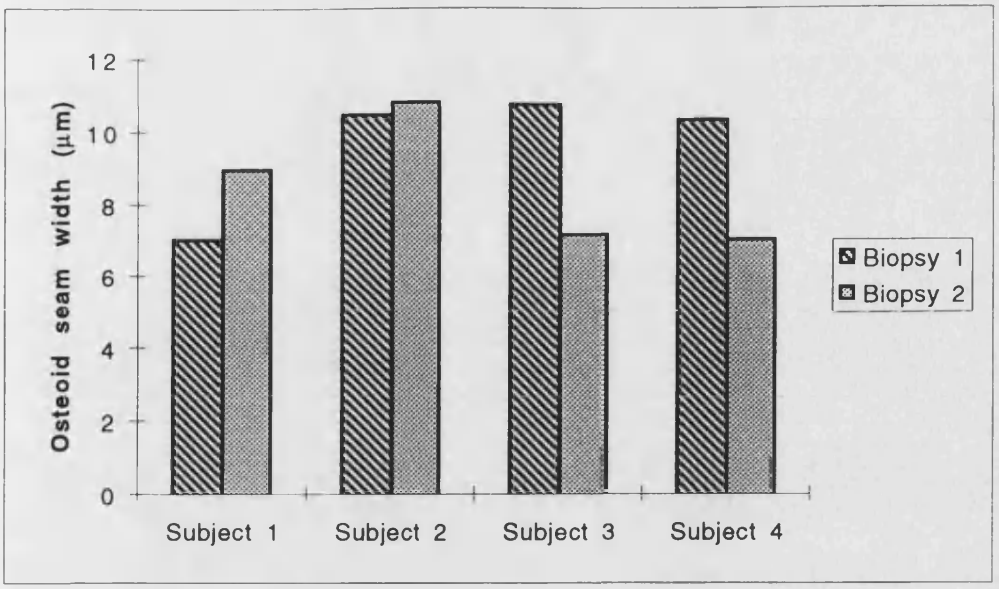
Results shown as cancellous bone area as % of medullary area

Figure 6.10 Mean osteoid perimeter



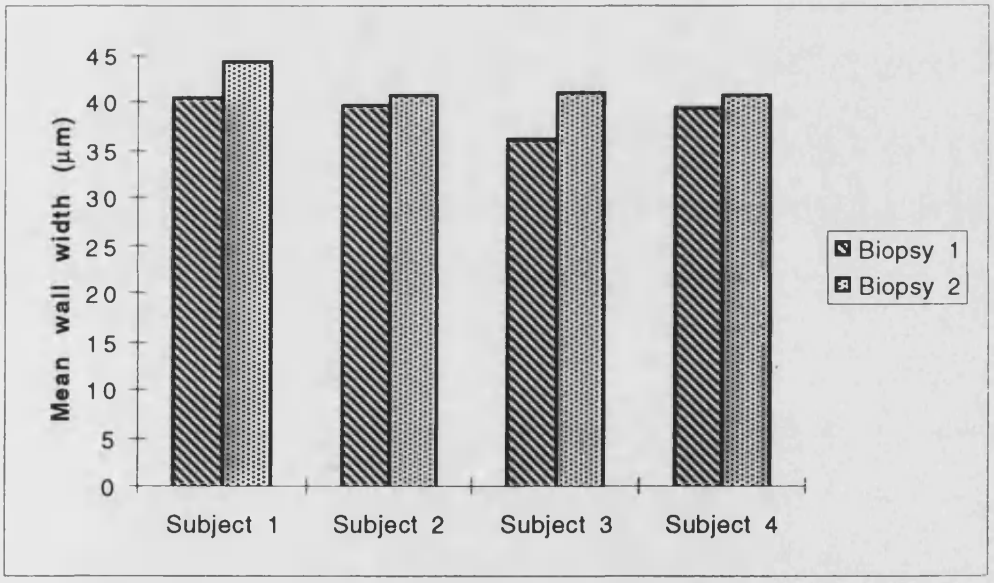
Results shown as % of total bone perimeter

Figure 6.11 Mean osteoid seam width



Results shown in µm

Figure 6.12 Mean wall width



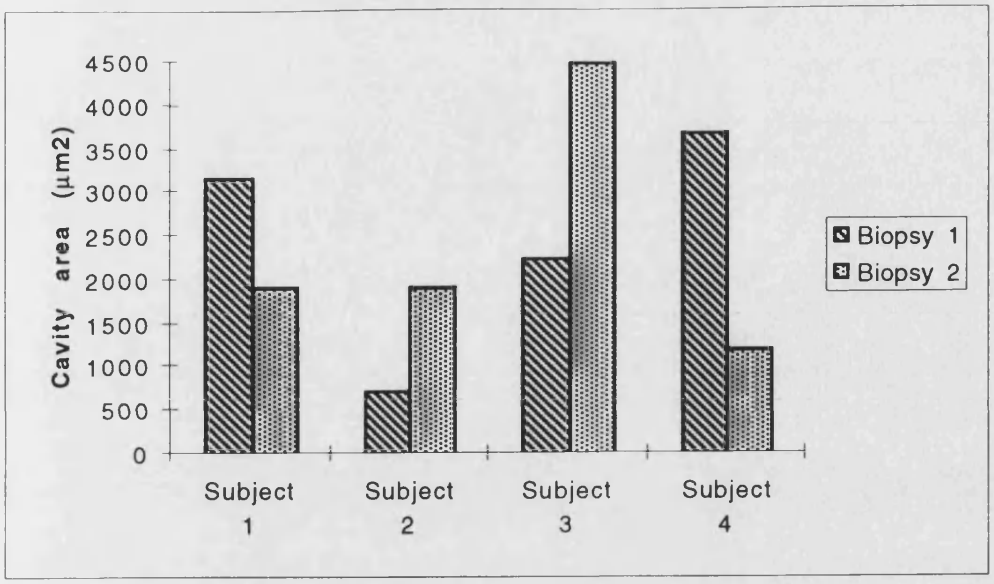
Results shown in µm

Resorption cavity measurements are given in Table 6.7 and shown in Figures 6.13 to 6.16. Figure 6.13 shows the mean resorption cavity area, Figure 6.14 the mean cavity depth and Figure 6.15 the maximum cavity depth for the eight biopsies. The reconstructed cavity length is shown in Figure 6.16 and is a measure of the endosteal surface extent of the cavity. There were no clear trends apparent in the resorption cavity measurements made in this study. For cavity area, maximum cavity depth and reconstructed cavity length, there were equal increases and decreases in the subjects (two increased and two decreased for each). For the measurement of mean cavity depth, there was an increase in the measurement in three out of the four subjects.

Table 6.7 Resorption cavity measurements

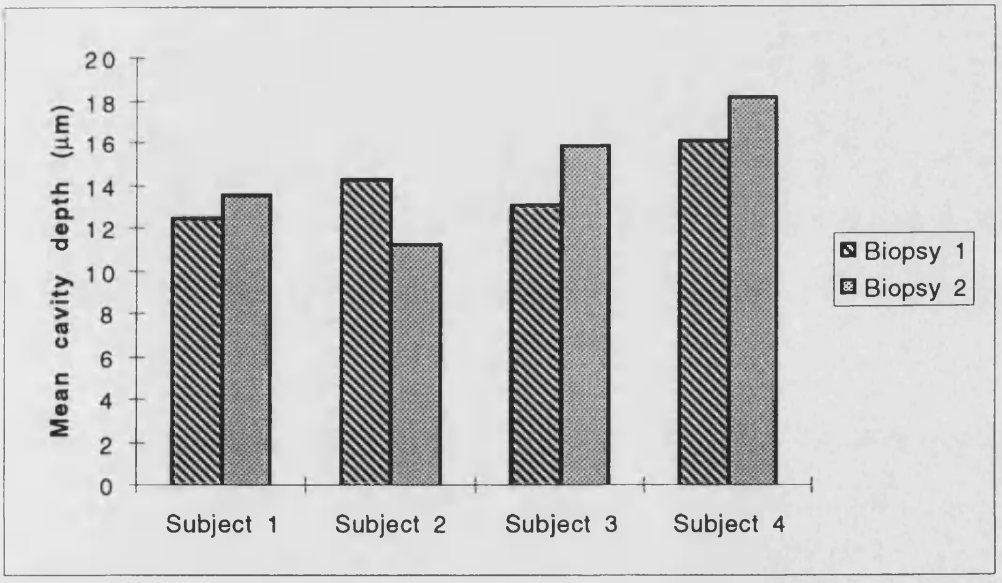
	Mean cavity depth (μm)	Maximum cavity depth (μm)	Reconstructed cavity length (μm)	Area of cavity (μm^2)
Subject 1 1st biopsy	12.51 ± 6.24	30.01	157.10	3162.09
Subject 1 2nd biopsy	13.52 ± 5.12	22.59	136.75	1900.16
Subject 2 1st biopsy	14.36 ± 6.33	16.34	83.75	713.32
Subject 2 2nd biopsy	11.26 ± 3.56	27.39	120.58	1912.17
Subject 3 1st biopsy	13.15 ± 3.87	30.90	168.44	2249.85
Subject 3 2nd biopsy	15.85 ± 6.62	36.30	205.88	4480.02
Subject 4 1st biopsy	16.14 ± 3.68	41.87	168.55	3656.22
Subject 4 2nd biopsy	18.15 ± 4.51	37.38	99.85	1181.76
Mean ± standard deviation of all 1st biopsies	14.040 ± 1.596	29.78 ± 10.458	144.710 ± 41.027	2445.370 ± 1293.325
Mean ± standard deviation of all 2nd biopsies	14.695 ± 2.969	30.915 ± 7.130	140.765 ± 45.962	2368.528 ± 1448.499

Figure 6.13 Mean resorption cavity area



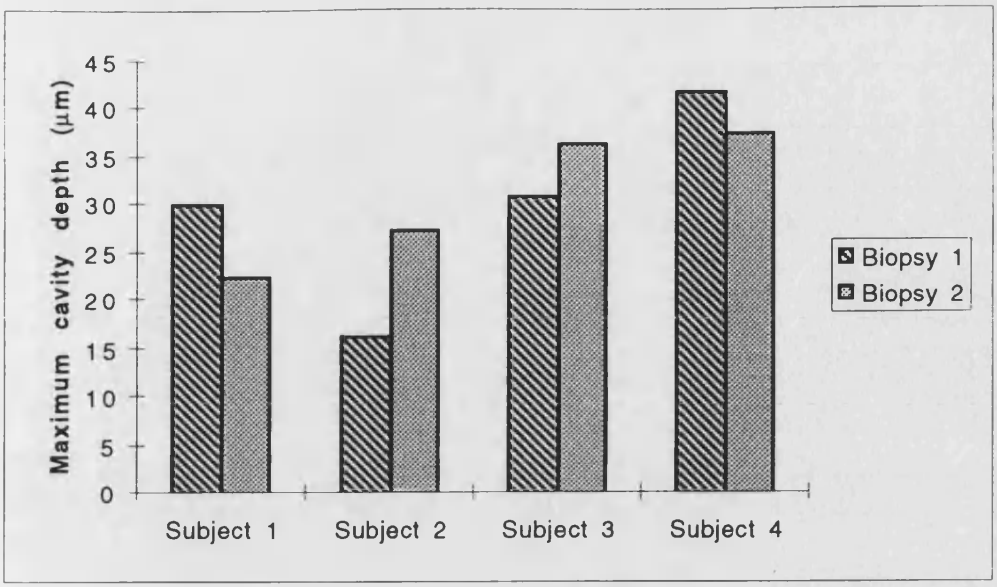
Results shown as μm^2

Figure 6.14 Mean resorption cavity depth



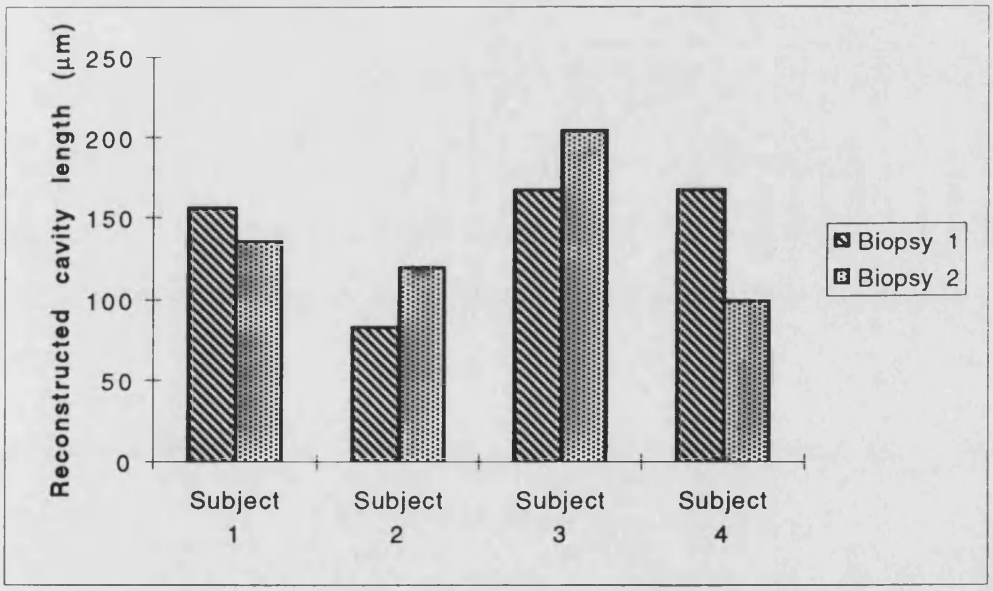
Results shown as µm

Figure 6.15 Mean maximum cavity depth



Results shown as µm

Figure 6.16 Mean reconstructed cavity length



Results shown as µm

6.7.2 Dynamic Measurements

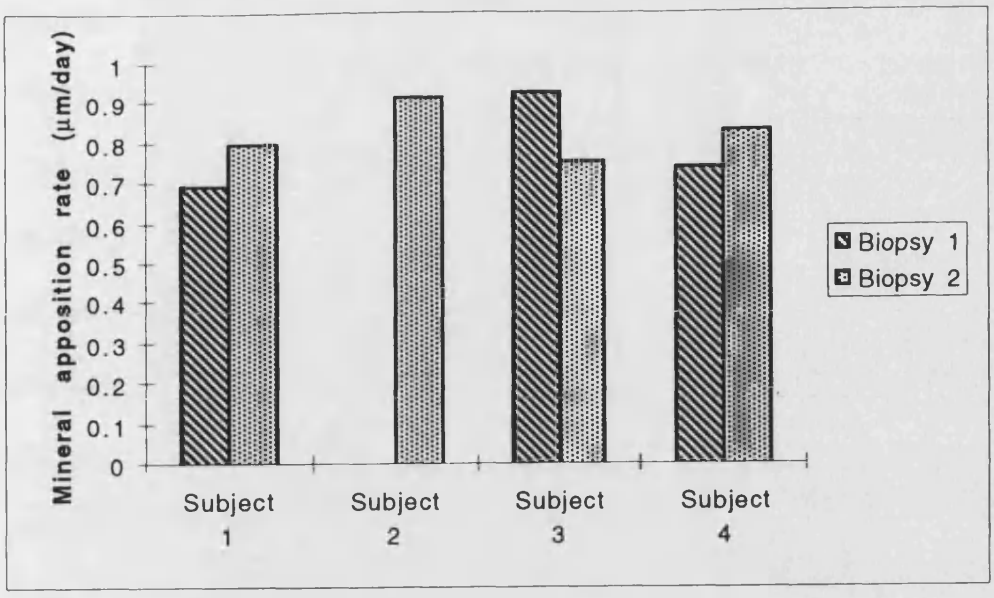
Dynamic measurements could be made on only seven out of eight biopsies. Despite examination of many sections from different depths, no tetracycline label could be found in the pre-treatment biopsy of subject 2. This biopsy was unfortunately fractured during its removal from the subject, and much of the trabecular bone was rubbed off during the processing. Thus only three paired biopsies had dynamic measurements made on them from which derived indices can be calculated (Section 6.7.3). The mean results and standard deviation of the mean are given in Table 6.8 and are shown graphically in Figure 6.17 (mineral apposition rate) and Figure 6.18 (mineralising perimeter). For each measurement, there was an increase in two out of three subjects.

Table 6.8 Results for dynamic measurements

	Mineral apposition rate (μm/day)	Mineralising perimeter (%)
Subject 1 1st biopsy	0.69 ± 0.02	5.5 ± 3.27
Subject 1 2nd biopsy	0.80 ± 0.08	5.57 ± 1.19
Subject 2 1st biopsy	No label	No label
Subject 2 2nd biopsy	0.91 ± 0.04	5.44 ± 0.98
Subject 3 1st biopsy	0.93 ± 0.06	1.54 ± 0.37
Subject 3 2nd biopsy	0.75 ± 0.01	4.67 ± 1.72
Subject 4 1st biopsy	0.74 ± 0.17	2.66 ± 0.95
Subject 4 2nd biopsy	0.83 ± 0.02	1.50 ± 0.50

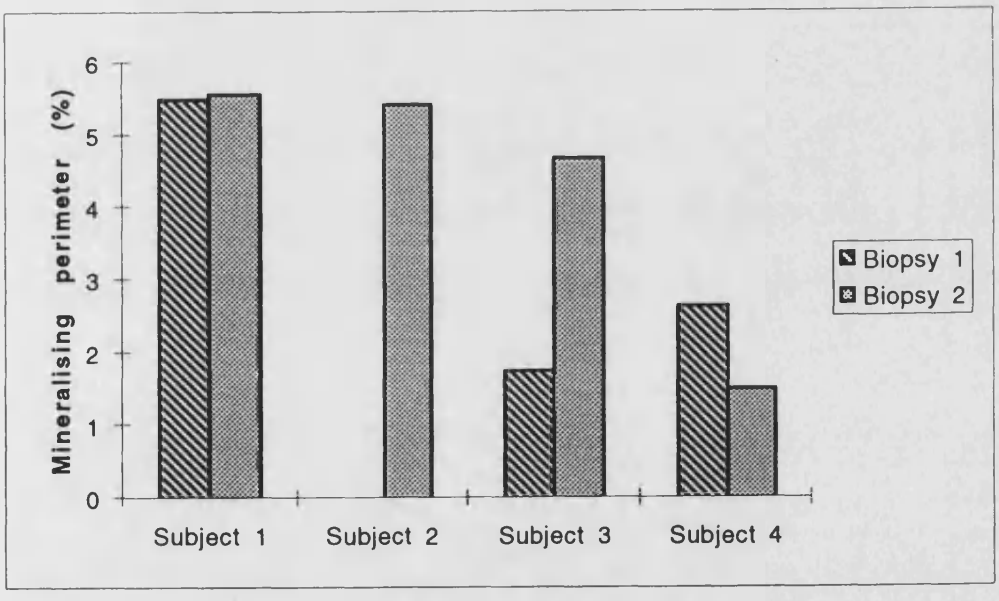
Results shown as mean ± standard deviation of the mean. No label was present in the first biopsy of subject 2

Figure 6.17 Mean mineral apposition rate



Results shown as µm/day

Figure 6.18 Mean mineralising perimeter



Results shown as % of total perimeter

6.7.3 Definitions of the Derived Indices

Derived indices of bone formation were calculated from the static and dynamic measurements given above.

The derived indices are calculated from the following formulae.

AjAR (μm/day) = MAR x MP/OP,

where MAR is the mineral apposition rate (μm/day, Section 6.5.7), MP is the mineralising perimeter (%, Section 6.5.8) and OP is the osteoid perimeter (%, Section 6.5.3).

BFR/BP ($\mu m^2/\mu m$/day) = MAR x MP/BP,

where MAR is the mineral apposition rate (μm/day, Section 6.5.7), MP is the mineralising perimeter (% of the bone perimeter, Section 6.5.8) and BP is the bone perimeter (always 100%).

Acf (/year) = (BFR/BP) / (365 x W.Wi),

where BFR is the bone formation rate ($\mu m^2/\mu m$/day, see above), BP is the bone perimeter (μm) and W.Wi is the mean wall width (μm, Section 6.5.5).

FP (days) = W.Wi/AjAR,

where W.Wi is the mean wall width (μm, Section 6.5.5) and AjAR is the adjusted apposition rate (μm/day, see above).

6.7.4 Results of the Derived Indices

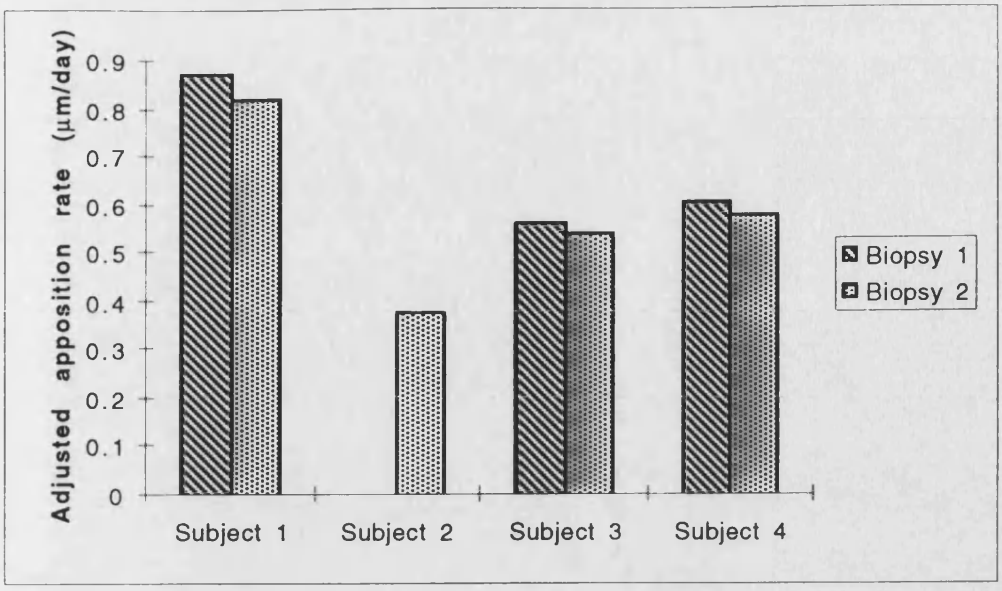
These results are given in Table 6.9. The adjusted apposition rate (AjAR, μm/day) is shown in Figure 6.19, the bone formation rate (BFR, $\mu m^2/\mu m$/day) in Figure 6.20, the activation frequency (Acf, /day) in Figure 6.21 and the formation period (FP, days) in Figure 6.22.

The adjusted apposition rate decreased slightly in all three subjects for whom paired results were available, whilst the BFR and Acf increased in two out of three subjects. The FP increased in all three subjects.

Table 6.9 Derived indices of bone turnover

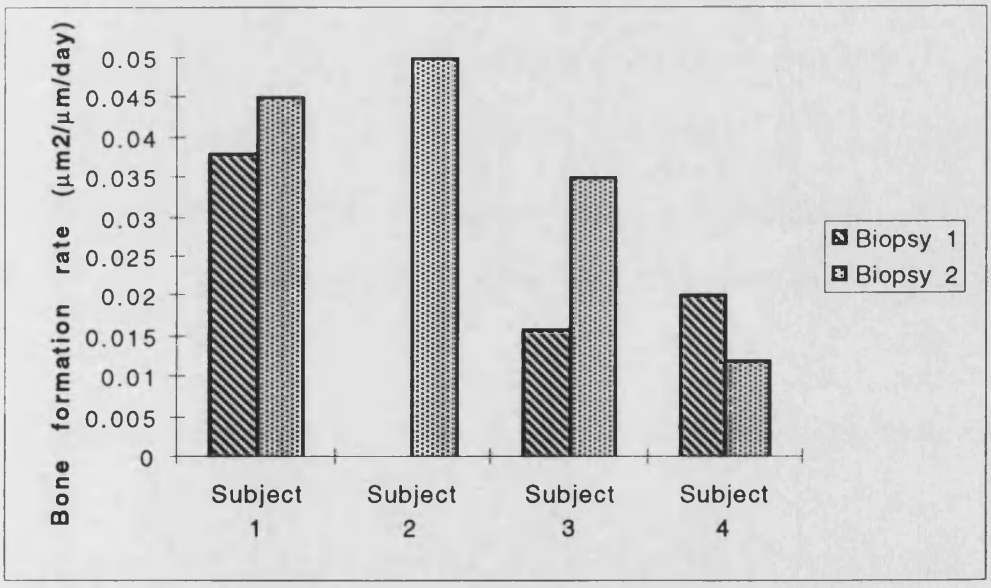
	Adjusted Apposition Rate ($\mu m.day^{-1}$)	Bone Formation Rate ($\mu m^2/\mu m.day^{-1}$)	Activation Frequency ($year^{-1}$)	Formation Period (days)
Subject 1 1st biopsy	0.871	0.038	0.00093151	46.7
Subject 1 2nd biopsy	0.82	0.045	0.0010137	54.12
Subject 2 1st biopsy	No label	No label	No label	No label
Subject 2 2nd biopsy	0.378	0.05	0.00120548	108.39
Subject 3 1st biopsy	0.56	0.016	0.00043836	64.92
Subject 3 2nd biopsy	0.537	0.035	0.00084932	76.92
Subject 4 1st biopsy	0.606	0.02	0.00049315	65.33
Subject 4 2nd biopsy	0.574	0.012	0.00030137	71.1

Figure 6.19 Adjusted apposition rate



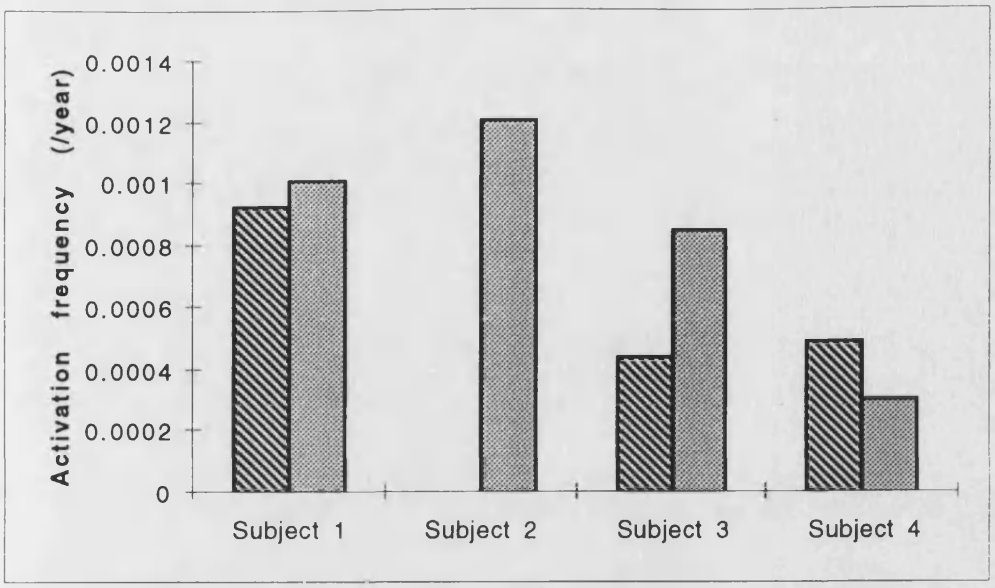
Results shown as µm/day

Figure 6.20 Bone formation rate



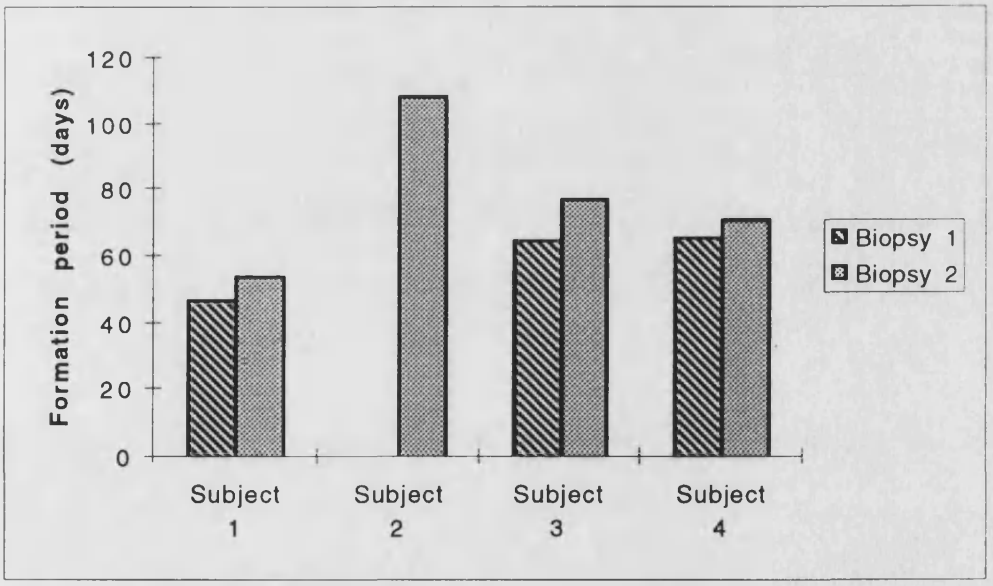
Results shown as $\mu m^2/\mu m/day$

Figure 6.21 Activation frequency



Results shown as events / year

Figure 6.22 Formation period



Results shown as days

6.7.5 Analysis of Results

The mean differences between pre-treatment and post-treatment values for all the measurements described above are shown in Table 6.10, together with the p values by paired t-test at the 95% confidence level. The only difference to reach significance was the derived index of formation period (p = 0.0459). The mean difference in formation period was eight days, which is probably not clinically relevant.

Table 6.10 Analysis of results

Measurement	Mean difference (Biopsy 2 minus Biopsy 1)	P value for paired t-test, 95% confidence interval
B.Ar	6.207	0.0869
OP	1.573	0.2256
OW	-1.173	0.4512
W.Wi	2.778	0.0569
MAR	0.007	0.9485
MP	0.613	0.6630
Mean cavity depth	0.655	0.6488
Maximum cavity depth	1.135	0.8087
Reconstructed cavity length	-3.945	0.8865
Cavity area	-76.842	0.9480
AjAR	-0.035	0.0505
BFR	0.006	0.5227
Acf	0.037	0.6225
FP	8.397	0.0459

6.7.6 Structural Analysis Results

Structural analysis was only possible on two paired biopsies. This was because the first biopsy of subject two was fractured and the second biopsy of subject four was very oblique, with a rim of cortex in a semi-circle around the trabecular bone in the centre. The available results are shown in Table 6.11. The node to node distance in the second biopsy was calculated as -0.47 which is clearly impossible. Statistical analysis on such a small sample (n of two) was not attempted.

Table 6.11 Structural analysis

	Subject 1 Biopsy 1	Subject 1 Biopsy 2	Subject 3 Biopsy 1	Subject 3 Biopsy 2	Subject 4 Biopsy 1
Free end count	0.97	2.02	1.44	1.53	1.39
Node count	0.56	2.47	0.60	1.25	2.08
Total Strut count	1.27	3.67	1.52	2.55	3.66
Node / Free end (% total strut length)	22.10	30.17	29.33	44.92	19.03
Node / Node (% total strut length)	24.87	39.86	15.98	-0.47	27.79
Node / Free end ratio	0.57	1.22	0.42	0.82	1.5

6.8 Summary of Results

Histomorphometric analysis of this small sample revealed no toxic effect of MTX on bone formation. Indeed, cancellous bone area and mean wall width which is an indicator of osteoblast function (Croucher et al, 1989) increased in all four subjects during the treatment period. There were no differences in resorption cavity

parameters and osteoid width and extent measurements did not suggest a defect of mineralisation. Statistical analysis of the measures found only one significant difference: the mean formation period which increased by a mean of eight days. This is unlikely to be significant clinically.

In conclusion, no effect of low-dose MTX was found in this study of four women with early RA. This is reassuring and is in keeping with other findings of bone mineral density and markers of bone turnover in the larger cohort of subjects (Chapter 3). However, because the sample size was very small, only consistent and relatively toxic effects are likely to be demonstrated.

7. DISCUSSION

7.1 Introduction

The inflammatory joint disease rheumatoid arthritis (RA) predisposes to increased bone loss and ultimately osteoporosis (Goldring, 1996). The anti-folate drug methotrexate (MTX) is used increasingly to treat RA (Weinblatt, 1996). MTX is generally well tolerated by patients and often has beneficial effects on symptoms such as joint pain, joint swelling and early morning stiffness. However, there are reports of osteoporosis and fragility fractures developing in patients given MTX for a variety of conditions (Preston et al, 1993). This is of concern as many patients on MTX for RA have additional risk factors for osteoporosis such as reduced mobility or oestrogen deficiency in postmenopausal women. Any medication which may increase bone loss in this vulnerable population should therefore be avoided.

7.2 Study Design

This study was designed to determine whether MTX has a measurable and clinically relevant effect on bone mineral density (BMD) and bone turnover in subjects taking it for RA. The null hypothesis was that MTX had no effect on these parameters. The research methods used were (i) a large clinical study of subjects with RA, (ii) histomorphometry of bone biopsies from a sub-group of the RA subjects and (iii) *in vitro* studies of human bone cell cultures. Thus, the effects of MTX were studied at the level of the whole organism, the remodelling unit and the bone-forming cell population.

7.3 The Clinical Study

The clinical study was a pragmatic, observational study as it was not feasible for a single investigator to undertake a double-blind or single-blind, randomised study in the research setting of the Royal National Hospital for Rheumatic Diseases. The study would have been more rigorous had these conditions been imposed, but many fewer subjects would then have been recruited.

The first year was spent recruiting one hundred and sixteen subjects into the four study groups: those starting MTX; those starting sulphasalazine (SPZ); those who had been on MTX for at least five years; and those who had been on other disease-modifying anti-rheumatic drugs (DMARDs) for at least five years. None had taken oral corticosteroids for RA. It was not known at the outset how many patients attending the Royal National Hospital for Rheumatic Diseases would fulfil the entry criteria for any group and so the notes were reviewed for every out-patient clinic to identify suitable subjects. Statistical advice obtained before commencing the study had indicated that to find a potentially clinically relevant difference of 5% in BMD at any given site between the groups treated with MTX and other DMARDs would require at least one hundred subjects in each group. The numbers recruited fell short of this target but, with the benefit of hindsight, it is clear that the author's time constraints would have precluded the satisfactory assessment over one or two years of a larger cohort.

7.3.1 Analysis of the Clinical Data

The clinical data set was studied by a statistician (Dr Daphne Kounali). Her help was sought as the analysis between the four groups had to take account of many potentially confounding factors present in the subjects. The primary outcome

group after they had been split by sex and menopausal status was between five and nineteen. The only category of subject in which there were enough subjects in all four treatment groups to undertake a detailed regression and discriminant analysis was the postmenopausal women. This is the group at most risk of adverse effects of treatment due to the higher prevalence of osteoporosis following oestrogen withdrawal.

7.3.2 Demographic Features of Subjects

There were no significant differences between the four groups in prevalence of osteoporotic risk factors such as previous fragility fracture, family history of osteoporosis, smoking or alcohol use. Postmenopausal females predominated in each group. Subjects starting either MTX or SPZ tended to be younger and had much shorter RA disease duration. The group continuing MTX treatment was different from those continuing other DMARDs as they had 'failed' between one and six DMARDs previously and may have had more aggressive RA which was thus difficult to treat.

7.3.3 Rheumatoid Arthritis Disease Activity

Most subjects in all four treatment groups had high or moderate disease activity (disease activity score, DAS) throughout the study. Both MTX and SPZ caused a rapid and statistically significant reduction in DAS from baseline over the first year of treatment, which was maintained at two years, but there was no difference betweeen groups. The DAS did not change significantly over the year of the study in those subjects continuing on MTX or another DMARD.

7.3.4 Functional Status

The functional status was very similar between the four groups when assessed by the length of time spent walking outdoors each day. The mean Health Assessment Questionnaire (HAQ) score was higher at baseline in the groups continuing on their DMARD suggesting that longer duration of RA was associated with more disability, although the differences did not reach significance. In the groups starting a DMARD, there was a significant difference between those starting MTX and SPZ by repeated measures ANOVA. The HAQ in the group starting SPZ was reduced whilst the mean HAQ did not significantly change over the first two years of treatment in the group starting MTX. The unresponsiveness of the HAQ in the group starting MTX may be related to the longer mean duration of RA in this group.

7.3.5 Radiographic Score of Damage

Unsurprisingly, subjects who had a longer history of RA (groups continuing MTX or other DMARDs) had more erosions than those with a shorter history (those starting MTX or SPZ). There was, however a difference between the groups; those who had taken MTX for more than five years had significantly more erosions at the hand and elbow (but not wrist) joints than those continuing other DMARDs. It is worthy of note that the group starting MTX developed significantly more erosions at the wrist (but not hand or elbow) over the first year of the study when compared to the group starting SPZ. In the subjects with early RA who started MTX there was a significant increase in both hand and wrist Larsen scores at both the one and two year points, whilst there was no significant increase during the study in the wrist scores in subjects starting SPZ. Unfortunately, at the time of the study, it was hospital policy to

do oblique radiographic views of the feet, precluding scoring of these films. It was not felt justifiable (ethically or financially) to obtain additional feet radiographs as most subjects had had recent hand and feet films taken during a clinic visit.

7.3.6 Bone Mineral Density Results

Analysis of the unadjusted whole data set revealed significantly reduced age- and sex-adjusted BMD (Z scores) at the proximal third forearm site in the group continuing MTX compared to the other three treatment groups ($p < 0.0001$). The BMD was also significantly lower in this group at both the total and mid-portion regions of the forearm. In contrast, the ultra-distal portion of the forearm was spared.

When the BMD results from the postmenopausal women were examined by regression and discriminant analysis by, the only region where MTX treatment was associated with lower BMD after adjustment for confounding factors, was the midportion region of the forearm. This suggested that the reduced BMD at the proximal third forearm site in those on MTX for more than five years could be explained by more aggressive RA disease activity.

In the subgroups with early RA of less than two years' duration, the baseline BMD of subjects starting MTX were well-matched with those starting SPZ. During the first year of treatment there was a reduction in BMD at all sites measured in the group starting MTX. However, compared to the group starting SPZ the percentage change was not significant at any site. There were no significant differences in the percentage change in BMD between the two groups either during the second year of follow-up or at two years compared to baseline.

The subgroup of male subjects behaved differently from the other groups and from the data set as a whole. The numbers in each group were very small (six to eight), but there were no differences between the groups at baseline for any site where age- and sex-adjusted BMD was estimated. Similarly, there was no significant difference between treatment groups for the rate of change of age- and sex-adjusted BMD over the first year of the study.

7.3.7 Bone Turnover Results

In the subjects with early RA, there were no significant changes found over one or two years in any of the three markers of bone turnover between the groups starting MTX and SPZ.

When all the subjects were considered together, serum osteocalcin (OC) and bone-specific alkaline phosphatase (AP) were significantly higher in the group continuing MTX. There were no differences between the four groups in urinary deoxypyridinoline corrected for urinary creatinine (Dpd). In the groups continuing treatment, the only significant change in the markers of bone turnover after one year was for AP where the group continuing MTX had a reduction of 11% whilst the group continuing other DMARDs had an increase of 5%. Again, there was no significant change in Dpd.

In the men, the group starting MTX had significantly lower OC at baseline than the group continuing on MTX ($p < 0.05$), and those starting MTX had a significant increase in OC over the first year of the study.

In the subgroup of postmenopausal women, the group continuing on MTX had significantly higher markers of bone formation compared to both those continuing other DMARDs and those starting SPZ at baseline and at one year. Both OC and AP decreased significantly compared to baseline in all four groups.

Although OC is termed a marker of bone formation, it is also released during bone resorption and so significant changes must be interpreted with caution. The introduction of either MTX or SPZ was associated with an increase in mean Dpd during the first year and a return to near the baseline at two years which may represent regression to the mean. No difference in treatment effect was found for either OC or AP during the two years of the study for those subjects with early RA. This may be because there was no treatment effect or perhaps a larger study would be necessary to detect small differences between MTX and SPZ, given the inherent variability of the assays.

7.4 Histomorphometry of Bone Biopsies

It had been planned to obtain twenty paired bone biopsies for analysis by histomorphometry from women who were premenopausal, had RA of less than two years' duration and were commencing MTX. Although eight women did agree to the procedure, only four paired samples were eventually available for analysis, which was disappointing. This limits the conclusions that can be drawn. However, there were no toxic effects of MTX on cancellous bone formation as osteoid seam width and osteoid perimeter were not significantly different between the pre- and post-treatment biopsies. Also the resorption cavity measurements were unchanged. Large and consistent adverse effects of MTX would probably be detected in such a small sample, but a larger sample would be necessary to detect more subtle effects.

A longer treatment period before the second biopsy may be necessary to reveal differences, as the remodelling cycle takes 200 days in trabecular (cancellous) bone. Given the finding in the clinical study of an effect of MTX on the mid-portion forearm BMD, examination of the amount and structure of cortical bone in these biopsies might prove informative.

7.5 Bone Cell Culture

The series of experiments performed to study the effect of clinically relevant doses of MTX on culture human bone-derived revealed inhibitory rather than toxic effects of the drug on their activity. The number of colonies formed from marrow cell suspensions and the proportion which were osteoblast-like (expressing alkaline phosphatase) were reduced by MTX in a generally dose-dependent manner. It is not clear whether this is due to decreased attachment of marrow precursors in the presence of MTX, increased apoptosis or delayed growth. As MTX interrupts the metabolism of folate, thus impairing DNA synthesis, the inhibition of proliferation is likely to be an important mechanism.

MTX also had an effect on the surface expression of the differentiation markers Stro-1 and B4-78 (alkaline phosphatase) which were detected by flow cytometry. By reducing proliferation, MTX, like dexamethasone, appeared to behave as a promoter of differentiation to the osteoblast-like phenotype.

It was hoped to study effects of MTX on the synthesis of collagen type I but unfortunately time did not allow for this.

7.6 Conclusions

This ambitious study has demonstrated that there is unlikely to be a clinically relevant adverse effect of MTX on *cancellous* (trabecular) bone formation and turnover when used at the doses commonly used to treat RA. The histomorphometry study did not demonstrate an adverse effect of MTX on cancellous bone formation *in vivo* and this is in agreement with the serum markers of bone formation, which were highest in subjects treated with MTX. After controlling for confounding factors, regression analysis of the BMD and markers of bone turnover in the postmenopausal women subjects found a significant treatment effect of MTX only in the midportion of the forearm region, which is not prone to osteoporotic fracture. The mid-portion region has a higher cortical bone content than, for example, the ultra-distal region of the forearm. This study provides prospective data in support of the proposition made by Dequeker's group that MTX affects cortical bone preferentially when used in RA (Maenaut et al, 1996).

Bone cell culture experiments found that MTX did influence the colony-forming efficiency, proliferation and differentiation of normal human bone-derived cells, with dose-dependent reduction in proliferation.

Overall, these results suggest that the most important effect of MTX on the skeleton in RA is beneficial through its modulation of the immune system which suppresses the RA disease activity. No independent effect of MTX was seen on markers of bone turnover. This study is reassuring for clinicians and patients using MTX, as cancellous (trabecular) bone is unaffected. The mechanism of cortical bone loss is unknown, but it is becoming clear that neuropeptides are expressed in both synovium and bone (Rosen et al, 1998). It is tempting to speculate that MTX may

inhibit neuropeptide synthesis which could result in envelope-specific effects on remodelling preferentially affecting cortical bone.

7.7 Proposals for Further Study

A larger, double-blind clinical study over a longer time period would have had more power and may have demonstrated subtle differences between treatments, although they may not necessarily have had clinical relevance. In view of the reports of fragility fractures occurring in the distal tibia (Preston et al, 1993), serial total body BMD estimation may be informative as variations in the different regions of the axial and appendicular skeleton could be studied. Radiographic scores of damage in the knees, ankles and feet joints would then be important confounders, as would mobility and time spent weight-bearing.

A larger sample, treated for longer and including analysis of cortical parameters in addition to the cancellous (trabecular) bone would be necessary to confirm the lack of adverse effect demonstrated by histomorphometry in this study.

Finally, the bone cell culture work should be confirmed and extended. If a more normal source tissue of bone and marrow cells could be identified, studies could include the effect of MTX on collagen synthesis and expression of messenger RNA for relevant bone proteins including osteocalcin, alkaline phosphatase, oestrogen and the receptors for vitamin D_3. Any effect of MTX on programmed cell death (apoptosis) and cell-cell and cell-matrix membrane interactions could also be studied.

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