



**Bone loss in osteoporosis and rheumatoid arthritis diseases: The effects of  
disease mechanisms, age, gender and ethnic origin on responsiveness to  
treatment**

**Mohammed Mater Albogami**

**A thesis submitted to the University of London  
for the degree of Doctor of Philosophy  
in the Faculty of Medicine**

**August 2014**



**Bone and Joint Research Unit  
William Harvey Research Institute  
Barts and The London School of Medicine and Dentistry  
Queen Mary University of London**

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The author

Mohammed Mater Albogami

The author:

PhD, Bone loss in osteoporosis and rheumatoid arthritis diseases – present  
Queen Mary University of London, London, UK

BSc, Medical Applied sciences (2001) – graduation rank: 2<sup>nd</sup> of class  
King Saud University, Riyadh, KSA

MSc, Medical science (2007)  
Surrey University, Guildford, UK

## **Acknowledgments**

Firstly, I would like to extend my sincere thanks to Riyadh Medical Services, for sponsoring the program of my PhD studies at University of London. I would like to express my greatest gratitude for this opportunity to achieve my ambitions towards the advancement of science and medicine.

My special thanks go to my supervisor Professor Rizgar A. Mageed who always supported, encouraged and motivated me throughout the PhD journey and also for his helping me to learn the necessary techniques to carry out my research. My thanks also go to my supervisor Professor Ali S Jawad for his suggestions and offering his time to help in clinical works and recruiting patients for my study.

I would also like to acknowledge my department members for their support. I must express my special gratitude to Drs Jonas Bystrom and Taher Taher for their grateful time with me in the lab to finish part of my PhD study work. Special thanks to Mrs Gill Adams and my colleague Christopher Schultz for their assistance with proofreading of the thesis.

Special thanks to Drs Nasra Al-Adhoubi and Stephen Kelly at the Rheumatology clinics in Mile End for providing me with the data of patients required in part of my PhD study work.

I would also like to thank Dr Funso Akanle at the BMD department in London Royal Hospital for his support and important time he gave me to work on the machine and print all required DEXA scans report.

My special thanks to Miss Jacqui Frith for all the paperwork, her continuous advice and support and caring.

My completion of this project could not have been accomplished without the support of my parents, wife, and kids.

Mohammed Mater Albogami

## Abstract

Bone makes up a framework that provides protection for internal body organs. The homeostasis of bone is maintained by a balanced process involving old bone degradation and new bone formation. However, this balance can be altered in pathophysiological conditions such as in postmenopausal osteoporosis and in patients with rheumatoid arthritis (RA). In recent years, new therapies have been developed to reduce bone resorption. However, there is disparity in patients' response to these therapies. The reasons are unclear although age, gender, ethnic background and lifestyle have all been suggested to play a part. For patients with chronic inflammatory conditions, treatment was revolutionised by the discovery and application of biologic therapies that target pro-inflammatory proteins and/or pathways. However, whilst the anti-inflammatory effect of these biologic agents is well-established, their effect on bone loss is just emerging. In RA, it is not clear whether the beneficial anti-inflammatory effects of biologic anti-tumour necrosis factor alpha (TNF $\alpha$ ) agents are accompanied by parallel improvements in bone erosion/density, whether there are differences between patient groups and what factors influence the response. In order to address these issues, a database on the factors that influence responsiveness of patients with osteoporosis to bisphosphonates, a treatment that suppresses bone resorption, was established. Based on the outcome of this study, the influence of the key factor(s) that affect bone response to treatment in combination with excess pro-inflammatory cytokine production on bone response in RA patients was determined. Significant improvement in bone mineral density (BMD) and plasma levels of bone biomarkers has been shown in this study with biologic anti-TNF $\alpha$  agents. The improvement in BMD was not always consistent with improvement the clinical response to treatment as assessed by changes in disease activity score 28(DAS28). The study also provides a mechanistic explanation for how blockade of TNF $\alpha$  in patients can reverse the balance of bone loss in patients with RA. Thus, the data show that treatment of patients with biologic anti-TNF $\alpha$  agents reduces the number of osteoclast precursors (OCs) in the blood.

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## Abbreviations

|        |  |
|--------|--|
| Ab     | Antibody   |
| A-CCPs | Autoantibodies to cyclic citrullinated peptides            |
| ACR50  | American College of Rheumatology 50                        |
| AP     | Anteroposterior  |
| APC    | Allophycocyanin  |
| BlyS   | B-lymphocyte stimulator                                    |
| BMD    | Bone mineral density                                       |
| BMC    | Bone mineral content                                       |
| BSA    | Bovine Serum Albumin                                       |
| BUA    | Broadband ultrasonic attenuation                           |
| CCPs   | Cyclic citrullinated peptides                              |
| CD11b  | Cluster of Differentiation molecule 11b                    |
| CD14   | Cluster of Differentiation molecule 14                     |
| CIA    | Collagen-Induced Arthritis                                 |
| COREC  | Central Office for Research Ethics Committee               |
| CRP    | C-reactive Protein   |
| CT     | Calcitonin   |
| CTLA-4 | Cytotoxic T-lymphocyte Antigen 4                           |
| CTX    | Carboxyterminal cross-linking telopeptide of bone collagen |
| CV     | Coefficient of Variation                                   |
| DAS28  | Disease Activity Score 28                                  |
| DCE    | Dynamic contrast enhanced                                  |
| DMARDs | Disease Modifying Anti-Rheumatic Drugs                     |
| DEXA   | Dual energy X-ray absorptiometry                           |
| DKK1   | Dickkopf-related protein 1                                 |
| DNA    | Deoxyribonucleic Acid                                      |
| DXR    | Digital X-ray Radiogrammetry                               |
| EDTA   | Ethylene Diamine Tetraacetic Acid                          |
| ELISA  | Enzyme-Linked Immunosorbent Assay                          |
| ER     | Oestrogen Receptor   |
| ESR    | Erythrocyte Sedimentation Rate                             |
| Fab    | Fragment antigen-binding                                   |

|                |  |
|----------------|--|
| FACS           | Fluorescence Activated Cell Sorting              |
| FBS            | Foetal Bovine Serum                              |
| Fc             | Fragment crystallisable region                   |
| FCS            | Foetal Calf Serum                                |
| FDA            | Food and Drug Administration                     |
| FITC           | Fluorescein Isothiocyanate                       |
| GCs            | Glucocorticoids                                  |
| GM-CSF         | Granulocyte-Macrophage Colony-Stimulating Factor |
| HRP            | Horseradish Peroxidase                           |
| HRT            | Hormone Replacement Therapy                      |
| Ig             | Immunoglobulin                                   |
| IL             | Interleukin                                      |
| IFN $\gamma$   | Interferon gamma                                 |
| IGF            | Insulin-like Growth Factor                       |
| LDL            | Low-density lipoprotein                          |
| LPS            | Lipopolysaccharide                               |
| LT $\alpha$    | Lymphotoxin alpha                                |
| MCI            | Metacarpal Index                                 |
| MRI            | Magnetic Resonance Imaging                       |
| MCP-1          | Monocyte chemoattractant protein-1               |
| M-CSF          | Macrophage-Colony-Stimulating Factor             |
| MFI            | Mean Fluorescence Intensity                      |
| MSCs           | Mesenchymal Stem Cells                           |
| MSD            | Meso Scale Discovery                             |
| mTOR           | mammalian Target Of Rapamycin                    |
| NHS            | National Health Service                          |
| NICE           | National Institute of Clinical Excellence        |
| NF- $\kappa$ B | Nuclear factor kappa B                           |
| OCPs           | Osteoclast Precursors                            |
| OPG            | Osteoprotogerin                                  |
| OCs            | Osteoclasts                                      |
| OD             | Optical Density                                  |
| PE             | Phycoerythrin                                    |
| PBMNCs         | Peripheral Mononuclear Cells                     |
| PMA            | Phorbol Myristate Acetate                        |

|        |   |
|--------|---|
| PBS    | Phosphate-Buffered Saline                   |
| QCT    | Quantitative Computed Tomography            |
| QUS    | Quantitative Ultrasound                     |
| RA     | Rheumatoid Arthritis                        |
| RANK   | Receptor Activator of the NFκB              |
| RANK-L | Receptor Activator of the NFκB -Ligand      |
| RF     | Rheumatoid factor                           |
| RPMI   | Roswell Park Memorial Institute medium      |
| RT     | Room Temperature                            |
| PINP   | Procollagen type I N-terminal Propeptide    |
| PTHrP  | Parathyroid Hormone related Peptide         |
| PTH    | Parathyroid Hormone                         |
| PDEXA  | Peripheral Dual Energy X-ray Absorptiometry |
| SD     | Standard Deviation                          |
| SEM    | Standard Error of Mean                      |
| SERMs  | Selective Oestrogen Receptor Modulators     |
| SXA    | Single X-ray Absorptiometry                 |
| TB     | Tuberculosis                                |
| TCR    | T Cell Receptor                             |
| TNF    | Tumour Necrosis Factor                      |
| TNFR1  | Tumour Necrosis Factor Receptor 1           |
| TRAP   | Tartrate-Resistant Acid Phosphatase         |
| US     | Ultrasound                                  |
| WHO    | World Health Organization                   |

# **Chapter One**

## **Introduction and reviews of the literature**

## **1.1) Bone**

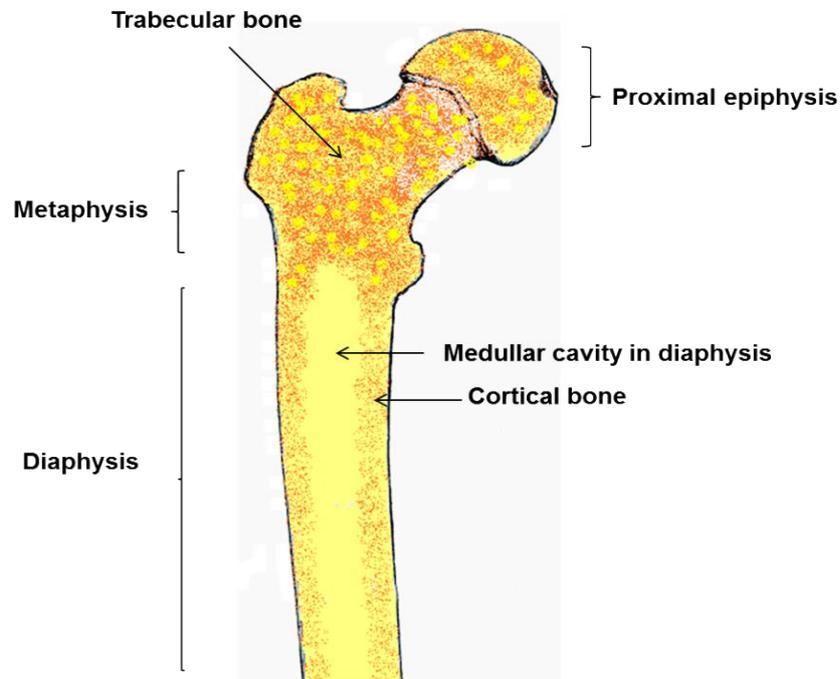
Bone is a specialised connective tissue involved in numerous vital functions in the body. These include giving the body a structure, protecting vital internal organs and acting a milieu for the bone marrow. For example, the skull acts as a helmet and protects the eyes, ears and brain. The ribs form a cage that surrounds and protects the lungs and the heart. Furthermore, bone has an essential metabolic function acting as a mineral reservoir of calcium, magnesium and phosphates that are required for the maintenance of blood homeostasis. In addition, bone forms a framework for the muscular system to facilitate movement (1).

## **1.2) Structure and organisation of bone**

The human skeleton is comprised mainly of osseous (bone) tissue, which is composed of mineralised matrix with both organic and inorganic components. The inorganic matrix is mainly crystalline mineral salts and calcium phosphate present in the form of hydroxyapatite,  $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_6$  on which bone hardness depends (2). Fewer amounts of manganese, chlorine, potassium are also present in bone crystals. The organic part of the matrix is primarily type I collagen, which forms more than 25% of bone. This collagen is in a fibre form with a triple helical molecular structure that provides bone with flexibility. Other organic components of bone are mucopolysaccharides, glycoproteins and lipids (1).

Several types of bone exist within the human skeleton. The two major types are cortical bone, which makes up to 80% of all bone tissues and trabecular bone which makes up to 20%. Cortical, or compact bone, is dense and forms the protective exterior shell of long bones and vertebrae. It supports long bones to resist the stress of weight placed on them. In contrast, trabecular bone is a spongy-like structure which contains a network of fine and interlacing partitions, the trabeculae. Trabecular bone forms the main part of the vertebral body and the epiphyses of the long bones (Figure 1.1). Any bone loss is likely to initially

affect areas composed primarily of trabecular bone due to its higher rate of bone turnover (approximately eight times higher) than cortical bone (1,2).



**Figure 1.1: Types of bone that exist within the human femur.** The figure depicts the distribution of cortical (compact) and trabecular (spongy) bone. Cortical bone forms the protective exterior shell of long bones and vertebrae while trabecular bone forms the main part of the vertebral body and the epiphyses of the long bones. The Figure is obtained and redrawn from reference number (1).

### 1.3) Bone homeostasis

Bone homeostasis is tightly regulated by remodelling, in which old bone is removed (bone resorption) and replaced with new bone (bone formation) (1-3). The two processes involve a number of bone cell types. The key cell types within the bony matrix are osteoclasts, osteoblasts and osteocytes (2). The cells that are responsible for bone formation and for producing bone tissue are osteoblasts. They are relatively immature cells line the bone and produce hormones, such as prostaglandins, enzymes such as alkaline phosphatase and

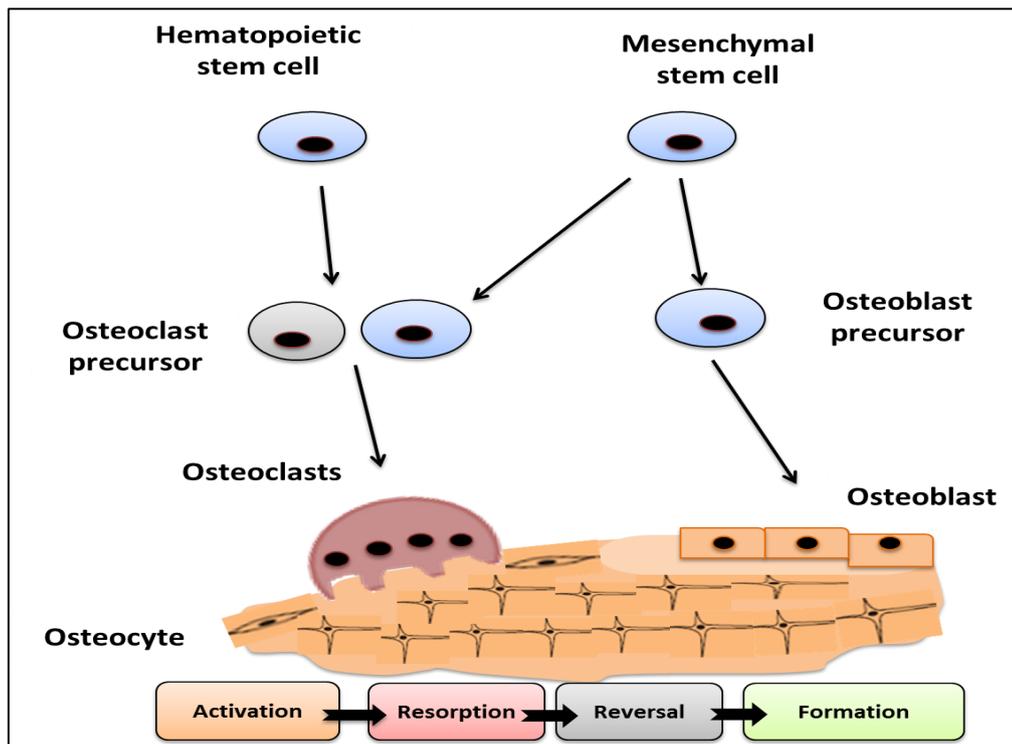
other matrix proteins such as osteocalcin, osteonectin, glycosaminoglycans, bone growth factors and sialoproteins (3).

Osteocytes are generated from osteoblasts that have migrated into the bone matrix and work as mature bone cells which function as mechano-sensory receptors to adjust bone responses to stress and also control bone formation, matrix maintenance and calcium homeostasis (4). They are the most abundant bone cells and are distributed throughout the matrix. Despite being embedded in bone, osteocytes stick to the bone surface as well as to each other via cytoplasmic processes that go through the bone in tunnels known as canaliculi. In addition to its essential function during mechanical loading, osteocytes play a key role in bone homeostasis via the production of sclerostin, an inhibitor of bone formation (5). Recently, investigators revealed that osteocytes are important sources of receptor activator for nuclear factor  $\kappa$ B ligand (RANK-L), a main factor for osteoclast development and bone loss, suggesting that they are key players in bone remodelling (6,7).

Osteoclasts are large multinucleated cells derived from mononuclear precursor cells of the monocyte/macrophage lineage that absorb bone matrix and cause bone resorption. The cells are generated under the influence of mesenchymal cells, which provide signals essential for their differentiation. These signals involve RANK, RANK-L, osteoprotegerin (OPG) and macrophage-colony-stimulating factor (M-CSF). RANK is receptor bound to the membrane of osteoclasts. It binds to RANK-L and this engagement triggers signaling cascades that lead to osteoclast-mediated bone resorption. RANK-L exists in soluble as well as tissue-bound forms. It belongs to the tumour necrosis factor (TNF) superfamily of ligands and receptors and is the main osteoclastogenic cytokine. RANK-L is inhibited by its natural decoy receptor osteoprotegerin (OPG). OPG is a key inhibitor of bone resorption that exists in a soluble non-signaling form (2-4,8). In both mouse and human, deletion of the gene that encodes OPG causes profound osteoporosis. In contrast, over expression of the gene that encodes OPG,

*TNFRSF11B* under a hepatic promoter in transgenic mice results in severe osteopetrosis as a consequence of a lack of osteoclasts. OPG expression is inhibited by pro-inflammatory cytokines such as TNF $\alpha$ , interleukin (IL)-1, IL-6, and IL-17 which, at the same time, enhance RANK-L expression with the net result being a rise in osteoclast formation and function (8).

The bone remodelling process occurs at highly regulated stages that depend on two cell lineages interactions, the hematopoietic osteoclastic lineage and the mesenchymal osteoblastic lineage. The first stage involves activation and this includes the interaction of osteoclast and osteoblast precursor cells (Figure 1.2). This results in the differentiation, fusion and migration of the large multinucleated osteoclasts. These cells stick to the mineralised bone surface and start the resorption stage by releasing hydrogen ions and enzymes, particularly cathepsin K, which can resorb all the components of bone matrix including collagen resulting in the formation of cavities on the bone surface. After osteoclasts complete their work in bone removal, the reversal stage begins through the appearance of mononuclear cells on the bone surface with the release of growth factors which initiate the formation stage. Throughout the formation stage of the remodelling processes, the cavity formed by resorption can totally be filled in by successive layers of osteoblasts, which differentiate from their mesenchymal precursors (3).



**Figure 1.2: Bone remodelling processes.** A cartoon depicting the remodelling cycle that consists of four stages: activation, which includes recruitment and activation of mononuclear monocyte/macrophage osteoclast precursors from the circulation; resorption, in which old bone is digested by osteoclasts; reversal, which involves the appearance of mononuclear cells on bone surface and formation, in which osteoblasts generate new bone until the resorbed bone is completely replaced. Osteocytes are generated from osteoblasts and are the most abundant bone cells. The RANK, RANK-L and OPG system regulate osteoclast formation, activation, and resorption. The Figure is procured and modified from reference number (3).

#### 1.4) Factors that influence changes in bone metabolism

Bone continuously remodels itself to maintain optimal mass and repair small and large damages such as fractures. There are a number of factors that affect bone metabolism and mass including deficient intake of calcium and vitamin D, sedentary lifestyle, smoking and excessive alcohol consumption (9). Bone mass is also influenced by genetic factors and ethnicity. For example, African-American females are more likely to achieve higher peak bone mass than Caucasian females. Furthermore, there is evidence that vertebral fractures are

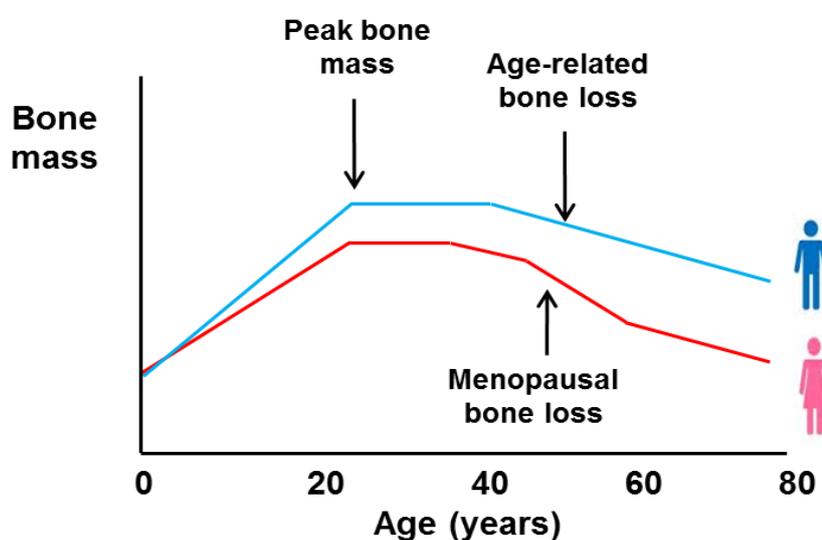
less common in Afro-Caribbean than Caucasian women and that the incidence of these fractures in Japanese women is similar to that in Caucasian populations (10, 11). The scientific basis for this observation is unknown.

The risk of bone loss is also enhanced by aging, menopausal status, diet, drugs, stress, injuries and by chronic inflammatory diseases, such as rheumatoid arthritis (RA) (9). In menopausal women, levels of oestrogen play an important role in the remodelling process. Oestrogen is, thus, responsible for maintaining bone density by stimulating osteoblasts to form new bone. The hormone is produced by the ovaries before menopause. After menopause, the ovaries stop producing oestrogen but the adrenal gland secretes small amounts of androgens and these are converted to oestrogen by the enzymes aromatase. As a result of reduced oestrogen levels in post-menopausal women, osteoblasts become less active resulting in decreased bone mass (12). Furthermore, long-term treatment of patients with medications such as corticosteroids causes bone loss by several mechanisms, which will be discussed in more detail in section 1.6.1.2 on pathological conditions leading to bone loss.

Bone metabolism is also influenced by major calcium-regulating hormones, including parathyroid hormone (PTH), calcitonin and 1,25-dihydroxy vitamin D. PTH is an effective stimulator of bone resorption and affects bone formation. Further, PTH tends to rise with age, which may lead to an increase in bone turnover and bone loss. In contrast, calcitonin (produced by the thyroid) is a potent inhibitor of bone resorption and osteoclast activity. 1,25-dihydroxy vitamin D is essential for the differentiation of both osteoclasts and osteoblasts and can stimulate bone resorption and formation. Furthermore, bone formation and resorption can be stimulated by growth hormones which act systemically and also through the induction of local insulin-like growth factor (IGF) production (13).

### 1.4.1 Changes in bone metabolism with age

In children, bone formation is most rapid as a consequence of more bone growth and lengthening. Before the age of 20 years, 90% of bone growth takes place. Bones grow their final 10% between the ages of 20 and 30 but become thicker rather than longer. At the age of 30 to 35, bones reach their largest and strongest levels. This level is called the “peak bone mass”. The process of bone loss starts from the age of 35 to 40, as bone resorption exceeds bone formation (Figure 1.3) (3). It has been shown that the rate of bone loss accelerates to about 2% per year in the early postmenopausal years in females, where bone loss from the spine is greater than of other sites of the skeleton. It has also been demonstrated that approximately, 35% and 50% of cortical and trabecular bone is lost from the skeleton, respectively (14).



**Figure 1.3: Age-related changes in bone mass in men and women.** The graph shows that at about 30 years of age bone density peaks. The process of bone loss begins from around the age of 35-40 years. In women, bone loss is more rapid than in men. The graph is redrawn from reference number (15).

## **1.5) Methods of assessment for changes in bone density**

A range of clinical and laboratory protocols are routinely used to monitor pathological changes in bone structure and density. Early diagnosis of pathological changes is essential to limit bone loss and effectively treat diseases such as osteoporosis. Bone mineral density (BMD) is a measure of the amount of bone minerals (calcium hydroxyapatite) per unit volume of bone tissue. It is calculated as grams per square centimetre of bone tissue and is determined using a number of methods including dual energy X-ray absorptiometry (DEXA) and peripheral dual energy X-ray absorptiometry, among others. Laboratory tests for changes to bone conditions include measurement of serum levels of bone-specific alkaline phosphatase, osteocalcin, deoxypyridinoline and carboxyterminal cross-linking telopeptide of bone collagen (CTX) (3,16).

BMD and bone mineral content (BMC) are used as standard units of measurement. BMD signifies total bone mineral mass (calcium hydroxyapatite) per unit area ( $\text{g}/\text{cm}^2$ ) of bone whereas BMC represents the total bone mineral mass in a specific region of the bone. Measurement of BMD at any skeletal site provides a predictive value of fracture incidence. However, measurement at the particular skeletal site of interest is usually most predictive for fractures at that site. Moreover, in order to predict osteoporotic fractures, assessment of the axial skeletal sites of the spine and femur has proved to be the most useful approach (17,18). The methods in general use for assessing bone are summarised below.

### **1.5.1 Radiographic methods:**

#### **a) Dual energy X-ray absorptiometry (DEXA)**

DEXA is currently the most widely used technique for the measurement of BMD in the diagnosis and follow-up of patients with osteoporosis due to its precision, accuracy and low radiation dose. The World Health Organization (WHO) standards for the diagnosis of

osteoporosis are based on DEXA (summarised in Table 1.1). DEXA, therefore, has become the gold standard for clinical bone densitometry measurements of the central and peripheral skeletal BMD (18-19). DEXA uses two X-ray beams of different energy levels that are absorbed differently by bone mineral and soft tissues. The method depends on measurement of the transmission of X-rays with high and low photon energies through various tissues in the body. The principle is based on the fact that absorption of X-rays in any material such as bone mineral and soft tissue (fat and muscle) is dependent on the X-ray energy as well as on the elemental composition of the attenuating material. Therefore, DEXA can accurately measure bone within soft tissue by separating the attenuation of transmission due to bone from that due to soft tissue. DEXA results, therefore, represent a composite measure of both cortical and trabecular bones in  $\text{g/cm}^2$  (19,20). DEXA scan of the lateral spine assesses mainly the vertebral body and avoids some measurement errors that can occur on anteroposterior (AP) spine testing, which also evaluates the posterior elements of the vertebrae, such as the pedicles, articulating and spinous processes (21).

**Table 1.1: The World Health Organization (WHO) classification of osteoporosis.**

|                     |  |
|---------------------|--|
| <b>Normal</b>       | BMD within 1 SD of young adult reference range.  |
| Osteopenia          | BMD more than 1 SD below the young adult mean but less than 2.5 SD of this value.            |
| Osteoporosis        | BMD 2.5 SD or more below the young adult mean.   |
| Severe Osteoporosis | BMD 2.5 SD or more below young adult mean and the presence of 1 or more fragility fractures. |

Data in the Table are for the WHO guidelines for assessment of fracture risk and its application to screening for postmenopausal osteoporosis (19).

**b) Peripheral dual energy X-ray absorptiometry (PDEXA)**

This technique measures peripheral skeletal sites using dual energy X-ray. While PDEXA of the wrist and calcaneus is more suitable and less costly than conventional DEXA, variations between peripheral and central sites are common and may result in the underestimation of fracture risks. However, PDEXA is a more useful procedure for older patients because at that stage bone loss in the peripheral skeleton would have reached central skeletal sites (21,22).

**c) Single X-ray absorptiometry (SXA)**

This protocol uses the same general principles as DEXA. However, SXA does not allow for adjustment for soft tissues as it utilizes a single energy beam only. Therefore, the use of SXA is limited to distal appendicular skeletal structures that have little interfering soft tissue. The most common sites for SXA are the calcaneus, the distal radius and ulna. This technology can be a useful and an inexpensive predictor of fracture risks particularly in older women (21).

**d) Quantitative ultrasound (QUS)**

QUS is one of the latest technologies approved for the assessment of bone density. The technology uses non-ionizing radiation; hence, it has the potential of becoming a low-cost alternative to bone densitometry. Ultrasound is transmitted from a transducer with frequencies in the range 0.2-1.0 MHz through regions of interest, e.g. calcaneus where both broadband ultrasonic attenuation (BUA) and the speed of sound are measured and are used to estimate BMD. Several prospective studies indicate that the predictive capability of QUS for hip fracture is as good as DEXA prediction of the femoral neck and that QUS and DEXA both predict hip fractures better than DEXA of the lumbar spine. Presently, the most important limitation of QUS use is the time needed to observe a significant change in BMD. Because the results of most clinical studies of therapy depend on the DEXA BMD, many researchers

believe that more information is needed before diagnosis of individuals can rely on QUS measures. At present, QUS is recommended as a screening instrument to help identify patients who should undergo further BMD testing. In the next few years, QUS could become a reproducible, inexpensive and noninvasive measure of osteoporosis risk (21,22).

**e) Quantitative computed tomography (QCT)**

QCT is an X-ray absorptiometric technique that uses a clinical computed tomography scanner to determine the true volumetric density ( $\text{mg}/\text{cm}^3$ ) of trabecular, or cortical bone in three dimension (3D). BMD can be calculated using this technology through the selection of bone regions of interest by the mean Hounsfield number (a quantitative scale for describing radiodensity) in the area selected. The ability of QCT to selectively assess the metabolically-active and structurally-important trabecular bone in the vertebral body provides for an excellent ability to differentiate vertebral fractures from healthy vertebrae and to also measure bone loss.

Currently, QCT is performed on the spine only but QCT measurement of the proximal femur is being developed in a few research centers. It has generally better sensitivity than other methods such as DEXA. However, its use is limited due to cost, high radiation dose and difficulty in being utilized when sequential measurements are needed (21).

**f) Radiographic absorptiometry**

This is a technique used for bone mass measurements from radiographs of peripheral sites, most commonly the hand, or heel. The technique relies on taking 2 X-rays of the site at slightly different angles and then analysing the data. Before imaging, for instance the hand, an aluminum alloy reference wedge is placed next to the middle phalanx of the index finger. The developed films are then sent to a central analysis unit and the average density of the middle phalanx of the middle 3 fingers is determined in radiographic absorptiometry units. These

units correlate with other bone density measurement methods. Despite the ease of application of this technology to existing x-ray technology, the usefulness of radiographic absorptiometry in predicting site-specific fracture risk remains to be established (21).

### **1.5.2 Laboratory biomarkers of bone metabolism:**

The development of biomarkers to assess the rate of bone remodelling is one of the important advances in managing bone loss. The process of bone remodelling can be assessed using biomarkers of bone resorption and synthesis with measure of static bone density. Bone formation can be evaluated by proteins that are released from osteoblasts such as osteocalcin, specific alkaline phosphate and pro-collagen peptides while bone resorption is generally indicated by products of collagen breakdown. When osteoclasts degrade collagen, cross-links of collagen and their adjacent peptides are released into the circulation and excreted in urine (e.g. urinary N-telopeptide cross-links of collagen and C-telopeptide cross-links of collagen). Cross-linked type I collagen is the most widely used biomarker of bone resorption. Since bone resorption and formation are linked, biomarkers of turnover change in sequence. In response to therapy, for example, changes in bone resorption biomarkers precede those of bone formation (22).

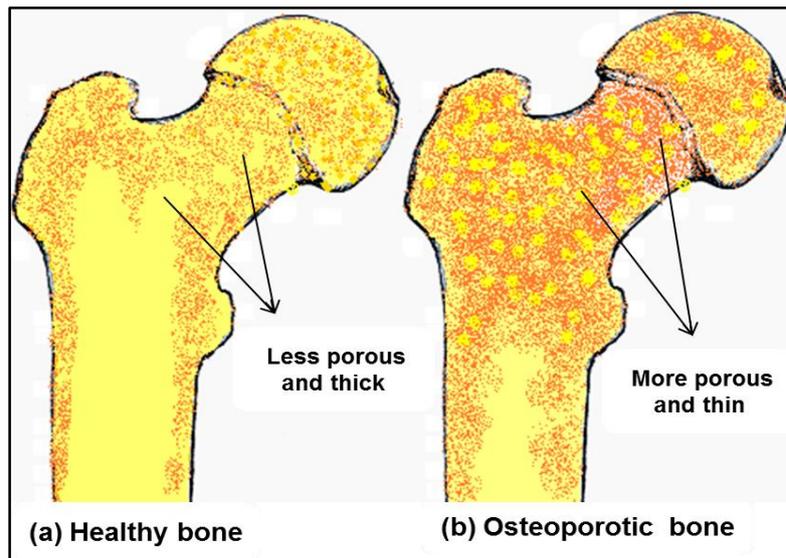
### **1.6) Pathological conditions associated with changes in bone metabolism**

Bone is maintained in a healthy state by balanced metabolism involving resorption and new bone formation. Alteration to this balance causes metabolic bone diseases including osteoporosis, rickets, osteomalacia, osteopetrosis and Paget's disease of bone. These metabolic bone diseases may result in bone pain and loss of height (due to compression of vertebrae) and they predispose patients to fractures.

### 1.6.1) Osteoporosis

Osteoporosis is a systemic skeletal disease characterised by low bone mass and microarchitectural deterioration of bone tissue with a consequent increase in bone fragility that could lead to fractures (16). This is a condition often seen in postmenopausal women with a rate of one in three over the age of 50 years worldwide affected (Figure 1.4) (17,21).

Osteoporosis starts when the regular processes of bone formation and resorption become unbalanced culminating in net bone loss. Fractures can occur as a result of decreased bone mass and its strength. Vertebra, hips and wrists are the most common sites where osteoporotic fractures occur. Osteoporosis can be categorised as primary or secondary (21).



**Figure 1.4: Trabecular bone tissue in a healthy adult and a patient with osteoporosis.** In osteoporosis, bone mass and its microarchitecture deteriorate and become porous leading to bone fragility and fracture. The Figure is obtained and redrawn from reference number (1).

### **1.6.1.1) Primary osteoporosis**

Reduction in bone mass not mediated by chronic illnesses or by medication and is a consequence of aging and decreased gonadal function is referred to as primary osteoporosis. This is subdivided into two types: postmenopausal osteoporosis (also known as type I osteoporosis) and age-related osteoporosis (also referred to as type II osteoporosis) (21).

#### **a) Postmenopausal Osteoporosis**

Postmenopausal osteoporosis (type I) is the most common osteoporosis condition mediated by reduced levels, or lack of oestrogen production during the postmenopause (23). Oestrogen deficiency promotes osteoclasts degradation of bone without sufficient new bone formation by osteoblasts, resulting in osteoporosis. Normally, osteoclasts have receptors for oestrogen, oestrogen receptor alpha and beta (ER $\alpha$  and ER $\beta$ ), which play a key role in the synthesis of inhibitory proteins and inhibit osteoclast formation when they bind to oestrogen. However, their role is affected when oestrogen production is deficient post-menopause, resulting in a large pool of osteoclast progenitors (23). Furthermore, osteoclast apoptosis is inhibited with all events together culminating in bone resorption (23). It has been demonstrated that osteoclast formation is inhibited by oestrogen *in vitro* when osteoclast precursors are co-cultured with osteoblastic cells. However, oestrogen did not directly change precursors of osteoclast when they were cultured alone (24). The outcome of this study strongly indicated that oestrogen regulation of osteoclast activity is controlled by osteoblasts (24).

#### **b) Age-related osteoporosis**

Age-related osteoporosis occurs both in men and in women. However, prevalence of the disease is 2-3 times higher in women than in men. Age-related osteoporosis in women involves two stages, a rapid stage that starts at menopause and takes 4–8 years followed by a slower continuous stage that continues throughout life (23). In contrast, men go only through

the slow continuous stage. Therefore, women normally lose more bone quicker than men. The slower rate of bone loss result from a group of factors including age-related deficiency of bone formation, reduced calcium and vitamin D intake, reduced physical activity and a decrease in calcium absorption through the intestine and the kidney due to the loss of oestrogen's positive effects on calcium balance. As a result of the reduction in blood calcium, parathyroid hormone levels will increase leading to calcium depletion from bone to make up for the loss. The outcome of this process is an increase in bone resorption (23). In aging men, sex steroid deficiency is a key factor in age-related osteoporosis (23).

#### **1.6.1.2) Secondary osteoporosis**

In addition to primary osteoporosis, bone loss can occur secondary to a number of chronic conditions such as, endocrine disturbances, cancer, gastrointestinal diseases, renal failure and inflammatory diseases such as RA. The condition could also be induced with long-term treatment with glucocorticoids. Therefore, secondary osteoporosis can occur at any age and affect men and women similarly (21). Some common examples of secondary osteoporosis are discussed below in more detail.

##### **a) Bone loss induced by cancer**

Bone cancer and, more frequently, metastasised cancers from outside of the skeleton to the bone can lead to osteoporosis. The outcome of both types of cancers is generally bone loss but some metastatic cancers can cause increase of bone formation. Breast and prostate cancers often metastasise to the bone leading to breaks (osteolytic lesions) or the increase in new bone formation (osteoblastic lesion). Cancer cells can secrete parathyroid hormone related peptide (PTHrP), which induces osteoclast differentiation via up-regulation of RANK-L, thereby causing bone destruction (25). Tumour-induced bone resorption, in turn, induces the secretion of growth factors from bone which further promotes tumour growth (26). Bone destruction also occurs in most patients with multiple myeloma, leukemia, Burkitt's

lymphoma and non-Hodgkins's lymphoma, in which tumour cells secrete bone-resorbing cytokines (25). Severe bone pain, pathologic fractures, spinal cord compression and life-threatening increases in blood calcium levels can be the consequences of bone destruction (26).

#### **b) Bone loss induced by Glucocorticoids**

Glucocorticoids (GCs) are effective immunomodulatory drugs frequently used to treat a wide range of inflammatory conditions including autoimmune diseases, such as RA, asthma, allergies and multiple sclerosis. However, several clinical complications including bone loss are caused by treatment with GCs (27). Bone loss is commonly noted within the first few months of treatment with GCs. With prolonged GC-based treatment a significant reduction in bone mass and an increased risk of fracture occurs (28). Treatment with GCs promotes bone loss mainly by targeting osteoblasts and reducing their function and promoting their apoptosis. High GC levels induce apoptosis of osteocytes (29) and increase osteoclast formation and function by increasing RANK-L expression and decreasing OPG production by osteoblasts (27). Treatment with GCs also affects other body processes such reduction of calcium resorption in the intestine and an increase excretion in the kidneys. This causes an imbalance in calcium levels and a gradual depletion from the bone. In addition, GCs reduce sex hormones, which play key roles in bone metabolism (27).

#### **c) Bone loss induced by chronic inflammatory diseases**

Patients with chronic inflammatory joint diseases such as RA manifest periarticular bone loss due to erosions, generalised bone loss and are at a higher risk of fractures compared with matched healthy controls (30,31). Although, there are multiple causes for bone loss in inflammatory diseases, many animal models of arthritis as well as evidence from patients to indicate that inflammatory mediators, including TNF $\alpha$  play a major role in local and generalised bone loss (32). TNF $\alpha$ , a key cytokine in human inflammatory disorders, causes

bone erosions in experimental models and these effects are induced through the induction of osteoclasts. RANK, its ligand, RANK-L and OPG are significant mediators in inflammatory processes and are critically-involved in the pathophysiology of bone loss (32). Since RA represents a typical example of systemic inflammatory diseases that causes significant changes in bone metabolism, it will be discussed in more detail. In addition, the key role of TNF $\alpha$  plays in bone loss will be further explored.

### **(i) Rheumatoid arthritis (RA)**

RA is a chronic symmetrical inflammatory destructive joint disease of unidentified etiology that affects 0.5-1% of the world's population. The production of autoantibodies and pro-inflammatory cytokines such as TNF $\alpha$  causes chronic inflammation, which leads to joint damage, but generalised bone damage is also a feature of the disease (33). Bone damage in RA occurs very early in the course of the disease, progresses rapidly and is not repaired as it would be under physiological conditions (34). The detrimental effect of chronic inflammation on bone is evident in the increase of fracture risks in RA patients; the more inflammation that is present the greater the risk of fracture. Indeed, decreases of 2.5% in vertebral and 5% femoral neck BMD are apparent just in the first year of RA disease and such loss doubles in the second year if disease activity remains uncontrolled (35). With regards to mechanisms of bone loss in RA, the available evidence indicates that pro-inflammatory mediators, including TNF $\alpha$ , promote osteoclastogenesis. Although the perception has been that bone loss in RA due to pro-inflammatory mediators is most likely to be confined to the periarticular region of affected joints, emerging evidence from the use of biological anti-inflammatory agents provide compelling evidence for their involvement in generalised osteoporosis too. This evidence highlights the overlap between inflammatory pathways in RA and mechanisms of bone resorption including direct and indirect effects on osteoclastogenesis. Thus, mice engineered to lack osteoclasts do not develop bone erosion in arthritis induced by TNF $\alpha$  (36).

Further, this appears to occur through the ability of TNF $\alpha$  to induce RANK-L expression of T-lymphocytes and osteoclasts (37,38). Indeed, when RANK-L is inhibited, the formation of osteoclasts in arthritic joints is inhibited (39,40). Interestingly, however, inhibition of RANK-L does not appear to have any impact on the inflammatory process (41,42). Thus, a phase 2 clinical trial of denosumab in RA showed that the agent reduced bone erosion, but had no effect on disease activity (43). This data indicates that interfering with the RANK/RANK-L/OPG pathways have few detrimental effects on the immune system.

In contrast, data on the effect of biologic inhibitors of inflammation suggests that the inflammatory response in RA promotes RANK-L expression and bone resorption, not only in the periarticular region, but also generalised bone loss. Support for this notion is provided by observations in which treatment with biologic anti-inflammatory agents including anti-TNF $\alpha$  agents reduced RANK-L expression and increased OPG production (44). Furthermore, treatment of psoriatic arthritis patients with biologic anti-TNF $\alpha$  agents reduced the number of peripheral osteoclast precursors (45).

The association between chronic inflammation and bone erosion in RA is clouded by the fact that a significant proportion of patients are treated with glucocorticoids (GCs). GCs are used for their anti-inflammatory efficacy, but their long-term use leads to bone loss as cited above. Therefore, it has been difficult to discern exactly how much each of these two events play in bone loss in RA. In this respect, it is interesting to note that a recent study revealed that osteoclast induction in RA is inhibited with a compound modulator of the monomer glucocorticoid receptor (46). This compound does not induce glucocorticoid receptor dimerisation but suppresses the production of pro-inflammatory cytokines in fibroblast-like synovial cells from patients with RA and in osteoblasts (46). This observation further highlights the direct relationship between chronic inflammation and bone loss in RA.

## **(ii) Early diagnosis of patients with RA**

In recent years, early diagnosis of RA has become feasible. This has been achieved as a result of the development of better diagnostic laboratory tests including new more predictive biomarkers and refined combinations of radiographic and surgical protocols (47,48). Thus, criteria for the diagnosis of RA currently includes the presence of elevated inflammatory mediators, erosions detected by radiography, the presence of subcutaneous nodules and positive tests for rheumatoid factor (RF) and autoantibodies to cyclic citrullinated peptides (CCPs). Previously, the lack of specific and reliable sensitive biomarkers has meant that differential diagnosis of early RA has been difficult. The current clinical approach for RA management is to achieve remission and prevent joint and bone damage by applying early diagnosis and aggressive early treatment (47,48).

### **a) Anti-CCP autoantibodies**

Laboratory tests for anti-CCP autoantibodies are the most relevant among the recently-employed laboratory measures to aid in the early diagnosis of RA. These autoantibodies have a high specificity for RA of ~96% and are different from RF as they are not detectable in the blood of healthy individuals nor in patients with other connective tissue diseases. The specificity of RA diagnosis reaches ~100% when both RF and anti-CCP are positive in patients with early undifferentiated arthritis (49). In addition, recent studies have indicated that high levels of anti-CCP autoantibodies can predict disease chronicity and the likelihood of developing erosions. However, despite their high specificity, tests for anti-CCP autoantibodies are not very sensitive as they are detected only in up to 50% of patients with RA (49).

In addition to their predictive diagnostic values, anti-citrullinated protein antibodies (ACPA) have been reported to be associated with bone resorption in RA. These autoantibodies help in the migration of cells to the synovium and contribute to the production of inflammatory cytokines which stimulate osteoclastogenesis. In addition, a recent study

provided evidence for a direct role of these autoantibodies in the stimulation of osteoclasts and their precursors independent of inflammation (50). Citrullinated vimentin, a common target for autoantibodies found in RA patients, has been shown to be expressed on the cell surface of osteoclasts and their precursors where it can be targeted by ACPA (51). The binding of ACPA to pre-osteoclasts leads to increased osteoclastogenesis due to the production of TNF $\alpha$  which results in increased bone resorption *in vitro* and *in vivo*. These findings suggest that these autoantibodies can directly influence bone metabolism.

Although treatments that directly target ACPA or B-lymphocytes that produce such autoantibodies have not been developed yet, total ablation of B-cells by anti-CD20 monoclonal antibody (rituximab) has been highly successful and has been approved by the National Institute of Clinical Excellence (NICE) for use in treatment of RA patients who have not responded to treatment with biologic anti-TNF $\alpha$  agents (50). Rituximab causes B-cell depletion and has been shown to lead to a significant reduction in autoantibody levels and bone loss after treatment (51).

**b) Radiographic procedures for the early detection and assessment of bone erosions in RA**

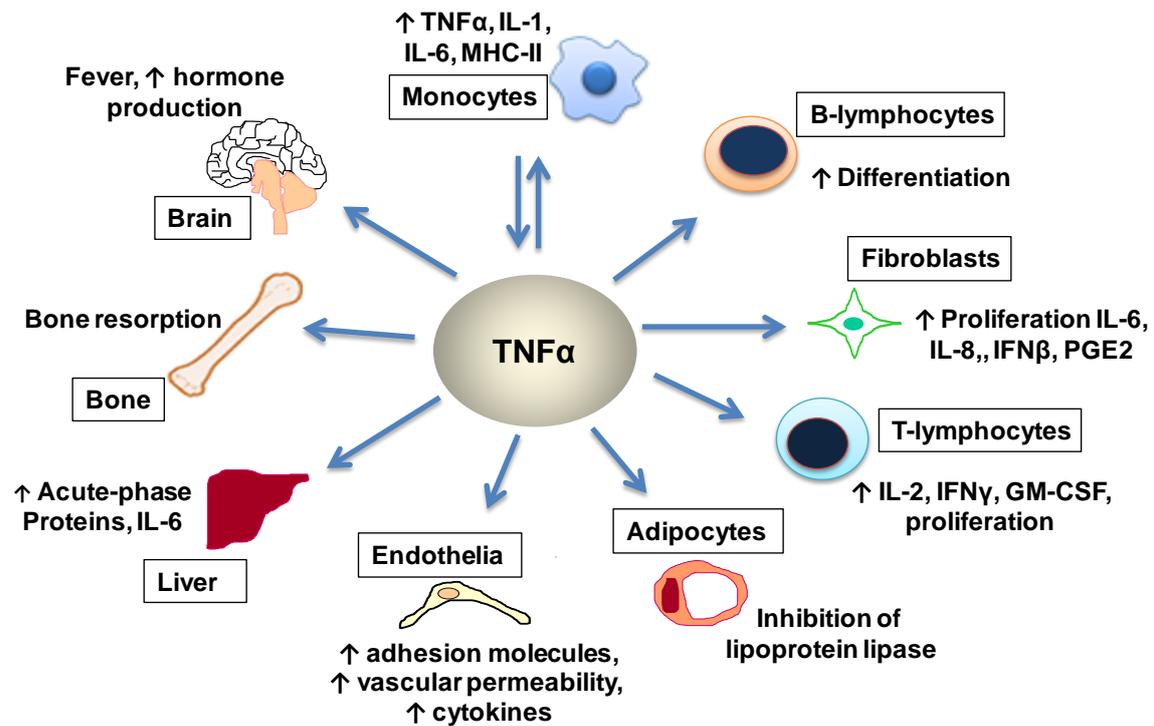
Conventional radiographic procedures are not able to help in the early identification of bone erosion in RA because they only show joint damage. However, the use of MRI has made it possible to detect early inflammatory changes in RA synovia including synovitis and joint effusion. Using MRI, bone erosions can be seen within the first four months of the onset of disease symptoms (52). However, there are some disadvantages with the routine use of MRI including limited availability, long times for investigation and high costs (53).

### **c) Ultrasound (US) techniques**

The application of US for assessing disease in patients with RA includes monitoring of response to treatment and for guiding intra-articular procedures. In recent years, US was widely used for the evaluation of the response to treatment with different drugs, especially in patients treated with biological therapies (54). The usefulness of joint examination by US has been evident in enabling early diagnosis of patients with undifferentiated arthritis (55). Active inflammatory changes such as synovitis and increased synovial blood flow can be detected using the Doppler ultrasound technique. This technique is more sensitive than clinical examination, especially for evaluating synovitis in early RA and is better than X-ray radiography for detecting erosive changes (55). Furthermore, US is a quick, inexpensive and bedside imaging technique are accepted by young patients as it does not require anaesthesia, as with magnetic resonance, and does not involve radiation (56).

### **(iii) Bone loss in RA: The role of the TNF $\alpha$**

TNF $\alpha$  is a pleiotropic cytokine that affects a range of biological responses, cells, tissues and organs including the immune system (Figure 1.5) (57). In response to injury or infections, TNF $\alpha$  is initially produced by macrophages. This helps the immune system mount a response to the injury/infection and amplify the response further by attracting and activating adaptive immune cells and relevant proteins to the site resulting in acute inflammation. TNF $\alpha$  achieves its pro-inflammatory and immune effector functions by promoting lymphocyte and neutrophil adhesion, haemopoiesis, collagenase and prostaglandin E2 production and the induction of other pro-inflammatory cytokines. TNF $\alpha$  also contributes to proteoglycan breakdown, acute tubular necrosis and bone resorption (8).

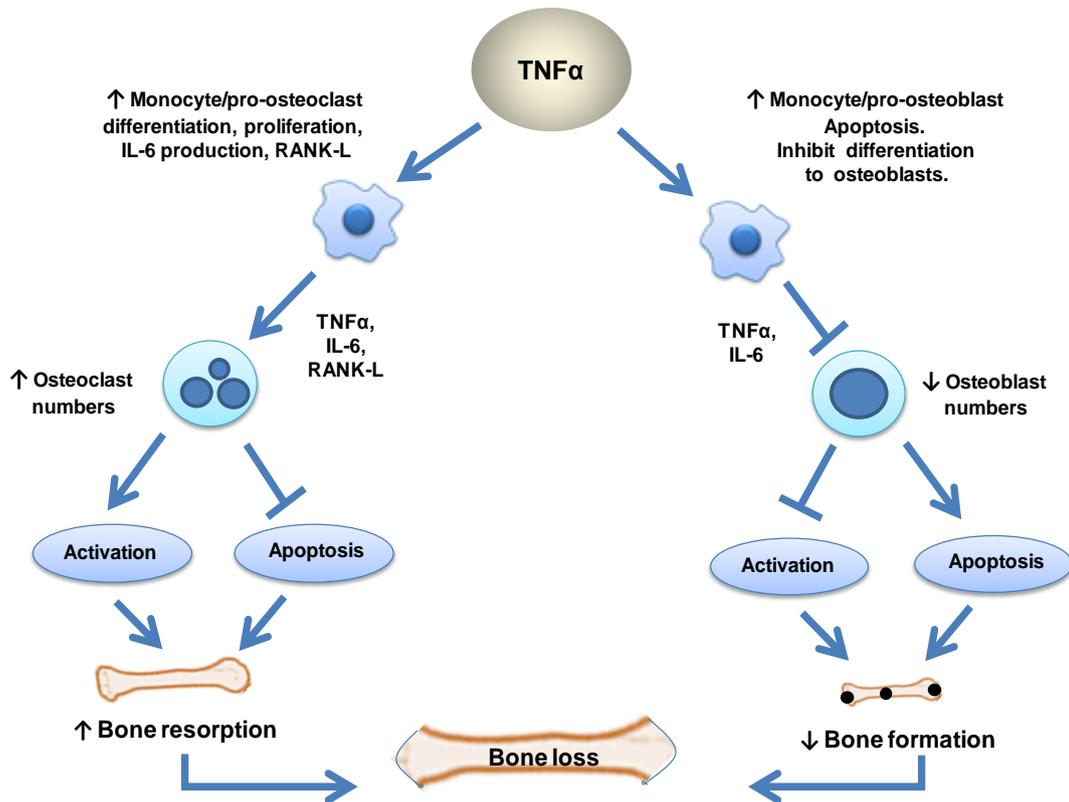


**Figure 1.5: Biological responses effected by TNF $\alpha$ .** A cartoon summarising the wide ranging effects of TNF $\alpha$  on cells, organs and tissues. The outcome of TNF $\alpha$  binding to its receptors on its targets is indicated. The figure is drawn based on information published in reference number (57).

#### a) Osteoclastogenic effects of TNF $\alpha$

Bone resorption results from the activation of NF- $\kappa$ B transcription factor. NF- $\kappa$ B is primarily an inflammatory response transcription factor that is induced, amongst other stimuli, by RANK/RANK-L signaling. Activation of NF- $\kappa$ B is a key target for TNF $\alpha$  signaling through TNF receptor 1 (TNFR1), which is expressed on macrophages and osteoclast precursors. In this respect, TNF $\alpha$  is an established inducer of osteoclast formation and bone resorption (58). Thus, TNF $\alpha$  can promote the differentiation of pro-osteoclasts into mature osteoclasts even in the absence of signaling through RANK (59). Moreover, it has been shown that TNF $\alpha$  enhances osteoclast differentiation indirectly by increasing the expression of M-CSF and RANK-L in osteoblasts during inflammation (60). Furthermore, TNF $\alpha$  inhibits osteoclast apoptosis by activating the mammalian target of rapamycin/S6

kinase (mTOR) (61). These processes lead, ultimately, to increased bone resorption due to increases in the number and, potentially, long-lived osteoclasts in pro-inflammatory conditions (Figure 1.6).

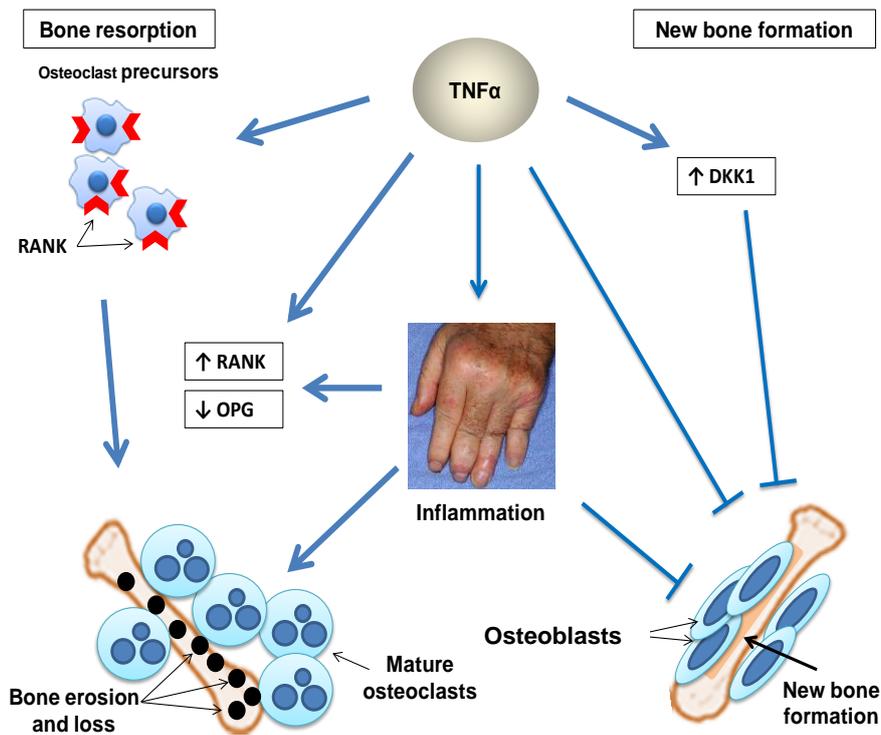


**Figure 1.6: Pathways of bone loss influenced by TNF $\alpha$ .** TNF $\alpha$  promotes osteoclastogenesis by directly activating macrophage and the differentiation of pre-osteoclast. It also activates mature osteoclasts and inhibits apoptosis. TNF $\alpha$  also promotes bone degradation through inducing IL-6 and RANK-L production. Finally, TNF $\alpha$  suppresses osteoblastogenesis by inhibiting the proliferation and differentiation of mesenchymal stem cell (MSCs) into osteoblasts. TNF $\alpha$  also promotes osteoblast apoptosis. The Figure is adapted from reference number (60).

## **b) The effects of TNF $\alpha$ on osteoblastogenesis**

Excess TNF $\alpha$  production has been shown, both in *in vitro* and in animal models, to inhibit the proliferation and differentiation of osteoblasts and to promote their apoptosis. These effects appear to be induced through the TNFR1 and the activation of NF- $\kappa$ B, which is also involved in inhibiting osteoblast differentiation and activity (62-64). TNF $\alpha$  inhibits osteoblasts differentiation through reduced osterix expression, a key regulator of the initial stages of osteoblast differentiation (65). Furthermore, it has been reported that inhibition of the Wnt- $\beta$ -catenin pathway by TNF $\alpha$  promotes the upregulation of the Wnt inhibitor, Dickkopf-related protein 1 (DKK1) (Figure 1.7) (66,67).

In general, TNF $\alpha$  induces its biological effects by binding to either of its two cell-bound receptors, TNF-RI and II. It binds to these receptors and induces its biological effects as a trimeric molecule and initiates a cascade of signaling pathways. The two TNF $\alpha$  receptors can also be activated by lymphotoxin  $\alpha$  (LT $\alpha$ ), which has a similar structure and biological activities to TNF $\alpha$ . Soluble forms of the two TNF $\alpha$  receptors are, however, known to act as endogenous TNF $\alpha$  inhibitors including inhibiting bone resorption (8). Therefore, therapeutic inhibitors of TNF $\alpha$  have been believed at the outset to have a beneficial effect on BMD reduction and bone erosions in RA (68). This proposition has been verified by the use of biologic anti-TNF $\alpha$  agents in clinical practice to treat patients with RA. Currently, there are 3 main biologic anti-TNF $\alpha$  agents routinely used in the clinic. These biologic anti-TNF $\alpha$  agents are more effective when used in combination with methotrexate.



**Figure 1.7: TNF $\alpha$  promotes bone loss through impacting and activating a number of osteoclastogenic pathways and the production of proteins.** TNF $\alpha$  promotes bone resorption through enhancing osteoclastogenesis and RANK+ osteoclast precursor cell proliferation. It also induces RANK-L production. TNF $\alpha$  also inhibits osteoblast maturation and function, in part, by increasing the expression of the Wnt antagonist DKK1 resulting in impaired bone formation. The figure is based on information published in reference number (62).

### 1.6.2) Osteopetrosis

Osteopetrosis is a group of rare genetic bone-remodelling disorders characterised by the failure of osteoclasts to resorb bone, leading to increased bone density. The reduction of bone resorption is a result of mutations that affect osteoclast function and formation. Many gene mutations of osteopetrosis have been demonstrated in molecular genetic studies. All of the identified gene mutations are encoded in proteins that engaged in bone loss controlled by osteoclasts (69).

### **1.6.3) Paget's disease**

Paget's disease is a chronic bone remodelling disorder characterised by high bone turnover, leading to unbalanced bone formation and resorption (70). Paget's disease is thought to be a result of abnormal activation of osteoclasts, to which there is an observable increase in osteoblastic activity. Therefore, bone density is increased in Paget's disease, however, because of the irregular bone pattern, the strength is reduced and fractures may occur. Furthermore, Paget's disease has a genetic component that may increase frequency of osteosarcoma in the affected areas of the bone (70).

### **1.7) Treatment of patients with osteoporosis**

The aim in treating patients with osteoporosis is to inhibit and/or restrict the activity of osteoclasts, the enhancement of osteoblast activity and the regulation of bone marrow adipogenesis. The ultimate objective is to reduce risk factors of bone fracture. At present, key classes of agents include anti- resorption agents, which target osteoclasts and anabolic agents which modulate osteoblasts. There are three classes of drugs currently in use for treating osteoporosis: hormone replacement therapy (HRT), selective oestrogen receptor modulators (SERMs) and bisphosphonates.

#### **a) Hormone replacement therapy (HRT)**

HRT is used to reduce chances of osteoporosis and fractures occurring in postmenopausal women. HRT helps to avoid discomfort and health problems caused by reduced circulating oestrogen and progesterone levels. The binding of oestrogen to its receptors in bone stimulates the proliferation of osteoprogenitors and osteoblast-like cells and enhance the production of growth factors. The productions of hormones that influence bone metabolism (vitamin D3, parathyroid hormone, and calcitonin) are also stimulated by oestrogen (71). The use of HRT has been controversial, however, as risks of HRT may surpass its benefits. For example, a number of studies have shown that HRT could increase the risk of breast cancer,

thromboembolism and cardiovascular disease. Nevertheless, HRT is effective in preventing osteoporosis and, thus, reduces the risk of fractures (71-74).

In order to obtain the greatest beneficial effects of HRT on bone status, treatment with HRT needs to start before the age of 60 and to be taken for at least 5 years. Generally, the effect of HRT on bone varies with dose and dosage requirement varies with age. A typical dosage of 40-60pg/ml slows down the rate of bone resorption. This translates to a 5-10% increase in BMD within 1-3 years regardless of the time since menopause (71,73).

### **b) Bisphosphonates**

Bisphosphonates are used clinically to inhibit resorption through a mechanism that differs from that of other anti-resorptive treatment (75). Bisphosphonates bind to hydroxyapatite binding sites on bony surfaces, particularly areas exposed to active resorption. When bone resorption is mediated by osteoclasts, bisphosphonates reduce the ability of osteoclasts to create ruffled borders, to stick to the bony surface and to secrete proteins required for continued bone resorption. Furthermore, bisphosphonates reduce the number of the osteoclasts and their activity by promoting their apoptosis (75,76).

There are many generations of bisphosphonates, all of which are synthetic analogues of cellular inorganic pyrophosphonates. The first generation includes non-nitrogen containing agents whereas the latest generations are aminobisphosphonates that are administered orally or intravenously (77).

Bisphosphonates are also used for the treatment of patients with metastatic bone disease resulting from breast cancer to reduce fractures and hypercalcemia. Alendronate (Fosamax) and risedronate (Actonel) are the most commonly used bisphosphonates in clinical practice. Both have been shown to increase bone mass and significantly reduce fracture risks by approximately 50% in the spine and 30% at other sites in patients. However, as with other

drugs, bisphosphonates has side-effects, such gastrointestinal tract disturbance and, more seriously, as osteonecrosis of the jaw (77,78).

Recent studies of bisphosphonate use have revealed additional effects for this class of drugs. For example, a study by Kobayashi and colleagues of Japanese patients reported inhibitory effects by bisphosphonates on glucocorticoid-induced RANK-L expression in human cells (79). These investigators concluded that bisphosphonates, but not non-steroidal anti-inflammatory drugs (cyclooxygenase inhibitors) suppressed RANK-L upregulation. In addition, a study by Valleala and colleagues reported that recurrent cyclical therapy of the bisphosphonate (Etidronate) has a beneficial effect on general bone metabolism as indicated by reduced serum levels of both N-terminal propeptide (PINP), an indicator of bone formation, and crosslinked C-telopeptide (ICTP), a marker of collagen degradation (80). Etidronate did not, however, prevent radiographic progression in patients with inflammatory disease such as RA.

### **c) Selective oestrogen receptor modulators (SERMs)**

SERMs are used for their ability to bind oestrogen receptors and act either as oestrogen-agonists, or as oestrogen-antagonists depending on the targeted tissue and hormonal status. The first generation of SERM was tamoxifen, for which oestrogen-like agonist activity on bone was seen to occur at the same time with oestrogen antagonist activity on the breast. A drawback of tamoxifen use has been its oestrogen-like action on the endometrium. Second-generation SERMs have since been developed, such as raloxifene, which has oestrogen-like actions on bone, lipids and the coagulation system and oestrogen antagonist effects on the breast and uterus. Raloxifene has been used as an alternative to HRT in the treatment of osteoporosis without deleterious effects on the endometrium. The effect of raloxifene is dependent on dosage. Patients on raloxifene have a significant increase in BMD

at the lumbar spine, hip and whole body. Raloxifene can also cause a decrease in total cholesterol and LDL levels, with added benefits for patients with cardiovascular risk (81,82).

#### **d) Calcitonin (CT)**

CT is an endogenous polypeptide hormone that suppresses osteoclast-mediated bone resorption. It binds to its receptor on osteoclasts, leading to a fast loss of the osteoclast's ruffled border, termination of motility and suppression of the ability of osteoclasts to release essential enzymes and protons that partake in bone resorption. The reduction of osteoclast bone resorption by calcitonin is dose-dependent but is not paralleled by a reduced activity of osteoblasts (83). Similarly to oestrogen, CT promotes an increase in bone mass, enhances intestinal calcium absorption and has a positive effect on calcium balance (17).

The analgesic effects of CT have added beneficial effects for patients treated with hormones. Calcitonin can be administered either by injection or as a nasal spray. Injections of CT are normally given only as a short-term treatment for painful vertebral fractures, but the nasal spray may be used as a long-term treatment for osteoporosis (84). However, serious allergic reactions have been reported and skin testing before treatment should be considered (85). Injected calcitonin may cause hypocalcemic tetany and parenteral calcium is recurrently required during the first several administrations. Possible side effects of the nasal spray include hot flushes, nausea and blocked or runny nose (84). A Food and Drug Administration (FDA) advisory group advised that the CT benefits in postmenopausal osteoporosis is uncertain and is associated with serious side effects (86). A newly developed oral formulation of CT has been shown to have increased efficacy in phase II and III clinical trials. The introduction of this new formulation is likely to enhance patient compliance as it has fewer side effects (87,88).

#### **e) Teriparatide (Forsteo)**

Teriparatide is the first bone anabolic agent with established anti-fracture effects and an acceptable safety profile (89). It is a polypeptide synthesised using recombinant DNA technology and functions as an active part of human parathyroid hormone (PTH). PTH is the key regulator of calcium/phosphate metabolism in the bone and kidneys.

Unlike anti-resorption agents, teriparatide increases bone formation, cortical thickness and trabecular bone connectivity (90). However, due to excessive new bone formation and osteosarcoma in long-term toxicology studies in rats, this treatment has been limited to a maximum of 24 months until further data is available (91). At present, teriparatide is used mainly as a final treatment option for established osteoporotic patients, particularly patients who have had fractures despite using other treatments (92).

#### **f) New anti-osteoporosis therapies**

Denosumab is an effective anti-resorptive agent that was approved recently by the FDA for treatment of postmenopausal women at high risk of fracture and to increase bone mass in patients who are at high risk for fracture due to treatments as in patients with cancer and patients with chronic inflammatory diseases such as RA. The agent is injected subcutaneously with long dosing intervals and no limitations relating to renal function. Denosumab is a fully human monoclonal antibody that binds to and inhibits RANK-L. When denosumab binds to RANK-L, it prevents RANK-L–RANK interaction in much a similar way to OPG, causing a reduction in osteoclast-mediated bone resorption (93).

There has been an ever-increasing number of studies to show that denosumab increases BMD levels and reduces bone turnover biomarker levels in postmenopausal women with low BMD. It also increases BMD greater than alendronate does and decreases fracture risk at the lumbar spine, non-vertebral sites and the hip (94,95).

## **1.8) Biologic anti-inflammatory agents and their effect on bone loss in RA**

### **a) Etanercept**

Etanercept was the first biological agent to be approved for the treatment of patients with RA and, subsequently, for refractory juvenile RA. It is a recombinant dimeric form of the soluble TNF $\alpha$  receptor II that binds with high affinity to and inhibits the activity of both TNF $\alpha$  and LT $\alpha$  (8). It is administered subcutaneously twice weekly. In 2004, the TEMPO study reported that the combination of methotrexate and etanercept at 25mg twice weekly, resulted in improvement in disease activity and reduced bone erosion (96). Thus, 37% of the patients had improved DAS28 scores while 80% had no signs of joint damage (96,97).

### **b) Infliximab**

Infliximab is a chimeric mouse-human monoclonal antibody that binds to and neutralises TNF $\alpha$ . Infliximab was initially approved for treatment of Crohn's disease, but was subsequently used for the treatment of patients with RA, mostly in combination with methotrexate. Clinical evidence consistent with a beneficial effect of infliximab on BMD has started to appear in recent years (98,99). One of these studies was a case-control trial by Marotte and colleagues in which 99 patients with RA treated with infliximab had evidence for maintaining BMD in the lumbar spine and femoral neck after 1 year (99). In contrast, bone loss of 3.9% and 2.5% was seen at the same sites in the control group treated with methotrexate alone. Changes in biomarkers of bone turnover from baseline, or between groups, however, were not significantly different (99).

### **c) Adalimumab**

Adalimumab is a parenterally-administered fully human monoclonal antibody which binds and neutralises TNF $\alpha$ . In a study by Weinblatt and colleagues, it was observed that the use of adalimumab in combination with methotrexate resulted in significant improvement in the

American College of Rheumatology 50 (ACR50) and DAS28 scores indicative of remission with significantly less radiographic disease progression (100).

Although the introduction of biologic anti-TNF $\alpha$  agents has largely revolutionised the treatment of RA, a proportion of patients do not respond to these agents (around 35-40% at the end of 6 months) (101), experience toxicity or have contraindications such as cancers or previous TB.

#### **d) Rituximab**

Due to the key role B-lymphocytes play in the pathogenesis of RA, including acting as antigen presenting cells, activating T-cells and producing RF and anti-CCP autoantibodies, the depletion of these cells was used as another approach for treating RA patients. Rituximab is a chimeric human-murine monoclonal antibody that depletes B-cells by binding to CD20 on the surface of B-cells. In clinical trials, rituximab was shown to be efficacious in treating patients with RA (102,103). Rituximab was approved 4 years ago in NICE for use in combination with methotrexate for the treatment of patients with RA who are refractory to at least one biologic anti-TNF $\alpha$  agent.

Rituximab is administered by intravenous infusion on two separate occasions 14 days apart. The difference between biologic anti-TNF $\alpha$  agents and rituximab is that the latter is not associated with an increased risk of TB re-activation and previous history of cancer is not a contraindication. Emerging evidence indicates that rituximab substantially reduces autoantibody levels and bone loss in RA (104). Mechanistically, treatment of patients with rituximab decreases synovial osteoclast precursors and RANK-L expression and increases OPG/RANK-L ratio in blood (105,102).

#### **e) Abatacept**

Abatacept was recently introduced for the treatment of patients with RA. It is a selective inhibitor of cognate T-lymphocyte interaction with other immune cells. It is a recombinant fusion protein composed of the Fc region of human IgG1 and the extracellular domain of cytotoxic T-lymphocyte antigen 4 (CTLA-4). Abatacept binds with high avidity to CD80 (B7-1), CD86 (B7-2) and blocks their engagement with CD28 and T-lymphocyte activation. Clinical trials of abatacept in patients with RA have so far shown that patients refractory to methotrexate and biologic anti-TNF $\alpha$  agents respond well and have good ACR20 response rates at 6 months to 1 year. Moreover, the clinical response is maintained for up to 3 years (106).

In addition, studies using dynamic contrast enhanced (DCE) MRI and arthroscopy-acquired synovial biopsies have shown 15-40% improvement in MRI parameters together with increased plasma levels of OPG and reduced RANK-L in RA patients treated with abatacept (107). However, there are reports of higher rates of infection in abatacept-treated patients compared with patients treated with combination therapy involving biologic anti-TNF $\alpha$  agents and methotrexate (106). Recently, NICE approved the use of abatacept in RA in patients who have failed anti-TNF $\alpha$  therapy and are unable to receive rituximab, or have failed rituximab as a result of a side-effect/poor tolerability.

#### **f) Tocilizumab**

Tocilizumab is a monoclonal anti-interleukin 6 receptor (IL-6R) antibody that blocks IL-6 binding and disrupts the action. The AMBITION study evaluated the efficacy and safety of tocilizumab monotherapy versus methotrexate in patients with active RA refractory to methotrexate/biologic agents (108). The investigators concluded that tocilizumab monotherapy was more effective than methotrexate monotherapy leading to quick improvement in disease signs and symptoms and a favorable benefit-risk in patients for whom

treatment with methotrexate or other biological agents has previously failed (108). More recent evidence indicates that tocilizumab promotes the repair in bone erosions, particularly in large lesions with sclerosis (109).

**g) Anakinra**

Anakinra is a recombinant human interleukin-1 receptor (IL-1R) antagonist licensed for the treatment of patients with RA. In clinical trials, anakinra considerably reduced radiographic progression in RA patients compared with placebo. Furthermore, anakinra significantly decreased clinical symptoms of the disease when used as monotherapy, or in combination with methotrexate and it has been suggested that the agent protects bone and cartilage from progressive destruction in RA (110,111). However, more studies are needed to evaluate safety and efficacy of the agent.

In addition to the above cited therapies, a range of new biologic agents have recently been developed for treating inflammatory conditions such as RA. These include a number of biologic anti-TNF $\alpha$  agents, such certolizumab pegol and golimumab which were shown to be highly effective in treating patients with RA. Certolizumab pegol is characterised by rapid onset of action, longer half-life and reduced antigenicity compared with other biologic anti-TNF $\alpha$  agents. Since certolizumab pegol only contains of the Fab fragment (antigen binding fragment) of the anti-TNF $\alpha$  antibody and lacks the IgG FC fragment, it does not activate the complement system nor induce antibody-mediated cellular cytotoxicity (112).

Golimumab is a completely humanised monoclonal antibody shown to be effective in treating RA patients who fail other biologic TNF $\alpha$  agents. Golimumab has the advantage of being administered once monthly, subcutaneously or intravenously (113). A significant improvement in synovitis and bone erosion has been reported after a treatment with a combination therapy consisting of golimumab and methotrexate as compared with methotrexate alone (114).

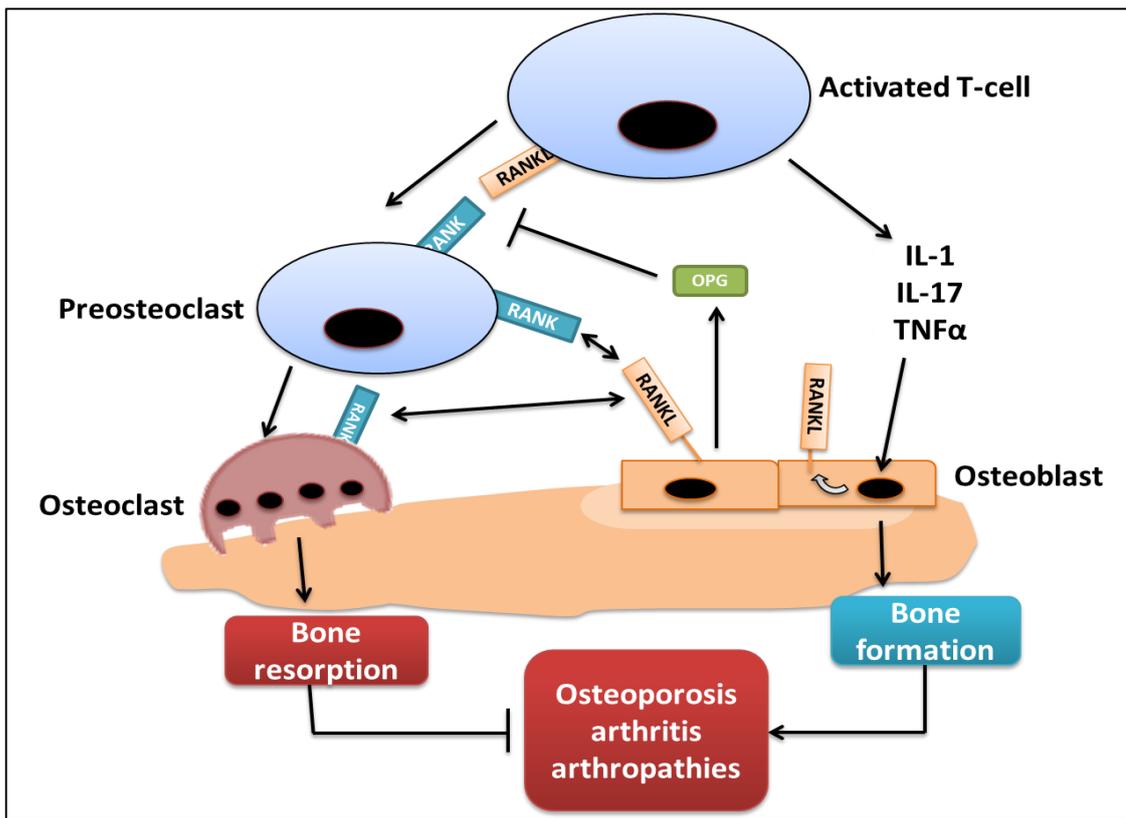
In addition to the established effects of TNF $\alpha$ , IL-1 and IL-6 on inflammation and bone loss, several other cytokines such as IL-15, IL-17, IL-32, lymphotoxins and B-lymphocyte stimulator (BlyS) could play roles in sustaining the inflammatory process and bone loss in RA. Each of these cytokines, therefore, presents a potential target for therapy. Furthermore, other than such drugs that target cytokines and receptors, small inhibitor molecules, such as kinase inhibitors, which target intracellular kinases involved in signal transduction, are also currently being assessed (115). In summary, in the next few years there will be new avenues for the development of novel therapies of RA and its associated bone erosions and joint destruction.

### **1.9) The relationship between Osteoporosis and RA**

As cited above, osteoclast activation is controlled by three principle components whose levels are influenced by TNF $\alpha$ : RANK, RANK-L and OPG. Higher levels of TNF $\alpha$  with inflammatory arthritides are associated with pathological changes to the bone. In RA, the pathological hallmark, however, is joint destruction with erosion of the bone, as a consequence of activated immune cells in the synovium which also promote osteoclasts generation and activation through TNF $\alpha$  signaling (116).

Although the effect of TNF $\alpha$ , produced by activated T-cells and monocytes, in osteoporosis driven by inflammatory conditions, or by infection is well recognised, only recently the role of TNF $\alpha$  in the postmenopausal and senile osteoporosis, as well as bone loss caused by a variety of endocrine conditions has begun to be recognised (117). The pathogenesis of bone loss in osteoporosis is multi-factorial; however, aging and menopause are the two key determinants of osteoporosis. *In vitro* and *in vivo*, oestrogen plays a protective role against osteoporosis via suppression of TNF $\alpha$ , IL-1 and IL-6 expression (117). Decreased production of TNF $\alpha$ , IL-1 and IL-6 has been reported in cultures of mononuclear cells derived

from either oestrogen-treated postmenopausal women with osteoporosis, or untreated premenopausal women. However, elevated levels of TNF $\alpha$  and IL-1 were reported in cultures of mononuclear cells derived from ovariectomised premenopausal women and osteoporosis patients (117). Elevated TNF $\alpha$  promotes osteoclast differentiation and activation by inducing RANK-L expression, suggesting an association between TNF $\alpha$  in postmenopausal women and osteoporosis too (118). Furthermore, knockout mice deficient in either TNF $\alpha$  or TNF $\alpha$  receptor I are resistant to ovariectomy-induced bone loss (119), indicating that TNF $\alpha$  a key player in oestrogen deficiency-induced bone loss (120). In addition, T-cells have been reported to play an essential role in regulating bone loss induced by oestrogen deficiency through their ability to produce TNF $\alpha$  (117) (Figure 1.8). T-cell- deficient nude mice and wild type mice in whom T-cells have been deleted are protected from ovariectomy-induced bone loss (120). This observation reinforces the proposition that circulating T-cells are the major source of oestrogen-regulated TNF $\alpha$ . In general, the available evidence confirms the involvement of TNF $\alpha$  in both of postmenopausal and RA related osteoporosis. Further, neutralisation of TNF $\alpha$  protects oestrogen deficiency-induced bone loss, which supports the hypothesis that neutralisation of TNF $\alpha$  could prove an efficient strategy in the treatment of inflammation-related osteoporosis in the future.



**Figure 1.8: The known roles of activated T-cells in promoting bone loss.** Pro-inflammatory cytokines such as  $\text{TNF}\alpha$ , IL-1 and IL-17 that are produced by T-cells induce RANK-L expression in osteoblasts and osteoclast differentiation from progenitors via RANK-L-RANK engagement and signaling leading to bone resorption. The soluble decoy receptor for RANK-L, OPG, blocks both pathways which could be a potential therapeutic approach for patients with osteoporosis and RA. The Figure is obtained and redrawn from reference number (121).

### 1.10) Reduced bone loss in patients treated with biologic anti- $\text{TNF}\alpha$ agents

Randomised controlled trials have shown that biologic anti- $\text{TNF}\alpha$  agents delay radiographic joint destruction (122,123). These findings raise possibility that these agents also have the ability to prevent generalised bone loss in the spine and hip and local bone loss in the joints in patients with RA.

Lange and colleagues studied 26 patients (19 women) with persistently active RA (duration of 9.8 years) who were treated with biologic anti- $\text{TNF}\alpha$  agents (124). After 12

months of treatment with infliximab these investigators observed that there was a significant increase in BMD at the femoral neck ( $0.84\pm 0.33$  to  $0.95\pm 0.15\text{g/cm}^2$ ) and the spine ( $1.07\pm 0.14$  to  $1.10\pm 0.23\text{g/cm}^2$ ). In addition, there was a trend for a correlation between improvement in BMD and reduced in DAS28 but not with changes in biomarkers of bone remodelling (124). In another study of 102 patients, 53 years of age on average, including 82 females with active and severe RA treated with infliximab for 1 year, spine and hip BMD were unchanged (125).

The Behandel–Strategieën (BeSt) study prospectively compared the efficacy of 4 treatment strategies in RA: sequential monotherapy of several disease modifying anti-rheumatic drugs (DMARDs), step-up combination therapy, initial combination therapy with tapered high-dose prednisone and initial combination therapy with infliximab (126). These investigators reported that there were no significant changes in BMD after 1 year. Of importance is the strategy that underpinned this study: sequential assessment of patients with therapies aiming at complete control of inflammation. Other studies of biologic anti-TNF $\alpha$  agents reported that reduction of bone loss was associated with improvement in disease activity and a reduction in inflammation (100,125,127,128). These observations indicate that effective control of inflammation, rather than any specific drug therapy, is a likely mechanism by which bone loss is prevented.

Despite accumulating evidence for improvements in radiographic progression of RA disease in patients treated with biologic anti-TNF $\alpha$  agents, especially etanercept and infliximab, the exact mechanism by which bone loss is reduced or prevented remains unknown. One study by Catrina and colleagues examined the effect of treatment with biologic anti-TNF $\alpha$  agents on the expression of OPG and RANK-L in synovial tissues of RA patients (129). These investigators established that treatment with infliximab or etanercept altered the ratio of OPG to RANK-L by osteoblasts and endothelial cells in *ex vivo* RA synovial biopsies and cultured cells. The investigators concluded that biologic anti-TNF $\alpha$  agents slowed down the development of RA-related joint damage and bone loss (129). Other recent studies have

suggested that bone loss in RA can be prevented by the combination of biologic anti-TNF $\alpha$  agents and systemic recombinant OPG administration (8,130). The efficacy of combination therapy with OPG and biologic anti-TNF $\alpha$  agents on inflammation and bone erosion was also studied in collagen-induced arthritis models in mice using urinary deoxypyridinoline, BMD measurements and histological scoring (130). These studies suggested that combination therapy with OPG and biologic anti-TNF $\alpha$  agents acts through multiple pathways to decrease bone resorption and maintain bone formation and, therefore, prevent loss in inflammatory diseases (130).

In addition to the above cited studies, the effect of treatment of RA patients with biologic anti-TNF $\alpha$  agents on bone turnover biomarkers was studied. Osteocalcin (a bone formation biomarker) was considerably increased in 6 studies involving treatment of patients with biologic anti-TNF $\alpha$  agents (124,125,131-134). Three studies reported no changes in osteocalcin levels (99,135,136). Studies of the level of CTX (a biomarker of bone resorption) revealed that the level of this biomarker decreased in 5 of the studies (124,125,136-138) while another 2 studies reported that the level remained unchanged (99,134). The level of soluble RANK-L and OPG in RA patients treated with biologic anti-TNF $\alpha$  agents was also studied. Published reports indicate inconsistencies in the level of soluble RANK-L between no change (122) to reduction after treatment (125, 139). For OPG, levels are also noted to be consistent in (125,133) but reduced in another (139).

Earlier studies of the effect of biologic TNF $\alpha$  antagonists on bone loss in RA have, at times, been contradictory because of patient heterogeneity and how baseline levels of BMD and which biomarkers were measured. Most studies have focused on studying the effect of treatment with infliximab with limited data on changes in bone loss or erosions. In addition, many studies do not indicate whether patients assessed were on potentially relevant medications, such as calcium or Vitamin D supplementation. This is important as these may impact changes in bone turnover. Several studies reported data only on baseline use of

corticosteroid and DMARDs without indications about alterations to dosages of these agents during the study, clinical change or adverse events. In addition, for most studies, the periods covered were in general less than 54 weeks which is relatively short for follow up studies of changes in bone turnover. Moreover, most of these studies didn't evaluate the effect of treatment with biologic anti-TNF $\alpha$  agents on fractures but were involved in measuring bone loss in the spine and hip but not in the hands. For these reasons, the current study was conceived to use a range of clinical procedures and biomarkers to assess changes in bone. These include DEXA scans to measure generalised bone loss in lumbar spine and hip, digital X-ray radiogrammetry (DXR) to measure localised bone loss in hands and ELISAs to measure changes in bone turnover biomarkers including osteocalcine and CTX and bone metabolism biomarkers including OPG and RANK-L.

The key pro-inflammatory cytokines TNF $\alpha$ , IL-1, IL-6 and IL-17 have effect on bone metabolism and have been known to induce generalised bone loss and subchondral bone resorption in arthritis. These pro-inflammatory cytokines act on osteoblasts to promote RANK-L expression and, thus, stimulate osteoclastogenesis (140-143). Furthermore, inflammatory osteoclastogenesis can be induced by granulocyte-macrophage colony-stimulating factor (GM-CSF) which plays an important role in the differentiation of osteoclasts from its precursors (144,145). In addition, IL-20 and IL-22, which are members of the IL-10 cytokine family, have been shown to enhance RANK-L expression with the net effect being a rise in osteoclast formation and function (146,147). All these cytokines that are involved in bone resorption have been assessed in the current study. In addition, the current study will explore new insights into the anti-catabolic effects of neutralising TNF $\alpha$  on bone tissues and determine how the level of osteoclast precursors (OCPs), RANK, RANK-L and OPG are affected by the treatment.

## **Hypothesis of the study**

The hypothesis that underlies the current study is that treatment of RA patients with biologic anti-TNF $\alpha$  agents reduces localised as well as generalised bone loss in the patients. If this proves to be correct, the data would, thus, suggest that there are clear overlaps between mechanisms that sustain chronic inflammation and those that drive osteoporosis. Because of the manner in which disease activity is measured in RA, with DAS28, improvements in bone turnover in RA may, or may not, be consistent with changes in the DAS28. Furthermore, it is likely that not all patients will show similar improvements due to a range of factors such as ethnic origin and lifestyle effects on bone loss. This study, therefore, is aimed at assessing the effect of age, gender, ethnic origin and lifestyle on bone loss given limitation on the number of patient recruitment to determine the link between inflammation and bone loss in RA.

## **Aims and objectives**

Current studies provide compelling evidence for beneficial clinical response of the majority of patients with RA to treatment with biologic anti-TNF $\alpha$  agents. Thus, there is clear evidence for therapeutic efficacy of these agents on inflammation and improvement in the overall clinical response as measured by DAS28 in 60-70% of RA patients. However, there have been efforts at the re-appraisal of the clinical value of some of the subjective measures of responsiveness to biologic anti-TNF $\alpha$  agents in favour of more objective measures including improvement in bone metabolism and joint damage. There is, therefore, a need to assess if changes in bone density and erosion could be of higher, or of additional value to assess clinical responsiveness to biologic anti-TNF $\alpha$  agents. Furthermore, evidence that changes in key inflammatory cytokines including TNF $\alpha$ , IL-1, IL-6, IL-17, IL-20, IL-22 and GM-CSF promotes osteoclastogenesis but their role in bone turnover needs to be assessed in RA patients. There is also a need to determine if all RA patients treated with biologic anti-TNF $\alpha$  agents show improvement in their bone turnover and, if not, determine the factors that

influence such a lack of responsiveness in some patients. The aim of the study is to assess the effect of treatment with biologic anti-TNF $\alpha$  agents on bone metabolism in responder and non-responder RA patients in relation to the inflammatory response. The effect of age, gender, ethnic origin, lifestyle and the key inflammatory cytokines on changes in bone metabolism will be assessed in responder and non-responder RA patients.

To achieve these objectives the study will:

- 1- Verify whether, or not, beneficial clinical response to biologic anti-TNF $\alpha$  agents as measured by DAS28 corresponds with parallel improvement in bone density/erosion and turnover in RA patients.
- 2- Incorporate the effect of age, gender, ethnic origin and lifestyle on the degree of changes in bone density after treatment into any such findings.
- 3- Assess if excess production of pro-inflammatory cytokines in RA patients corresponds with a lack of, or reduced, improvement in bone density and erosion.
- 4- Assess potential biomarker(s) that best demonstrates improvement in bone density in RA patients treated with biologic anti-TNF $\alpha$  agents.
- 5- Analyse the effect of biologic anti-TNF $\alpha$  agents on the frequency of circulating OCPs cells in both *in vivo* and *in vitro* and correlate the data with the clinical response and with changes in biomarkers of bone turnover.

# **Chapter Two**

## **Patients and Methods**

## 2.1) Patients

To assess key factors that affect BMD and the response of patients to treatment, the effect of age, gender, ancestral origin and some aspects of lifestyle in a cohort of 647 patients with primary osteoporosis attending the osteoporosis clinics at the Royal London and St Bartholomew's Hospitals was studied. The patients included 533 females, 114 males with a mean age ( $\pm$ SD) of  $68.3\pm 11.6$  years (51-87 years) (Figure 2.1) . Information on the smoking habits of most of the patients was available with 121 of the cohort confirmed to be smokers.

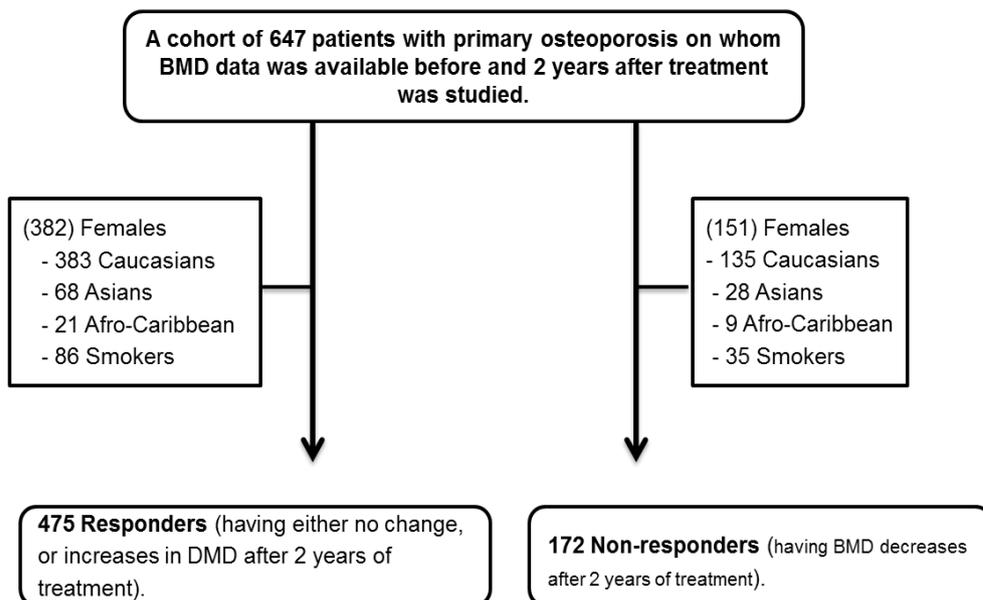
The study also involved recruiting 117 RA patients who were prescribed treatment with biologic anti-TNF $\alpha$  agents (Figure 2.1). These patients were studied to assess the effect of treatment with this class drugs on BMD at lumbar spine and the hip. The cohort included 62 patients on whom radiological data was available on their BMD before treatment with biologic anti-TNF $\alpha$  agents and at 2 years after treatment. This specific group included 49 females and 13 males. The mean age of patient group was  $69\pm 12$  years (40-79 years). All patients had active disease (mean DAS28 $\pm$ SD of  $5.96\pm 1.2$ ) at the time they were prescribed biologic anti-TNF $\alpha$  agents.

A another of 55 patients within the cohort of RA patients were studied to assess the status of their immune system including T- and B-lymphocyte and monocyte production of cytokines, plasma levels cytokines that have an effect on bone metabolism and also for the measurement of bone turnover biomarkers. The level of the following cytokines: IL-1 $\beta$ , IL-2, IL-6, IL-8, IL-10, IL-12, IL-17, IL-22, IL-23, GM-CSF, INF $\gamma$ , MCP-1 and TNF $\alpha$  as produced by T- and B-lymphocytes and monocytes enriched from the blood of the patients and then activated *ex vivo* were studied. The blood samples were obtained immediately before and after 1 and then 3 months after treatment with biologic anti-TNF $\alpha$  agents. Plasma levels of the following cytokine and bone turnover biomarkers from the same patients were also determined in this group of patients: TNF $\alpha$ , IL-1, IL-6, IL-17, IL-20, IL-22, GM-CSF,

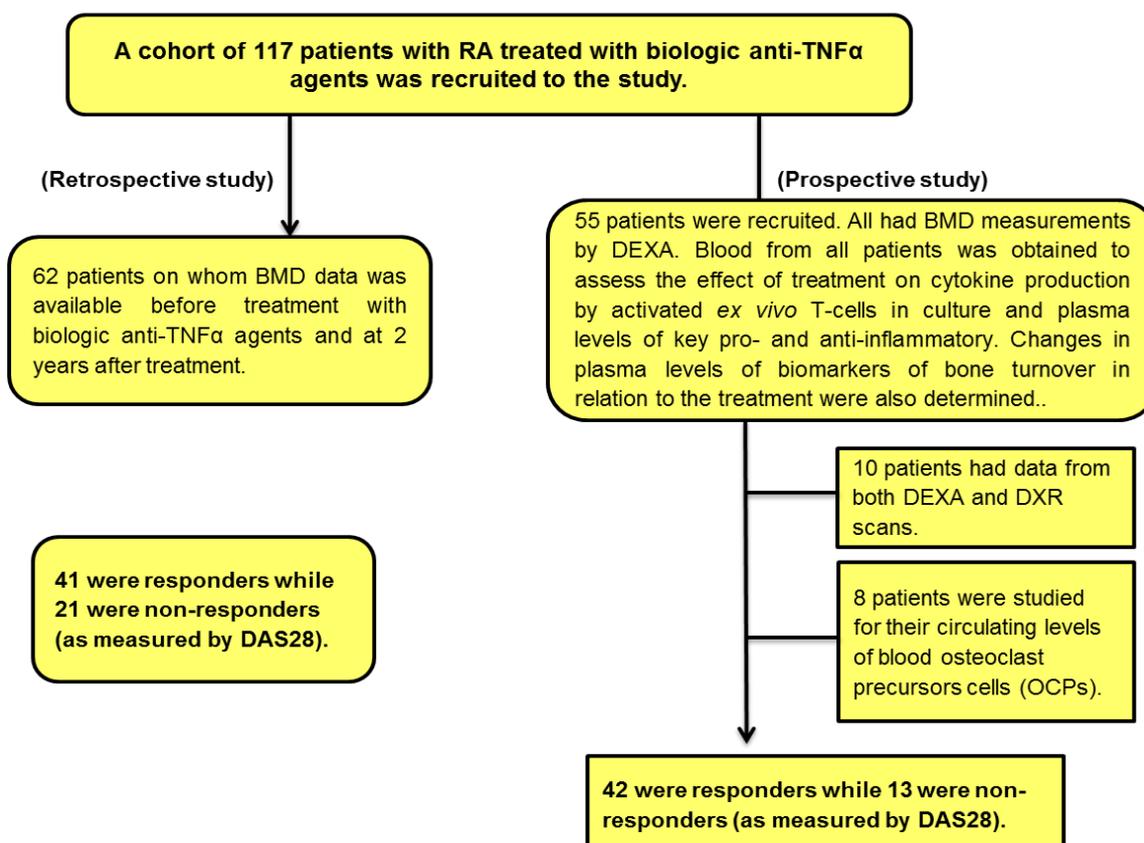
RANK-L, OPG, osteocalcin and CTX. The 55 patients included 41 females and 14 males with a mean  $\pm$ SD of age (range) of  $57\pm 14$  (24-79) years. The mean  $\pm$ SD of DAS28 of the patients was  $5.8\pm 0.8$  at the time they were prescribed the anti-TNF $\alpha$  agents. BMDs of lumbar spine and hip were determined together with DXR for 10 of the patients.

The effect of treatment with the biologic anti-TNF $\alpha$  agents on the circulating levels of blood osteoclast precursors cells (OCPs) in both *ex vivo* and *in vitro* experiments was also studied in 8 RA patients. Blood samples from the 8 patients were obtained immediately before treatment with the biologic anti-TNF $\alpha$  agent had started and at 1 and then 3 months after treatment. Blood mononuclear cells (PBMNCs) were isolated from these patients and studied by FACS analysis for *ex vivo* levels of the OCPs or cultures as described later. The patients included 7 females and 1 male with a mean  $\pm$ SD age (range) of  $54\pm 17$  (24-77) years. The mean  $\pm$ SD of DAS28 of the patients was  $5.4\pm 0.9$  at the time they were prescribed the treatment. This study also included obtaining blood samples from 12 healthy individuals with a mean  $\pm$ SD age (range) of  $45\pm 12$  (27-62) years. All healthy individuals were males.

a) **The effect of age, gender, ethnic origin and lifestyle of the response to treatment with anti-osteoporotic therapy.**



b) The effect of biologic anti-TNF $\alpha$  agents on changes in BMD and bone biomarkers in patients with RA and factors that impact the outcome.



**Figure 2.1:** Flowcharts of the studies involved in this thesis.

## 2.2) Methods

### 2.2.1) Dual energy X-ray absorptiometry (DEXA)

A Hologic Discovery QDR series Dual Energy X-ray Absorptiometry (DEXA) scanner located at Royal London Hospital at Whitechapel was used to measure BMD of the lumbar spine and the hip. The principles of this protocol are described in chapter 1. T and Z-scores are used with BMD as standard units for diagnosis and follow up of changes in bone density. The T-score refers to the number of standard deviations (SDs) above or below the average BMD value for young normal adults (aged 20-35) matched for sex and race. The T-score is calculated according to the following formula:

$$\text{T-score} = (\text{patient's BMD}) - (\text{mean BMD of young adult}) / \text{SD (young adult)}$$

The Z-score refers to the number of SDs above or below the average BMD value for people of the same age, sex and race. This value is used for assessing BMD in older patients (148-150).

The Z-score is calculated according to the following formula:

$$\mathbf{Z\text{-score} = (\text{patient's BMD}) - (\text{mean BMD of age matched}) / SD (\text{age matched})}$$

Variations in the precision of DEXA scanner should be less than 1.0% according to manufacturers' specifications (148). Precision and accuracy are two main considerations for assessing the performance of a DEXA scanner in order to analyse the results of patients with confidence.

#### **a) Precision and accuracy**

Since bone densitometry relies on ionizing radiation, care is always taken such that measurements are always reproducible in order to ensure maximum benefits for patients with least exposure to the radiation.

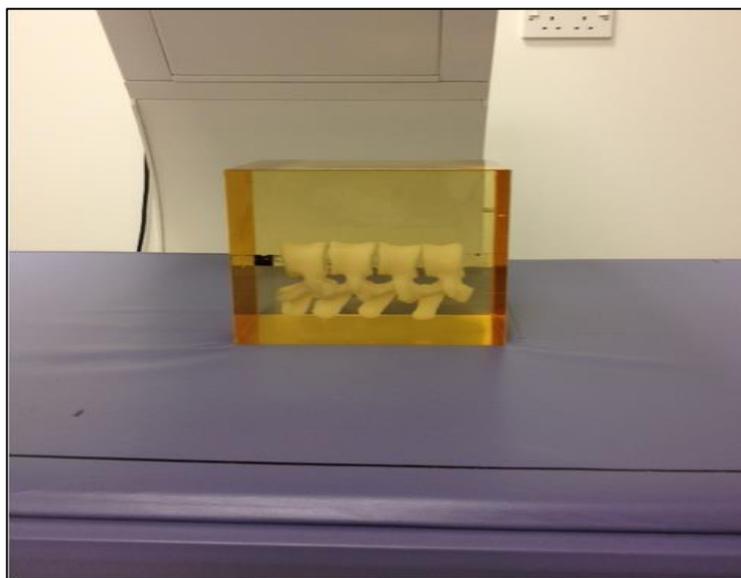
Precision of the DEXA system is defined as the ability to obtain consistent BMD values upon repeat measurements for the same patient. Accuracy, in contrast, is a measure of the actual BMD value and is highly important when this value is compared with a reference population. Accuracy is expressed in percent and is defined as the % difference between measurements of a quantity to that quantity's actual (true) value (151).

Clinical DEXA scanner precision is affected by a combination of short-term and long-term variability, patient movements, body size and operator-dependent factors. Short-term precision is essential in setting lower bound accuracy on a system while long-term precision is important in the assessment of follow-up scans carried out at later dates (152). The measured value of precision assessment is called the precision error. Understanding this error is

important in clinical diagnosis of serial BMD studies in patients with bone metabolic diseases and those requiring therapeutic interference (152). In a series of frequent BMD tests, precision can be stated as either the standard deviation (SD), or % coefficient of variation (% CV, defined as  $100 \times \text{SD}/\text{mean}$ ) (152). With a 1% precision system, results are interpreted as an increase, or a decrease at the 95% confidence level is only achieved if two readings obtained from a subject, within a year a part, are greater than  $2.8 \times \% \text{ CV}$  (151).

**b) *In vitro* short-term precision**

*In vitro* short-term precision was measured by scanning an anthropomorphic spine phantom, provided by the scanner's manufacturer, 20 times per day. The phantom contains a human-like spine segment made of calcium hydroxyapatite, enclosed in a block of water-simulant epoxy. This phantom has a BMD equal to  $0.995\text{g}/\text{cm}^2$  (148). The phantom was used so that errors due to patient positioning did not affect measurements used for the purpose of this study, as the key objective was to evaluate the performance of the scanner over a range of bone density values, and avoid operator and patient sources of error (Figure 2.2).



**Figure 2.2: Anthropomorphic spine phantom used for daily quality assurance.** The phantom contains a human-like spine segment made of calcium hydroxyapatite, enclosed in a block of water-simulant epoxy. This phantom has a BMD equal to  $0.995\text{g}/\text{cm}^2$ . The picture was taken during *in vitro* short-term precision testing performed for the current study.

Measurements were carried out by placing the phantom on the scanner's table with repositioning of the phantom between scans using the positioning lasers of the scanner. Using the manufacturer's software, BMD values of the spine phantom were evaluated by analysing images obtained from where bone edge determination was made automatically. To eliminate additional variability in precision caused by repositioning of the phantom, a second set of 20 scans was carried out without repositioning of the phantom between scans.

### **c) *In vitro* long-term precision**

The long-term precision of the scanner was assessed on a daily basis using BMD measurements of the spine phantom as a routine part of quality control. These measurements were carried out daily for 28 months (August 2011 to Dec. 2013), the duration of the current study. Similar to the short-term precision, long-term precision was expressed in coefficients of variation (CV).

#### **d) Subject positioning and site of measurements**

BMD measurements were carried out on two sites. The first was the anterior-posterior lumbar spine including the vertebral bodies L1 through L4, and the average calculated and used as total spine BMD. The second was for total hip where the BMD average was calculated for femoral neck, Ward's region, trochanter, and the shaft. The purpose of measuring BMD at these areas was because they provide best indications of bone loss (151). Measurement of femoral neck BMD is also essential as that of the lumbar spine can be unreliable because of inaccuracies introduced by calcification of the abdominal aorta, or by bony osteocytes (152,153).

Proper positioning of all patients was performed strictly in order to eliminate the impact of variability in positioning on BMD measurements. For hip positioning, the femoral neck was placed in a plane parallel to the scanner's table. This was achieved by placing the subject at the centre of the table. The hip was internally rotated 25° inwards and stabilised by a foot positioner as seen in Figure (2.3). The legs were then abducted from the midline of the body in order to straighten the femur. The lumbar spine scanning was carried out by positioning the subject comfortably and supine on the scanner's table to have their spine straight. A knee positioner as seen in Figure (2.4) was placed under the subject's lower legs to have the femurs as vertical as possible. This aids in straightening the lumbar lordosis.



**Figure 2.3: Patient positioning for DEXA absorptiometry of the hip.** The picture was taken during scanning one patient involved in current study.



**Figure 2.4: Patient positioning for lumbar spine BMD measurements using DEXA absorptiometry.** The picture was taken during scanning one patient involved in current study.

#### **e) BMD scan reports**

Data acquired for the study were obtained from the BMD report of each subject. The BMD report contains demographic data, an image of the area scanned, location of the region of interest superimposed on the image, the BMD in  $\text{g/cm}^2$  of the bone region scanned (automatically calculated by the software) the T-score, Z-score, a graph showing the patient's BMD compared with the manufacturer's reference database for healthy individuals and state of the bone according to WHO classification (normal, osteopenic, osteoporotic) (Figures 2.5 and 2.6).

**DEXA SCAN UNIT**  
**2ND FLOOR, MAIN BUILDING, THE ROYAL LONDON HOSPITAL, WHITECHAPEL**  
**LONDON E1 1BB**

Phone: 020 359 40195

Fax: 020 359 43206

|   |                                 |  |
|---|---------------------------------|--|
| Name: [REDACTED]<br>ID: [REDACTED]<br>DOB: [REDACTED] | Sex: Female<br>Ethnicity: White | Height: 164.6 cm<br>Weight: 88.6 kg<br>Age: 65 |
|---|---------------------------------|--|

Referring Physician: PROF JAWAD

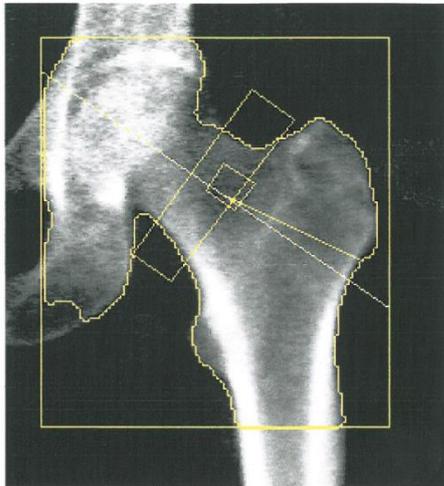
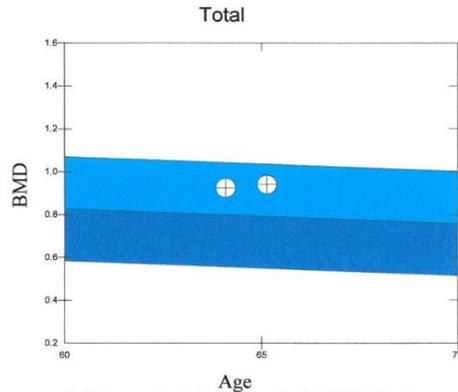


Image not for diagnostic use  
100 x 117

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 Analysis: 09 January 2012 12:51 Version 12.3  
 Left Hip  
 Operator: OA  
 Model: Discovery A (S/N 80933)  
 Comment:



Reference curve and scores matched to White Female

Source: NHANES

**Results Summary:**

| Date    | Age | BMD (g/cm <sup>2</sup> ) | T - Score | BMD Change vs Baseline | BMD Change vs Previous |
|---------|-----|--------------------------|-----------|------------------------|------------------------|
| 01.2012 | 65  | 0.940                    | -0.0      | 1.7%                   | 1.7%                   |
| 02.2010 | 64  | 0.924                    | -0.1      |                        |                        |

MD CV 1.0%

Notes significant change at the 95% confidence level.

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**Figure 2.5: BMD scan report of the hip.** BMD average of femoral neck, Ward's region, trochanter and shaft was calculated and used as the total for the hip.

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**LONDON E1 1BB**

Phone: 020 359 40195

Fax: 020 359 43206

|  |                                 |  |
|--|---------------------------------|--|
| Name: SANDRA<br>ID: A0109120C<br>Patient No: 1 | Sex: Female<br>Ethnicity: White | Height: 164.6 cm<br>Weight: 88.6 kg<br>Age: 65 |
|--|---------------------------------|--|

Referring Physician: PROF JAWAD

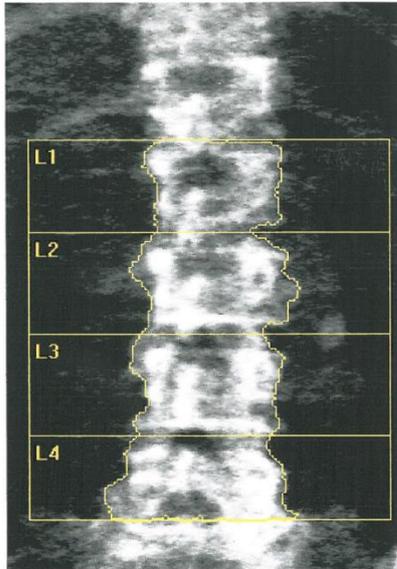
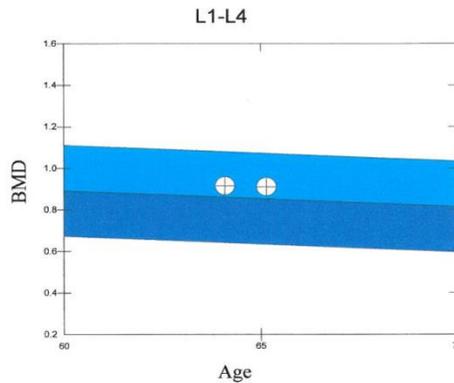


Image not for diagnostic use  
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 Analysis: 09 January 2012 12:50 Version 12.3  
 Lumbar Spine  
 Operator: OA  
 Model: Discovery A (S/N 80933)  
 Comment: DAP 10.3



Source: Hologic

**Results Summary: L1-L4**

| Date | Age | BMD (g/cm <sup>2</sup> ) | T - Score | vs Baseline | BMD Change vs Previous |
|------|-----|--------------------------|-----------|-------------|------------------------|
| 2012 | 65  | 0.911                    | -1.2      | -0.5%       | -0.5%                  |
| 2010 | 64  | 0.915                    | -1.2      |             |                        |

CV 1.0%  
 indicates significant change at the 95% confidence level.

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**Figure 2.6: BMD scan report of the anterior-posterior view of the spine.** Views are of L1 through L4. The BMD of all four vertebrae was measured and the average calculated and used as total spine BMD.

### **2.2.2) Digital X-ray radiogrammetry (DXR)**

DXR is a new operator-independent diagnostic protocol that permits automated measurements of cortical BMD and of metacarpal index (MCI), based on estimates of cortical thickness using digitised radiography. The protocol provides quantitative data on periarticular osteoporosis in patients with RA and serves as a promising supplement to the X-ray scoring methods, allowing earlier diagnosis, which is essential for optimal and timely treatment. The computer algorithms employed in the DXR technique automatically define regions of interest around the narrowest parts of the second, third and fourth metacarpals and then identify the outer and inner cortical edges of the cortical bone parts examined. The mean of the cortical thickness and overall bone cortical thickness of the second, third and fourth metacarpals are estimated. Subsequently, the cortical volume per area is calculated for each bone (154).

Because of the potential advantages the DXR will provide for the current study in enabling accurate quantification of changes in bone, Sectra Imtec AB, the company that carries out such analyses was approached for assistance with the study. It was agreed that the company carry out quantitative measurements of changes in bone density in the RA cohort for whom digital X-ray images were obtained. The company set up the parameters for acquiring the images at our end and awarded that lab a grant to cover half of the costs incurred based on the analysis of 3 images for up to 60 treated patients at 3 separate visits to the clinic: before treatment and 6 and 12 months after treatment. The coefficient of variation for this method was determined to be 0.25%.

#### **DXR precision:**

##### **a) *In vitro* short-term precision**

Short-term precision was measured by scanning a cadaver forearm phantom, provided by the scanner's manufacturer, 31 times per day. The phantom contains a human-like hand

segment made of calcium hydroxyapatite, enclosed in a block of water-simulant epoxy. The phantom was used so that errors due to patient positioning did not affect measurements used for the purpose of this study.

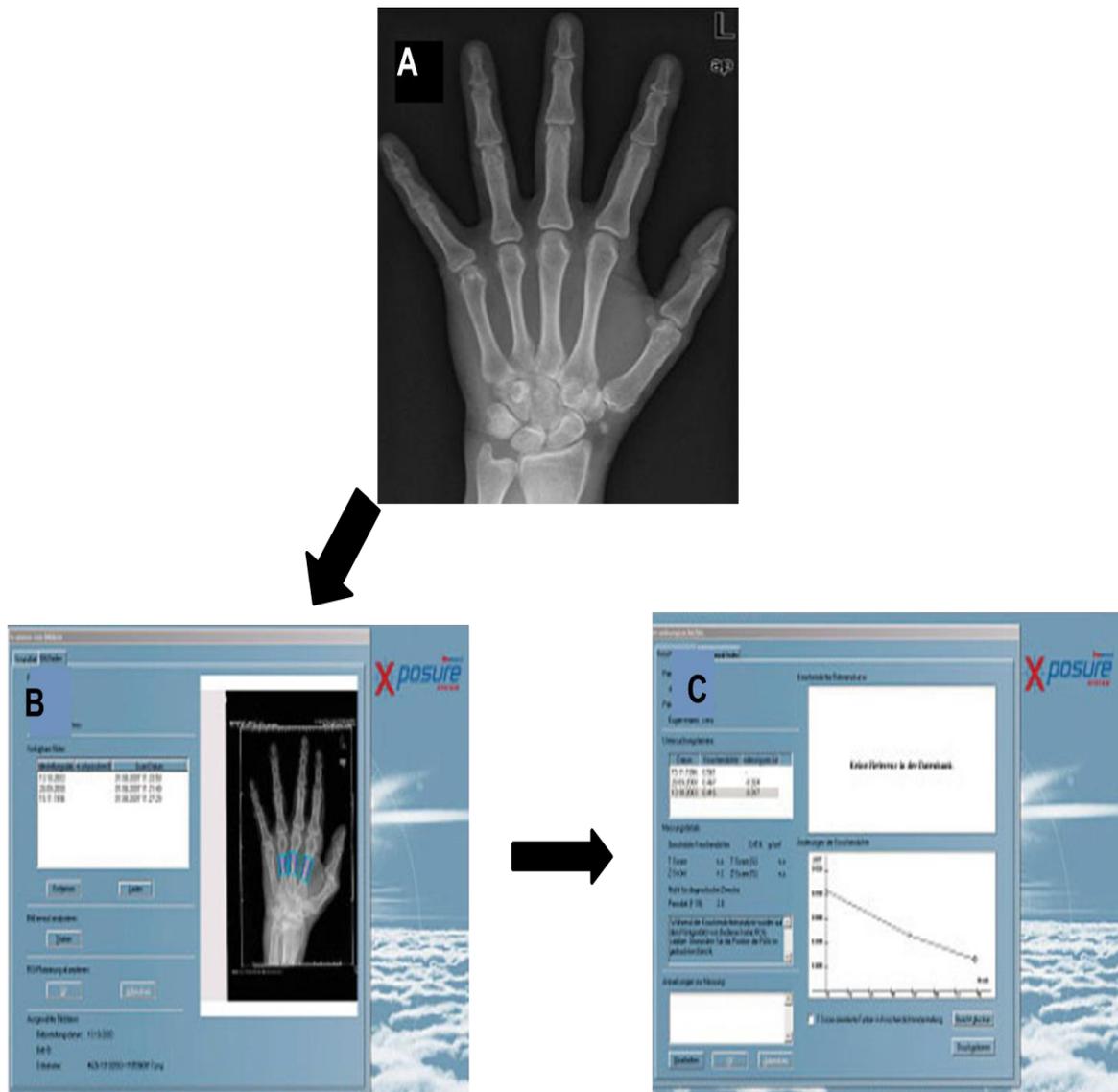
DXR calculates BMD from digital hand radiographs. The parameters for acquiring hand radiographs were film focus distance (FFD) 100cm; tube voltage 50kV; exposure dose 5mAs. All the digital radiographs were analysed by the same computer software program at the manufacturer's lab. To measure DXR-BMD, the system requires hand radiographs from a digitised X-ray system with known resolution. The computer automatically determines regions of interest (ROI) around the narrowest part of the 3 metacarpal bones in hand radiographs and measures cortical thickness, bone width, and porosity (Figure 2.7).

DXR-BMD is defined as:  $c \times VPA_{\text{comb}} \times (1-p)$ , where  $c$  is a density constant,  $VPA$  is volume per unit area, and  $p$  is porosity. To eliminate additional variability in precision caused by repositioning of the phantom, a second set of 31 scans was carried out without repositioning of the phantom between scans.

#### **b) Long-term precision**

The long-term precision of the DXR was assessed on a daily basis using BMD measurements of the cadaver forearm phantom as a routine part of quality control. This procedure was performed at baseline, 3 months and 6 months. Similar to the short-term precision, long-term precision was expressed in coefficients of variation (CV).

The precision (defined as the BMD data variability occurring with repeated measurements over time) of the DXR technique for the measurement of BMD has shown short-term precision with a CV of 0.25% and long-term precision with CV of 0.28% (154).



**Figure 2.7: BMD scan report of the anterior-posterior view of the hand.** For DXR assessment of BMD, the system requires hand radiographs from a digitised X-ray system with known high resolution (A). The computer automatically identifies regions of interest (ROI) around the narrowest part of the second, third, and fourth metacarpal bones on hand radiographs (B) and measures cortical thickness, bone width, and porosity in the region (C).

### **2.2.3) Separation and stimulation of T- and B-lymphocytes and monocytes**

T-lymphocytes, B-lymphocytes and monocytes were isolated from peripheral blood by negative selection, using RosetteSep enrichment cocktails kits (Stem Cell Technologies). Blood samples were mixed with the different cocktails depending on which cell was intended for enrichment at 50µl of the cocktail per ml blood. The cocktails contain bi-specific antibodies, with one part reacting with membrane proteins specific for unwanted immune cells and the other part reacting with glycoprotein A on red blood cells (RBCs). Thus, the cocktail used for enriching T-lymphocytes contained antibody with specificity for CD16, CD19, CD36, CD56, CD66b, while the cocktail to enrich B cell cocktail contain antibodies recognising CD2, CD3, CD16, CD36, CD56, CD66b and the monocyte cocktail contained antibodies recognising CD2, CD3, CD8, CD19, CD56, CD66b and CD123. The blood/cocktail mixture was left for 20 minutes and subsequently mixed 1 to 1 with RPMI (cell culturing medium) containing 2% foetal bovine serum (FBS). For monocyte separation additionally 1 mM EDTA was included to stop monocytes attaching to the tube walls. Subsequently the cell separation mixtures were layered on top of Ficoll-Paque (GE Healthcare Amersham, UK) and subjected to centrifugation at 1200g for 20 minutes without brakes. Enriched immune cells were then re-suspended in RPMI supplemented containing 10% FBS, 100units/ml penicillin and 10µg/ml streptomycin and simulated. T-lymphocytes were stimulated with 10µg/ml anti-CD3 pre-coated onto the plates and then 10µg/ml anti-CD28 added to the cells. After 48 hours of culture, supernatants were collected for determination of cytokine production. Separated B-lymphocytes were stimulated with 10µg/ml antibody to human membrane immunoglobulin coated onto the plate together and with 10µg/ml antibody to human CD40. Enriched monocytes were stimulated for 48hrs with 1µg/ml lipopolysaccharide (LPS). In parallel, T-lymphocytes were also stimulated with 0.1µg/ml PMA and Ionomycin with addition of 1µl/ml golgiplug<sup>TM</sup> (BD Bioscience) overnight according to the manufacturer's instructions for further assessment of total intracellular

cytokine levels. Supernatants and cell pellets from cell stimulations were stored at  $-80^{\circ}\text{C}$  until analysed. T-lymphocytes stimulated with PMA + ionomycin and protected from secretion with golgi-plug were stained extracellularly using anti-CD4 antibody for 20 min, permeabilised using Leucoperm (ABD Serotec) and stained for intracellular cytokines using specific fluorophore labelled antibodies detecting IL-17,  $\text{INF}\gamma$ ,  $\text{TNF}\alpha$  and GM-CSF according to the manufacturer's instructions. The cells were fixed using 0.5% paraformaldehyde in PBS prior to further analysis.

#### **2.2.4) Enrichment of peripheral blood mononuclear cells (PBMNCs) for evaluating the effect of treatment with biologic anti- $\text{TNF}\alpha$ agents on osteoclastogenesis**

PBMNCs were enriched from whole blood obtained from 8 RA patients and 12 healthy individuals by centrifugation on Ficoll-Paque gradients. The number of PBMNCs was determined using an equal volume of 0.5% Trypan blue mixed with the cell suspension and numbers and viability determined under an inverted light microscope. One million of the PBMNCs were then placed in a well of a 24-well culture plates with RPMI1640 medium containing 10% foetal calf serum (FCS) and antibiotics.

The cultured cells were incubated in a humidified incubator at  $37^{\circ}\text{C}$  with 5%  $\text{CO}_2$  in the air for 14 days. The culture medium was replenished every 3 days and supernatants collected, centrifuged and stored at  $-20^{\circ}\text{C}$  until tested to study the effects of treatment of the patients with biologic anti- $\text{TNF}\alpha$  agents on osteoclasts precursor differentiation. The cultured cells were treated with monocyte colony stimulating factor (M-CSF) at 25ng/ml, OPG (1 $\mu\text{g}$ ) and RANK-L (100ng/ml) served as positive controls (all from R&D).

After 14 days of culture, the cells were stained for tartrate-resistant acid phosphatase (Acid Phosphatase, Leukocyte (TRAP) Kit; Sigma-Aldrich, UK) and viewed and quantified by light microscopy. The number of TRAP-positive cells with 3 or more nuclei was counted as osteoclasts in cultures for monocytes from the RA patients and the healthy controls. The

scoring system used showed the data as the number of osteoclasts per  $1 \times 10^6$  PBMNCs recorded as the number of TRAP-positive multinuclear cells.

### **2.2.5) Enzyme-linked immunosorbent assay (ELISA)**

ELISA is a highly sensitive and specific technique widely used to measure proteins and other biologically-relevant molecules including antibodies, cytokines and bone biomarkers in biological and clinical samples at ranges that vary from pg/ml to mg/ml (155,156). For the purpose of the studies reported in this thesis, a range of ELISA protocols were established for the measurement of bone turnover biomarkers and cytokines involved in regulating bone resorption and their sensitivity, specificity and reproducibility assessed.

#### **2.2.5.1) ELISA for protein involved in regulating bone resorption**

Plasma levels of RANK-L and OPG are measured as indicators of osteoclast activity. IL-20 cytokine was recently identified as an important pro-inflammatory cytokine from the IL-10 family of proteins that is involved in promoting bone resorption (146). Levels of RANK-L, OPG and IL-20 in the plasma of RA patients were measured using ELISA before and after treatment with biologic anti-TNF $\alpha$  agents in order to assess potential improvement in BMD. The capture ELISA protocol was carried out using sets of two monoclonal antibodies, one to coat the ELISA wells with to capture the protein of interest and the second was biotinylated and used to detect proteins captured in plasma from the patients. Bound RANK-L, OPG and IL-20 in the tested plasma samples were then revealed with horseradish peroxidase (HRP) conjugated streptavidin that specifically binds to the biotin on the detection Ab. The protocol was carried out by first attaching the coating Ab to the walls of ELISA microtiter plates by incubation at room temperature (RT) overnight. Next day, the plates were emptied of unbound coating Ab and washed 3 times with phosphate buffered saline containing 0.05% Tween 20 (PBS/Tween). Unoccupied sites in the wells were blocked with

blocking buffer (1% bovine serum albumin, BSA in PBS) at RT for 1 hour. The plates were then washed again 3 times with PBS/Tween and diluted plasma samples (1:10) or serial dilutions of recombinant proteins, used as standards, 1% BSA in PBS added to each well according to a prepared map. The plates were then incubated at RT for a further 2hrs. The samples were aspirated and plates washed 3 times with PBS/Tween. The biotinylated detection Abs were diluted to a concentration of 2ng/ml and added to each well and plates incubated for a further 2hrs at RT. The plates were aspirated again, washed 3 more times with PBS/Tween and Streptavidine-HRP (diluted 1:200 in BSA/PBS/Tween) added to each well and incubated for 20min at RT. The plates were aspirated and washed as before and freshly-prepared substrate added to each well and incubated at RT for 20min. The reactions were stopped by adding the stop solution (4M H<sub>2</sub>SO<sub>4</sub>) with gently-tapping of the plate. Finally, the optical density values were measured by using the ELISA reader at 492nm. Concentrations of RANK-L, OPG, and IL-20 in each plasma were determined by interpolation from the standard curve constructed from the known input of recombinant proteins.

#### **2.2.5.2) ELISA of bone turnover biomarkers**

Bone turnover can be evaluated by measuring the level of biomarkers, proteins and/or polypeptides released from osteoblasts and osteoclasts such as osteocalcin, and collagen breakdown polypeptides. When osteoclasts degrade collagen, cross-links of collagen and their adjacent peptides are released into the circulation and excreted in urine (e.g. C-telopeptide cross-links of collagen type I, CTX). Levels of osteocalcin and CTX were measured using ELISA plates coated in the lab with specific antibodies to the target proteins and also by using commercially available kits (Serum CrossLaps® (CTX-I) and N-MID® Osteocalcin, Immunodiagnostic Systems PLC). ELISA plates were coated with specific monoclonal or polyclonal antibodies at 2-10µg/ml in PBS, depending on the specific biomarker detected, by incubation at room temperature for 2 hrs followed by overnight incubation at 4°C. The plates were washed 3 time with PBS/Tween, dried and then unoccupied sites on the plates quenched

with 1% BSA in PBS by incubation at RT for 2hrs. The plates were washed 3 more time with PBS/Tween and dilutions of plasma samples (1:10) and corresponding standards at a range of concentrations all diluent (1% BSA in PBS/Tween) added to the plates and incubated for 2hrs at RT. Bound biomarkers were detected with a detection antibody conjugated with biotin. The detection antibody was always specific for the target biomarkers and bound detected using HRP-conjugated Streptavidin. The plate was then washed 5 times with the washing buffer and substrate solution added to each well and incubated for 15min at RT. The reactions were stopped by adding the stop solution (4M H<sub>2</sub>SO<sub>4</sub>) with gently-tapping of the plate. Finally, the optical density values were measured by using the ELISA reader at 450nm. The level of each of the detected biomarker was determined by extrapolation from the standard curve constructed from the known inputs of the standard.

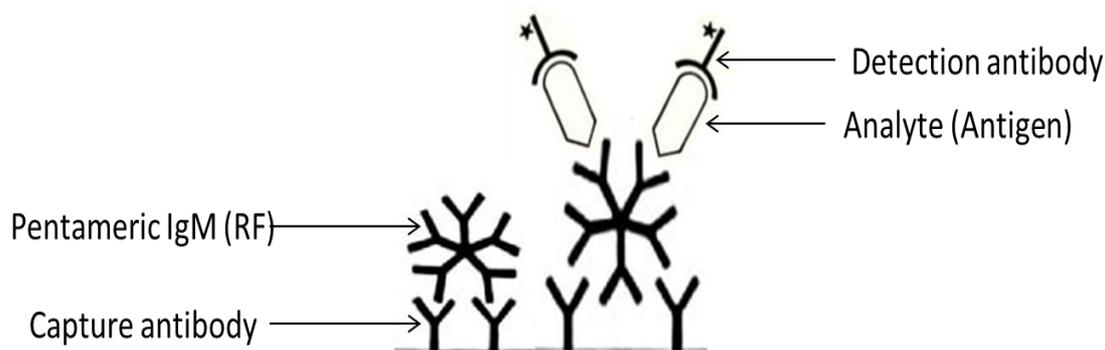
### **2.2.5.3) Reproducibility and accuracy of ELISA**

#### **a) The effect of rheumatoid factor (RF) autoantibody interference on the reproducibility/accuracy of ELISA for bone biomarkers**

RFs are auto-antibodies with specificity for IgG. These autoantibodies are mostly of the IgM isotype, although IgG, IgA and IgE RFs have can also be detected in the blood of patients with RA. These autoantibodies have specificity for antigenic epitopes in the Fc region of IgG. RF autoantibodies are found in the blood of about 80% of patients with RA and high levels have long been associated with the disease (157).

Because of their reactivity with IgG of many species, it was critical that their effects on and interference with the detection of cytokines and other biomarkers be assessed. Thus, RFs present in the plasma of patients with RA could bind to the coating antibodies and interfere with the ability of the capture antibody to capture its target protein and hence the

amount of protein measured underestimated, or not detected even when present. Alternatively, it is possible, theoretically at least, that IgM-RF non-specifically bind to the coated antibody and to capture the biotinylated detection antibody and, thus, give false positive results (158,159) (Figure 2.8). For these reasons, it was imperative that a comprehensive evaluation of the effect of RFs on the ELISA protocols be carried out.



**Figure 2.8: An illustration of the potential interference of RF that could bind to the coating antibody and causing false negative or positive results.** RF, by binding to capture and/or detection antibodies has the potential to give both false negative and positive readings.

To study the effect of RFs on the assay systems used, magnetic beads (Dynabeads<sup>®</sup> M-270 Epoxy-life technologies) were coated with rabbit IgG at 25mg/ml and used to remove RF from diluted plasma samples. Plasma samples before and after their content of RF were removed/partially removed with IgG-coated magnetic beads were then tested in the ELISA protocols to assess the effect of the procedure on the level of proteins of interest. The efficiency with which RF was removed/partially removed was also assessed by carrying out ELISA for measuring the level of RF before and after the depletion from plasma with the IgG-coated magnetic beads. The data was then analysed and compared using Student's t-test.

## **b) ELISA for the measurement of RF**

The ELISA protocol for measuring RF is based on a protocol routinely used in the laboratory of Prof. Mageed. The assay is based on using rabbit-IgG bound on the surface of microtiter ELISA plate. The plates are coated with 50µl of rabbit IgG solution at 20µg/ml by incubation overnight at 4°C. The plates are then washed 3 times with PBS/Tween 20. Diluted RA patient plasma (1:10 final dilution) with and without pre-incubation with the rabbit IgG-coated plates and standard were then pipetted into the wells. The added plasma samples were then titrated 1:2 across the plate. After 2 hours incubation at room temperature, the plates were washed with PBS/Tween to remove unbound proteins and bound IgM-RF detected using peroxidase conjugated F(ab<sub>2</sub>) of goat IgG anti-IgM (1:5000) added to the plates and incubated for 2 hrs. The plates were washed 3 more times with PBS/Tween and bound peroxidase revealed with its substrate (o-phenylene di-amine) solution and the reaction stopped with H<sub>2</sub>SO<sub>4</sub>. The titer of the RF in the plasma with and without pre-incubation with rabbit IgG-coated beads was then determined using an ELISA reader at wavelength of 492nm.

### **2.2.6) Measurement of cytokine levels produced by immune cells from RA patients treated with biologic anti-TNF $\alpha$ agents in relation to changes in BMD**

The effect of treating RA patients with biologic anti-TNF $\alpha$  agents on the immune system and the relation of these to changes in BMD was studied by isolating T- and B-lymphocytes and monocytes and studying cytokine production by these cells *ex vivo*. The experiments were carried out with the help of Drs. Taher and Bystrom in Prof. Mageed's lab. Blood monocytes, B-lymphocytes and T-lymphocytes were enriched to high purity using negative selection protocols that rely on using RosetteSep kits from Stem Cell Technologies (160). These cells were then assessed for their purity by flow cytometry and stimulated *ex vivo* as cited above and level of key cytokines determined by a multiplex-based protocol from Meso Scale Discovery (MSD).

### **a) Cytokine analysis**

Plasma and cell culture supernatants, from *in vitro* activated enriched B- and T-lymphocytes and monocytes, were diluted (plasma used undiluted for GM-CSF detection or diluted 1:5 for plasma TNF $\alpha$ , IL-1 $\beta$ , IL-6 and MCP-1 detection supernatants in assay buffers and plates from MSD (StemCell Technologies) according to the manufacturer's instructions. TNF $\alpha$ , IL-6, IL-1 $\beta$ , GM-CSF, INF $\gamma$  and IL-10 were assessed using a multiplex assay on MSD plates while IL-17 and MCP-1 were assessed as separate ELISA. The IL-17 assay was carried out using MDS plates while measurement of MCP-1 levels was carried out by ELISA using antibodies from R&D systems (Minneapolis, MN, Oxford, UK) and standard binding plates from MSD technologies.

#### **2.2.7) MSD protocols for the measurement of cytokines**

Cytokines produced by immune cells activated *in vitro* were measured using the MSD assay platform (MSD, Gaithersburg, MD) according to the manufacturer's instructions. The MSD platform is based on the proprietary combination of electrochemiluminescence detection and patterned arrays. The MSD assay is highly sensitive and simultaneously provides quantitative data on multiple cytokines using small volumes of clinical samples including supernatants from cultured cells (25 $\mu$ l). The assay has a detection limit 4000 to 1.2 pg/ml. Briefly, 25 $\mu$ l of supernatants (and recombinant human cytokine standards) were incubated for 2 hours on a custom-designed human cytokine 96-well microtiter plate bearing a patterned array of capture antibodies on the bottom of the wells. After washing, a mixture of specific anti-cytokine detection antibodies (each at 1 $\mu$ g/ml) conjugated to the ruthenium-based MSD Sulfo-TAG<sup>TM</sup> reagent were added. The plates were washed 3 times and the MSD Read Buffer T (2x) added and results read on a Sector Imager 6000 incorporating a CCD. Cytokine concentrations were determined with Softmax Pro Version 4.6 software, using curve fitting models (log-log or four-parameter log-logistic) as suggested by the manufacturer for the specific cytokine (161). For the purpose of the study, part of which described in this

thesis, 14 different cytokines were measured, 10 using multiplex MSD plates and 4 including IL-17, IL-23, MCP-1 and IL-22 on single cytokine measurement plates.

### **2.2.8) Inhibition of osteoclastogenesis by OPG and anti-TNF $\alpha$**

PBMNCs from 3 healthy controls were seeded into 96-well flat-bottomed culture plates at  $1 \times 10^6$  cells per well with 50% culture supernatants of PBMNCs obtained from RA patients cultured for the purpose of assessing the effect of treatment of patients on the production of osteoclastogenic factors. The culture supernatants were mixed with an equal volume of fresh culture medium in a total of 200 $\mu$ l. To further assess the effect of inhibiting TNF $\alpha$  in the culture supernatants from the RA patients, anti-TNF $\alpha$  antibody (Infliximab; courtesy of Prof. A. Jawad) was added at a final concentration of 2.5 $\mu$ g/ml. To assess the effect OPG on reducing the differentiation of osteoclast in the culture system, OPG-Fc (R&D Systems Inc.) was added at a final concentration of 1.0 $\mu$ g/ml. The medium was replenished twice weekly and after 14 days with the same mix in culture. The cultured cells were then stained for TRAP and multinucleated cells were counted as described section 2.1. The results were compared for PBMNCs from healthy controls cultured with supernatants from the RA cells before and after treatment with biologic anti-TNF $\alpha$  agents and compared with the same cells cultured in the presence of TNF $\alpha$  inhibitors or OPG.

### **2.2.9) Fluorescence-activated cell sorting (FACS) analysis for measuring the frequency of OCPs before and after treatment of RA patients with biologic anti-TNF $\alpha$ agents**

PBMNCs were isolated from the blood of patients with RA and healthy controls by centrifugation on Ficoll-Paque gradients. The cells were washed with and resuspended in PBS containing 4% foetal bovine serum (FBS). Aliquots of  $0.1 \times 10^6$  cells were incubated with anti-human CD11b (clone ICRF44), anti-human CD14 (clone M5E2) (both from BD Pharmingen TM, UK) and RANK (R&D) antibodies. The latter staining was carried out to

determine the percentage of cells expressing RANK within the cell population because RANK is a key marker of OCPs. The cells were incubated for 30 minutes at 4°C, washed with 4% FBS-PBS 3 times and fixed using 4% paraformaldehyde and stored at 4°C until analysed (within 24-48hrs). Data was acquired using a FACS Caliber flow cytometer and analysed by CellQuest software version 3.1 (both from Becton Dickinson Immunocytometry Systems, Bedford, Massachusetts, USA).

#### **2.2.10) Disease activity score 28 (DAS28) measurements**

DAS28 is a scoring scheme developed for assessing the severity of disease in patients with RA. It is used as a guide for treatment decisions, for monitoring the response of patients to such treatments as well in clinical trials to assess the efficacy of new drugs. DAS28 is calculated by using a formula that includes counts for tender and swollen joints (28 joints), an evaluation of general health by the patient (on a scale of 0 to 100) and a measure of blood inflammatory markers (ESR or CRP). DAS28 greater than 5.1 is considered to be a marker of high disease activity, between 5.1 and 3.2 of moderate disease activity and less than 3.2 of low disease activity. In addition, a patient with DAS28 less than 2.6 is defined as been in remission (162).

#### **2.2.11) RA patient selection**

The study of the effect of treating patients with biologic anti-TNF $\alpha$  agents on bone in RA patients was carried using data from patients routinely treated at the Department of Rheumatology at Barts and Royal London NHS Trust. Patients that met the American College of Rheumatology (ACR) criteria for RA who were at least 18 years of age, had the disease for at least 3 years and had a no response to, or developed resistance to non-steroidal anti-inflammatory drugs (NSAID) and disease-modifying anti-rheumatic drugs (DMARDs) one of which is always methotrexate were included. A number of patients were studied in the laboratory of Prof. Mageed for their clinical and immunological responses to treatment with

biologic anti-TNF $\alpha$  agents. The study was approved by the local NHS and Central Office for Research Ethics Committee (COREC). The study protocol and information leaflets were prepared and routinely given to all patients studied. For the routine measurement of laboratory serological markers of RA (RF and anti-citrullinated peptide antibodies, CRP, ESR), cytokine production and bone metabolism, 25 ml of blood was drawn at the commencement of the treatment and at 1 month and then 3 month after treatment. Plasma levels of OPG, RANK-L, IL-1, IL-6, IL-17, IL-20, IL-22, TNF $\alpha$ , GM-CSF and bone turnover biomarkers were determined at similar time points. The data was compared between responder and non-responder patients, responders from different ethnic backgrounds and different age, gender and with different lifestyles.

#### **2.2.12) Statistical Methods**

For studying the impact of age, gender, ethnic origin and lifestyle on the response of osteoporosis patients to treatment, differences between the groups were determined using t-test for continuous variables and chi squared test for category variables. Subgroup analyses were carried out to investigate differences in changes in BMD of the spine and the hip in different groups. Changes in BMD between these subgroups were analysed using an independent Student's t-test or the Mann-Whitney U test, as necessary (Appendix). Associations between changes in BMD and risk factors were also assessed in a multivariate linear regression model adjusted for age, gender, ancestral origins and lifestyle.

Changes of lumbar spine and hip BMD and DAS28 in RA patients were compared using the Wilcoxon signed rank sum test (Appendix). Correlations between changes of BMD and DAS28 were also tested using the Spearman correlation coefficient.  $p < 0.05$  values were considered significant for all analysis. Data were analysed by using SPSS 19 program. For the statistical training and continued advice I was ably helped and kindly supported by Ms Janice Thomas and Mr. Peter Brown.

# **Chapter Three**

## **Methodology assessment and refinement**

### **3.1) Assessment of the DEXA protocol used in the study**

#### **3.1.1) *In-vitro* short-term precision**

Before each set of measurements of BMD for patients, 20 scans were performed on the spine phantom provided by the manufacturer with a BMD of  $0.995 \text{ g/cm}^2$  (mean BMD  $0.996 \text{ g/cm}^2$ ,  $SD= 0.003 \text{ g/cm}^2$ ). For repositioning of the phantom between scans, short-term precision errors were expressed in percent coefficient of variation (CV) which was found to be 0.33%. For measurements obtained without repositioning of the phantom (mean BMD  $0.994 \text{ g/cm}^2$ ,  $SD=0.003 \text{ g/cm}^2$ ), the CV of short-term precision was 0.32%. With 99% confidence level, no significant differences ( $p=0.3$ ) were found between the two values (Tables 3.1 and 3.2).

**Table 3.1: Determination of short-term precision of the DEXA with repositioning.**  
 Twenty DEXA scans of a spine phantom were performed with repositioning of the phantom in-between the scans to assess short-term precision.

| Scan number | BMD (g/cm <sup>2</sup> ) | Mean  | Standard Deviation | CV % |
|-------------|--------------------------|-------|--------------------|------|
| 1           | 1                        | 0.996 | 0.0033             | 0.33 |
| 2           | 0.995                    |       |                    |      |
| 3           | 1                        |       |                    |      |
| 4           | 1                        |       |                    |      |
| 5           | 0.995                    |       |                    |      |
| 6           | 0.997                    |       |                    |      |
| 7           | 0.992                    |       |                    |      |
| 8           | 0.995                    |       |                    |      |
| 9           | 0.998                    |       |                    |      |
| 10          | 0.994                    |       |                    |      |
| 11          | 0.998                    |       |                    |      |
| 12          | 0.999                    |       |                    |      |
| 13          | 0.998                    |       |                    |      |
| 14          | 0.996                    |       |                    |      |
| 15          | 0.99                     |       |                    |      |
| 16          | 0.998                    |       |                    |      |
| 17          | 0.995                    |       |                    |      |
| 18          | 0.99                     |       |                    |      |
| 19          | 0.994                    |       |                    |      |
| 20          | 0.99                     |       |                    |      |

Data analysed as described in the Material and Methods. Results of the full statistical analyses are provided in Appendix1, Tables 1 and 2.

**Table 3.2: Determination of short-term precision of the DEXA without repositioning.**

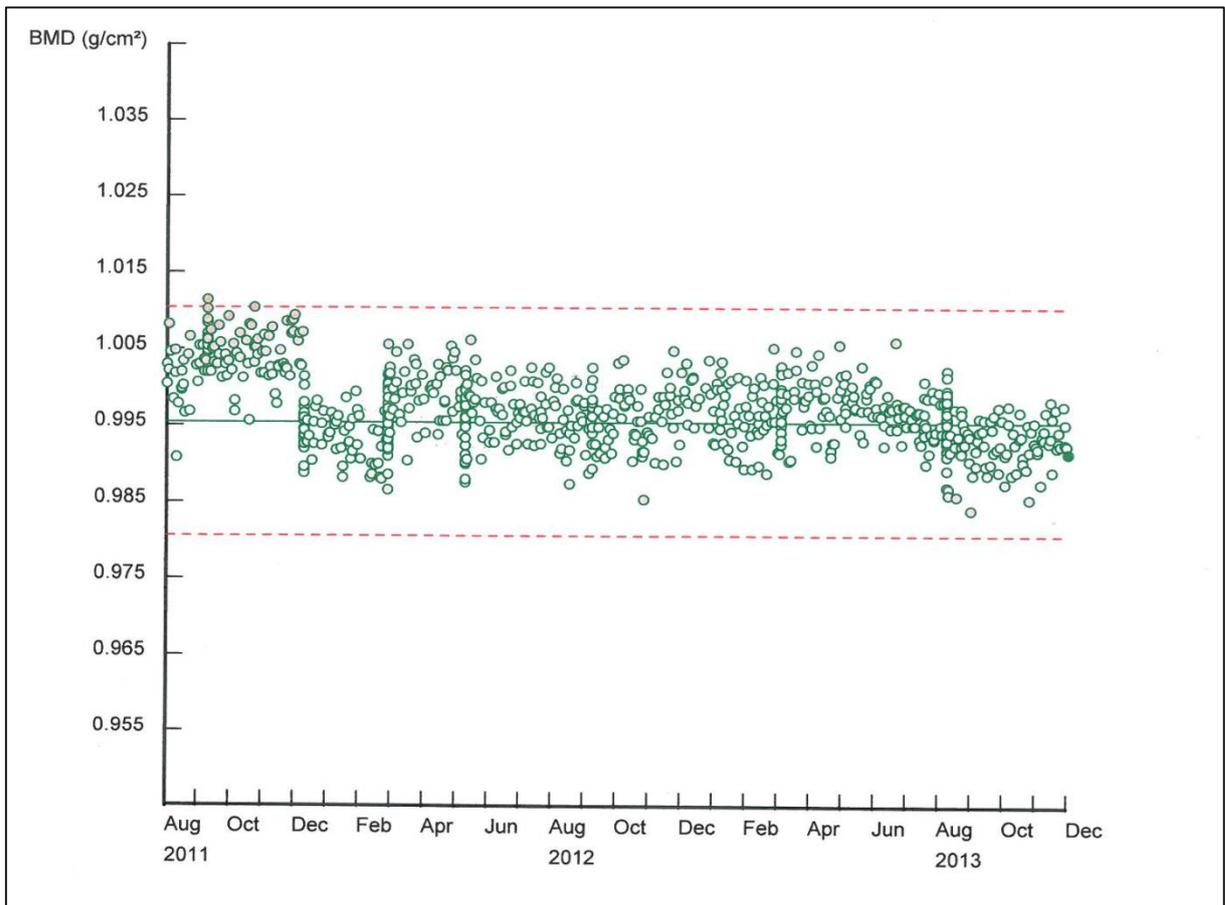
Twenty DEXA scans of a spine phantom were performed without repositioning to assess short-term precision.

| Scan number | BMD (g/cm <sup>2</sup> ) | Mean  | Standard Deviation | CV % |
|-------------|--------------------------|-------|--------------------|------|
| 1           | 0.999                    | 0.994 | 0.0032             | 0.32 |
| 2           | 0.993                    |       |                    |      |
| 3           | 1.000                    |       |                    |      |
| 4           | 0.993                    |       |                    |      |
| 5           | 0.994                    |       |                    |      |
| 6           | 0.993                    |       |                    |      |
| 7           | 0.993                    |       |                    |      |
| 8           | 0.998                    |       |                    |      |
| 9           | 0.996                    |       |                    |      |
| 10          | 0.997                    |       |                    |      |
| 11          | 0.992                    |       |                    |      |
| 12          | 0.993                    |       |                    |      |
| 13          | 0.993                    |       |                    |      |
| 14          | 0.987                    |       |                    |      |
| 15          | 0.997                    |       |                    |      |
| 16          | 0.993                    |       |                    |      |
| 17          | 0.997                    |       |                    |      |
| 18          | 0.990                    |       |                    |      |
| 19          | 0.994                    |       |                    |      |
| 20          | 0.998                    |       |                    |      |

Data analysed as described in the Material and Methods. Results of the full statistical analyses are provided in Appendix1, Tables 1 and 2.

### 3.1.2) *In vitro* long-term precision assessment

The *in vitro* long-term precision of the DEXA scanner was carried out during a period of 28 months. BMD measurements were derived from a spine phantom, as used for short-term precision, and errors in precision were expressed as CV. The mean BMD of the spine phantom was  $0.997\text{g/cm}^2$ ,  $\text{SD}= 0.005\text{g/cm}^2$  and CV 0.5%. As demonstrated in Figure (3.1), all BMD measurements were within limits of  $\pm 1.5\%$  of the mean (Table 3.3).



**Figure 3.1: Long-term *in vitro* precision of BMD measurements.** BMD precision measurements were carried out routinely on a daily basis using the spine phantom to verify the quality control of the scanner. These measurements were carried out for 28 months (August 2011 to Dec. 2013).

**Table 3.3: Results of long-term *in vitro* precision of BMD measurements.**

| <b>Setup</b>                                     | <b>Reference values</b>  | <b>Plot statistics</b>   |
|--|--|--|
| Lumbar Spine phantom #12691<br>System S/N: 80933 | <b>Limits:</b> $\pm 1.5\%$ of mean<br><b>Mean:</b> 0.995 (g/cm <sup>2</sup> )<br><b>SD:</b> 0.003 (g/cm <sup>2</sup> ) | <b>Number of Points:</b> 798<br><b>Mean:</b> 0.997 (g/cm <sup>2</sup> )<br><b>SD:</b> 0.005 (g/cm <sup>2</sup> )<br><b>CV:</b> 0.470 % |

Data obtained from long-term *in vitro* precision report. BMD precision measurements were carried out routinely on a daily basis using the spine phantom to verify the quality control of the scanner.

### **3.1.3) Outcome of precision assessments**

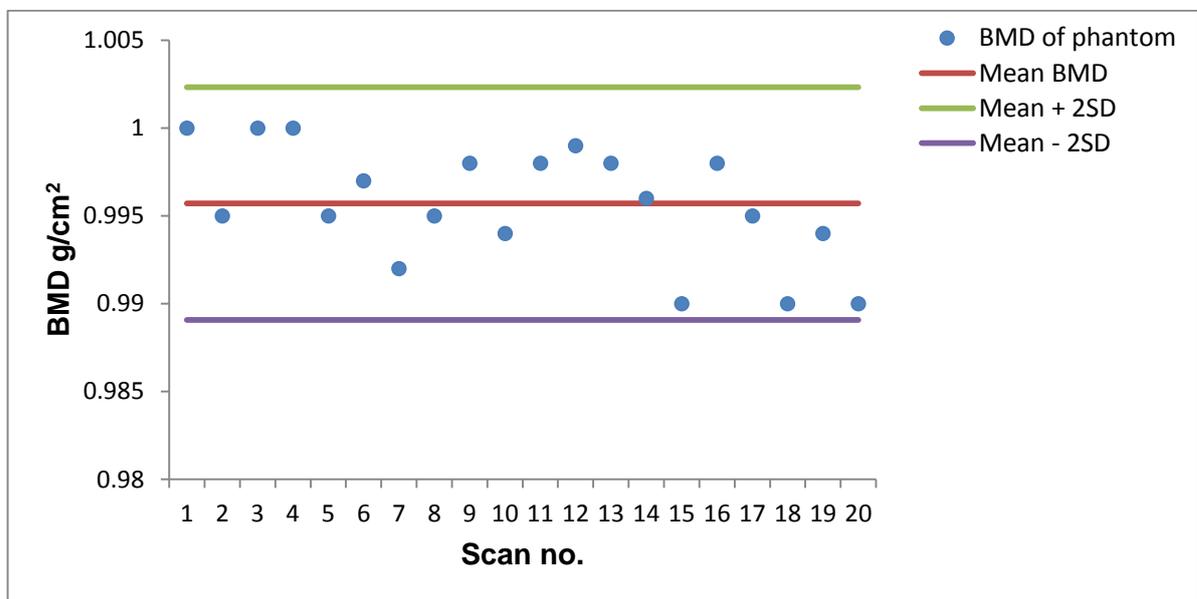
Two sets of assessments were carried out to verify the precision of the DEXA machine: short-term and long-term precision. For short-term precision, repositioning of the phantom was performed in order to determine possible effects of operator-positioning on the precision of BMD measurements. For measurements of the long-term precision, the phantom was kept constant on the scanning table.

As expected, operator-positioning slightly affected precision of the machine as there was a 0.01% difference from 0.33% in the first position to 0.32% in the second. However, with a 99% confidence interval, the differences were not statistically significant ( $p=0.8$ ).

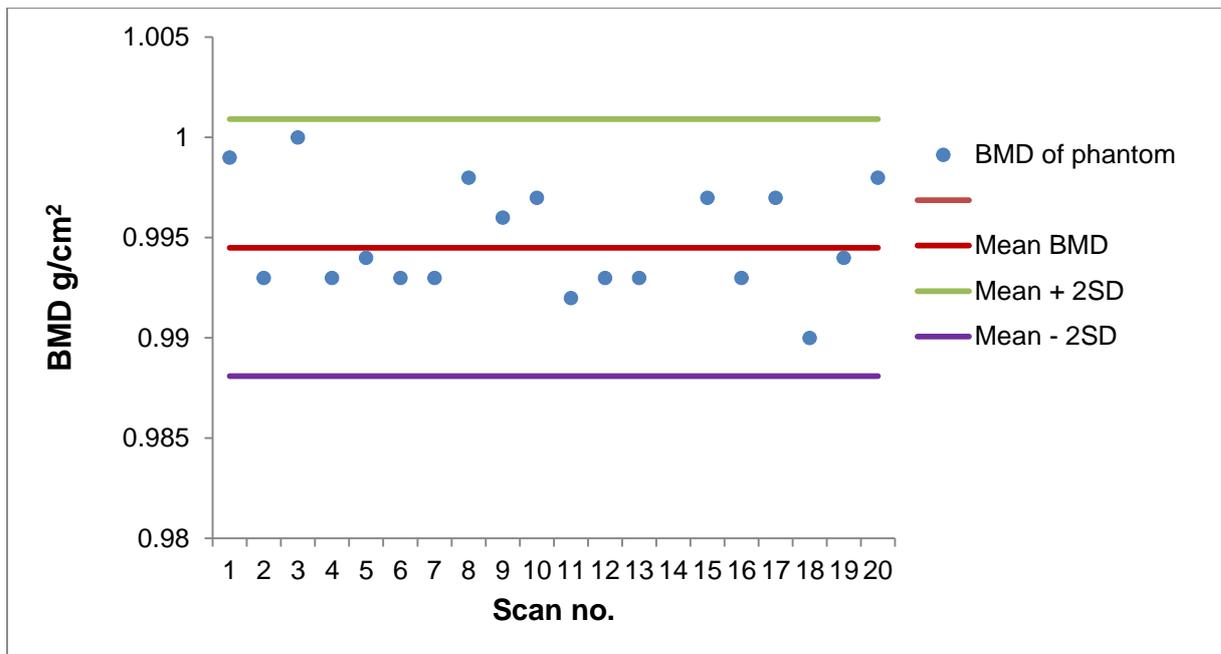
In general, the short-term precision values for the Hologic QDR series DEXA scanner used in the studies were consistent with the precision values provided by the manufacturer stated to be less than 1% (1). With expert personnel, a change of approximately 0.05g/cm<sup>2</sup> in BMD is normally observed as being significant at a 95% confidence interval (1,2). As seen in Tables 3.1 and 3.2, the differences between all BMD measurements were less than 0.05g/cm<sup>2</sup> which confirms the reproducibility of the results. Furthermore, the number of scans performed

when plotted against the BMD in  $\text{g}/\text{cm}^2$ , all BMD measurements were within the limits of  $\pm 2$  SD (Figures 3.2 and 3.3).

By comparing the CV resulted from long-term precision ( $\text{CV}=0.5\%$ ) to that of short-term precision ( $\text{CV}=0.33\%$ ) for the same scanner, the higher value for the former may be due to the influence of variability in the instrument performance through the months of long-term precision observations. However, as long as the value obtained is within the limits of the manufacturer's specifications of the machine, the performance of this scanner was considered reliable in interpreting series of measurements for patients on follow-up studies.



**Figure 3.2: BMD measurements of short-term precision.** Twenty DEXA scans of a spine phantom were performed with repositioning of the phantom in between the scans.



**Figure 3.3: BMD measurements of short-term precision.** Twenty DEXA scans of a spine phantom were performed without repositioning of the phantom in all scans.

### 3.2) Assessment of accuracy and reproducibility of ELISA protocols

#### 3.2.1) The potential interference of RFs in the ELISA protocols for the measurement of bone biomarkers

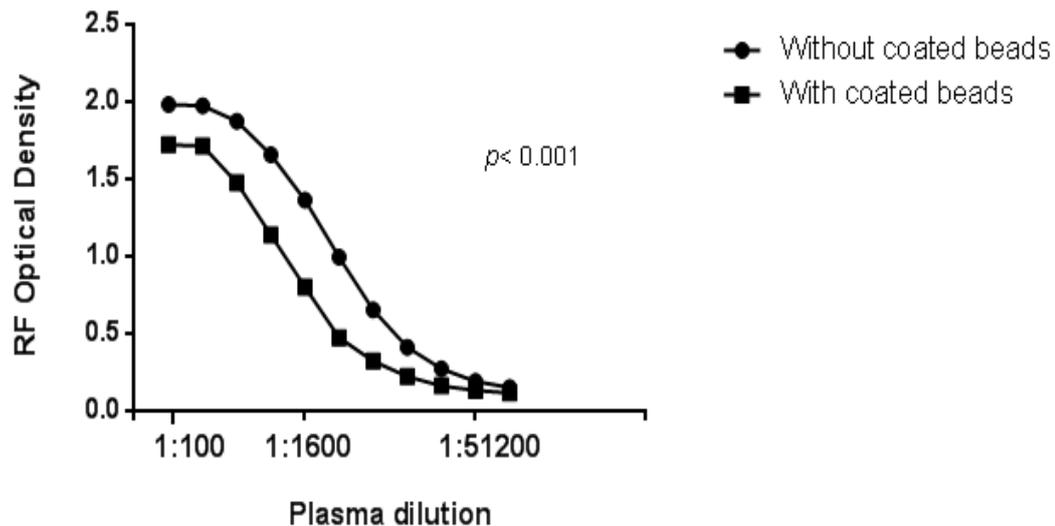
In order to assess if and by how much the presence of RFs in the plasma of patients with RA could interfere with the detection of bone biomarkers and other proteins studied such as cytokines, plasma dilutions were incubated with magnetic beads coated with rabbit IgG as a known target antigen for human RFs. Before testing the efficiency with which the removal of RF could impact the measurement outcome, the ability of the beads to remove RFs from plasma was determined. For this purpose, a microtiter ELISA plate was coated with rabbit IgG at 20µg/ml in BPS and a plasma sample from a patient with RA known to be positive for RF was diluted 1:100 and tested for RF content with and without pre-incubation with 25µl of the coated beads. The magnetic beads were then removed before adding to the plate by attaching the tube with the diluted plasma to a big collection magnet and carefully removing the diluted plasma without the magnetic beads which were attached to the big magnet. The

two diluted plasma samples were then added to the plates and titrated across the plate. Bound RF was revealed with horseradish peroxidase (HRP) conjugated goat F(ab)<sub>2</sub> anti-human IgM. The results revealed that there was a notable reduction in optical density (OD) values obtained for RF binding in the diluted plasma that was pre-incubated with the rabbit IgG coated magnetic beads (Table 3.4 and Figure3.4). Overall, there was a mean reduction of 33% ( $p=0.0004$ ) in the OD of RF with the beads.

**Table 3.4: Assessment of the efficiency of rabbit IgG-coated magnetic beads in removing IgM RF from diluted plasma.**

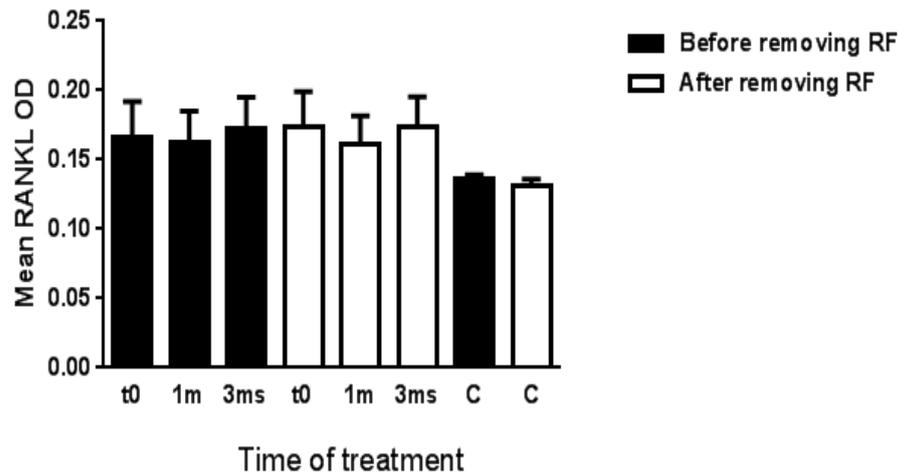
| Plasma dilution | OD for RF without pre-incubation with coated magnetic beads | OD for RF binding after incubation with coated magnetic beads |
|-----------------|---|---|
| 1:100           | 1.983   | 1.722   |
| 1:200           | 1.974   | 1.714   |
| 1:400           | 1.873   | 1.476   |
| 1:800           | 1.658   | 1.138   |
| 1:1600          | 1.364   | 0.801   |
| 1:3200          | 0.994   | 0.475   |
| 1:6400          | 0.654   | 0.321   |
| 1:12800         | 0.412   | 0.221   |
| 1:25600         | 0.272   | 0.162   |
| 1:51200         | 0.191   | 0.133   |
| 1:102400        | 0.152   | 0.116   |

A plasma samples from a patient with RA known to have high levels of IgM RF was diluted 1:100 in PBS and either left without further manipulation or mixed with 25 $\mu$ l of magnetic beads coated with rabbit IgG. The plasma was incubated at 37°C with constant mixing before removing the beads and adding the diluted plasma to the ELISA plate coated with rabbit IgG (Materials and Methods). OD values for the binding of IgM RFs were obtained with HRP-conjugated goat F(ab)<sub>2</sub> anti-human IgM at the wavelength of 492 nm.



**Figure 3.4: The effect of magnetic beads on the removal of RF from diluted RA plasma.** OD values obtained for RF binding to rabbit IgG coated ELISA plate after pre-incubation of the diluted plasma with rabbit IgG coated magnetic beads.

In order to assess the possibility that RF could interfere with the detection of biomarkers such as RANK-L, the level of RANK-L was determined in plasma of 8 RA patients and 5 healthy controls with and without pre-incubation with rabbit IgG coated magnetic beads. In plasma from patients with no pre-incubation with rabbit IgG coated magnetic beads, the mean OD values of RANK-L before and 1 and 3 months after treatment with biologic anti-TNF $\alpha$  agents were 0.166, 0.162, and 0.173 nm (SD=0.1), respectively. After the partial removal of RF with the beads, the mean OD values of RANK-L were 0.173, 0.163 and 0.174, suggesting an increase in OD values by 4.5% before treatment and 0.35% and 0.6% after 1 and 3 months of treatment ( $p=0.83$ ) (Figure 3.5).



**Figure 3.5: The effect of IgM RF in plasma on the ability to detect RANK-L by ELISA.** RANK-L was measured by ELISA in plasma of 8 RA patients and 5 healthy controls (C). Plasma from the patients were tested either with pre-incubation with or without rabbit IgG coated magnetic beads. The plasma samples from the 8 patients were collected either before treatment with biologic anti-TNF $\alpha$  agents or at 1 month and then 3 months after treatment.

### 3.2.2) Conclusions on the effect of RF interference in ELISA for RANK-L measurements

In order to assess, and then reduce, the potential interference of RF in ELISA for RANK-L measurements, magnetic beads were coated with rabbit IgG and used to remove RF from plasma samples and compare RANK-L levels in treated and untreated plasma. Before assessing the effect of the removal of RF, the efficiency of the magnetic beads to remove IgM RF from the plasma was determined.

The results of measuring IgM RF in plasma with and without pre-incubation with the beads revealed a significant reduction in OD for RF. The mean reduction in the OD of RF was 33% ( $p=0.0004$ ) after incubation with the beads. These results, thus, indicated that the beads did manage to reduce the level of RF although by no means all the IgM RFs were removed. However, the level of IgM RF removed could still be important as these RFs were removed with IgG from another species, in this case rabbit, which are the RFs that are likely to

interfere with the ELISAs in which IgG from species other than humans are used to capture the biomarkers of interest, in this case RANK-L.

The OD values for RANK-L measurement by ELISA were 4.5%, 0.35, and 0.6% higher in the plasma of patients before, after 1 and 3 months of the treatment that were pre-incubated with the rabbit IgG coated magnetic beads. However, with a 99% confidence interval, there was no significant difference between measurements of RANK-L whether the plasma samples were incubated with the beads or not ( $p=0.8$ ).

### **3.3) Evaluation of factors that influence the response of patients to anti-osteoporotic therapy**

As cited in chapter one, changes in hormonal status with age leads to increased bone resorption. In addition, in chronic inflammatory conditions of joints, such as seen in patients with RA, chronic inflammation promotes bone resorption and erosion. In contrast to the significant generalised bone loss in osteoporosis, however, the pathology of bone loss in RA that is associated with the disease is generally thought to be mainly focal but there is a significant body of evidence that these patients also suffer from generalised bone loss. In recent years, new therapies have been developed to reduce bone resorption in patients with osteoporosis. However, there is disparity in patient response to these therapies. The reasons are unclear although age, gender, ancestral origin and lifestyle have all been cited as potential factors. In parallel there has been a profound improvement in the clinical care of patients with RA with the use of new biologic anti-inflammatory agents but it remains unclear whether these agents are accompanied by parallel improvements in bone erosion/density whether local or generalised. It was therefore essential to evaluate the factors that determine the response of patients with osteoporosis to anti-osteoporotic agents to be able to provide an unbiased assessment of biologic anti-inflammatory agents on BMD in RA. In order to address these issues, an extensive audit was carried out to assess the factors that influence responsiveness of

patients with osteoporosis to bisphosphonates, a treatment that suppresses bone resorption. Based on the outcome of this audit, it was hoped that the influence of the key factor(s) that affect bone response would be incorporated into unbiased studies of the impact of biologic anti-inflammatory agents on bone response in RA patients.

### **3.3.1) Changes in BMD of osteoporosis patients treated with bisphosphonates**

#### **a) Patient enrolment**

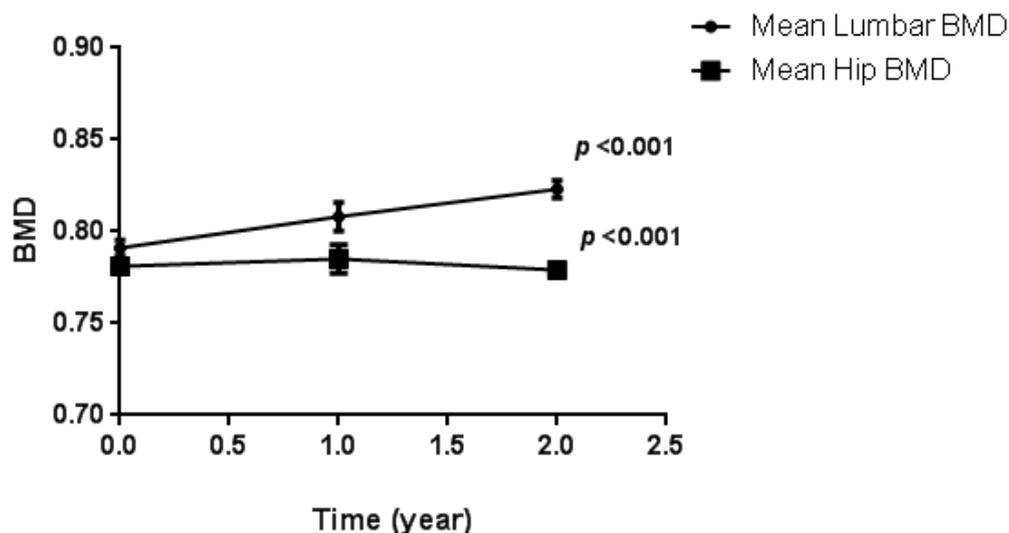
A cohort of 647 patients (mean age  $68.3 \pm 11.6$  years) with primary osteoporosis attending the osteoporosis clinics at the Royal London and St Bartholomew's Hospitals between April 2011 and August 2013 were studied. In this study of patients receiving bisphosphonates, changes in BMD (spine and hip) were measured in daily clinical practice during 2 years of treatment. For the purpose of the study, the impact of age, gender, ancestral origin and smoking on bone response was evaluated. Patients with osteoporosis were selected for a number of reasons including the availability of a large cohort of patients and that most were treated primarily for their bone disease with anti-osteoporotic medications (bisphosphonates). Patients who were bedridden, using steroids or anti-epileptics drugs, with diabetes mellitus, had renal disease, hyperthyroidism or cancers were excluded. Lumbar spine (L1-L4) and hip were assessed by DEXA scans (Hologic Discovery QDR series). Both sites were scanned simultaneously and were monitored for the progress of their response annually. Duration of bisphosphonate treatment was  $1.95 \pm 0.1$  years (mean  $\pm$  SD). For the purpose of this study, BMD "nonresponse" was defined as having BMD decrease from before treatment (baseline) at two of the measured sites at 2 years after treatment has started. BMD "response" was defined as having either no change, or increase from before treatment (baseline) at the measured sites at 2 years post treatment. The coefficient of variation (CV), measured with a local spine phantom for DEXA scan was acceptable (described in chapter 2). All patients received oral daily calcium (1000mg) and vitamin D (800IU). Demographic and clinical

characteristics were collected by interviewer-administered questionnaire before treatment and after 1 and 2 years during DEXA scan appointments. Data collected was comprised of age, lifestyle (smoking, alcohol consumption for more than 2 servings a day and physical exercise), personal and family history of fractures and current medications.

### **b) Changes in BMD**

Mean changes in BMD in both lumbar spine and hip after one year were  $0.017\text{g/cm}^2$  (2.14%,  $p<0.001$  vs baseline) and  $0.006\text{g/cm}^2$  (0.5%,  $p<0.001$  vs baseline). Furthermore, changes in BMD after 2 years of treatment were  $0.032\text{g/cm}^2$  (4.1%,  $p<0.001$  vs baseline) and  $-0.002\text{g/cm}^2$  (-0.25%,  $p<0.001$  vs baseline) in both lumbar spine and the hip respectively (Figure 3.6).

Of the 647 patients included in this study 475 responded to treatment with bisphosphonates while 172 did not. The BMD of the lumbar spine and hip increased in responder patients by  $0.056\text{g/cm}^2$  (7.1%,  $p<0.001$  vs. baseline) and by  $0.004\text{g/cm}^2$  (0.51%,  $p<0.001$  vs. baseline), respectively. For non-responders, BMD decreased by a mean of  $-0.031\text{g/cm}^2$  (-3.9%,  $p<0.001$  vs. baseline) and  $-0.018\text{g/cm}^2$  (-2.3%,  $p<0.001$  vs. baseline) in lumbar spine and the hip, respectively. Epidemiological data on the patient cohort studied, their brief medical history, lifestyle and BMD baseline are summarised in Tables 3.5 and 3.6. There were no statistically-significant differences between responder and non-responder patients in age, gender, risk factors and baseline BMD.



**Figure 3.6: Changes in BMD after treatment of osteoporosis patients with bisphosphonates.** A total of 647 patients treated with bisphosphonates were included in this analysis. Mean lumbar spine and hip BMD were measured using DEXA scan at baseline (time 0) and at 1 and 2 years post treatment. The error bars represent +/- 2 standard deviations (SD).

**Table 3.5: Baseline BMD prior to initiation of the treatment for responder and non-responder osteoporosis patients.**

| Parameter                | Responder<br>n=475 | Non-responder<br>n=172 | All patients<br>n=647 |
|--------------------------|--------------------|------------------------|-----------------------|
| <b>Lumbar spine</b>      |                    |                        |                       |
| BMD (g/cm <sup>2</sup> ) | 0.793(0.11)        | 0.787 (0.1)            | 0.791(0.1)            |
| t-score                  | -2.88(1.0)         | -2.87 (0.9)            | -2.88 (0.96)          |
| <b>Hip</b>               |                    |                        |                       |
| BMD (g/cm <sup>2</sup> ) | 0.784(0.12)        | 0.772 (0.11)           | 0.781 (0.11)          |
| t-score                  | -2.43(0.85)        | -2.46 (0.84)           | -2.44 (0.85)          |

BMD values [mean (SD)] were measured by DEXA scan. According to WHO criteria, a patient with t-score  $\leq -2.5$  at any skeletal sites is diagnosed as having primary osteoporosis

**Table 3.6: Epidemiological data on osteoporosis patients studied.**

| <b>Parameter</b>                                   |           | <b>Responder<br/>n=475</b> | <b>Non Responder<br/>n= 172</b> | <b>Total<br/>647</b> |
|--|-----------|----------------------------|---------------------------------|----------------------|
| <b>Age</b>   | Mean (SD) | 68.6 (11.8)                | 67.5 (11)                       | 68.3 (11.6)          |
| <b>Gender (female)</b>                             | n (%)     | 382 (80.4)                 | 151 (87.8)                      | 533                  |
| Post-menopausal                                    | n (%)     | 366 (96)                   | 144 (95)                        | 510                  |
| <b>Ancestral origin</b>                            |           |                            |                                 |                      |
| Caucasians   | n (%)     | 383 (81)                   | 135 (79)                        | 520                  |
| Asians   | n (%)     | 68 (14)                    | 28 (16)                         | 97                   |
| Afro-Caribbean                                     | n (%)     | 21 (5)                     | 9 (5)                           | 30                   |
| <b>Calcium Intake (mg/day),<br/>Vitamin D (µg)</b> | Mean (SD) | 586 (370)                  | 600 (360)                       | 590 (367)            |
|  | Mean (SD) | 5.6 (3.7)                  | 5.7 (3.6)                       | 5.6 (3.7)            |
| <b>Age group</b>                                   |           |                            |                                 |                      |
| Fifty  | n (%)     | 89 (19)                    | 30 (17)                         | 119                  |
| Sixty  | n (%)     | 139 (29)                   | 75 (44)                         | 214                  |
| Seventy  | n (%)     | 159 (33)                   | 39 (23)                         | 198                  |
| Eighty   | n (%)     | 88 (19)                    | 28 (16)                         | 116                  |
| <b>Fracture history (vertebral and peripheral)</b> | n (%)     | 79 (17)                    | 38 (22)                         | 117                  |
| <b>Familial fracture</b>                           | n (%)     | 60 (13)                    | 27 (16)                         | 87                   |
| <b>History of medically induced menopause</b>      | n (%)     | 29 (6)                     | 14 (8)                          | 43                   |
| <b>Been on HRT,</b>                                | n (%)     | 51 (11)                    | 22 (13)                         | 73                   |
| <b>Low Body Mass</b>                               | n (%)     | 42 (8)                     | 25 (15)                         | 67                   |
| <b>OP treatment</b>                                |           |                            |                                 |                      |
| Alendronic Acid (70 mg once/w)                     | n (%)     | 278 (58)                   | 89 (52)                         | 367                  |
| IV Zoledronic Acid 5mg/year                        | n (%)     | 133 (28)                   | 46 (27)                         | 179                  |
| Risendronic Acid (35 mg/w)                         | n (%)     | 37 (8)                     | 31 (18)                         | 68                   |
| Pamidronate (30 mg/3months)                        | n (%)     | 27 (6)                     | 6 (3)                           | 36                   |
| <b>Lifestyle (history)</b>                         |           |                            |                                 |                      |
| Smoker   | n (%)     | 86* (18)                   | 35 (20)                         | 121                  |
| Non-smoker   | n (%)     | 266 (56)                   | 94 (55)                         | 360                  |
| Alcohol consumption (more than 2 servings)         | n (%)     | 38 (8)                     | 6 (3)                           | 44                   |
| Physical (weight bearing exercises)                | n (%)     | 117 (25)                   | 39 (23)                         | 156                  |

Six hundred and forty seven patients were included in the study to assess the effect of age, gender, ancestral origin and smoking habits on the response to treatment with bisphosphonates. All patients whose data were analysed were from the cohort that attended the rheumatology clinics at the Royal London and St Barts Hospitals between April 2011 and August 2013. \* Information on smoking habits of only 481 patients was available.

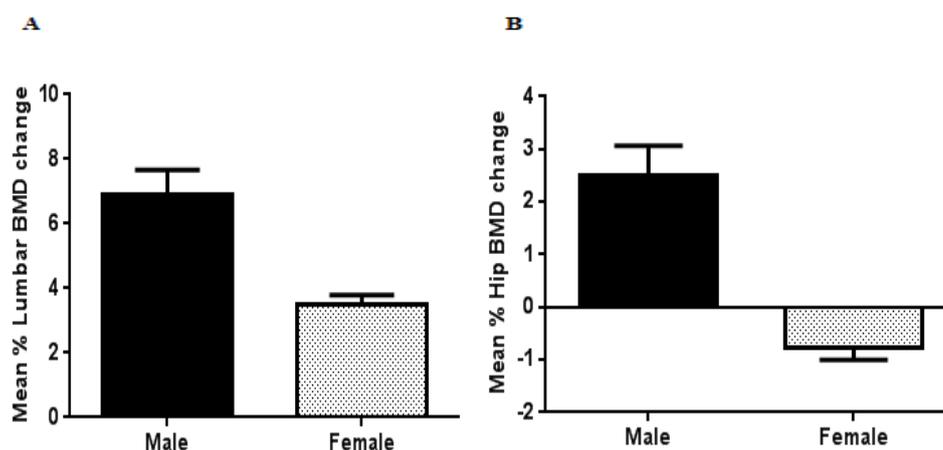
**c) Factors that influence changes in BMD in patients treated with bisphosphonates**

In order to assess the impact of individual factors on the response of osteoporosis patients to treatment with bisphosphonates, the effect of gender on changes in BMD was first investigated. Improvement in BMD among all male patients was statistically significant after 2 years of treatment when compared with baseline values (Table 3.7). The BMD of the lumbar spine and the hip increased in males by 0.057g/cm<sup>2</sup> (6.9%, *p*<0.001 vs. before treatment) and by 0.021g/cm<sup>2</sup> (2.48%, *p*<0.001 vs. before treatment), respectively. For female patients, changes in BMD of the lumbar spine and hip were 0.028g/cm<sup>2</sup> (3.58%, *p*<0.001 vs. before treatment) and -0.006g/cm<sup>2</sup> (-0.78%, *p*<0.001 vs. before treatment), respectively (Figure 3.7).

**Table 3.7: Change in BMD in male and female osteoporosis patients treated with bisphosphonates.**

| <b>Gender</b>               | <b>N</b>      | <b>BMD values before treatment (SD)</b> | <b>Mean % BMD change</b> | <b>Std. Error of Mean</b> |
|-----------------------------|---------------|---|--------------------------|---------------------------|
| <b>Change in lumbar BMD</b> | <b>Male</b>   | 114                                     | 0.828(0.11)              | 0.8                       |
|                             | <b>Female</b> | 533                                     | 0.783(0.11)              | 0.3                       |
| <b>Change in hip BMD</b>    | <b>Male</b>   | 114                                     | 0.846(0.13)              | 0.57                      |
|                             | <b>Female</b> | 533                                     | 0.767(0.11)              | -0.78                     |

Group statistics and independent samples test for changes in BMD in male and female patients are presented as the mean and data analysed as described in the Materials and Methods. Full statistical analyses are described in Appendix 1, Table 3 and 4.



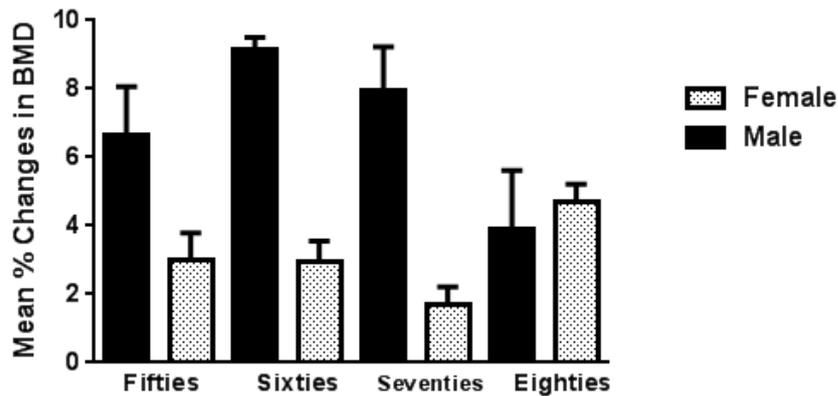
**Figure 3.7: Changes in BMD at lumbar spine (A) and hip (B) in male and female osteoporosis patients after 2 years post-treatment with bisphosphonates.** Data on 533 females and 114 male patients were analysed as described in the Materials and Methods chapter. BMD values are presented as the mean +/- SEM.

Analysis of effect of gender within the same age groups showed that male patients had a higher improvement in BMD as compared with female patients in the same age group (Table 3.8). However, the change in BMD of male patients in the eighties age groups was lower than female patients, but, caution should be taken in over interpretation of these results due to the low number of male patients in this age group (10 male patients only) (Figure 3.8).

**Table 3.8: Change in BMD in male and female osteoporosis patients according to age treated with bisphosphonates.**

| Change in lumbar BMD |                  | N   | Mean % BMD change | Std. Error of Mean (SEM) |
|----------------------|------------------|-----|-------------------|--------------------------|
| <b>Male</b>          | <b>Fifties</b>   | 25  | 6.66              | 1.4                      |
|                      | <b>Sixties</b>   | 33  | 9.14              | 1.9                      |
|                      | <b>Seventies</b> | 46  | 7.9               | 1.3                      |
|                      | <b>Eighties</b>  | 10  | 3.9               | 1.7                      |
| <b>Female</b>        | <b>Fifties</b>   | 94  | 3.1               | 1                        |
|                      | <b>Sixties</b>   | 181 | 2.95              | 0.6                      |
|                      | <b>Seventies</b> | 152 | 1.7               | 0.5                      |
|                      | <b>Eighties</b>  | 106 | 4.7               | 0.5                      |

Group statistics and independent sample tests for changes in BMD in osteoporosis patients divided according to gender and age groups are presented as the mean and data analysed as described in the Materials and Methods chapter.



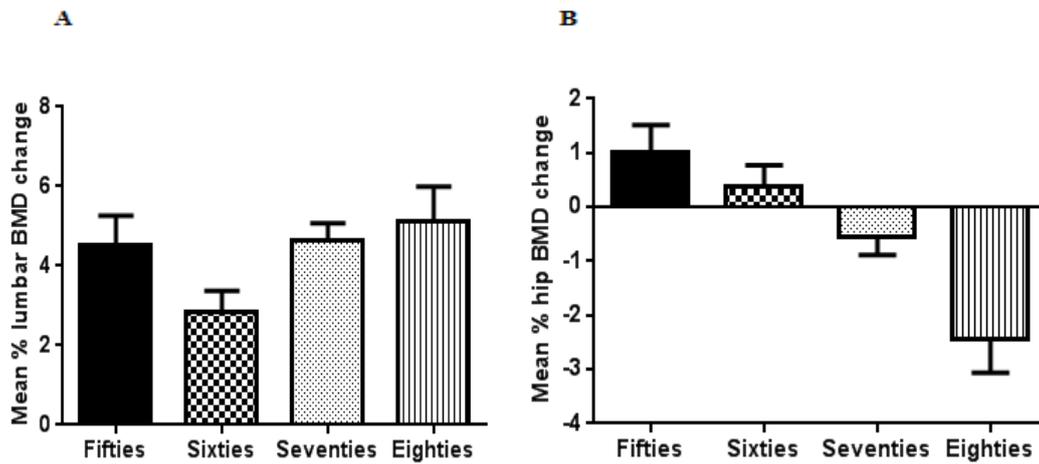
**Figure 3.8: Changes in BMD in osteoporosis patients in relation to age in males and females.** The 647 male and female osteoporosis patients were divided into subgroups based on age range to assess the effect of age in males and females on changes in lumbar BMD after treatment with bisphosphonates.

Variation in BMD among different age groups was analysed by ANOVA (Table 3.9). Lower improvement in hip BMD among older patients was statistically significant after 2 years of treatment when compared with younger patients. In contrast, significant differences between the age groups in the lumbar spine BMD could not be demonstrated. Furthermore, patients in their sixties responded less well than other groups (Figure 3.9).

**Table 3.9: Changes in BMD in response to bisphosphonates according to age.**

| Age                         | N   | Mean % BMD change | Std. Error of Mean (SEM) |
|-----------------------------|-----|-------------------|--------------------------|
| <b>Change in lumbar BMD</b> |     |                   |                          |
| <b>Fifties</b>              | 119 | 4.54              | 0.72                     |
| <b>Sixties</b>              | 214 | 2.86              | 0.52                     |
| <b>Seventies</b>            | 198 | 4.6               | 0.65                     |
| <b>Eighties</b>             | 116 | 5.1               | 0.87                     |
| <b>Change in hip BMD</b>    |     |                   |                          |
| <b>Fifties</b>              | 119 | 1.1               | 0.5                      |
| <b>Sixties</b>              | 214 | 0.4               | 0.4                      |
| <b>Seventies</b>            | 198 | -0.54             | 0.3                      |
| <b>Eighties</b>             | 116 | -2.44             | 0.6                      |

Group statistics and independent sample tests for changes in BMD in osteoporosis patients divided according to age are presented as the mean and data analysed as described in the Materials and Methods chapter.



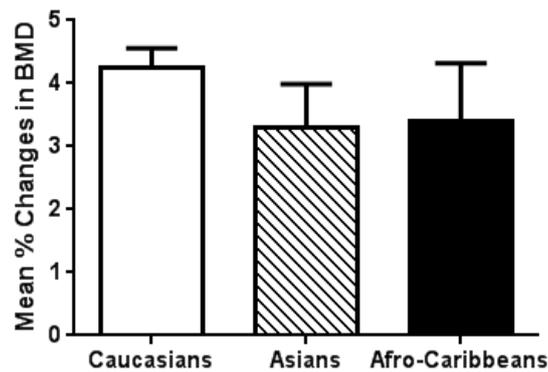
**Figure 3.9: Changes in BMD in osteoporosis patients shown on the basis of differences according to age groups.** The analyses include 119 patients in their fifties, 214 in their sixties, 198 in their seventies and 116 in their eighties.

Differences in BMD in the lumbar spine and the hip between patients from different ethnic origins were not statistically significant (Table 3.10). There was more improvement in BMD in Caucasian patients than in Asian and Afro-Caribbean patients. However, the number of Afro-Caribbean patients available to include in the analysis was low which has impacted the statistical analysis. Generally, however, Afro-Caribbean patients had higher BMD before treatment (0.855 and 0.914 g/cm<sup>2</sup>) compared with Caucasian (0.796 and 0.773g/cm<sup>2</sup>) and Asian patients (0.746 and 0.783g/cm<sup>2</sup>) for lumbar spine and hip respectively (Figure 3.10).

**Table 3.10: Changes in BMD of osteoporosis patients divided according to ethnic origin in response to bisphosphonates.**

| Ethnic origin            | N   | Baseline BMD (mean SD) | Mean BMD change | Std. Error (SEM) |
|--------------------------|-----|------------------------|-----------------|------------------|
| <b>Lumbar BMD change</b> |     |                        |                 |                  |
| Caucasias                | 520 | 0.795 (0.1)            | 4.24            | 0.32             |
| Asians                   | 97  | 0.748 (0.1)            | 3.3             | 0.69             |
| Afro-Caribbean           | 30  | 0.855 (0.1)            | 3.4             | 0.92             |
| <b>Hip BMD change</b>    |     |                        |                 |                  |
| Caucasias                | 520 | 0.773 (0.1)            | -0.4            | 0.3              |
| Asians                   | 97  | 0.783 (0.1)            | 0.96            | 0.7              |
| Afro-Caribbean           | 30  | 0.914 (0.1)            | -0.2            | 0.9              |

Group statistics and independent sample tests for changes in BMD in osteoporosis patients divided according to ethnic origin. The data is presented as the mean and SEM and analysed as described in the Materials and Methods.



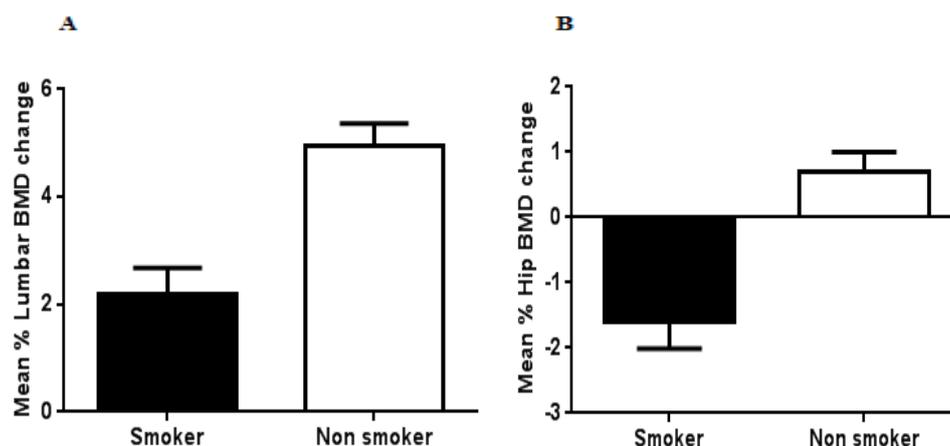
**Figure 3.10: Changes in lumbar spine BMD in patients based on ethnic origin.** A total of 520 Caucasian, 97 Asian and 30 Afro-Caribbean osteoporosis patients treated with bisphosphonates were included in this analysis. The data are for all patients irrespective of whether they were responders or non-responders to the treatment.

With respect to the effect of smoking on changes in BMD, a considerably lower improvement in BMD of the lumbar spine and the hip was observed in smoker patients compared with non-smokers ( $p=0.001$ ) (Table 3.11). Changes in BMD of smokers' lumbar spine and hip were  $0.017\text{g}/\text{cm}^2$  (2.18%) and  $-0.01\text{g}/\text{cm}^2$  (-1.61%), respectively, and  $0.04\text{g}/\text{cm}^2$  (4.96%) and  $0.006\text{g}/\text{cm}^2$  (0.7%) for BMD of lumbar spine and hip of non-smokers (Figure 3.11).

**Table 3.11: Changes in BMD in smoker and non-smoker osteoporosis patients.**

|                              | Smoke      | N   | Baseline BMD<br>(mean SD) | Mean BMD<br>change | Std. Error<br>(SEM) |
|------------------------------|------------|-----|---------------------------|--------------------|---------------------|
| <b>Lumbar BMD<br/>change</b> | Smoker     | 121 | 0.781(0.1)                | 2.18               | 0.5                 |
|                              | Non smoker | 360 | 0.80(0.1)                 | 4.96               | 0.4                 |
| <b>Hip BMD<br/>change</b>    | Smoker     | 121 | 0.761(0.11)               | -1.61              | 0.4                 |
|                              | Non smoker | 360 | 0.787(0.11)               | 0.7                | 0.3                 |

Group statistics and independent sample tests for changes in BMD in osteoporosis patients divided according to smokers and non-smokers. Full statistical analysis is provided in Appendix 1, Tables 5 and 6.



**Figure 3.11: The effect of smoking on changes in lumbar and hip BMD in osteoporosis patients treated with bisphosphonates.** BMD data on 121 smoker and 360 non-smoker osteoporosis patients treated with bisphosphonates were analysed. BMD values after 2 years of treatment were compared with baseline (before treatment). Error bars: +/- SEM.

In addition, Spearman's correlation coefficient analyses were performed to assess the relationship between patient characteristics (age, gender, ancestral origin and smoking) and changes in BMD at both lumbar spine and the hip (Table 3.12). Being female and smoking correlated significantly with lower improvement in BMD at the lumbar spine as well as in the hip ( $p < 0.001$ ). Changes in BMD at the hip were significantly higher in younger patients. The ethnic origin was not significantly related to changes in BMD levels. Finally, there were no significant correlations between changes in lumbar spine and hip BMD and other clinical characteristics such as history of medically-induced menopause, been on HRT and having low body mass ( $p > 0.05$ ).

**Table 3.12: Correlation between lumbar spine and hip BMD in osteoporosis patients treated with bisphosphonates with age, gender, ethnic origin and smoking.**

| Patient characteristics | Hip BMD Changes |                 | Lumbar spine BMD changes |                 |
|-------------------------|-----------------|-----------------|--------------------------|-----------------|
|                         | <i>r</i>        | <i>p</i> -value | <i>r</i>                 | <i>p</i> -value |
| <b>Age</b>              | -0.20           | <0.001          | 0.05                     | 0.22            |
| <b>Gender (female)</b>  | -0.46           | <0.001          | -0.36                    | <0.001          |
| <b>Ethnic origin</b>    | 0.06            | 0.11            | 0.05                     | 0.24            |
| <b>Smoking</b>          | -0.26           | <0.001          | - 0.34                   | <0.001          |

Correlations were analysed using Spearman's correlation coefficient analysis.

**d) The effect of drug formulation for route of administration and patient compliance on the response to anti-osteoporotic agents**

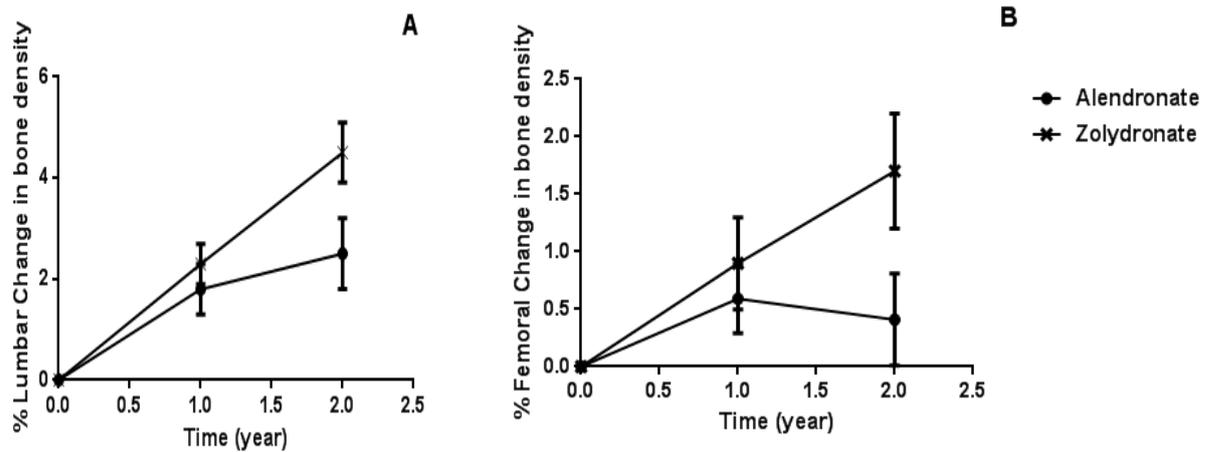
It is established that due to side effects associated with oral administration of bisphosphonates that patient compliance could be a factor in determining the beneficial therapeutic effects of these agents. Therefore, an audit was carried out to assess the impact of drug route and possible compliance of patients on the effect of bisphosphonates. To address these issues, data from patients receiving the orally-administered bisphosphonate agent alendronate and the intravenously-administered zoledronate were compared. There were no significant differences in age, gender, ethnicity and lifestyle between the two groups of patients. The mean age of patients studied was  $67.5 \pm 12.5$  years for the alendronate group and  $69.6 \pm 11.1$  for the zoledronate group. The proportion of post-menopausal women in each group was 94 and 98% for the alendronate and the zoledronate group, respectively. Baseline BMD in both treatment groups at the hip and lumbar spine sites were comparable (Table 3.13). BMD at the lumbar spine increased from baseline after two years in patients receiving either alendronate or zoledronate. However, the increase was significantly greater in patients

receiving zoledronate (% BMD change 3.58% for alendronate vs. 5.69% for zoledronate;  $p<0.001$ ) (Figure 3.12A). BMD of the hip in patients receiving alendronate increased by 0.41% from baseline after two years of treatment, whereas the zoledronate group increased by 1.7%;  $p<0.001$  (Figure 3.12B).

**Table 3.13: Epidemiological data, medication and life style of the patient cohort studied for treatment efficacy with alendronate and zoledronate.**

| <b>Parameter</b>  | <b>Oral alendronate<br/>70mg once weekly<br/>n=118</b> | <b>IV zoledronate<br/>5mg/year<br/>n= 116</b> |
|---|--|---|
| <b>Age</b> , mean in years (SD)                           | 67.5 (12.5)  | 69.6 (11.1)                                   |
| <b>Gender</b> (female), n (%)                             | 98 (83)  | 100 (86)                                      |
| Post-menopausal, n (%)                                    | 92 (94)  | 98 (98)                                       |
| <b>Fracture history</b> (vertebral and peripheral), n (%) | 19 (16)  | 28 (24)                                       |
| <b>Familial fracture</b> , n (%)                          | 16 (14)  | 17 (15)                                       |
| <b>Calcium Intake</b> (mg/day), mean (SD)                 | 692 (266)  | 697 (259)                                     |
| <b>Vitamin D</b> ( $\mu\text{g}$ ), mean (SD)             | 6.40 (2.9)   | 6.45 (2.9)                                    |
| <b>History of medically induced menopause</b> , n (%)     | 10 (9)   | 9 (8)   |
| <b>Been on HRT</b> , n (%)                                | 8 (7)  | 9 (8)   |
| <b>Low Body Mass</b> , n (%)                              | 6 (5)  | 9 (8)   |
| <b>Lifestyle (history)</b> , n (%)                        |  |   |
| Smoking   | 17 (15)  | 17 (15)                                       |
| Alcohol consumption (more than 2 servings)                | 7 (6)  | 5 (5)   |
| Physical (weight bearing exercises)                       | 12 (10)  | 20 (17)                                       |
| <b>Ethnic origin</b> , n (%)                              |  |   |
| Caucasian   | 92 (78)  | 98 (84)                                       |
| Asian   | 20 (17)  | 14 (12)                                       |
| Afro-Caribbean  | 6 (5)  | 4 (4)   |
| <b>Lumbar spine BMD</b> ( $\text{g}/\text{cm}^2$ ) (SD)   | 0.82 (0.1)   | 0.75 (0.1)                                    |
| <b>Total hip BMD</b> ( $\text{g}/\text{cm}^2$ ) (SD)      | 0.79 (0.1)   | 0.75 (0.1)                                    |

No statistically significant differences were observed between the treatment groups.



**Figure 3.12: Change in BMD from baseline in response to treatment with alendronate and zoledronate.** The assessment was carried out after two years of treatment at: (a) the lumbar spine and (b) the hip sites in patients with osteoporosis receiving oral alendronate or intravenous zoledronate. BMD of the hip and lumbar spine were determined by DEXA scan at baseline (time 0) and at 1 and 2 years post treatment initiation.

**e) Conclusions on the assessment of factors that influence the response of patients to anti-osteoporotic therapy**

Of patients with osteoporosis treated with bisphosphonates, 27% were non-responders with evidence of reduction in BMD 2 years after start of treatment at the measured sites while 73% were responders with stable, or increased BMD at the measured sites 2 years after start of treatment.

Mean changes in BMD in both lumbar spine and the hip in all patients after 2 years of treatment were  $0.032\text{g/cm}^2$  (+4.1%,  $p < 0.001$  vs. baseline) and  $-0.002\text{g/cm}^2$  (-0.25%,  $p < 0.001$  vs baseline), respectively. The difference in values of BMD at the hip and lumbar spine sites would be due to the higher rate of bone remodelling in cancellous bones, more abundant in the lumbar spine than cortical bones, abundant in hips (163). In this study, the results of assessing the impact of bisphosphonates on BMD are consistent with results of earlier studies (164-167).

The study also revealed a greater increase in BMD in male patients compared with female patients. This finding can be explained by the fact that postmenopausal women between the ages of 50-60 are prone to higher rates of osteoporosis due to ovarian failure and significant oestrogen reduction that accelerate bone resorption by 90% at menopause. These observations have been confirmed by biomarkers, whereas bone formation markers increase by only 45% and this imbalance between bone resorption and bone formation leads to lower improvement in BMD in females (163,168). In contrast, testosterone hormone in males has beneficial effects on BMD improvements with therapy (168).

The data on smoking were interesting as they showed a significant association with lower BMD improvement. Association between smoking and changes in BMD has been documented previously (169-171). Thus, many investigators have linked reduced improvement in BMD after treatment with smoking habit both in treated as well as untreated patients (169-171).

Overall, findings from this study are consistent with those of other investigators (172,173). Thus, data showing higher baseline BMD in the lumbar spine and the hip of Afro-Caribbean patients compared with Caucasian and Asian patients is consistent with previous reports (172). However, the current study did not have a large cohort of patients of Afro-Caribbean origin to make the data comprehensive and significant.

Interestingly, data on improvement in BMD in osteoporosis patients showed that the route of administration of the drug and, hence, possible compliance with the agent could also impact the response to treatment. In general, however, the data indicates that age, gender, ancestral origin and smoking habits underpin variations in the response of patients to treatment directed as osteoporosis; in this case bisphosphonate. These factors will, therefore,

should be taken into consideration when analysing the effect of treating RA patients with biologic anti-inflammatory agents.

Finally, although the results of these methodological assessments and refinements have revealed important benchmarks for consideration when analysing the effect of biologic anti-inflammatory on BMD in RA patient, the data, nevertheless, have limitations. Firstly, the analysis of ethnicity in relation to the response to bisphosphonates is likely to have been affected by the small sample size of Afro-Caribbean patients. Furthermore, sample size was also a consideration when assessing the relationship between changes in BMD and fractures, the main endpoint of interest for clinicians. Secondly, the analyses could have been stronger had it been combined with measurements of blood and urine biomarkers of bone formation and resorption including the level of sex hormones. A third limitation of the study is the duration of time between assessments of changes to BMD as we had to wait for 1-2 years to assess a patient's response to the bisphosphonates (174). This could be important as some studies have shown significant reductions in blood biomarkers of bone turnover within days to months of starting treatment with bisphosphonates (175).

# **Chapter Four**

**The effect of biologic anti-TNF $\alpha$  agents on bone  
in patients with RA: clinical and laboratory  
evidence**

## 4.1 Introduction

RA, perhaps uniquely, manifests local and systemic bone pathology (68). Local bone damage in RA patients appears as juxta-articular osteopenia and subchondral bone erosion, the hallmark of joint destruction, which essentially influences the functional outcome of the disease. In contrast, generalised bone loss in RA represents a silent and slowly evolving counterpart to local bone loss (176,131). In the recent literature, there is a clear association between localised bone loss, most notably in the hands, in RA patients and generalised bone loss, such as seen in the spine and hip, suggesting a common pathway for this loss of bone in RA. Changes to the homeostasis of the RANK-L and OPG system provide a theoretical backdrop for such a common mechanism (177). RANK-L expression is one among the key factors that is upregulated by several pro-inflammatory cytokines including TNF $\alpha$ , IL-1, IL-6, IL-17, IL-20 and IL-22 (146,178). In addition to its role in initiating and maintaining chronic inflammation, TNF $\alpha$  has osteoclastogenic properties and, thus, can promote bone resorption (179). Indeed, the assessment of biomarkers of bone turnover in RA shows an increase in bone resorption as the dominant pathological mechanism mediated by TNF $\alpha$  (180). Although biologic anti-TNF $\alpha$  agents have been suggested to successfully slow down, or even arrest, local bone resorption as well as inhibiting inflammation in mice (181), their potential to interfere with local and, more importantly, generalised bone loss in RA patients has not been adequately explored. Furthermore, it is unclear if bone metabolism is affected in all patients treated with biologic anti-TNF $\alpha$  agents, the degree of variability in responsiveness and the factors that determine such variability. This chapter aims to address these issues and also study changes in biomarkers of bone turnover, including RANK-L and OPG before and following treatment with biologic anti-TNF $\alpha$  agents.

## **4.2 Materials and Methods:**

### **4.2.1 Retrospective study on the effects of treatment with biologic anti-TNF $\alpha$ on BMD:**

#### **a) Patient enrolment**

In the first phase of this study, the effect of biologic anti-TNF $\alpha$  agents on patients on whom BMD data was available was studied in 62 patients. This cohort of patients included 49 females and 13 males. The mean age of patients was  $69\pm 12$  years (40-79 years). All patients had active disease (mean DAS28 $\pm$ SD of  $5.96\pm 1.2$ ) at the time they were prescribed treatment with biologic anti-TNF $\alpha$  agents. Patients receiving, or having received bisphosphonates or hormone replacement therapy were excluded from the analysis. Twenty seven of the patients were treated with etanercept, 26 with infliximab and 9 with adalimumab. Lumbar and hip BMD were measured by DEXA scan before the start of treatment and 1 and 2 years later. In combination with the biologic anti-TNF $\alpha$  agents, 47 patients received methotrexate (12.5mg/day), 8 patients received leflunamide (10mg/day) and 14 patients received prednisolone (7.5 mg/day). Nine patients were taking calcium (1g/day) and vitamin D (800 IU/day).

Table 4.1 summarises the baseline information on patients included in the analysis. The patients were divided according to their clinical response to the treatment with biologic anti-TNF $\alpha$  agents to identify further effects on changes in BMD. There were no significant differences between responder and non-responder patients in age, on the use of disease modifying anti-rheumatic drugs (DMARD) and DAS28. However, non-responder patients included postmenopausal females.



received, bisphosphonates or hormone replacement therapy were excluded from the study. Eighteen patients were treated with etanercept, 16 with certolizumab, 9 with adalimumab, 7 with infliximab and 5 with golimumab. In combination with the biologic anti-TNF $\alpha$  agents, 51 patients were receiving methotrexate (12.5mg/day) and 16 patients were also receiving prednisolone (7.5 mg/day). Thirteen patients were taking calcium (1g/day) and vitamin D (800IU/day). Lumbar and hip BMD was measured by DEXA scan before the start of treatment and 12 months later. BMDs of the hand at baseline and after six months were measured on plain radiographs of the right hand using digital X-ray radiogrammetry (DXR; Pronosco X posture system, system 2.0, Sectra, Linköping, Sweden).

**b) Lymphocyte isolation and measurement of blood biomarkers of immunity and bone turnover:**

For assessing status of the immune system and its impact on bone turnover and response to the treatment and the level of cytokines and bone turnover biomarkers, 25ml of blood were drawn from the patients at the commencement of the treatment and at 1 month and then 3 months after treatment. At similar time points, plasma levels and the level of cytokines produced by enriched and *ex vivo* stimulated T- and B-lymphocytes and monocytes in culture supernatants were measured using multiplex MSD kits. The level of the following cytokines was measured: TNF $\alpha$ , IL-1, IL-6, IL-17, IL-22 and GM-CSF using the MSD kits. Plasma levels of IL-20 and the following bone turnover biomarkers were measured by ELISA: C-telopeptide cross-links of collagen type I (CTX), osteocalcin and osteoclast-regulating proteins including RANK-L and OPG, (Described in detail in the Materials and Methods chapter).

The recruitment of patients and experimental procedures were approved by the local NHS and Central Office for Research Ethics Committee (COREC) (REC reference number: 06/Q0605/8). The study protocol and information leaflets were prepared and were

routinely handed to all patients recruited. Demographic information and details of the 55 RA patients included in the study are summarised in Table 4.2.

**Table 4.2: Demographic information and details of RA patients included in this study.**

| <b>Parameters</b>                   | <b>Responder<br/>n=42</b> | <b>Non-<br/>responder<br/>n=13</b> | <b>All<br/>patients<br/>n=55</b> |
|-------------------------------------|---------------------------|------------------------------------|----------------------------------|
| <b>Gender</b>                       |                           |                                    |                                  |
| <b>Male (%)</b>                     | 12 (29)                   | 2 (15)                             | 14                               |
| <b>Female (%)</b>                   | 30 (71)                   | 11 (85)                            | 41                               |
| - (Post-menopausal) (%)             | 17 (57)                   | 6 (55)                             | 23                               |
| <b>Age (year)</b>                   | Mean (range)              | 55 (32-78)                         | 58                               |
| <b>Disease activity score 28</b>    | Mean (SD)                 | 5.6 (0.8)                          | 5.8 (0.8)                        |
| <b>Calcium and Vitamin D intake</b> | n (%)                     | 8 (19)                             | 13                               |
| <b>Smoking*</b>                     |                           |                                    |                                  |
| - Past                              | n (%)                     | 6 (14)                             | 7                                |
| - Never                             | n (%)                     | 7 (17)                             | 10                               |
| - Current                           | n (%)                     | 2 (5)                              | 4                                |
| <b>Ethnic origin**</b>              |                           |                                    |                                  |
| - White                             | n (%)                     | 17 (40)                            | 21                               |
| - Asian                             | n (%)                     | 13 (31)                            | 19                               |
| - Black                             | n (%)                     | 2 (5)                              | 3                                |
| <b>Methotrexate use</b>             | n (%)                     | 39 (93)                            | 51                               |
| <b>Use Prednisone</b>               | n (%)                     | 12 (29)                            | 16                               |

Patients were divided according to their clinical response to the treatment. Fifty five RA patients were recruited for the study to determine if and how biologic anti-TNF $\alpha$  agents affects bone turnover and association between bone and the immune system in responder and non-responder RA patients.\* Information on the smoking habits of only 21 patients was available.

\*\* Information on ethnic origin of 12 patients wasn't available.

## 4.3 Results

### 4.3.1 Retrospective study:

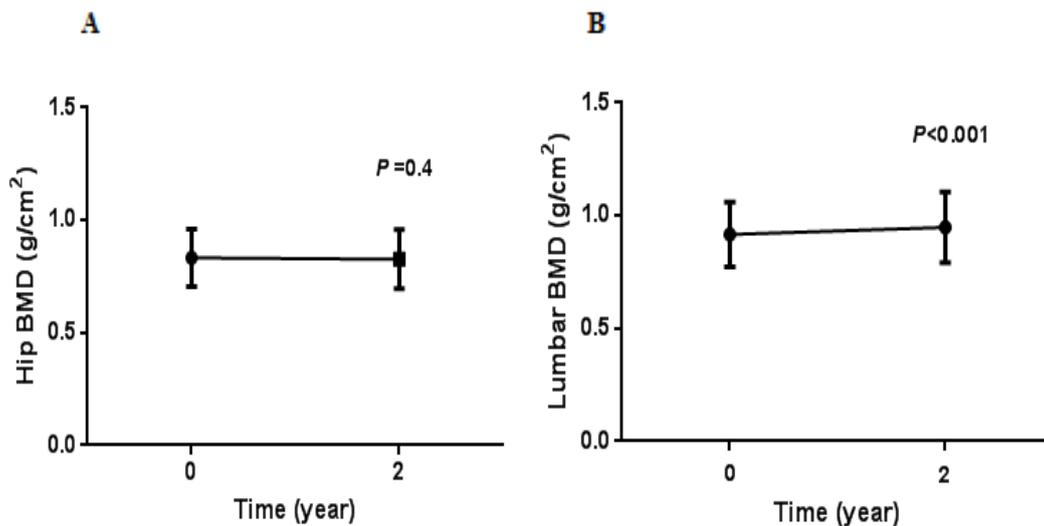
#### a) Changes in BMD

Measurement of changes in BMD in the patients before treatment and 2 years after, revealed significant changes in BMD (Table 4.3) with mean changes of BMD in both lumbar spine and the hip of 3.49% and -0.6%, ( $p<0.001$  and  $p=0.4$ ), respectively (Figure 4.1). BMD of the lumbar spine and hip increased in responder patients after treatment by  $0.0396\text{g/cm}^2$  (3.96%,  $p<0.001$  vs. before treatment) and  $0.001\text{g/cm}^2$  (0.11%,  $p<0.001$  vs. before treatment), respectively. Interestingly, clinically non-responder patients, according to their DAS28, showed an improvement in BMD of the lumbar spine with mean of  $0.03\text{g/cm}^2$  (3.03%,  $p<0.001$  vs. before treatment) and minor reductions in BMD values at the hip with a mean of  $-0.008\text{g/cm}^2$  (-0.78%,  $p<0.001$  vs. before treatment)) (Figure 4.2). This data suggests that biologic anti-TNF $\alpha$  agents have beneficial effects on BMD in RA patients. This beneficial effect may not parallel changes in DAS28 measures.

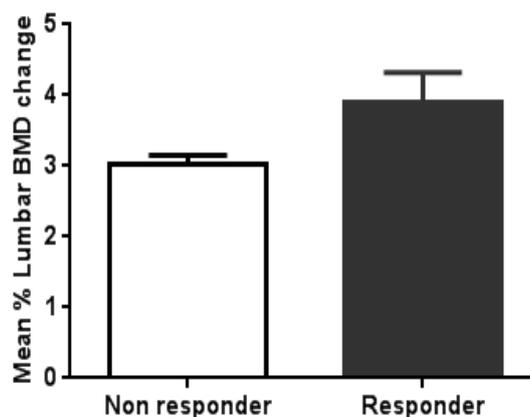
**Table 4.3: Changes in BMD in RA patients treated with biologic anti-TNF $\alpha$  agents at 2 years after treatment.**

|  | <b>Before treatment<br/>(Mean<math>\pm</math>SD)</b> | <b>2 years after treatment<br/>(Mean<math>\pm</math>SD)</b> | <b><i>P</i></b> |
|--|--|---|-----------------|
| <b>BMD of L1-4 in g/cm<sup>2</sup></b> | 0.917 $\pm$ 0.14                                     | 0.949 $\pm$ 0.16  | <0.001          |
| <b>BMD of hip in g/cm<sup>2</sup></b>  | 0.832 $\pm$ 0.11                                     | 0.827 $\pm$ 0.11  | 0.4             |

Data presented in the Table were obtained as described in the Materials and Methods chapter. The difference between before and after treatment was analysed using Wilcoxon signed rank test.  $P$  values  $<0.05$  were considered statistically significant. The detailed approach used in the statistical analysis is provided in Appendix 2, Table 1 and 2.



**Figure 4.1: Change in BMD from before to 2 years after treatment of RA patients with biologic anti-TNF $\alpha$  agents.** (A) Measurement of BMD at the hip and (B) the lumbar spine sites in RA patients. BMD of the hip and lumbar spine were determined by DEXA scan before treatment (time 0) and after 2 years of treatment initiation.  $P$  values refer to the significance of differences in BMD from before to 2 years after treatment had started.

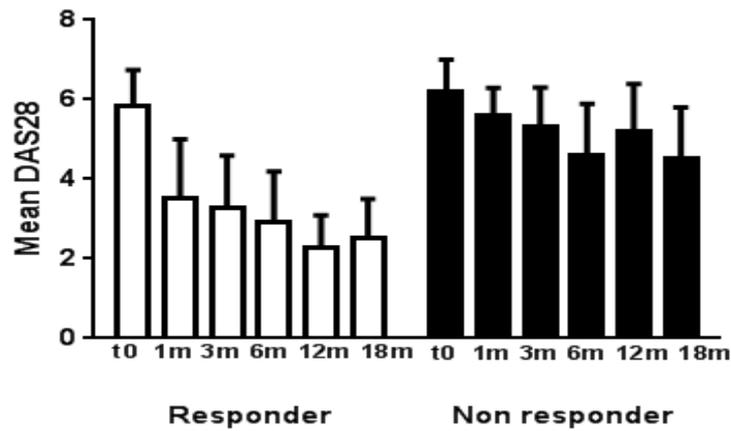


**Figure 4.2: Changes in BMD in responder and non-responder RA patients treated with biologic anti-TNF $\alpha$  agents.** Data on changes in BMD in 62 RA patients treated with biologic anti-TNF $\alpha$  agents were compared between responder and non-responder patients. Response to biologic anti-TNF $\alpha$  agents was based on improvement in DAS28 while BMD was measured using DEXA scans immediately before treatment and then 2 years after treatment had started. The error bars represent:  $\pm$  2 SEM.

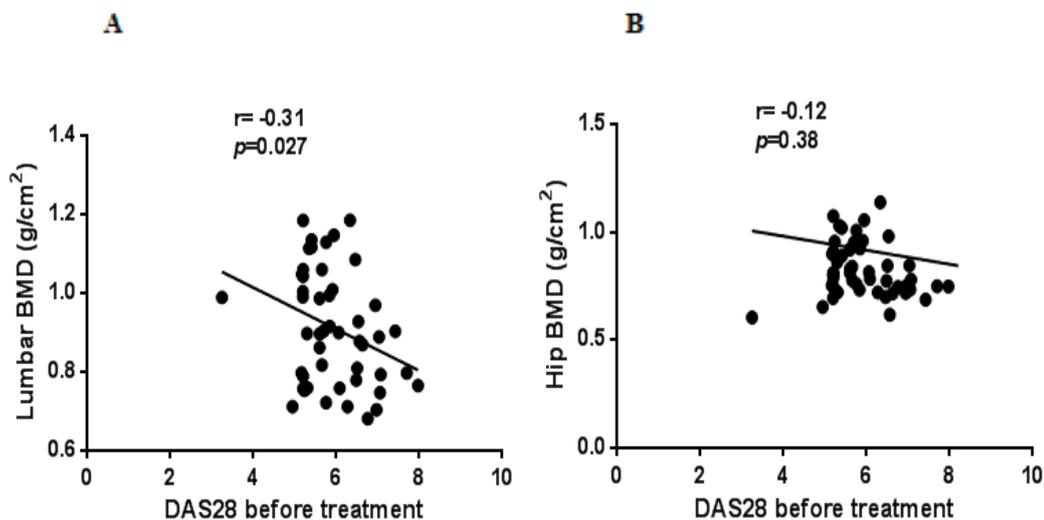
**b) Correlation between patients characteristics and BMD values:**

On assessing correlations between BMD and patient personal data, interesting observations were made using Spearman's correlation coefficient. Negative correlations were observed between postmenopausal female and BMD at the lumbar spine and the hip ( $r=-0.31$ ,  $p=0.026$  and  $r=-0.29$ ,  $p=0.035$ , respectively).

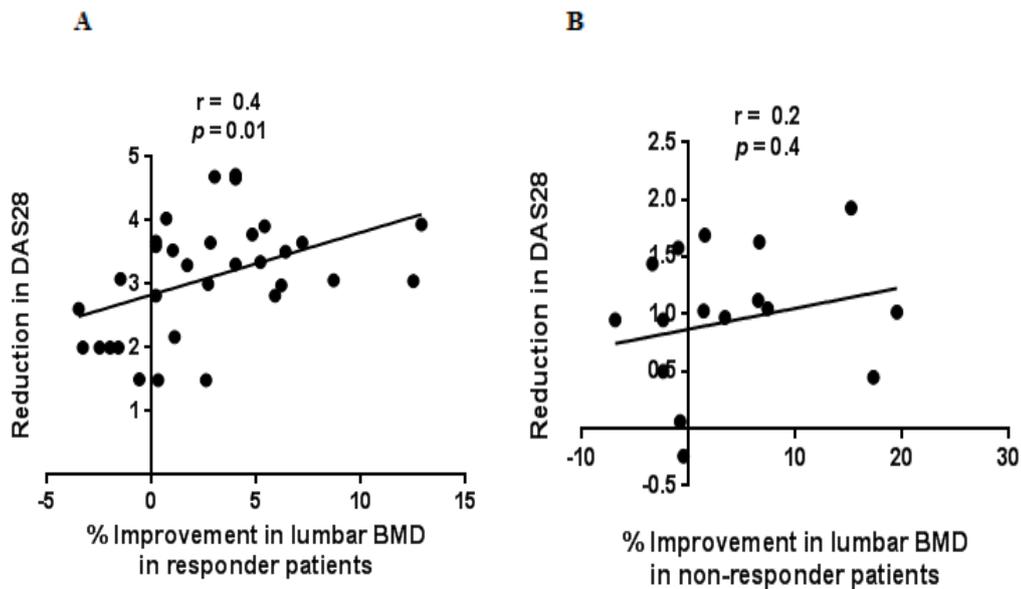
Changes in DAS28 at 3, 6, 12, and 18 months after treatment were significantly different from before treatment at  $p<0.01$  (Figure 4.3). DAS28 and BMD at lumbar spine and the hip were correlated. The analysis revealed that increased DAS28 before treatment correlated significantly with reduction in BMD at the lumbar spine but not the hip, although there was a trend in that direction ( $r=-0.31$ ,  $p=0.027$ , and  $r=-0.12$ ,  $p=0.38$  with lumbar spine and hip BMD, respectively) (Figure 4.4). Positive correlations were observed between reduction in DAS28 (initial DAS28 - DAS28 at 24 months post treatment) and improvement in BMD at the lumbar spine in responder patients but not the non-responder ( $r=0.4$ ,  $p=0.01$  and  $r=0.2$ ,  $p=0.4$ , respectively) (Figure 4.5). No significant correlations between changes in the lumbar spine and the hip BMD and other medications taken by the patients such as calcium, vitamin D or prednisone and DMARDS were observed ( $p>0.05$ ).



**Figure 4.3: Changes in DAS28 in responder and non-responder RA patients treated with biologic anti-TNF $\alpha$  agents at different time points during the clinical assessment process.** The figure depicts changes in DAS28 at different time point following treatment with biologic anti-TNF $\alpha$  agents in 62 RA patients whose mean BMD changes are presented in Figure 4.2. DAS28 was measured before treatment and at 1 month (1m), 3m, 6m, 12m and 18m after treatment had started.



**Figure 4.4: Correlations between DAS28 and BMD at lumbar spine (A) and hip (B) before treatment with biologic anti-TNF $\alpha$  agents.** Data on BMD in 62 RA patients were correlated with DAS28 before treatment with biologic anti-TNF $\alpha$  agents. BMD was measured using DEXA scan while DAS28 was measured as described in the Materials and Methods chapter. The data are for all patients irrespective of whether they were responders or non-responders.



**Figure 4.5: Correlations between reduction in DAS28 at 24 months of treatment with biologic anti-TNF $\alpha$  agents and improvement in BMD at lumbar spine in responder (A) and non-responder patients (B).** Improvement in BMD in responder and non-responder patients, based on improvement in DAS28, were correlated with changes in DAS28. BMD was measured using DEXA scan immediately before treatment and then 24 months after treatment had started. Correlations were calculated by using Spearman's correlation coefficient (r).

The impact of individual factors on changes in the BMD of RA patients was also investigated. For that reason, patients were divided into responders and non-responders to treatment with biologic anti-TNF $\alpha$  agents based on changes in their DAS28, and then the patients were divided into subgroups according to gender and age group. Variation in BMD among different groups was analysed by using contingency Tables (Fishers exact test/Chi<sup>2</sup> test) (Table 4.4).

Better improvement in BMD was observed among responder patients when compared with non-responder patients; BMD improved in 27 responder patients (66%) compared with 11 non-responder patients (52%), however this difference in BMD improvement was not statistically significant ( $p=0.4$ ).

Although the cohort of studied responder patients included less males (10) compared with females (31), there was a tendency for better improvement in BMD, as expected from the analysis of osteoporosis patients cited in Chapter 3, among male patients compared with female patients. Thus, BMD of responder patients improved in 9 males (90%) compared to 18 females (58%), however, the difference was not statistically significant ( $p=0.1$ ). Regarding BMD in non-responder patients, no significant difference was observed in BMD between male and female patients. BMD improved in 2 males (67%) compared with 9 females (50%) ( $p=1.0$ ). The mean change in BMD in the lumbar spine and the hip in male patients was greater than in female patients,  $0.036\text{g}/\text{cm}^2$  (3.57%) and  $0.009\text{g}/\text{cm}^2$  (0.95%) vs.  $0.025\text{g}/\text{cm}^2$  (2.53%) and  $-0.005\text{g}/\text{cm}^2$  (-0.5%) ( $p=0.58$ ,  $p=0.3$ ), respectively.

Furthermore, more improvement in BMD was observed in  $\leq 50$  year old patients compared with  $>50$  year old patients. BMD in responder patients improved in 7  $\leq 50$  year old patients (88%) compared with 20  $>50$  year old patients (61%). However, there was no significant difference in BMD improvement between the two age groups ( $p=0.2$ ). In addition, BMD improved in 1  $\leq 50$  year old patient and 10  $>50$  year old patients who did not respond to the treatment as measured by DAS28, with no significant changes between the two groups ( $p=0.6$ ) (Table 4.4). The mean change in BMD in the lumbar spine and the hip in  $\leq 50$  year old patients was greater than in  $>50$  year old patients,  $0.027\text{g}/\text{cm}^2$  (2.7%) and  $0.009\text{g}/\text{cm}^2$  (1.18%) vs.  $0.02\text{g}/\text{cm}^2$  (2.2%) and  $-0.004\text{g}/\text{cm}^2$  (-0.4%) ( $p=0.8$ ,  $p=0.2$ ), respectively. It was not possible to study the effect of ethnic origin on changes in BMD in the RA patients since 95% of the treated patients were Caucasians. In addition, no data was available on smoking habits of the patients and, therefore, the effect of smoking on changes in BMD in the RA patients treated with biologic anti-TNF $\alpha$  agents could not be assessed.

**Table 4.4: Patients with RA studied for the effect of treatment with biologic anti-TNF $\alpha$  agents on BMD.**

| Parameters              | Improved BMD | Non-improved BMD | Total |
|-------------------------|--------------|------------------|-------|
| <b>Response</b>         |              |                  |       |
| ▪ <b>Responders</b>     |              |                  |       |
| - Male n (%)            | 9 (90)       | 1 (10)           | 10    |
| - Female n (%)          | 18 (58)      | 13 (42)          | 31    |
| ▪ <b>Non responders</b> |              |                  |       |
| - Male n (%)            | 2 (67)       | 1 (33)           | 3     |
| - Female n (%)          | 9 (50)       | 9 (50)           | 18    |
| ▪ <b>Responders</b>     |              |                  |       |
| - $\leq 50$ n (%)       | 7 (88)       | 1 (12)           | 8     |
| - $>50$ n (%)           | 20 (61)      | 13 (39)          | 33    |
| ▪ <b>Non responders</b> |              |                  |       |
| - $\leq 50$ n (%)       | 1 (33)       | 2 (67)           | 3     |
| - $>50$ n (%)           | 10 (56)      | 8 (44)           | 18    |

RA patients were divided according to their response to treatment with biologic anti-TNF $\alpha$  agents, gender and age group. Improved BMD was defined as having either no change or an increase from before treatment at the measured sites at 2 years post treatment. Non-improved BMD was defined as having BMD decrease from baseline at the measured sites at 2 years after treatment has started. The differences in BMD among patient groups were assessed by using contingency tables.

**c) The effect of treating RA patients with biologic anti-TNF $\alpha$  agents on BMD:**

**Conclusions**

Of 62 RA patients treated with biologic anti-TNF $\alpha$  agents, 41 patients (66%) responded to the treatment, as defined by improvement of at least 1.2 in DAS28 score after 1 year while 21 did not respond. Before start of treatment, no significant differences were observed in BMD at the lumbar spine and the hip between responder and non-responder patients. However, the majority of non-responder patients were postmenopausal females, which may explain their noted lower mean BMD before treatment compared with responder patients.

Although most of the patients included in this audit were at relatively high risk of osteoporosis, not only because of their chronic inflammation but also more advanced age, being mostly female and using steroids, the results, nevertheless, showed notable improvement in BMD with biologic anti-TNF $\alpha$  agents. This improvement in BMD in the treated patients was not always consistent with improvements in DAS28 scores. This finding suggests that the effect of biologic anti-TNF $\alpha$  agents on bone overlaps but is not always consistent with their anti-inflammatory effects. However, this audit needs to be further verified in a larger cohort, but is, nevertheless, consistent with findings in experimental arthritis. Interestingly, TNF $\alpha$  blockade in arthritic mice has been shown to have a greater effect on cartilage degradation and joint destruction than on inflammation (182,183). Furthermore, the data may raise an issue about the role of other cytokines and pro-inflammatory pathways in joint pathogenesis in RA and the possible involvement of these in the incomplete response of some patients with RA to treatment with biologic anti-TNF $\alpha$  agents. This suggests that other targeted therapeutic approaches, such as anti-IL-17 which has been shown to correlate with the absence of clinical response to TNF $\alpha$  antagonists in RA patients which may enhance the beneficial bone response to treatment (184).

Although this analysis provides preliminary evidence to indicate the potential effect of biologic anti-TNF $\alpha$  agents on bone, it would be of interest if data on serum and urine biomarkers of bone turnover could be made available to confirm the association between treatments with biologic anti-TNF $\alpha$  agents, BMD and bone turnover.

### **4.3.2 A prospective study to assess the effect of biologic anti-TNF $\alpha$ agents on bone turnover and correlation with inflammation:**

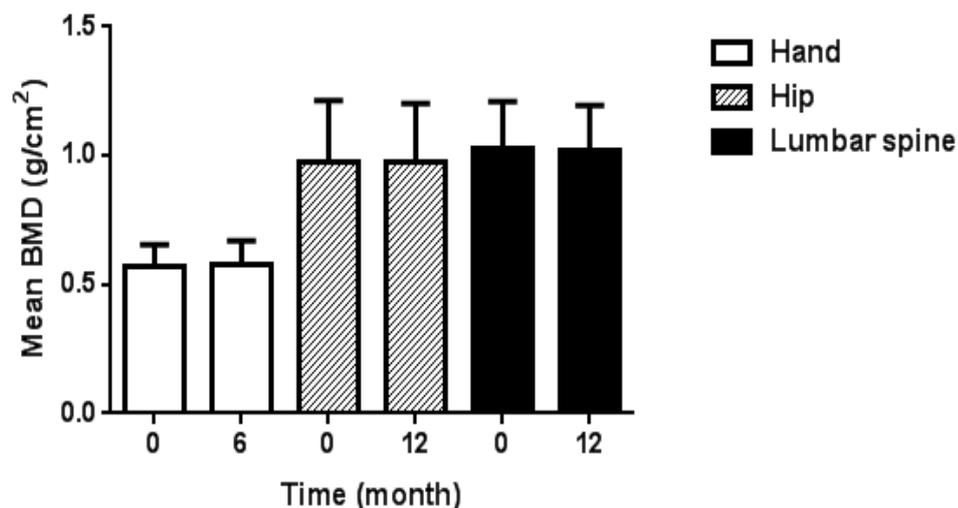
#### **a) Changes in disease activity**

The mean and standard deviation (SD) of DAS28 for the patients before treatment was 5.8 (0.8). This was reduced in the responder patients to 3.8 (0.9) after 1 month and 3.5 (1.4) after 3 months of the start of treatment ( $p < 0.001$  at all-time points compared with before treatment). Forty two patients responded to treatment with the biologic anti-TNF $\alpha$  agents and with a reduction in DAS28 of  $\geq 1.2$ . Thirteen of the patients did not respond to the treatment.

#### **b) Changes in BMD**

Ten of the treated 55 patients had DEXA scans before treatment had started and then another scan 1 year later. Before the start of treatment, mean BMD ( $\pm$ SD) values were 1.031 (0.2) and 0.972 (0.2) at lumbar spine and hip, respectively. Eight patients (14.5%) had osteopenia (T-score  $< -1.0$  but  $> -2.5$ ) at either spine or hip. BMD of the lumbar spine and the hip remained unchanged during treatment with the biologic anti-TNF $\alpha$  agents (Figure 4.6). These values were  $1.03 \pm 0.2 \text{ g/cm}^2$  and  $0.97 \pm 0.2$  before treatment and  $1.02 \pm 0.2$  and  $0.97 \pm 0.2 \text{ g/cm}^2$  1 year after treatment for lumbar spine and the hip, respectively.

A further 10 of the patients had conventional radiographs before treatment had started and then another radiograph 6 months later. BMD of the hand, which was quantified by DXR, remained stable during the treatment with the biologic anti-TNF $\alpha$  agents (Figure 4.6), being  $0.57 \pm 0.1 \text{ g/cm}^2$  before treatment started and  $0.57 \pm 0.1 \text{ g/cm}^2$  after treatment.



**Figure 4.6: Changes in BMD of the hand, hip and lumbar spine before and after treatment with biologic anti-TNF $\alpha$  agents.** Ten RA patients treated with biologic anti-TNF $\alpha$  agents had DEXA scans of the lumbar spine and the hip before treatment started and 12 months after treatment. BMDs of the hand of the same patients were measured on plain radiographs of the right hand (anteroposterior view) before treatment and 6 months after treatment and data analysed using digital X-ray radiogrammetry (DXR; Pronosco X posure system 2.0, Sectra, Linkaping, Sweden). The error bars represent +/- SD units.

Before treatment had started, differences in BMD between responder and non-responder patients, as defined by changes in DAS28, were not significant. However, all non-responder patients who had BMD scans were postmenopausal females, which may explain, at least partly, their noted lower mean BMD before start of treatment compared with responder patients (Table 4.5).

**Table 4.5: BMD prior to initiation of the treatment with biologic anti-TNF $\alpha$  agents for responder and non-responder RA patients.**

| <b>Parameters</b>  | <b>Responders<br/>n=7</b> | <b>Non-responders<br/>n=3</b> | <b>All patients<br/>n=10</b> |
|--|---------------------------|-------------------------------|------------------------------|
| <b>BMD of the L. spine (g/cm<sup>2</sup>) Mean (SD) before treatment</b> | 1.069 (0.2)               | 0.941 (0.1)                   | 1.03 (0.2)                   |
| <b>BMD of the hip (g/cm<sup>2</sup>) Mean (SD) before treatment</b>      | 0.992 (0.3)               | 0.926 (0.1)                   | 0.972 (0.2)                  |
| <b>BMD of the hands (g/cm<sup>2</sup>) Mean (SD) before treatment</b>    | 0.58 (0.1)                | 0.52 (0.03)                   | 0.57 (0.1)                   |

Ten RA patients treated with biologic anti-TNF $\alpha$  agents had DEXA scans of the lumbar spine and the hip before treatment started and 12 months after treatment. BMDs of the hand of the same patients were measured on plain radiographs of the right hand before treatment and 6 months after treatment and data analysed using DXR.

No significant changes were observed in BMD of the lumbar spine and the hip in responder patients after one year of treatment. These values were  $1.069 \pm 0.2 \text{ g/cm}^2$  and  $0.992 \pm 0.3 \text{ g/cm}^2$  before treatment and  $1.063 \pm 0.2 \text{ g/cm}^2$  and  $0.989 \pm 0.3 \text{ g/cm}^2$  1 year after treatment for lumbar spine and the hip, respectively. Interestingly, clinically non-responder patients, according to their DAS28, showed no significant changes in BMD of lumbar spine and the hip. These values were  $0.941 \pm 0.1 \text{ g/cm}^2$  and  $0.926 \pm 0.1$  before treatment and  $0.91 \pm 0.1$  and  $0.931 \pm 0.1 \text{ g/cm}^2$  1 year after treatment for lumbar spine and the hip, respectively. Furthermore, no significant changes were observed in BMD of the hand in both responder and non-responder patients after 6 months of treatment. These values were  $0.58 \pm 0.1 \text{ g/cm}^2$  and  $0.52 \pm 0.03 \text{ g/cm}^2$  before treatment and  $0.60 \pm 0.1 \text{ g/cm}^2$  and  $0.52 \pm 0.03 \text{ g/cm}^2$  6 months after treatment for responders and non-responder patients respectively (Table 4.6).

**Table 4.6: Changes in BMD of lumbar spine, hip and hand following treatment with biologic anti-TNF $\alpha$  agents.**

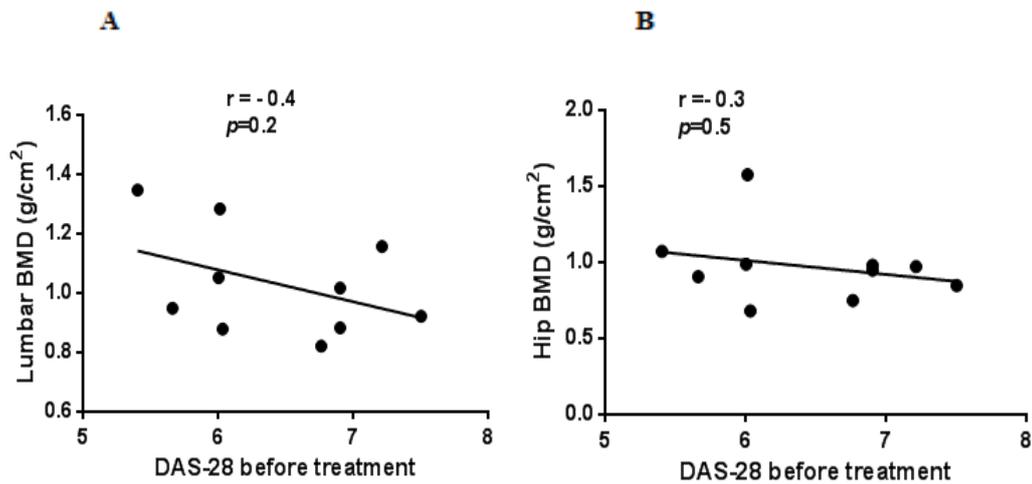
| BMD (g/cm <sup>2</sup> ) | Before start of treatment | After 6 months | After 12 months | <i>p</i> value |
|--------------------------|---------------------------|----------------|-----------------|----------------|
| <b>Lumbar spin</b>       |                           |                |                 |                |
| - Responders             | 1.069 (0.2)               |                | 1.063 (0.2)     | 0.6            |
| - Non responders         | 0.941 (0.1)               |                | 0.91 (0.1)      | 0.06           |
| <b>Hip</b>               |                           |                |                 |                |
| - Responders             | 0.992 (0.3)               |                | 0.989 (0.3)     | 0.8            |
| - Non responders         | 0.926 (0.1)               |                | 0.931 (0.1)     | 0.8            |
| <b>Hand</b>              |                           |                |                 |                |
| - Responders             | 0.58 (0.1)                | 0.60 (0.1)     |                 | 0.9            |
| - Non responders         | 0.52 (0.03)               | 0.52 (0.03)    |                 | 0.3            |

Full BMD measurements, including the hands in addition to the traditional lumbar spine and the hip, were available from 10 patients for this analysis. All 10 RA patients were treated with biologic anti-TNF $\alpha$  agents. BMD values are shown as mean and (SD). Full statistical analysis is provided in Appendix 3, Tables 1, 2 and 3.

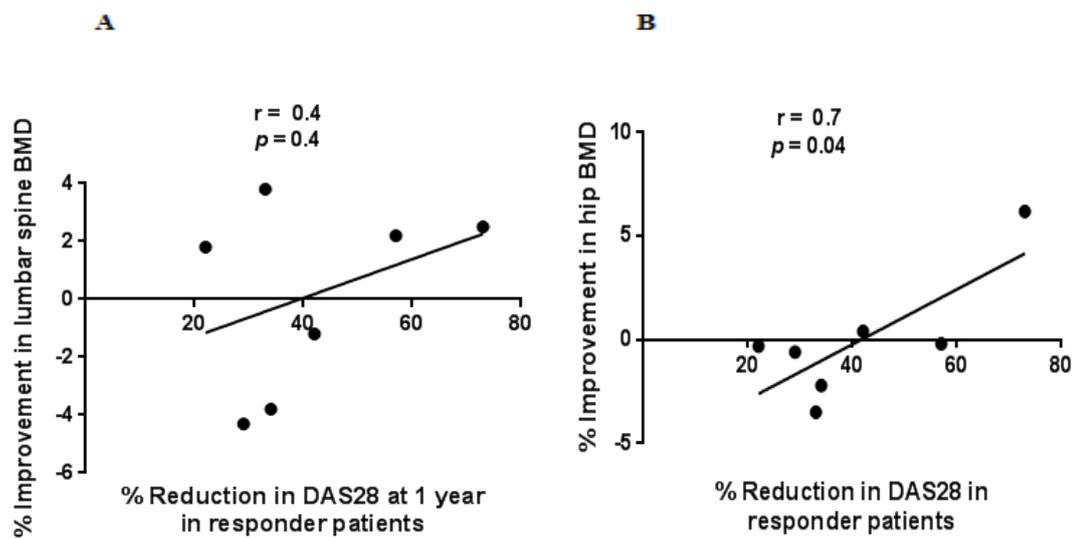
#### **4.3.3 Correlation between patients characteristics and BMD values:**

DAS28 and BMD at lumbar spine and hip were correlated. The analysis showed that increased DAS28 correlated with lower values of BMD at lumbar spine and hip although this was not statistically significant due to the effect of sample size ( $r=-0.4$ ,  $p=0.2$  and  $r=-0.3$ ,  $p=0.5$  with lumbar spine and hip BMD, respectively) (Figure 4.7).

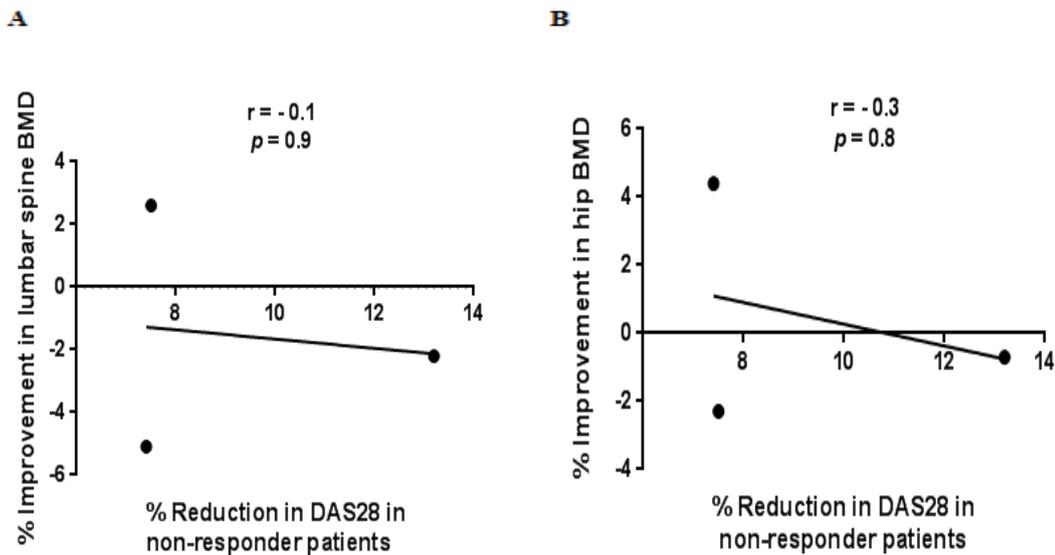
Positive correlations were observed between reduction in DAS28 and improvement in BMD of the lumbar spine and hip in responder patients compared with the non-responders ( $r=0.4$ ,  $p=0.4$  and  $r=0.7$ ,  $p=0.04$  vs.  $r=0.-1$ ,  $p=0.9$  and  $r=-0.3$ ,  $p=0.8$ , respectively) (Figures 4.8 and 9). However, most of these correlations did not reach statistically-significant levels due to the effect of sample size.



**Figure 4.7: Relationships between DAS28 and BMD at lumbar spine (A) and the hip (B) before treatment with biologic anti-TNF $\alpha$  agents.** BMD of 10 RA patients were correlated with DAS28 before treatment with biologic anti-TNF $\alpha$  agents. BMD was measured using DEXA scan while DAS28 was measured as described in the Materials and Methods chapter. The data are for all patients irrespective of whether they were responders or non-responders.



**Figure 4.8: Relationship between reduction in DAS28 and BMD in responder patients at lumbar spine (A) and hip (B) after 1 year of treatment with biologic anti-TNF $\alpha$  agents.** BMD in 7 responder patients, based on improvement in DAS28, were correlated with reduction in DAS28 after one year. BMD was measured using DEXA scan. Correlations were calculated using Spearman's correlation coefficient ( $r$ ).



**Figure 4.9: Relationships between reduction in DAS28 and improvement in BMD of non-responder patients at lumbar spine (A) and hip (B) after 1 year of treatment with biologic anti-TNF $\alpha$  agents.** BMD in 3 non-responder patients were correlated with reduction in DAS28 after one year. BMD was measured using DEXA scan. Correlations were calculated by using Spearman's correlation coefficient ( $r$ ).

Variation in improvement of BMD among different patient groups was analysed using contingency tables (Table 4.7). BMD improved in 3 responder patients (43%) compared with 1 non-responder patient (33%) but, perhaps expectedly, this difference was not statistically significant ( $p=1.0$ ), given the small sample size. Better improvements in BMD were observed, as expected from the analysis of in the analysis of both osteoporosis and RA patients cited above, among male patients when compared with female patients. However, the patient cohort scanned for their BMD included fewer males ( $n=2$ ) compared with females ( $n=8$ ). BMD improved in 1 male patients (50%) compared to 3 female patients (38%) but the difference in BMD improvement was not statistically significant ( $p=1.0$ ) (Table 4.7). Mean BMD ( $\pm$ SD) values in the male patients were  $1.22\pm 0.1$ ,  $1.27\pm 0.1$  and  $0.63\pm 0.1\text{g/cm}^2$  before treatment and  $1.22\pm 0.1$ ,  $1.25\pm 0.1$  and  $0.64\pm 0.1\text{g/cm}^2$  after treatment for lumbar spine, hip and hand, respectively. In contrast, BMD values in the female patients were  $0.984\pm 0.2$ ,  $0.897\pm 0.1$  and  $0.56\pm 0.03\text{g/cm}^2$  before treatment and  $0.967\pm 0.2$ ,  $0.903\pm 0.1$  and  $0.55\pm 0.03\text{g/cm}^2$  after treatment for lumbar spine, hip and hand, respectively.

Although the sample size included 6 younger ( $\leq 50$  years) and 4 older patients ( $>50$  years), no significant differences in BMD were observed among  $\leq 50$  year old patients compared with  $>50$  year old patients. BMD improved in 3 of the  $\leq 50$  year old patients (50%) compared with 1  $>50$  year old patient (25%) ( $p=0.6$ ) (Table 4.7). It was not possible to study the effect of ethnic origin on changes in BMD in the RA patients since 95% of the patients who had BMD scans were Caucasians. In addition, no adequate data was available on smoking habits for those patients and, therefore, the effect of smoking on changes in BMD in the RA patients could not be assessed.

**Table 4.7: Patients with RA studied for the effect of treatment with biologic anti-TNF $\alpha$  agents on BMD.**

| Parameters       |       | Improved BMD | Non-improved BMD | Total |
|------------------|-------|--------------|------------------|-------|
| <b>Response</b>  |       |              |                  |       |
| ▪ Responders     | n (%) | 3 (43)       | 4 (57)           | 7     |
| ▪ Non responders | n (%) | 1 (33)       | 2 (67)           | 3     |
| <b>Gender</b>    |       |              |                  |       |
| ▪ Male           | n (%) | 1 (50)       | 1 (50)           | 2     |
| ▪ Female         | n (%) | 3 (38)       | 5 (62)           | 8     |
| <b>Age</b>       |       |              |                  |       |
| ▪ $\leq 50$      | n (%) | 3 (50)       | 3 (50)           | 6     |
| ▪ $> 50$         | n (%) | 1 (25)       | 3 (75)           | 4     |

RA patients were divided according to their response to treatment with biologic anti-TNF $\alpha$  agents, gender and age group. Improved BMD was defined as having BMD either no change or increased at the measured sites after 1 year post treatment. Non-improved BMD was defined as having a BMD decreased at the measured sites after 1 year post treatment. The differences in BMD among the RA patient groups were assessed using contingency tables.

In general, this analysis indicates that biologic anti-TNF $\alpha$  agents have beneficial effects on BMD in RA patients, as expected from the retrospective study of RA patients cited above but this beneficial effect may not parallel changes in DAS28 measures. Moreover,

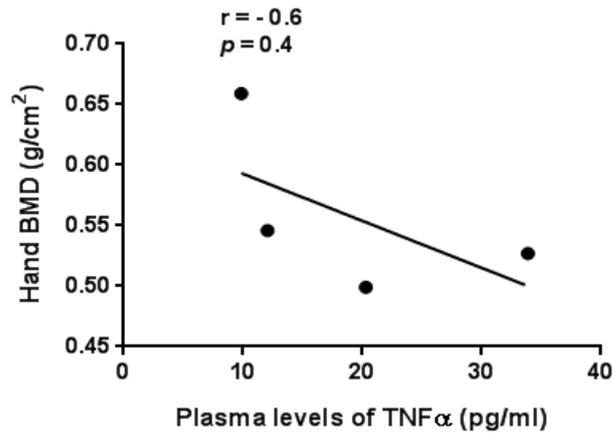
despite all of the non-responder patient group were postmenopausal females, treatment with biologic anti-TNF $\alpha$  agents prevented both generalised bone loss, measured by DEXA scan of lumbar spine and the hip and localised bone loss, measured by the DXR scan of the hand.

#### **4.3.4 Association of changes in BMD with pro-inflammatory and bone resorptive cytokines.**

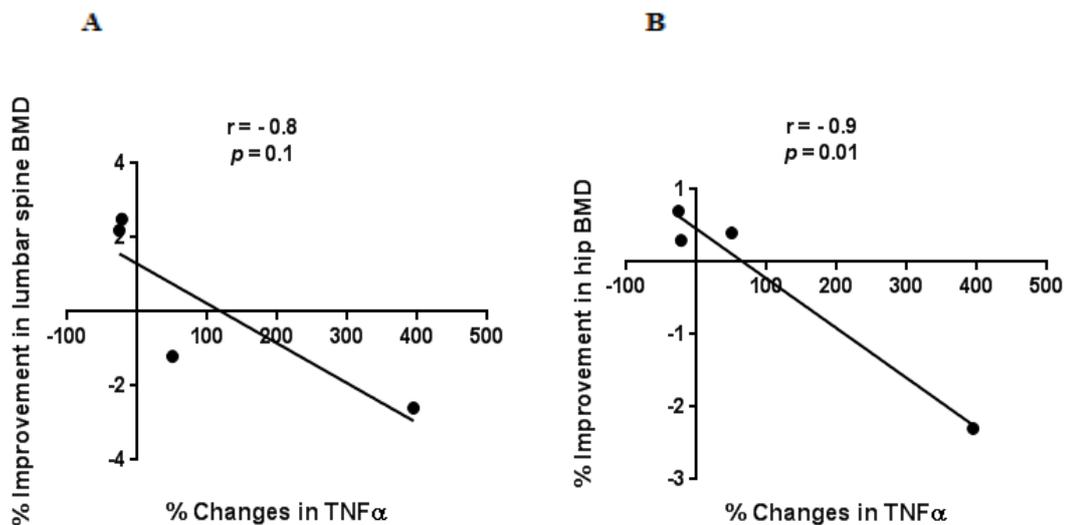
As cited in chapter one, the key pro-inflammatory cytokines TNF $\alpha$ , IL-1, IL-6 and IL-17 contribute to generalised bone loss as well as subchondral bone resorption in RA. These pro-inflammatory cytokines act on osteoblasts to promote RANK-L expression and stimulate osteoclastogenesis. Furthermore, inflammatory osteoclastogenesis can be induced by GM-CSF which plays an important role in the differentiation of osteoclasts from their precursors (144,145). In addition, IL-20 and IL-22 have been shown to be involved in bone loss through enhancing RANK-L expression with the net result being a rise in osteoclast formation and functions (146,147). Therefore, analysing the data on the level of key disease promoting cytokines with changes in BMD will help to identify the key cytokine(s) that are involved in bone damage in RA.

##### **a) Relationship between BMD with plasma levels of key pro-inflammatory and bone resorptive cytokines before and after treatment.**

BMD and changes in plasma level of TNF $\alpha$  was correlated. The analysis of the level of TNF $\alpha$  before treatment revealed a correlation between lower values of BMD at hand ( $r=-0.6$ ,  $p=0.4$ ) with high levels of the cytokines. However, this correlation was not statistically significant, probably, due to the effect of sample size (Figure 4.10). Changes in plasma levels of TNF $\alpha$  were negatively correlated with improvement in BMD at lumbar spine and hip ( $r=-0.8$ ,  $p=0.1$  and  $r=-0.9$ ,  $p=0.01$ , respectively) (Figure 4.11).

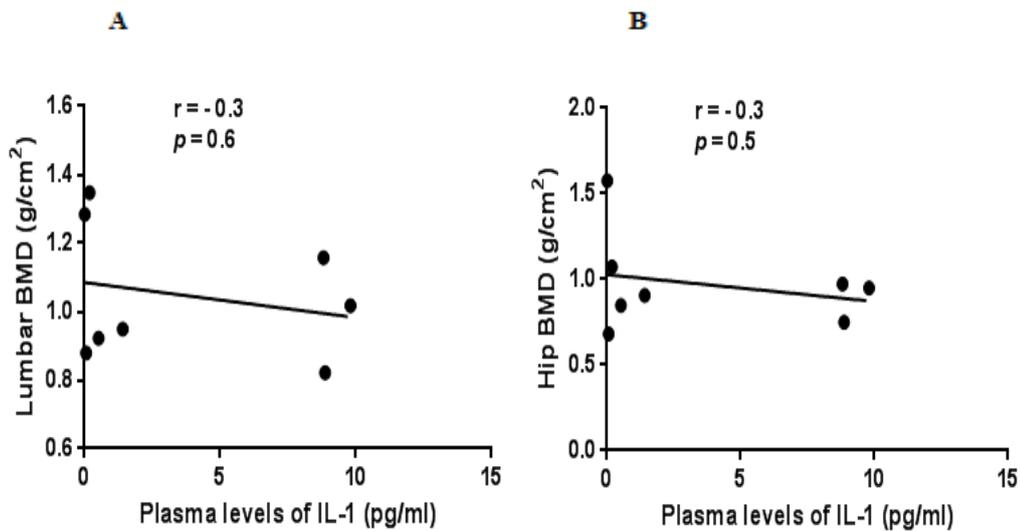


**Figure 4.10: Relationship between plasma levels of TNF $\alpha$  and BMD of the hand.** Plasma levels of TNF $\alpha$  before treatment with biologic anti-TNF $\alpha$  agents were correlated with hand BMD of treated patients. BMD was assessed using DXR scan while TNF $\alpha$  levels were determined using ELISA. Plasma levels of TNF $\alpha$  were correlated with BMD using Spearman's correlation coefficient ( $r$ ).

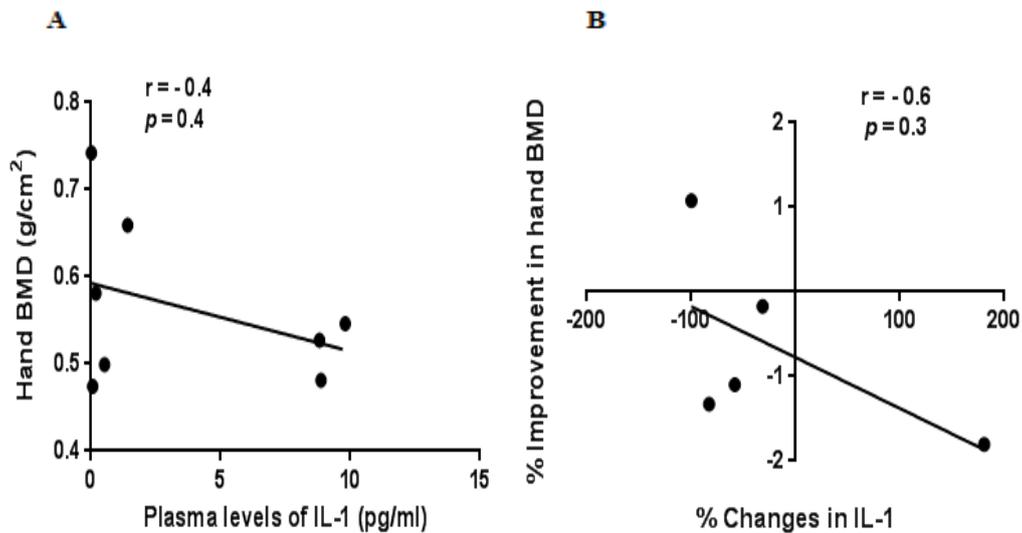


**Figure 4.11: The relationships between improvement in BMD at lumbar spine (A) and hip (B) with changes in plasma levels of TNF $\alpha$  after treatment with biologic anti-TNF $\alpha$  agents.** Changes in plasma levels of TNF $\alpha$  from before treatment to 3 months after treatment were determined using ELISA. BMD was assessed using DEXA scan as described in the Materials and Methods chapter. Correlations were with Spearman's correlation coefficient ( $r$ ).

There was also a negative correlation, before treatment had started, between the level of plasma IL-1 and BMD of the lumbar spine, hip and the hand ( $r=-0.3$ ,  $p=0.6$ ;  $r=-0.3$ ,  $p=0.5$  and  $r=-0.4$ ,  $p=0.4$ , respectively). Changes in plasma level of IL-1 were negatively correlated with improvement in BMD of the hand after treatment with biologic anti-TNF $\alpha$  agents ( $r=-0.6$ ,  $p=0.3$ ) (Figures 4.12 and 4.13).

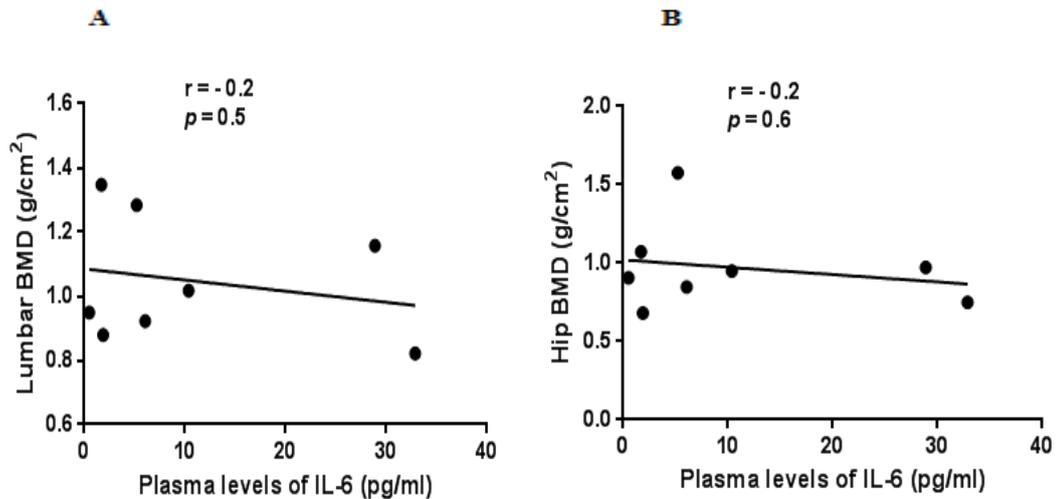


**Figure 4.12: Relationship between plasma levels of IL-1 and BMD at lumbar spine and hip.** IL-1 levels were determined in the plasma of treated patients using ELISA, while BMD of the Lumbar spine (A) and hip (B) was measured using DEXA. Levels of IL-1 were correlated with BMD before treatment with biologic anti-TNF $\alpha$  agents had started. Correlations were with Spearman's correlation coefficient ( $r$ ).

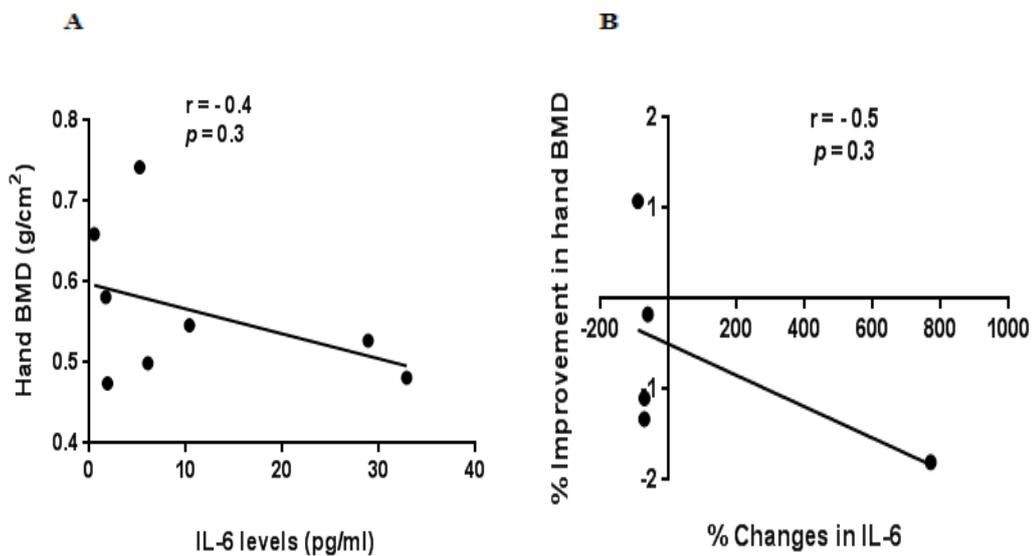


**Figure 4.13: Relationships between plasma levels of IL-1 and BMD of the hand.** IL-1 levels were determined in plasma using ELISA. BMD of the hand was measured using DXR. Levels of IL-1 were correlated with BMD before treatment with biologic anti-TNF $\alpha$  agents had started (A) then changes in IL-1 were correlated with improvement in hand BMD after treatment (B). Correlations were with Spearman's correlation coefficient ( $r$ ).

There was a trend for correlations between reduced levels of IL-6 before treatment and increased BMD at the lumbar spine, hip and hand but the correlations were not statistically significant ( $r = -0.2$ ,  $p = 0.5$ ;  $r = -0.2$ ,  $p = 0.6$  and  $r = -0.4$ ,  $p = 0.3$ , respectively) (Figure 4.14). The analysis of the level of IL-6 after treatment revealed a correlation between improvement of BMD at hand ( $r = -0.5$ ,  $p = 0.3$ ) with changes in the levels of the cytokine (Figure 4.15).

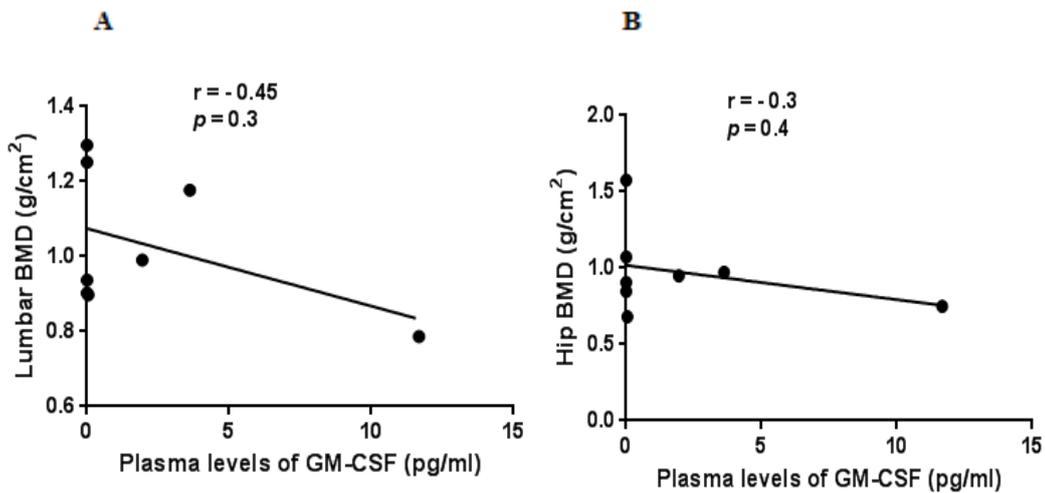


**Figure 4.14: Relationship between plasma levels of IL-6 before treatment and BMD at lumbar spine and hip.** The level of plasma IL-6 was determined using ELISA. BMD of the Lumbar spine (A) and hip (B) were measured using DEXA. Correlations were assessed using Spearman's correlation coefficient ( $r$ ).

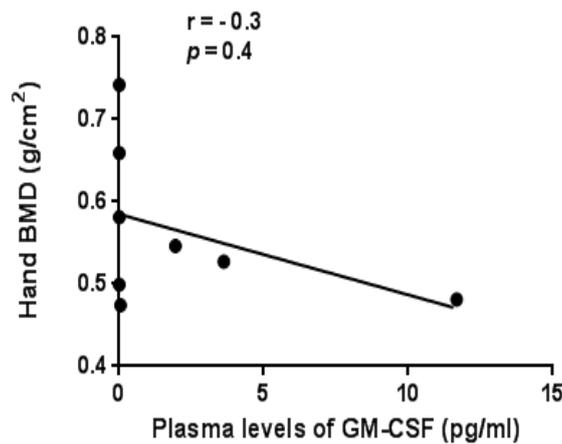


**Figure 4.15: Relationship between plasma levels of IL-6 and BMD of the hand.** Plasma levels of IL-6 were determined using ELISA. BMD of the hand was measured using DXR. Levels of IL-6 were correlated with BMD before treatment with biologic anti-TNF $\alpha$  agents had started (A) then changes in IL-6 were correlated with improvement in hand BMD after treatment (B). Correlations were with Spearman's correlation coefficient ( $r$ ).

Similar to IL-6, plasma levels of GM-CSF were also negatively correlated with BMD before treatment at the lumbar spine, hip and the hand ( $r=-0.5$ ,  $p=0.3$ ;  $r=-0.3$ ,  $p=0.4$ ; and  $r=-0.3$ ,  $p=0.4$ ), respectively (Figure 4.16 and 17).



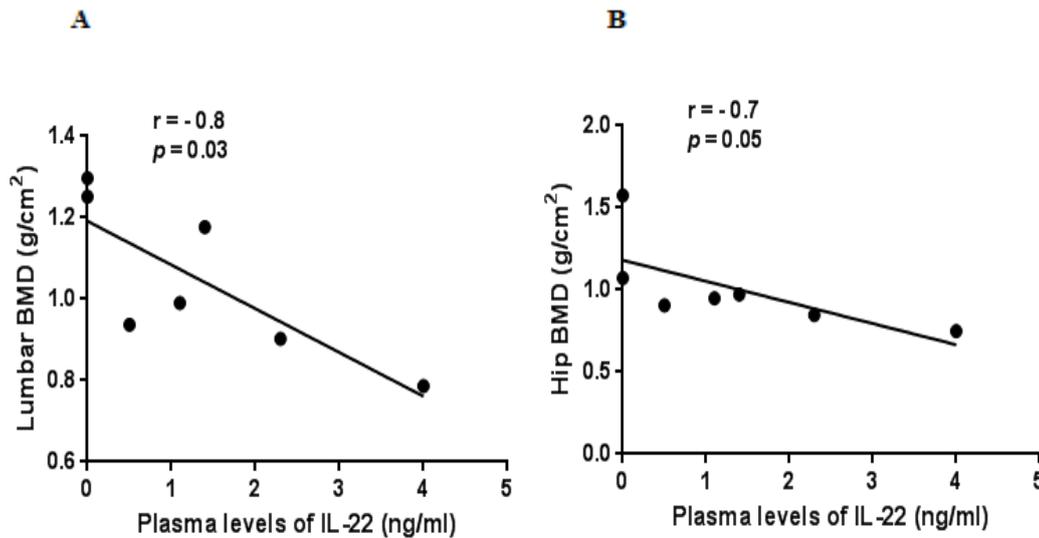
**Figure 4.16: Relationships between plasma levels of GM-CSF and BMD at lumbar spine (A) and hip (B).** Plasma levels of GM-CSF were determined before treatment using ELISA. BMD was measured using DEXA scan. Correlations were with Spearman's correlation coefficient (r).



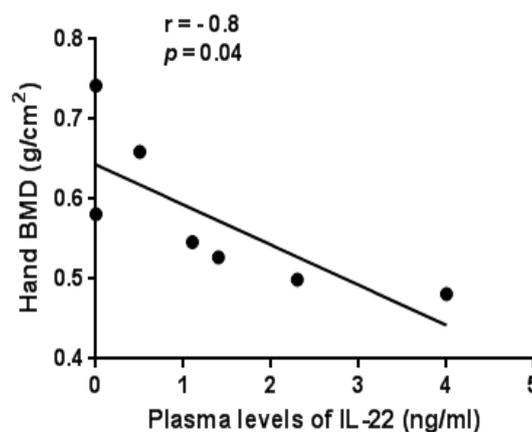
**Figure 4.17: Relationship between plasma levels of GM-CSF and BMD at the hand.** BMD of the hand was measured using DXR. Plasma GM-CSF level and the statistical analyses were as described in the legend to Figure 4.16.

The relationship between plasma levels of IL-20 and BMD could not be analysed as the developed ELISA method could only help detect level of this cytokine only in 2 patients.

Plasma levels of IL-22 negatively correlated with BMD of the lumbar spine, hip and the hand. These correlations were statistically significant ( $r=-0.8$ ,  $p=0.03$ ;  $r=-0.7$ ,  $p=0.05$  and  $r=-0.8$ ,  $p=0.04$  respectively) (Figures 4.18 and 4.19).



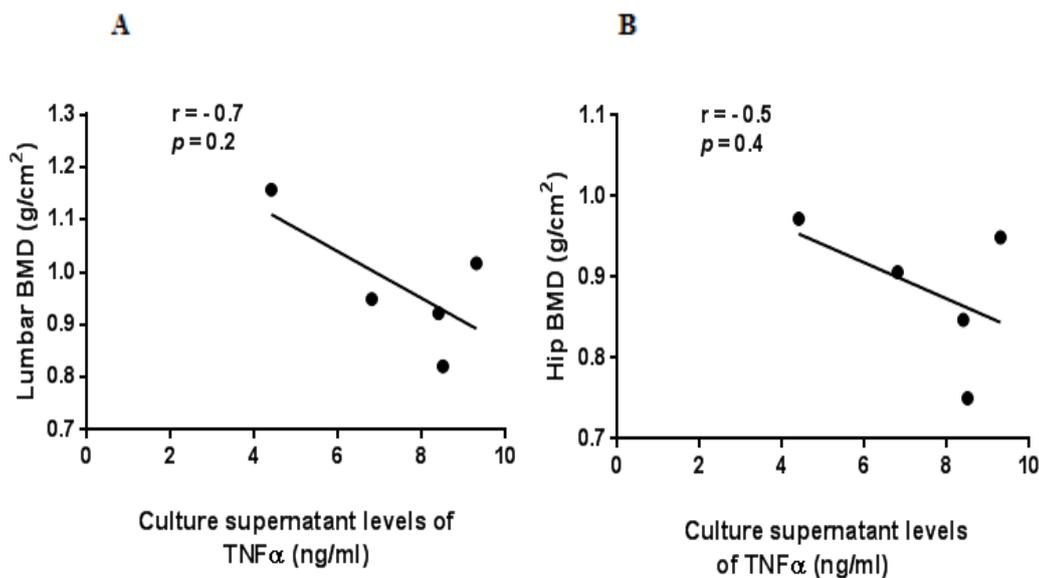
**Figure 4.18: Relationship between plasma levels of IL-22 and BMD at lumbar spine and hip.** Plasma IL-22 levels were determined prior to treatment by ELISA. BMD and the statistical analyses were carried out as described for the previous correlation figures.



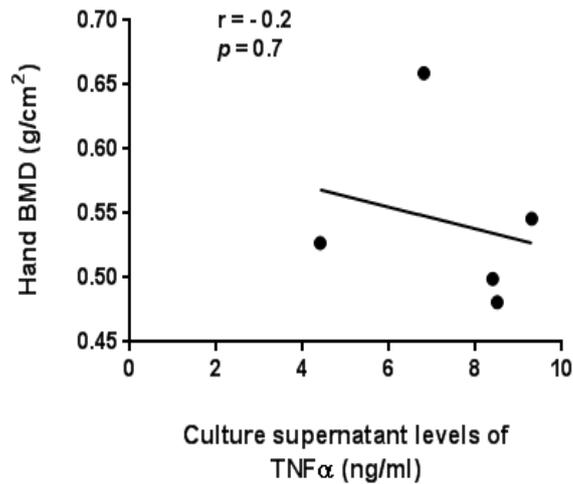
**Figure 4.19: Relationship between plasma levels of IL-22 and BMD at the hand.** Plasma IL-22 levels were determined by ELISA. BMD of the hand was measured using DXR and correlation determined by Spearman's correlation coefficient (r).

**b) Relationship between BMD and pro-inflammatory and bone resorptive cytokines produced by *ex vivo* stimulated immune cells**

The potential of immune cells to produce TNF $\alpha$  in RA patients treated with biologic anti-TNF $\alpha$  agents and relationship of such levels with improvement in BMD after treatment were correlated. The analysis involved enriching immune cells from the patients to high purity and stimulating the cells *ex vivo* for 48 hours before measuring the level of TNF $\alpha$  in culture supernatants from the cells in relation with changes in the BMD at the lumbar spine, hip and the hand. This analysis revealed a negative relationship although the data did not reach statistically significant levels, probably due to small sample size ( $r=-0.7$ ,  $p=0.2$ ;  $r=-0.5$ ,  $p=0.4$  and  $r=-0.2$ ,  $p=0.7$ , respectively) (Figures 4.20 and 4.21).



**Figure 4.20: Relationships between levels of TNF $\alpha$  produced by *ex vivo* T-lymphocytes and BMD at lumbar spine and hip.** The level of TNF $\alpha$  produced by *ex vivo* T-lymphocytes enriched before treating the patients was by MSD kits. BMD was measured using DEXA. Correlations were with Spearman's correlation coefficient ( $r$ ).

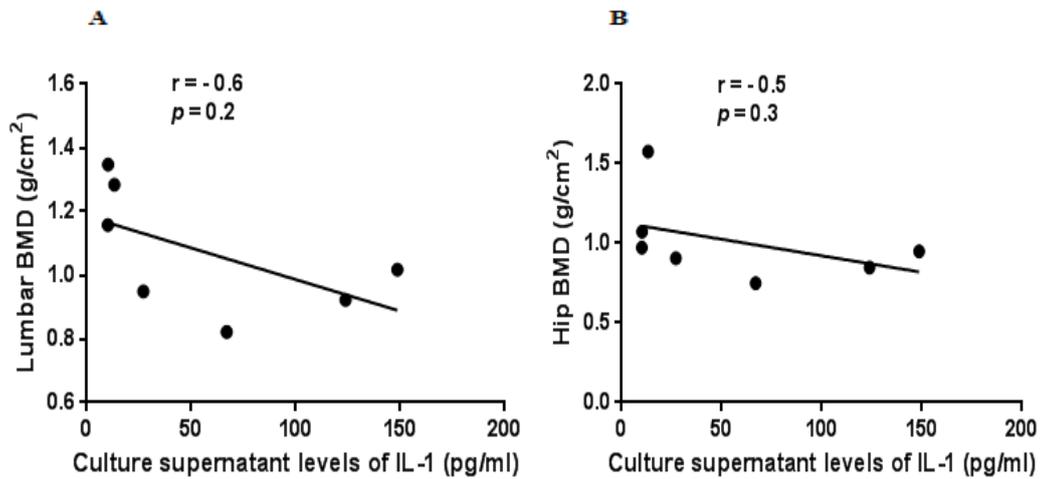


**Figure 4.21: Relationship between culture supernatant levels of TNF $\alpha$  and BMD at the hand.** TNF $\alpha$  levels in culture supernatants of activated T-lymphocytes was determined by MSD kits, BMD of the hand was measured using DXR and correlation determined by Spearman's correlation coefficient (r).

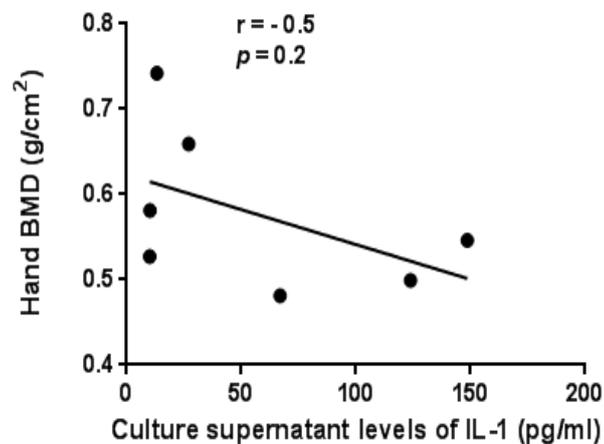
There was also a negative correlation between culture supernatant levels of IL-1 and BMD of the lumbar spine, hip and the hand ( $r=-0.6$ ,  $p=0.2$ ;  $r=-0.5$ ,  $p=0.3$  and  $r=-0.5$ ,  $p=0.2$ , respectively) (Figures 4.22 and 4.23). Furthermore, there was a trend for correlations between reduced levels of IL-1 after treatment and improvement in BMD at the lumbar spine and hip but the correlations were not statistically significant ( $r=-0.3$ ,  $p=0.6$  and  $r=-0.6$ ,  $p=0.1$ , respectively) (Figure 4.24).

There were no correlations between levels of IL-6, IL-17, IL-22 and GM-CSF in culture supernatants of the activated T-lymphocytes and BMD at lumbar spine, hip and hand.

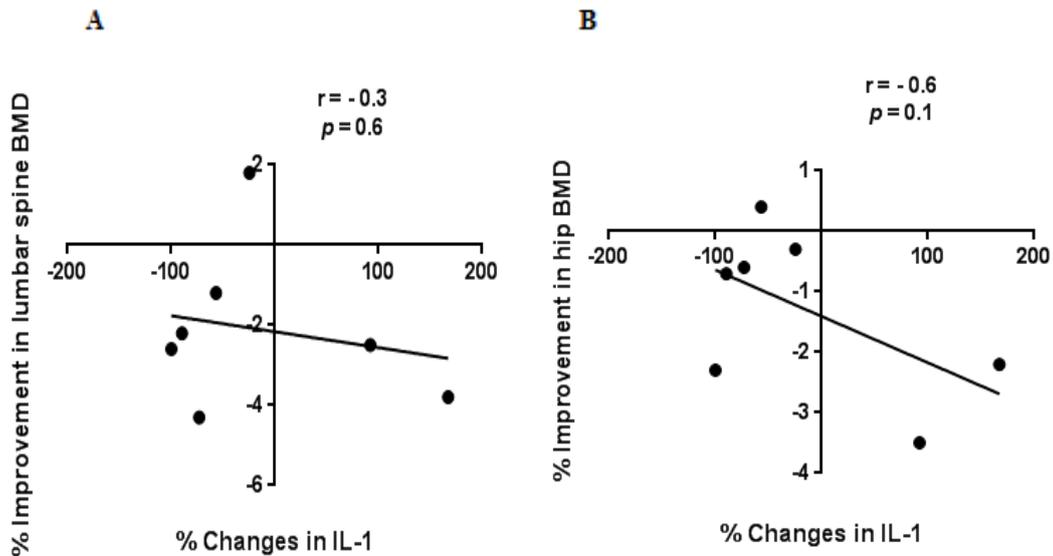
The findings summarised in this section, therefore, provide further evidence for a relationship between increased disease-promoting cytokines and accelerated bone loss. This, in turn, suggests that the inflammation characteristic of RA patients is associated with the generalised bone loss noted in the disease.



**Figure 4.22: Relationship between culture supernatant levels of IL-1 and BMD at the lumbar spine and hip.** IL-1 levels produced by *ex vivo* T-lymphocytes enriched before treating the patients was by MSD kits. BMD of the lumbar spine (A) and of hip (B) was measured using DEXA and correlations were by Spearman's correlation coefficient.



**Figure 4.23: Relationship between culture supernatant levels of IL-1 and BMD at the hand.** IL-1 levels in culture supernatants of activated T-lymphocytes, BMD and the statistical analyses were as described in the legend to Figure 4.21.



**Figure 4.24: The relationships between improvement in BMD at lumbar spine (A) and hip (B) with changes in culture supernatant levels of IL-1 after treatment with biologic anti-TNF $\alpha$  agents.** IL-1 levels in culture supernatants of activated T-lymphocytes, BMD and the statistical analyses were as described in the legend to Figure 4.22.

#### 4.3.5 Assessment of the relationship between changes in plasma bone biomarkers and the clinical response to treatment with biologic anti-TNF $\alpha$ agents.

Plasma levels of bone turnover biomarkers including RANK-L and OPG and pro-inflammatory cytokines including TNF $\alpha$ , IL-1, IL-6, IL-17, IL-20, IL-22 and GM-CSF were measured for the 55 RA patients included in this study.

Levels of CTX and osteocalcin before treatment were higher in responder patients ( $164 \pm 125$  pg/ml and  $15.5 \pm 10.5$  ng/ml) compared with non-responder patients ( $127 \pm 103$  pg/ml and  $11.6 \pm 8.1$  ng/ml). However, the differences did not reach statistically significant levels ( $p = 0.2$  and  $p = 0.7$ , respectively). There were also no statistically significant differences between levels of RANK-L and OPG in both responder and non-responder patients before treatment had started (Table 4.8).

**Table 4.8: Plasma levels of bone turnover biomarkers in responder and non-responder patients before the initiation of the treatment with biologic anti-TNF $\alpha$  agents.**

| Parameters          |           | Responders  | Non-responders | All patients |
|---------------------|-----------|-------------|----------------|--------------|
| CTX (pg/ml)         | Mean (SD) | 164 (125)   | 127 (103)      | 155 (120)    |
| Osteocalcin (ng/ml) | Mean (SD) | 15.5 (10.5) | 11.6 (8.1)     | 14.5 (10)    |
| RANK-L (pg/ml)      | Mean (SD) | 197 (187)   | 186 (206)      | 195 (189)    |
| OPG (ng/ml)         | Mean (SD) | 2.87 (1.5)  | 2.83 (1.2)     | 2.86 (1.5)   |

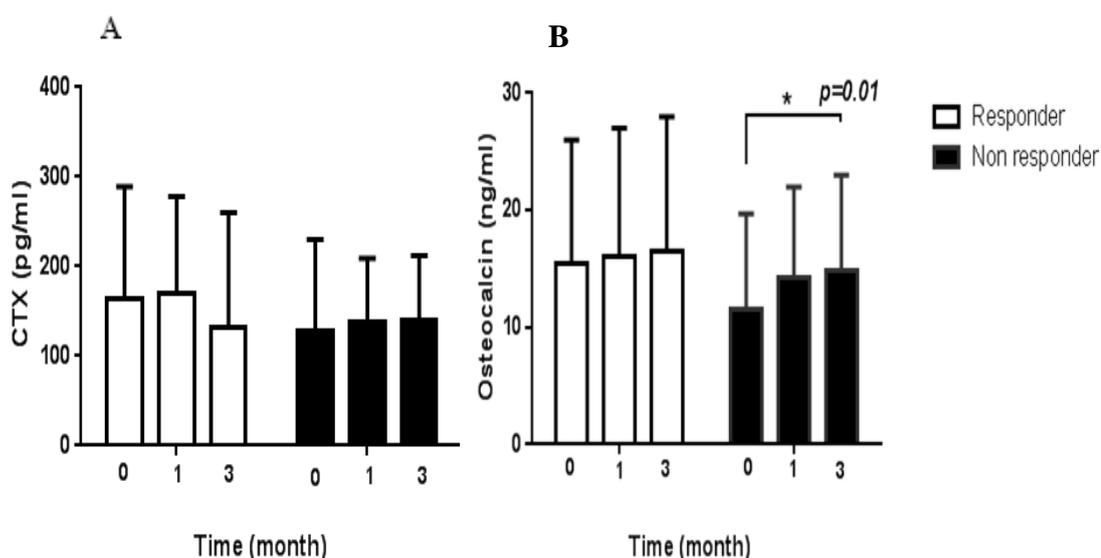
Plasma levels of bone turnover biomarkers, RANK-L and OPG for the 55 RA patients included in this study were measured by ELISA as described in the Materials and Methods chapter. The data was then evaluated to obtain as comprehensive insight into bone response to the treatment with biologic anti-TNF $\alpha$  agents as possible. The patients were divided into responder and no-responders to the treatment based on changes in their DAS28. The values shown are for the mean and (SD).

The level of CTX decreased in responder patients after 3 months of treatment compared with before treatment, 164 $\pm$ 125pg/ml to 131 $\pm$ 129pg/ml. However, the difference was not statistically significant ( $p=0.45$ ). This is, probably, due to the small patient number within the cohort. In contrast, non-responder patients had an increase in the plasma level of CTX 3 months after treatment compared with before treatment, 127 $\pm$ 103pg/ml compared with 140 $\pm$ 72pg/ml ( $p=0.58$ ) (Table 4.9). Interestingly, the level of osteocalcin increased significantly after 3 months of treatment in non-responder patients compared with before treatment, from 11.6 $\pm$ 8.1ng/ml to 14.9 $\pm$ 8.1ng/ml ( $p=0.01$ ). However, there was no statistically significant difference in the level osteocalcin in the patients after 1 month of treatment. Thus, plasma levels of osteocalcin were 11.6 $\pm$ 8.1ng/ml before treatment and 14.3 $\pm$ 7.7ng/ml ( $p=0.09$ ) after 1 month of treatment. The level of osteocalcin also increased in responder patients from 15.5  $\pm$ 10.5ng/ml before treatment to 16.5  $\pm$ 11.5ng/ml 3 months after treatment but the difference was not statistically different ( $p=0.19$ ) (Figure 4.25).

**Table 4.9: Changes in the level of bone turnover biomarkers after treatment with biologic anti-TNF $\alpha$  agents**

| Parameters                 | Before treatment | 1 month after treatment | 3 months after treatment |
|----------------------------|------------------|-------------------------|--------------------------|
| <b>CTX (pg/ml)</b>         |                  |                         |                          |
| - Responders               | 164 (125)        | 170 (108)               | 131 (129)                |
| - Non responders           | 127 (103)        | 137 (72)                | 140 (72)                 |
| <b>Osteocalcin (ng/ml)</b> |                  |                         |                          |
| - Responders               | 15.5 (10.5)      | 16 (11)                 | 16.5 (11.5)              |
| - Non responders           | 11.6 (8.1)       | 14.3 (7.7)              | 14.9 (8.1)               |

Plasma levels of CTX and osteocalcin were measured for 55 RA patients by ELISA. The patients were divided into responder and no-responders to treatment with biologic anti-TNF $\alpha$  agents based on changes in their DAS28. The values shown are for the mean and (SD). Full statistical analysis is provided in Appendix 4, Tables 1 and 2.



**Figure 4.25: Changes in plasma levels of CTX and osteocalcin in RA patients treated with biologic anti-TNF $\alpha$  agents.** Levels of CTX (A) and osteocalcin (B) were measured in the plasma of 55 RA patients by ELISA. The patients were divided into responder and non-responder groups based on changes in their DAS28 values. \* indicates  $p \leq 0.05$  for values after 3 months of treatment compared with before treatment.

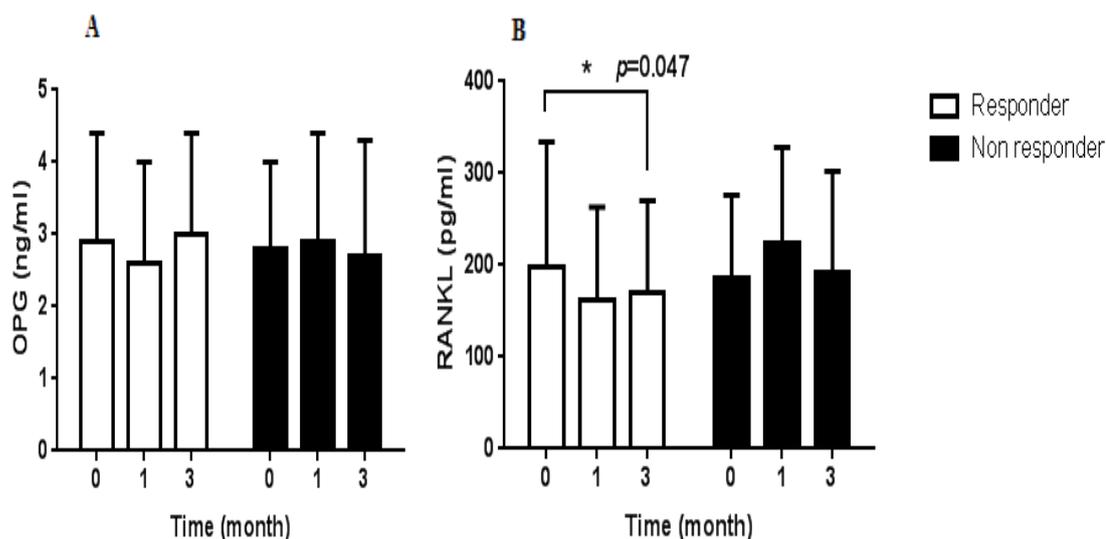
Levels of OPG increased in responder patients from their levels before treatment  $2.87 \pm 1.5 \text{ ng/ml}$  to  $2.64 \pm 1.4 \text{ ng/ml}$  ( $p=0.08$ ) and  $2.93 \pm 1.4 \text{ ng/ml}$  ( $p=0.4$ ) 1 and 3 months after treatment, respectively. The levels of OPG did not change markedly in non-responder patients (from  $2.83 \pm 1.2 \text{ ng/ml}$  before treatment to  $2.86 \pm 1.5 \text{ ng/ml}$  ( $p=0.9$ ) and  $2.80 \pm 1.6 \text{ ng/ml}$  ( $p=0.6$ ) 1 and 3 months after treatment) (Table 4.10) (Figure 4.26A).

The level of RANK-L decreased significantly in responder patients after 3 months of treatment compared with its levels before treatment from  $197 \pm 137 \text{ pg/ml}$  to  $170 \pm 100 \text{ pg/ml}$  ( $p=0.047$ ). In contrast, there was an increase in RANK-L level in non-responder patients after treatment but the difference was not statistically significant. The mean level of RANK-L was  $186 \pm 90 \text{ pg/ml}$  before treatment and  $223 \pm 105 \text{ pg/ml}$  ( $p=0.12$ ) after 1 month and  $192 \pm 110 \text{ pg/ml}$  ( $p=0.8$ ) after 3 months of treatment (Table 4.10) (Figure 4.26B).

**Table 4.10: Changes in the level of OPG and RANK-L after treatment of RA patients with biologic anti-TNF $\alpha$  agents**

| Parameters            | Before treatment | After 1 month | After 3 months |
|-----------------------|------------------|---------------|----------------|
| <b>OPG (ng/ml)</b>    |                  |               |                |
| - Responders          | 2.87 (1.5)       | 2.64 (1.4)    | 2.93 (1.4)     |
| - Non responders      | 2.83 (1.2)       | 2.86 (1.5)    | 2.80 (1.6)     |
| <b>RANK-L (pg/ml)</b> |                  |               |                |
| - Responders          | 197 (137)        | 161 (102)     | 170 (100)      |
| - Non responders      | 186 (90)         | 223 (105)     | 192 (110)      |

Plasma levels of OPG and RANK-L for the 55 RA patients studied were measured by ELISA. The patients were divided into responder and no-responders based on changes in their DAS28. The values shown are for the mean and (SD). Full statistical analysis is provided in Appendix 4, Tables 3 and 4.



**Figure 4.26: Changes in plasma levels of OPG and RANK-L in RA patients treated with biologic anti-TNF $\alpha$  agents.** The cohort of 55 RA patients studied was divided into responders and non-responders based on changes in their DAS28. Means OPG (A) and RANK-L (B) were measured by ELISA. The error bars represent SD. Results were compared with values before to after treatment using paired Student's t-test.\* indicates  $p \leq 0.05$  for values before to after treatment.

#### 4.3.6 Assessment of changes in pro-inflammatory and bone resorptive cytokines in RA patients treated with biologic anti-TNF $\alpha$ agents.

##### a) Changes in plasma levels of pro-inflammatory and bone resorptive cytokines:

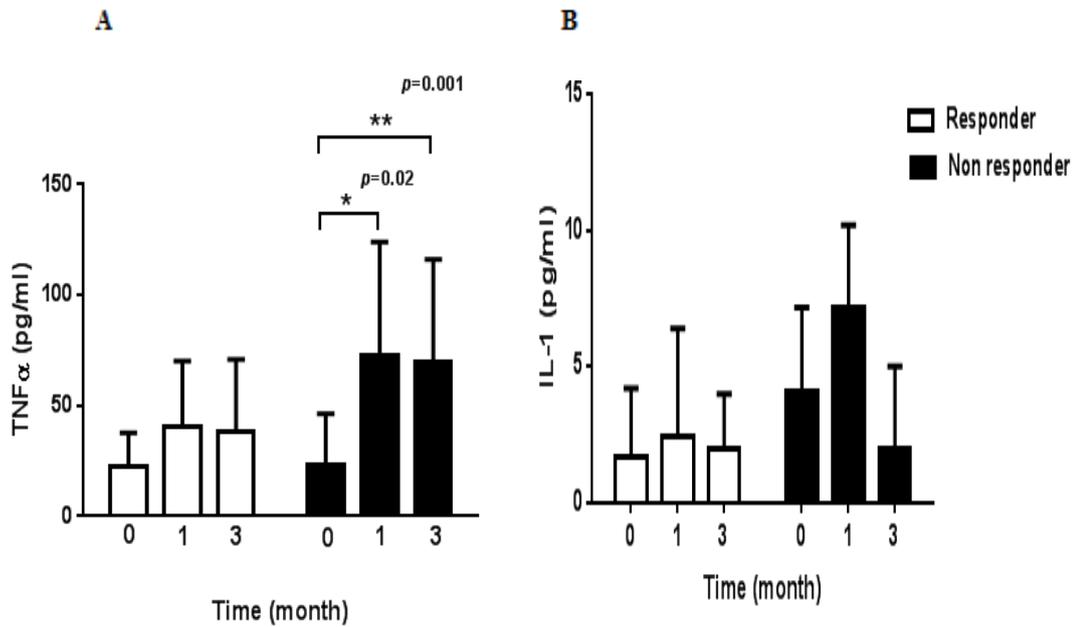
There were no statistically significant differences in plasma levels of TNF $\alpha$  between responder and non-responder patients before the start of treatment. Thus, the mean and SD level of plasma TNF $\alpha$  in responder patients before treatment was  $22.8 \pm 15$  pg/ml compared with  $23.3 \pm 23$  pg/ml in non-responder patients. Interestingly, the plasma levels of TNF $\alpha$  increased in the responder patients after treatment. Thus, levels of plasma TNF $\alpha$  were  $40.2 \pm 30$  pg/ml ( $p=0.3$ ) and  $37 \pm 33$  pg/ml ( $p=0.13$ ) after 1 and 3 months of treatment, respectively. In non-responders patients, plasma levels of TNF $\alpha$  increased significantly after 1 and 3 months of treatment to  $73 \pm 51$  pg/ml and  $70 \pm 46$  pg/ml, respectively (both  $p < 0.05$ ) (Table 4.11) (Figure 4.27A). Plasma level of IL-1 was lower in responder patients at  $1.7 \pm 2.5$  pg/ml

compared with  $4.1 \pm 3$  pg/ml in non-responder patients before treatment. Level of plasma IL-1 in non-responder patients increased to  $7.2 \pm 3$  pg/ml ( $p=0.3$ ) after 1 month of treatment but then declined to  $2.4 \pm 3$  pg/ml ( $p=0.3$ ) 3 months after treatment. There were no significant differences in plasma levels of IL-1 among responders patients from before to after 1 and 3 months of treatment at  $2.5 \pm 4$  pg/ml ( $p=0.7$ ) and  $2 \pm 2.7$  pg/ml ( $p=0.4$ ), respectively (Table 4.11) (Figure 4.27B).

**Table 4.11: Changes in TNF $\alpha$  and IL-1 cytokine levels after treatment with biologic anti-TNF $\alpha$  agents**

| Parameters                            | Before treatment | After 1 month | After 3 months |
|---------------------------------------|------------------|---------------|----------------|
| <b>TNF<math>\alpha</math> (pg/ml)</b> |                  |               |                |
| - Responders                          | 22.3 (15)        | 40.2 (30)     | 37 (33)        |
| - Non responders                      | 23.3 (23)        | 73 (51)       | 70 (46)        |
| <b>IL-1 (pg/ml)</b>                   |                  |               |                |
| - Responders                          | 1.7 (2.5)        | 2.5 (4)       | 2 (2.7)        |
| - Non responders                      | 4.1 (3)          | 7.2 (3)       | 2.4 (3)        |

Plasma levels of TNF $\alpha$  and IL-1 cytokines for 55 RA patients included in this study were measured by ELISA. The patients were divided into responder and no-responders to treatment with biologic anti-TNF $\alpha$  agents based on changes in their DAS28. The values shown are for the mean and (SD).



**Figure 4.27: Changes in plasma levels of TNF $\alpha$  and IL-1 in RA patients treated with biologic anti-TNF $\alpha$  agents.** Levels of TNF $\alpha$  and IL-1 were measured by ELISA. Response to treatment was based on changes of more than 1.2 in DAS28 scores. Means and SD of TNF $\alpha$  and IL-1 values in responder and non-responder patients are presented in pg/ml. The analysis was carried out using paired Student's t-test. \* indicates  $p \leq 0.05$ .

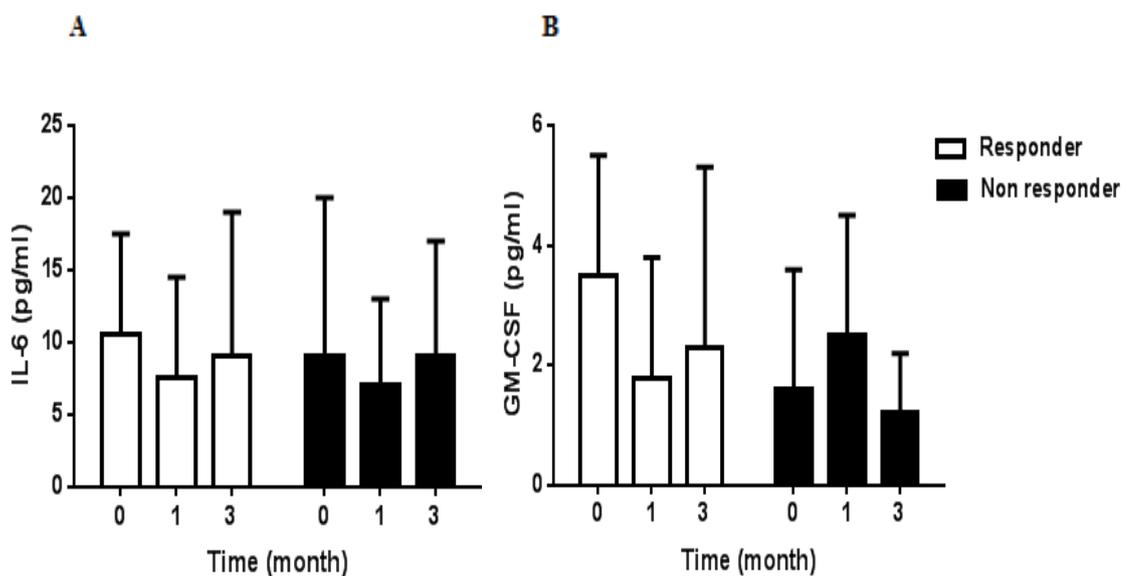
Plasma levels of IL-6 before treatment were  $11 \pm 7$  pg/ml in responder and  $9 \pm 11$  pg/ml in non-responder patients (Table 4.12). The mean level of IL-6 decreased in responder patients to  $8 \pm 10$  pg/ml and  $9 \pm 10$  pg/ml at 1 and 3 months post treatment, respectively. However, these differences did not reach statistically significant levels ( $p=0.8$  and  $p=0.2$ ). There were also no significant differences in plasma levels of IL-6 in non-responder patients after 1 and 3 months of treatment with biologic anti-TNF $\alpha$  agents from before treatment ( $8 \pm 6$  pg/ml and  $10 \pm 8$  pg/ml respectively) (Figure 4.28A). Plasma levels of GM-CSF were higher in responder patients compared with non-responders,  $3.5 \pm 2$  pg/ml compared to  $1.6 \pm 2$  pg/ml before treatment. There were reductions in plasma levels of GM-CSF in responder patients after 1 and 3 months of treatment to  $1.7 \pm 2.5$  pg/ml ( $p=0.2$ ) and  $2.3 \pm 3.7$  ( $p=0.3$ ), respectively. In non-responder patients, plasma levels of GM-CSF increased after 1 month of treatment but not after 3 months. Thus, plasma levels of GM-CSF in non-responder

patients were  $2.5 \pm 2$  pg/ml ( $p=0.5$ ) and  $1.2 \pm 1.7$  pg/ml ( $p=0.5$ ) after 1 and 3 months of treatment, respectively (Figure 4.28B).

**Table 4.12: Changes in plasma levels of IL-6 and GM-CSF after treatment with biologic anti-TNF $\alpha$  agents.**

| Parameters            | Before treatment | After 1 month | After 3 months |
|-----------------------|------------------|---------------|----------------|
| <b>IL-6 (pg/ml)</b>   |                  |               |                |
| - Responders          | 11 (7)           | 8 (10)        | 9 (10)         |
| - Non responders      | 9 (11)           | 8 (6)         | 10 (8)         |
| <b>GM-CSF (pg/ml)</b> |                  |               |                |
| - Responders          | 3.5 (2)          | 1.7 (2.5)     | 2.3 (3.7)      |
| - Non responders      | 1.6 (2)          | 2.5 (2)       | 1.2 (1.7)      |

Plasma levels of IL-6 and GM-CSF were measured by ELISA as described in the legend to the other figures. The values shown are for the mean and (SD).



**Figure 4.28: Changes in plasma levels of IL-6 and GM-CSF in RA patients treated with biologic anti-TNF $\alpha$  agents.** Levels of IL-6 and GM-CSF were measured by ELISA. Patient response was based on changes of more than 1.2 in the DAS28 scores. The data were analysed using paired Student's t-test.

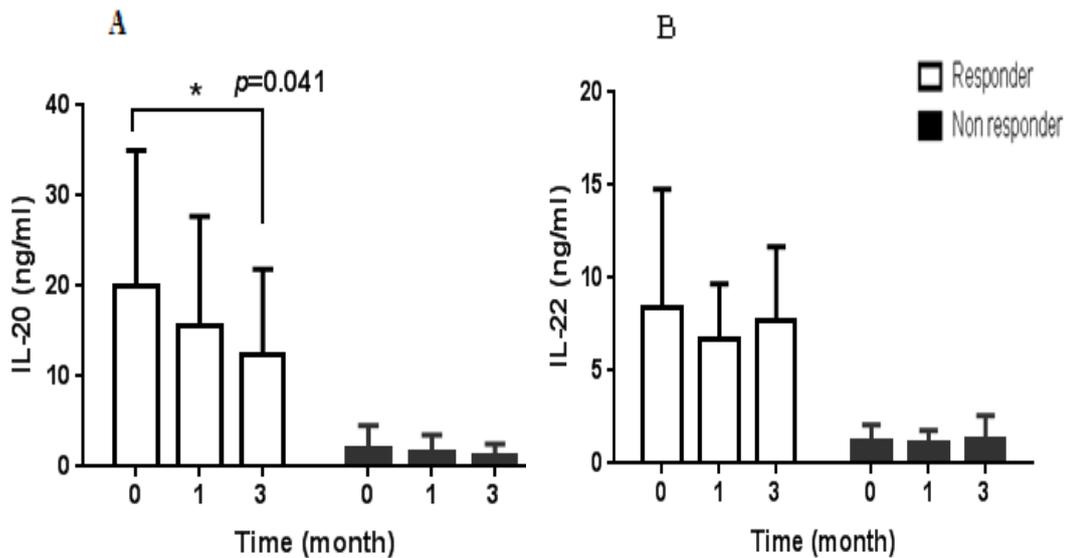
The measurement of plasma levels of IL-20 showed significant reduction in responder patients after 3 months of treatment with biologic anti-TNF $\alpha$  agents compared with before treatment. Thus, the mean and SD level of IL-20 was 12.4 $\pm$ 9.5ng/ml at 3 months after treatment compared with 20 $\pm$ 15ng/ml before treatment ( $p=0.041$ ). Somewhat unexpectedly, however, the level of IL-20 was lower before the start of treatment in non-responder patients at 2 $\pm$ 2.6ng/ml compared with responder patients (Table 4.13). However, caution may be applied to avoid over interpretation of this observation since the number of non-responder patients was relatively small (IL-20 could only be detected in the plasma of 2 of 13 non-responder patients studied) (Figure 4.29A).

The level of IL-22 was also lower in non-responder patients (1.2 $\pm$ 0.9ng/ml) compared with responder patients before the start of treatment (8.4 $\pm$ 6.4ng/ml). After treatment, the mean level of IL-22 decreased in responder patients after 1 and 3 months of treatment. However, these differences did not reach statistically significant levels. Thus, plasma levels of IL-22 were 8.4 $\pm$ 6.4ng/ml before treatment and 6.7 $\pm$ 3 ( $p=0.7$ ) after 1 month and 7.7 $\pm$ 4ng/ml after 3 months of treatment ( $p=0.18$ ). Further, no significant differences were observed in plasma levels of IL-22 in non-responder patients from before treatment to after 1 and 3 months of treatment, 1.1 $\pm$ 0.7ng/ml ( $p=0.2$ ) and 1.3 $\pm$ 1.3 ( $p=0.9$ ), respectively (Figure 4.29B).

**Table 4.13: Changes in plasma levels of IL-20 and IL-22 cytokines after treatment with biologic anti-TNF $\alpha$  agents**

| Parameters           | Before treatment | After 1 month | After 3 months |
|----------------------|------------------|---------------|----------------|
| <b>IL-20 (ng/ml)</b> |                  |               |                |
| - Responders         | 20 (15)          | 15.8 (12)     | 12.4 (9.5)     |
| - Non responders     | 2 (2.6)          | 1.5 (2)       | 1.1 (1.4)      |
| <b>IL-22 (ng/ml)</b> |                  |               |                |
| - Responders         | 8.4 (6.4)        | 6.7 (3)       | 7.7 (4)        |
| - Non responders     | 1.2 (0.9)        | 1.1 (0.7)     | 1.3 (1.3)      |

Plasma levels of IL-20 and IL-22 were measured by ELISA and patients divided into responder and no-responders to treatment with biologic anti-TNF $\alpha$  agents based on changes in their DAS28.



**Figure 4.29: Changes in plasma levels of IL-20 and IL-22 in RA patients treated with biologic anti-TNF $\alpha$  agents.** Levels of IL-20 and IL-22 were determined by ELISA. Error bars represent +/- SD. \* indicates  $p \leq 0.05$  at 1 or 3 months after treatments compared with the values before treatment had started.

**b) Changes in the levels of pro-inflammatory cytokines produced in culture of activated T-lymphocytes and bone resorptive biomarkers:**

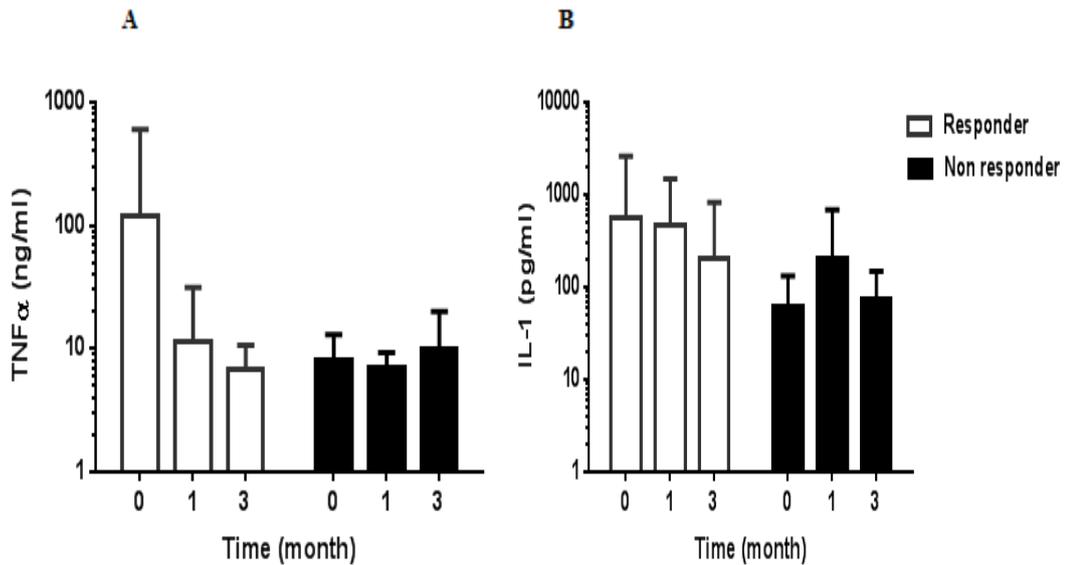
Quantification of the levels of TNF $\alpha$  from before to after treatment revealed that these were reduced in responder patients after 1 and 3 months of treatment. Thus, the mean and SD level of TNF $\alpha$  in the culture supernatants before treatment was  $120 \pm 483$  ng/ml and this decreased to  $11.4 \pm 20$  ng/ml and  $6.7 \pm 4$  ng/ml after 1 and 3 months of treatment. However, these differences were not statistically significant at  $p=0.3$  and  $0.4$ , respectively, perhaps due to the sample number. The level of TNF $\alpha$  was lower in non-responder patients at  $8.2 \pm 5$  ng/ml before treatment compared with responder patients ( $120 \pm 483$  ng/ml; Table 4.14). In addition, the level of TNF $\alpha$  in culture supernatants increased in the non-responder patients after 1 and 3 months to  $7 \pm 2$  ng/ml ( $p=0.3$ ) and  $10 \pm 10$  ng/ml ( $p=0.8$ ), respectively (Figure 4.30A).

The level of IL-1 in the culture supernatants also decreased in the responder patients after 1 and 3 months. However, these reductions did not reach statistically significant levels. Thus, the the mean and SD level of IL-1 in the culture supernatants of stimulated T-lymphocytes before treatment was 550±500pg/ml and this decreased to 472±500pg/ml ( $p=0.8$ ) and 205±250pg/ml ( $p=0.4$ ) after 1 and then 3 months of treatment. Similar to what was seen with the level of TNF $\alpha$  in culture supernatants of activated T-lymphocytes, the level of IL-1 in non-responder patients increased from 63±69pg/ml before treatment to 202±483pg/ml ( $p=0.7$ ) and 73±75pg/ml ( $p=0.3$ ) after 1 and 3 months of treatment, respectively (Table 4.14) (Figure 4.30B).

**Table 4.14: Changes in the levels of TNF $\alpha$  and IL-1 in culture supernatants of *ex vivo* activated T-lymphocytes from RA patients treated with biologic anti-TNF $\alpha$  agents.**

| Parameters                            | Before treatment | After 1 month | After 3 months |
|---------------------------------------|------------------|---------------|----------------|
| <b>TNF<math>\alpha</math> (ng/ml)</b> |                  |               |                |
| - Responders                          | 120 (483)        | 11.4 (20)     | 6.7 (4)        |
| - Non responders                      | 8.2 (5)          | 7 (2)         | 10 (10)        |
| <b>IL-1(pg/ml)</b>                    |                  |               |                |
| - Responders                          | 550 (500)        | 472 (500)     | 205 (250)      |
| - Non responders                      | 63 (69)          | 202 (483)     | 73 (75)        |

Levels of TNF $\alpha$  and IL-1 in culture supernatants of T-lymphocytes from treated RA patients stimulated *ex vivo* with anti-CD3 and anti-CD28 (both at 10 $\mu$ g/mL). Levels of the two cytokines were determined using multiplex MSD kits. Patients were divided into responder and no-responders based on changes in their DAS28. The values shown are for the mean and (SD).



**Figure 4.30: Changes in the levels of TNF $\alpha$  and IL-1 in culture supernatants of *ex vivo* activated T-lymphocytes from RA patients treated with biologic anti-TNF $\alpha$  agents.** The T-lymphocytes were activated with anti-CD3/CD28 as described in the legend to Table 4.14 and TNF $\alpha$  and IL-1 content of the culture supernatants quantified using MSD kits. Patients were considered responders or non-responders depending on changes in their DAS28 scores.

The mean  $\pm$  SD level of IL-6 in culture supernatants of activated *ex vivo* T-lymphocytes before treatment was  $3.2 \pm 7$  ng/ml in responder patients compared with  $0.9 \pm 1$  ng/ml in non-responder patients (Table 4.15). The mean level of IL-6 increased in both groups of patients after 1 month of treatment with biologic anti-TNF $\alpha$  agents. Thus, the mean  $\pm$ SD level of IL-6 after 1 month was  $3.5 \pm 5$  ng/ml ( $p=0.8$ ) in responder patients and  $2 \pm 2.7$  ng/ml ( $p=0.08$ ) in non-responder patients. The level of IL-6 decreased after 3 months of treatment in the responder patients to  $1.5 \pm 2$  ng/ml ( $p=0.3$ ) but remained higher than before treatment in non-responder after 3 months of treatment at  $1.1 \pm 1$  ng/ml ( $p=0.6$ ) (Figure 4.31A).

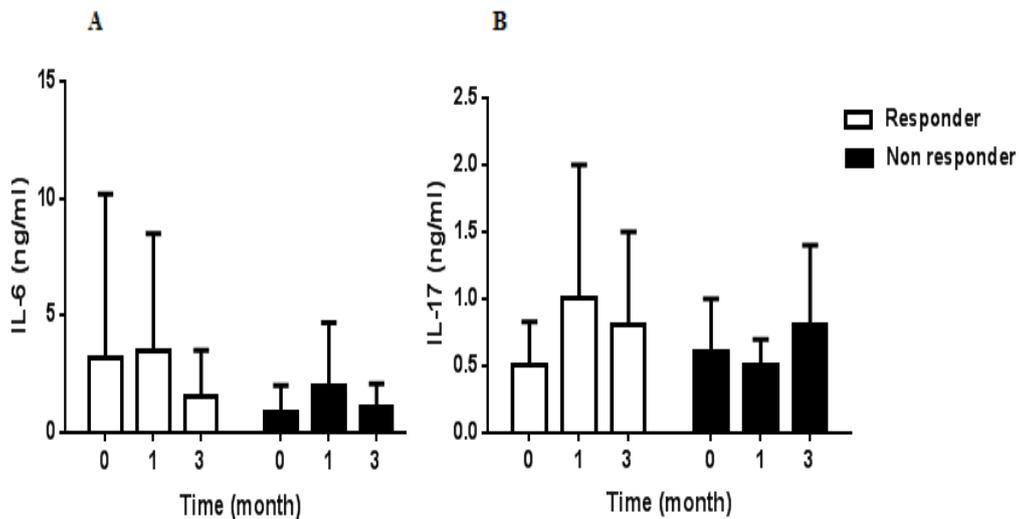
The mean and SD level of IL-17 in culture supernatant of the activated T-lymphocytes increased in the responder patients after 1 and 3 months of treatment. Thus, the mean and SD levels of IL-17 were  $0.5 \pm 0.3$  ng/ml before treatment and  $1 \pm 1$  ng/ml ( $p=0.3$ ) and  $0.8 \pm 0.7$  ng/ml ( $p=0.4$ ) after 1 and 3 months of treatment. The level of IL-17 decreased in the non-responder patients after 1 month but increased after 3 months of treatment. Thus, the mean and SD

levels of IL-17 were  $0.6 \pm 0.4$  ng/ml before treatment,  $0.5 \pm 0.2$  ng/ml ( $p=0.5$ ) and  $0.8 \pm 0.6$  ng/ml ( $p=0.5$ ) after 1 and 3 months of treatment (Table 4.15) (Figure 4.31B).

**Table 4.15: Levels of IL-6 and IL-17 in culture supernatants of activated *ex vivo* T-lymphocytes from RA patients treated with biologic anti-TNF $\alpha$  agents.**

| Parameters           | Before treatment | After 1 month | After 3 months |
|----------------------|------------------|---------------|----------------|
| <b>IL-6 (ng/ml)</b>  |                  |               |                |
| - Responders         | 3.2 (7)          | 3.5 (5)       | 1.5 (2)        |
| - Non responders     | 0.9 (1)          | 2.0 (2.7)     | 1.1 (1)        |
| <b>IL-17 (ng/ml)</b> |                  |               |                |
| - Responders         | 0.5 (0.3)        | 1.0 (1)       | 0.8 (0.7)      |
| - Non responders     | 0.6 (0.4)        | 0.5 (0.2)     | 0.8 (0.6)      |

Levels of IL-6 and IL-17 were measured using MSD multiplex kits as described in Table 4.14. The values shown are for the mean and (SD).



**Figure 4.31: Changes in the levels of IL-6 and IL-17 produced into culture supernatants by activated *ex vivo* T-lymphocytes from RA patients treated with biologic anti-TNF $\alpha$  agents.** Levels of the two cytokines and patient classification were as described in the legend of Table 4.14.

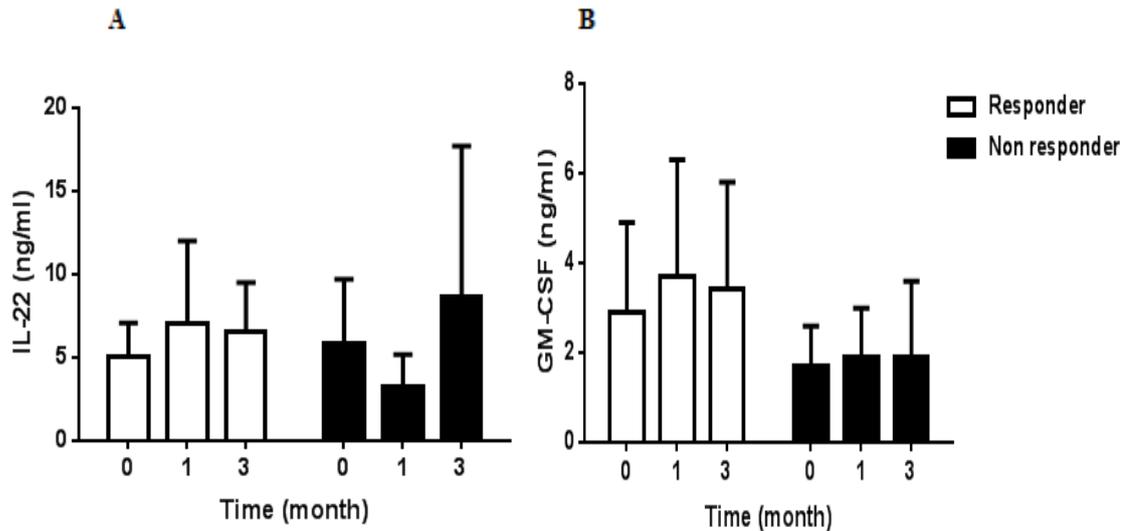
The level of IL-22 in the culture supernatants increased after treatment in the responder patients at 1 and 3 months of treatment. However, the differences did not reach statistically significant levels. Thus, the mean and SD levels of IL-22 were  $5.1 \pm 2$  ng/ml before treatment and  $7 \pm 5$  ng/ml ( $p=0.5$ ) and  $6.5 \pm 3$  ng/ml ( $p=0.7$ ) at 1 and 3 months after treatment. In contrast, the level of culture supernatant IL-22 decreased in non-responder patients from  $5.8 \pm 4$  ng/ml before treatment to  $3.3 \pm 2$  ng/ml ( $p=0.2$ ) after 1 month then increased after 3 months of treatment to  $8.7 \pm 9$  ng/ml ( $p=0.5$ ) (Table 4.16) (Figure 4.32A).

The level of GM-CSF was higher at  $2.9 \pm 2$  ng/ml in responder patients than in non-responder patients at  $1.7 \pm 0.9$  ng/ml before treatment. The mean level of GM-CSF increased in both groups of patients after 1 and 3 months of treatment but the differences were not statistically significant. Thus, the mean and SD levels of GM-CSF after 1 and 3 months of treatment were  $3.7 \pm 2.6$  ng/ml ( $p=0.06$ ) and  $3.4 \pm 2.4$  ng/ml ( $p=0.2$ ) in responder patients and  $1.9 \pm 1.1$  ng/ml ( $p=0.4$ ) and  $1.9 \pm 1.7$  ng/ml ( $p=0.9$ ) in non-responder patients (Table 4.16) (Figure 4.32B).

**Table 4.16: Levels of IL-22 and GM-CSF in culture supernatants of *ex vivo* activated T-lymphocytes from RA patients treated with biologic anti-TNF $\alpha$  agents.**

| Parameters            | Before treatment | After 1 month | After 3 months |
|-----------------------|------------------|---------------|----------------|
| <b>IL-22 (ng/ml)</b>  |                  |               |                |
| - Responders          | 5.1 (2)          | 7 (5)         | 6.5 (3)        |
| - Non responders      | 5.8 (4)          | 3.3 (2)       | 8.7 (9)        |
| <b>GM-CSF (ng/ml)</b> |                  |               |                |
| - Responders          | 2.9 (2)          | 3.7 (2.6)     | 3.4 (2.4)      |
| - Non responders      | 1.7 (0.9)        | 1.9 (1.1)     | 1.9 (1.7)      |

Levels of the two cytokines and patient classification were as described in the legend to Table 4.14. The values shown are for the mean and (SD).



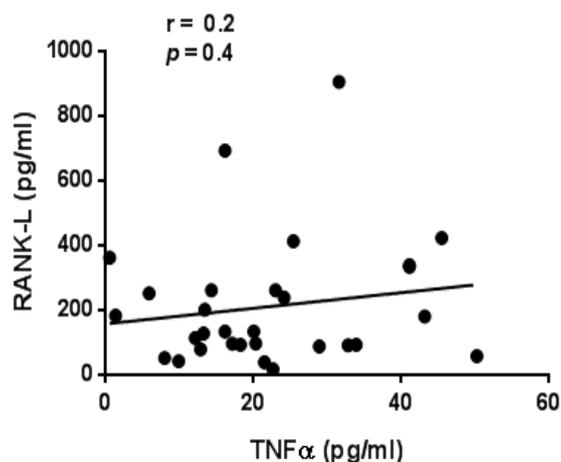
**Figure 4.32: Changes in the levels of IL-22 and GM-CSF in culture supernatants of activated *ex vivo* T-lymphocytes from RA patients treated with biologic anti-TNF $\alpha$  agents.** Levels of the two cytokines and patient classification were as described in the legend to Table 4.14. Means and SD of IL-22 and GM-CSF are presented in ng/ml. Levels of the two cytokine after treatment were compared with before treatment using paired Student's t-test.

#### **4.3.7 Relationship between levels of bone turnover biomarkers with pro-inflammatory cytokines in RA patients treated with biologic anti-TNF $\alpha$ agents.**

To further assess the relationship between inflammation and bone loss in RA and the impact of biologic anti-TNF $\alpha$  agents, the relationship between bone turnover biomarkers and the level of key cytokines affected by the treatment was studied. The analysis involved correlating the level of cytokines in plasma and culture supernatant from activated T-lymphocytes and bone turnover biomarkers in plasma. The results are summarised below.

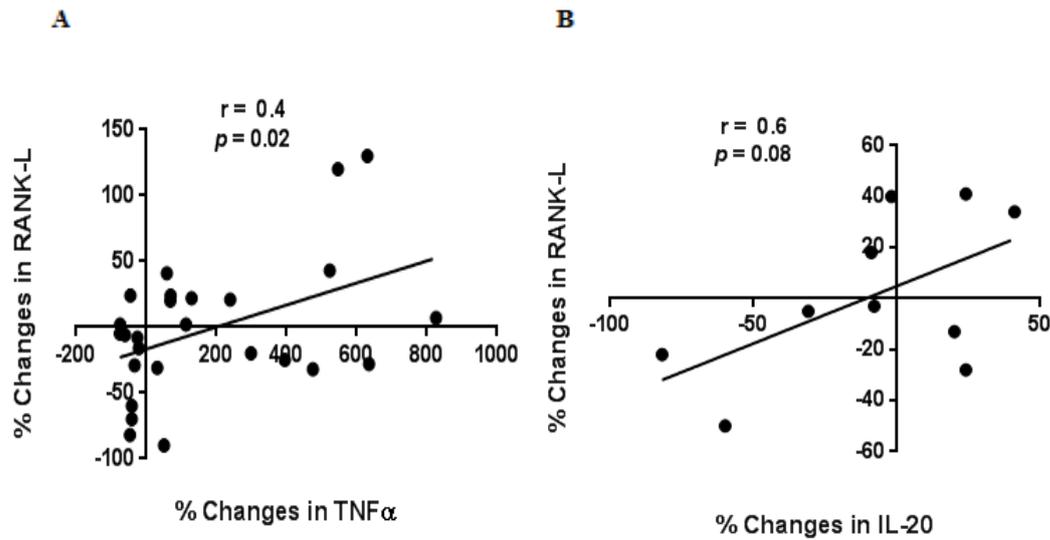
**a) Relationship between plasma levels of bone turnover biomarkers and pro-inflammatory and bone resorptive cytokines.**

Analysis of the relationship before treatment between TNF $\alpha$  and RANK-L showed a positive trend for a correlation between plasma levels of the two proteins. However, the correlation was not statistically significant ( $r=0.2$ ,  $p=0.4$ ) (Figure 4.33).



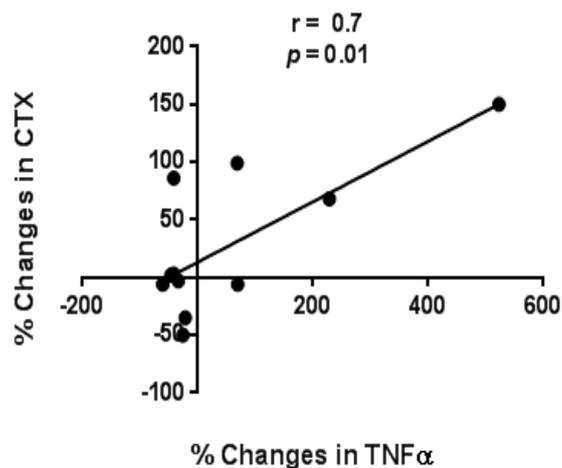
**Figure 4.33: The relationship between plasma levels of RANK-L and TNF $\alpha$  before treatment with biologic anti-TNF $\alpha$  agents.** Plasma levels of RANK-L were determined in the treated patients by ELISA while the level of TNF $\alpha$  was determined using multiplex MSD kits. Correlations made using Spearman's correlation coefficient ( $r$ ).

After treatment, the analysis of the correlation between changes in RANK-L and TNF $\alpha$  revealed a direct positive correlation. This correlation was statistically significant,  $r=0.4$ ,  $p=0.02$  (Figure 4.34A). There was also a positive correlation between changes in RANK-L and IL-20 levels after 3 months of treatment,  $r=0.6$ ,  $p=0.08$ . Thus, the level of RANK-L decreased with reduction in IL-20 after treatment with biologic anti-TNF $\alpha$  agents (Figure 4.34B).



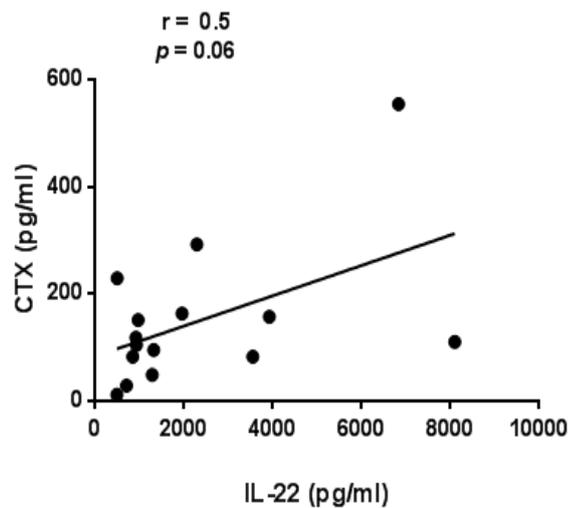
**Figure 4.34: The relationship between changes in plasma level of RANK-L with changes in levels of TNFα (A) and IL-20 (B) after 3 months of treatment with biologic anti-TNFα agents.** The levels of RANK-L, TNFα and IL-20 were determined by ELISA. Correlations were with Spearman's correlation coefficient (r).

A strong positive correlation was observed between changes in plasma levels of the bone resorption marker CTX and TNFα after 3 months of treatment with biologic anti-TNFα agents, ( $r=0.7, p=0.01$ ) indicating an involvement of TNFα in bone erosion (Figure 4.35).

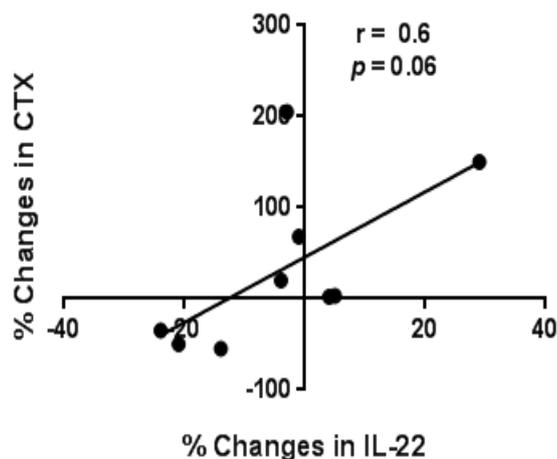


**Figure 4.35: The relationship between changes in plasma levels of CTX and TNFα in RA patients after 3 months of treatment with biologic anti-TNFα agents.** The levels of CTX and TNFα were determined by ELISA and correlations were calculated by Spearman's correlation coefficient (r).

Although, a strong positive correlation was observed before treatment between CTX and plasma levels of IL-22 this did not reach statistically significant levels ( $r=0.5$ ,  $p=0.06$ ) (Figure 4.36). The analysis of the correlation between changes in CTX and IL-22 after 3 months of treatment revealed a direct positive correlation,  $r=0.6$ ,  $p=0.06$  (Figure 4.37).

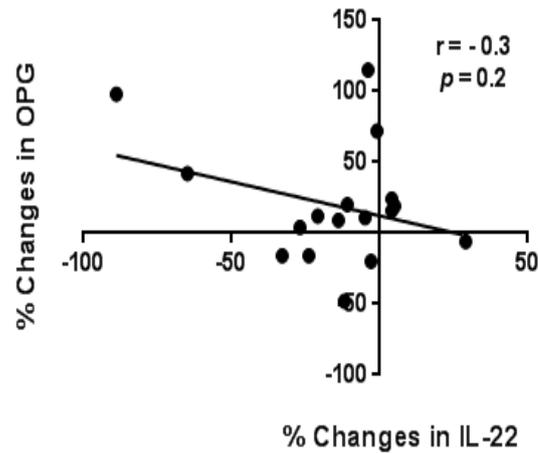


**Figure 4.36: The relationship between plasma levels of CTX and IL-22 in RA patients before treatment with biologic anti-TNF $\alpha$  agents. CTX and IL-22 levels were measured by ELISA and correlations calculated by Spearman's correlation coefficient (r).**

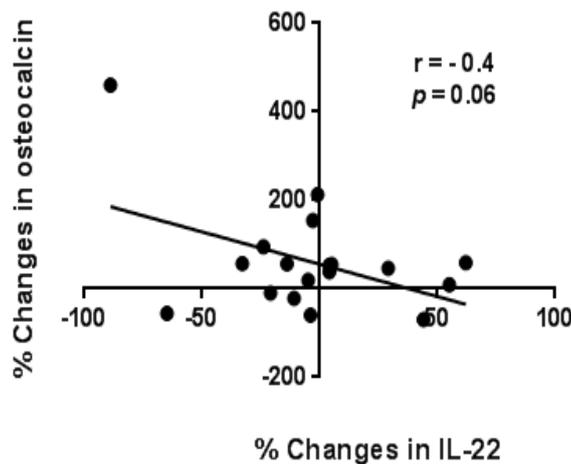


**Figure 4.37: The relationship between changes in plasma level of CTX and changes in IL-22 level after 3 months of treatment with biologic anti-TNF $\alpha$  agents. The levels of CTX and IL-22 were determined by ELISA. Correlations were with Spearman's correlation coefficient (r).**

In contrast to CTX, a negative correlation was observed between changes in plasma levels of the OPG and osteocalcin with changes in IL-22 level after 3 months of treatment with biologic anti-TNF $\alpha$  agents, ( $r=-0.3, p=0.2$  and  $r=-0.4, p=0.06$ ) (Figures 4.38 and 4.39).



**Figure 4.38: The relationship between changes in plasma level of OPG and changes in IL-22 level after 3 months of treatment with biologic anti-TNF $\alpha$  agents.** The levels of OPG and IL-22 were determined by ELISA. Correlations were with Spearman's correlation coefficient ( $r$ ).

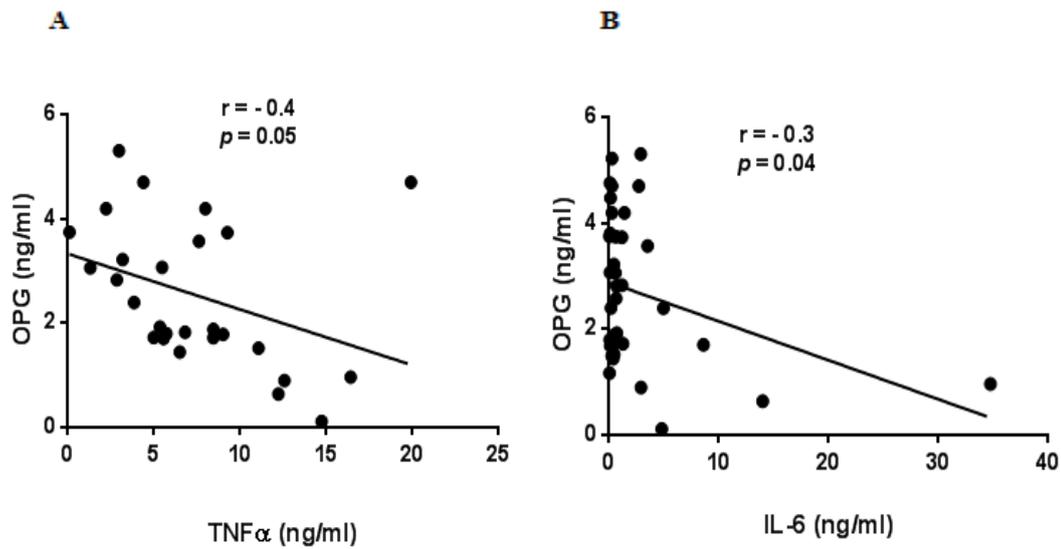


**Figure 4.39: The relationship between changes in plasma level of osteocalcin and changes in IL-22 level after 3 months of treatment with biologic anti-TNF $\alpha$  agents.** The levels of osteocalcin and IL-22 were determined by ELISA. Correlations were with Spearman's correlation coefficient ( $r$ ).

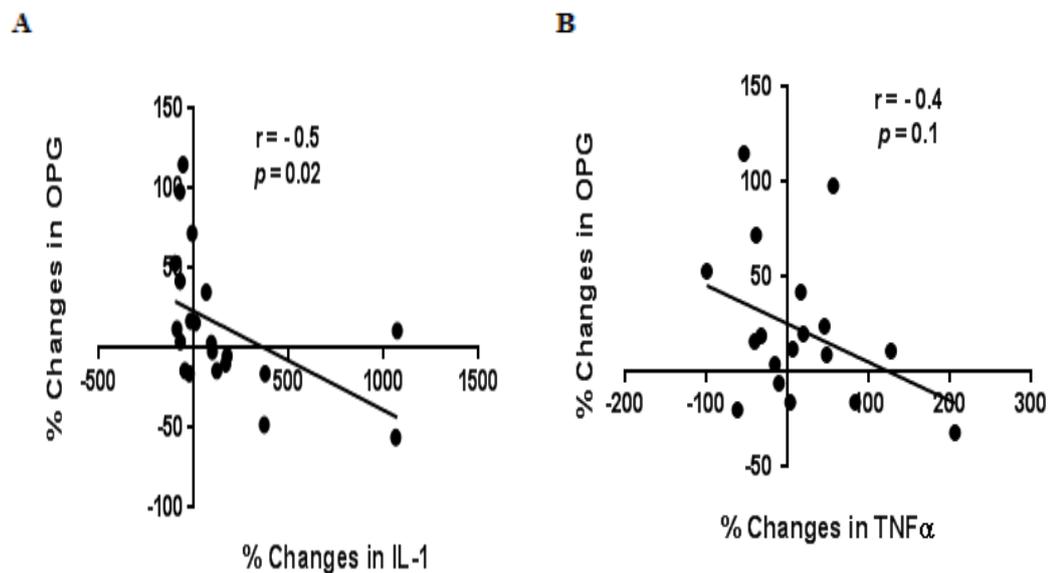
**b) Relationship between the levels of pro-inflammatory and bone resorptive cytokines produced by *ex vivo* T-lymphocytes and bone turnover biomarkers.**

The ability of T-lymphocytes from treated RA patients to produce pro-bone resorption cytokines before and after treatment with biologic anti-TNF $\alpha$  was correlated with the level of bone turnover biomarkers. This was carried out with two questions in mind: 1) whether the production of these cytokines influenced bone loss in RA patients, and 2) whether changes in the level of these cytokines after treatment correlated with similar changes in the bone biomarkers which would confirm the effect of TNF $\alpha$  on both sets of variables. The ability of T-lymphocytes to produce cytokines was studied using enriched *ex vivo* T-lymphocytes stimulated with anti-CD3/CD28. The level of cytokines secreted into the culture supernatants of the cultured T-lymphocytes was measured using a combination of MSD kits and ELISA protocols.

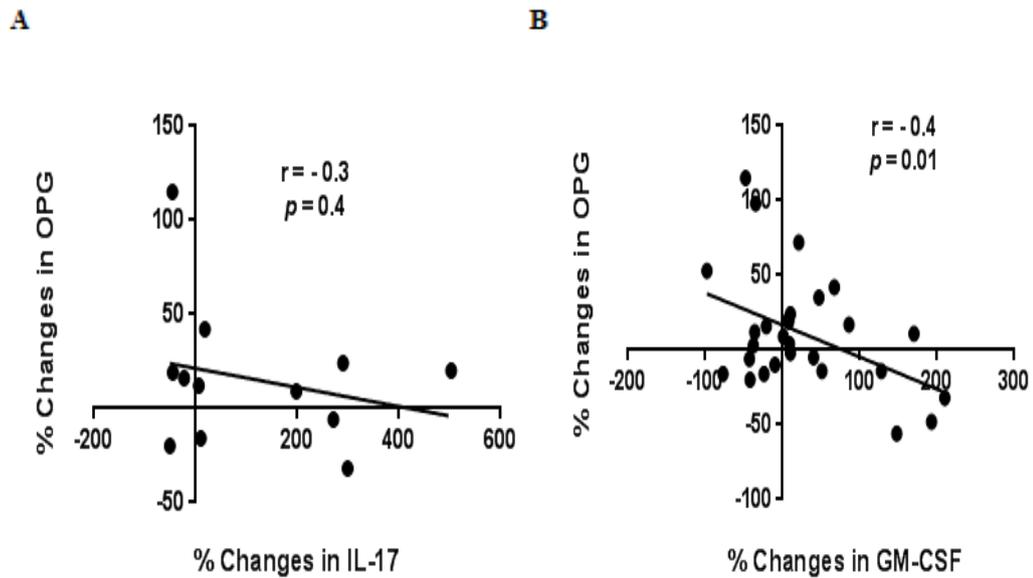
Analysis of the level of OPG in plasma with the level of TNF $\alpha$  and IL-6 in culture supernatants of the T-lymphocytes before treatment showed that the level of OPG inversely correlated with the levels of TNF $\alpha$  and IL-6 in the culture supernatants ( $r=-0.4$ ,  $p=0.05$ ; and  $r=-0.3$ ,  $p=0.04$ , respectively) (Figure 4.40). The changes in level of OPG also inversely correlated with changes in IL-1, TNF $\alpha$ , IL-17 and GM-CSF levels after 3 months of treatment with biologic anti-TNF $\alpha$  agents,  $r=-0.5$ ,  $p=0.02$ ;  $r=-0.4$ ,  $p=0.1$ ;  $r=-0.3$ ,  $p=0.4$  and  $r=-0.4$ ,  $p=0.01$ , respectively (Figures 4.41 and 4.42).



**Figure 4.40: The relationship between plasma level of OPG with TNF $\alpha$  and IL-6 produced *ex vivo* by activated T-lymphocytes from RA patients.** Levels of TNF $\alpha$  (A) and IL-6 (B) were determined by a multiplex MSD kit. Plasma OPG levels were determined by ELISA. The correlations are for patients before treatment with biologic anti-TNF $\alpha$  agents had started. Correlations were calculated by Spearman's correlation coefficient (r).

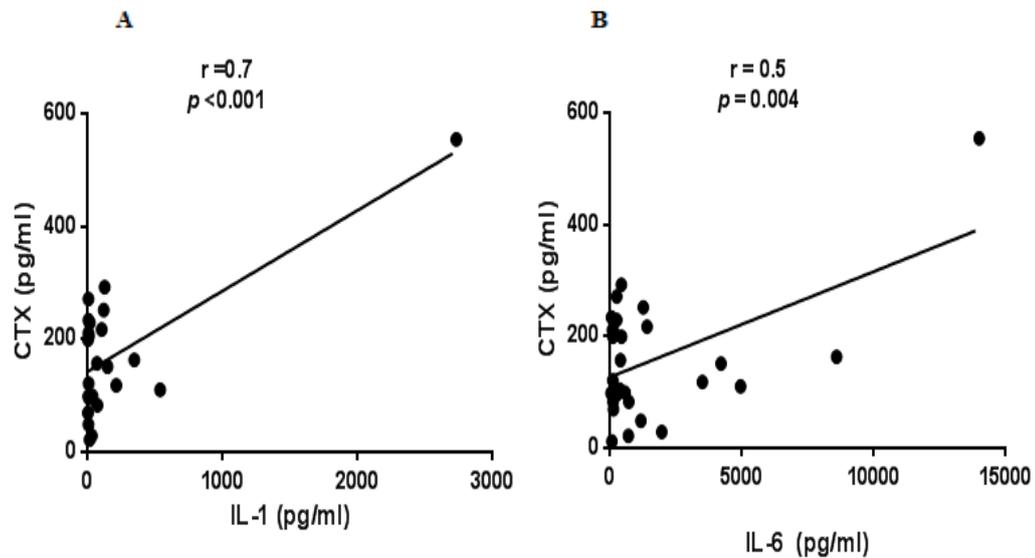


**Figure 4.41: Relationship between changes in plasma level of OPG and changes in IL-1 and TNF $\alpha$  after 3 months of treatment with biologic anti-TNF $\alpha$  agents.** Plasma levels of OPG were determined by ELISA. Levels of IL-1 and TNF $\alpha$  in culture supernatants of enriched and *ex vivo* activated T-lymphocytes were determined using MSD kits. Correlations were by Spearman's correlation coefficient (r).

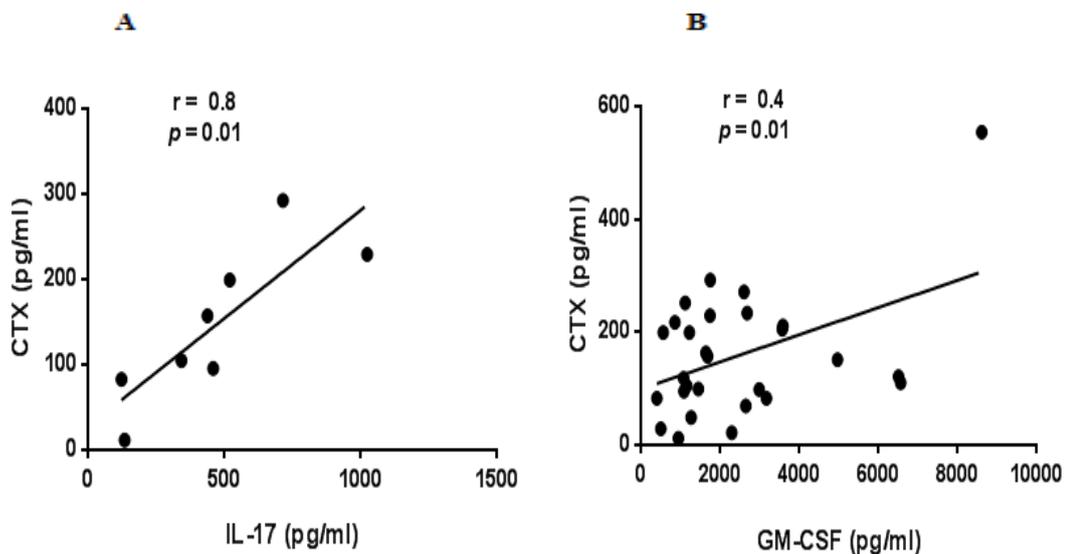


**Figure 4.42: Relationship between changes in plasma level of OPG and changes in IL-17 and GM-CSF after 3 months of treatment with biologic anti-TNF $\alpha$  agents.** Plasma levels of OPG were determined by ELISA. Levels of IL-17 and GM-CSF in culture supernatants of enriched and *ex vivo* activated T-lymphocytes were determined using MSD kits. Correlations were by Spearman's correlation coefficient ( $r$ ).

There were significant correlations between plasma level of CTX and the level of IL-1, IL-6, IL-17 and GM-CSF ( $r=0.7$ ,  $p<0.001$ ;  $r=0.5$ ,  $p=0.004$ ;  $r=0.8$ ,  $p=0.01$ ; and  $r=0.4$ ,  $p=0.01$ , respectively). These correlations again support the existent of a relationship between the potential of T-lymphocytes in RA patients to produce pro-inflammatory cytokines and bone loss (Figures 4.43 and 4.44).

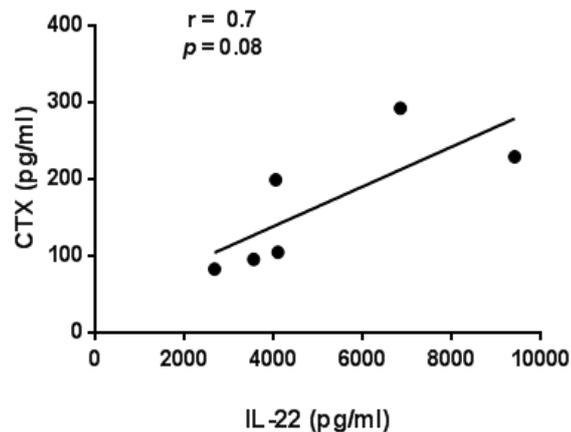


**Figure 4.43: Relationship between plasma levels of CTX with IL-1 and IL-6 in culture supernatants of *ex vivo* activated T-lymphocytes from RA patients.** Levels of IL-1 (A) and IL-6 (B) were determined in culture supernatants of enriched and activated T-lymphocytes from RA patients before the start of treatment with biologic anti-TNF $\alpha$  agents using multiplex MSD kits. Plasma levels of CTX were determined by ELISA. Correlations were determined by Spearman's correlation coefficient ( $r$ ).



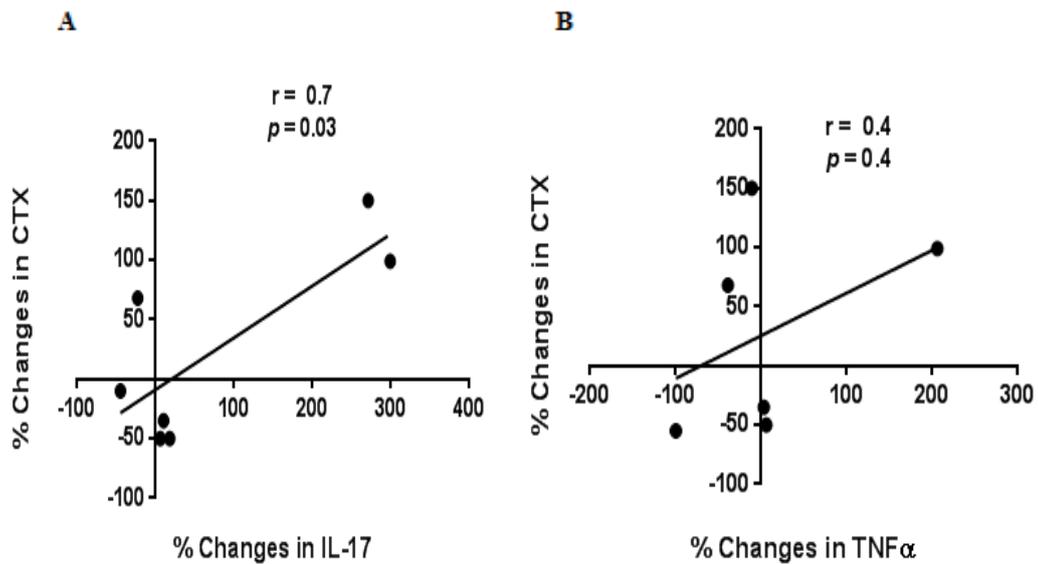
**Figure 4.44: Relationship between plasma levels of CTX with IL-17 and GM-CSF in culture supernatants of *ex vivo* activated T-lymphocytes from RA patients.** Levels of IL-17 (A) and GM-CSF (B) were determined in culture supernatants of enriched and activated T-lymphocytes using multiplex MSD kits. Correlations were determined as described in the legend to the previous figures.

The analyses also revealed a positive correlation between plasma levels of CTX and IL-22 in the culture supernatants of the enriched and activated T-lymphocytes from the patients before the start of treatment. This correlation, however, was not statistically significant ( $r=0.7$  and  $p=0.08$ ) (Figure 4.45).



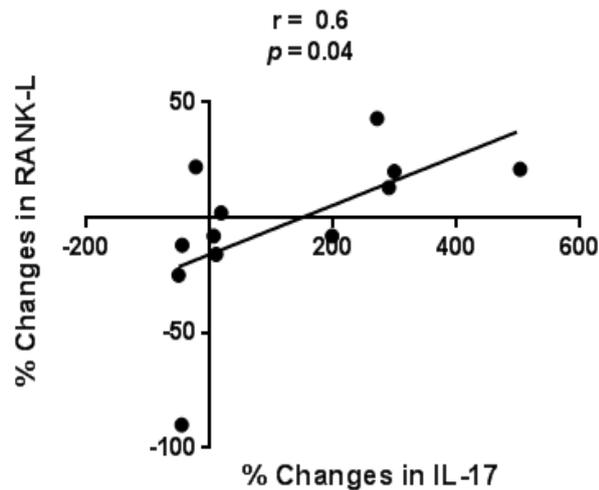
**Figure 4.45: Relationship between plasma levels of CTX with IL-22 in culture supernatants of *ex vivo* activated T-lymphocytes from RA patients.** Levels of IL-22 in the culture supernatants were determined using ELISA.

In addition to the direct relationship between the level of pro-inflammatory cytokines and biomarker of bone resorption before treatment, there was also a strong positive relationship between changes in the plasma levels of CTX with changes in the levels of IL-17 and TNF $\alpha$  produced by activated *ex vivo* T-lymphocytes in culture after 3 months of treatment at the same time point,  $r=0.7$ ,  $p=0.03$  and  $r=0.4$ ,  $p=0.4$ , respectively (Figure 4.46).



**Figure 4.46: Relationships between changes plasma levels of CTX with changes in levels of IL-17 (A) and TNFα (B) produced by enriched and activated T-lymphocytes obtained from RA patients after 3 months of treatment with biologic anti-TNFα agents.** The level of CTX in plasma was determined by ELISA. The level of IL-17 and TNFα in culture supernatant was by MSD. Correlations were by Spearman's correlation coefficient (r).

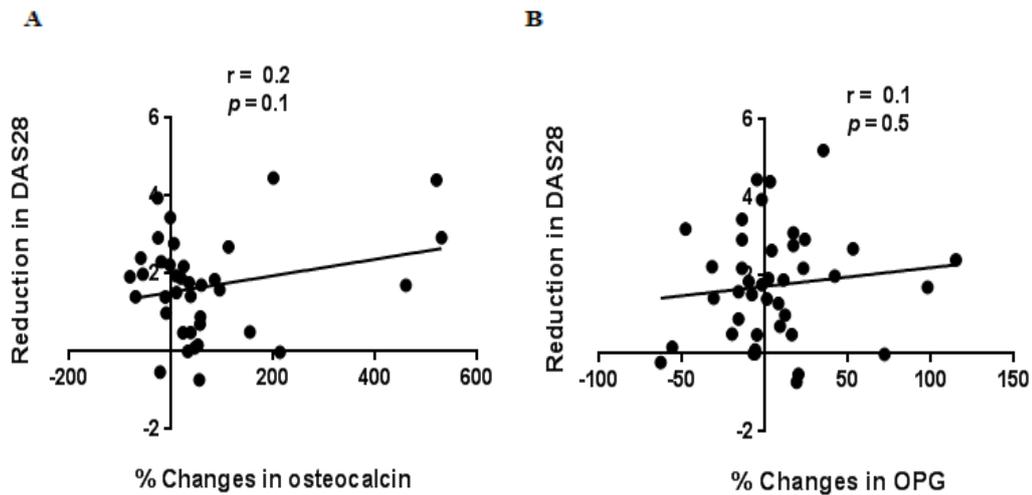
The analyses also revealed a significant positive correlation between plasma levels of RANK-L and IL-17 in the culture supernatants of the enriched and activated T-lymphocytes from the patients after 3 months of treatment,  $r=0.6$  and  $p=0.04$  (Figure 4.47).



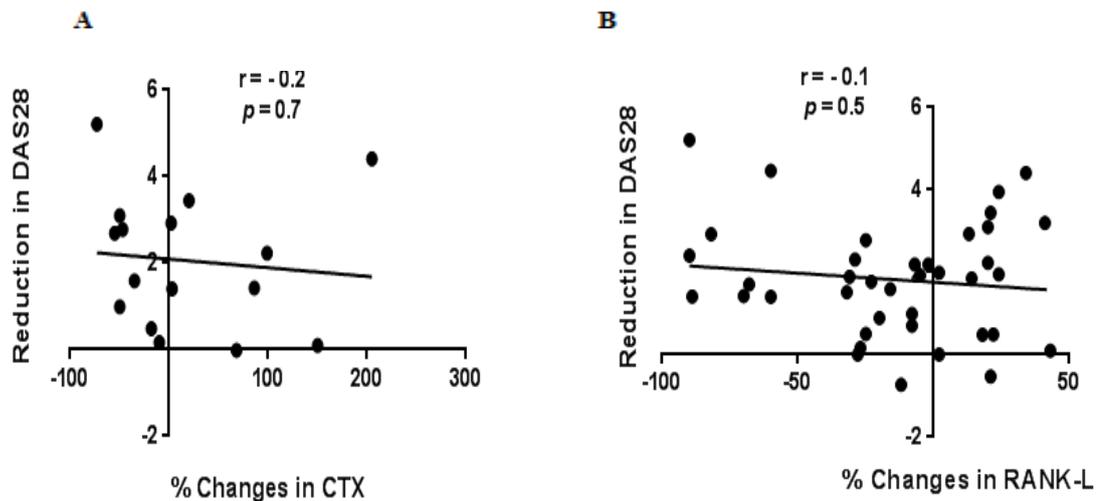
**Figure 4.47: Relationships between changes plasma levels of RANK-L with changes in levels of IL-17 produced by enriched and activated T-lymphocytes obtained from RA patients after 3 months of treatment with biologic anti-TNF $\alpha$  agents.** The level of RANK-L in plasma was determined by ELISA. The level of IL-17 in culture supernatant was by MSD. Correlations were by Spearman's correlation coefficient (r).

#### **4.3.8 The relationship between the clinical response of patients with RA to biologic anti-TNF $\alpha$ agents and their bone response to the treatment.**

The clinical response of patients to treatment with biologic anti-TNF $\alpha$  agents as measured in DAS28 at 3 months after treatment was correlated with bone turnover biomarkers. The analyses revealed a trend for positive correlation between increased levels of osteocalcin and OPG with reduction in DAS28 (initial DAS28 - DAS28 at 3 months post treatment) after 3 months of treatment ( $r=0.2$ ,  $p=0.1$ ; and  $r=0.1$ ,  $p=0.5$ ), respectively, (Figure 4.48). In contrast, a trend for negative correlation between increased levels of CTX and RANK-L with reduction in DAS28 after 3 months of treatment ( $r=-0.2$ ,  $p=0.7$ ; and  $r=-0.1$ ,  $p=0.5$ ), respectively, (Figure 4.49).



**Figure 4.48: The relationships between changes in the levels of osteocalcin and OPG with reduction in DAS28 after treatment with biologic anti-TNF $\alpha$  agents.** The figure depicts the relationship between reduction in disease activity measured in DAS28 and levels of osteocalcin and OPG after 3 months of treatment with biologic anti-TNF $\alpha$  agents. Osteocalcin and OPG levels were determined by ELISA. The correlations were by Spearman's correlation coefficient (r).



**Figure 4.49: The relationships between changes in the levels of CTX and RANK-L with reduction in DAS28 after treatment with biologic anti-TNF $\alpha$  agents.** The figure depicts the relationship between reduction in disease activity measured in DAS28 and levels of CTX and RANK-L after 3 months of treatment with biologic anti-TNF $\alpha$  agents. CTX and RANK-L levels were determined by ELISA. The correlations were by Spearman's correlation coefficient (r).

The effect of age, gender and ethnic origin on improvement in the level of bone turnover biomarkers in the treated RA patients was also investigated. For that reason, patients were divided into responder and non-responders to treatment with biologic anti-TNF $\alpha$  agents based on changes in their DAS28. These patients were then divided into subgroups according to gender, age groups and ethnic origin. Variations in the level of the bone turnover biomarkers among the different groups were then analysed using contingency tables.

**a) The impact of gender, age and ethnic origins on changes in the level of osteocalcin following treatment of RA patients with biologic anti-TNF $\alpha$  agents.**

Patients were assessed for the effect of gender on changes in the level of osteocalcin after treatment. No significant differences in the level of osteocalcin before treatment were observed between the male and female patients. Thus, mean osteocalcin ( $\pm$ SD) levels in male and female patients were  $13\pm 9$ ng/ml and  $15\pm 11$ ng/ml, respectively. The mean increase in osteocalcin level in male patients was greater than in female patients after 1 and 3 months of treatment, 2ng/ml (15%) and 6ng/ml (46%) ( $p=0.1$  and  $p=0.06$ ) vs. 1ng/ml (6.6%) and 1ng/ml (6.6%) ( $p=0.2$  and  $p=0.5$ ), respectively. The plasma level of osteocalcin was improved in 9 of 11 responder males (82%) compared with 14 of 27 responder female patients (52%) ( $p=0.1$ ). Further, changes in the level of osteocalcin in non-responder patients was compared between females ( $n=11$ ) and males ( $n=2$ ). Osteocalcin level increased in the plasma of all male patients and in 8 (73%) female patients ( $p=1.0$ ) (Table 4.17).

To study the effect of age on changes in the level of osteocalcin following treatment, levels of osteocalcin was assessed by determining increases in its level in 12  $\leq 50$  year old patients and 39  $> 50$  year old patients. Plasma levels of osteocalcin before treatment were  $12\pm 9$ ng/ml in  $\leq 50$  year old patients compared with  $14\pm 11$ ng/ml in  $> 50$  year old patients. The mean levels of osteocalcin increased in both groups of patients after 1 and 3 months of treatment with biologic anti-TNF $\alpha$  agents, however, these differences did not reached the

statistically significant levels. Thus, the mean difference and percentage of increase in osteocalcin levels after 1 and 3 months of treatment were 1ng/ml (8%) and 1ng/ml (8%) ( $p=0.5$  and  $p=0.6$ ) in  $\leq 50$  year old patients and 1ng/ml (7%) and 2ng/ml (14%) ( $p=0.1$  and  $p=0.07$ ) in  $>50$  year old patients.

No significant differences were observed in changes in the level of osteocalcin between the two subgroups of patients. The level of osteocalcin increased in 5 of 8  $\leq 50$  year age responder patients (63%) and 18 of 30  $>50$  year responder patients (60%) ( $p=1.0$ ). In non-responder patients, the level of osteocalcin increased after treatment with the biologic anti-TNF $\alpha$  agents in 2 of 4  $\leq 50$  year old non-responder patients (50%) and in 8 of 9  $>50$  year old patients (89%) ( $p=0.2$ ) (Table 4.17).

Changes in the level of osteocalcin after treatment with biologic anti-TNF $\alpha$  agents in patients from different ethnic groups were also investigated. Plasma levels of osteocalcin before treatment were  $12\pm 8$ ng/ml in Caucasian patients compared with  $13\pm 9$ ng/ml in Asian patients. The increase in osteocalcin level in Caucasian patients was greater than in Asian patients after 1 and 3 months of treatment, 3ng/ml (25%) and 3ng/ml (25%) ( $p=0.01$  and  $p=0.02$ ) vs. 1ng/ml (7%) and 1ng/ml (7%) ( $p=0.3$  and  $p=0.07$ ), respectively. In responder patients, the osteocalcin level increased in the plasma of 13 of 17 Caucasian patients (76%) and 8 of 12 Asian patients (67%) ( $p=0.7$ ). In non-responder patients, osteocalcin was increased in the plasma of all 5 Caucasian patients and in the plasma of 3 of 6 Asian patients (50%) (Table 4.17).

**Table 4.17: Changes in the level of osteocalcin in RA patients treated with biologic anti-TNF $\alpha$  agents.**

| <b>Parameters for patients classification</b> | <b>Increased osteocalcin</b> | <b>No change in osteocalcin levels</b> | <b>Total of patients studied</b> |
|---|------------------------------|--|----------------------------------|
| <b>Gender</b>                                 |                              |  |                                  |
| ▪ <b>Responder</b>                            |                              |  |                                  |
| - Male n (%)                                  | 9 (82)                       | 2 (18)                                 | 11                               |
| - Female n (%)                                | 14 (52)                      | 13 (48)                                | 27                               |
| ▪ <b>Non responder</b>                        |                              |  |                                  |
| - Male n (%)                                  | 2 (100)                      | 0 (0)                                  | 2                                |
| - Female n (%)                                | 8 (73)                       | 3 (27)                                 | 11                               |
| <b>Age group</b>                              |                              |  |                                  |
| ▪ <b>Responder</b>                            |                              |  |                                  |
| - ≤50 years old n (%)                         | 5 (63)                       | 3 (47)                                 | 8                                |
| - >50 years old n (%)                         | 18 (60)                      | 12 (40)                                | 30                               |
| ▪ <b>Non responder</b>                        |                              |  |                                  |
| - ≤50 years old n (%)                         | 2 (50)                       | 2 (50)                                 | 4                                |
| - >50 years old n (%)                         | 8 (89)                       | 1 (11)                                 | 9                                |
| <b>Ethnic origin*</b>                         |                              |  |                                  |
| ▪ <b>Responder</b>                            |                              |  |                                  |
| - Caucasians n (%)                            | 13 (76)                      | 4 (24)                                 | 17                               |
| - Asians n (%)                                | 8 (67)                       | 4 (33)                                 | 12                               |
| ▪ <b>Non responder</b>                        |                              |  |                                  |
| - Caucasians n (%)                            | 5 (100)                      | 0 (0)                                  | 5                                |
| - Asians n (%)                                | 3 (50)                       | 3 (50)                                 | 6                                |

RA patients treated with biologic anti-TNF $\alpha$  agents were divided into responder and non-responder patients based on changes in their DAS28. The patients from the two groups were further subdivided into subgroups according to gender, age and ethnic origin. Changes in osteocalcin levels were defined as having an increase in its level after treatment compared with its level before treatment. No change in the level of osteocalcin was defined as having level similar to or reduced after treatment compared with its level before treatment. The differences in osteocalcin among patient groups were assessed by using contingency tables. Information on ancestral origin of 11 patients wasn't available.

**b) The effect of gender, age and ethnic origin on changes in the level of CTX following treatment of RA patients with biologic anti-TNF $\alpha$  agents.**

Patients were assessed for the effect of gender on changes in the level of CTX after treatment. No significant differences in the level of CTX before treatment were observed between the male and female patients. Thus, mean CTX ( $\pm$ SD) levels in male and female patients were 164 $\pm$ 166pg/ml and 158 $\pm$ 100pg/ml, respectively. The mean reduction in CTX levels in male patients was greater than in female patients after 1 and 3 months of treatment, 5pg/ml (4%) and 65pg/ml (40%) ( $p=0.4$  and  $p=0.5$ ) vs. 1pg/ml (0.6%) and 8pg/ml (4%) ( $p=0.3$  and  $p=0.6$ ), respectively. The level of CTX decreased in the plasma of 5 of 9 male patients (56%) and 11 of 19 female patients (58%) within the responder group ( $p=1.0$ ). In non-responder patients, plasma levels of CTX did not change in both male patients but decreased in the plasma of 4 of the 7 (57%) female patients studied (Table 4.18).

The effect of age on change in plasma levels of CTX was assessed by determining reduction in its level in 7  $\leq$ 50 year old patients and 30  $>$ 50 year old patients. Plasma levels of CTX before treatment were 169 $\pm$ 99pg/ml in  $\leq$ 50 year old patients compared with 158 $\pm$ 131pg/ml in  $>$ 50 year old patients. The mean levels of CTX decreased in both groups of patients after 1 and 3 months of treatment with biologic anti-TNF $\alpha$  agents, however, these differences did not reached the statistically significant levels. Thus, the mean difference and percentage of reduction in CTX levels after 1 and 3 months of treatment were 21pg/ml (12%) and 26pg/ml (15%) in  $\leq$ 50 year old patients and 2pg/ml (1.3%) and 29pg/ml (18%) in  $>$ 50 year old patients. The level of CTX decreased in the plasma of 3 of 4  $\leq$ 50 year old responder patients (75%) compared to 13 of 24  $>$ 50 year old responder patients (54%) ( $p=0.6$ ). CTX was detectable in the plasma of 3  $\leq$ 50 year old and 6  $>$ 50 year old non-responder patients. The analysis revealed a higher level of reduction in plasma levels of CTX in the  $\leq$ 50 year patients compared with the  $>$ 50 old ( $p=0.04$ ) (Table 4.18).

Changes in the level of CTX in patients from different ethnic origins were also assessed. No differences were observed in the level of CTX before treatment between Caucasian and Asian patients. Thus, the mean plasma levels of CTX before treatment were  $159\pm 134$ pg/ml in Caucasian patients compared with  $161\pm 91$ pg/ml in Asian patients. The reduction in CTX levels in Asian patients were greater than Caucasian patients after 1 and 3 months of treatment, 1pg/ml (0.6%) and 63pg/ml (39%) ( $p=0.8$  and  $p=0.01$ ) vs. 1pg/ml (0.6%) and 4pg/ml (3%) ( $p=0.9$  and  $p=0.2$ ), respectively. The plasma levels of CTX decreased in 7 of 12 Caucasian responder patients (58%) and 8 of 10 Asian responder patients (80%) ( $p=0.4$ ). In the non-responder group, the level of CTX did not decrease in the plasma of 3 Caucasian patients but decreased in plasma of 4 of 5 Asians patients (80%) ( $p=0.1$ ) (Table 4.18).

**Table 4.18: Changes in the level of CTX in RA patients treated with biologic anti-TNF $\alpha$  agents.**

| <b>Parameters for patients classification</b> | <b>Decreased CTX levels</b> | <b>No change in CTX levels</b> | <b>Total of patients studied</b> |
|---|-----------------------------|--------------------------------|----------------------------------|
| <b>Gender</b>                                 |                             |                                |                                  |
| ▪ <b>Responder</b>                            |                             |                                |                                  |
| - Male n (%)                                  | 5 (56)                      | 4 (44)                         | 9                                |
| - Female n (%)                                | 11 (58)                     | 8 (42)                         | 19                               |
| ▪ <b>Non responder</b>                        |                             |                                |                                  |
| - Male n (%)                                  | 0 (0)                       | 2 (100)                        | 2                                |
| - Female n (%)                                | 4 (57)                      | 3 (43)                         | 7                                |
| <b>Age group</b>                              |                             |                                |                                  |
| ▪ <b>Responder</b>                            |                             |                                |                                  |
| - ≤50 year old n (%)                          | 3 (75)                      | 1 (25)                         | 4                                |
| - >50 year old n (%)                          | 13 (54)                     | 11 (46)                        | 24                               |
| ▪ <b>Non responder</b>                        |                             |                                |                                  |
| - ≤ 50 year old n (%)                         | 3 (100)                     | 0 (0)                          | 3                                |
| - >50 year old n (%)                          | 1 (17)                      | 5 (83)                         | 6                                |
| <b>Ethnic origin*</b>                         |                             |                                |                                  |
| ▪ <b>Responder</b>                            |                             |                                |                                  |
| - Caucasians n (%)                            | 7 (58)                      | 5 (42)                         | 12                               |
| - Asians n (%)                                | 8 (80)                      | 2 (20)                         | 10                               |
| ▪ <b>Non responder</b>                        |                             |                                |                                  |
| - Caucasians n (%)                            | 0 (0)                       | 3 (100)                        | 3                                |
| - Asians n (%)                                | 4 (80)                      | 1 (20)                         | 5                                |

Patients were divided into responder and non-responders described in the legend to Table 4.17. Reduction the level of CTX was based on lower levels after treatment compared with before treatment. Significant differences in reductions in the level of CTX among patient groups were assessed by using contingency tables. Information on the ethnic origin of 7 patients was not available.

**c) The effect of gender, age and ethnic origin on changes in plasma levels of RANK-L in RA patients treated with biologic anti-TNF $\alpha$  agents.**

Patients were separately assessed for the effect of gender on changes in the level of RANK-L after treatment. No significant differences in the level of RANK-L were observed between the male and female patients before treatment. Thus, mean RANK-L ( $\pm$ SD) levels in male and female patients were  $161\pm 136$ pg/ml and  $178\pm 171$ pg/ml, respectively. The mean reduction in RANK-L levels in female patients was greater than in male patients after 1 and 3 months of treatment, 32pg/ml (18%) and 30pg/ml (17%) ( $p=0.03$  and  $p=0.8$ ) vs. 4pg/ml (2%) and 5pg/ml (4%) ( $p=0.3$  and  $p=0.6$ ), respectively. In the responder group, RANK-L level decreased after treatment in the plasma of 5 of 11 male patients (45%) and in 17 of 28 females (61%) ( $p=0.5$ ). In the non-responder group the level of RANK-L did not decrease in the plasma of 2 male patients but decreased in plasma of 7 of 9 female patients ( $p=0.1$ ) (Table 4.19).

The effect of age on change in plasma levels of RANK-L was assessed by determining reduction in its level in 13  $\leq 50$  year old patients and 37  $> 50$  year old patients. Plasma levels of RANK-L before treatment were  $142\pm 107$ pg/ml in  $\leq 50$  year old patients compared with  $162\pm 128$ pg/ml in  $> 50$  year old patients. The mean levels of RANK-L decreased in both groups of patients after 1 and 3 months of treatment with biologic anti-TNF $\alpha$  agents, however, these differences did not reached the statistically significant levels. Thus, the mean difference and percentage of reduction in RANK-L levels after 1 and 3 months of treatment were 10pg/ml (7%) and 17pg/ml (12%) in  $\leq 50$  year old patients and 6pg/ml (4%) and 11pg/ml (8%) in  $> 50$  year old patients. The level of RANK-L in plasma of responder patients decreased in 6 of 9  $\leq 50$  year old group patients (67%) and in 16 of 30  $> 50$  year old patients (53%) ( $p=0.7$ ) (Table 4.19). In non-responder patients, the level of RANK-L

decreased in the plasma of 3 of 4  $\leq 50$  year old patients (75%) and in 4 of 7  $> 50$  year old patients (57%) but this difference was not statistically significant ( $p=1.0$ ) (Table 4.19).

Changes in the level of RANK-L after treatment in relation to ethnic origin of the patients were also assessed. Plasma levels of RANK-L before treatment were  $165 \pm 169$  pg/ml in Caucasian patients compared with  $134 \pm 111$  pg/ml in Asian patients. The mean difference and percentage of reduction in RANK-L levels after 1 and 3 months of treatment were 6 pg/ml (4%) and 7 pg/ml (4%) in Caucasian patients and 10 pg/ml (7%) and 12 pg/ml (9%) in Asian patients. The level of RANK-L decreased in the plasma of 8 of 18 Caucasian responder patients (44%) and in 6 of 11 Asian responder patients (55%) ( $p=0.7$ ). In the non-responder patients, the level of RANK-L decreased in the plasma of 2 of 3 Caucasian non-responder patients (67%) and in 4 of 6 Asian non-responders (67%) ( $p=1.0$ ) (Table 4.19).

**Table 4.19: Changes in the level of RANK-L after treatment of RA patients with biologic anti-TNF $\alpha$  agents.**

| <b>Parameters for patients classification</b> | <b>Decreased RANK-L levels</b> | <b>No change in RANK-L levels</b> | <b>Total</b> |
|---|--------------------------------|-----------------------------------|--------------|
| <b>Gender</b>                                 |                                |                                   |              |
| ▪ <b>Responder</b>                            |                                |                                   |              |
| - Male n (%)                                  | 5 (45)                         | 6 (55)                            | 11           |
| - Female n (%)                                | 17 (61)                        | 11 (39)                           | 28           |
| ▪ <b>Non responder</b>                        |                                |                                   |              |
| - Male n (%)                                  | 0 (0)                          | 2 (100)                           | 2            |
| - Female n (%)                                | 7 (78)                         | 2 (22)                            | 9            |
| <b>Age group</b>                              |                                |                                   |              |
| ▪ <b>Responder</b>                            |                                |                                   |              |
| - $\leq$ 50 years old n (%)                   | 6 (67)                         | 3 (33)                            | 9            |
| - >50 years old n (%)                         | 16 (53)                        | 14 (47)                           | 30           |
| ▪ <b>Non responder</b>                        |                                |                                   |              |
| - $\leq$ 50 years old n (%)                   | 3 (75)                         | 1 (25)                            | 4            |
| - >50 years old n (%)                         | 4 (57)                         | 3 (43)                            | 7            |
| <b>Ancestral origin*</b>                      |                                |                                   |              |
| ▪ <b>Responder</b>                            |                                |                                   |              |
| - Caucasians n (%)                            | 8 (44)                         | 10 (56)                           | 18           |
| - Asians n (%)                                | 6 (55)                         | 5 (45)                            | 11           |
| ▪ <b>Non responder</b>                        |                                |                                   |              |
| - Caucasians n (%)                            | 2 (67)                         | 1 (33)                            | 3            |
| - Asians n (%)                                | 4 (67)                         | 2 (33)                            | 6            |

Patients were divided into responder and non-responders as described above. Improvement in RANK-L was defined as having RANK-L decrease after treatment. Non-improved RANK-L was defined as having RANK-L remaining the same or increased after treatment. The differences in RANK-L among patient groups were assessed by using contingency tables. Information on ancestral origin of 12 patients wasn't available.

**d) The effect of gender, age and ethnic origin on changes in plasma level of OPG after treatment of RA patients with biologic anti-TNF $\alpha$  agents.**

Patients were separately assessed for the effect of gender on changes in the level of OPG after treatment. The plasma level of OPG in female patients was greater than in male patients before treatment. Thus, mean OPG ( $\pm$ SD) levels in female and male patients were  $3.1\pm 1.4$ ng/ml and  $2.4\pm 1.7$ ng/ml, respectively. There were higher increases in the level of OPG in male patients compared with female patients after 1 and 3 months of treatment. However, the difference did not reach statistically significant levels. The increase and percentage of changes in OPG level in male patients after 1 and 3 months of treatment were  $0.1$ ng/ml (4%) and  $5$ ng/ml (21%) ( $p=0.6$  and  $p=0.2$ ), respectively. In contrast, OPG level in female patients decreased after 1 and 3 months of treatment by  $0.2$ ng/ml (-6%) and  $0.1$ ng/ml (-3%) ( $p=0.2$  and  $p=0.9$ ), respectively. In responder group the level of OPG increased after treatment in 7 of 11 male patients (64%) and in 13 of 28 female patients (46%) ( $p=0.7$ ). In non-responder patients, the level of OPG increased more in male patients compared with the female patients. Thus, the level of OPG increased in the plasma of all male patients and in 7 of the 11 female patients (64%) (Table 4.20).

Analysis of the level of OPG in plasma in relation to age of patients was carried out in 40 >50 year old patients and 12  $\leq$ 50 year old patients. The plasma level of OPG in >50 year old patients was greater than in  $\leq$ 50 year old patients before treatment. Thus, mean OPG ( $\pm$ SD) levels in >50 year old patients and  $\leq$ 50 year old patients were  $3\pm 1.6$ ng/ml and  $2.4\pm 0.9$ ng/ml, respectively. There were reductions in plasma levels of OPG in the  $\leq$ 50 year old patients after 1 and 3 months of treatment to  $2.1\pm 0.6$ ng/ml ( $p=0.2$ ) and  $2.2\pm 0.8$  ( $p=0.3$ ), respectively. The plasma level of OPG in >50 year old patients did not change after 1 and 3 months of treatment. In the responder patients, the level of OPG increased after treatment in the plasma of 17 >50 year old patients (55%) and 3  $\leq$ 50 year old patients (38%), but this difference was not statistically significant ( $p=0.5$ ). In the non-responder patients, the levels of

OPG increased in 3 of 4  $\leq 50$  year olds (73%) compared with 6 of 9  $> 50$  year olds patients (67%) ( $p=1.0$ ) (Table 4.20).

Changes in the level of OPG in relation to ethnic origin was analysed in 23 Caucasian and 17 Asian patients. There were no significant differences in the level of OPG before and after treatment between the two ethnic groups. Thus, mean OPG ( $\pm$ SD) levels before treatment in Caucasian and Asian patients were  $2.6\pm 1.4$ ng/ml and  $2.7\pm 1.5$ ng/ml, respectively. The plasma levels of OPG after 1 and 3 months of treatment were  $2.4\pm 1.2$ ng/ml and  $2.8\pm 1.6$ ng/ml, respectively ( $p=0.1$  and  $p=0.2$ ) in Caucasian patients and  $2.7\pm 1.7$ ng/ml and  $2.5\pm 1.4$ ng/ml, respectively ( $p=0.9$  and  $p=0.8$ ) in Asian patients. The level of OPG increased in the plasma of 11 of 18 responder Caucasian patients (61%) and in the plasma of 5 of 11 responder Asian patients (42%) ( $p=0.5$ ). In non-responder patients, the level of OPG increased after treatment in the plasma of 2 of 5 (40%) treated Caucasian patients and in 4 out of 6 (67%) Asian patients ( $p=0.6$ ) (Table 4.20).

**Table 4.20: Changes in the level of OPG after treatment of RA patients with biologic anti-TNF $\alpha$  agents.**

| <b>Parameters of patients classification</b> | <b>Increased OPG levels</b> | <b>No change in OPG levels</b> | <b>Total</b> |
|--|-----------------------------|--------------------------------|--------------|
| <b>Gender</b>                                |                             |                                |              |
| ▪ <b>Responder</b>                           |                             |                                |              |
| - Male      n (%)                            | 7 (64)                      | 4 (36)                         | 11           |
| - Female    n (%)                            | 13 (46)                     | 15 (54)                        | 28           |
| ▪ <b>Non responder</b>                       |                             |                                |              |
| - Male      n (%)                            | 2 (100)                     | 0 (0)                          | 2            |
| - Female    n (%)                            | 7 (64)                      | 4 (36)                         | 11           |
| <b>Age group</b>                             |                             |                                |              |
| ▪ <b>Responder</b>                           |                             |                                |              |
| - $\leq$ 50 year old   n (%)                 | 3 (38)                      | 5 (62)                         | 8            |
| - $>$ 50 year old    n (%)                   | 17 (55)                     | 14 (45)                        | 31           |
| ▪ <b>Non responder</b>                       |                             |                                |              |
| - $\leq$ 50 year old   n (%)                 | 3 (75)                      | 1 (25)                         | 4            |
| - $>$ 50 year old    n (%)                   | 6 (67)                      | 3 (33)                         | 9            |
| <b>Ancestral origin*</b>                     |                             |                                |              |
| ▪ <b>Responder</b>                           |                             |                                |              |
| - Caucasians n (%)                           | 11 (61)                     | 7 (39)                         | 18           |
| - Asians     n (%)                           | 5 (42)                      | 6 (58)                         | 11           |
| ▪ <b>Non responder</b>                       |                             |                                |              |
| - Caucasians n (%)                           | 2 (40)                      | 3 (60)                         | 5            |
| - Asians     n (%)                           | 4 (67)                      | 2 (33)                         | 6            |

Patients were divided into responder and non-responders as described above. Improvement in plasma levels of OPG was defined as having OPG increased after treatment compared with before treatment. No change in OPG was defined as having OPG remaining relatively the same or decreasing in the patients after treatment compared with before treatment. The differences in OPG among patient groups were assessed by using contingency tables. Information on the ethnic origin of 12 patients was not available.

#### 4.4 Discussion

Key findings from studies reported in this chapter are that treatment of RA patients with biologic anti-TNF $\alpha$  agents reduces generalised bone loss and alters the balance of bone turnover in favour of new bone formation as evidenced by increased BMD at lumbar spine and total hip, and by improvement in plasma levels of a range of bone biomarkers. In addition, the study provides preliminary evidence for decreased localised bone loss in the hand of patients with RA treated with the biologic anti-TNF $\alpha$  agents.

Previous studies in this field have shown that generalised bone loss in patients with RA usually starts as a result of disease activity and the resulting immobilisation of the patients (35,185). Bone loss of up to 0.7% at the spine and 1.1% at the hip after 1 year of disease have been reported in historical control group studies of patients with RA when treated with conventional DMARDs (186,187). Furthermore, in early RA, generalised annual bone loss is even greater than the values cited above at 4.2% at the spine and 2.1% at the hip (189). Comparing the results of the present study with those of the earlier historical control group studies of patients with RA clearly indicates that bone loss is far less in patients treated with biologic anti-TNF $\alpha$  agents compared with patients treated with other agents. In addition to the effect of biologic anti-TNF $\alpha$  agents on BMD in the spine and the hip, the current study provides preliminary evidence for improvement in localised BMD of the hand as measured by DXR. Thus, the results show that no reduction in hand BMD was seen after six months of treatment with biologic anti-TNF $\alpha$  agents. Since there are no DXR data on hand BMD in patients with RA treated with DMARDs, it is unclear how this effect by biologic anti-TNF $\alpha$  agents on localised bone loss in the hand compares with the effect of DMARDs. A double-blind randomised controlled trial revealed that the rate of hand bone loss measured by DXR was significantly reduced in patients with RA treated with prednisolone compared with patients treated with placebo (190). However, this improvement decreased considerably over time (190). In contrast to our findings, a group of investigators reported a decrease in hip and

hand BMD of RA patients treated with adalimumab (124). However, these investigators measured hand BMD after one year of treatment and 22% of their patients were treated with bisphosphonates, while in this study, hand BMD was measured six months after treatment had started and patients receiving bisphosphonates were excluded from the study.

As expected, the bone resorption biomarker, CTX decreased in responder patients after 3 months of treatment compared with its levels before treatment. In contrast, non-responder patients showed an increase in the level of CTX after 3 months of treatment compared with before treatment. This supports the view that treatment with biologic anti-TNF $\alpha$  agents reduces disease activity and thereby suppresses bone loss. However, there was no significant association between changes in disease activity and CTX levels. Reduction of CTX in patients treated with biologic anti-TNF $\alpha$  agents was reported by other investigators (124,136,138) indicating That treatment with biologic anti-TNF $\alpha$  has potential to inhibit generalised bone loss in patients with RA in addition to its clinical efficacy in reducing disease activity.

Measurement of osteocalcin levels, in contrast, revealed significant increases 3 months after treatment compared with before treatment in non-responder patients but not after one month. This data can be explained by an initial inhibitory effect of TNF $\alpha$  on osteoblast metabolic function (191). Levels of osteocalcin also increased in responder patients. However, the differences between the two response groups did not reach statistical significance. The effect of biologic anti-TNF $\alpha$  agents after 1 year of treatment was reported in other studies (124,131-134). These studies reported persistent increases in osteocalcin levels, which are consistent with the results presented in this study.

The osteoclast-activating RANK-L decreased significantly after three months of treatment in responder patients compared with its levels before treatment ( $197\pm 137$ pg/ml vs.  $170\pm 100$ pg/ml;  $p=0.047$ ). However, there was an increase in the level of RANK-L in non-responder patients although this was not statistically significant ( $186\pm 90$ pg/ml vs.

192±110pg/ml;  $p=0.8$ ). The decrease in plasma levels of RANK-L in responder patients, which was paralleled by an increase in OPG levels, and the tendency for increased RANK-L in non-responders suggests that RANK-L production is regulated by TNF $\alpha$  in RA patients (192,193). In this respect, it seems possible that the production of RANK-L is induced through several pathways one of which involves TNF $\alpha$ . In RA patients in whom TNF $\alpha$  is not the prime inducer of RANK-L, treatment with biologic anti-TNF $\alpha$  agents do not induce remission nor reduce RANK-L levels since inflammatory pathways other than TNF $\alpha$  drive disease and RANK-L. This proposition is based on the observation that non-responder patients have a preponderance of T-lymphocytes that produce IL-17 (184). Overall, changes in bone biomarkers during treatment with biologic anti-TNF $\alpha$  agents support the effect of this treatment on bone-protection.

Previous studies have indicated that pro-inflammatory cytokines other than TNF $\alpha$ , such as IL-1, IL-6, IL-17, IL-20 and IL-22 are involved in inhibiting of OPG production but promote RANK-L production with the net result being an obvious rise in osteoclasts formation and functions (144-147). The results reported in this chapter were consistent with these studies. Positive correlations were observed between changes in plasma levels of RANK-L and CTX with levels of TNF $\alpha$ , IL-20 and IL-22. In addition, GM-CSF and pro-inflammatory cytokines including IL-1, IL-6, IL-17 showed significant positive correlations with the level of CTX, ( $r=0.7$  ( $p<0.001$ ),  $r=0.5$  ( $p=0.004$ ),  $r=0.8$  ( $p=0.01$ ) and  $r=0.4$  ( $p=0.01$ ), respectively, indicating a strong relationship between inflammation and bone loss. In contrast, OPG levels increased with reductions in TNF $\alpha$ , IL-1, IL-17 and IL-22 levels. Negative correlations were also observed between changes in plasma levels of osteocalcin and IL-22. These observations can be explained as being a consequence of the involvement of pro-inflammatory cytokines in the suppression of OPG and promoting RANK-L upregulation as it was reported in earlier studies (144-147).

The relationship between inflammation and bone loss has been clearly established in numerous clinical and experimental models (144-147,180,194). Key pro-inflammatory cytokines including TNF $\alpha$ , IL-1, IL-6 and IL-17 as well as IL-20, IL-22 and GM-CSF have bone actions to induce generalised bone loss and subchondral bone resorption in inflammatory diseases such as RA. Results presented in this study are consistent with these studies. BMD values of the lumbar spine, hip and hand negatively correlated with increases in TNF $\alpha$ , IL-1, IL-6, IL-22 and GM-CSF, indicating that a good control of inflammation would prevent further bone loss.

Although many studies of the effect of biologic anti-TNF $\alpha$  agents on markers of bone turnover have been carried out (132-134,138), the impact of individual factors on the improvement of bone turnover biomarkers in RA patients have rarely been evaluated and most of these studies have focused on the relation between disease severity and low BMD. Lems and colleagues (195) measured variables associated with BMD in subgroups of patients and they found that low BMD improvement was associated with older age, longer duration of menopause and lower body mass in female patients. Similarly, findings in this study are consistent with results from an audit (retrospective study) cited at the beginning of this chapter and results from the analysis of changes in BMD among osteoporosis patients cited in chapter 3. Better improvement in both bone turnover biomarkers and BMD was observed among male patients compared with female patients, as well as younger ( $\leq 50$  years) compared with older patients ( $> 50$  years). However, the differences did not reach statistically significant levels due, probably, to the effect of sample size. Importantly, a beneficial effect was also observed in the apparently non-responder patients despite the effect of being female and older.

# **Chapter 5**

**The effect of biologic anti-TNF $\alpha$  agents on bone in patients with RA: direct effects on osteoclast precursor cell generation and differentiation**

## 5.1 Introduction

There is compelling evidence to indicate that local and systemic bone loss in RA result from osteoclast-mediated bone resorption mediated by increased production of TNF $\alpha$  (179,183,195). Furthermore, TNF $\alpha$  has been shown to act as key factor in the development and differentiation of osteoclast precursor cells (OCPs) in TNF $\alpha$ -transgenic mice (183). In addition, TNF $\alpha$  promotes osteoclast differentiation from progenitor cells in cooperation with RANK-L, an essential growth factor for osteoclast lineage cell generation (38,59,196). In support of these observations in humans, treatment of psoriatic arthritis patients with biologic anti-TNF $\alpha$  agents decreases the frequency of OCPs (197). Furthermore, these agents also suppress RANK expression on peripheral blood mononuclear cells (PBMNCs) and RANK-L on synovial fibroblasts in patients with RA in parallel with an increase in the number of OPGs (198). Indeed, there are studies showing that treatment of RA patients with biologic anti-TNF $\alpha$  agents reduces joint damage (199). The aim of this study was to determine the direct effect of blocking TNF $\alpha$  in patients with RA on the frequency of OCPs. The effect of treatment with biologic anti-TNF $\alpha$  agents on the frequency of *ex-vivo* blood OCPs cells and on the differentiation of blood monocytes/macrophages *in-vitro* were studied. The data were then correlated with the clinical response of patients with RA treated with the biologic anti-TNF $\alpha$  agents. In addition, the data on the direct effect of the agents on changes in bone biomarkers were determined. In order to determine the ability of biologic anti-TNF $\alpha$  agents on the production of other potential factors that promote OCP generation in blood, the ability of culture supernatants of PBMNCs from RA patients to promote the differentiation of monocytes from healthy control to OCPs after treatment was also determined. The effect of TNF $\alpha$  and RANK-L blockade *in vitro* on suppressing osteoclast differentiation was also studied.

## **5.2 Patients and Methods:**

Blood samples from 8 RA patients treated with biologic anti-TNF $\alpha$  agents were obtained for the study. The samples were collected just before treatment had commenced and at 1 and 3 months after treatment. The mean and SD of their ages were 54 $\pm$ 18 years with a range of 24-77 years old. All patients with severe disease and a mean  $\pm$ SD of DAS28 scores of 5.4 $\pm$ 0.9 at the time they were prescribed biologic anti-TNF $\alpha$  agents. All patients were prescribed the treatment after they have failed other disease-modifying anti rheumatic drugs one of which was always methotrexate. The patients included 7 females and 1 male. None of the patients were receiving bisphosphonates or hormone replacement therapy. The patients received combination therapy including biologic anti-TNF $\alpha$  with methotrexate (12.5mg/day); 6 received etanercept and 2 adalimumab. Three patients were taking calcium (1g/day) and vitamin D (800 IU/day). A total of 12 healthy controls were also recruited for the study, with the mean and SD of their age been 44 $\pm$ 12 years with a range of 27-62 years old. All healthy controls were males.

### **a) Enrichment and analyses of PBMNCs:**

Peripheral bloods were obtained by venous puncture into EDTA/heparin containing tubes. PBMNCs were obtained from 25ml blood by centrifugation on Ficoll-Paque, as described in the Materials and Methods' chapter. Plasma samples from the blood were obtained and stored until tested for the level of RANK-L, OPG, and bone turnover biomarkers including osteocalcin and CTX using adapted ELISA protocols. The number of PBMNCs was counted using an equal volume of 0.5% Trypan blue under an inverted light microscope. One million of the PBMNCs were then placed in a well of a 24-well culture plates with RPMI1640 medium containing 10% FCS and antibiotics.

The cultured cells were incubated in a humidified incubator at 37°C with 6% CO<sub>2</sub> in the air for 14 days. Culture media were replenished every 3 days and culture supernatants collected, filtered, and stored at -20°C until used to study the effects of TNF $\alpha$  inhibition on OCP differentiation. Cultured cells incubated with monocyte colony stimulating factor (M-CSF) at 25ng/ml and RANK-L (100ng/ml) served as positive controls (all from R&D).

After 14 days of culture, the cells were stained for tartrate-resistant acid phosphatase (Acid Phosphatase, Leukocyte (TRAP) Kit; Sigma-Aldrich, UK) and viewed and quantified by light microscopy. The number of TRAP-positive cells with three or more nuclei was counted as osteoclasts in cultures of monocytes from the RA patients and matched healthy controls.

**b) Fluorescence-activated cell sorting (FACS) analysis for measuring the frequency of OCPs before and after treatment of RA patients with biologic anti-TNF $\alpha$  agents**

Flow cytometry (or FACS) analysis was carried to calculate the frequency of OCPs before and after treatment of RA patients with the biologic anti-TNF $\alpha$  agents. For this purpose, the cells were washed and resuspended in cold PBS containing 4% FBS. Aliquots of  $0.1 \times 10^6$  cells were incubated with anti-human CD11b (clone ICRF44), anti-human CD14 (clone M5E2) (both from BD Pharmingen TM, UK) and antibody to RANK (R&D). The latter staining was carried out to determine the percentage of cells expressing RANK within the cell population because RANK is a key marker of OCPs. The cells were washed with 4% FBS-PBS and fixed using paraformaldehyde and stored at 4°C until analysed (within 24-48hrs). Data were acquired using a FACS Calibur flow cytometer and analysed by CellQuest software, version 3.1 (both from Becton Dickinson Immunocytometry Systems, Bedford, Massachusetts, USA).

**c) Inhibition of osteoclastogenesis by OPG and anti-TNF $\alpha$**

PBMNCs from 3 healthy controls were seeded in 96-well flat-bottomed culture plates at  $1 \times 10^6$  cells per well with 50% culture supernatants obtained from the cultured PBMNCs obtained from RA patients and mixed with an equal volume of fresh culture medium in a total of 200 $\mu$ l. To assess the effect of inhibiting TNF $\alpha$  in the culture supernatants from the RA patients, anti-TNF $\alpha$  antibody (infliximab; courtesy of Prof. A. Jawad) was added to parallel wells at a final concentration of 2.5 $\mu$ g/ml. To assess the effect of OPG on reducing the differentiation of osteoclast in the culture system, OPG-Fc (R&D Systems Inc.) was added at a final concentration of 1.0 $\mu$ g/ml. The medium was replenished every 3 days and after 14 days in culture, the cells were stained for TRAP and osteoclasts counted as described previously. The results were compared for PBMNCs from healthy controls cultured with supernatant from the RA cells to similar cells cultured in the presence of TNF $\alpha$  inhibitors and OPG. Personal and clinical details of the 8 RA patients involved in the study are summarised in Table 5.1.

**Table 5.1: Clinical and demographic details of RA patients included in the study of OCP.**

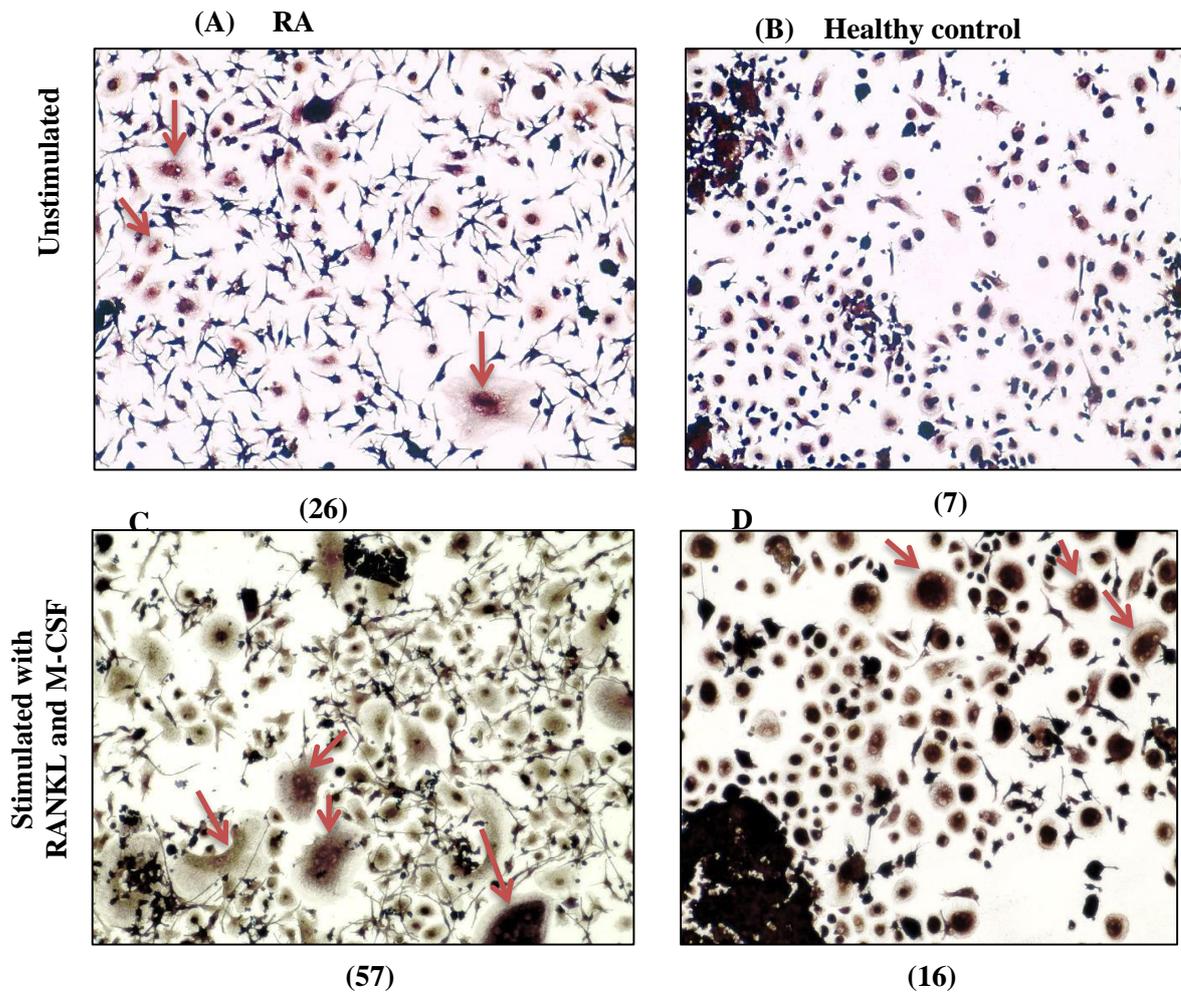
| Parameters of patient assessment    |                         | Patients<br>n=8 |
|-------------------------------------|-------------------------|-----------------|
| <b>Gender</b>                       | Male (%)                | 1 (13)          |
|                                     | Female (%)              | 7 (87)          |
|                                     | - (Post-menopausal) (%) | 5 (71)          |
| <b>Age (year)</b>                   | Mean (range)            | 54 (24-77)      |
| <b>Disease activity score 28</b>    | Mean (SD)               | 5.4 (0.9)       |
| <b>Calcium and Vitamin D intake</b> | n (%)                   | 3 (38)          |
| <b>Ethnic origin</b>                | - White n (%)           | 3 (38)          |
|                                     | - Asian n (%)           | 4 (50)          |
|                                     | - Black n (%)           | 1 (12)          |
| <b>Methotrexate use</b>             | n (%)                   | 8 (100)         |

Eight RA patients were recruited to determine the direct effect of blocking TNF $\alpha$  on the *in vivo* and *in vitro* OCP frequency.

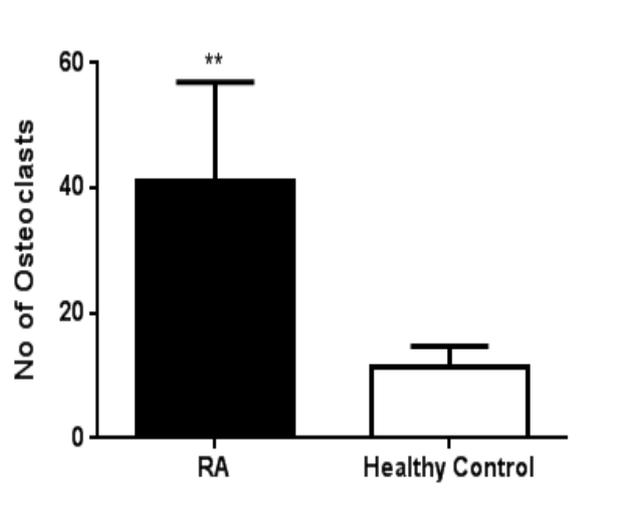
### 5.3 Results

#### 5.3.1 Patients with RA manifest increased numbers of osteoclasts in unstimulated *in vitro* cultures of PBMNCs before treatment

Large numbers of multinucleated TRAP positive cells, which are markers of osteoclasts, were identified in the unstimulated cultures of PBMNCs from RA patients before treatment with the biologic anti-TNF $\alpha$  agents. These observations were made without the addition of RANK-L or M-CSF, which have been shown to promote the generation of osteoclast (200,201). The number of these cells was significantly higher than osteoclasts seen in cultures of PBMNCs from healthy controls (mean  $\pm$ SD, 41 $\pm$ 15.9 vs. 11.4 $\pm$ 3.3 osteoclasts per 10<sup>6</sup> PBMNCs;  $p$ < 0.001) (Figure 5.1A and B). The addition of RANK-L and M-CSF increased the number and size of osteoclasts in PBMNC cultures from RA patients (222.5 $\pm$ 64 osteoclasts per 10<sup>6</sup> PBMNCs;  $p$ < 0.001) (Figure 5.1C) and, to a lesser extent, in cultures of PBMNC from the healthy controls (16 $\pm$ 9 osteoclasts per 10<sup>6</sup> PBMNCs;  $p$ < 0.001) (Figure 5.1D). These results suggested that more OCPs circulate in the peripheral blood of RA patients compared with healthy controls (Figure 5.2). Moreover, OCPs in the blood of RA patients but not the controls progressed to mature osteoclasts without the addition of RANK-L or M-CSF to the cultures.



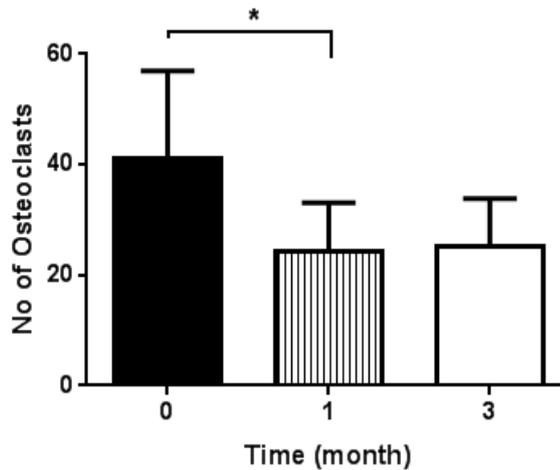
**Figure 5.1: Multinucleated TRAP<sup>+</sup> cells (osteoclasts) in cultured, unstimulated peripheral PBMCs of patients with RA before treatment and healthy controls.** PBMCs were enriched from the blood of 8 RA patients and 12 healthy controls and cultured in the absence (A and B) or presence (C and D) of M-CSF (25ng/ml) and RANK-L (100 ng/ml) for 14 days. The cultured cells were then fixed and stained for TRAP, and photographed at  $\times 20$  magnification. Numbers in parentheses under each figure represent the total number of osteoclasts per  $10^6$  PBMCs for each sample shown.



**Figure 5.2: The number of osteoclast in peripheral blood of RA patients and healthy controls.** More osteoclasts differentiate from unstimulated PBMNCs of patients with RA compared with healthy controls. PBMNCs were enriched from the blood of 8 RA patients and 12 healthy controls. The cells were cultured and stained for TRAP as described in the legend to Figure 5.1. The number of TRAP-positive multinucleated cells (osteoclasts) was counted and is presented as osteoclasts per million PBMNCs that were cultured at the start of the experiment. The results are presented as Mean and SD. \*\* indicates  $p < 0.001$ .

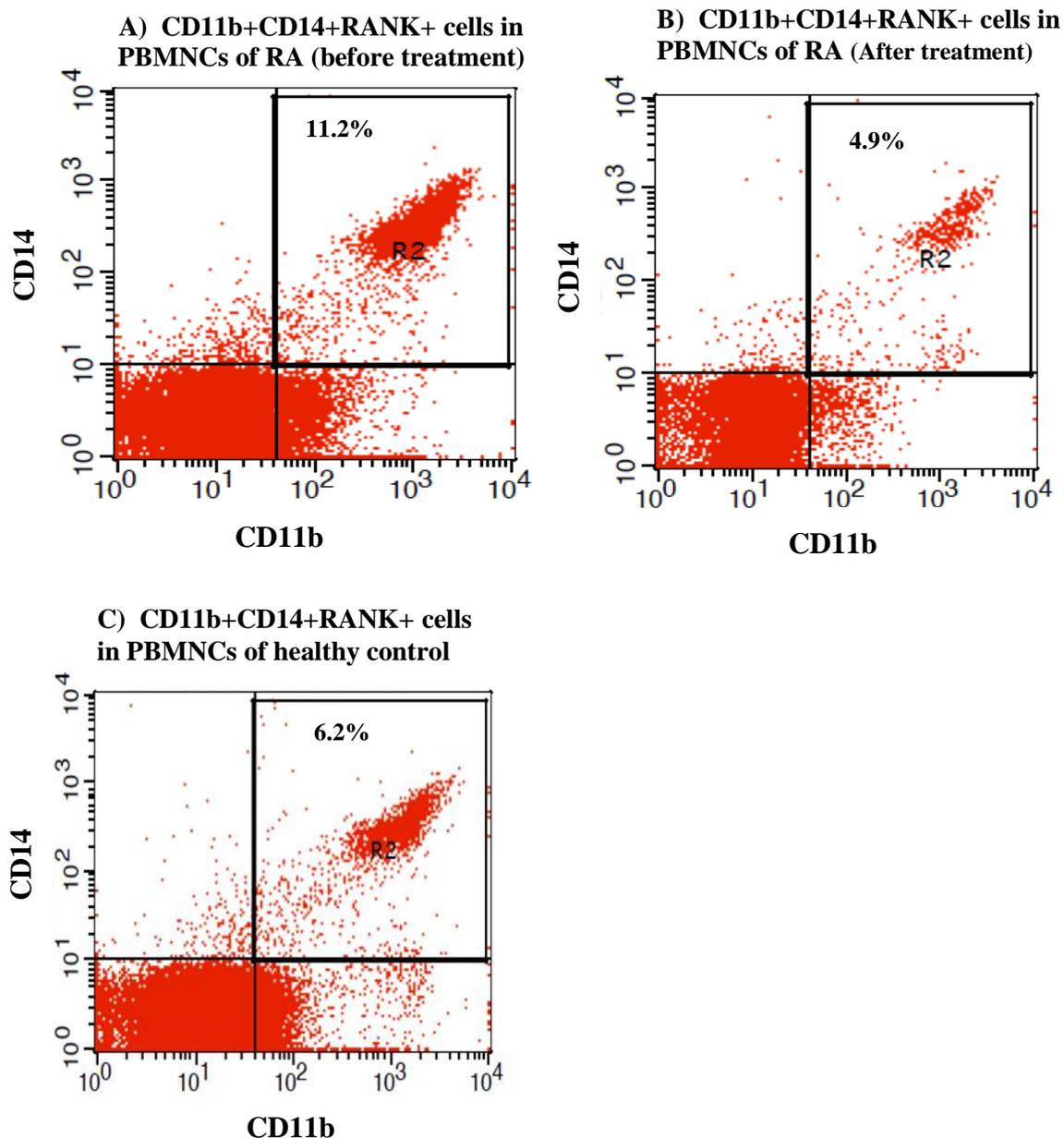
### **5.3.2 Treatment with biologic anti-TNF $\alpha$ agents reduces the differentiation and frequency of OCPs in the blood of patients with RA**

To assess the effect of TNF $\alpha$  on osteoclasts *in vivo*, the frequency of OCPs was determined in the blood of 8 RA patients treated with biologic anti-TNF $\alpha$  agents before treatment and at 1 and then 3 months after treatment. PBMNCs were cultured as described in the Methods and the number of TRAP-positive multinucleated cells (osteoclasts) determined after 14 days of culture. As mentioned above, 6 of the patients were treated with etanercept while 2 were treated with adalimumab. All patients were responders to treatment according to their clinical assessment by DAS28. Osteoclasts number reduced significantly in all patients after treatment with the biologic anti-TNF $\alpha$  agents (41% and 56% after 1 and 3 months post treatment, respectively;  $p=0.03$ ) (Figure 5.3).



**Figure 5.3: Treatment of RA patients with biologic anti-TNF $\alpha$  agents results in the reduction of osteoclast numbers *in vitro*.** PBMNCs from the blood of 8 RA patients treated with biologic anti-TNF $\alpha$  agents were cultured for 14 days, as described in the text, to determine the impact of this treatment on the frequency of osteoclast generation *in vitro*. Data are expressed as mean osteoclasts per 10<sup>6</sup> PBMNCs. The error bars represent +/- standard deviation (SD). \*  $p \leq 0.05$  compared with before treatment.

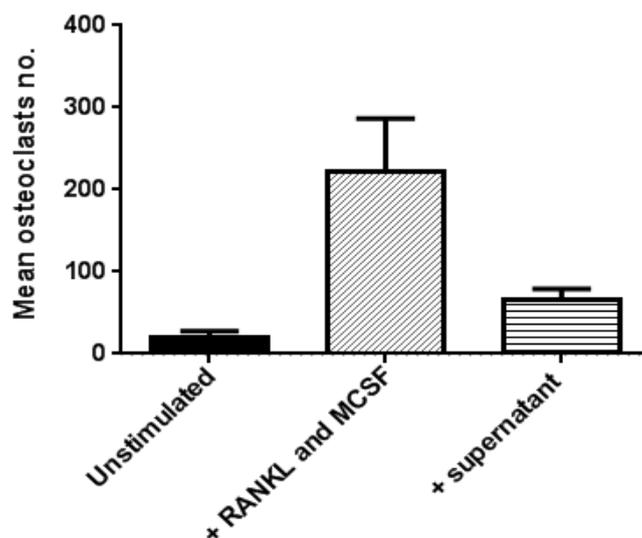
To confirm the noted effect of treatment with biologic anti-TNF $\alpha$  agents on the generation of OCPs an alternative approach was also used. This approach was based on FACS analysis of the frequency of mononuclear OCPs, as compared with multinucleated TRAP-positive cells in culture. This approach was based on the knowledge that blood mononuclear OCPs express RANK as well as CD11b and CD14. For this purpose, freshly enriched PBMNCs from 8 RA patients and 12 healthy controls were stained for CD11b, CD14 and RANK and analysed by flow cytometry. The mean fluorescence intensity (MFI) of RANK on CD11b<sup>+</sup>CD14<sup>+</sup> cells decreased in the RA patients by 42% after 1 month and by 44% after 3 months after treatment. This finding confirms that treatment with biologic anti-TNF $\alpha$  agents has a significant impact on reducing the levels of RANK<sup>+</sup> cells and the generation of OCPs in RA patients and that more OCPs circulate in the peripheral blood of RA patients compared with healthy controls (Figure 5.4).



**Figure 5.4: Treatment of RA patients with biologic anti-TNF $\alpha$  agents reduces the frequency of blood mononuclear OCPs.** The frequency of mononuclear OCPs in the blood of RA patients and healthy controls was determined by FACS analysis of PBMNCs from 8 RA patients and 12 healthy controls. PBMNCs were stained for CD11b (APC mouse anti-human CD11b), CD14 (FITC mouse anti-human CD14) and RANK (PE anti-human RANK). The Figure depicts percentages of PBMNCs (R2) expressing CD11b and CD14 in a RA patient before (A) and post treatment with etanercept (B) and in a healthy control (C).

### **5.3.3 Supernatants of cultured RA patients' PBMNCs before treatment with biologic anti-TNF $\alpha$ agents enhanced the differentiation of PBMNCs from healthy controls to osteoclasts**

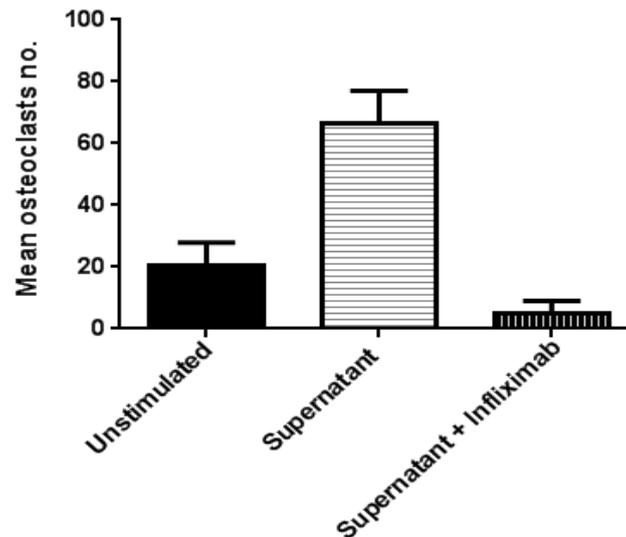
Previous studies have shown that systemic TNF $\alpha$  in TNF $\alpha$ -transgenic mice directly increases the frequency of OCPs while treatment of these mice with anti-TNF $\alpha$  reduces the number of OCPs (183). Results described in the previous sections revealed that the frequency of mononuclear OCPs is significantly higher in the blood of patients with RA compared with healthy controls. Furthermore, there was evidence for increased differentiation of cultured RA PBMNCs into osteoclasts compared with cultured PBMNCs from healthy controls. Furthermore, treatment of the RA patients with biologic anti-TNF $\alpha$  agents resulted in a significant reduction in the frequency of blood mononuclear OCPs and differentiated multinucleated osteoclasts in culture. These data indicate that TNF $\alpha$  is involved in the increases of differentiated osteoclasts. To provide further evidence that the increase in the number of osteoclasts is related to elevated levels of TNF $\alpha$  in RA patients, supernatants collected from the culture of PBMNCs from 2 RA patients were added to PBMNCs from 2 healthy control and the cultures maintained for 14 days (Figure 5.5). When unstimulated PBMNCs from the healthy controls were cultured for two weeks without added supernatants from the RA PBMNCs, there were  $20 \pm 7.7$  osteoclasts per 1 million of the cultured PBMNCs. When PBMNCs from the healthy controls were cultured in the presence of supernatants obtained from the PBMNCs from the patients obtained before treatment, the osteoclast numbers increased to  $65 \pm 14$  per 1 million. Although the number of osteoclasts was less than those obtained when the PBMNCs were cultured in the presence of recombinant RANK-L and M-CSF at  $222.5 \pm 64$  per 1 million of the cultured PBMNCs, the results, nevertheless, indicate that culture supernatants of PBMNCs from the RA patients before treatment contained factors that promote the differentiation of healthy PBMNCs into osteoclasts.



**Figure 5.5: Supernatants of cultured PBMCs from patients with RA before their treatment with biologic anti-TNF $\alpha$  agents promotes the differentiation of osteoclasts.** PBMCs from 2 healthy controls were seeded in 96-well flat-bottomed culture plates at  $1 \times 10^6$  cells per well with 50% culture supernatants obtained from the PBMCs obtained from 2 RA patients and mixed with an equal volume of fresh culture medium in a total of 200 $\mu$ l. The cultures were maintained for 14 days to induced osteoclastogenesis as described in the Methods. The mean number of differentiated osteoclasts from the unstimulated PBMCs from the healthy controls was compared with PBMCs from the healthy controls cultured in the presence of RANK-L and M-CSF, or supernatant from cultured PBMCs of RA patients. The results are presented as the mean  $\pm$  SD.

To confirm that of TNF $\alpha$  in culture supernatants of PBMCs from the RA patients is one of the factors that contribute to the differentiation of PBMCs from the healthy controls, experiments were carried out in which 2.5 $\mu$ g/ml of anti-TNF $\alpha$  antibody (infliximab) was added to the supernatants before initiating the culture systems. The results revealed significant inhibition of the differentiation of PBMCs from healthy controls to osteoclasts under the influence of culture supernatants from the RA patients. Thus, the addition of infliximab reduced the number of osteoclasts to  $5 \pm 4$  cells per 1 million of the cultured PBMCs (Figure 5.6). These experiments indicate that cultured PBMCs from RA

patients untreated with biologic anti-TNF $\alpha$  agents secrete TNF $\alpha$  that promote the differentiation of PBMCs to osteoclasts and that TNF $\alpha$  is an important element in the differentiation of osteoclasts.

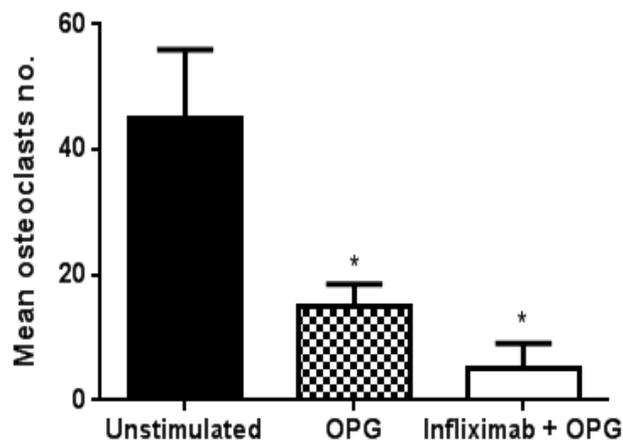


**Figure 5.6: Anti-TNF $\alpha$  antibody inhibits the differentiation of PBMCs from healthy controls to osteoclasts under the influence of RA PBMCs supernatants.** Supernatants obtained from culturing PBMCs of 2 RA patients were added to cultured PBMCs of two healthy controls in the presence or absence of anti-TNF $\alpha$  antibody (infliximab). Data are presented as the mean  $\pm$  SD.

#### 5.3.4 OPG inhibits osteoclastogenesis

As cited earlier, it is established that OPG reduces osteoclastogenesis and prevents bone erosion as has been shown in the collagen-induced arthritis (CIA) model in mice (202,203). Indeed, results presented in the previous chapter have shown that there was an increase in the level of OPG in RA patients treated with biologic anti-TNF $\alpha$  agents. To test if the reduction in the frequency of mononuclear OCPs in the blood of patients and of differentiated multinucleated osteoclasts in the culture system was likely to have been influenced by OPG, experiments were carried out to assess the effect of recombinant OPG on the number of

osteoclasts generated in the culture of PBMCs from 3 RA patients. In unstimulated cultures, the average number of osteoclasts was  $45 \pm 11$  per  $1 \times 10^6$  PBMCs. However, this number was reduced considerably to  $15 \pm 3.5$  cells when PBMCs were cultured with  $1.0 \mu\text{g/ml}$  of OPG in the culture. The effect of OPG on the number of osteoclasts was enhanced in combination with infliximab. Thus, the number of osteoclasts cells declined to  $5 \pm 4$  osteoclasts per  $1 \times 10^6$  PBMCs with this combination (Figure 5.7). However, the results indicated that the effect of the combined OPG and infliximab on the number of osteoclasts was additive rather than synergistic which may indicate that both act, probably, through the same or related pathways.

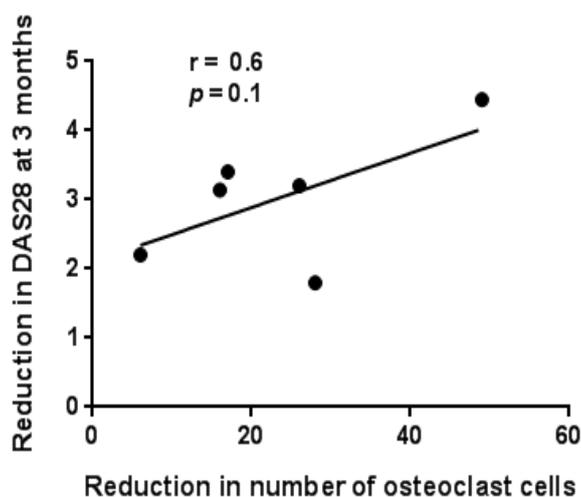


**Figure 5.7: OPG inhibits the differentiation of PBMCs to osteoclasts in culture.**

The number of multinucleated TRAP<sup>+</sup> cells (osteoclasts) was measured as described in the legend to Figure 5.2. The number of these cells was determined in cultures of PBMCs from 3 RA patients with further stimulation in the presence or absence of recombinant OPG-Fc ( $1 \mu\text{g/ml}$ ) alone or in combination with  $1 \mu\text{g/ml}$  infliximab. Data are expressed as the mean  $\pm$  SD of four independent wells (\*  $p < 0.05$ ).

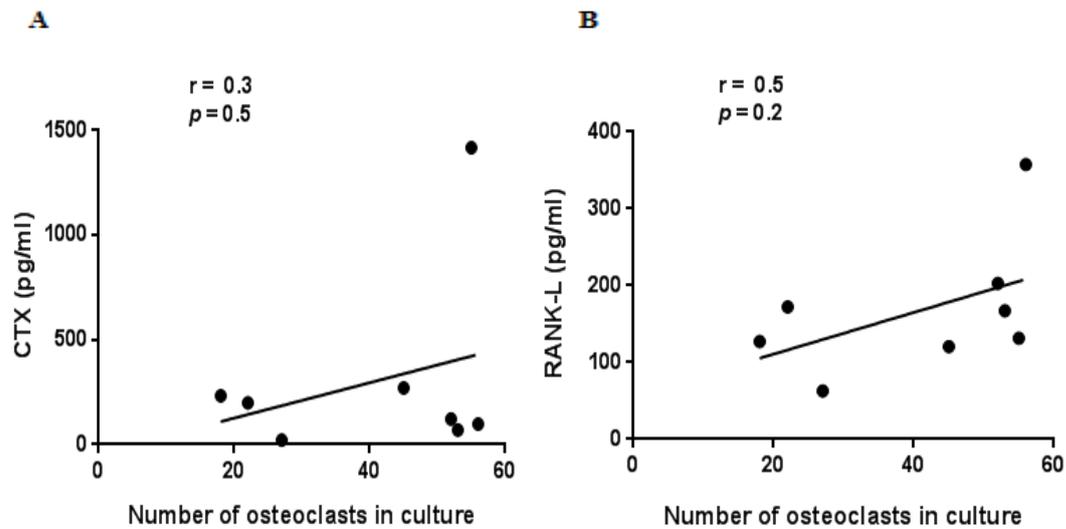
### **5.3.5 Reduction in the frequency and differentiation of OCPs in RA patients correlate with their responsiveness to treatment with biologic anti-TNF $\alpha$ agents and with improvements in bone biomarkers**

Data presented in Chapter 4 indicated that RA patients treated with biologic anti-TNF $\alpha$  agents had beneficial bone responses whether they had improvements in their DAS28 scores or not. Data presented in this chapter indicated that there was a potential reduction osteoclastogenesis in RA patients treated with biologic anti-TNF $\alpha$  agents as suggested by the reduction in the number of CD11b<sup>+</sup>CD14<sup>+</sup>RANK<sup>+</sup> cells in blood and of TRAP-positive cells (osteoclasts) in cultured PBMNCs from the patients. Due to the limited number of patients that could be recruited for the study of OCPs and that all were responder patients to treatment with the biologic anti-TNF $\alpha$  agents it was not possible to confirm whether or not the reduction in frequency and differentiation of OCP was or was not related to the clinical response. However, the data were analysed to assess the correlation between reduction in DAS28 (initial DAS28 – DAS28 at 3 months) and the reduction in number of cultured osteoclasts. This analysis revealed a positive correlations between reduction in DAS28 and number of cultured osteoclasts ( $r=0.6$ ,  $p=0.1$ ) (Figure 5.8).

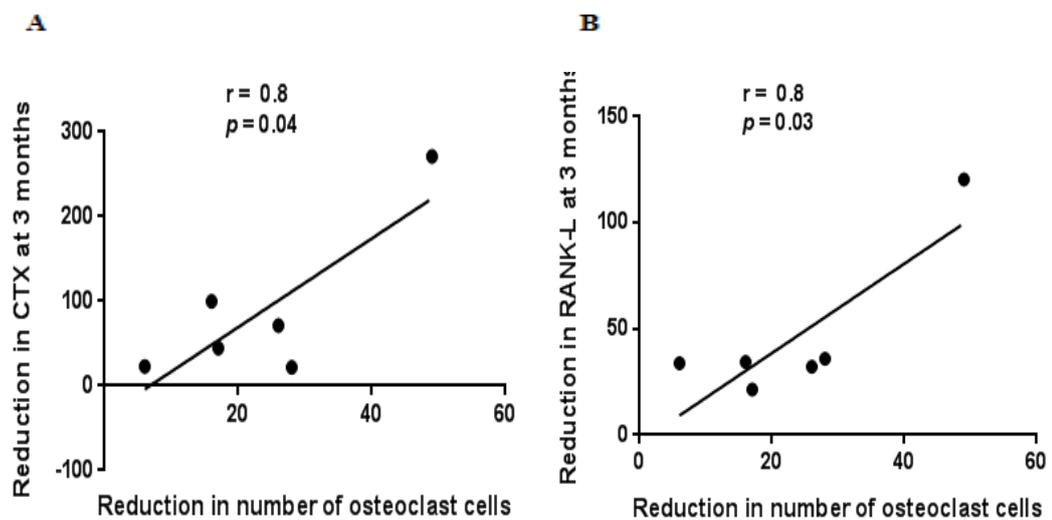


**Figure 5.8: Correlations between the reduction in number of differentiated osteoclasts and reduction in DAS28 at 3 months of treatment with biologic anti-TNF $\alpha$  agents.** Osteoclasts were obtained from culturing PBMNCs of RA patients as described in the legend to Figure 5.2, while DAS28 was measured before treatment and at 3 months after treatment had started as described in Materials and Methods chapter. Correlations were calculated using Spearman's correlation coefficient ( $r$ ). PBMNCs were not cultured for two patients at 3 months post treatment.

Next, reduction in number of differentiated osteoclasts was compared with blood biomarkers of bone turnover in plasma measured using the ELISA-based protocol described in Chapter 4. The data revealed that number of differentiated osteoclasts in culture correlated with plasma levels of both CTX and RANK-L ( $r=0.3$ ,  $p=0.5$  and  $r=0.5$ ,  $p=0.2$ , respectively) (Figure 5.9). Furthermore, a significant positive correlation was observed between reduction in numbers of cultured osteoclasts and plasma levels of CTX and RANK-L after 3 months of treatment ( $r=0.8$ ,  $p=0.04$  and  $r=0.8$ ,  $p=0.03$ , respectively) (Figure 5.10). No statistically significant correlations were observed between levels of OCPs and plasma levels of osteocalcin and OPG before treatment had started and then at 1 month and 3 months after treatment with the biologic anti-TNF $\alpha$  agents ( $p>0.05$ ).



**Figure 5.9: The relationships between the number of differentiated osteoclasts in culture and plasma levels of CTX (A) and RANK-L (B).** Osteoclasts were obtained from cultured PBMCs of 8 RA patients before treatment had started as described in the legend to Figure 5.2, while plasma levels of CTX and RANK-L were determined by ELISA as described in Materials and Methods chapter. The correlation was with Spearman's correlation coefficient ( $r$ ).



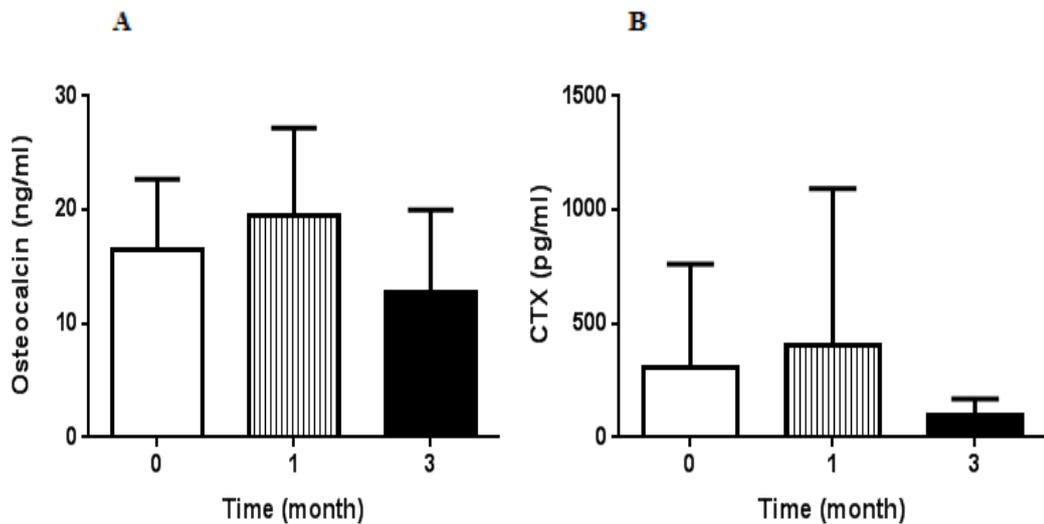
**Figure 5.10: Relationships between the reduction in number of differentiated osteoclasts in culture and reduction in plasma levels of CTX (A) and RANK-L (B).** Reduction in plasma levels of CTX and RANK-L were correlated with reduction in number of differentiated osteoclasts in culture of PBMCs from 8 RA patients 3 months after treatment with biologic anti-TNF $\alpha$  agents. The correlations were made using Spearman's correlation coefficient ( $r$ ). Data of both CTX and RANK-L were only available for 5 patients.

Although plasma levels of osteocalcin increased 1 month after treatment had started, there were no significant differences in the level of osteocalcin in patients cohort included in this study after treatment with biologic anti-TNF $\alpha$  agents. Thus, plasma levels of osteocalcin were 17 $\pm$ 6.2ng/ml before treatment and 20 $\pm$ 8ng/ml ( $p=0.2$ ) and 13 $\pm$ 7.3 ( $p=0.9$ ) 1 and 3 months after treatment, respectively. Furthermore, plasma levels of CTX were increased 1 month after treatment but declined after 3 months. However, the difference was not statistically significant, probably due to patient numbers. Thus, plasma levels of CTX were 304 $\pm$ 458pg/ml before treatment and 406 $\pm$ 687pg/ml ( $p=0.3$ ) and 101 $\pm$ 68 pg/ml ( $p=0.3$ ) 1 and 3 months after treatment, respectively (Table 5.2) (Figure 5.11).

**Table 5.2: Changes in bone turnover biomarkers after treatment with biologic anti-TNF $\alpha$  agents.**

| <b>Parameters of patient assessment</b> | <b>Before treatment</b> | <b>1 month</b> | <b>3 months</b> |
|---|-------------------------|----------------|-----------------|
| <b>Osteocalcin (ng/ml)</b>              | 17 (6.2)                | 20 (8)         | 13 (7.3)        |
| <b>CTX (pg/ml)</b>                      | 304 (458)               | 406 (687)      | 101 (68)        |

Plasma levels of bone turnover biomarkers for 8 RA patients included in this study were measured by ELISA before and then at 1 and 3 months after treatment as described in the Materials and Methods chapter. The values shown are for the mean and (SD).



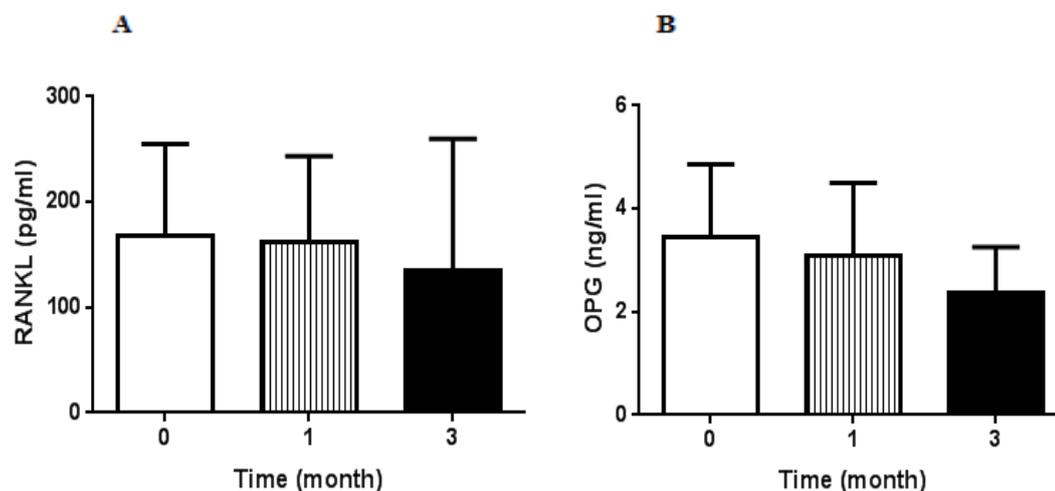
**Figure 5.11: Changes in plasma levels of osteocalcin and CTX in RA patients treated with biologic anti-TNF $\alpha$  agents.** Levels of osteocalcin (A) and CTX (B) were measured in the plasma of 8 RA patients treated with biologic anti-TNF $\alpha$  agents. Osteocalcin and CTX levels were determined using ELISA. The data presented are for the values of the two biomarkers from before treatment and after 1 and 3 months of treatment. The values shown are for the mean and the error bars represent +/- standard deviation (SD).

Plasma levels of RANK-L decreased at 1 and 3 months after treatment compared with their levels before treatment had started ( $162 \pm 81$  pg/ml ( $p=0.9$ ) and  $135 \pm 124$  pg/ml ( $p=0.6$ ) 1 and 3 month after treatment compared with  $168 \pm 87$  pg/ml before treatment. There were no significant differences in levels of OPG after treatment with biologic anti-TNF $\alpha$  agents, which probably a result of sample size. Thus, plasma levels of OPG were  $3.4 \pm 1.4$  ng/ml before treatment and  $3.1 \pm 1.4$  ng/ml ( $p=0.2$ ) and  $2.4 \pm 0.9$  ng/ml ( $p=0.6$ ) 1 and 3 months after treatment, respectively (Table 5.3) (Figure 5.12).

**Table 5.3: Changes in RANK-L and OPG after treatment with biologic anti-TNF $\alpha$  agents.**

| Parameters of patient assessment | Before treatment | 1 month   | 3 months  |
|----------------------------------|------------------|-----------|-----------|
| <b>RANK-L (pg/ml)</b>            | 168 (87)         | 162 (81)  | 135 (124) |
| <b>OPG (ng/ml)</b>               | 3.4 (1.4)        | 3.1 (1.4) | 2.4 (0.9) |

Plasma levels of osteoprotegerin (OPG) and RANK-L for the 8 RA patients included in this study were measured by ELISA as described in the Materials and Methods. The values shown are for the mean and (SD).



**Figure 5.12: Changes in plasma levels of RANK-L and OPG in RA patients treated with biologic anti-TNF $\alpha$  agents.** Mean RANK-L (A) and OPG (B) levels were determined using ELISA for plasma samples obtained from patients before treatment and at 1 and then 3 months after treatment. The errors bars represent +/- SD. Results were compared with values of the biomarkers before treatment using paired Student's t-test.

## 5.4 Discussion

The results presented in this chapter reveal the impact of inhibiting TNF $\alpha$  in patients with RA on the frequency and potential for differentiation of cells that mediate bone erosion. Furthermore, the data throw light on the relationship between the number of circulating OCPs and differentiation potential of PBMNCs in patients and healthy controls with blood biomarkers of bone including RANK-L, OPG, CTX and osteocalcin.

The results reported in the chapter show that the frequency of mononuclear OCPs is significantly increased in the blood of RA patients compared with healthy controls. These blood cells express surface markers CD11b, CD14 and RANK and have been identified as mononuclear OCPs (59,204,205). A key role for TNF $\alpha$  in stimulating OCP formation is supported by the observations that blocking TNF $\alpha$  *in vivo* significantly reduces the number of circulating OCPs. The results presented in the chapter also showed that the number of these cells was increased in RA patients compared with the healthy control before the patients had started treatment with biologic anti-TNF $\alpha$  agents but declined significantly after treatment. In addition, neutralisation of TNF $\alpha$  in the *in vitro* cultures with infliximab reduced the differentiation of PBMNCs into multinucleated TRAP-positive (osteoclasts) cells.

A number of early studies have shown that systemic TNF $\alpha$  directly increases the frequency of blood OCPs in mice and this increase is inhibited by treatment with biologic anti-TNF $\alpha$  agents (59,206). In the experiments described in this chapter, this proposition was shown to also be true for RA patients, as the frequency of TRAP+ cells decreased markedly in 6 of 8 patients treated with biologic anti-TNF $\alpha$  agents. Interestingly, this reduction in the number of TRAP+ cells in culture paralleled the clinical improvement of the patients.

Results of culturing PBMNCs from healthy controls with culture supernatants of PBMNCs from the RA patients before treatment with biologic anti-TNF $\alpha$  agents, indicated

that the cultured RA PBMNCs produced factors sufficient for the differentiation of PBMNCs from healthy controls into multinucleated TRAP<sup>+</sup> cells (osteoclasts) *in vitro*. This is important as the data highlights the relevance of studying peripheral blood from patients with RA to assess the factors that determine disease progression and response of RA patients to treatment of a systemic disease that primarily affects joints. Subsequent inhibition experiments confirmed that one key factor in the culture supernatant of the PBMNCs from the patients that promoted the differentiation of PBMNCs from healthy controls to OCPs was TNF $\alpha$ .

The additional finding in this study was that the decrease in osteoclasts obtained from culturing PBMNCs correlated with reduction in disease activity, measured by DAS28, indicating that disease activity may be a dependent factor in bone loss in RA. This result is consistent with findings from numerous animal model studies of inflammation, together with clinical evidence, showing that disease activity plays a key role in inducing bone loss (32,59,207,208).

To gain further insights into how blocking TNF $\alpha$  could inhibit/suppress the differentiation of PBMNCs to osteoclasts, parallel experiments were carried out to inhibit RANK-L with OPG. The experiments revealed that OPG was capable of reducing the number of differentiated osteoclasts in culture, presumably, through reducing the level of RANK-L which is also partly induced by TNF $\alpha$ . Therefore, these results may suggest that TNF $\alpha$  causes a systemic increase in the number of circulating OCPs through enhancing RANK-L expression. This finding is in agreement with our finding that positive correlations were observed by analysing the relationship between plasma levels of RANK-L and the number of cultured osteoclasts. In general, these findings are consistent with a previous study (207) in which TNF $\alpha$  was shown to induce bone loss directly by stimulating osteoclast precursors, and indirectly through releasing RANK-L which activates osteoclasts. Furthermore, this finding is

consistent with findings by Lam and colleagues (59) that suggested a local regulation of the differentiation of OCPs into osteoclasts by RANK-L. Therefore, the observed reduction in bone resorption upon treatment with biologic anti-TNF $\alpha$  agents may also be caused by the blockade of the RANK-L-independent stimulatory effects of TNF $\alpha$  on the number of circulating OCPs. In addition, blocking TNF $\alpha$  resulted in a strong reduction in OCPs numbers and of cultured osteoclasts. This effect corresponded with a decrease of plasma CTX levels. This result is in line with a previous study (133), which had provided a description on the cellular levels for the anti-catabolic effects of TNF neutralisation on bone.

In summary, the discovery of osteoclastogenic mediators, including OPG–RANK–RANK-L system, provided an important step in identifying mechanisms involved in chronic inflammation-induced bone loss. Based on the literature and the outcomes of this study it is possible to conclude that treatment with biologic anti-TNF $\alpha$  agents provides reduced osteoclastogenesis in RA patients. This is suggested by the reduction in the number of CD11b<sup>+</sup>CD14<sup>+</sup>RANK<sup>+</sup> cells in blood and in TRAP-positive cells (osteoclasts) in cultured PBMNCs from patients.

**Chapter Six**  
**General Discussion, Conclusions**  
**and Future Perspectives**

## 6.1) General discussion

One of the most significant risk factors for bone fracture is low bone mineral density (BMD) (209). Key objectives of treating patients suffering from low BMD are suppressing osteoclast activity with anti-resorptive agents, enhancing osteoblast activity with anabolic agents and regulating bone marrow adipogenesis. Other approaches include reducing the level of factors that contribute to bone loss (210). Bisphosphonates are the most widely-used first-line anti-resorptive agents. They are recommended for the treatment of patients with low BMD following the success of numerous randomised trials in which these agents demonstrated significant anti-fracture efficacy and good safety profiles (211,212).

Results reported in this thesis on changes in BMD after treatment with bisphosphonates in relation to factors that influence such changes are in line with earlier studies (164-167,213). Furthermore, results on differences in the pattern of BMD change in the lumbar spine versus the hip are also consistent with previous studies. These regional differences in the effect of bisphosphonate may reflect differences in the composition of bone in the lumbar spine, which has a larger component of trabecular than cortical bone compared with the hips (163,209).

The results presented in the thesis have also revealed that there is a disparity in patients' response to treatment that relate to differences in age, gender, ethnic origin and lifestyle. More than two folds increase in BMD at the lumbar spine and hip was observed in men compared with women after two years of treatment with bisphosphonates. Postmenopausal women, especially those who are 50-60 years of age are prone to osteoporosis because the level of their oestrogen drops, thus, accelerating bone resorption by 90%. In contrast, the level of bone formation in these individuals increases by only 45% and this imbalance between resorption and new bone formation leads to lower improvement in overall BMD in women treated with anti-resorptive agents (163,214). In men, testosterone has

beneficial effects on BMD and on their responsiveness to treatment (214). Similar to results presented in this thesis on lower improvement in BMD in female patients at 50-60 years of age compared with other age groups, a previous study observed that bone loss is greatest among women aged 50–54 who were transitioning from premenopausal to postmenopausal status (209).

In addition to the effect of age and gender on responsiveness to anti-resorptive agents, this study provides data on the effect of ethnic origin on responsiveness to treatment with anti-resorptive agents. Thus, results reported in the study show that individuals of Afro-Caribbean origin of similar age and gender to Caucasian and Asian patients with osteoporosis have significantly higher BMD before treatment with anti-resorptive agents. This data is consistent with findings by *Hochberg et al.* and *Yates et al.* on BMD in the lumbar spine and hips in individuals from different ethnic origins (172,173). However, due to the small number of patients examined in the current study, the data does not lend itself to statistically meaningful comparisons for the effect of ethnic origin on responsiveness to anti-resorptive agents. Interestingly, and within the same context, the data has also revealed that the average change in BMD at the lumbar spine in Caucasians is higher than in Asians. However, this variation in change following treatment in BMD between Caucasians and Asians was not statistically significant. This is in line with previous reports (215) revealing that variations in change in BMD after treatment between Caucasian and Asian patients can be partially related to the different ethnic genetic background.

In attempting to evaluate the effect of lifestyle on the response of osteoporosis patients to treatment with anti-resorptive agents, information was collected on the effect of smoking. This data showed a significant association between lower improvement in BMD after treatment of smokers compared with non-smoker patients of similar age, gender and ethnic background. This is consistent with data that has established smoking as a risk factor for reduced bone health. Current evidence suggest that this effect is likely to be due to the effect

of smoking on hormone metabolism, levels of vitamin D, blood circulation, body weight, calcium absorption, elevation in oxidative stress, local hypoxia, free radical generation and the production of pro-inflammatory cytokines. Changes in the balance of these factors affect both bone resorption and formation leading ultimately to osteoporosis and reduced healing from fractures (216-218).

By and large, results presented in this thesis on factors that affect the response of patients to treatment with anti-resorptive agents are consistent with previous studies (209,172,215). However, the current study has limitations, as do earlier studies of changes in BMD, including low samples size, limited age ranges of patients examined and different inclusion and exclusion criteria with most studies excluding men (219-222). Another limitation to the study of using BMD as a measure of responsiveness to anti-resorptive agents is the need to wait for 1-2 years before been able to assess a patients' response to therapy (174). This highlights the potential benefit of using biomarkers of bone turnover which have been suggested to provide indications on responsiveness within days to months of starting treatment (175).

Elevated levels of TNF $\alpha$  have been reported in cultures of mononuclear cells derived from the blood of postmenopausal women and osteoporosis patients, suggesting an association between high levels of TNF $\alpha$  and osteoporosis (68). Therefore, identifying factors that affect the response of patients suffering from bone density loss in the local area with its diversity, while highly beneficial, has the added benefit of providing the backdrop for studying the effect of treatment with biologic anti-TNF $\alpha$  agents on bone in patients with RA. The study of factors that impact bone in patients with RA and the beneficial effects of treatment are clouded by the complex nature of factors that affect changes in bone. Thus, bone loss in RA is generally thought to come in two forms: periarticular (local) and general. Periarticular bone loss in RA is first seen at the end of inflamed joints, which is a hallmark of

early disease. In contrast, generalised bone loss is observed as an extra-articular outcome of the inflammatory response in RA which ultimately leads to fractures (68). This generalised bone loss results from three main causes: inflammation, long-term treatment with suppressive medications such as corticosteroids and disability as a result of the disease (35,185). Treatment with corticosteroids and disease-modifying anti-rheumatic agents has two contradictory effects: increase the risk of osteoporosis, but also inhibit inflammatory activity, which this thesis argues is an important risk factor for generalised bone loss in RA.

Biologic anti-TNF $\alpha$  agents have been developed and successfully used to inhibit inflammation, but they have also been shown to slow, or even arrest local bone resorption in mice (177). However, their potential to impact bone loss in RA has not been adequately analysed. Furthermore, it is unclear if bone turnover is affected in all patients treated with biologic anti-TNF $\alpha$  agents, the degree of variability in responsiveness and whether the beneficial/lack of clinical response to biologic anti-TNF $\alpha$  agents is consistent with any beneficial effects on bone.

In addition, there is a clear lack of direct evidence as to the relationship between localised bone loss (e.g. in the hands) and generalised bone loss (in the spine and hip) in patients with RA. Available data suggests that a common pathway could underlie both. The RANK-L/OPG system provides a theoretical background for such a common mechanism (177). RANK-L expression is key, perhaps, among others upregulated by several pro-inflammatory mediators including TNF $\alpha$ , IL-1, IL-6, IL-17, IL-20 and IL-22 (146,178,182,184). The data presented in this thesis revealed that treatment with biologic anti-TNF $\alpha$  agents reduces generalised bone loss as shown by improvement in BMD at the lumbar spine and hip of RA patients. Interestingly, the results showed that this beneficial effect of biologic anti-TNF $\alpha$  agents did not parallel improvement in DAS28 in all patients. This data is consistent with findings in experimental arthritis in mice in that blockade of TNF $\alpha$  has a greater effect on cartilage degradation and joint destruction than on inflammation (182). This

data may raise the prospect that whereas TNF $\alpha$  is the key pro-inflammatory mediator in both local and generalised bone loss, that the inflammatory response in different patients may be driven by other mediators instead of, or in addition to TNF $\alpha$ . For example, there is evidence that IL-17 could be a key pro-inflammatory mediator in patients who do not respond to biologic anti-TNF $\alpha$  agents although the role of this cytokine in bone loss in the disease is to be determined (184). Indeed, data on assessing the effect of treatment with biologic anti-TNF $\alpha$  agents on localised bone loss have shown that these agents have arrested localised bone loss in RA. This data, however, is inconsistent with observations by Vis and his colleagues who have reported a decrease in hand BMD as measured by DXR in RA patients treated with infliximab (125). However, these investigators have measured hand BMD after one year of treatment and 84% of their patients were females.

When the effect of biologic anti-TNF $\alpha$  agents on bone was assessed by quantifying the level of bone turnover biomarkers, the data were consistent with previous studies (124,125,131-134,137,139,223). Although, there are a number of studies addressing the effect of biologic anti-TNF $\alpha$  agents on bone turnover biomarkers, comparative studies in responder and non-responder patients are limited. Results presented in the thesis are based on dividing the RA patients treated with biologic anti-TNF $\alpha$  agents into responder and non-responder patients based on changes in their DAS28 scores. The results strongly support the proposition that biologic anti-TNF $\alpha$  agents have beneficial effects on bone which is not entirely in line with its anti-inflammatory properties. Thus, the results show beneficial bone effects also in clinically non-responder RA patients.

Previous studies have documented that pro-inflammatory cytokines, such as TNF $\alpha$ , IL-1, IL-6, IL-17, IL-20, IL-22 and GM-CSF are associated with enhanced osteoclastogenesis through affecting the OPG-RANK-RANK-L circuit with the net result being an obvious rise in osteoclast formation and functions (144-147,179,192). In the current report, the studies revealed that levels of TNF $\alpha$ , IL-1, IL-6, IL-20, IL-22 and GM-CSF are reduced in patients

with RA in response to the treatment with biologic anti-TNF $\alpha$  agents. Furthermore, GM-CSF and the other pro-inflammatory cytokines including IL-1, IL-6 and IL-17 showed significant positive correlations with CTX indicating a strong relationship between inflammation and bone loss in RA. In addition, BMD values for the lumbar spine, hips and hands negatively correlated with increases in TNF $\alpha$ , IL-1, IL-6, IL-22 and GM-CSF. This can be explained as being a consequence of the involvement of pro-inflammatory cytokines in suppressing OPG and promoting RANK-L upregulation. The results presented also confirmed the firm relationship between inflammation and bone loss which has been clearly established in many clinical and experimental models before (131,178,203).

The available evidence indicates that the key determining factor in bone loss by the inflammatory pathways operating in RA is osteoclastogenesis (183,203). The results presented in the thesis clearly show that treatment of RA with biologic anti-TNF $\alpha$  agents are consistent with a beneficial effect on bone as also supported by the suppressive effect on osteoclast precursor (OCPs) generation and reduction in the level of RANK and RANK-L and increasing OPG levels. TNF $\alpha$  acts as a key factor in inducing the development and differentiation of OCPs in TNF $\alpha$ -transgenic mice (224). This observation in mice was confirmed in the current study in humans and also showing that blocking TNF $\alpha$  in patients significantly reduces the number of circulating OCPs. Indeed, the addition of infliximab to cultured PBMNCs from patients with RA inhibited osteoclast formation as shown by reduced positive TRAP staining and multinuclearity. This data is consistent with previous studies (128,179) which showed that osteoclasts can grow from unstimulated PBMNCs from healthy individuals when grown in the presence of RANK-L and M-CSF, or by TNF $\alpha$  and this is inhibited after the addition infliximab to cultured PBMNCs (179,224).

The results described in the thesis also showed that the decrease in the frequency of OCPs in cultured PBMNCs from RA patients treated with biologic anti-TNF $\alpha$  agents

correlated with a reduction in disease activity in the treated patients suggesting, perhaps, that disease activity may correspond with the extent of bone resorption in RA. The reduction in bone loss was in correlation with reduction in disease activity and inflammation was also reported in previous studies (125,128) indicating that the beneficial effects of TNF $\alpha$  agents could be related to their anti-inflammatory activity.

The difference between treatment with biologic anti-TNF $\alpha$  agents and traditional pharmaceutical DMARDs on bone loss have been studied in only two randomised clinical trials, the Behandelstrategieen RA (BeSt) and the PREMIER. In BeSt, the effect of four treatment strategies was examined (126). Monotherapy, step-up therapy, initial combination of DMARD with high dose prednisone and combination of DMARD with infliximab were assessed. The study reported higher bone loss in patients treated with traditional DMARDs compared with combination therapy involving infliximab. In PREMIER trial, combination of methotrexate (MTX) with adalimumab led to less hand bone loss compared with monotherapy of either adalimumab or MTX alone. Hand bone loss was correlated with treatments not involving adalimumab, with age and with level of inflammation (225). Patients with high/moderate DAS28 scores had greater hand bone loss compared with those with low DAS28. The findings of this trial indicated that the advantages of treatment with biologic anti-TNF $\alpha$  agents may not be restricted to the control of inflammation but also to the ability to suppress osteoclast activation.

Treatment with biologic anti-TNF $\alpha$  agents has also been demonstrated to suppress bone loss in the spine and hip in a number of studies (124,126,225). Thus, Marotte and colleagues reported that hip and spine BMD was reduced in patients treated with MTX alone, but was conserved in patients treated with the combination of infliximab and MTX (99). Furthermore, these investigators observed that changes in BMD did not correlate with the clinical response of the patients to the combination therapy. However, in other studies of treatment with biologic anti-TNF $\alpha$  agents, suppression of bone loss paralleled improvements

in DAS28 (125-128), suggesting that a good control of inflammation could prevent bone loss in RA patients.

Despite previous studies and the results reported in this thesis, larger sample size studies are required in order to establish a definitive database on the effect of age, gender, ethnic origin and lifestyle such as smoking on the bone effects of biologic anti-TNF $\alpha$  agents in patients with RA. Interestingly, however, the current investigation whose results are summarised in this thesis has clearly identified a slight disparity in beneficial response of bone and the chronic inflammation. Thus, the data indicated that in some patients who did not benefit clinically from treatment with biologic anti-TNF $\alpha$  there was evidence for improvement in bone turnover.

## **6.2) Study limitations**

Results reported in this thesis provide interesting and potentially important new insights into the relationship between chronic inflammation and bone loss in patients with RA. The initial part of the study established statistically-relevant and scientifically meaningful information and the relationships between patient demographics and lifestyle with responsiveness to treatment with anti-osteoporotic agents. These studies were made possible because of the cooperation between and support from the Rheumatology and Radiology departments at Barts Health NHS Trust. This enabled the study to recruit a relatively large cohort of patients with osteoporosis. The outcome provided a good foundation on which to build the next stage of the study which involved applying the findings from the first part of the study to gain insights into mechanistic relationships between chronic inflammation in RA and bone loss in the patients. The key strategy for this part of the study was to assess the relationship between chronic inflammation and bone loss based on the outcome of treating patients with biologic anti-TNF $\alpha$  agents. TNF $\alpha$  is a principal pro-inflammatory and pleiotropic cytokine widely implicated in chronic inflammation in, at least, a majority of

patients with RA. Although, it is established that TNF $\alpha$ , in combination with other cytokines, is involved in local bone loss in the joint of RA patients, its role in generalized bone loss in the disease is only emerging. This proposition is based on the ability of TNF $\alpha$  to upregulate RANK/RANK-L expression and the induction of osteoclasts.

The design of the second part of the study was for a two stage analysis. The first was to be based on a retrospective analysis of the general relationship between the clinical response of patients to treatment with anti-TNF $\alpha$  based on changes in DAS28 and BMD measured by DEXA scans at 1 and 2 years after treatment. This part of the study also enabled limited application of the model established from the osteoporosis study to assess the effect of patient demographics on clinical and bone response to treatment with biologic anti-TNF $\alpha$ . The second part of the analysis was to explore the question in depth by assessing changes in physiology in patients including changes in BMD, bone biomarkers and the immune response that is known to be directly influenced by TNF $\alpha$  in RA patients following treatment with anti-TNF $\alpha$  agents. For this purpose, efforts were directed at recruiting RA patients treated with biologic anti-TNF $\alpha$  agents from September 2011 to April 2014. These patients were to be recruited mainly from the rheumatology clinics at Barts Health NHS trust at the Mile End Hospital. However, due to the large number of clinical trials that were either ongoing or to be started by the Professor Costantino Pitzalis and his group that were recruiting patients from the same cohort, it was decided to approach colleagues at the Royal Free Hospital NHS to recruit patients from their cohort. This was carried out after obtaining all necessary ethical approval from the local committee of the Health Research Authority and the Trust itself. The objective was to recruit patients prescribed treatment with biologic anti-TNF $\alpha$  agents who were also to be assessed for changes in BMD by DEXA scans. For this group, it was also decided to carry out parallel assessments of changes in BMD by DXR to obtain quantitative data and also compare the protocol with data obtained by DEXA. Barts and the London

Hospitals are among the largest centres in England in treating RA patients with biologic anti-TNF $\alpha$  agents with more than 50 patients treated annually. In addition, 25-30 RA patients are prescribed anti-TNF $\alpha$  agents at the Royal Free Hospital NHS Trust. It was, therefore, expected that enough patients will be recruited to render the results statically significant. However, a number of factors contributed to limiting the number of patients recruited to the study. These factors related primarily to the large number of clinical trials and studies carried out by Professor Pitzalis and his group who granted themselves priority access to the patients because the research set up was established and managed by Professor Pitzalis and his colleagues. Another limitation on recruitment of RA patients treated with anti-TNF $\alpha$  agents to the study described in the thesis was that only patients who were sent for BMD density analyses by DEXA were to be recruited to obtain the necessary information for the study. These could not be artificially increased because of NHS budgetary limitations and conditions set for our ethical permission. Furthermore, the study faced serious problems in that specific conditions were required to carry out hand X-rays of single hand for the information to be properly analysed by DXR. For these conditions to be set up there were numerous processes and approvals that had to be granted which took lengthy periods of time.

Due to the relatively large number of studies by Prof. Pitzalis' group recruitment for the current study were seriously restricted and were generally advised to recruit from patients that did not fit the criteria set for the current study and only from patients that were surplus to studies by Prof. Pitzalis. Unfortunately, it was subsequently discovered that most of these latter groups of patients were those more inclined not to consent to participate in research. Any attempts by other staff at the clinic to help us recruit patients to the study were not looked at favourably by Prof. Pitzalis and his staff. In addition, attempts to obtain ethical approval to recruit patients from the Royal Free Hospital NHS Trust did take an unusually lengthy time to be put in place.

### 6.3) Conclusions

One of the most significant risk factors for fracture in patients with osteoporosis or chronic inflammatory conditions is low BMD (209). Anti-resorptive agents have been used for the treatment of low BMD in several randomised controlled trials, and the results have shown that their usefulness is related to their ability to increase, or stabilise bone density (211,212). However, it has been established that there is disparity in patients' response to treatment with anti-resorptive agents including bisphosphonates. Most of the reasons for this disparity, but not all, are known and these include age, gender, ethnic origin and lifestyle. However, the extent of the effect of biologic anti-inflammatory agents, such as anti-TNF $\alpha$  agents on bone in patients with RA and whether such effects are consistent with their anti-inflammatory effects are not been adequately explored.

The pathogenesis of bone loss in osteoporosis is multi-factorial including aging and the menopause. Thus, oestrogen deficiency promotes T-cell activation and the production of TNF $\alpha$ . Subsequently, TNF $\alpha$  is established as a key promoter of bone resorption acting through the expression of RANK-L which, in turn, leads to the activation and differentiation of osteoclasts through binding to RANK (62,118). TNF $\alpha$ , however, also promotes bone loss through the inhibition of bone formation by promoting osteoblast apoptosis and reducing the differentiation and proliferation of osteoblasts and their precursors (62,226).

Bone loss in patients with RA is observed in two forms: peri-articular (local) bone loss near inflamed joints, which is a hallmark of early disease and generalised bone loss as one of the extra-articular features of RA which causes increased risks of fractures (68). Thus, bone loss in RA is a risk factor for secondary osteoporosis and it is thought to be a result of three main factors: inflammation, long-term treatment with medications such as corticosteroids and disability as a result of the disease (35,185).

In the recent literature, there has been reports of a clear association between localised bone loss (in the hands) in RA patients and generalised bone loss (in the spine and hip), suggesting a common pathway. The RANK-L and OPG circuit provides a theoretical background for such a common mechanism (177). RANK-L upregulation is one among a number of factors influenced by pro-inflammatory cytokines such as TNF $\alpha$ , IL-1, IL-6, IL-17, IL-20 and IL-22 (146,147,179,191,192). All these cytokines activate osteoclasts through influencing the RANK/RANK-L/OPG circuit.

Findings reported in this thesis support the notion that treatment with biologic anti-TNF $\alpha$  agents arrests both generalised bone loss at lumbar spine and hip and localised bone loss in the hand of patients with RA. Interestingly, the beneficial effects of biologic anti-TNF $\alpha$  agents on BMD did not completely parallel improvements in the patients' DAS28 scores. The beneficial effects of biologic anti-TNF $\alpha$  agents were also consistent with changes in plasma levels of biomarkers of bone turnover and regulatory proteins.

In general, this study and several previous studies highlight the beneficial effects of biologic anti-TNF $\alpha$  agents on bone. However, the precise mechanism(s) through which such beneficial effect is achieved remains unclear partly due to the relatively small sample size, short follow-up periods and lack adequate controls. Studies of the molecular correlates and targets of the beneficial effects of biologic anti-TNF $\alpha$  agents requires large sample size, longer and sustained disease control to identify such factors and targets. However, similar to patients with osteoporosis, factors such as gender, age and ethnic origin all impact bone response to the treatment with biologic anti-TNF $\alpha$  agents.

#### 6.4) Future Perspectives

Biologic anti-TNF $\alpha$  agents are highly effective in suppressing disease in numerous inflammatory conditions (223). However, whilst the anti-inflammatory and disease-modifying effects on biologic anti-TNF $\alpha$  agents have been shown to suppress bone loss *in vivo* in animal models and *in vitro* using human cells, their effectiveness in reducing osteoporosis and fractures has not been universally recognised.

There are many pathways through which osteoclasts can be activated in addition to pathway(s) triggered by TNF $\alpha$  and these include pathways mediated, or triggered by IL-1, IL-6 and IL-17 (227). However, the results presented in this thesis clearly indicate TNF $\alpha$  is the major inducer of bone loss in RA. Nevertheless, despite the limited evidence provided herein, further larger studies will be required to identify the exact pathways and targets through which inhibiting TNF $\alpha$  with the biologic agents could bring about such outcome.

There is some evidence to indicate that treatment with biologic anti-TNF $\alpha$  agents have indirect anti-resorptive effects through controlling inflammation. However, several studies that have addressed this issue have relatively short follow-up periods, small sample size, or lack suitable controls (118,126,128,225). Thus, to provide evidence for such mechanisms the effect of biologic anti-TNF $\alpha$  agents would require longer and continued disease control to clarify how such effects benefit bone in patients with RA.

As part of the national research programme in the Kingdom of Saudi Arabia, part of the current work will be expanded to include RA patients from the country. Furthermore, the study will form a platform for identifying the factors that impact patients with osteoporosis since this disease has a high prevalence in that area with, potentially, additional more risk factors, particularly lifestyle.

In recent years, advanced imaging techniques have been explored to evaluate bone quality at multi levels, such as microarchitecture of trabecular bone, mineralisation, micro-damage and bone remodelling rates (228). At present, the microarchitecture of trabecular bone can be evaluated *in vivo* using high-resolution peripheral quantitative computed tomography (CT). Combining bone mineral density measurements by DEXA with CT images may offer an alternative option to assess bone response and treatment progress. Part of my future work will involve using CT to measure BMD in parallel with DEXA to link that with DEXA scan department of the Royal London Hospital under the supervision Dr. Akanle.

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# Appendices

## Appendix 1

**Table 1: Group statistics and independent samples test of BMD for short-term precision assessments.**

| Case                                      | N  | Mean  | Std. Deviation | Std. Error Mean |
|---|----|-------|----------------|-----------------|
| <b>Short-term precision</b>               |    |       |                |                 |
| - <b>With repositioning of phantom</b>    | 20 | 0.996 | 0.003          | 0.001           |
| - <b>Without repositioning of phantom</b> | 20 | 0.994 | 0.003          | 0.001           |

The spine phantom was scanned 20 times with and without repositioning between scans. The data was analysed as described in the Materials and Methods.

**Table 2: Levene's test and independent sample t-test assessment for measuring the equality of BMD means of short-term precision.**

|                                      | Levene's Test for Equality of Variances |      | t-test for Equality of Means |       |                 |            |                 |   |       |
|--------------------------------------|---|------|------------------------------|-------|-----------------|------------|-----------------|---|-------|
|                                      | F                                       | Sig. | T                            | df    | Sig. (2-tailed) | Mean Diff. | Std. Err. Diff. | 99% Confidence interval of the Difference |       |
|                                      |   |      |                              |       |                 |            |                 | Lower                                     | Upper |
| <b>Short-term precision</b>          |   |      |                              |       |                 |            |                 |   |       |
| - <b>Equal Variances assumed</b>     | .057                                    | .813 | 1.983                        | 38    | .055            | .002       | .001            | -.001                                     | .005  |
| - <b>Equal Variances not assumed</b> |   |      | 1.983                        | 37.94 | .055            | .002       | .001            | -.001                                     | .005  |

The spine phantom was scanned 20 times with and without changing its position between scans. Data analysed as described in the Material and Methods.

**Table 3: Group statistics and independent samples test of changes in lumbar and hip BMD for male and female patients.**

|                                   | Gender | N   | Mean | Std. Deviation | Std. Error Mean |
|-----------------------------------|--------|-----|------|----------------|-----------------|
| <b>BMD change at lumbar spine</b> | Male   | 114 | 6.90 | 8.075          | .756            |
|                                   | Female | 533 | 3.54 | 6.839          | .295            |
| <b>BMD change at hip</b>          | Male   | 114 | 2.48 | 6.066          | .568            |
|                                   | Female | 533 | -.78 | 4.990          | .215            |

Group statistics and independent samples test for changes in BMD in male and female patients are presented as the mean and data analysed as described in the Materials and Methods.

**Table 4: Levene's test and independent sample t-test assessment to measure the equality of changes in BMD of lumbar spine and hip between male and female patients.**

|                                  |                             | Levene's Test for Equality of Variances |      | t-test for Equality of Means |     |                 |            |                  |   |       |
|----------------------------------|-----------------------------|---|------|------------------------------|-----|-----------------|------------|------------------|---|-------|
|                                  |                             | F                                       | Sig. | t                            | df  | Sig. (2-tailed) | Mean Diff. | Std. Error Diff. | 95% Confidence Interval of the Difference |       |
|                                  |                             |   |      |                              |     |                 |            |                  | Lower                                     | Upper |
| <b>BMD change a lumbar spine</b> | Equal variances assumed     | 6.06                                    | .014 | 4.61                         | 651 | .000            | 3.36       | .729             | 1.931                                     | 4.793 |
|                                  | Equal variances not assumed |   |      | 4.14                         | 149 | .000            | 3.36       | .812             | 1.759                                     | 4.966 |
| <b>BMD change at hip</b>         | Equal variances assumed     | .105                                    | .746 | 6.09                         | 650 | .000            | 3.26       | .535             | 2.209                                     | 4.312 |
|                                  | Equal variances not assumed |   |      | 5.37                         | 147 | .000            | 3.26       | .607             | 2.060                                     | 4.461 |

**Table 5: Group statistics and independent samples test of changes in lumbar and total hip BMD for smoker and non-smoker patients.**

| Smoking                           |            | N   | Mean  | Std. Deviation | Std. Error Mean |
|-----------------------------------|------------|-----|-------|----------------|-----------------|
| <b>BMD change at lumbar spine</b> | Smoker     | 121 | 2.18  | 5.739          | .522            |
|                                   | non smoker | 360 | 4.96  | 7.656          | .401            |
| <b>BMD change at hip</b>          | Smoker     | 121 | -1.61 | 4.771          | .434            |
|                                   | non smoker | 360 | 0.7   | 5.600          | .293            |

Group statistics and independent samples test for changes in BMD in smoker and non-smoker patients are presented as the mean and data analysed as described in the Materials and Methods.

**Table 6: Levene’s test and independent sample t-test assessment to measure the equality of changes in BMD of lumbar spine and total hip between smoker and non-smoker patients.**

|                                   |                             | Levene's Test for Equality of Variances |      | t-test for Equality of Means |     |                 |                 |                       |   |        |
|-----------------------------------|-----------------------------|---|------|------------------------------|-----|-----------------|-----------------|-----------------------|---|--------|
|                                   |                             | F                                       | Sig. | t                            | df  | Sig. (2-tailed) | Mean Difference | Std. Error Difference | 95% Confidence Interval of the Difference |        |
|                                   |                             |   |      |                              |     |                 |                 |                       | Lower                                     | Upper  |
| <b>BMD change at lumbar spine</b> | Equal variances assumed     | 10.725                                  | .001 | -3.67                        | 484 | .000            | -2.783          | .758                  | -4.272                                    | -1.293 |
|                                   | Equal variances not assumed |   |      | -4.23                        | 272 | .000            | -2.783          | .658                  | -4.078                                    | -1.487 |
| <b>BMD change at hip</b>          | Equal variances assumed     | .344                                    | .001 | -2.97                        | 484 | .003            | -1.683          | .567                  | -2.797                                    | -.568  |
|                                   | Equal variances not assumed |   |      | -3.22                        | 238 | .001            | -1.683          | .523                  | -2.714                                    | -.652  |

## Appendix 2

**Table 1a: Paired samples test of changes in lumbar spine BMD for RA patients treated with biologic anti-TNF $\alpha$  agents.**

| Lumbar spine                          | Mean | N  | Std. Deviation | Std. Error Mean |
|---------------------------------------|------|----|----------------|-----------------|
| BMD before treatment                  | .917 | 62 | .143993        | .019968         |
| Pair 1 BMD after 2 years of treatment | .949 | 62 | .156637        | .021722         |

Data on changes in BMD in 62 RA patients treated with biologic anti-TNF $\alpha$  agents were compared before and 2 years after treatment.

**Table 1b: Paired sample t-test to measure the equality of changes in BMD of lumbar spine for RA patients before and 2 years after treatment with biologic anti-TNF $\alpha$  agents.**

|   | Paired Differences |                |                 |   |         | T     | df | Sig. (2-tailed) |
|---|--------------------|----------------|-----------------|---|---------|-------|----|-----------------|
|   | Mean               | Std. Deviation | Std. Error Mean | 95% Confidence Interval of the Difference |         |       |    |                 |
|   |                    |                |                 | Lower                                     | Upper   |       |    |                 |
| Pair 1 BMD lumbar before<br>-BMD lumbar after 2 yrs | -.0314             | .05056         | .00701          | -.04552                                   | -.01737 | -4.49 | 51 | .000            |

**Table.1c: Wilcoxon signed rank test to measure changes in lumbar spine BMD of RA patients treated with biologic anti-TNF $\alpha$  agents.**

|   | Null Hypothesis  | Test                                      | Sig. | Decision                    |
|---|--|---|------|-----------------------------|
| 1 | The median of differences between BaselineBMDLumbar and BMDLumbarafterTwoYrs equals 0. | Related-Samples Wilcoxon Signed Rank Test | .000 | Reject the null hypothesis. |

Data on changes in BMD of 62 RA patients treated with biologic anti-TNF $\alpha$  agents were compared before and 2 years after treatment.

**Table 2a: Paired samples test of changes in hip BMD for RA patients treated with biologic anti-TNF $\alpha$  agents.**

| Total hip                          | Mean | N  | Std. Deviation | Std. Error Mean |
|------------------------------------|------|----|----------------|-----------------|
| <b>BMD before treatment</b>        | .832 | 62 | .112484        | .017851         |
| <b>BMD after treatment (2 yrs)</b> | .827 | 62 | .112180        | .018509         |

Data on changes in BMD in 62 RA patients treated with biologic anti-TNF $\alpha$  agents were compared before and 2 years after treatment.

**Table 2b: Paired sample t-test to measure the equality of changes in BMD of total hip for RA patients before and 2 years after treatment with biologic anti-TNF $\alpha$  agents.**

|   | Paired Differences |                |                 |   |       | t    | df | Sig. (2-tailed) |
|---|--------------------|----------------|-----------------|---|-------|------|----|-----------------|
|   | Mean               | Std. Deviation | Std. Error Mean | 95% Confidence Interval of the Difference |       |      |    |                 |
|   |                    |                |                 | Lower                                     | Upper |      |    |                 |
| <b>Total hip BMD before</b><br><b>Pair 1 -BMD after treatment</b><br><b>(2 yrs)</b> | .0053              | .03469         | .00486          | -.0044                                    | .0151 | 1.09 | 50 | .412            |

**Table 2c: Wilcoxon signed rank test to measure changes in total hip BMD RA patients treated with biologic anti-TNF $\alpha$  agents.**

|          | Null Hypothesis  | Test                                      | Sig. | Decision                    |
|----------|--|---|------|-----------------------------|
| <b>1</b> | The median of differences between hipBMDbefore and hipBMDafter2yrs equals 0. | Related-Samples Wilcoxon Signed Rank Test | .412 | Retain the null hypothesis. |

Data on changes in BMD in 62 RA patients treated with biologic anti-TNF $\alpha$  agents were compared before and 2 years after treatment.

### Appendix 3

**Table 1a: Changes in lumbar spine BMD for RA patients treated with biologic anti-TNF $\alpha$  agents.**

| Lumbar spine (responder patients) |                               | Mean  | N | Std. Deviation | Std. Error Mean |
|-----------------------------------|-------------------------------|-------|---|----------------|-----------------|
| Pair 1                            | BMD before treatment          | 1.069 | 7 | .20147         | .07615          |
|                                   | BMD after 1 year of treatment | 1.063 | 7 | .19328         | .07305          |

Data on changes of BMD in 7 responder patients, based on improvement in DAS28 were compared before and 1 year after treatment.

**Table 1b: Paired sample t-test to measure the equality of changes in BMD of lumbar spine for responder RA patients before and 1 year after treatment with biologic anti-TNF $\alpha$  agents.**

| Lumbar spine BMD | Paired Differences                                     |                |                 |   |        | t     | df   | Sig. (2-tailed) |      |
|------------------|--|----------------|-----------------|---|--------|-------|------|-----------------|------|
|                  | Mean   | Std. Deviation | Std. Error Mean | 95% Confidence Interval of the Difference |        |       |      |                 |      |
|                  |  |                |                 | Lower                                     | Upper  |       |      |                 |      |
| Pair 1           | BMD before treatment<br>-BMD after 1 year of treatment | .0071          | .03390          | .0128                                     | -.0242 | .0385 | .557 | 6               | .597 |

**Table 1c: Changes in lumbar spine BMD for RA patients treated with biologic anti-TNF $\alpha$  agents.**

|  | Mean | N | Std. Deviation | Std. Error Mean |
|--|------|---|----------------|-----------------|
| <b>Lumbar spine (non-responder patients)</b> |      |   |                |                 |
| <b>Pair 1 BMD before treatment</b>           | .941 | 3 | .068923        | .039793         |
| <b>BMD after 1 year of treatment</b>         | .91  | 3 | .076788        | .044333         |

Data on changes of BMD in 3 non-responder patients, based on improvement in DAS28 were compared before and 1 year after treatment.

**Table 1d: Paired sample t-test to measure the equality of changes in BMD of lumbar spine for non-responder RA patients before and 1 year after treatment with biologic anti-TNF $\alpha$  agents.**

|   | Paired Differences |                |                 |   |        | t     | df | Sig. (2-tailed) |
|---|--------------------|----------------|-----------------|---|--------|-------|----|-----------------|
|   | Mean               | Std. Deviation | Std. Error Mean | 95% Confidence Interval of the Difference |        |       |    |                 |
|   |                    |                |                 | Lower                                     | Upper  |       |    |                 |
| <b>Lumbar spine BMD</b>                                 |                    |                |                 |   |        |       |    |                 |
| <b>Pair 1 BMD before -BMD after 1 year of treatment</b> | .0310              | .01345         | .00777          | -.00242                                   | .06442 | 3.991 | 2  | .057            |

**Table 2a: Changes in hip BMD for RA patients treated with biologic anti-TNF $\alpha$  agents.**

| Hip (responder patients) |                               | Mean | N | Std. Deviation | Std. Error Mean |
|--------------------------|-------------------------------|------|---|----------------|-----------------|
| Pair 1                   | BMD before treatment          | .992 | 7 | .29175         | .11027          |
|                          | BMD after 1 year of treatment | .989 | 7 | .27443         | .10373          |

Data on changes of BMD in 7 responder patients, based on improvement in DAS28 were compared before and 1 year after treatment.

**Table 2b: Paired sample t-test to measure the equality of changes in BMD of hip for responder RA patients before and 1 year after treatment with biologic anti-TNF $\alpha$  agents.**

| Hip BMD  | Paired Differences |                |                 |   |        | t    | df | Sig. (2-tailed) |
|--|--------------------|----------------|-----------------|---|--------|------|----|-----------------|
|  | Mean               | Std. Deviation | Std. Error Mean | 95% Confidence Interval of the Difference |        |      |    |                 |
|  |                    |                |                 | Lower                                     | Upper  |      |    |                 |
| Pair 1 BMD before treatment – BMD after 1 year | .0033              | .03487         | .01318          | -.02897                                   | .03554 | .249 | 6  | .811            |

**Table 2c: Changes in hip BMD for RA patients treated with biologic anti-TNF $\alpha$  agents.**

|                                     | Mean | N | Std. Deviation | Std. Error Mean |
|-------------------------------------|------|---|----------------|-----------------|
| <b>Hip (non-responder patients)</b> |      |   |                |                 |
| <b>Pair 1 BMD before treatment</b>  | .926 | 3 | .069981        | .040404         |
| <b>BMD after 1 year</b>             | .931 | 3 | .092065        | .053154         |

Data on changes of BMD in 3 non-responder patients, based on improvement in DAS28 were compared before and 1 year after treatment.

**Table 2d: Paired sample t-test to measure the equality of changes in BMD of hip for non-responder RA patients before and 1 year after treatment with biologic anti-TNF $\alpha$  agents.**

|   | Paired Differences |                |                 |   |        | t     | df | Sig.<br>(2-tailed) |
|---|--------------------|----------------|-----------------|---|--------|-------|----|--------------------|
|   | Mean               | Std. Deviation | Std. Error Mean | 95% Confidence Interval of the Difference |        |       |    |                    |
|   |                    |                |                 | Lower                                     | Upper  |       |    |                    |
| <b>Total hip</b>  |                    |                |                 |   |        |       |    |                    |
| <b>Pair 1 BMD before –BMD after 1 year of treatment</b> | -.005              | .034429        | .0199           | -.09086                                   | .08019 | -.268 | 2  | .814               |

**Table 3a: Changes in hand BMD for RA patients treated with biologic anti-TNF $\alpha$  agents.**

| Hand BMD (responder patients) |                           | Mean | N | Std. Deviation | Std. Error Mean |
|-------------------------------|---------------------------|------|---|----------------|-----------------|
| Pair 1                        | BMD before treatment      | .58  | 6 | .09214         | .04607          |
|                               | BMD after 6m of treatment | .60  | 6 | .09684         | .04842          |

Data on changes of BMD in 6 responder patients, based on improvement in DAS28, were compared before and 6 months after treatment.

**Table 3b: Paired sample t-test to measure the equality of changes in BMD of hand for responder RA patients before and 6 months after treatment with biologic anti-TNF $\alpha$  agents.**

| Hand BMD                                      | Paired Differences |                |                 |   |        | t     | df | Sig. (2-tailed) |
|---|--------------------|----------------|-----------------|---|--------|-------|----|-----------------|
|   | Mean               | Std. Deviation | Std. Error Mean | 95% Confidence Interval of the Difference |        |       |    |                 |
|   |                    |                |                 | Lower                                     | Upper  |       |    |                 |
| Pair 1 BMD before – BMD after 6m of treatment | -.0002             | .00487         | .00244          | -.00793                                   | .00758 | -.073 | 3  | .947            |

**Table 3c: Changes in hand BMD for RA patients treated with biologic anti-TNF $\alpha$  agents.**

| Hand BMD (non-responder patients)     | Mean   | N | Std. Deviation | Std. Error Mean |
|---------------------------------------|--------|---|----------------|-----------------|
| <b>Pair 1</b><br>BMD before treatment | .52254 | 3 | .032919        | .023277         |
| BMD after 6m of treatment             | .51876 | 3 | .035268        | .024938         |

Data on changes of BMD in 3 non-responder patients, based on improvement in DAS28 were compared before and 6 months after treatment.

**Table 3d: Paired sample t-test to measure the equality of changes in BMD of hand for non-responder RA patients before and 6 months after treatment with biologic anti-TNF $\alpha$  agents.**

|  | Paired Differences |                |                 |   |        | t    | df | Sig. (2-tailed) |
|--|--------------------|----------------|-----------------|---|--------|------|----|-----------------|
|  | Mean               | Std. Deviation | Std. Error Mean | 95% Confidence Interval of the Difference |        |      |    |                 |
|  |                    |                |                 | Lower                                     | Upper  |      |    |                 |
| <b>BMD before – BMD</b><br><b>Pair 1 after 6m of treatment</b> | .0038              | .002349        | .00166          | -.01734                                   | .02488 | 2.27 | 1  | .264            |

## Appendix 4

**Table 1a: Paired sample t-test to measure changes in osteocalcin for RA patients treated with biologic anti-TNF $\alpha$  agents.**

|        |                   | Mean<br>(pg/ml) | N  | Std.<br>Deviation | Std. Error<br>Mean |
|--------|-------------------|-----------------|----|-------------------|--------------------|
| Pair 1 | Osteocalcin (t0)  | 15566.1         | 37 | 10493.1           | 1708.6             |
|        | Osteocalcin (t1m) | 15916.7         | 37 | 10960.8           | 1801.9             |
| Pair 2 | Osteocalcin (t0)  | 14510.3         | 31 | 11102.8           | 1994.1             |
|        | Osteocalcin (t3m) | 16454.7         | 31 | 11533.8           | 2071.5             |

Data on changes of osteocalcin in responder patients were compared before (t0) and 1 (t1m) and 3 months (t3m) after treatment.

**Table 1b: Paired sample t-test to measure the paired differences in osteocalcin for responder RA patients before and 1 and 3 months after treatment with biologic anti-TNF $\alpha$  agents.**

|        |   | Paired Differences |                   |                       |   | t      | df    | Sig. (2-tailed) |       |
|--------|---|--------------------|-------------------|-----------------------|---|--------|-------|-----------------|-------|
|        |   | Mean               | Std.<br>Deviation | Std.<br>Error<br>Mean | 95% Confidence<br>Interval of the<br>Difference |        |       |                 |       |
|        |   |                    |                   |                       | Lower   |        |       |                 | Upper |
| Pair 1 | Osteocalcin (t0) –<br>Osteocalcin (t1m) | -850.6             | 4554.5            | 748.76                | -2369.18  | 667.93 | -1.14 | 36              | .263  |
| Pair 2 | Osteocalcin (t0) –<br>Osteocalcin (t3m) | -1944.3            | 8240.4            | 1480.1                | -4966.94  | 1078.3 | -1.31 | 30              | .199  |

**Table 1c: Paired sample t-test to measure changes in osteocalcin for RA patients treated with biologic anti-TNF $\alpha$  agents.**

|        |                   | Mean<br>(pg/ml) | N  | Std. Deviation | Std. Error<br>Mean |
|--------|-------------------|-----------------|----|----------------|--------------------|
| Pair 1 | Osteocalcin (t0)  | 11614.8         | 12 | 8078.6         | 2332.1             |
|        | Osteocalcin (t1m) | 14266.1         | 12 | 7740.2         | 2234.4             |
| Pair 2 | Osteocalcin (t0)  | 10775.8971      | 10 | 8416.1         | 2661.4             |
|        | Osteocalcin (t3m) | 14854.6042      | 10 | 8060.3         | 2548.9             |

Data on changes of osteocalcin in non-responder patients were compared before (t0) and 1 (t1m) and 3 months (t3m) after treatment.

**Table 1d: Paired sample t-test to measure the paired differences in osteocalcin for non-responder RA patients before and 1 and 3 months after treatment with biologic anti-TNF $\alpha$  agents.**

|   | Paired Differences |                |                 |   |          | t      | df | Sig.<br>(2-tailed) |
|---|--------------------|----------------|-----------------|---|----------|--------|----|--------------------|
|   | Mean               | Std. Deviation | Std. Error Mean | 95% Confidence Interval of the Difference |          |        |    |                    |
|   |                    |                |                 | Lower                                     | Upper    |        |    |                    |
| Pair 1<br>Osteocalcin (t0) -<br>Osteocalcin (t1m) | -2651.2            | 5605.1         | 1618.1          | -6212.6                                   | 910.13   | -1.639 | 11 | .130               |
| Pair 2<br>Osteocalcin (t0) -<br>Osteocalcin (t3m) | -4068.7            | 3958.5         | 1251.8          | -6900.5                                   | -1236.95 | -3.250 | 9  | .010               |

**Table 2a: Paired sample t-test to measure changes in CTX for RA patients treated with biologic anti-TNF $\alpha$  agents.**

|               |                  | Mean<br>(pg/ml) | N  | Std.<br>Deviation | Std. Error<br>Mean |
|---------------|------------------|-----------------|----|-------------------|--------------------|
| <b>Pair 1</b> | <b>CTX (t0)</b>  | 163.8           | 28 | 125.2             | 24.1               |
|               | <b>CTX (t1m)</b> | 169.9           | 28 | 108.1             | 20.4               |
| <b>Pair 2</b> | <b>CTX (t0)</b>  | 146.8           | 23 | 107.4             | 22.4               |
|               | <b>CTX (t3m)</b> | 131.8           | 23 | 129.9             | 27.1               |

Data on changes of CTX in responder patients were compared before (t0) and 1 (t1m) and 3 months (t3m) after treatment.

**Table 2b: Paired sample t-test to measure the paired differences in CTX for responder RA patients before and 1 and 3 months after treatment with biologic anti-TNF $\alpha$  agents.**

|              |                                | Paired Differences |                   |                    |   | t     | df    | Sig.<br>(2-<br>tailed) |       |
|--------------|--------------------------------|--------------------|-------------------|--------------------|---|-------|-------|------------------------|-------|
|              |                                | Mean               | Std.<br>Deviation | Std. Error<br>Mean | 95% Confidence<br>Interval of the<br>Difference |       |       |                        |       |
|              |                                |                    |                   |                    | Lower   |       |       |                        | Upper |
| <b>Pair1</b> | <b>CTX (t0)-<br/>CTX (1m)</b>  | -8.28              | 78.47             | 14.83              | -38.71  | 22.15 | -.558 | 27                     | .581  |
| <b>Pair2</b> | <b>CTX (t0) -<br/>CTX (3m)</b> | 15.1               | 94.54             | 19.71              | -25.88  | 55.86 | .761  | 22                     | .455  |

**Table 2c: Paired sample t-test to measure changes in CTX for RA patients treated with biologic anti-TNF $\alpha$  agents.**

|               |                  | Mean (pg/ml) | N | Std. Deviation | Std. Error Mean |
|---------------|------------------|--------------|---|----------------|-----------------|
| <b>Pair 1</b> | <b>CTX (t0)</b>  | 127.1        | 9 | 103.6          | 34.5            |
|               | <b>CTX (t1m)</b> | 137.1        | 9 | 72.4           | 24.1            |
| <b>Pair 2</b> | <b>CTX (t0)</b>  | 132.6        | 8 | 109.3          | 38.9            |
|               | <b>CTX (3m)</b>  | 140.4        | 8 | 72.7           | 25.7            |

Data on changes of CTX in non-responder patients were compared before (t0) and 1 (t1m) and 3 months (t3m) after treatment.

**Table 2d: Paired sample t-test to measure the paired differences in CTX for non-responder RA patients before and 1 and 3 months after treatment with biologic anti-TNF $\alpha$  agents.**

|               |                             | Paired Differences |                |                 |   | t     | df    | Sig. (2-tailed) |       |
|---------------|-----------------------------|--------------------|----------------|-----------------|---|-------|-------|-----------------|-------|
|               |                             | Mean               | Std. Deviation | Std. Error Mean | 95% Confidence Interval of the Difference |       |       |                 |       |
|               |                             |                    |                |                 | Lower                                     |       |       |                 | Upper |
| <b>Pair 1</b> | <b>CTX (t0) - CTX (t1m)</b> | -10.1              | 93.18          | 31.1            | -81.69                                    | 61.56 | -.324 | 8               | .75   |
| <b>Pair 2</b> | <b>CTX (t0) - CTX (t3m)</b> | -7.8               | 93.3           | 32.98           | -85.8                                     | 70.2  | -.236 | 7               | .58   |

**Table 3a: Paired sample t-test to measure changes in RANK-L for RA patients treated with biologic anti-TNF $\alpha$  agents.**

|               |                     | Mean<br>(pg/ml) | N  | Std. Deviation | Std. Error<br>Mean |
|---------------|---------------------|-----------------|----|----------------|--------------------|
| <b>Pair 1</b> | <b>RANK-L (t0)</b>  | 197.3           | 38 | 148.3          | 24.1               |
|               | <b>RANK-L (t1m)</b> | 160.6           | 38 | 102.2          | 24.7               |
| <b>Pair 2</b> | <b>RANK-L (t0)</b>  | 212.3           | 33 | 193.8          | 33.7               |
|               | <b>RANK-L (t3m)</b> | 170.4           | 33 | 100.4          | 26.1               |

Data on changes of RANK-L in responder patients were compared before (t0) and 1 (t1m) and 3 months (t3m) after treatment.

**Table 3b: Paired sample t-test to measure the paired differences in RANK-L for responder RA patients before and 1 and 3 months after treatment with biologic anti-TNF $\alpha$  agents.**

|               |                                       | Paired Differences |                   |                    |   |       | t     | df | Sig.<br>(2-tailed) |
|---------------|---------------------------------------|--------------------|-------------------|--------------------|---|-------|-------|----|--------------------|
|               |                                       | Mean               | Std.<br>Deviation | Std. Error<br>Mean | 95% Confidence<br>Interval of the<br>Difference |       |       |    |                    |
|               |                                       |                    |                   |                    | Lower   | Upper |       |    |                    |
| <b>Pair 1</b> | <b>RANK-L (t0) –<br/>RANK-L (t1m)</b> | 17.8               | 63.83763          | 10.36              | -3.21   | 38.76 | 1.717 | 37 | .094               |
| <b>Pair 2</b> | <b>RANK-L (t0) –<br/>RANK-L (t3m)</b> | 41.9               | 116.65653         | 20.30730           | .51   | 83.23 | 2.062 | 32 | .047               |

**Table 3c: Paired sample t-test to measure changes in RANK-L for RA patients treated with biologic anti-TNF $\alpha$  agents.**

|               |                     | Mean<br>(pg/ml) | N  | Std. Deviation | Std. Error<br>Mean |
|---------------|---------------------|-----------------|----|----------------|--------------------|
| <b>Pair 1</b> | <b>RANK-L (t0)</b>  | 185.6           | 10 | 89.7           | 65.4               |
|               | <b>RANK-L (t1m)</b> | 222.7           | 10 | 104.7          | 91                 |
| <b>Pair 2</b> | <b>RANK-L (t0)</b>  | 200.5           | 9  | 213.5          | 71.2               |
|               | <b>RANK-L (t3m)</b> | 192.5           | 9  | 110.4          | 70.3               |

Data on changes of RANK-L in non-responder patients were compared before (t0) and 1 (t1m) and 3 months (t3m) after treatment.

**Table 3d: Paired sample t-test to measure the paired differences in RANK-L for non-responder RA patients before and 1 and 3 months after treatment with biologic anti-TNF $\alpha$  agents.**

|               |                                   | Paired Differences |                |                 |   | t    | df    | Sig.<br>(2-tailed) |       |
|---------------|-----------------------------------|--------------------|----------------|-----------------|---|------|-------|--------------------|-------|
|               |                                   | Mean               | Std. Deviation | Std. Error Mean | 95% Confidence Interval of the Difference |      |       |                    |       |
|               |                                   |                    |                |                 | Lower                                     |      |       |                    | Upper |
| <b>Pair 1</b> | <b>RANK-L (t0) – RANK-L (t1m)</b> | -37.1              | 106.3          | 33.6            | -113.1                                    | 39   | -1.10 | 9                  | .12   |
| <b>Pair 2</b> | <b>RANK-L (t0) – RANK-L (t3m)</b> | 7.9                | 38.5           | 12.8            | -21.6                                     | 37.5 | .619  | 8                  | .83   |

**Table 4a: Paired sample t-test to measure changes in OPG for RA patients treated with biologic anti-TNF $\alpha$  agents.**

|        | Mean (pg/ml) | N  | Std. Deviation | Std. Error Mean |
|--------|--------------|----|----------------|-----------------|
| Pair 1 | OPG (t0)     | 38 | 1529.6         | 248.1           |
|        | OPG (t1m)    | 38 | 1387.9         | 225.1           |
| Pair 2 | OPG (t0)     | 33 | 1508.5         | 262.6           |
|        | OPG (t3m)    | 33 | 1438.9         | 250.5           |

Data on changes of OPG in responder patients were compared before (t0) and 1 (t1m) and 3 months (t3m) after treatment.

**Table 4b: Paired sample t-test to measure the paired differences in OPG for responder RA patients before and 1 and 3 months after treatment with biologic anti-TNF $\alpha$  agents.**

|                             | Paired Differences |                |                 |   |       | t    | df | Sig. (2-tailed) |
|-----------------------------|--------------------|----------------|-----------------|---|-------|------|----|-----------------|
|                             | Mean               | Std. Deviation | Std. Error Mean | 95% Confidence Interval of the Difference |       |      |    |                 |
|                             |                    |                |                 | Lower                                     | Upper |      |    |                 |
| Pair 1 OPG (t0) – OPG (t1m) | 190.3              | 657.7          | 106.7           | -25.8                                     | 406.5 | 1.78 | 37 | .083            |
| Pair 2 OPG (t0) – OPG (t3m) | -119.6             | 732.7          | 127.5           | -379.4                                    | 140.2 | -.94 | 32 | .355            |

**Table 4c: Paired sample t-test to measure changes in OPG for RA patients treated with biologic anti-TNF $\alpha$  agents.**

|        |           | Mean<br>(pg/ml) | N  | Std. Deviation | Std. Error<br>Mean |
|--------|-----------|-----------------|----|----------------|--------------------|
| Pair 1 | OPG (t0)  | 2829.4          | 12 | 1208.4         | 348.8              |
|        | OPG (t1m) | 2861.9          | 12 | 1462.2         | 422.1              |
| Pair 2 | OPG (t0)  | 2911.8          | 11 | 1231.5         | 371.3              |
|        | OPG (t3m) | 2791.3          | 11 | 1588.1         | 478.8              |

Data on changes of OPG in non-responder patients were compared before (t0) and 1 (t1m) and 3 months (t3m) after treatment.

**Table 4d: Paired sample t-test to measure the paired differences in OPG for non-responder RA patients before and 1 and 3 months after treatment with biologic anti-TNF $\alpha$  agents.**

|                             | Paired Differences |                |                 |   |        | t     | df | Sig. (2-tailed) |
|-----------------------------|--------------------|----------------|-----------------|---|--------|-------|----|-----------------|
|                             | Mean               | Std. Deviation | Std. Error Mean | 95% Confidence Interval of the Difference |        |       |    |                 |
|                             |                    |                |                 | Lower                                     | Upper  |       |    |                 |
| Pair 1 OPG (t0) – OPG (t1m) | -32.6              | 920.1          | 265.6           | -617.2                                    | 551.9  | -.123 | 11 | .904            |
| Pair 2 OPG (t0) – OPG (t3m) | 195.5              | 1338.3         | 403.5           | -703.6                                    | 1094.6 | .485  | 10 | .638            |

## Scientific Communications

### Publications:

Mohammed M. Albogami, Ali S. Jawad, Rizgar A. Mageed. Bone loss in osteoporosis and arthritis, pathogenesis and therapeutic strategies. Saudi Med J 2014; 35 (3):224-33.

Bystrom J, Al-Adhoubi N, Al-Bogami M, Jawad AS, Mageed RA. Th17 lymphocytes in respiratory syncytial virus infection. Viruses. 2013;5(3):777-91

M. M. Al-Bogami, M. A. Alkhorayef, O. A. Akanle, A. S. Jawad, R. A. Mageed. The effects of smoking on response of osteoporosis treatment using dual energy X-ray absorptiometry scans. Transactions of the American Nuclear Society 109: (1) 51-52 (2013).

### Article under revision (as requested by the journal):

Mohammed Al-Bogami, Mohammed Alkhorayef, Jonas Bystrom, Olufunso A. Akanle, Nasra Al-Adhoubi, Ali S. Jawad, Rizgar A. Mageed. Superior bone mineral density improvement in osteoporosis patients treated with zoledronate compared with alendronate: The outcome of a 2 year study. Int Osteoporosis Journal.

### Article in preparation

Jonas Bystrom, Taher Taher, Mohammed Al-Bogami, Richard O Williams, Ali S. Jawad, Rizgar A. Mageed. Cytokine profile from anti-TNF treated rheumatoid arthritis patients reveals a responsive T lymphocyte population.