By
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Changes in bone density and bone turnover in patients with rheumatoid arthritis treated with rituximab, a B cell depleting monoclonal antibody

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ABSTRACT

Background
The role of B-lymphocytes regarding bone turnover is controversial (1). Some studies showed that treatment with tumor necrosis factor alpha (TNFα) blockers could prevent structural bone damage and improve bone mineral density (BMD) in RA (2). Hence, we set up this study to determine rituximab will have similar effect on bone turnover and the possible mechanisms.

Aim
To assess the effect of rituximab on bone turnover and to correlate these changes with circulating B cells in RA.

Materials & Methods
45 RA patients starting rituximab were assessed over 5 visits for one year. Patients were treated with two courses of rituximab. Patients were assessed clinically using DAS28, BMD using DXA scan, bone formation markers (BAP, P1NP), osteocyte markers (DKK1 and sclerostin), resorption markers (TRAP5b and CTX) and flow cytometry of peripheral blood CD19+ cells including subsets.

Results
Subjects were 59.3±12.1 years old mostly females (80%). 57.8% were postmenopausal. Patients had severe active disease at baseline DAS28=6.1±1.3. 68.9% of patients had vitamin D deficiency at baseline. 15(33.3%) of patients took prednisolone 11.2±9.8 mg. DAS28 score at 12 months decreased to 4.7±1.4 compared to 6.1±1.3 at baseline (P<0.001). Median (95%CI) P1NP levels increased from 41.9 (34.1-46.4) to 47.7 (42.0-61.4) after 12 months. Yet, the change was not statistically significant. BAP increased significantly from 19.0±6.5 to 21.3±7.7 (P=0.007). There was insignificant small reduction in TRAP5b and CTX levels during follow up. Osteocyte markers (sclerostin and DKK1) were stable during follow up. After performing Bonferroni correction, the increase in sclerostin levels between 6 and 12 months was found to be statistically significant (p=0.019). Depletion of most of CD19 B cells following baseline with increase at 6th month then continued to decrease following the second course of rituximab. After 12 months, BMD slightly decreased at all sites. However, the reduction was statistically significant only at total hip (p=0.041). There was significant positive independent association of BMD L2-L4 at 12 months with CD19 B-lymphocytes at baseline.

Conclusion
Based on results obtained from bone markers, B cell depletion using rituximab increased bone formation and slightly reduced bone resorption markers. However, sclerostin slightly increased from 6th to 12 month. BMD results were inconsistent with bone markers findings, as there was a reduction in all sites mostly at total hip. Furthermore, CD19+ B cells were found to have a direct positive association with BMD at LS suggesting that B cell depletion is not protective for bone metabolism as previously expected.
Key Words: “bone markers”, “bone resorption”, “bone formation”, “osteocyte biomarkers”, “bone mineral density”, “bone turnover”, “rheumatoid arthritis” and “disease activity indices”.

References
Dedication

I dedicate my dissertation work to my father, professor Dr. Hassan El Shahaly, who has been a wonderful father, a great friend and an amazing mentor. Thanks to his guidance, words of encouragement and push for tenacity, this work has been finished. He was wishing to see this work after completion. Though, his shocking death did not allow this to happen. I am indebted to him with everything in my life.

I also dedicate this work to my mother, my wife and my kids for bearing me during the hard times over the last five years. A special feeling of gratitude belongs to my loving grandmother for always being there for me.
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<tr>
<td>AGEs</td>
<td>Advanced glycation end products</td>
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<tr>
<td>ALP</td>
<td>Alkaline phosphatase</td>
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<td>ANA</td>
<td>Antinuclear antibodies</td>
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<tr>
<td>BAP</td>
<td>Bone specific alkaline phosphatase</td>
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<tr>
<td>βCTX</td>
<td>Beta-isomerised carboxy terminal telopeptide of type I collagen</td>
</tr>
<tr>
<td>BMD</td>
<td>Bone mineral density</td>
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<td>BMI</td>
<td>Body mass index</td>
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<td>BMU</td>
<td>Basic multicellular unit</td>
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<tr>
<td>BPM</td>
<td>Beats per minute</td>
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<td>CI</td>
<td>Confident interval</td>
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<tr>
<td>CNS</td>
<td>Central nervous system</td>
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<td>CRP</td>
<td>C-reactive protein</td>
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<tr>
<td>CTX</td>
<td>Carboxy-terminal cross-linked telopeptides of type 1 collagen</td>
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<tr>
<td>CVD</td>
<td>Cardiovascular disease</td>
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<tr>
<td>CV,</td>
<td>Total analytical imprecision</td>
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<tr>
<td>CV,</td>
<td>Within-subject coefficient of variation</td>
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<tr>
<td>DAS28</td>
<td>Disease activity score 28 joints</td>
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<tr>
<td>DBP</td>
<td>Diastolic blood pressure</td>
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<tr>
<td>DEXA</td>
<td>Dual-energy X-ray absorptiometry</td>
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<tr>
<td>DKK-1</td>
<td>Dickkopf-related protein 1</td>
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<tr>
<td>DM</td>
<td>Diabetes mellitus</td>
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<tr>
<td>DMARD</td>
<td>Disease modifying anti-rheumatic drug</td>
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<td>Disease modifying anti-rheumatic drugs</td>
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<td>DMP1</td>
<td>Dentin matrix protein 1</td>
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<tr>
<td>DPD</td>
<td>Dioxypyridinoline</td>
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<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<tr>
<td>ESR</td>
<td>Erythrocyte sedimentation rate</td>
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<td>EULAR</td>
<td>European League Against Rheumatism</td>
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<td>FZ</td>
<td>Frizzled protein</td>
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<td>GFR</td>
<td>Glomerular filtration rate</td>
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<td>GH</td>
<td>Growth hormone</td>
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<td>GM-CSF</td>
<td>Granulocyte-macrophage colony-stimulating factor</td>
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<td>HAQ</td>
<td>Heath assessment questionnaire</td>
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<td>HB</td>
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<td>HCQ</td>
<td>Hydroxychloroquine</td>
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<td>Hematocrit</td>
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<td>Heart rate</td>
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<td>HRT</td>
<td>Hormone replacement therapy</td>
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<td>IDS</td>
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<td>IFN-γ</td>
<td>Interferon-gamma</td>
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<td>Ig</td>
<td>Immunoglobulin</td>
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<td>IGF1</td>
<td>Insulin-like growth factor 1</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>KG</td>
<td>Kilogram</td>
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<tr>
<td>KG/M2</td>
<td>Kilogram per square meter</td>
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<tr>
<td>LRP</td>
<td>Low density lipoprotein receptor-related protein</td>
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<tr>
<td>L2-4</td>
<td>Lumbar vertebrae 2-4</td>
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<tr>
<td>MEPE</td>
<td>Matrix extracellular phosphoglycoprotein</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>µg/L</td>
<td>Microgram per liter</td>
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<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
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<tr>
<td>NICE</td>
<td>National institute for clinical excellence</td>
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<td>NMOL</td>
<td>Nanomole</td>
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<tr>
<td>NTX</td>
<td>Amino-terminal cross-linked telopeptide of type 1 collagen</td>
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<td>NSAIDs</td>
<td>Non-steroidal anti-inflammatory drugs</td>
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<tr>
<td>OB</td>
<td>Osteoblast</td>
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<td>OC</td>
<td>Osteocalcin</td>
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<td>OCY</td>
<td>Osteocyte</td>
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<td>OPG</td>
<td>Osteoprotegerin</td>
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<td>OPN</td>
<td>Osteopontin</td>
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<tr>
<td>PhGA</td>
<td>Physician global assessment</td>
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<tr>
<td>PICP</td>
<td>Procollagen type 1 carboxy-terminal propeptide</td>
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<tr>
<td>PINP</td>
<td>Procollagen type 1 amino-terminal propeptide</td>
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<tr>
<td>PtGA</td>
<td>Patient global assessment</td>
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<tr>
<td>PTH</td>
<td>Parathyroid hormone</td>
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<tr>
<td>p-value</td>
<td>Probability value</td>
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<td>RA</td>
<td>Rheumatoid arthritis</td>
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<tr>
<td>RANK</td>
<td>Receptor activator of nuclear factor kappa B</td>
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<tr>
<td>RANKL</td>
<td>Receptor activator of nuclear factor kappa B ligand</td>
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<tr>
<td>RTX</td>
<td>Rituximab</td>
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<tr>
<td>SBP</td>
<td>Systolic blood pressure</td>
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<td>SCL</td>
<td>Sclerostin</td>
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<tr>
<td>SD</td>
<td>Standard deviation</td>
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<tr>
<td>SERMs</td>
<td>Selective estrogen receptor moderators</td>
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<tr>
<td>SJC</td>
<td>Swollen joint count</td>
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<tr>
<td>t</td>
<td>Student (paired) t-test</td>
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<tr>
<td>TGFβ-1</td>
<td>Transforming growth factor beta-1</td>
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<tr>
<td>Th</td>
<td>T-helper</td>
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<tr>
<td>TJC</td>
<td>Tender joint count</td>
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<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor-alpha</td>
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<td>TRAP</td>
<td>Tartrate resistant acid phosphatase</td>
</tr>
<tr>
<td>TSH</td>
<td>Thyroid stimulating hormone</td>
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<tr>
<td>UK</td>
<td>United Kingdom</td>
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<tr>
<td>WBC</td>
<td>White blood count</td>
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<td>WHO</td>
<td>World Health Organization</td>
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INTRODUCTION AND RATIONALE

Osteoporosis is a disease of increased bone fragility leading to increased risk of fracture (1). Decreased bone mineral density is the hallmark of the disease. However, micro architectural alterations represent a major pathogenic factor leading to what is called fragility fractures (low trauma fractures). Women are more commonly affected than men with 50% of women above the age of 50 years are at risk of fracture compared to 20% for men (2, 3).

RA is a chronic inflammatory joint disease that may lead to various extra-articular symptoms. While there has been progress in defining the etiology and pathogenesis of RA, these are still incompletely understood. There is synovial inflammation, subsequent pannus formation and intra-articular bone erosions, resulting in joint destruction. Joint damage is the major reason for disability in RA patients. In addition, the disease can affect the whole musculoskeletal system including bones, cartilage, ligaments and tendons. The bone system is particularly affected resulting in localized, peri-articular bone loss as well as generalized bone loss (4-9).

An intricate interaction between the immune system and the skeleton is evidenced by the significant bone loss shown in RA (10). This interaction is thought to be occurring through the cell surface receptors, cytokines and signaling pathways. Furthermore, it is becoming clear that immune cells influence bone remodeling and vice versa. It is thought that there is a direct interaction between immune cells and bone cells due to their proximity in the bone marrow. The role of T cells is well established in inflammatory bone resorption and osteoclastogenesis (12). T cells have the ability to regulate bone turnover through direct cell-cell interaction with bone cells and indirectly through the production of inflammatory and other cytokines that modulate osteoclastogenesis. Osteoblasts and osteoclasts can express major histocompatibility complex (MHC) class II molecules under inflammatory conditions. Therefore, it is plausible that T cell:bone cell interaction may contribute to the autoimmune response as well as
accentuate bone turnover.

On the other hand, the role of B-lymphocytes in osteoclast (OC) formation is controversial (13). B-lymphocytes can inhibit and stimulate osteoclastogenesis by their ability to secrete different cytokines (13). B lymphocytes produce pro-osteoclastogenic cytokines including receptor activator of NF-kappa-B ligand (RANKL), as well as cytokines that inhibit osteoclast differentiation from the progenitor cells, such as osteoprotegerin (OPG), transforming growth factor-β (TGF-β) and interleukin (IL)-7. Furthermore, B cells can stimulate the activation of other immune cells including T cells leading to further bone loss. Additionally, a subset of B cells named “regulatory” B cells can inhibit the inflammatory response and down-regulate the function of T cells (14).

Therefore, the net effect of B cell depletion on bone turnover may depend on the relative depletion of effector versus regulatory B cell subsets. Studies have shown that B-lymphocytes stimulate osteoclastogenesis; while others have shown that B cells inhibit bone resorption (15). Knocking out B cells in mice led to severe osteoporosis indicating that B cells have an important role in bone metabolism (12).

This thesis is to study the effects of rituximab, a B cell depleting monoclonal antibody, on bone turnover in RA patients in the UK. NICE has licensed rituximab for use in patients with RA who are unresponsive to treatment with second-line anti-rheumatic medication and a TNF-inhibitor. Rituximab proved its efficacy in controlling RA disease activity in many studies. This success has demonstrated that B cells play a key role in perpetuation of the disease.

The study builds on pilot data obtained from RA patients, which suggested that B cell depletion has a beneficial effect on bone turnover in RA. The pilot study demonstrated a 50% decrease (p<0.001) in beta-crosslaps (CTX), a biochemical marker of bone resorption, and a
small but statistically significant increase in P1NP, a biochemical marker of bone formation, in sera of 46 RA patients 6 months after a single treatment course of rituximab (11).

However, the pilot study had limitations as it was mainly based on assessment of serum levels of biochemical markers of bone turnover but not bone mineral density (BMD) measurements. Furthermore, the serum samples were collected for a different study aim and were randomly collected during the day and non-fasting. We therefore set up a new prospective clinical study (named ‘Horus trial’) to investigate bone mineral density measurements and markers of bone formation, resorption and osteocytes, in addition to markers of inflammation and disease activity in RA patients treated with rituximab.

This multicenter, open-label, single treatment arm, prospective clinical trial aimed to determine the effects of B cell depletion using rituximab on bone density and biochemical markers of bone turnover in RA patients.

We postulated that the presumed bone-protective effects of rituximab on bone density and turnover may be either due to a direct effect of B cell depletion on osteoclastogenesis, or due to reduction of disease activity, modulation of autoreactivity or all of the above (11).
AIM OF THE WORK

This study is based on a multicenter prospective clinical trial aiming to assess the effects of B cell depletion using rituximab on disease activity, BMD and biochemical markers of bone turnover including bone formation markers, bone resorption markers and osteocyte markers in RA patients. We aim to determine whether this effect is related to remission of disease activity by the drug or a direct effect of B cell depletion on bone turnover. Additionally, we wanted to determine the effect of changes in disease activity on bone metabolism in RA.

STUDY OBJECTIVES

Primary Goal
To determine the effect of B cell depletion using rituximab treatment on bone mineral density (BMD) measurements, disease activity and bone turnover (biochemical markers of bone resorption and formation) in RA patients.

Secondary Goals
To correlate changes in bone density with changes in biochemical markers of bone turnover; changes in biomarkers of inflammation and changes in circulating B cells. Additionally, we wanted to determine the effect of changes in disease activity on bone metabolism in RA.

STUDY HYPOTHESIS

B cell depletion has a beneficial effect on bone loss resulting from RA. We hypothesize that RTX is able to stop bone resorption and enhance bone formation by blocking inflammation and a possible direct effect on bone cells.
CHAPTER 1. RHEUMATOID ARTHRITIS

1.1. EPIDEMIOLOGY

RA is a common disease affecting between 0.5-1% of the adult population worldwide (4). Osteoporosis is a leading co-morbidity in RA. Almost one third of women with RA will develop a fracture within 5 years of their diagnosis. These data clearly show that bone loss in RA is a significant public health problem (6).

1.2. RISK FACTORS

The cause of RA is still unknown. Risk factors for RA include female sex, positive family history for RA, presence of the shared epitope HLA DR beta chains most commonly HLA DR4 and DR14, smoking, increased BMI, infection, presence of other autoimmune diseases and the presence of auto-antibodies rheumatoid factor (RF) and anti-citrullinated peptide antibodies (ACPA). Other risk factors include heavy alcohol consumption, artificial-feeding and low socioeconomic status (7, 9, 14).

1.3. PATHOGENESIS

Despite the fact that the cause of RA is not completely understood, some data on RA pathogenesis exist. Environmental factors such as infections and smoking stimulate the immune system in the genetically susceptible patients to initiate the disease. This activation of the immune response leads to the production of citrullinated peptides. These peptides activate the production of anti-citrullinated peptides antibodies (ACPA) leading to immune complexes formation, which in turn activate the complement system. This activation of the immune system attracts further antigen presenting cells (APC), T and B cells into the joints through different mechanisms. These cells produce many pro-inflammatory cytokines including IL-1,
IL-6 and TN-α. These cytokines further aggravate the inflammatory response in the joint space causing synovial inflammation (7).

Synovial neovascularization helps recruit the inflammatory cells into the joint space producing more pro-inflammatory cytokines. These cytokines stimulate the expression of adhesion molecules on the surface of endothelial cells leading to diapedesis of further inflammatory cells into the synovial space. This inflamed synovium start to creep under the cartilage destroying it and eroding the subchondral bone and ligaments through the production of proteases. (9, 16) (Figure 1).
1.4. CLINICAL FEATURES

1.4.I. Articular manifestations

Although RA is a systemic disease and can affect all systems of the human body, it tends to present with inflammation of the joints leading to swelling, redness, hotness and loss of function of the affected joints. RA has an insidious onset in most of the patients. It usually starts with arthritis of the metacarpophalangeal (MCP), proximal interphalangeal (PIP), wrists and metatarsophalangeal (MTP) joints. Then it may affect larger joints like elbows, shoulders, ankles and knees (17-19).

RA may have an acute onset with body aches, fatigue, low-grade fever, weight loss and depression. In other cases, it may present with polymyalgia rheumatica features such as pain in the shoulder and hip girdles associated with morning stiffness. Characteristic RA features may develop later. Intermittent disease with mono- or oligo-arthritis for few hours or days then settle down for weeks or months can also be a presenting feature. This picture is suggestive of palindromic rheumatism which some consider as an early phase of RA (17-19).

Typically, the arthritis tends to be symmetrical affecting mainly the small joints of the hands sparing the DIPs. Morning stiffness for more than 1 hour is a common complaint in active disease. According to the diagnostic criteria, these symptoms should be present for at least 6 weeks (20).

1.4.II. Extra-articular manifestations

Extra-articular manifestations might be a presentation of RA. Furthermore, RA can be complicated by other rheumatic diseases such as Sjogren syndrome, vasculitis, Felty’s syndrome, amyloidosis, serositis, splenomegaly, neuropathy, renal, lung, bone, muscle, skin and eye diseases (21).
**1.4.II.A- Muscle disorders**

RA can affect the muscles by different mechanisms including synovitis, inflammatory myositis, vasculitis, and steroid induced myopathy. Disuse weakness and atrophy resulting from chronic arthritis is common. For example, chronic knee arthritis leads to weakness and atrophy of the quadriceps muscle. This leads to joint instability and further damage (22).

Infiltration of inflammatory cells is common in muscles of RA patients leading to muscle necrosis. Moreover, some patients may suffer from frank polymyositis (23, 24).

**1.4.II.B- Skin manifestations**

Skin lesions in RA may include rheumatoid nodules, mucocutaneous ulcers, corticosteroid induced skin atrophy and rarely pyoderma gangrenosum and rheumatoid neutrophilic dermatosis (25).

**1.4.II.C- Vasculitis**

RA vasculitis is an unusual but serious complication. It may present as digital or skin ulcers, mononeuritis multiplex, myositis, retinal vasculitis, purpura, coronary, pulmonary, renal, and gastrointestinal tract (GIT) vasculitis. It may also mimic polyarteritis nodosa. Some risk factors are known for RA vasculitis. These include long standing erosive disease, high titer of RF and systemic disease. Rheumatoid nodules are common on patients with vasculitis. It is thought that both conditions are of the same pathogenesis (26).

**1.4.II.D- Eye disorders**

Eye manifestations in RA may include dry eyes, episcleritis and rarely scleritis. However, RA can also cause uveitis, keratitis and corneal melt (27).
Eye dryness is not related to RA disease activity. However, other conditions such as scleritis and corneal melts usually occur in active systemic disease (27).

1.4.II.E- **Pulmonary manifestations**

RA commonly affects the pleura presenting as pleuritis or pleural effusion that can be either asymptomatic or may present with chest pain, fever and dyspnea (28).

Lungs could also be affected in RA leading to ILD. The most common presentations are usual interstitial pneumonia and non-specific interstitial pneumonia. Other forms of ILD that are less common include cryptogenic organizing pneumonia, lymphocytic and desquamative interstitial pneumonia. Symptoms can be fever, dyspnea, cough, malaise and weight loss (29).

Pulmonary infections represent a major cause of morbidity and mortality in RA. Rheumatoid nodules may occur in the lungs. When associated with pneumoconiosis is called Kaplan syndrome. Pulmonary vasculitis maybe a part of RA vasculitis (30).

1.4.II.F- **Cardiac manifestations**

RA is a significant independent risk factor for coronary heart disease. Cardiac involvement includes pericarditis, myocarditis, myocardial infarction, arrhythmias, heart failure and sudden death. Pericarditis is common in RA patients with active systemic disease and high RF titer. Myocarditis and heart failure are less common (31).

1.4.II.G- **Peripheral vascular disease**

RA is also a significant independent risk factor for atherosclerosis and peripheral vascular disease in the same way as the coronary artery disease (32).

1.4.II.H- **Renal manifestations**
RA can cause glomerulonephritis (GN), renal vasculitis and secondary AA amyloidosis leading to nephrotic syndrome that may occur in patients with longstanding active RA. Iatrogenic renal compromise is the most common cause of renal affection in RA by the medications used in the treatment of RA such as gold, penicillamine, cyclosporine and non-steroidal anti-inflammatory drugs (NSAIDs) (33).

1.4.II.I- Sjogren Syndrome

Sjogren syndrome (SS) is characterized by dryness of the mucous membranes such as dry eyes and dry mouth. It is also named sicca syndrome. It can be either primary or secondary. Primary SS occur when there is no underlying disease while secondary SS occur in patients with pre-existing rheumatic diseases such as RA. SS is manifested as gritty or foreign body sensation in the eyes and difficulty in swallowing of dry food with dental caries and oral candidiasis (34).

1.4.II.J- Neurological manifestations

Entrapment neuropathies are the most common form of neurological disease in RA. Carpal tunnel syndrome is the most common entrapment neuropathy found in RA. Peripheral nerves can also be affected by joint damage, pannus formation and rheumatoid vasculitis. Neuropathies may include ischemic neuropathy, mono-neuritis multiplex and peripheral neuropathy. Spinal cord affection due to atlanto-axial subluxation is a life-threatening complication of longstanding active disease.

Rheumatoid vasculitis may rarely affect the CNS as a part of the systemic condition leading to stroke, seizures, or intracranial hemorrhage. The risk of CNS ischemic events generally increases in RA as a result of the enhanced atherosclerosis (35-37).

1.4.II.K- Hematologic manifestations
The most common hematologic abnormality in RA is anemia. It can be either normocytic normochromic due to chronic inflammation, microcytic hypochromic due to iron deficiency, macrocytic or hemolytic anemia. Hypersplenism and neutropenia in longstanding active RA represent the triad of Felty syndrome.

Disease-modifying anti-rheumatic drugs (DMARDs) may also cause pancytopenia and leucopenia including methotrexate, sulphasalazine, gold and azathioprine. On contrary, leukocytosis and thrombocytosis occurs in active disease (38-40).

1.4.II.L- Skeletal manifestations

RA induced osteoporosis is multifactorial including increased inflammatory cytokines, immobility, and drugs such as corticosteroids, old age and low body mass index (41, 43).

RA patients suffer from osteoporosis twice as much as age matched healthy individuals (43, 44). RA patients lose 2.4% of their bone density at the spine and 4.3% at the hip (45). Additionally, the rate of vertebral and non-vertebral fractures in RA patients is higher than the age and gender matched healthy controls (46).

Furthermore, patients with high disease activity had 2.1% decrease in BMD of the lumbar spine compared to 0.2% in patients with low disease activity (47). This section is discussed in further details in chapter 2.

1.5. DIAGNOSIS

RA should be suspected in any patient presenting with polyarthritis. For research purposes, diagnostic criteria have been set by the American college of rheumatology (ACR) in 1987 (Table I). These criteria were also used in clinical practice to help diagnose RA. In 2010, new criteria for the diagnosis of RA were developed by the ACR and the EULAR (Table II).
However, the old criteria of 1987 have the problem of omitting ACPA. While the new criteria omitted rheumatoid nodules and radiographic changes which are absent in early RA (48-50). The new criteria allow identification of early stages of RA, which allows early aggressive management of the disease to prevent its complications. This is in contrast to the old criteria that defined the disease late based on symmetrical arthritis, x-ray findings and rheumatoid nodules. The old criteria allowed only late diagnosis of well-established disease which delayed proper treatment.

*Table (1)* showing the ACR classification criteria for diagnosis of RA (1987) (49)

<table>
<thead>
<tr>
<th>Diagnosis of RA is defined as having 4 or more out of the 7 following parameters:</th>
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<tbody>
<tr>
<td>1- Morning stiffness: Morning stiffness in and around the joints, lasting at least one hour before maximal improvement.</td>
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<tr>
<td>2- Arthritis of 3 or more joint areas: At least 3 joint areas (out of 14 possible areas; right or left PIP, MCP, wrist, elbow, knee, ankle, MTP joints) simultaneously have had soft-tissue swelling or fluid (not bony overgrowth alone) as observed by a physician.</td>
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<tr>
<td>3- Arthritis of hand joints: At least one area swollen (as defined above) in a wrist, MCP, or PIP joint.</td>
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<tr>
<td>4- Symmetric arthritis: Simultaneous involvement of the same joint areas (as defined above) on both sides of the body (bilateral involvement of PIPs, MCPs, or MTPs, without absolute symmetry is acceptable).</td>
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<tr>
<td>5- Rheumatoid nodules: Subcutaneous nodules over bony prominences or extensor surfaces, or in juxta-articular regions as observed by a physician.</td>
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<tr>
<td>6- Serum rheumatoid factor: Demonstration of abnormal amounts of serum rheumatoid factor by any method for which the result has been positive in less than 5 percent of normal control subjects.</td>
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7- Radiographic changes: Radiographic changes typical of rheumatoid arthritis on posterior anterior hand or wrist radiographs, which must include erosions or unequivocal bony decalcification localized in, or most marked adjacent to, the involved joints (osteoarthritis changes alone do not qualify).

Table (2) showing ACR/EULAR Criteria for diagnosis of RA (50)

The ACR/EULAR 2010 criteria defined patients with definite RA who has arthritis of at least one joint and a total score of 6 or more out of 10 in four domains as follows:

<table>
<thead>
<tr>
<th>A. Number and site of inflamed joints</th>
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<tr>
<td>1 point for 2 to 10 large joints such as the knees, the shoulders, the elbows, the ankles and the hips,</td>
</tr>
<tr>
<td>2 points for 1 to 3 small joints such as PIP, MCP, wrist, MTP except of the big toe and the interphalangeal (IP) joint of the thumb.</td>
</tr>
<tr>
<td>3 points for 4 to 10 small joints</td>
</tr>
<tr>
<td>5 points for more than 10 joints including at least one small joint</td>
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<table>
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<tr>
<th>B. Serological abnormality including RF or ACPA</th>
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<tbody>
<tr>
<td>2 points for low positive titer</td>
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<tr>
<td>3 points for high positive titer</td>
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<tr>
<th>C. Acute phase reactants</th>
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<tr>
<td>1 point for elevated serum levels of ESR or CRP</td>
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<tr>
<th>D. Symptom duration</th>
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<tr>
<td>1 point for symptoms of at least 6 weeks</td>
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</table>

1.6. ASSESSMENT OF SEVERITY/PROGRESSION

1.6.1. Assessment of disease activity
Active RA can cause major complications such as joint deformities, joint destruction, functional limitation and impaired activities of daily living. Accordingly, tight control of RA disease activity is mandatory to prevent such problems. Proper control of the disease starts with an accurate assessment of disease activity. The new strategy adopted by ACR and EULAR focuses on treat to target aimed at induction and maintenance of remission. Assessment of RA disease activity can be done using various tools which include tender joint count, swollen joint count, patient global assessment of disease activity and acute phase reactants such erythrocyte sedimentation rate (ESR) or CRP (51).

Multiple indices have been developed using the above-mentioned parameters including the disease activity score (DAS), its modification DAS 28 joints (DAS28), simplified disease activity index (SDAI) and clinical disease activity index (CDAI). The DAS28 is currently the most commonly used index. Disease activity can be categorized in high disease activity (DAS28>5.1), moderate disease activity (DAS28 score between 3.2 and 5.1), low disease activity (DAS28 score between 2.6 to 3.2), and remission (DAS28<2.6) (51).

1.6.II. Assessment of physical health status

Functional impairment is a characteristic hallmark in RA. Hence, functional assessment is crucial in measuring treatment response in addition to DAS28, which is greatly affected by the acute phase reactants. This might be deceiving sometimes specially that these acute phase reactants rise in multiple conditions such as infections, malignancies…etc. (52).

In many cases, patients have low acute phase response but suffer significant impairment of activities of daily living. So, assessment of functional capacity is as important as measuring the disease activity in RA follow-up. The health assessment questionnaire (HAQ) is the most commonly used in clinical trials. It consists of 20 questions divided into 8 categories dressing, rising, eating, walking, hygiene, reach, grip, and usual activities. Each question has a score
ranging from 0 to 3 with 0 = no difficulty; 1 = some difficulty; 2 = much difficulty; and 3 = inability to do (51).

The score for each category is given to the question with highest score. Using an assistive device or help from another person increases the score to 2 unless it is 3, then it scores 3 as it is. The HAQ score is the mean of the eight categories ranging from 0 to 3 with 0 being the best health status and 3 is the worst (51).

1.6.III. Response criteria

Response criteria determine the change in disease activity in response to treatment. These criteria compare the disease activity between baseline and upcoming visits or the achievement of remission and low disease activity. The most common criteria are the ACR and EULAR response criteria. The ACR response criteria are ACR20, ACR50 and ACR70. ACR20 improvement criteria is defined as at least 20% improvement in swollen and tender joint counts and three of the following five variables: Patient’s global assessment (by visual analogue scale [VAS]), Physician’s global assessment (by VAS), Pain assessment by the patient (by VAS), Functional assessment (by HAQ), Acute phase reactants (ESR or CRP).

In the same way, ACR50 and ACR70 are at least 50% and 70% improvement in swollen and tender joint counts and three of the previously mentioned variables respectively (53, 54).

The EULAR response criteria are based on DAS28 score. EULAR response can be either moderate or good response. For good response, there is a reduction of at least 1.2 in the DAS28 score and result in the achievement of low disease activity (DAS28 <3.2). While moderate response is a reduction of more than 1.2 but not reaching low disease activity or a reduction of DAS28 between 0.6 and 1.2 and achieving at least moderate disease activity (DAS28<5.1) (53, 54).
1.7. MANAGEMENT OF RA

Management of RA includes pharmacological and non-pharmacological treatments. Care of RA patients should be done by a multidisciplinary team including rheumatologists, orthopedic surgeons, nurses, occupational therapists, orthotists and prosthetists. Treat to target is the general goal of RA treatment. The aim for management is inducing RA disease remission. This can be achieved by early diagnosis and introduction of traditional and biological DMARDs to control the disease activity. Prevention and early management of comorbidities such as deformities help patients cope with activities of daily life (55-60).

1.7.I. Non-pharmacological management of RA

Non-pharmacological management includes rest of inflamed joints and bed rest during flare-ups; patient education about the nature of the disease and its treatments. Pacing and a relaxed schedule can be useful in patients complaining of fatigue. Physical therapy can ameliorate complications such as disuse atrophy, contractures and joint instability (60).

Exercise is a crucial part of in the management of such patients. Isometric strengthening exercise can be helpful in preventing muscle weakness without worsening the joint damage (55).

Physical therapy is an important part of management of RA. It aims to decrease inflammation and pain, improve joints’ range of motion (ROM) and ameliorate the patients’ physical fitness. Different modalities can be used including superficial heat such as hot packs and infrared; deep heat such as short wave diathermy, ultrasound; electrical stimulation to strengthen weak muscles and reduce the joint effusion. Last but not least, exercise is a crucial part of physical therapy including aerobics; isometric strengthening and stretching exercises (56, 57).
Assistive devices including orthoses and prostheses play a key role in correction of deformities and improving the function of the affected limb (56, 57). These help patients to better perform activities of daily living without pain. Occupational therapy has a major role in helping patients cope with their daily activities including jar opener, key holder, appliances for reach, and built up utensils (57).

1.7.II. Pharmacological management of RA

The strategy of RA treatment is currently based on the early introduction of disease modifying drugs to stop joint damage and disease progression. Medications include painkillers, non-steroidal anti-inflammatory drugs (NSAIDs), corticosteroids and DMARDs. Supplementation with vitamins such as calcium and vitamin D is of great importance to protect against RA induced osteoporosis (60).

Different management strategies have been set to stop RA disease progression but they all aim to induce remission of the disease. For instance, NICE advised that a combination of traditional DMARDs should be started immediately after confirmation of diagnosis including methotrexate and at least one other DMARD, in addition to bridge therapy with corticosteroids for three months in order to inhibit the disease activity quickly till DMARDs’ effect can be obtained (60).

Biological DMARDs should only be started in patients who fail to respond to at least two traditional DMARDs including methotrexate or side effects cannot be tolerated. Traditional DMARDs have to be tried for at least 6 months, unless side effects limited their use. Disease activity should be assessed on two different visits 1 month apart. Recently, NICE introduced many options as first line biological therapies such as TNF-α inhibitors, abatacept and tocilizumab. The choice of drug is based according to the patient and physician’s preferences. Methotrexate should be continued with biological DMARDs whenever tolerated. If patients
fail to respond to a biological agent, an alternative biological agent can be tried. Patients should continue using the biological drug only if DAS28 score improved by at least 1.2 after 6 months of initiation of the drug. In patients failing to respond to at least one TNFα inhibitor can be shifted to rituximab. Rituximab should be used in combination with methotrexate and should only be continued in a similar way to the above-mentioned biological DMARDs (60).
CHAPTER 2. BONE METABOLISM

2.1. OVERVIEW

Bone is a metabolically active organ and is in continuous turnover during our life. Interestingly, 20% of bone tissue is renewed every year (61). Bone turnover starts with bone resorption by osteoclasts. Bone deposition by osteoblasts follows. The terminally differentiated osteoblasts lie in lacunae forming osteocytes. Many factors orchestrate bone remodeling. These include hormones, cytokines, systemic diseases, drugs, age, sex and nutritional status. All these factors can affect bone metabolism through regulating the activity of different bone cells (10).

In normal circumstances, there is a balance between bone resorption and formation. Abnormal bone architecture results from uncoupling of this balance resulting in low bone quality. Osteoporosis occurs in conditions where there is increased bone resorption compared to bone deposition leading to increased bone fragility resulting in increase in fracture risk (62).

Fragility fracture is the most feared complication of osteoporosis. Osteoporotic fractures are common especially in postmenopausal women (62). Treatment of osteoporotic fractures is a significant economical problem with medical costs estimated at £1.8 billion in the UK in addition to the costs resulting from turning active community members into disabled patients (63). Osteoporosis is more prevalent in females than males and incidence increases with age (64, 65).

World Health Organization (WHO) defined osteoporosis as a decrease of BMD equal to or more than 2.5 standard deviations (SD) below the mean peak bone mass of a young adult of the same sex and ethnicity (T-score ≤ -2.5 SD) (66).
BMD is currently the gold standard for diagnosis of osteoporosis worldwide. However, BMD should not be the sole element used in treatment decision due to the lack of sensitivity. BMD for example gives false negative results in patients with spondylosis. Furthermore, BMD is a good estimate of bone mass but not bone quality and does not provide data on the rate of bone turnover i.e. whether bone resorption exceeds formation or vice versa (67).

2.2. BONE PROPERTIES

Bone quality depends on three factors including mineralization, bone proteins and activity of bone cells. Bone should be strong but resilient at the same time to allow for weight bearing without being fractured (10). If the bone is stony hard, then it will fail to adsorb shocks and will fracture. This happens in a disease called osteopetrosis. On the contrary, if bone is soft and flexible, this will also end up with a fracture such as in rickets in children and osteomalacia in adults. Bone also should be light in weight to allow easy mobility of the body (10).

2.2.1. Bone structure

Two types of bone are present in the human body. The outer cortical bone forms three quarters of bone mass while the inner trabecular bone represents one quarter of the bone mass. Compact bone consists of osteons made up of lamellae of bone tissue surrounding the central haversian canal which hosts the nerves and blood vessels. Between the osteons lie the interstitial lamellae and lacunae containing osteocytes (68). Hematopoiesis takes place in the bone marrow. Cancellous bone lines the cortex of long and flat bones. It looks like a sponge with pores to give lightness and resilience to bone structure (10).

2.2.1.1. Matrix
Bone matrix contains mainly collagen type I with almost 90% of the matrix in addition to proteoglycan, osteocalcin and osteonectin representing the remaining 10%. Osteoblasts, the bone forming cells, produce pro-collagen which is then converted to collagen. Collagen fibers are made up of fibrils grouped together. Each fibril contains two \( \alpha-1 \) chains and one \( \alpha-2 \) chain connected together forming a triple helix (10).

### 2.2.1.II. Minerals

Bone minerals are mainly calcium hydroxyapatite lying among the matrix collagen fibers. Bone mineralization is the main determinant of bone strength. In utero, primary mineralization happens fast within days producing most of the matrix minerals; while secondary mineralization is slower and occurs within months producing the mature calcium crystals (69).

### 2.2.1.III. Cells

There are three types of bone cells: osteoclasts, osteoblasts and osteocytes. Osteoclasts are the bone resorbing cells; osteoblasts are the bone forming cells; mature osteoblasts transform into osteocytes after they end up with bone formation. Thus, osteoclasts and osteoblasts are only present in areas with active bone turnover; while osteocytes are the main cells in stable bone (69).

### 2.3. MECHANISM OF PHYSIOLOGICAL BONE TURNOVER AND ITS DETERMINANTS

Under physiological conditions, there is a balance between bone formation by osteoblasts and bone resorption by osteoclasts. Many factors including hormones, cytokines, immune signaling and bone cells maintain this balance. An imbalance in bone turnover cycle result in abnormal bone that predispose to fractures and deformities (68).
2.3.A. Role of osteocytes

Osteocytes, the fully differentiated osteoblasts, lie in lacunae in the mineralized matrix and the osteoid tissue of bone. Osteocytes are thought to be able to regulate their own proliferation through their ability to control the differentiation of their derivative osteoblasts. However, the exact mechanism of osteocytogenesis is still largely unknown (68).

Over the last decades, research has focused on the role of osteoclasts and osteoblasts in osteoporosis. Osteocytes have been found to have a potential key role in the regulation of both bone formation and resorption. Osteocytes are able to detect changes in bone morphology particularly micro-fractures through their sensitivity to detect mechanical strain acting like bone mechanoreceptors (69).

They act accordingly by regulation of bone turnover through direct physical contact with other bone cells and their ability to produce various factors such as dentin matrix protein 1 (DMP1), sclerostin and dickkopf-related protein 1 (DKK1). In vivo studies showed that osteocyte depletion results in profound loss of trabecular bone mass. The authors thought that these results might be due to the enhanced activity of osteoclasts and inhibition of osteoblasts (70).

In a different model, reduction of bone mechanical pressure resulted in increased osteocyte expression of osteopontin (OPN) known also as bone sialoprotein I (BSP-1), which is an important osteoclastogenic cytokine. OPN is thought to be responsible for the activation and development of osteoclasts promoting them to acquire their ruffled border (71). Moreover, bone resorption by osteoclasts is directed by the site of osteocyte apoptosis in a different model (72).
These results suggest the close interaction between osteocytes and the other bone cells and highlight their role in bone turnover regulation. The osteocyte markers can be used in vitro as markers of osteocyte function for research purposes but yet to be validated for daily clinical application for the monitoring of treatment response in osteoporosis and inflammatory arthritis. DMP1 is a secreted protein present on the surface of osteocytes and to lesser extent osteoblasts (73).

DMP1 deficiency led to decreased mineralisation of bones with increased mineral crystals formation in a murine model that suggests that it is essential for mineralisation and osteocyte maturation (74). Additionally, phosphate homeostasis is determined by DMP1 through its ability to modify its renal reabsorption (75).

Matrix extracellular phosphoglycoprotein (MEPE) is a protein markedly expressed by osteocytes (76). Like DMP1, MEPE is also capable of regulating phosphate homeostasis and hence affect bone mineralisation (77). Deletion of MEPE from osteoblasts in mice resulted in inhibition of bone formation (78).

The wingless-int(Wnt)/beta-(β-)catenin signalling pathway has been studied thoroughly over the past few years as many factors have been found to target this pathway to affect bone formation by osteoblasts. The Wnt proteins bind to the frizzled (Fz) receptors and the low-density lipoprotein receptor-related protein 5 (LRP5) and 6 (LRP6) co-receptors on the surface of osteoblasts. This interaction activates the dishevelled family proteins that lead to the phosphorylation of β-catenin. On the other hand, the activated dishevelled proteins inhibit the activity of the complex axin, and the protein APC that inhibits the β-catenin signalling. The last step is critical to stabilize the β-catenin which then diffuses from the cytoplasm to the nucleus to interact with T cell factor/lymphoid enhancer binding factor (TCF/LEF) transcription factors to promote the effects of Wnts on gene transcription (79) (Figure 2).
More interestingly, Wnt signalling pathway was found to affect the osteoblastic expression of both OPG and RANKL (80). Another study showed that the activation of Wnt signalling pathway increased the bone mass in mice, while β-catenin ablation resulted in marked osteoporosis in mice due to inhibited osteoblast proliferation and activity (81, 82). Research focused on treatments targeting the Wnt signalling pathway in the management of osteoporosis and related bone diseases (83).

DKK1 is a secreted osteocyte marker acting as an inhibitor to the Wnt signalling pathway through binding to LRP5/6 and hence blocking the Wnt effects on osteoblasts decreasing bone formation (83). Murine studies showed that DKK1 deletion increased Wnt signalling leading to activation of osteoblasts and increased both trabecular and cortical bone mass (84). In a human model with thalassemia-induced osteoporosis, Serum DKK1 levels increased in these patients and correlated with the disease severity. Furthermore, DKK1 levels were suppressed following administration of zoledronic acid suggesting its role in bone formation in addition to the already known mechanism in bone resorption (85).

DKK1 has been also found to have a role in the pathogenesis of post-menopausal and steroid induced osteoporosis as well as bone loss in malignant diseases. The implication of DKK1 levels as a valid bone marker for clinical practice is challenging and needs to be validated due to its variable ranges among different enzyme-linked immunosorbent assay (ELISA) methods as well as its variability according to physical effort. Also, DKK1 needs to be further tested in response to other factors that might affect its levels in blood such as diet and diurnal variations (86, 87).

Sclerostin is another inhibitory protein of bone formation secreted by osteocytes. It is the product of the SclerOSTin (SOST) gene located on the chromosome 17 (88). It acts in a relatively similar way as DKK1 by blocking the Wnt signalling pathway. Sclerostin bind to
LRP5/6 on the osteoblasts preventing the coupling of the Fz/LRP complex and hence inhibiting osteoblast activation (89). Mice expressing high levels of human SOST gene suffered from osteoporosis (90). While ablation of the SOST gene resulted in increased bone mass in another mice model (91).

Parathyroid home (PTH) is capable of decreasing sclerostin formation that explains the mechanism of how PTH is able to stimulate bone formation as part of its mode of action in the treatment of bone loss related conditions. This has directed the researchers that targeting sclerostin can be a therapeutic option in the treatment of osteoporosis. However, the use of serum sclerostin levels for monitoring of osteoporosis and patient compliance to treatment in daily practise is still questionable and needs to be validated (92).

![Figure (2)](image)

**Figure (2)** showing the mechanism of blockade of the Wnt signaling pathway by osteocytes.

Osteocytes detect changes in bone morphology through their sensitivity to mechanical forces, thereby regulating bone turnover through direct physical contact with osteoblasts. Osteocytes
produce OPN, DMP, MEPE, sclerostin and DKK-1. DKK-1 and sclerostin act as inhibitors of the Wnt signalling pathway through binding to LRP5 and 6 and hence blocking the Wnt effects on osteoblasts decreasing bone formation (83).

DKK-1: dickkopf-related protein-1; DMP: dentin matrix protein 1; FZ: frizzled protein; LRP 5/6: low density lipoprotein receptor-related proteins 5/6; MEPE: matrix extracellular phosphoglycoprotein; OB: osteoblast; OCY: osteocyte; OPN: osteopontin; SCL: sclerostin.

2.3.B. Role of Osteoblasts

Osteoblasts are specialized stromal cells responsible for the production and mineralization of the bone osteoid matrix. Collagen type I is the main protein found in the osteoid matrix together with other proteins such as OPN, osteonectin, osteocalcin and bone sialoprotein. Furthermore, osteoblasts are the main regulators of osteoclastogenesis by virtue of their ability to produce the pro-osteoclastogenic RANKL and IL-6 and the anti-osteoclastogenic cytokine OPG. (10)

Osteoblasts are derived from the pool of mesenchymal stem cells (MSC) that reside on the outer surfaces of blood vessels and sinuses in the bone marrow and periosteum (93). MSC tend to migrate to areas of bone formation (94).

The interaction between Wnt/β-catenin in osteoblasts and osteocytes and other growth factors such as transforming growth factor-beta (TGF-β) are critical for osteoblastic differentiation and hence bone deposition. The canonical Wnt signaling pathway is now established as the main mechanism of osteoblastic differentiation. Wnts are a group of secreted glycoproteins essential for embryonic development, determination of cell polarity and cellular differentiation (95).
These glycoproteins are involved in three signaling pathways of osteoblast maturation; the Wnt/β-catenin, the bone morphogenetic proteins (BMP’s) and the transcription factor RUN X2 signalling pathways. Wnt/β-catenin, another name for the canonical Wnt signalling pathway, starts with binding of the Wnt proteins to the frizzled family of receptors (Sfrps) and to LRP5/6 leading to the activation of a part of the Wnt receptor complex called disheveled protein and resulting in the inhibition of glycogen synthase kinase-3b (GSK-3). This inhibits the phosphorylation and degradation of the β-catenin intracellular signaling molecule leading to an increase in the amount of β-catenin in the nucleus, which interacts with transcription factors of the TCF/LEF family (96).

BMPs are a group of growth factors essential for bone homeostasis; they belong to the tumor growth factor superfamily. BMPs act by binding to their cell surface receptors resulting in activation of the signaling cascade; this interaction initiates the phosphorylation of the Smads that are cytoplasmic signaling molecules. These phosphorylated molecules migrate to the cell nucleus leading to transcription of BMP response genes inducing bone and cartilage formation (97).

Dkk family members (Dkk1 and Dkk2) and secreted Sfrps are families of extracellular proteins that bind to LRP-5/6, resulting in suppression of the Wnt signaling pathway; Sfrps bind directly to Wnts and prevent their association with LRP and Fzd receptors (98).

Whereas, Dkk1 sequesters the LRP5/6 receptors with the transmembrane protein Kremens (Kringle-containing protein marking the eye and the nose) and destroys these receptors with lysosomal enzymes; hence decreasing their binding to Wnt proteins (99).

Sclerostin secreted by osteocytes has a negative metabolic effect on osteoblast development by blocking the LRP5/6 receptors thus blocking the Wnt signaling pathway (99).
Bone formation by osteoblasts is regulated by several factors including hormones, BMP’s, growth factors, cells, cytokines, and mechanical forces. Many hormones can affect osteoblastic function and differentiation and are essential in certain concentrations for the development of the skeleton; these include cortisol, sex hormones such as estrogen and testosterone, PTH, calcitriol, calcitonin, growth hormone (GH), thyroid hormones, insulin and leptin (100-107).

Additionally, estrogen is now an established regulator of both the skeletal and immune systems (108). The osteoblast-stimulating factor (OSF-1) also known as pleiotrophin (PTN); is chemotactic for the osteoprogenitor cells and stimulates mature osteoblast activity (109). Fibroblast growth factors (FGFs), platelet-derived growth factor (PDGF), insulin-like growth factors (IGFs) and epidermal growth factor all promote the renewal of MSC’s and hence stimulate bone formation by osteoblasts (110-113).

Many inflammatory cells are capable of affecting osteoblastic activity, particularly activated T cells exerting their effects directly or indirectly either by cytokines (114) or by their interaction with dendritic and B cells (115).

Bone cells can also regulate osteoblastogenesis. Osteocytes can produce this effect by either direct cross talking by gap junctions or via signaling pathways (116).

Osteoclasts were also found to be able to stimulate alkaline phosphatase (ALP) expression by osteoblasts. Mechanical stress is also one of the most powerful inducers to osteoblastic differentiation via different signaling pathways and activation of the transcription factor RUNX2 (117).

2.3.C. Role of Osteoclasts
Osteoclasts are multinucleated giant cells specialized in bone resorption, derived from the hematopoietic progenitor cells of the monocyte/macrophage cell lineage in the bone marrow. Bone resorption starts with binding of osteoclasts to bone through various mediators including integrins, OPN, fibronectin, collagen and bone sialoprotein. The next step involves polarization of osteoclasts to form the specialized ruffled border. This step is crucial for increasing the surface area for bone resorption. The ruffled border then releases acidic hydrogen ions, lysosomal enzymes such as cathepsin K and matrix metalloproteinases (MMPs) to dissolve the bone. Tartrate-resistant acid phosphatase (TRAP) further digests the bone matrix degradation products (*Figure 3*) (118).

Calcitonin inhibits bone resorption by osteoclasts. The macrophage colony-stimulating factor (M-CSF) produced by osteoblasts and the RANKL are the major cytokines stimulating osteoclastogenesis. RANKL binds to its receptor RANK on the surface of osteoclast precursors to promote their growth and maturation. OPG is the natural decoy receptor of RANKL. Osteoblasts and stromal cells primarily produce it. OPG acts by blocking the bond between RANKL and its receptor RANK to regulate bone turnover. Other regulators of osteoclastogenesis include different cytokines, vitamins, hormones and other molecules (118).
Osteoclastogenesis regulators mostly act by regulating the activity of RANKL and its decoy OPG as follows:

![Diagram showing the mechanism of bone turnover and the origin of biochemical markers of bone formation and resorption. The bone remodeling cycle indicating the origin of markers of bone turnover that express the metabolic activity of either osteoclasts (resorption) or osteoblasts (formation) and cell to cell communication between the two. BAP: bone specific alkaline phosphatase; CTX: carboxy-terminal cross-linked telopeptides of type 1 collagen; DPD: deoxypyridinoline; NTX: amino-terminal cross-linked telopeptide of type 1 collagen; OPG: osteoprotegerin; OC: osteocalcin; PICP: procollagen type 1 carboxy-terminal propeptide; P1NP: procollagen type 1 amino-terminal propeptide; PYD: pyridinoline; RANK: receptor activator of nuclear factor kappa B; RANKL: receptor activator of nuclear factor kappa B ligand; TRAP: tartrate resistant acid phosphatase.]

Figure (3) showing the mechanism of bone turnover and the origin of biochemical markers of bone formation and resorption. The bone remodeling cycle indicating the origin of markers of bone turnover that express the metabolic activity of either osteoclasts (resorption) or osteoblasts (formation) and cell to cell communication between the two. BAP: bone specific alkaline phosphatase; CTX: carboxy-terminal cross-linked telopeptides of type 1 collagen; DPD: deoxypyridinoline; NTX: amino-terminal cross-linked telopeptide of type 1 collagen; OPG: osteoprotegerin; OC: osteocalcin; PICP: procollagen type 1 carboxy-terminal propeptide; P1NP: procollagen type 1 amino-terminal propeptide; PYD: pyridinoline; RANK: receptor activator of nuclear factor kappa B; RANKL: receptor activator of nuclear factor kappa B ligand; TRAP: tartrate resistant acid phosphatase.
Stimulators of RANKL production are IL-1β, IL-6, IL-7, TNF-α, 1, 25-dihydroxycholecalciferol (1,25(OH)2D3); PTH and glucocorticoids (GCs); activated T cells, and leptin. On the other hand, RANKL inhibitors include interferon-γ and OPG stimulators such as lipopolysaccharide (LPS) released by fibroblasts; estrogens and tumor growth factor (TGF). Osteoclast precursor cells migrate to the endosteal and periosteal surfaces of bones to proliferate and differentiate into osteoclasts. Three signal pathways may be involved in osteoclastic development; including the cyclic adenosine monophosphate (cAMP) mediated pathway, the glycoprotein130 (gp130) mediated pathway and the 1,25(OH)2D3 receptor mediated pathway (119).

Osteoblasts and marrow stromal cells also play a critical role in the regulation of osteoclast activity and differentiation either by direct cell-cell contact or through their ability to express the main inducers of osteoclastogenesis. Osteoclasts are principally activated by the canonical pathway, which is mediated mainly by RANKL and M-CSF. RANKL is a member of the TNF superfamily; it binds to its receptor activator of nuclear factor kappa B (RANK) on the surface of osteoclasts inducing bone resorption. RANKL is essential for normal bone development and its deficiency results in osteopetrosis due in part to a breakdown in the crosstalk between osteoblasts and osteoclasts. Under normal conditions, a balance between RANKL and its antagonist (OPG) maintain bone homeostasis. OPG is a soluble decoy receptor of RANKL and is becoming the main physiological inhibitor of osteoclastogenesis. It acts by competing with RANKL to bind with RANK thus blocking the RANK-RANKL interaction (120).

RANK-RANKL interaction recruits TNF receptor-associated factor (TRAF) proteins which in turn activates nuclear factor of kappa B (NF-κB) and nuclear factor of activated T cells, cytoplasmic, calcineurin-dependent 1 (NFATc1) leading to an increase in intracellular calcium (Ca2+) and tyrosine phosphorylation (120).
RANKL activates the immuno-receptor tyrosine-based activation motif (ITAM) bearing co-stimulatory molecules DNAX activation protein 12 (DAP12) and Fc receptor gamma chain (FcRγ). Deficiency of DAP12 & FcRγ in mice led to a marked impairment of osteoclastogenesis, resulting in severe osteopetrosis (121). RANKL is responsible for formation of mature osteoclasts, stimulating their bone resorption activity and suppressing their apoptosis. (122).

M-CSF is a cytokine involved in the proliferation and differentiation of osteoclasts. M-CSF binds to its osteoclast transmembrane receptor, colony-stimulating factor 1 receptor (c-fms), leading to the activation of spleen tyrosine kinase (Syk) and osteoclast activation (123).

Additionally two other inducers of osteoclastogenesis are known; osteoclast-associated receptor (OSCAR) and triggering receptor expressed on myeloid cells 2 (TREM-2). OSCAR is a collagen receptor involved in osteoblast-osteoclast interaction and is critical for the formation and function of osteoclasts. RANKL increases the expression of OSCAR which acts as a co-stimulatory molecule of the FcRγ-associated pathway and by binding to specific motifs involved in osteoclast maturation, results in enhanced osteoclastogenesis (124).

TREM-2 plays a central role in regulating the function of myeloid cells; it is expressed on macrophages, microglia and pre-osteoclasts and has been found to be essential for osteoclastogenesis (125).

New evidence has revealed that osteoclastogenesis can also occur in a RANKL independent manner, referred to as non-canonical osteoclastogenesis. Five of these RANKL substitutes have recently been described which are capable of osteoclast differentiation and activation; a proliferation inducing ligand (APRIL); B cell activating factor (BAFF); nerve growth factor (NGF) and insulin like growth factor (IGF) I and II, although their role in pathological bone resorption has yet to be established. BAFF also known as B lymphocyte stimulator (BLyS) is
a protein that belongs to the tumor necrosis factor superfamily, which is expressed on a variety of cells predominantly leukocytes. BAFF is a potent activator of B cell proliferation and differentiation and an essential step in their maturation (126).

Other well-documented surrogates include TNF-α (127), LIGHT, (homologous to lymphotoxins exhibiting inducible expression and competing with herpes simplex virus glycoprotein D for herpes virus entry mediator) HVEM, is a receptor expressed by T lymphocytes (TNFSF14), IL-6 and TGF-β (128-130).

Additionally, it has been demonstrated that β-catenin and Wnt signaling can inhibit osteoclast differentiation; however, its negative regulatory role on osteoclasts is due to modulation of RANKL/OPG ratio in the osteoblasts and osteocytes (131).

Another critical modulator of osteoclastogenesis is the large zinc finger protein ZAS3 encoded by the HIVEP3 gene. ZAS3 has been shown to both inhibit osteoblast differentiation and stimulate osteoclastic activity and differentiation (132).

Osteoclasts express a specific marker named TRAP; its role is still unknown but its deficiency in mice leads to decreased osteoclastic activity and osteopetrosis (133). It is hypothesized that TRAP is capable of dephosphorylating OPN and bone sialoprotein and the production of oxygen radicals leading to osteoclast differentiation. Integrins expressed by osteoclasts are now confirmed to be essential for bone resorption of calcified matrix (134).

Resorption of bone starts with osteoclastic recognition of the target matrix, followed by cellular morphological changes including polarization and organisation of podosomes forming a tight sealing ring. Podosomes are formed of a shell of vinculin, talin, actinin, fimbrin, gelsolin and vimentin and contain a cytoskeleton core of F-actin. Adherence of osteoclasts to
the bone surface is followed by secretion of vesicles containing proteinases leading to bone resorption (135).
CHAPTER 3. BONE LOSS IN RA

Bone loss resulting from RA has been extensively studied over the last years. However, its mechanism is still poorly understood. Moreover, trials to prevent its progression are still unsatisfactory as they only aim to treat it rather than prevent it. It has been well established that RA leads to increased bone resorption, but it has more recently been demonstrated that there is also substantially reduced bone formation.

3.1. The role of Cytokines

The increased bone resorption in RA has been explained by the increased osteoclastic activity under the effect of various inflammatory cytokines. Both the canonical and non-canonical osteoclastogenesis pathways are thought to play a role in the increased bone turnover in RA. Overproduction of RANKL by a wide range of cells, including osteoblasts, endothelial cells, T cells and B cells is a characteristic feature of RA (136).

RANKL binds to its receptor RANK on the osteoclasts activating their proliferation and stimulating osteoclastogenesis. Human studies revealed that RANKL play a crucial role in the pathogenesis of bony erosions and peri-articular osteoporosis in RA. Synovial levels of RANKL positively correlate with RA disease activity and bone resorption in RA patients. The source of RANKL in synovium is thought to be mainly due to synovial fibroblasts and T cells (136).

Moreover, higher serum levels of RANKL were found in patients with high bone resorption markers and low bone mineral density of hips. Also, levels of serum RANKL was able to predict joint destruction (137).

In another study, it was suggested that both increased RANKL and decreased OPG in peripheral blood is the main mechanism of osteoporosis in RA (138)
Interestingly, many other cytokines such as TNF-α, IL-1, IL-4, IL-6, IL-7, IL-10, IL-11, IL-12, IL-13, IL-17, IL-18, IFN-γ and GM-CSF have variable effects on bone turnover and act via different mechanisms. TNF-α is a key player in RA osteoclastogenesis by both RANKL-dependent and independent mechanisms. TNF-α blocking agents have recently been proven to reduce the local and generalized bone loss in RA (139).

Moreover, TNF-α is thought to inhibit bone formation by increasing Dkk-1 expression, thereby inhibiting bone formation by blocking the Wnt signaling pathway and leading to apoptosis of osteoblasts through induction of the Fas-FasL system (140).

IL-1 is capable of increasing bone resorption by increasing the release of MMPs and other degradative products and by promoting osteoclast differentiation and activation (141). In addition to their ability to stimulate bone cells to secrete RANKL, IL-6 and IL-11 can also induce bone loss by a RANKL-independent mechanism (142).

IL-6 induces osteoclastogenesis via the activation of TH cells to secrete RANKL and IL-17 (143). IL-17 is a major inducer of osteoclastic activity in inflammatory arthritis secreted by TH17 cells (144).

IFN-γ functions as a modulator of bone turnover as it has a dual effect on osteoclasts; IFN-γ can block the formation of osteoclasts directly by inhibiting the differentiation of osteoclast precursors, but can also stimulate osteoclastogenesis indirectly by activating the T cells to produce RANKL and TNF-α, however, the end result is bone loss (145).

In contrast other cytokines, such as IL-4, IL-10, IL-12, IL-13, IL-18, IFN-γ and GM-CSF are protective of bone by either inhibiting osteoclastogenesis, or stimulating bone formation by osteoblasts. As explained earlier, IL-4 inhibits the osteoclastogenic cytokines TNF-α, IL-1, IL-6, and IL-11 in RA, additionally IL-4 and IL-13 can stimulate osteoblasts and suppress prostaglandin E2 (PGE2) synthesis (146).
IL-10 is secreted by many cells, primarily monocytes and to a lesser extent by lymphocytes and is known to have an immunoregulatory effect in RA. It is presumed that it may have an anabolic effect on bone turnover by decreasing the production of TNF-α, IL-1, GM-CSF and the expression of HLA class II by monocytes (147).

Moreover, IL-10 is able to suppress the inflammatory effects of TH17 cells and promote the immunoregulatory Treg formation in RA patients (148). GM-CSF has been thought to inhibit osteoclast formation and activity; nevertheless, *in vivo* studies are contradictory (149).

IFN-γ has been found to play a pivotal role in bone formation *in vivo*. IFN-γ knockout mice developed osteoporosis and suffered from a marked decrease in both osteoblast and osteoclast numbers (150).

IL-12 is capable of inhibiting osteoclastogenesis through its ability to stimulate immune cells particularly T cells and dendritic cells to secrete IFN-γ (151).

IL-18 is capable of inhibiting osteoclast formation by the ability to stimulate the release of GM-CSF by TH cells (152). Additionally, it has been recently found to inhibit TNF-α mediated osteoclastogenesis *in vivo* by a T cell independent mechanism (153).

RA patients showed increased OSCAR expression in peripheral blood monocytes (PBMC’s) compared to healthy controls leading to enhanced differentiation of these monocytes into osteoclasts. Additionally, there was a positive correlation between OSCAR expression and disease activity suggesting an increased activity of osteoclastogenesis co-stimulatory signals in RA (154).

On the other hand, decreased bone formation in RA has been suggested to be mainly due to increased expression of Dkk-1. This causes blocking of the LRP-5 and LRP-6 receptors on the surface of osteoblasts, thereby inhibiting the Wnt signaling pathway (98).
In a mouse model of RA, inhibition of Dkk-1 by a neutralizing antibody led to a reversal of bone erosion and resulted in new bone formation but no change in markers of inflammation was noted (98).

IL-1, TNF-α and IFN gamma are also capable of suppression of bone formation by inhibiting osteoblast collagen formation (155). LIGHT also increases in RA patients suggesting that it may play a role in the pathogenesis of RA localized and systemic bone loss (156).

3.2. The role of T cells

T cells are involved in most of the autoimmune diseases including RA. For many decades RA was considered to be a T-cell dependent disease as evidenced by large numbers of CD4+ T cells infiltrating the synovial tissues of RA patients (157). T cells are activated by antigen presenting cells via MHC class II and trigger the immune response by secreting a wide range of inflammatory cytokines and thereby activating the whole immune system. T cells are a major source of RANKL and TNF-α and are therefore capable of regulating bone turnover in RA by activating osteoclastogenesis (158).

On the other hand, T cells may have an inhibitory effect on osteoclastogenesis by increasing OPG production by a mechanism that involves increased vitamin D3 activity (159).

TH1 cells produce IFN-γ and IL-2 and activate the cell-mediated immune response, whereas the TH2 subset produces IL-4 and stimulates humoral immunity (160). Imbalance between these two subsets results in inappropriate production of their respective cytokines and was found to correlate with disease activity (161).

Despite the inflammatory effects of T cells in RA, there is also a protective subset named regulatory T cells or Tregs that have anti-inflammatory effects. They act by regulating both TH1 and TH2 cells (162). However, in RA it is still not established if there is a defect in the
number or function of these Tregs. Interestingly, TNF-α has been found to suppress the Tregs response and this might be another mechanism by which TNF blocking agents act to control the disease activity in RA (163). Notably, both Tregs and CD8⁺ T cells have anti-osteoclastogenic effects (164).

A third type of helper T cells, the TH17 cells, has recently also been implicated in RA bone loss. TH17 cells secrete IL-17 that is capable of inducing osteoclastogenesis resulting in damage to cartilage and bones (165). T cells are also capable of interacting either directly with osteoblasts and osteoclasts or indirectly via dendritic cells and B cells (166).

3.3. Effects of anti-rheumatic medication

RA predisposes to both localized and generalized bone loss and increased fracture risk. This bone loss is multi factorial. Some of the drugs used to treat the condition can themselves cause bone loss such as glucocorticoid. Corticosteroids have been considered the cornerstone of the treatment of RA and most of the autoimmune diseases. Long-term use of corticosteroids is an established cause of generalized bone loss in RA. Nowadays, corticosteroid use has been minimized with the tendency to more aggressive use of DMARDs and the development of biological therapy (167).

RA can be significantly disabling leading to marked reduction in mobility and even loss of the patient’s ability to feed themselves which results in disuse atrophy of the muscles and a consequent reduction in bone mass. Muscle atrophy and joint destruction will also increase falls risk. Inflammation and autoimmunity are the main mechanisms of osteoporosis in RA. Chronic systemic inflammation leads to generalized osteoporosis and localized inflammation with regional osteoporosis. It was therefore presumed that treating inflammation would be sufficient to stop bone loss in RA, but this view is now thought too simplistic (167).
With the introduction of biological drugs, the rate of generalized and localized bone loss has been markedly reduced specially with the use of TNF blockers that are effective in controlling the disease activity and inhibiting osteoclastogenesis directly (168).

In a study of 102 RA patients, the use of infliximab for 1 year resulted in a stable BMD of the spine and hip but decreased BMD of the wrists. These results were supplemented with a significant decrease of serum levels of CTX and RANKL without a significant change in osteocalcin and osteoprotegerin (OPG) (168).

Another study showed prevention of bone loss in the spine and hips after 1 year of treatment with adalimumab (169). More recently, treatment for more than 3 years with infliximab resulted in an increase of 2.55% of the spine BMD and a slight decrease of -0.12% of the hip BMD (170).

Moreover, Schett et al showed that TNF blockers are able to decrease bone resorption induced by RANKL and to increase bone formation by reducing the levels of the Wnt signaling pathway inhibitor DKK1 (171).

**3.4. The role of B cells**

This will be discussed in details in chapter 4.
CHAPTER 4. B CELLS

4.1. B cells in RA

B cell development

B cells are formed in the bone marrow where hematopoietic stem cells differentiate initially through the pro-B to pre-B cell stage and later transform into immature B cells then complete their maturation in the spleen through three developmental stages; transitional type 1; type 2; then mature B cells (172). Mature B cells then differentiate into antibody secreting plasma cells, also known as effector B cells. B cells are subdivided into two main types; B1 cells are characterized by expressing mainly immunoglobulin M (IgM) and present in low numbers in the lymph nodes and spleen and more abundantly in the peritoneal and pleural cavities; while B2 cells are present in the peripheral circulation and the bone marrow. Recent studies also indicate the coexistence of a distinct B cell subset called B regulatory cells (Bregs), they can down-regulate the immune system and consequently are protective against autoimmune diseases (173).

Regulation of B cell function

B cell regulation in physiological and pathological conditions is a complex mechanism under the influence of many cells. T cells are thought to play a major role in the activation of B cells by two mechanisms. The first through direct cell to cell contact in which the primary signal is the B cell receptor (BCR) binding to antigen and then presenting the antigen via MHC class II to the T cell receptor (TCR) on the surface of T cells, these in turn provide the B cells with the secondary signal via co-stimulatory binding of CD40L on T cells to CD40 on B cells to complete the B cell activation. The antigen-presenting function of B-lymphocytes is thought to be relevant mainly in the late phase of infections and in secondary immune responses, due to the low number of clonogenic antigen-specific B cells that are present in homeostatic
conditions. The second mechanism by which T cells regulate B cell activity is by the release of lymphokines acting as growth factors for B cells (174, 175).

B cells have multiple functions in the pathogenesis of RA; they secrete autoantibodies such as rheumatoid factor (RF) and the highly specific anti-cyclic citrullinated peptides antibodies (ACPA). RFs are antibodies directed against the Fc portion of IgG, thus leading to formation of immune complexes.

Additionally, B cells are involved in processing and presentation of autoantigen and activation of T cells (176). B cells are also able to secrete different pro-inflammatory cytokines and promote differentiation of follicular dendritic cells in secondary lymphoid organs (177).

In RA, effector B cells for example secrete TNFα, IL-6, IL-12 and IFNγ (178-180). TNFα plays a critical role in the pathogenesis of RA and its inhibition is currently one of the most successful treatments available for this disease. TNFα has a wide variety of functions; including complement activation and stimulation of synovial fibroblasts and macrophages to secrete pro-inflammatory cytokines such as IL-6 (181), prostaglandin E2 (PGE2) and matrix metalloproteinases (MMPs) (182), inducing inflammation, cartilage and bone damage, in addition to transforming growth factor-beta (TGF-β), granulocyte/monocyte-colony stimulating factor (GM-CSF) and other growth factors that induce neovascularization and promote pannus formation characteristic of RA.

IL-6, another major pro-inflammatory mediator secreted by many cells including B cells, has a pivotal role in RA. IL-6 shares many effects with TNF-α, including stimulation of neovascularization, promotion of acute phase response, infiltration of inflammatory cells, synovial hyperplasia and damage to cartilage and bone (183).
B cells also produce IL-12 that stimulates the release of IFN-γ by T lymphocytes and natural killer (NK) cells and activates the T cell immune response leading to cellular infiltration, inflammation, cartilage and bone destruction (184).

IL-12 levels in serum and in synovium correlate with disease activity in RA revealing the definite role of this mediator in RA pathology (187). On the other hand, IFN-γ is found to have a dual action on chronic inflammation as it is capable of both inducing chronic inflammation and regulating the immune responses (185).

Interestingly, B cells can also secrete anti-inflammatory mediators such as IL-4 and IL-13. IL-4 inhibits the activity of pro-inflammatory TNF-α, IL-1, IL-11 and IL-6, in addition to up-regulating the inhibitory mediator IL-1 receptor antagonist in RA (186). IL-13, similar to IL-4, is capable of suppressing the inflammation in RA (187).

Recently, a new subtype of regulatory B-lymphocytes known as Bregs has been reported to regulate the immune system by producing the inhibitory cytokine IL-10. IL-10 has pleiotropic effects in immuno-regulation and inflammation. It down-regulates the proliferative responses and pro-inflammatory cytokine production by T helper cells, MHC class II antigen expression and co-stimulatory molecules on macrophages. Moreover Bregs can promote differentiation of T cells into regulatory IL-10 producing T cells and hence permit vigorous immune suppression (188).

Despite these anti-inflammatory effects, IL-10 has also been found to be an activator of B cell proliferation and antibody production. Moreover, Bregs were found to have other potential inhibitory mechanisms including secretion of the inhibitory cytokine TGF-β, interactions with Tregs and production of regulatory antibodies (189).
4.2. B cells and bone

Crosstalk between bone cells and B-lymphocytes is bidirectional, in that bone cells can regulate the development and maturation of B cells and B cells can regulate both osteoblastic and osteoclastic activity. The mechanisms that underlie these interactions are only partially understood, as is the precise role of B cells in bone turnover.

B cells have the capacity to both stimulate and inhibit bone turnover by direct and indirect mechanisms. Additionally, B cells appear to be capable of affecting bone formation and resorption under different physiological and pathological conditions.

4.2.A. B cells and RANK-RANKL-OPG system

Mature B cells secrete a number of different cytokines including RANKL, the key cytokine of bone breakdown and its inhibitor OPG, this mechanism works correctly under normal physiological conditions.

The bone protective role of B cells is mainly achieved through OPG production. In RA, this balance is disturbed by the increased B and T cell activity, leading to a marked increase in the production of RANKL by both cells in addition to other pro-inflammatory cytokines such as TNF-α. This shifts the bone turnover balance towards bone loss.

In an RA human model, the cytokine messenger-RNA expression by CD4 and CD8 T cells, B cells, macrophages and neutrophils were evaluated to identify the source of RANKL in the synovial fluid and peripheral blood. They found that B cells were the major source of RANKL in RA (190).
4.2.B. *B cells and cytokines*

In addition to the bone specific cytokines mentioned above, other cytokines secreted by B cells can affect bone turnover positively or negatively. TNF-α, IL-6 and IFN-γ for example are pro-osteoclastogenic while TGF-β, IL-4, IL-10, IL-12 and IL-13 promote bone formation (191).

4.2.C. *B cells and osteoclasts*

B cells are able to stimulate, both directly and indirectly, the conversion of monocytes to active osteoclasts. More interestingly, studies have shown that osteoclasts and B-lymphocytes may both arise from pro-B cells. Pro-B cells from osteopetrotic mice for example expressed markers from the B-lymphoid (CD19, CD43 and CD5) and the myeloid (F4/80) lineages. When stimulated with RANKL and M-CSF, these cells could grow into osteoclasts, while they were able to differentiate into B cells when stimulated by IL-7 (192).

Furthermore, B cells have been found capable of transforming into osteoclasts. Recently it has been found in a murine model that a subset of CD19+ B lymphocytes named B1 cells, can differentiate into mononuclear phagocytes forming osteoclast-like multinucleated giant cells. These cells express RANK and the macrophage colony-stimulating factor receptor (M-CSFR) and when stimulated with RANKL and M-CSF, these cells transformed into tartrate resistant acid phosphatase (TRAP) positive osteoclasts and have osteoclastic properties leading to formation of lacunae when allowed to grow on a calcium phosphate analog. Deficiency of B1 cells resulted in failure of bone resorption. Moreover, reconstitution of these cells resulted in regaining of osteoclastogenesis (193).

In a human model of multiple myeloma, nuclei of malignant B cells were found in osteoclasts that might suggest that myeloma cells can differentiate into osteoclasts explaining the mechanism of increased osteoclastogenesis in these patients (194).
Additionally, deletion of any of the transcription factors Ebf-1, paired box protein 5 (Pax5) and PU.1, which are essential for B cell development, resulted in marked changes in bone turnover in vivo. The transcription factor PU.1 is a protein encoded by the SPI1 gene, it is a specific factor for gene expression during myeloid and B-lymphoid cell development (195, 196).

Levels of PU.1 positively correlated with osteoclastic differentiation in vitro and deletion of PU.1 in vivo led to the arrest of osteoclast and macrophage differentiation. More recently, reduction in PU.1 activity resulted in impaired B-cell development (195, 196).

Ebf-1 deficiency in mice, another key regulator of B cell development, led to increased osteoclastogenesis and impaired B cell development, but despite this there was an overall increased bone mass. The authors explained that the mechanism of increased bone formation was related to increased numbers and activity of osteoblasts. However, there is a hypothesis that B cell depletion may share in this anabolic effect on bone (197).

4.2.D. B cells and estrogen

Estrogens are known to be important regulators of both the skeletal and immune systems. Estrogen is able to inhibit the autoimmune disturbances in RA evidenced by decreased disease activity during pregnancy and a flare up after delivery. Accordingly, estrogens are capable of affecting the disease course and bone density in RA. Replacing estrogen deficiency is an established treatment for postmenopausal osteoporosis.

Estrogen has the ability to both inhibit the cellular immune response by T cells and stimulate the humoral response by B cells. In fact in postmenopausal women, it has been found that down regulation of estrogen receptor 1 (ESR1) and mitogen activated protein kinase 3 (MAPK3) in B cells, regulates secretion of factors leading to either increased
osteoclastogenesis or decreased osteoblastogenesis, while estrogen deficiency stimulates the hematopoietic synthesis of B cells (198).

Indeed, the increased osteoclastogenesis resulting from estrogen deficiency might be partly due to increased B cell proliferation and activity. The increased B cell numbers may either directly affect bone turnover, if B cell precursors are actually able to differentiate into active osteoclasts, or act indirectly through the release of different cytokines (199).

Mouse studies showed that B cells are able to stimulate the conversion of monocytes to active osteoclasts both indirectly through the production of RANKL and M-CSF and directly through their proximity in the bone marrow. However, the mechanisms of direct stimulation is not clearly understood (200). Other studies suggested that osteoclasts and B-lymphocytes might both arise from pro-B cells (201).

Pro-B cells from osteopetrotic mice for example expressed markers from the B-lymphoid (CD19, CD43 and CD5) and the myeloid (F4/80) lineages. When stimulated with RANKL and M-CSF these cells could differentiate into osteoclasts, while they were able to differentiate into B cells when stimulated by IL-7 (202).

In a different study, depletion of a subset of B cells named B-1 cells in mice with induced periodontitis resulted in impaired osteoclastogenesis and bone resorption. However, reconstitution of B-1 cells in these mice led to increased bone resorption to near normal values. This subtype of B cells express RANK and M-CSFR. Stimulation of B cells with RANKL and M-CSF transformed these cells into functional TRAP positive osteoclast-like multi-nucleated giant cells (203).

These cells express RANK and the macrophage colony-stimulating factor receptor (M-CSFR) and when stimulated with RANKL and M-CSF, these cells differentiated into tartrate resistant acid phosphatase (TRAP) positive osteoclasts, which formed lacunae when allowed to grow
on a calcium phosphate analog. Deficiency of B1 cells resulted in failure of bone resorption in these mice (203). Moreover, reconstitution of these cells resulted in restoration of osteoclastogenesis.

Other mice studies showed that deletion of any of the transcription factors Ebf-1, paired box protein 5 (Pax5) and PU.1, which are essential for B cell development, resulted in marked changes in bone turnover. Expression of Pax5, for example, is restricted to B-lymphocytes (203).

In Pax5 depleted mice, B cell development was stopped at the pro-B cell stage. These mice developed severe osteoporosis, with 60% bone loss compared to baseline. The diminished bone mass was explained by doubling of the numbers of osteoclasts together with a mild reduction in osteoblast numbers. The result was a marked decrease in bone volume, specifically trabecular bone in tibiae and femur. However, the authors suggested that the bone loss was not due to B cell depletion, because a similar effect could not be found in other strains of B cell-deficient mice (203).

The transcription factor PU.1 is a protein encoded by the SPI1 gene; it is a specific factor for gene expression during myeloid and B-lymphoid cell development. Levels of PU.1 positively correlated with osteoclastic differentiation in vitro and deletion of PU.1 in mice led to the arrest of osteoclast and macrophage differentiation. More recently, reduction in PU.1 activity resulted in impaired B-cell development (204, 205).

Ebf-1 deficiency in mice, another key regulator of B cell development, led to increased osteoclastogenesis and impaired B cell development, but despite this there was an overall increased bone mass. The authors explained that the mechanism of increased bone formation was the concomitant increase in the numbers and activity of osteoblasts. However, there is a
hypothesis that B cell depletion may share in this anabolic effect on bone (197). These data support the close connection between B cells and osteoclasts development.

In another murine model, quantification of OPG in the bone marrow using ELISA and real time PCR showed that B cells are responsible for the production of 64% of total BM OPG. In the same study, depletion of B cells in these mice developed osteoporosis due to the deficiency of OPG in the bone marrow. Moreover, reconstitution of B cells in these mice reversed the bone loss (206).

In humans with multiple myeloma, examining the bone sections revealed the presence of osteoclasts which contain transcriptionally active nuclei of malignant myeloma-B cells. These hybrid cells are thought to either develop from the fusion of osteoclasts and malignant B cells or due to the conversion of the malignant B cells into functioning osteoclasts in multiple myeloma patients. This explains the marked increase of osteoclastogenesis in these patients (190). Whether this mechanism is found only in malignant B cells or stimulated healthy B cells are capable of attaining the same capabilities needs to be further investigated.

In summary, literature review confirms the presence of a close interaction between B cells and bone cells. However, the results are conflicting. Additionally, there is a clear lack of evidence of these effects in humans and the underlying mechanisms behind them.

4.3. B cell depletion in RA osteoporosis

In one study, 31 active RA patients with long-standing disease treated with rituximab were assessed at baseline and after 12 weeks using DAS 28 & HAQ as well as laboratory parameters using ESR, CRP, RF levels in addition to flow cytometry of CD19, CD27, CD38 and CD45 in peripheral blood and bone marrow. Results showed a significant improvement of disease activity indices and RF concentrations. RTX markedly reduced CD19+ B cells in peripheral blood and to a lesser extent in the bone marrow. Additionally, it was found that
there was a significant decrease in CD19+CD27+ memory B cells in both compartments in patients who had a good response to RTX treatment compared to increased or stable levels of memory cells in the ones who did not have a favorable clinical response (207).

In another study, 13 active RA patients were assessed every 3 months over 1 year post RTX treatment. Samples were tested for Type I C-terminal collagen propeptide (CICP) and tartrate resistant acid phosphatase isoform 5b (TRAP 5b), Osteoprotegerin (OPG) and total sRANKL, Bone-specific alkaline phosphatase. The authors found no significant effect of RTX on the measured biomarkers. However, the sample was small and there were no bone mineral density measurements to support the results (208).

28 patients with active RA treated with RTX were assessed by X-ray of hands and feet at baseline and after 1 year of treatment using the Sharp–van der Heijde score (SHS), immunohistochemistry and immunofluorescence of synovial biopsies, serum levels of bone markers including OPG, RANKL, osteocalcin and cross-linked N-telopeptides of type I collagen (NTx) were measured by ELISA obtained at baseline and 16 weeks after treatment. There was an improvement in the mean (SD) of SHS of 1.4 (10.0) after 1 year (209).

After 16 weeks of treatment, there was a decrease of 99% in RANK-positive osteoclast precursors and a decrease of 37% of RANKL expression in the synovium associated with 25% reduction in synovial OPG expression. In serum, both OPG and RANKL levels were significantly reduced by 20% and 40% respectively after 16 weeks, but the OPG/RANKL ratio increased by 157% (209).

Additionally, osteocalcin levels increased but not significantly while NTx concentrations did not change. It was concluded from this study that B cell depletion reduces the synovial osteoclast precursors and RANKL expression and increases bone formation by increasing the OPG/RANKL ratio in serum (210).
CHAPTER 5. BONE TURNOVER ASSESSMENT

The identification of cellular components of bone matrix led to development of specific markers to aid the management of osteoporosis. Bone biomarkers are formation markers, resorption markers or the newly discovered osteocyte markers. Though, this distinction is not clear cut as some markers may represent both processes e.g. osteocalcin. Some of these biomarkers are now reliable, sensitive, specific and cost-effective methods to detect bone turnover. These markers have been used in research for a long time. Currently, there are great efforts to improve their accuracy to allow them to be used in clinical practice. During recent years, methods of detection of these markers have greatly improved and become reliable in measuring these markers.

These bone markers can be found in blood or urine. Under physiological conditions, bone-remodeling processes are coupled. Measurement of a combination of formation and resorption markers is crucial to be able to determine the metabolic activity of bone cells. However, these markers are not disease specific, yet they can give a good idea of what is going on in bone turnover processes (211).

Unfortunately, some bone markers are really not bone-specific and can change in non-skeletal diseases as well. Accordingly, translation of their levels should be with great caution in coordination with the clinical picture of the patient (211).

5.1. MARKERS OF BONE FORMATION

Markers of bone formation are either by-products of active developing osteoblasts or enzymes generated by osteoblasts during their activity. The most popular markers of bone formation nowadays are serum bone specific alkaline phosphatase (BAP), osteocalcin and the carboxy- and amino-terminal propeptides of type 1 collagen (P1CP, P1NP). BAP is a component of the cellular membrane of osteoblasts. During bone mineralization, BAP is cleaved by
phospholipase and enters the circulation. BAP activity and concentration can both be measured in serum with almost similar results (212).

Osteocalcin is the second most abundant protein in the bone matrix after collagen. It is produced by osteoblasts during bone deposition and can also be found in the serum that can be measured. Interestingly, osteocalcin is also released during bone resorption and is therefore considered a marker of bone turnover rather than pure marker of bone formation. The half-life of osteocalcin in serum is very short lasting about few minutes; then it is rapidly cleared in the urine (213, 214).

Type 1 collagen is manufactured in bone, skin, dentin, cornea, fibrocartilage and tendons (211). It is synthesized by fibroblasts and osteoblasts from pro-collagen. This collagen derivative is cleaved by proteases. The peptides released by the cleavage of pro-collagen are P1CP and P1NP as the C-terminal or the N-terminal propeptides respectively. Both markers are derivatives of collagen formation; hence they can be used as markers of bone formation. Both peptides are metabolized by the liver. In clinical trials, P1NP is more commonly used than P1CP (215-217).

5.2. MARKERS OF BONE RESORPTION

On the contrary, all bone resorption markers are derived from type I collagen degradation except TRAP. The collagen triple helix is stabilized by cross-links that bind the neighboring collagen molecules. Two major cross-links are present: pyridinoline (PYD) and deoxypyridinoline (DPD). They are formed extracellularly after collagen is deposited in bone matrix. During bone collagen degradation, these cross-links are released into the circulation and are excreted in the urine. DPD is more specific for bone than PYD, which is also found in type II collagen (218).
These cross-links are released in the circulation in the form of CTX and NTX. Both CTX and NTX are produced by cathepsin K activity. Both markers can be detected by specific immunoassays. Though, CTX is more widely used. Increase in serum levels of CTX and NTX indicate acceleration of bone resorption rate (214).

TRAP is an acid phosphatase expressed by different tissues and cells in the body. It is cleaved by proteases into two subunits 5a and 5b. TRAP5a is expressed by macrophages and TRAP5b is present in the ruffled border of osteoclasts. Activated osteoclasts secrete TRAP5b, which leads to degradation of type 1 collagen (219).

The OPG/ RANKL system is a major determinant of bone turnover. RANKL is produced by osteoblasts in response to signals, which induce osteoclastogenesis, such as pro-inflammatory cytokines (220).

RANKL binds to RANK receptors found on the surface of osteoclasts leading to their activation and proliferation. On the other hand, OPG, the RANKL decoy receptor, is also produced by osteoblasts. However, it inhibits osteoclastogenesis by binding to RANKL preventing its effects on osteoclasts. Laboratory methods used to measure OPG and RANKL are available but their results need to be validated before they can be used in clinical practice (221).

5.3. OSTEOCYTE MARKERS

Osteocytes play a key role in the regulation of bone turnover. Osteocytes are fully differentiated osteoblasts and lie in lacunae in the mineralized matrix and osteoid tissue of bone (222).

Osteocytes are capable of regulating their own proliferation through their ability to control the differentiation of their derivative osteoblasts; however, the exact mechanism of
osteocytogenesis is still largely unknown. Osteocytes are able to detect changes in bone morphology, particularly micro-fractures through their sensitivity to mechanical forces, acting like bone mechanoreceptors (223), thereby regulating bone turnover through direct physical contact with other bone cells. In vivo studies have shown that osteocytes directly inhibit the activity of osteoclasts and stimulate the activity of osteoblasts, their depletion therefore results in profound loss of trabecular bone mass (224-226).

Moreover, the site of osteocyte apoptosis directs osteoclasts recruitment. On the other hand, increased osteocyte activity resulted in increased bone resorption by osteoclasts through the enhanced expression of osteopontin by active osteocytes. Additionally, osteocytes produce various factors, which affect bone balance such as DMP1, MEPE, sclerostin and DKK1. Research has focused on the Wnt signaling pathway in the management of osteoporosis and related bone diseases. DKK1 is a secreted osteocyte marker acting as an inhibitor to the Wnt signaling pathway through binding to LRP5/6 and hence blocking the Wnt effects on osteoblasts decreasing bone formation (227).

The use of DKK1 as a bone marker for clinical practice needs to be validated due to both its analytical and biological variability. In addition, its response to other factors such as diet and diurnal variations needs to be explored. Sclerostin is another inhibitory protein of bone formation, secreted by osteocytes. It is the product of the SOST gene located on the chromosome 17 (228). It also blocks the Wnt signaling pathway by binding to LRP5/6 on osteoblasts, preventing the coupling of the Fz/LRP complex and thus inhibiting osteoblast activation (229).

PTH decreases sclerostin formation explaining in part how exogenous PTH is able to stimulate bone formation in the treatment of bone loss conditions. This has targeted research into the application of sclerostin as a therapeutic option for the treatment of osteoporosis.
However, its use as a diagnostic marker or for monitoring patient compliance to treatment is still under investigation (230).
CHAPTER 6. PATIENTS AND METHODS

6.1. INTRODUCTION

The study aims were investigated in a multicentre, open-label, single treatment arm, prospective clinical trial. The effect of RTX on bone turnover was investigated in patients with RA eligible to start RTX for the first time during their lifetime according to NICE guidelines. Changes of B cell numbers, bone densitometry, bone markers and changes in disease activity were assessed in these patients to determine whether RTX has any effect on bone turnover similar to anti-TNF blockers and if present, whether the mechanism is indirect through inhibition of disease activity or there is a direct effect of B cell depletion on bone cells.

6.2. STUDY SAMPLE

Forty-five adult RA patients were recruited into the study between the period of August 2011 and September 2012. Patients were recruited on the basis of being RA patients eligible for starting rituximab (RTX) treatment.

According to NICE guidelines, RTX is a second line biological agent; which means that most of these patients failed at least one TNF blocking agent unless it was contraindicated or not tolerated. We excluded any patient who was using treatment for osteoporosis such as bisphosphonates, strontium ranelate, raloxifene, denosumab or teriparatide within three months prior to joining the study.

However, patients using calcium and vitamin D were allowed to join our study. This is because according to NICE guidelines, any patient using long-term corticosteroids should be prescribed calcium and vitamin D. Additionally; these supplements do not affect the functions
of bone cells so are considered as replacement of deficiency of these elements. We also excluded patients who used RTX or any other B cell depleting agent before joining the study.

6.3. Methods

Eligible patients were invited to join the study and were given the study patient information leaflet (PIL). Patients were allowed 2-4 weeks to read the PIL and discuss it with their physician and relatives. All patients who accepted to be enrolled in the study signed an informed consent of approval.

6.3.A. Clinical assessment

Patients who fulfilled the eligibility criteria described above were assessed on 3 monthly bases for one year from starting RTX. At baseline, a full patient history and detailed physical examination were done. History involved basic demographic data, duration of RA, disease activity, comorbidities and detailed drug history. Past history of past surgeries, hospitalizations, previous fractures, risk factors for osteoporosis. Family history of RA and osteoporosis (either a definite diagnosis or a low trauma hip fracture in a first degree relative), smoking, excess alcohol consumption (defined as greater than 21 units per week).

Detailed list of medications and other medical conditions were taken. Recorded data included intake of calcium, alfacalcidol, calcitriol, warfarin, corticosteroids, anticonvulsants or testosterone supplementation. Subjects were checked if they suffered from asthma, epilepsy, kidney disease, hyperthyroidism, eating disorders, coeliac disease, liver disease, gastrectomy, inflammatory bowel disease or other concomitant diseases. Subjects were asked if they had any of the secondary causes of osteoporosis such as long-term immobilization, gastrectomy, liver disease, inflammatory bowel disease, coeliac disease, anorexia nervosa and hypogonadism.
History involved clinical assessment of disease activity using DAS28 score. Health assessment questionnaire (HAQ) was completed by the patient in each visit to measure the functional and disability status.

6.3.B. Laboratory assessment

Underlying causes of osteoporosis were excluded by questionnaires, medical history, physical examination and blood tests. All blood was taken in the morning (9 am) to avoid diurnal variations of bone markers. Blood investigations included: full blood count, ESR, biochemical profile, thyroid function tests (thyroid stimulating hormone (TSH) and free thyroxin), parathyroid hormone (PTH), serum testosterone, estradiol ($E_2$), SHBG, gonadotropins (follicular stimulating hormone (FSH), and luteinizing hormone (LH)) and serum and urine electrophoresis. Biochemical investigations included bilirubin, total protein, albumin, globulin, corrected calcium (adjusted according to albumin levels), phosphate, alanine aminotransferase (ALT), alkaline phosphatase (ALP) and creatinine.

For the purposes of this study, steroid therapy was defined as the use of oral corticosteroids for at least three months. The use of corticosteroids during this study was considered unavoidable as patients were in RA flare. This was considered appropriate at the time of the study design. Regarding the risk of corticosteroid induced osteoporosis; the Royal College of Physicians of London stated that corticosteroid use for more than three months is considered to increase the risk of fractures (Royal College of Physicians of London, 2002). Hypogonadism was considered a diagnosis in the presence of low serum sex hormones and inappropriately raised or decreased gonadotropins. A past history of any gastric surgery or use of anticonvulsants was also regarded as a potential risk factor for osteoporosis.
6.3.C. BMD assessment

BMD was measured in lumbar spine L2-L4, both hips (total and neck of femur) and both wrists using DXA at baseline and after 12 months. All 12 months visit scans were done using the same DXA machine that was used in the baseline scan. DXA scanning was performed using GE lunar prodigy advance compact scanner in the following hospitals: James Cook University hospital (Middleborough), City Hospital Sunderland (Sunderland), North Tyneside General Hospital (Northumbria), Darlington Memorial Hospital (Darlington), Warwick hospital (Warwick) and Cannock Chase Hospital (Cannock). While Hologic Discovery QDR 2000 machine (Hologic Instruments, Waltham, MA, USA) was used in University Hospital of North Tees (North Tees), Queen Elizabeth Hospital (Gateshead) and Dewsbury and district hospital (Dewsbury). The machines had a coefficient of variation (CV%) of 1.0-1.5% at lumbar spine, total hip, forearms (UD) and 1.5-2.0% at femoral neck.

BMD results were obtained as an area density in g/cm², but T and Z scores were also recorded. The T-score is the number of standard deviation units above or below the mean for a healthy 30-year-old adult of the same sex and ethnicity, whilst the Z-score is the number of standard deviation units above or below the normal mean of the same age, sex and ethnicity (calculated using the manufacturer’s standard normal reference database).

6.3.D. General assessment

Further assessment of the patients was done using X-ray of the hands and feet to detect erosions and joint deformities. Laboratory assessment of the patients included routine follow-up investigations such as full blood count, ESR, electrolytes, liver and kidney function tests. Additionally, we tested the parameters that may affect bone turnover such as serum levels of parathyroid hormone, sex hormones and vitamin D levels. Immune profile for each patient
was done and included rheumatoid factor (RF), anti-nuclear antibodies (ANA), and immunoglobulins.

6.3.E. Flow cytometric analysis

Whole blood samples were analyzed using flow cytometry to determine the numbers of CD19+ B cells and its subsets CD24+, CD27+ and CD38+ cells on each visit to correlate the changes in the different B cell subsets with the changes of bone markers and markers of disease activity. FACS analysis of the patients’ samples was done using FACSCanto 1 machine in the institute of cellular medicine, the medical school, Newcastle university.

6.3.F. Bone markers assessment

All samples were taken fasting because of the effects of food intake and the diurnal variation of markers on bone turnover. Serum and plasma samples were stored in -80°C within 1 hour of collection till the end of the study. These samples were used for assessment of the markers of bone formation (Bone specific alkaline phosphatase, P1NP), markers of bone resorption (bCTX, TRAP5b) and markers of osteocyte functions (sclerostin and DKK1). The following biochemical markers were quantified in human serum by a manual solid phase, monoclonal antibody immunoenzymatic assay: BAP (ImmunoDiagnostics, IDS), sclerostin (Biomedica, Austria, CAT. NO. BI-20492), DKK1 (Biomedica, Austria, CAT.NO. BI-20412) and TRAP5b The BoneTRAP® Assay Kit (IDS, Inc., UK), while P1NP and CTX were quantified in plasma by an electrochemiluminescence immunoassay on an automated analyser, Roche - Elecsys 2010. Evaluation of the manual ELISA kits was done my myself and my colleague Gill Wheater during the study. However, these assays were completed by Ms Wheater alone after the end of follow-up period in December 2013.
6.3.G. Determination of the Sample Size

The determination of the sample size was based on the comparison of the BMD at baseline and 12 months. Assuming the true change in BMD of the lumbar spine is 0.01 g/cm2 (based on previous unpublished data from Dr S Tuck) and that the DXA scan is reproducible with a standard deviation of 0.02 g/cm2 (based on data from the manufacturer); then the study should have 80% power to detect a statistically significant difference (at the 5% confidence level) with 33 patients in the final analysis. A meta-analysis on anti-resorptive agents in osteoporosis concluded that a 1% gain in bone density of the spine was associated with a statistically significant reduction of 8% in non-vertebral fractures and a study in a large population cohort showing a decline of bone density over time in RA patients not treated with anti-resorptive agents (13,14). To allow for 25% dropout (based on the treatment criteria of NICE which mean some patients will discontinue therapy early due to treatment failure or complete disease resolution or incompliance with the study) 45 patients were needed based on a one sample T-test.

A 2.77% increase in bone density in lumbar spine and total hip and 3% increase in femoral neck would show that RTX improved BMD based on the minimum coefficient of variation of the DXA machines as provided by the participating centers (1-1.5% for LS and total hip and 1.1-2% for femoral neck). The calculation of the least significant change (LSC) is calculated as 2.77xCV% (ISCD).

6.3.H. Analysis plan:

Our study subjects were grouped into a single study group. Descriptive statistics were used to quantitatively summarise the distribution, central tendency and dispersion of data. Normality testing for each data set was done to determine whether median (95%CI) or means (SD) to be used. The primary endpoint; change in BMD of the lumbar spine and secondary endpoints;
changes in BMD of the hips (total hip and femoral neck) and forearms; bone turnover markers; inflammatory markers and disease activity between baseline and 12 months were investigated using one-way ANOVA if normally distributed or Freidman test if not normally distributed among the five visits.

Pearson’s and spearman correlation coefficient were used to correlate the changes in BMD with changes in biochemical markers of bone turnover, changes in biomarkers of inflammation and changes in circulating B-cells. Multivariate regression analyses were done to determine the best predictors at baseline of BMD and its changes after 12 months.

Patients were divided into two groups at baseline according the presence or absence of erosions and BMD was compared between the subgroups using T-test.

P values <0.05 were considered statistically significant, P values <0.01 were considered highly significant and a trend towards significance were defined as p<0.10.

6.3.1. Accrual:

Fifty-two patients were assessed for eligibility. Five patients were excluded as they did not meet eligibility criteria and 2 patients were excluded due to being on bisphosphonates. Forty-five patients were enrolled in the study group (Figure 4). Patients’ enrolment took place in ten participating centers in England.
Patients were treated with 2x 1,000 mg rituximab i.v. after a bolus of i.v. methylprednisolone 100 mg in accordance with NICE-guidelines. Patients who responded to the first course received a second course at 6 months unless they have attained a state of low disease activity, in accordance with clinical practice. Retreatment was delayed in patients with low disease activity at 6 months until a state of moderate disease activity has been documented in 2 consecutive visits one month apart.

The dose of NSAIDs was adjusted during the study if deemed necessary, e.g. because of a flare, but the dose of DMARDs and corticosteroids remained stable over the study period. We aimed for attainment of a state of low disease activity as measured by DAS score less than 3.2. All patients who fulfilled the eligibility criteria were assessed in 5 visits with 3 months interval as follows:

6.4. BASELINE EVALUATION

- Comprehensive medical patient’s history with particular attention to RA specifics, treatment history.
- Detailed physical and rheumatologic examinations.
Disease activity and health assessment indices (DAS28 and HAQ).

Anthropometric measures (weight, height and body mass index [BMI]).

**Laboratory assessment:**

- **Acute phase response:** ESR and CRP. Normal reference ranges: Males ESR 2-10 mm/hr; Females ESR 3-15 mm/hr; CRP 0-5 mg/L.
- **Chemistry:** PTH and 25OHD. Normal manufacturer’s reference range for 25OHD: Deficient <25; Insufficient 25-50; Adequate 50-75; Optimum >75 nmol/L; PTH 12 - 72 ng/L.
- **Immunology:** serum immunoglobulins including IgM RF and ACPA. Normal reference range RF <20 IU/mL; ACPA = <7U/mL

The following bone biochemical markers were quantified in human serum by a manual solid phase, monoclonal antibody immunoenzymatic assay:

- **BAP** (ImmunoDiagnostics, IDS). Reference range based on manufacturer for premenopausal females 2.9-14.5 µg/L; Postmenopausal females 3.8-22.6 µg/L; Males >50yrs 3.7-20.9 µg/L).
- **P1NP** and CTX were quantified in human serum by an electrochemiluminescence immunoassay on an automated analyzer, Roche - Elecsys 2010. Manufacturer’s reference ranges (Premenopausal females P1NP 15.1-58.6 µg/L, βCTX 25-573 ng/L; Postmenopausal females; P1NP 16.3-73.9 µg/L, βCTX 104-1008 ng/L; Males >50yrs; P1NP 15.1-58.6 µg/L, βCTX 0-854 ng/L,)
- **TRAP5b** (The Bone-TRAP® Assay Kit IDS, Inc., UK; CV = 9% to 13%). Normal manufacturer’s range is for premenopausal females 1.0-4.2 U/L; Postmenopausal females 1.5-4.9 U/L; Males >50yrs 1.9-4.8 U/L.
- **Sclerostin** (Biomedica CAT. NO. BI-20492). Normal range according to manufacturer: Females (21.6-68.1) pmol/L), Males (26.4-68.0) pmol/L.
- DKK1 (Biomedica CAT.NO. BI-20412). Normal range according to manufacturer: Females (12.4-72.2 pmol/L), Males (15.5-80.8) pmol/L.

Serum samples were cryopreserved at -80 degrees for biomarker studies till the end of the study. Because of the effects of food intake and the diurnal variation of markers of bone turnover, morning fasting blood samples were collected.

**6.5. FOLLOW-UP EVALUATION**

- Follow-up was performed at 3, 6, 9, 12 months after baseline.
- Patient’s history, concomitant medication, detailed physical and rheumatologic examinations.
- DAS28 and HAQ.
- ESR and CRP.
- Biochemical markers of bone turnover (BAP, P1NP, TRAP5b, CTX, sclerostin and DKK1).

**6.6. STUDY PROCEDURE**

The treatment protocol followed the routine clinical practice. No change in DMARDs or steroids dose were allowed during the study. The studied patients were followed up over one year. Patients were subjected to full history taking and thorough clinical examination (systemic and articular). Full laboratory investigations were done to confirm the diagnosis and to rule out patients with any of the exclusion criteria. Clinical assessments included DAS28 and HAQ at baseline, 3, 6, 9, and 12 months were recorded. Height and weight were recorded at baseline for all subjects. All patients were aimed for tight control of disease activity using traditional and biological DMARDs.
A comprehensive analysis of the changes in bone markers in serum was done using CTX and TRAP5b as markers of bone resorption; on the other hand, BAP, P1NP and the osteocyte markers (sclerostin and DKK1) levels in serum as markers of bone formation.

Changes in biomarkers and disease activity were analysed using descriptive statistics in both groups. Results of biomarkers measurements were correlated with disease activity parameters including CRP, ESR, PtGA, PhGA, DAS28, and HAQ.

BMD was measured in lumbar spine L2-L4, both hips (total and neck of femur) and both wrists using DXA scan at baseline and after 12 months. All 12 months visit scans were done using the same DXA machine that was used in the baseline scan. The first baseline scan was done on 8th of August 2011 and the last baseline scan was done on the fourth of September 2012.

6.7. ADMINISTRATIVE AND ETHICAL CONSIDERATIONS

The National Research Ethics Service (NRES), United Kingdom (UK) have approved the study. All documents related to the study have been prepared according to Good Clinical Practice (GCP) standards including PIL, consent forms, case record forms (CRF), standard operation procedures (SOP).

Research and development (R&D) department approval have been granted in participating centers. An electronic and a hard copy master file have been made. The study database has been made to include all patients’ data from different centers.

Each study subject was anonymized by an ID made of four digits, where the first two digits represent the center’s ID and the second two digits represent the patient’s number.

6.8. DATA MANAGEMENT AND STATISTICAL ANALYSIS
Upon completion of data collection, variables included in each data collection sheet were organized and tabulated then coded prior to computerized data entry using Microsoft Excel software. The data were then imported into Statistical Package for the Social Sciences (SPSS version 20.0) software for statistical analysis.

6.9. TIME PLAN

![Time plan of the study](image.png)

*Figure (5)* Time plan of the study.

6.10. Bone markers methods evaluation

6.10.A) DKK1 & sclerostin assays

Sclerostin (Biomedica, Austria, CAT. NO. BI-20492) and DKK1 (Biomedica, Austria, CAT.NO. BI-20412) manual ELISA kits were used to analyze 24 healthy control samples (12 females & 12 males) within 24 hours from being taken and 10 samples (males and females) stored in -80 C for 2 weeks. The aim was to evaluate these kits and to check the difference in detection between fresh and stored samples. Additionally, we wanted to evaluate the difference in serum levels of these markers among males and females. The full details of the methods used are attached in the index.
Males had higher levels of DKK1 compared to females using fresh samples with mean (SD) 37.3(21.3) pmol/l and 32.7(21.7) pmol/l respectively. Though, the difference was not statistically significant (P value = 0.68). Similarly, serum levels of sclerostin were higher in males compared to females with levels 24.3(5.7) pmol/l and 17.2(6.6) pmol/l respectively. The difference was statistically significant (P=0.01). Sclerostin levels were almost similar in both fresh and stored samples. Although there was a slight difference between fresh and stored samples for DKK1, the difference was not statistically significant (P=0.1).

Table 3 showing results of serum levels of DKK1 and sclerostin in 24 healthy control samples (12 females & 12 males) within 24 hours from being taken and 10 samples (males and females) stored in -80 C for 2 weeks.

<table>
<thead>
<tr>
<th></th>
<th>Sclerostin Mean (SD)</th>
<th>DKK1 Mean (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>24.3 (5.7)</td>
<td>37.3 (21.3)</td>
</tr>
<tr>
<td>Female</td>
<td>17.2 (6.7)</td>
<td>32.7 (21.7)</td>
</tr>
<tr>
<td>Fresh</td>
<td>20.8 (7.0)</td>
<td>39.3 (21.0)</td>
</tr>
<tr>
<td>Stored</td>
<td>20.5 (5.2)</td>
<td>36.0 (8.9)</td>
</tr>
</tbody>
</table>

6.11. Pilot study to compare serum levels of osteocyte markers among seropositive versus seronegative RA patients

Rationale

It is well established that plasma cells, a subset of B cells, are the main producer of autoantibodies such as RF and ACPA. However, the effect of hyperactive B cells and disease activity on bone turnover markers is still controversial. So, the aim of this study was to determine the effect of positivity of RF and the effect of RA disease activity on serum levels
of osteocyte markers such as sclerostin and DKK1 in RA patients. The availability of serum samples of RA patients from a prior study provided us with a unique opportunity to determine the effect of pathological autoantibodies and disease activity on bone turnover.

Methods

Our group (Myself and my colleague Gill Wheater) used stored serum samples of 291 RA patients collected during a previous study aiming to evaluate the reliability of self-reported smoke exposure in comparison with serum cotinine measurement for identifying smokers among patients attending the JCUH outpatient department. We analyzed the serum levels of osteocyte markers (sclerostin and DKK1) in these samples using Sclerostin (Biomedica, Austria, CAT. NO. BI-20492) and DKK1 (Biomedica, Austria, CAT.NO. BI-20412) manual ELISA kits. RF IgM levels were similarly analyzed using manual ELISA (Immunodiagnostics (IDS)). Manual ELISA assays were done in the research laboratory, JCUH. While CRP levels were analyzed in the clinical pathology department, JCUH using Roche automated analyzer.

Results

We divided our samples into two groups according to their CRP levels. Patients with CRP levels higher than 20 mg/l were considered to be having high disease activity while patients with CRP levels lower than 20 mg/l were considered to have low disease activity based on previous unpublished data from our group. Descriptive statistics were done to compare between the two groups. 162 patients had high disease activity were tested for sclerostin while 45 patients had low disease activity. 84 samples were excluded from analysis, as the amount of serum was not enough for analysis. Patients with high disease activity had higher levels of sclerostin but the difference was not statistically significant (P=0.418). For DKK1, 220 patients had high disease activity compared to 65 patients with low disease activity. 6 samples were excluded due to deficient amount of serum left. DKK1 was significantly higher in
patients with high disease activity compared to the patients with low disease activity and the difference was statistically significant (P=0.02).

**Table 4** shows the serum levels of sclerostin and DKK1 among patients with high and low disease activity

<table>
<thead>
<tr>
<th>Bone marker</th>
<th>High disease activity Mean (SD)</th>
<th>Low disease activity Mean (SD)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sclerostin</td>
<td>35.8 (17)</td>
<td>38.8 (23)</td>
<td>P=0.418</td>
</tr>
<tr>
<td>DKK1</td>
<td>36 (14.5)</td>
<td>31.7 (15.7)</td>
<td>P=0.02</td>
</tr>
</tbody>
</table>

Furthermore, we divided our patients into two groups according to their RF level. We categorized them as patients with seronegative RA (RF IgM < 20U/l) versus patients with seropositive RA (RF IgM > 20 U/L).

**Table 5** shows the bone markers levels in seronegative RA and seropositive RA patients.

<table>
<thead>
<tr>
<th>Bone marker</th>
<th>Seronegative patients Mean (SD)</th>
<th>Seropositive patients Mean (SD)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sclerostin</td>
<td>37.7 (23.3)</td>
<td>38.3 (21.1)</td>
<td>P=0.862</td>
</tr>
<tr>
<td>DKK1</td>
<td>30.2 (11.9)</td>
<td>35.0 (16.4)</td>
<td>P=0.014</td>
</tr>
</tbody>
</table>

Descriptive statistics were used to compare the sclerostin and DKK1 levels between the two groups. For sclerostin analysis, 69 patients had seronegative RA; 138 patients had seropositive RA and 84 samples were excluded due to insufficient amount of serum available for testing. Sclerostin levels were similar in the two groups with mean (SD) levels 37.7(23.3) and 38.3(21.1) respectively (P=0.862). For DKK1, 86 patients had seronegative RA, 199 patients had seropositive RA. 6 samples were excluded from analysis due to lack of amount of
serum available for testing. DKK1 was found to be higher in the seropositive (35 pmol/l) compared to the seronegative RA patients (30.2 pmol/l) (P=0.014).

**Discussion**

Our results are consistent with a previous study that suggested that serum levels of DKK-1 were significantly higher in patients with RA than in healthy controls and those with other rheumatic diseases. Moreover, lower serum levels of DKK1 were found in patients receiving treatment with TNF inhibitors and IL-1 Ra compared to patients receiving traditional DMARDs. However, the mechanism for this is still under investigation (237-239).

**Conclusion from the pilot study**

Patients with higher disease activity (CRP>20 mg/l) tend to have significantly higher levels of DKK1 compared to patients with low disease activity (CRP<20 mg/l). Moreover, RA patients with positive RF have significantly higher levels of DKK1 compared to RA patients with negative RF. However, sclerostin levels were similar in these groups. This suggests that patients with high disease activity and patients with positive RF had inhibited bone formation through increased production of DKK1 by osteocytes compared to patients with low disease activity and patients with seronegative RA respectively. This may suggest that hyperactive plasma cells, a subset of B cells, as well as increased inflammation may inhibit bone formation through increasing DKK1 production by osteocytes. This may explain part of the mechanism of bone loss in RA.

**Limitations of the pilot study**

In this study, we used stored serum samples that were collected for a different aim so samples were randomly taken during the day and non-fasting. We did not have data about the medications taken by these patients. So, we presume some of these patients might be using bisphosphonates or other drugs that may affect the bone markers levels.
CHAPTER 7. RESULTS

This multicenter, open-label, single treatment arm clinical study was designed to investigate bone density measurements using DEXA scan, disease activity using DAS28 and bone turnover using changes in serum levels of bone formation (BAP and P1NP), bone resorption (TRAP5b and CTX) and osteocyte biomarkers (sclerostin and DKK1) in patients with rheumatoid arthritis treated with rituximab over one year follow-up.

This chapter will examine changes in BMD, disease activity, flow cytometric analysis of B cells and bone markers in response to rituximab treatment over 1 year of treatment.

As presented in table 6, the mean age of patients was 59.3±12.1 years. Concerning the gender, 36 (80%) of the patients were females. 26 (57.8%) females were postmenopausal. 43 (95.6%) subjects were from white origin while 2 patients were Asians. The mean body mass index was 29.6±7.5. Fifteen subjects (33.3%) were current smokers, 16 (36%) were ex-smokers and the rest were non-smokers. Our patients had a severe active disease at baseline DAS28=6.1±1.3 as per the indication for rituximab according to NICE guidelines. Several comorbidities were recorded for the study participants, including diabetes mellitus (1 patient, 2.2%), thyroid diseases (8 patients, 17.8%), hypertension (18 patients, 40.0%) and CVD (19 patients, 42.2%). Nodules were found in 17 (37.8%) of participants.

Out of the 45 patients who joined the study, 9 patients had an early withdrawal from the study; three patients had to start bisphosphonates for being found to have osteoporosis at baseline as decided by their treating physician. Three patients had inappropriate response to RTX and were shifted to other biological therapies. One patient developed chronic lymphocytic leukemia (CLL), another patient developed secondary renal amyloidosis and a third patient had to be withdrawn due to non-compliance with 2 consecutive study visits. The
baseline characteristics included all patients who joined the study, while other comparative and follow-up analyses included only the 36 patients who successfully completed the study. Thirty-two patients received their second course of RTX as planned at the 6th month while four patients were postponed. Three patients were having DAS28 score less than 3.2; so we agreed with their treating physicians that it would be appropriate to postpone their second RTX course and one patient refused to take the second course based on a discussion with her treating physician that she was feeling well and she wanted to postpone it. All study participants have never used any treatment for osteoporosis during their lifetime. Missing data at any timepoint were excluded from analysis at all visits.
Table 6. Baseline characteristics of the study participants

<table>
<thead>
<tr>
<th>Baseline characteristics</th>
<th>No. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (mean±SD, range)</td>
<td>59.3± 12.1 (33-86)</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>36(80.0%)</td>
</tr>
<tr>
<td>Male</td>
<td>9(20.0%)</td>
</tr>
<tr>
<td>Ethnicity</td>
<td></td>
</tr>
<tr>
<td>White</td>
<td>43(95.6%)</td>
</tr>
<tr>
<td>Asian</td>
<td>2(4.4%)</td>
</tr>
<tr>
<td>Smoking status</td>
<td></td>
</tr>
<tr>
<td>Non-smoker</td>
<td>16(35.6%)</td>
</tr>
<tr>
<td>Current smoker</td>
<td>15 (33.3%)</td>
</tr>
<tr>
<td>Ex-smoker</td>
<td>14(31.1)</td>
</tr>
<tr>
<td>Post-menopausal – n(%)</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>10(22.2%)</td>
</tr>
<tr>
<td>Yes</td>
<td>26(57.8%)</td>
</tr>
<tr>
<td>BMI (Kg/m²) (mean±SD)</td>
<td>29.6 ±7.5</td>
</tr>
<tr>
<td>Baseline DAS28 (mean±SD)</td>
<td>6.1 (1.3)</td>
</tr>
<tr>
<td>Diabetes</td>
<td>1(2.2%)</td>
</tr>
<tr>
<td>Thyroid</td>
<td>8(17.8%)</td>
</tr>
<tr>
<td>Hypertension</td>
<td>18(40.0%)</td>
</tr>
<tr>
<td>CVD</td>
<td>19(42.2%)</td>
</tr>
<tr>
<td>Nodules</td>
<td>17(37.8%)</td>
</tr>
</tbody>
</table>
Serum levels of vitamin were sub-grouped as follows: Deficient <25; Insufficient 25-50; Adequate 50-75; Optimum >75 nmol/L. It was found that 68.9% of patients had low 25OHD (25 hydroxyvitamin D). These included patients deficient in 25OHD with levels <25 nmol/L and patients with insufficiency who had levels between 25-50 nmol/L at baseline. At baseline, the median (95% CI) for vitamin D3 was 31.15 (21.5-44.76). The median PTH at baseline was 34.0 (95%CI=34.6-46.4) ng/L as shown in table 7.

**Table 7.** Baseline PTH and 25OHD results of the study participants.

<table>
<thead>
<tr>
<th></th>
<th>Mean SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTH (ng/L) (Normal 12-72 ng/L)</td>
<td>34.0 (34.6-46.4)</td>
</tr>
<tr>
<td>Median (95%CI)</td>
<td></td>
</tr>
<tr>
<td>25OHD (nmol/L)</td>
<td></td>
</tr>
<tr>
<td>Median (95%CI)</td>
<td>31.15 (21.50-44.76)</td>
</tr>
<tr>
<td>Normal (50-125 nmol/L) (No., %)</td>
<td>14 (31.1%)</td>
</tr>
<tr>
<td>Low 25OHD (Deficiency &lt;25 nmol/L) + (Insufficiency 25-50 nmol/L) (No., %)</td>
<td>31 (68.9%)</td>
</tr>
</tbody>
</table>

At baseline, 33.3% of the study participants were using prednisolone during the study with a mean dose of (SD)=11.2(9.8) mg. We tried to keep the dose of steroids stable during the study unless decided inappropriate by the treating physician.

**Table 8.** Steroids use by the study participants.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Study subjects (N=45 patients)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prednisolone</td>
<td>15(33.3%)</td>
</tr>
<tr>
<td>Prednisolone dose (mg) (mean ± SD, range)</td>
<td>11.2±9.8 (5-40)</td>
</tr>
</tbody>
</table>
7.1. DISEASE ACTIVITY

CRP data were not normally distributed. Accordingly, Friedman test was used to compare CRP values among the five visits. Although there was a noticeable reduction of median CRP from 12.6 (95%CI = 6.3-27.1) mg/dl at baseline to 8.0 (95%CI = 3.4-13.1) mg/dl at 12 months, the change was not statistically significant (p=0.0698). Disease activity measure DAS28 was tested using one-way ANOVA as data were normally distributed. DAS28 score decreased significantly from highly active disease 6.08 (SD=1.298) at baseline to moderate disease activity score 4.66 (SD=1.417). It is clear that both measures improved after 1 year of rituximab treatment. Yet, improvement of DAS28 components tender joints count, swollen joints count, patient’s global assessment, physicians’ global assessment and ESR were more remarkable than the improvement of CRP in response to treatment.

Table 9. Changes of disease activity during follow-up

<table>
<thead>
<tr>
<th>Variables</th>
<th>Baseline</th>
<th>3 months</th>
<th>6 months</th>
<th>9 months</th>
<th>12 months</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRP (mg/dl)</td>
<td>12.6 (6.3-27.1)</td>
<td>8.9 (4.9-14.7)</td>
<td>9.5 (5.3-17.8)</td>
<td>11.5 (6.7-15.4)</td>
<td>8.0 (3.4-13.1)</td>
<td>0.0698***</td>
</tr>
<tr>
<td>Median (95% CI)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DAS28 Mean (SD)</td>
<td>6.08(1.298)</td>
<td>5.05(1.221)</td>
<td>4.95(1.583)</td>
<td>4.55(1.667)</td>
<td>4.66(1.417)</td>
<td><em>&lt;0.001</em>***</td>
</tr>
</tbody>
</table>

*Highly significant at p<0.01, **Statistically significant at p<0.05, *** Friedman test, ****one way ANOVA

CRP=C-reactive protein; DAS28=disease activity scale using 28 joints

After performing Bonferroni corrections, none of pairwise comparisons between CRP levels among different visits was statistically significant.
Figure 7. Changes of CRP values during the follow up period post-rituximab

The central box represents the values from the lower to upper quartile (25 to 75 percentile). The middle line represents the median. There are two sets of horizontal lines; the narrow horizontal lines represent the 95% confidence interval of the median, the wide horizontal lines extend from the minimum to the maximum value, excluding "far out" values which are displayed as separate points. A far out value is defined as a value that is smaller than the lower quartile minus 3 times the interquartile range, or larger than the upper quartile plus 3 times the interquartile range (wide horizontal lines set). These values are plotted with a different colour marker.
Figure 8. Changes of DAS28 score over 12 months post rituximab

The central box represents the values from the lower to upper quartile (25 to 75 percentile). The middle line represents the median. There are two sets of horizontal lines; the narrow horizontal lines represent the 95% confidence interval of the median, the wide horizontal lines extend from the minimum to the maximum value, excluding "far out" values which are displayed as separate points. A far out value is defined as a value that is smaller than the lower quartile minus 3 times the interquartile range, or larger than the upper quartile plus 3 times the interquartile range (wide horizontal lines set). These values are plotted with a different colour marker.

7.2. BONE MARKERS

A) Bone formation markers

Bone formation markers P1NP and BAP increased after one year of rituximab treatment. Median P1NP levels increased from 41.9 (95%CI=34.1-46.4) µg/L at baseline to 47.7 (95%=42.0-61.4) µg/L at the end of the study. However, the rise was not statistically significant (p=0.066) as was tested by Freidman test (data were not normally distributed). On the other hand, the mean BAP level increased from 18.996 (SD=6.496) µg/L at baseline to
21.256 (7.733) µg/L at 12 months. The change was statistically significant as tested using one-way ANOVA (p=0.022). As shown in figures 9 and 10, there is an increasing trend in both formation markers. This indicates that bone formation rate has improved during the study period in response to rituximab. Whether this is due to inhibition of inflammation and disease activity or due to a direct effect of B cell depletion on bone needs further analysis.

Table 10. Changes of bone formation markers (BAP and P1NP) during follow-up

<table>
<thead>
<tr>
<th>Variables</th>
<th>Baseline</th>
<th>3months</th>
<th>6months</th>
<th>9months</th>
<th>12months</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1NP (µg/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median (95%CI)</td>
<td>41.9</td>
<td>49.9</td>
<td>49.4</td>
<td>48.9</td>
<td>47.7</td>
<td>0.066**</td>
</tr>
<tr>
<td>BAP (µg/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean (SD)</td>
<td>18.996 (6.496)</td>
<td>20.967(8.481)</td>
<td>20.331(6.451)</td>
<td>20.274(6.939)</td>
<td>21.256(7.733)</td>
<td>0.022***</td>
</tr>
</tbody>
</table>

*Significant p-value <0.05, **Friedman test, ***one way ANOVA

P1NP=amino-terminal pro-peptide of type I pro-collagen; BAP=bone-specific alkaline phosphatase; µg/L= microgram per liter.

After performing Bonferroni corrections, none of pairwise comparisons between serum P1NP levels among different visits was statistically significant.
Figure 9. Changes in P1NP levels over 12 months follow-up post-rituximab.

The central box represents the values from the lower to upper quartile (25 to 75 percentile). The middle line represents the median. There are two sets of horizontal lines; the narrow horizontal lines represent the 95% confidence interval of the median, the wide horizontal lines extend from the minimum to the maximum value, excluding "far out" values which are displayed as separate points. A far out value is defined as a value that is smaller than the lower quartile minus 3 times the interquartile range, or larger than the upper quartile plus 3 times the interquartile range (wide horizontal lines set). These values are plotted with a different colour marker.
Figure 10. Changes in BAP levels over 1 year follow-up of rituximab treatment.

The central box represents the values from the lower to upper quartile (25 to 75 percentile). The middle line represents the median. There are two sets of horizontal lines; the narrow horizontal lines represent the 95% confidence interval of the median, the wide horizontal lines extend from the minimum to the maximum value, excluding "far out" values which are displayed as separate points. A far out value is defined as a value that is smaller than the lower quartile minus 3 times the interquartile range, or larger than the upper quartile plus 3 times the interquartile range (wide horizontal lines set). These values are plotted with a different colour marker.
7.3. BONE RESORPTION MARKERS

Serum levels of TRAP5b and CTX were not normally distributed. So, testing the change between the five visits was done using Friedman test. Although there was a reduction in both resorption markers as median TRAP5b decreased from 3.07 (95%CI=2.765-3.470) U/L at baseline to 2.88 (95%CI=2.756-3.464) U/L after 12 months; while median CTX decreased from 436.0 (95%CI=373.4-503.5) ng/L at baseline to 391.0 (299.0-469.7) ng/L after 12 months following treatment with rituximab. However, this reduction was not statistically significant (p=0.676, p=0.800 respectively).

Table 11. Changes of bone resorption markers in response to rituximab (TRAP5b and CTX) during follow-up:

<table>
<thead>
<tr>
<th>Variables</th>
<th>Baseline</th>
<th>3months</th>
<th>6months</th>
<th>9months</th>
<th>12months</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRAP5b (U/L) Median (95%CI)</td>
<td>3.07(2.765-3.470)</td>
<td>3.27 (2.884-3.441)</td>
<td>3.11 (2.783-3.620)</td>
<td>3.04 (2.795-3.458)</td>
<td>2.88 (2.756-3.464)</td>
<td>0.676</td>
</tr>
<tr>
<td>CTX (ng/L) Median (95%CI)</td>
<td>436.0 (373.4-503.5)</td>
<td>368.0 (271.5-446.7)</td>
<td>405.0 (309.5-471.2)</td>
<td>379.0 (302.0-428.7)</td>
<td>391.0 (299.0-469.7)</td>
<td>0.800</td>
</tr>
</tbody>
</table>

*Significant p-value <0.05, **Friedman test

TRAP5b=tartrate-resistant acid phosphatase 5b; CTX=cross-linked carboxy-terminal telopeptide of type I collagen; U/L= unit per liter; ng/L= nanogram per liter.

After performing Bonferroni corrections, none of pairwise comparisons between neither TRAP5b nor CTX levels among different visits was statistically significant.
Figure 11. Changes in serum levels of TRAP5b during the follow up period.

The central box represents the values from the lower to upper quartile (25 to 75 percentile). The middle line represents the median. There are two sets of horizontal lines; the narrow horizontal lines represent the 95% confidence interval of the median, the wide horizontal lines extend from the minimum to the maximum value, excluding "far out" values which are displayed as separate points. A far out value is defined as a value that is smaller than the lower quartile minus 3 times the interquartile range, or larger than the upper quartile plus 3 times the interquartile range (wide horizontal lines set). These values are plotted with a different colour marker.
Figure 12. Changes in serum levels of CTX during 3 monthly intervals.

The central box represents the values from the lower to upper quartile (25 to 75 percentile). The middle line represents the median. There are two sets of horizontal lines; the narrow horizontal lines represent the 95% confidence interval of the median, the wide horizontal lines extend from the minimum to the maximum value, excluding "far out" values which are displayed as separate points. A far out value is defined as a value that is smaller than the lower quartile minus 3 times the interquartile range, or larger than the upper quartile plus 3 times the interquartile range (wide horizontal lines set). These values are plotted with a different colour marker.
7.4. OSTEOCYTE MARKERS

The significance of osteocyte markers DKK1 and sclerostin is that they are inhibitors of the Wnt-ßcatenin pathway of bone formation. So, we predicted that there might be a reduction in their levels as part of the mechanism of increased bone formation rate evidenced by the rising bone formation markers shown above. However, there was no change in both osteocyte markers. This shows that there is another mechanism involved in osteoblastic activation other than the Wnt-ßcatenin pathway.

Data of DKK1 and sclerostin was not normally distributed. So, Freidman test was used to compare their levels among the five visits. There was no significant change among the five visits (p=0.226 and p=0.944 respectively). Median sclerostin level started with 54.50 (95%CI=48.437-58.802) pmol/L at baseline and ended the study with 55.65 (95%CI=50.630-62.368) pmol/L. While that of DKK1 started with 52.80 (95%CI=44.276-57.726) pmol/L at baseline and ended with 51.45 (95%CI=41.191-65.719) pmol/L at 12 months.

Table 12. Changes of osteocyte markers (sclerostin and DKK1) during follow-up

<table>
<thead>
<tr>
<th>Variables</th>
<th>Baseline</th>
<th>3months</th>
<th>6months</th>
<th>9months</th>
<th>12months</th>
<th>P value**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sclerostin (pmol/L)</td>
<td>54.50 (48.437-58.802)</td>
<td>53.10 (49.528-57.397)</td>
<td>51.50 (48.727-54.836)</td>
<td>54.20 (45.366-59.128)</td>
<td>55.65 (50.630-62.368)</td>
<td>0.226</td>
</tr>
<tr>
<td>DKK1 (pmol/L)</td>
<td>52.80 (44.276-57.726)</td>
<td>51.80 (46.684-55.741)</td>
<td>48.80 (37.684-54.812)</td>
<td>51.10 (40.421-59.137)</td>
<td>51.45 (41.191-65.719)</td>
<td>0.944</td>
</tr>
</tbody>
</table>

*Significant p-value <0.05, **Friedman test
After performing Bonferroni corrections, the increase in sclerostin levels between 6 and 12 months was found to be statistically significant (p=0.019). While pairwise comparisons between DKK1 levels among different visits was statistically significant.
Figure 13. Changes of Sclerostin levels during follow up.

The central box represents the values from the lower to upper quartile (25 to 75 percentile). The middle line represents the median. There are two sets of horizontal lines; the narrow horizontal lines represent the 95% confidence interval of the median, the wide horizontal lines extend from the minimum to the maximum value, excluding "far out" values which are displayed as separate points. A far out value is defined as a value that is smaller than the lower quartile minus 3 times the interquartile range, or larger than the upper quartile plus 3 times the interquartile range (wide horizontal lines set). These values are plotted with a different colour marker.
Figure 14. Changes of DKK1 levels during the 3 monthly follow-up visits

The central box represents the values from the lower to upper quartile (25 to 75 percentile). The middle line represents the median. There are two sets of horizontal lines; the narrow horizontal lines represent the 95% confidence interval of the median, the wide horizontal lines extend from the minimum to the maximum value, excluding "far out" values which are displayed as separate points. A far out value is defined as a value that is smaller than the lower quartile minus 3 times the interquartile range, or larger than the upper quartile plus 3 times the interquartile range (wide horizontal lines set). These values are plotted with a different colour marker.

Flow cytometric analysis (FACS) of CD19 B cells including subsets was done for 23 patients every follow-up visit to determine the efficacy of rituximab in depleting B cells and correlating their blood levels with disease activity and serum levels of bone markers. Five of these patients had an early withdrawal from the study.

Table 13 shows changes of absolute counts of CD19 B cells and their subset CD24hiCD38hi. We were mostly interested in this subset as they were assumed to represent the regulatory B
cells (B-reg). Flow cytometric analysis was done for 18 patients only from both Middlesborough and Newcastle upon-Tyne centers. There was a highly significant decrease in both CD19 B-lymphocytes (p<0.00001) and their subset CD24hiCD38hi (p<0.001) absolute counts through the follow up period with an increase in their count at the 6\textsuperscript{th} month then continued to decrease later on. This can be explained by the administration of the second course of rituximab at 6 months. Interestingly, as shown in figure 15, CD24hiCD38hi cells were the first to reappear at 3 and 6 months among the CD19 B cell subsets.

Table 13. Absolute counts of CD19 B cells and their subset CD24hiCD38hi (n=18)

<table>
<thead>
<tr>
<th>Variables</th>
<th>Baseline</th>
<th>3months</th>
<th>6months</th>
<th>9months</th>
<th>12months</th>
<th>P value**</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD19 B lymphocytes (cells/µl)</td>
<td>18.575 (13.916-23.330)</td>
<td>0.044 (.0341-.0626)</td>
<td>0.126 (0.996-2.919)</td>
<td>0.028 (0.023-0.055)</td>
<td>0.070 (0.050-0.134)</td>
<td>&lt;0.00001*</td>
</tr>
<tr>
<td>CD24hiCD38hi (cells/µl)</td>
<td>0.797 (0.177-1.100)</td>
<td>0.008 (0.001-0.012)</td>
<td>0.022 (0.014-1.562)</td>
<td>0.009 (0.002-0.012)</td>
<td>0.006 (0.002-0.018)</td>
<td>&lt;0.00001*</td>
</tr>
</tbody>
</table>

*Highly significant p-value <0.01, **Freidman test
Figure 15. Changes of CD19 B cells over 3 monthly intervals in response to rituximab

The central box represents the values from the lower to upper quartile (25 to 75 percentile). The middle line represents the median. There are two sets of horizontal lines; the narrow horizontal lines represent the 95% confidence interval of the median, the wide horizontal lines extend from the minimum to the maximum value, excluding "far out" values which are displayed as separate points. A far out value is defined as a value that is smaller than the lower quartile minus 3 times the interquartile range, or larger than the upper quartile plus 3 times the interquartile range (wide horizontal lines set). These values are plotted with a different colour marker.
Figure 16. Changes of CD24hiCD38hi B cells over 1 year in response to rituximab.

The central box represents the values from the lower to upper quartile (25 to 75 percentile). The middle line represents the median. There are two sets of horizontal lines; the narrow horizontal lines represent the 95% confidence interval of the median, the wide horizontal lines extend from the minimum to the maximum value, excluding "far out" values which are displayed as separate points. A far out value is defined as a value that is smaller than the lower quartile minus 3 times the interquartile range, or larger than the upper quartile plus 3 times the interquartile range (wide horizontal lines set). These values are plotted with a different colour marker.

7.5. BMD

Despite the promising results obtained from the bone turnover markers, BMD results obtained from DXA scans were relatively inconsistent with those findings. BMD of lumbar spine, femoral neck, total hip and forearms all decreased after 12 months in response to rituximab. Yet, the reduction was variable among the different regions. Median BMD of lumbar spine assessed at L2-L4 showed a slight decrease from 1.138 (95%CI=1.069-1.223) g/cm^3 at baseline to 1.137 (95%CI=1.067-1.216) g/cm^3 at 12 months. Femoral neck, total hip and
both ultra-distal radius were measured independently and the mean of both sides was used for every patient. However when a patient had a metal implant in one side, the affected side was excluded and the BMD of the other side was used for that particular site for this patient. A slight insignificant reduction (p=0.656) when tested using paired T-test was also noted at the femoral neck mean starting with 0.883 (SD=0.140) g/cm^3 and ending the study with 0.878 (SD=0.157) g/cm^3 (p=0.656). BMD of total hip showed a significant decrease from median BMD 0.964 (95%CI=0.914-1.001) g/cm^3 at baseline to 0.946 (95%CI=0.907-0.978) g/cm^3. The change was statistically significant as tested using Wilcoxon signed rank test (p=0.041). Median BMD of means of (UD) radius also decreased from 0.372 (95%CI=0.346-0.395) g/cm^3 at baseline to 0.353 (0.326-0.394) g/cm^3 after 12 months from initiation of rituximab. Yet this change was statistically insignificant (p=0.055) using wilcoxon signed rank test.

**Table 14.** Changes of BMD from baseline to 12th month following RTX

Total subjects = 35 patients

<table>
<thead>
<tr>
<th>Variables</th>
<th>Baseline</th>
<th>12months</th>
<th>P value**</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMD L2-L4</td>
<td>1.138 (1.069-1.223)</td>
<td>1.137 (1.067-1.216)</td>
<td>0.101**</td>
</tr>
<tr>
<td>Median (95% CI)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMD Neck femur (mean Rt&amp;Lt)</td>
<td>0.883 (0.140)</td>
<td>0.878 (0.157)</td>
<td>0.656***</td>
</tr>
<tr>
<td>Mean (SD)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMD Total Hip (mean Rt&amp;Lt)</td>
<td>0.964 (0.914-1.001)</td>
<td>0.946 (0.907-0.978)</td>
<td><em>0.041</em>*</td>
</tr>
<tr>
<td>Median (95% CI)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMD Radius (UD) (mean Rt&amp;Lt)</td>
<td>0.372 (0.346-0.395)</td>
<td>0.353 (0.326-0.394)</td>
<td>0.055**</td>
</tr>
<tr>
<td>Median (95% CI)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
*Significant p-value <0.05, **Wilcoxon signed rank test, Paired T-test***

**Figure 17.** Changes in BMD at different sites in response to RTX over 1 year follow-up

The central box represents the values from the lower to upper quartile (25 to 75 percentile). The middle line represents the median. There are two sets of horizontal lines; the narrow horizontal lines represent the 95% confidence interval of the median, the wide horizontal lines extend from the minimum to the maximum value, excluding "far out" values which are displayed as separate points. A far out value is defined as a value that is smaller than the lower quartile minus 3 times the interquartile range, or larger than the upper quartile plus 3 times the interquartile range (wide horizontal lines set). These values are plotted with a different colour marker.

Correlation analysis was done to examine any relationship between values on each visit and changes from baseline for the following: DAS28, bone makers, BMD at different sites and absolute counts of CD19+ B cells. Unfortunately, there was no significant correlation between each other on different visits not their changes from baseline (p>0.05) except that there was a significant negative correlation between CRP and each of BMD lumbar spine (-0.43, p=
0.011) on one hand and neck of femur (rho=-0.37, p=0.033) on the other hand. Also, total hip BMD showed a significant negative correlation with age (rho=-0.41, p=0.012), CRP (rho=-0.36, p=0.041) and a positive correlation with absolute counts of CD19 B lymphocytes (rho=0.44, 0.007) at 12 months. Radius BMD showed a significant negative correlation with age (rho=-0.37, p=0.035) and a positive one with BMI (rho= 0.43, p=0.013).

**Table 15.** Correlation between BMD L2-L4 and different parameters at 12th month visit

<table>
<thead>
<tr>
<th></th>
<th>BMD Lumbar 2-4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>rho</td>
</tr>
<tr>
<td>Age</td>
<td>0.11</td>
</tr>
<tr>
<td>BMI</td>
<td>0.11</td>
</tr>
<tr>
<td>DAS 28</td>
<td>0.28</td>
</tr>
<tr>
<td>CRP</td>
<td>-0.43</td>
</tr>
<tr>
<td>CD19.B lymphocyte</td>
<td>0.27</td>
</tr>
<tr>
<td>CD24hiCD38hi</td>
<td>-0.24</td>
</tr>
</tbody>
</table>

* Statistically significant at p<0.05
**Table 16.** Correlation between BMD Neck of Femur and different parameters at 12\textsuperscript{th} month visit

<table>
<thead>
<tr>
<th>Parameter</th>
<th>BMD</th>
<th>rho</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>-0.297</td>
<td>0.079</td>
<td></td>
</tr>
<tr>
<td>BMI</td>
<td>0.20</td>
<td>0.254</td>
<td></td>
</tr>
<tr>
<td>DAS 28</td>
<td>0.27</td>
<td>0.123</td>
<td></td>
</tr>
<tr>
<td>CRP</td>
<td>-0.37</td>
<td>0.033*</td>
<td></td>
</tr>
<tr>
<td>CD19.B lymphocyte</td>
<td>0.147</td>
<td>0.632</td>
<td></td>
</tr>
<tr>
<td>CD24hiCD38hi</td>
<td>0.34</td>
<td>0.238</td>
<td></td>
</tr>
</tbody>
</table>

* Statistically significant at p<0.05

**Table 17.** Correlation between BMD Total Hip and different parameters at 12\textsuperscript{th} month visit

<table>
<thead>
<tr>
<th>Parameter</th>
<th>BMD Total Hip</th>
<th>rho</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>-0.41</td>
<td>0.012*</td>
<td></td>
</tr>
<tr>
<td>BMI</td>
<td>0.62</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>DAS 28</td>
<td>0.27</td>
<td>0.118</td>
<td></td>
</tr>
<tr>
<td>CRP</td>
<td>-0.36</td>
<td>0.041*</td>
<td></td>
</tr>
<tr>
<td>CD19.B lymphocyte</td>
<td>0.44</td>
<td>0.007*</td>
<td></td>
</tr>
</tbody>
</table>

* Statistically significant at p<0.05
Table 18. Correlation between BMD radius and different parameters at 12th month visit

<table>
<thead>
<tr>
<th></th>
<th>BMD radius</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>-0.37</td>
<td>0.035*</td>
</tr>
<tr>
<td>BMI</td>
<td>0.43</td>
<td>0.013*</td>
</tr>
<tr>
<td>DAS 28</td>
<td>-0.162</td>
<td>0.376</td>
</tr>
<tr>
<td>CRP</td>
<td>0.29</td>
<td>0.102</td>
</tr>
<tr>
<td>CD19.B lymphocyte</td>
<td>-0.12</td>
<td>0.692</td>
</tr>
<tr>
<td>CD24hiCD38hi</td>
<td>-0.29</td>
<td>0.318</td>
</tr>
</tbody>
</table>

* Statistically significant at p<0.05

Based on the results from the correlation analysis, multivariate linear regression models were performed (backward procedure). Independent association between age, body mass index, CRP, Vitamin D, DAS28, CD19 B cells, PTH and CD24hiCD38hi B cells at baseline with BMD of different sites at 12 months was done in order to detect factors which may affect BMD after 12 months. There was a significant negative independent association between BMD L2-L4 at 12 months and each of age (standardized $\beta$=-0.577, p=0.013) and CD24hiCD38hi B cells (standardized $\beta$=-1.365, p=0.062) at baseline; while there was significant positive independent association of BMD L2-L4 at 12 months with CD19 B-lymphocytes (standardized $\beta$=1.467, p=0.049) and vitamin D levels (standardized $\beta$=1.085, p<0.001) at baseline (table 19). On the other hand, there was no significant association between the actual change in BMD after 12 months of treatment and the other parameters. This can be explained by that the change was very small to detect any significant association.
Table 19. Independent predictors of BMD L2-L4

<table>
<thead>
<tr>
<th></th>
<th>Unstandardized Coefficients</th>
<th>Standardized Coefficients</th>
<th>t</th>
<th>P value</th>
<th>95.0% Confidence Interval for B</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B</td>
<td>Std. Error</td>
<td>Beta</td>
<td>t</td>
<td>P value</td>
</tr>
<tr>
<td>(Constant)</td>
<td>1.414</td>
<td>0.143</td>
<td></td>
<td>9.908</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Age</td>
<td>-0.008</td>
<td>0.003</td>
<td>-0.577</td>
<td>-3.095</td>
<td>0.013</td>
</tr>
<tr>
<td>CD19 B-lymphocyte</td>
<td>0.578</td>
<td>0.253</td>
<td>1.467</td>
<td>2.279</td>
<td>0.049</td>
</tr>
<tr>
<td>CD24hiCD38hi</td>
<td>-1.858</td>
<td>0.871</td>
<td>-1.365</td>
<td>-2.132</td>
<td>0.062</td>
</tr>
<tr>
<td>Vitamin D</td>
<td>0.004</td>
<td>0.001</td>
<td>1.085</td>
<td>5.702</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Table 20 shows that there was a significant negative independent association between BMD neck femur at 12 months and each of age (standardized β = -0.872, p = 0.007), and PTH levels (standardized β = -0.630, p = 0.035) at baseline.

Table 20. Independent predictors at baseline for BMD neck femur at 12 months

<table>
<thead>
<tr>
<th></th>
<th>Unstandardized Coefficients</th>
<th>Standardized Coefficients</th>
<th>t</th>
<th>P value</th>
<th>95.0% Confidence Interval for B</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B</td>
<td>Std. Error</td>
<td>Beta</td>
<td>t</td>
<td>P value</td>
</tr>
<tr>
<td>(Constant)</td>
<td>1.548</td>
<td>0.186</td>
<td></td>
<td>8.34</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Age</td>
<td>-0.009</td>
<td>0.003</td>
<td>-0.872</td>
<td>-3.38</td>
<td>0.007</td>
</tr>
<tr>
<td>PTH</td>
<td>-0.030</td>
<td>0.012</td>
<td>-0.630</td>
<td>-2.44</td>
<td>0.035</td>
</tr>
</tbody>
</table>

As presented in table 21, there was an independent association between BMD total hip at 12 months and each of age (standardized β = -0.985, p = 0.001), and PTH (standardized β = -0.459,
p=0.061) at baseline. In other words, the higher the age at baseline, the lower the BMD of total hips at 12 months.

**Table 21.** Independent predictors at baseline for BMD total hip at 12 months

<table>
<thead>
<tr>
<th></th>
<th>Unstandardized Coefficients</th>
<th>Standardized Coefficients</th>
<th>95.0% Confidence Interval for B</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B</td>
<td>Std. Error</td>
<td>Beta</td>
</tr>
<tr>
<td>(Constant)</td>
<td>1.846</td>
<td>0.207</td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>-0.013</td>
<td>0.003</td>
<td>-0.985</td>
</tr>
<tr>
<td>PTH</td>
<td>-0.029</td>
<td>0.014</td>
<td>-0.459</td>
</tr>
</tbody>
</table>

Table 22 shows there were a significant negative independent association between BMD radius at baseline and DAS28 at baseline (standardized $\beta$=−0.597, p=0.031); while there was a highly significant positive association between BMD radius at 12 months and Vitamin D levels at baseline (standardized $\beta$=0.718, p=0.013).

**Table 22.** Independent predictors of BMD radius

<table>
<thead>
<tr>
<th></th>
<th>Unstandardized Coefficients</th>
<th>Standardized Coefficients</th>
<th>95.0% Confidence Interval for B</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B</td>
<td>Std. Error</td>
<td>Beta</td>
</tr>
<tr>
<td>(Constant)</td>
<td>0.016</td>
<td>0.126</td>
<td>0.128</td>
</tr>
<tr>
<td>DAS28</td>
<td>- 0.059</td>
<td>0.024</td>
<td>-0.597</td>
</tr>
<tr>
<td>Vitamin D</td>
<td>0.003</td>
<td>0.001</td>
<td>0.718</td>
</tr>
</tbody>
</table>
The presence of plain x-ray hands and feet reports was a great opportunity to study the effect of presence of erosions on BMD results. Patients were divided into two groups according to the presence of erosions in their report or their absence. Reports with vague terms such as (RA changes) were excluded from analysis. Group 1 refers to patients without erosions while group 2 refers to patients with erosions. Difference in numbers between both groups was not statistically significant. The mean (SD) BMD total hip for group 1 was 1.040 (0.145) compared to 0.871 (0.158) in group 2. The difference between both groups was highly significant (p=0.009). For femoral neck BMD, Group 1 mean BMD (SD) was 0.963 (0.134) compared to 0.838 (0.162) in group 2. Comparing both groups revealed a significant difference (p=0.043). To summarize these results, patients with erosions have lower BMD compared to patients without erosions in our sample.

**Table 23.** BMD difference between patients without and with erosions.

<table>
<thead>
<tr>
<th></th>
<th>Group 1 (12 subjects)</th>
<th>Group 2 (14 subjects)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total hip BMD</td>
<td>1.0403 (0.1452)</td>
<td>0.8711 (0.1577)</td>
<td>0.009</td>
</tr>
<tr>
<td>Femoral neck BMD</td>
<td>0.9634 (0.1338)</td>
<td>0.8376 (0.1621)</td>
<td>0.043</td>
</tr>
</tbody>
</table>
Figure 18. Total hip BMD difference between patients without and with erosions.

The central box represents the values from the lower to upper quartile (25 to 75 percentile). The middle line represents the median. There are two sets of horizontal lines; the narrow horizontal lines represent the 95% confidence interval of the median, the wide horizontal lines extend from the minimum to the maximum value, excluding "far out" values which are displayed as separate points. A far out value is defined as a value that is smaller than the lower quartile minus 3 times the interquartile range, or larger than the upper quartile plus 3 times the interquartile range (wide horizontal lines set). These values are plotted with a different colour marker.
**Figure 19.** Femoral neck BMD difference between patients without and with erosions.

The central box represents the values from the lower to upper quartile (25 to 75 percentile). The middle line represents the median. There are two sets of horizontal lines; the narrow horizontal lines represent the 95% confidence interval of the median, the wide horizontal lines extend from the minimum to the maximum value, excluding "far out" values which are displayed as separate points. A far out value is defined as a value that is smaller than the lower quartile minus 3 times the interquartile range, or larger than the upper quartile plus 3 times the interquartile range (wide horizontal lines set). These values are plotted with a different colour marker.
CHAPTER 8. DISCUSSION

RA is a common disease affecting between 0.5-1% of the adult population worldwide (234). Osteoporosis is a leading co-morbidity in RA (235). Bone loss resulting from RA has been extensively studied over the last years. However, its mechanism is still poorly understood. It has been well established that RA leads to increased bone resorption, but it has more recently been demonstrated that there is also substantially reduced bone formation (236).

The role of B-lymphocytes regarding bone turnover is controversial (237). The net effect of B cell depletion on bone turnover may depend on the relative depletion of effector versus regulatory B cell subsets. Studies have shown that B-lymphocytes stimulate osteoclastogenesis; while others have shown that B cells inhibit bone resorption (238). Knocking out B cells in mice led to severe osteoporosis indicating that B cells have an important role in bone metabolism (239).

The study described in this thesis is based on a multicenter prospective clinical trial of 45 RA patients to assess the effects of B cell depletion using rituximab on bone density measurements using DEXA scan and bone turnover using changes in serum levels of bone formation (BAP and P1NP), bone resorption (TRAP5b and CTX) and osteocyte biomarkers (sclerostin and DKK1) over 12 months follow-up.

Flow cytometric analysis of B cells including subsets was done to assess the direct effect of change in B cell numbers on different bone turnover parameters and disease activity. We aimed to confirm the effect of B cell depletion on BMD and bone turnover and to determine whether this effect is related to remission of disease activity by the drug or a direct effect of B cell depletion on bone turnover. Additionally, we aimed to determine the effect of changes in disease activity on bone metabolism in RA.
Patients were treated with 2x 1000 mg rituximab I.V. after a bolus of I.V. methylprednisolone 100 mg in accordance with NICE guidelines. Patients who responded to the first course received a second course at 6 months unless they have attained a state of low disease activity, in accordance with clinical practice. Retreatment was delayed in patients with low disease activity at 6 months until a state of moderate disease activity has been documented in 2 consecutive visits one month apart.

The dose of NSAIDs was adjusted during the study if deemed necessary such as in a flare, but the dose of DMARDs and corticosteroids remained stable over the study period. We aimed at attainment of a state of low disease activity as measured by DAS28 score less than 3.2

The results of this study showed contradictory results between bone turnover markers and BMD results. However, data obtained from bone turnover markers support the hypothesis that rituximab may have protective effects on bone. This study revealed a significant increase in bone formation markers in response to rituximab and a decrease in bone resorption, yet statistically insignificant along with steady levels of osteocyte markers over one year follow-up. On the other hand, BMD results showed a small statistically insignificant reduction at lumbar spine, femoral neck and wrists; while the decrease was statistically significant for the total hip BMD after 12 months.

Accordingly, we assume that B cell depletion by rituximab stimulated osteoblastic activity either directly through their proximity in bone marrow or indirectly through inducing disease remission more than affecting osteoclastogenesis. However, this effect may have not appeared at BMD results either due to precision error or small sample size.

Rituximab is a B cell depleting agent. It is an anti CD20 antibody. It acts by inducing depletion of different B cell populations with exception of the pro B cells and plasma cells. This therapy can improve signs and symptoms as well as physical function and radiological
progression in RA (240, 241). However, a potential influence of rituximab on systemic bone remodeling in RA has not been so far investigated. The crosstalk between immune system and the cells participating in bone remodeling (osteoblasts and osteoclasts) is extremely complex, and few experimental data indicate possible unfavorable effects of B cell depletion on bone remodeling (242).

The current study indicated that treatment with rituximab led to changes of disease activity during follow-up as our results showed that there was a significant decrease of mean (SD) DAS28 score from 6.08(1.298) at baseline to 4.66(1.417) at the end of the follow-up period. This reduction was highly significant (p<0.001). Concerning CRP, there was a decrease in CRP levels during follow-up compared to baseline values. Median (95%CI) CRP started with 12.6 (6.3-27.1) and declined to 8.0 (3.4-13.1) after 12 months. Though, the difference between groups was statistically insignificant (p=0.0698).

Regarding biochemical markers of bone turnover, this study revealed that changes of bone formation markers (BAP and P1NP) during follow-up showed an increasing trend. The median P1NP levels increase from 41.9 (95%CI=34.1-46.4) µg/L at baseline to 47.7 (95%=42.0-61.4) µg/L at the end of the study. However, the rise was not statistically significant (p=0.066). On the other hand, the mean BAP level increased from 18.996 (SD=6.496) µg/L at baseline to 21.256 (7.733) µg/L at 12 months. The change was statistically significant (p=0.022).

On the other hand, there was a reduction in both resorption markers as median TRAP5b decreased from 3.07 (95%CI=2.765-3.470) U/L at baseline to 2.88 (95%CI=2.756-3.464) U/L after 12 months; while median CTX decreased from 436.0 (95%CI=373.4-503.5) ng/L at baseline to 391.0 (299.0-469.7) ng/L after 12 months following treatment with rituximab. However, this reduction was not statistically significant (p=0.676, p=0.800 respectively).
Median sclerostin level started with 54.50 (95%CI=48.437-58.802) pmol/L at baseline and ended the study with 55.65 (95%CI=50.630-62.368) pmol/L. While that of DKK1 started with 52.80 (95%CI=44.276-57.726) pmol L at baseline and ended with 51.45 (95%CI=41.191-65.719) pmol/L at 12 months. However, there was no change in both osteocyte markers (p=0.226 and p=0.944 respectively).

Flow cytometric analysis (FACS) of CD19 B cells including subsets was done for 23 patients every follow-up visit to determine the efficacy of rituximab in depleting B cells and correlating their blood levels with disease activity and serum levels of bone markers. These patients were from Middleborough (The James Cook University hospital) and Newcastle upon Tyne (Freeman hospital) centers. Five of these patients had an early withdrawal from the study due to different reasons mainly starting bisphosphonates, inadequate response to rituximab and being diagnosed with other diseases, which may affect bone turnover (CLL). Additionally, one patient has been withdrawn due to non-compliance with the study visits.

The analysis for changes of absolute counts of CD19 B cells and their subset CD24hiCD38hi was for 18 patients only. There was a highly significant decrease in both CD19 B-lymphocytes (p<0.00001) and their subset CD24hiCD38hi (p<0.00001) absolute counts through the follow up period with an increase in their count at the 6th month then continued to decrease later on. This can be explained by the administration of the second course of rituximab at 6 months. This indicates that rituximab has successfully depleted almost all CD19 B cells and their subsets.

Despite the promising results obtained from the bone turnover markers, BMD results obtained from DXA scans were relatively disappointing. BMD of lumbar spine, femoral neck, total hip and forearms all decreased after 12 months in response to rituximab. Yet, the reduction was variable among the different regions. Median BMD of lumbar spine assessed at L2-L4 showed a slight decrease from 1.138 (95%CI=1.069-1.223) g/cm^3 at baseline to 1.137
A slight insignificant reduction (p=0.656) was also noted at the femoral neck mean starting with 0.883 (SD=0.140) g/cm^3 and ending the study with 0.878 (SD=0.157) g/cm^3 (p=0.656). BMD of total hip showed a significant decrease from median BMD 0.964 (95%CI=0.914-1.001) g/cm^3 at baseline to 0.946 (95%CI=0.907-0.978) g/cm^3 (p=0.041). Median BMD of means of (UD) radius also decreased from 0.372 (95%CI=0.346-0.395) g/cm^3 at baseline to 0.353 (0.326-0.394) g/cm^3 after 12 months from initiation of rituximab. Yet this change was statistically insignificant (p=0.055).

Although the reduction of total hip BMD was significant after 12 months of rituximab treatment, these changes were very small and less than the LSC calculated based on the CV% of DXA machines provided by each center. Accordingly, this change might be due to precision error rather than a real change in BMD. The presence of a control group could have been of great value for our study as it could detect such small changes. Another possible explanation is the short duration of the study, which may not be enough for the slow progression of BMD changes. Unfortunately, while reviewing the literature, some studies showed such small changes as significant without considering the precision error.

A study with 31 RA patients was done to assess bone turnover. The mean (SD) age of the patients was 50.6 (6.8). Their DAS28 score was 5.8 (1.2). All patients used prednisone (7.5 mg) and methotrexate 10 mg/week. 11 patients took concomitantly etanercept (25 mg twice/week) and 10 patients received infliximab 3mg/kg at 0, 2, 6 weeks then every 8 weeks. Ten patients receiving only prednisone and methotrexate were used as controls. BMD was assessed at baseline then after 6 months. BMD increased by 0.2% at LS and 0.1% at total hip in patients receiving TNF-alpha blockers compared to a reduction of 0.8% at LS and 0.6% at total hip in the control group. BMD changes were not significant similar to our study. However, the duration was very small to detect real BMD changes (243).
Femoral neck is well known by its sensitivity to minor position changes so very high precision is required to obtain accurate results. Though, femoral neck BMD may have slightly decreased in case of presence of persistent hip arthritis. Oppositely, BMD of total hip may have increased due to secondary osteoarthritis of the joint which may give false high BMD results.

Combining the BMD results with the bone turnover markers results may lead us to the fact that BMD failed to show the improvement noticed in bone formation and no worsening of bone resorption. This might be due to the short duration of the study or the reasons explained earlier. On the other hand, the results of bone formation markers may be encouraging that there is an anabolic effect of B cell depletion using rituximab on bone, as it did increased opposite to what is expected with severe RA patients such as our subjects. Obviously, B cell depletion led to improvement of bone formation and a slight reduction of bone resorption. Notably, the one-year follow-up period may not be enough for BMD to build up significantly to find an obvious change.

Correlation between different variables was tested to see if there was any significant association. Correlation was performed between mean change in DAS28 and changes in different parameters at follow up visits. Results revealed no persistent significant correlation between DAS28, bone markers, CD19+B cell numbers and BMD changes over 12 months.

However, there was a significant correlation between CRP and each of BMD lumbar 2-4 (-0.43, p= 0.011), BMD neck femur (rho=-0.37, p=0.033) and total hip CRP (rho=-0.36, p=0.041). Total hip BMD showed significant correlation with age (rho= -0.41, p=0.012). Radius BMD showed significant correlation with age (rho=-0.37, p=0.035) and BMI (rho= 0.43, p=0.013).
The finding that there is a significant negative correlation between CRP and BMD supports the hypothesis that inflammation has a negative effect on BMD. Age also negatively correlated with BMD.

Interestingly, we subdivided our patients based on the presence or absence of erosions on their hands and feet x-rays. We found a significant difference in BMD of total hip and femoral neck at baseline \( (p=0.009, p=0.043 \text{ respectively}) \). Patients with erosions had much lower BMD than patients without erosions. This may partly explain the worsening of BMD and suggests that patients with erosions should be routinely surveyed for osteoporosis and treated accordingly.

Interestingly, Depletion of CD19+ B cells led to worsening of BMD opposite to our hypothesis that the presence of B cells is pathogenic for bone homeostasis. So, this may suggest that not all B cells are harmful for bone. In fact, it might be the opposite, that the absence of B cells may lead to osteoporosis. This has been suggested in the literature by some studies. In a murine study, depletion of B cells resulted in development of osteoporosis. Moreover, reconstitution of B cells in these mice reversed bone loss (206).

Furthermore, we divided our patients into 2 subgroups based on the presence or absence of erosions in their x-ray reports at baseline. We found a significantly lower total hip BMD in patients with erosions compared to patients without erosions 1.040 (0.145) versus 0.871 (0.158). The difference between both groups was highly significant \( (p=0.009) \). Similarly, for femoral neck BMD, Group 1 mean BMD (SD) was 0.963 (0.134) compared to 0.838 (0.162) in group 2. Comparing both groups revealed a significant difference \( (p=0.043) \). Applying these results in clinical practice showed that for patients with erosions a vigilant and routine assessment of BMD should be done and osteoporosis should be treated accordingly if present.
Based on the results from the correlation analysis, multivariate linear regression models were performed (backward procedure). There was an independent association between age, body mass index, CRP, Vitamin D, CRP, DAS28, CD19 B-lymphocytes, PTH and CD24hiCD38hi B cell at baseline against BMD LS, total hip, femoral neck and wrist (UD) after 12 months. There was an independent association between BMD L2-L4 and each of age (standardized $\beta=-0.577$, $p=0.013$), CD19 B-lymphocytes (standardized $\beta=1.467$, $p=0.049$), CD24hiCD38hi B cells (standardized $\beta=-1.365$, $p=0.062$) and vitamin D (standardized $\beta=1.085$, $p<0.001$). The significant positive independent association found between CD19+ B cells and BMD lumbar spine at baseline suggests a direct effect of B cells on BMD. This may explain why BMD have slightly decreased in response to depleting B cells using rituximab. This suggests that rituximab may have a negative impact on BMD opposite to what we were expecting. The finding that CD24hiCD38hi B cells may have a noticeable but statistically insignificant negative independent association with BMD lumbar spine suggests that the presence of this subtype of B cells (previously considered as B-regs) is not actually protective but in fact may have a negative effect on BMD. Yet, these results were not found in BMD hips, femoral neck and forearms.

CD24hiCD38hi B cells were assumed to be B regulatory cells named Bregs. A recent study by Mauri et al. showed that in patients with active RA, CD19+Cd24hiCD38hi b cells failed to convert T helper cells into functioning T regulatory cells and lost their ability to suppress TH17 cells. On the other hand, healthy controls and patients with low disease activity had normal activity of CD24hiCd38hi B cells.
In our study, there was an independent association between BMD neck femur and each of age (standardized $\beta=-0.872$, $p=0.007$), and PTH (standardized $\beta=-0.630$, $p=0.035$). There was an independent association between BMD total hip and each of age (standardized $\beta=-0.985$, $p=0.001$), and PTH (standardized $\beta=-0.459$, $p=0.061$). Lastly, there was a significant independent correlation between BMD radius and DAS28 (standardized $\beta=-0.597$, $p=0.031$) and Vitamin D (standardized $\beta=0.718$, $p=0.013$).

These findings are in accordance with results obtained by multicenter, randomized, placebo-controlled, double-blinded trial conducted to evaluate efficacy and safety of rituximab in patients with active, longstanding RA over 24 weeks. Patients were categorized into 2 groups; 209 patients were randomized to placebo and 311 patients to receive rituximab I.V. 2x1000 mg at baseline. Mean DAS28 score and CRP levels were significantly decreased from baseline in rituximab-treated patients ($P=0.0001$, $P < 0.0001$ respectively) (244).

Keystone et al., reported that therapy with B cell depletion using rituximab, for patients with progressive RA led to decreased inflammatory activity and limited joint destructive process. Yet, potential effects on bone remodeling have not been studied (241).

Gough et al, reported that disease activity is consistently associated with low BMD. They concluded that generalized bone loss observed in RA is associated with disease activity as measured by CRP. So, suppression of the disease activity could stabilize this bone loss (241). Moreover, in a two-year follow up study, patients whose disease became inactive recovered partially from the initial bone loss. A similar relationship between sustained inflammation and BMD loss shown in ankylosing spondylitis, in which steroids are not used. Even in the general population, a mild elevation of CRP within the normal range increases non-traumatic fracture risk (246).
Results obtained by a prospective study conducted by Hein et al., on 13 patients with a follow-up of 3–15 months after administration of rituximab in dose of 2x1,000 mg revealed no significant change of the bone formation markers; BAP and c-terminal propeptide of collagen I, yet it revealed that rituximab had significant effect on bone resorption marker TRAP concentration after 15 months (247).

This is in accordance with what was reported by Fardellone et al., as they concluded that Rituximab lowered osteoclast activity in RA patients proven by a significant decrease of the bone resorption marker deoxypyridinoline, which was significantly reduced 15 months after treatment (244). These findings are contradictory to our results that bone resorption markers TRAP5b and CTX remained stable post RTX over 1 year.

A review of comparable studies that used infliximab to assess effects of TNF blockade on BMD revealed that dampening of inflammation and disease activity resulted in improvement of BMD (245-247). Earlier studies suggested also a beneficial effect of TNF blockade on osteoporosis among RA patients (246).

In a multicenter study of the effects of infliximab on bone, Chopin et al. showed a decrease in serum ICTP and CTX associated with a favorable change in ratio between markers of bone resorption and bone formation (252). So they suggested beneficial systemic and local bone effects of infliximab in patients with RA. The study included 48 women with mean age of 54.2±12.1 years old diagnosed with severe RA for 11.4±7.8 years. They all started infliximab after failure of other DMARDs. Assessment performed at baseline, 6, 22 and 54 weeks after initiating Infliximab therapy. The study results revealed that BMD remained stable over one year. Serum CTX-I levels decreased at week 6 and week 22 by 19% and 28%, respectively (p=0.032), returning to pre treatment levels at week 54. By contrast, ICTP levels declined progressively with a maximal decrease (25%) at week 54 (p=0.028). PINP levels remained
stable over time, leading to a 30-40% improvement in bone remodeling balance, as assessed by the ratios PINP/CTX and PINP/ICTP (p=0.05) (252).

Serio et al. reported that treatment with TNFα blockers was shown to prevent structural bone damage and improve BMD in RA patients. Whether this is a direct effect of TNFα inhibition on bone cells or an indirect effect through reducing RA disease activity, this could not be confirmed (251).

This is in accordance with Vis et al. who showed that patients with RA treated with infliximab could stop bone loss in both spine and hip, but not in hand bones. The outcome of their study also supported a relationship between clinical response, mirrored by reduced inflammatory activity, and bone loss changes of the spine, hip and hands. They conducted a cohort study on 102 patients with RA treated with infliximab over one year. Uncoupling between bone destruction and bone formation was observed in RA. The bone formation markers P1NP and osteocalcin increased 2 and 6 weeks after initiation of infliximab. On the other hand, serum CTX-I and sRANKL decreased significantly (250).

The BMD of the spine and hip using dual x ray absorptiometry and hands dual x ray radiogrammetry were measured before start of treatment and after one year. The BMD of the spine and hip were unchanged during treatment, whereas BMD of the hand significantly decreased by 0.8% (p=0.01). Serum RANKL and CTX were considerably decreased relative to baseline at all timepoints. The decrease in CTX was associated with the decrease in DAS28 and CRP during the 0 to 14 weeks interval (250).

Another study conducted on 20 patients with RA (infliximab and placebo group; 10 patients in each) with early active RA. BMD was assessed at hands; hips and lumbar spine (L2–4) by using DXA scan performed at baseline and after 12 months follow-up. The study revealed that BMD loss was reduced significantly in the infliximab group compared to placebo group at the
femoral neck (p=0.01) and total hip (p=0.03) but not at hands (p=0.82) or spine (p=0.71). Results revealed that the anti inflammatory effects of infliximab could arrest inflammatory bone loss at femoral neck and hip but could not at spine and hand (253).

Results of this study revealed no statistically significant correlation between change in BMD and age, gender, and methylprednisolone. In contrast, a significant correlation was detected between BMD and body mass index. DAS28 was significantly correlated with BMD loss at the femoral neck; CRP was significantly correlated with BMD loss (253).

Multivariate linear regression model was performed, the independent association between age, BMI, CRP, treatment groups, MRI synovitis and erosions with bone loss. An increase in CRP was found to be associated independently (standardised b=20.658, p=0.003) with hand bone loss. At the spine a high MRI synovitis score was found to be associated independently (standardized b=20.587, p=0.010) with bone loss. An increase in MRI erosions (standardized b=0.511, p=0.012) and CRP (standardized b=20.428, p=0.030) was found to be independently associated with bone loss at the femoral neck. Finally, at the total hip, MRI erosions were statistically significantly associated (standardized b=20.776, p=0.001) with bone loss (253).
Marotte and colleagues performed a case control study on 99 patients with RA treated with infliximab. After one year, patients receiving infliximab had preserved BMD of lumbar spine and femoral neck in contrast to bone loss of 3.9% and 2.5% observed at same sites, respectively, in the control group that was treated with methotrexate alone. Changes of biochemical markers of bone turnover either between the groups or from baseline were not statistically different. However, the trends in serum osteocalcin and serum CTX suggest greater decrease in bone remodeling activity among patients in infliximab group. Effects of infliximab on BMD appeared to occur independently of RA activity (254).

This is also in accordance with what is reported by Lange and colleagues as they studied 26 RA patients treated with 12 months of infliximab and observed a significant increase in BMD of spine and femoral neck of 2.7% and 1.3%, respectively. The study revealed a trend for a correlation between BMD changes and changes in DAS28 score but could not detect correlation between changes in BMD with bone markers changes (249).

Wang et al. reported that osteocyte marker DKK-1 correlated with inflammation and bone erosions in RA. So, the changes in DKK-1 levels may serve as a biomarker of disease activity and bone erosions (255).

Results obtained from the study conducted by Wang et al. on assessing osteocytes markers in patients with RA, revealed that serum levels of DKK-1 were significantly higher in patients with RA than in healthy controls and those with other rheumatic diseases (p<0.01). Serum DKK-1 level was correlated with levels of CRP (r = 0.488, p = 0.003) and ESR (r = 0.458, p = 0.002) and radiologic changes (r = 0.449, p = 0.001) in RA. DKK-1 was found to be significantly decreased in RA patients treated with either TNF-α inhibitor (p < 0.01) or IL-1Ra (p < 0.01) (255).
Blockade of Dkk-1 and sclerostin may serve to restore the balance between osteoblasts and osteoclasts and repair of bone erosions (252). This is in accordance with results obtained by a study conducted by Garnero et al. revealed that increased Dkk-1 levels were associated with a high risk of progression of bone erosions, independent of age, sex, baseline radiological damage, CRP, and disease activity in patients treated with etanercept (257).

Nakou et al. conducted a study on patients with active RA who failed treatment with TNFα blockers. Patients received rituximab at dose of 1,000 mg on days 1 and 15. Peripheral blood (n = 11) and BM (n = 8) aspirates were then collected at baseline and at 3 months. They used triple-color flow cytometer to assess B cell and T cell populations. This study revealed that rituximab significantly decreased peripheral blood CD19+ B cells from 2% to 0.9% (P = 0.022) but reduction was not significant for BM CD19+ B cells (4.6% to 3.8%, P = 0.273). These effects were specific to rituximab and not for TNF blocking therapy (258).

Results revealed no correlation between degree of peripheral B-cell reduction and clinical response. However, the authors denote that incomplete B-cell depletion could potentially be a reason for poor clinical response. This was noted in 4 out of 11 patients. It was found that responders to rituximab had a significant reduction of CD19+CD27+ memory B cells in peripheral blood and in BM. In non-responders, a similar reduction of memory B cells has not been found. Nevertheless, this group of RA patients had highly active disease refractory to previous treatments (258).

Cohen et al. suggested HLA-DR+ B cells are important for B cell mediated T cell activation. They concluded that depletion of HLA-DR+ cells could represent an additional mechanism for the beneficial effects of rituximab in RA patients (248).

Cohen et al. investigated the effects of rituximab on peripheral blood and BM lymphocyte subsets in patients with active RA. Results revealed that Rituximab therapy preferentially
depletes activated CD19+HLA-DR+ B cells in the peripheral blood and bone marrow, which was significant for only peripheral blood (P=0.022). Clinical response to rituximab was associated with CD19+CD27+ memory B cells depletion in peripheral blood and BM of RA patients (244).

That is also supported by results obtained from previous studies, which showed that rituximab was associated with complete depletion of CD19 B cells in the peripheral blood (259). The responses clearly support the hypothesis that B cells are main contributor to the immunopathogenesis of RA through several different potential mechanisms (260-262).

Wijbrandts et al. suggested the importance of controlling inflammation on progression of bone loss in RA. They conducted an open label prospective clinical study on 50 patients with active RA who started adalimumab and followed-up for one year. All patients used fixed dose of methotrexate and were allowed to use prednisone at ≤10 mg/day. The BMD of the lumbar spine and femur neck were measured before and one year after starting the treatment. Study results revealed that BMD of lumbar spine and femoral neck were correlated with disease activity and duration of RA at baseline. The mean BMD of femoral neck and lumbar spine remained unchanged after one year of adalimumab treatment. Actual increase in BMD of femoral neck (+2.5%) occurred in patients on prednisone in contrast to patients without concomitant prednisone (-0.7%). Lumbar spine also showed similar trend (263).

Our study had several limitations. First, the study was designed with no control group. Control group could be precious regarding comparing results at each timepoint of sequential treatment. The lack of a control group is a major limitation for predicting true estimation of the effect of rituximab treatment on bone. However, we could not have a control group because in the UK, rituximab can only be prescribed in RA patients with severe disease activity not responding to traditional DMARDs and only after failure of TNFα blockers.
according to NICE guidelines. So, there was no true control group that can match our study sample group in disease severity and duration.

The short duration of the study was another weakness point in our study. Because in only one year, it might be difficult to detect a significant change in the bone reflected as change in BMD over 12 months. A longer evaluation and follow-up of patients after subsequent treatment courses is needed to confirm these results.

Furthermore, our study had limited power to detect individual effects and interactions. Although all our study patients had high disease activity and fulfilled the criteria for treatment with rituximab, the group was heterogeneous, consisting of men, pre and postmenopausal women and different age groups.

Additionally, more than two-thirds of our patients had low serum vitamin D levels, which may have a significant impact on our BMD and bone markers results. Also, one third of patients were using steroids during the study, this can also be a good reason why BMD did not improve as we anticipated. Although in the study mentioned earlier, steroids were beneficial for BMD.
SUMMARY AND CONCLUSION

This multicenter, open-label, single treatment arm clinical study was designed to investigate bone density measurements using DEXA scan, disease activity using DAS28 and bone turnover using changes in serum levels of bone formation (BAP and P1NP), bone resorption (TRAP5b and CTX) and osteocyte biomarkers (sclerostin and DKK1) in patients with rheumatoid arthritis treated with rituximab over one year follow-up. Flow cytometric analysis of B cells was done to correlate these changes with changes in B cell numbers in blood during the study to determine whether B cells have any direct effect on bone turnover and the type of this effect whether it is towards bone formation or bone loss. We also tried to determine the reason behind this effect whether it is related to B cells themselves or it is the inhibition of inflammation and disease activity that caused this effect or a combination of both.

Our patients had severe active disease at baseline DAS28=6.08±1.298 6.08 as per the indication for rituximab. All patients failed at least one TNFα unless contraindicated according to NICE guidelines. That is why we could not find a matching control group to our patients with severe longstanding disease.

In our sample, we found that 68.9% of the patients had low vitamin D at baseline including patients with deficiency and insufficiency. Median PTH at baseline was 34.0 (95%CI=34.6-46.4) ng/L (Normal reference range Normal 12-72 ng/L). This finding may explain why our patients did not have a significant improvement in their bone densitometry results. Also, this should drive clinicians’ attention that vitamin D supplementation might be required to all white patients with RA. This deficiency may also contribute to the generalized musculoskeletal pain despite the inhibition of disease activity.
Additionally, 15(33.3%) of the study participants were using prednisolone concomitantly as part of their routine clinical practice during the study with a mean dose of 11.2±9.8 mg. We could not avoid them to do this, as it would have been unethical if their treating physicians think that steroids should be part of their management.

Mean DAS28 score decreased significantly from highly active disease 6.08 (SD=1.298) at baseline to moderate disease activity score 4.66 (SD=1.417). This reduction in disease activity was statistically significant (P<0.001). Although there was a noticeable reduction of median CRP from 12.6 (95%CI = 6.3-27.1) mg/dl at baseline to 8.0 (95%CI = 3.4-13.1) mg/dl at 12 months, the change was not statistically significant (p=0.0698). These results indicate that rituximab is a successful treatment for management of severe resistant RA.

In response to rituximab, there was almost depletion of all CD19+ B lymphocytes during the follow-up visits compared to baseline (p<0.00001) with an increase in their counts at the 6th month then continued to decrease later on. This is explained by the administration of the second course of rituximab at 6 months. Interestingly, CD24hiCD38hi B cells were the first to reappear at 3 and 6 months among the CD19+ B cell subsets. This B cell depletion was not associated with an increase in side effects such as infections during the study.

There was improvement in bone formation during the study in response to rituximab. Median P1NP levels increased from 41.9 (95%CI=34.1-46.4) µg/L at baseline to 47.7 (95%=42.0-61.4) µg/L at the end of the study with a median change of +12.16%. On the other hand, the mean BAP level increased from 18.996 (SD=6.496) µg/L at baseline to 21.256 (7.733) µg/L at 12 months with a median change of +10.63%. Interestingly, we found no correlation between DAS28 and any of the bone formation markers during all visits. This finding
suggests that there are other mechanisms for this anabolic bone effect other than inhibition of inflammation and disease activity.

Although there was a reduction in both resorption markers as median TRAP5b decreased from 3.07 (95%CI=2.765-3.470) U/L at baseline to 2.88 (95%CI=2.756-3.464) U/L after 12 months with a median change of -6.19%; while median CTX decreased from 436.0 (95%CI=373.4-503.5) ng/L at baseline to 391.0 (299.0-469.7) ng/L after 12 months following treatment with rituximab with a median change of -10.32%. However, the reduction was not statistically significant (p=0.676, p=0.800 respectively). This indicated that on the contrary of the known fact that inhibition of inflammation and reduction of disease activity lead to stoppage of bone resorption. The rate of bone formation versus breakdown ratio was estimated by 1.178 using P1NP/CTX change ratio. Similarly, the change of BAP/TRAP5b ratio was estimated by 1.717. Accordingly, our study shows that inhibition of disease activity affects mainly bone formation rather than osteoclastogenesis.

Osteocyte markers (sclerostin and DKK1) showed no significant change among the five visits (p=0.226 and p=0.944 respectively). Median sclerostin level started with 54.50 (95%CI=48.437-58.802) pmol/L at baseline and ended the study with 55.65 (95%CI=50.630-62.368) pmol/L. While that of DKK1 started with 52.80 (95%CI=44.276-57.726) pmol L at baseline and ended with 51.45 (95%CI=41.191-65.719) pmol/L at 12 months.

Our primary endpoint showed no significant change in Median BMD of lumbar spine (1.138 (95%CI=1.069-1.223) g/cm^3 at baseline to 1.137 (95%CI=1.067-1.216) g/cm^3 after 12 months) with a median change of -0.088%. Similarly, BMD of femoral neck and radius (UD) showed no significant change after 12 months (p=0.656 and p=0.055). However, BMD of total hip showed a significant decrease from median BMD 0.964 (95%CI=0.914-1.001) g/cm^3 at baseline to 0.946 (95%CI=0.907-0.978) g/cm^3 (p=0.041) with a median change of
-1.87%. However, the changes were very small and lower than the coefficient of variation calculated at the start of the study. Accordingly, further larger scale studies are required to confirm these results especially with the promising results obtained from bone markers.

Interestingly, we subdivided our patients based on the presence or absence of erosions on their hands and feet x-rays. We found a significant difference in BMD of total hip and femoral neck at baseline (p=0.009, p=0.043 respectively). Patients with erosions had much lower BMD than patients without erosions. This may partly explain the worsening of BMD and suggests that patients with erosions should be routinely surveyed for osteoporosis and treated accordingly.

Based on results obtained from bone markers, B cell depletion using rituximab increased bone formation and slightly reduced bone resorption markers. However, sclerostin slightly increased from 6th to 12 month. BMD results were inconsistent with bone markers findings, as there was a reduction at all sites mostly at total hip. Furthermore, CD19+ B cells were found to have a direct positive association with BMD at LS suggesting that B cell depletion is not protective for bone metabolism as previously expected. So, we believe that there is a direct effect of B cell depletion on bone cells that need to be further investigated. This effect is directed mainly to osteoblasts rather than osteoclasts as previously thought.
Recommendations for further work

The results and conclusions presented suggest a number of areas where further work is required.

1. There is a need for much larger studies with bigger sample size to confirm our results. There is also a strong need for a control group to compare the small changes we had with patients with similar circumstances.

2. Future work on cross sectional studies to detect the prevalence of vitamin D deficiency in the northeast of England and compare vitamin D incidence in RA and non-RA patients. This will help develop new guidelines to mandate vitamin D supplementation to RA patients even without the need to test its levels.

3. Newer biologic agents should be tested for ability to protect against RA induced osteoporosis as this information may make us prefer to use the biologic with the most bone-protective ability in patients at risk to osteoporosis (postmenauposal women).

4. The normative database, gender comparison, disease activity, bone markers and BMD have all shown promising results on the use of rituximab in RA. Therefore, further prospective studies with longer duration follow-up should take this into account with addition of fracture risk as well.

5. Further studies should include different genders and ethnic groups to standardise these results. More work is required to investigate the efficacy of rituximab on controlling RA disease activity and bone turnover in different age groups as well.

6. Further in-vitro studies are required to eliminate the other factors which may affect osteoporosis in RA patients to determine the precise effect of B cell depletion on bone turnover. This is important because some factors were unavoidable in our clinical study such as the use of steroids, vitamin D deficiency, age, sex …etc.
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APPENDIX

Study protocol

Changes in bone density and bone turnover in patients with rheumatoid arthritis treated with rituximab, a B cell depleting monoclonal antibody.

An investigator-led, industry supported multicenter, open-label, single treatment arm, prospective clinical trial.

1.0 INTRODUCTION

This multicenter, open-label, single treatment arm, prospective clinical trial aims to study the effects on bone turnover of rituximab, a B cell depleting monoclonal antibody, licensed for use in patients with rheumatoid arthritis who are unresponsive to treatment with second-line antirheumatic medication and a TNF-inhibitor. The effectiveness of rituximab in rheumatoid arthritis has demonstrated that B cells play a key role in perpetuation of rheumatoid arthritis. Recent investigations have provided abundant evidence for an intricate interaction between the immune and skeletal systems (1). This interaction is demonstrated by the fact that a number of cell surface receptors, cytokines and signaling pathways serve a critical role both in the immune and skeletal systems. Furthermore, it is becoming clear that immune cells influence bone remodeling and vice versa. The close interaction between immune progenitors and skeleton is facilitated by their proximity in the bone marrow. The role of the immune system, especially activated T cells, in inflammatory bone resorption and osteoclastogenesis is well established (2). In addition to indirect effect on bone turnover through the production of inflammatory and other cytokines that modulate osteoclastogenesis, T cells can also regulate bone turnover through direct cell-cell interaction with bone cells. Indeed, it has been shown that both osteoblasts and osteoclast can express major histocompatibility complex (MHC) class II molecules under inflammatory conditions, therefore, it is plausible that T cell:bone cell interactions may contribute to the autoimmune responses as well as accentuate bone turnover. The role of B-lymphocytes in osteoclast (OC) formation is controversial (3). Mature B-lymphocytes have the
capacity to both inhibit and stimulate osteoclastogenesis by virtue of their ability to secrete appropriate cytokines (3). B lymphocytes produce proosteoclastogenic cytokines including receptor activator of NFkappaB ligand (RANKL), as well as cytokines that inhibit osteoclast differentiation from the progenitor cells, such as osteoprotegerin (OPG), transforming growth factor-K (TGF-K) and interleukin (IL)-7. Furthermore, B cells can also activate autoreactive T cells and further aggravate bone loss through the mechanisms described earlier. It should be noted, however, recent data suggest that some B cells (often termed “regulatory” B cells) can modulate the function of CD4+ T cells to pathogens and autoantigens. Therefore, the net effect of B cell depletion on bone turnover and autoreactivity may depend on the relative depletion of effector versus regulatory B cell subsets. It is not therefore altogether surprising that while some in vitro studies have shown that B lymphocytes to stimulate osteoclastogenesis, others have shown that B cells are inhibitory. In vivo studies on the role of mature B cells in bone remodeling have been equally inconsistent (4). For instance, although estrogen withdrawal is associated with a significant expansion in mature B cell population, the role of these cells as mediators of ovariectomy-induced bone loss remains unclear. A further complicating factor is that ovariectomy-induced bone loss, which may be mediated by a variety of cytokines, has been shown to have selective effect on trabecular or cortical bone (5). B cell knockout mice are osteoporotic, but as these mice also lack plasma cells, it is unclear whether the osteoporotic phenotype can be ascribed to the absence of B cells (2).

Given the pathogenetic role of B cells in RA, it is tempting to speculate that B cell depletion has a beneficial effect on bone loss. We have recently generated pilot data demonstrating a 50% decrease (p<0.001) in beta-crosslaps (CTX), a biochemical marker of bone resorption, and a small but statistically significant increase in P1NP, a biochemical marker of bone formation, in sera of 46 RA patients 6 months after a single treatment course of rituximab, but these data need to be confirmed in a new prospective study. Biochemical markers of bone turnover such as beta-crosslaps are predictive of clinically relevant bone loss and future fracture risk. We anticipate
that some of these effects may be indirect through attenuation of systemic inflammation, while others may be direct as a result of the absence of B cells in osteoclasto- and osteoblastogenisis.

An accurate assessment of bone metabolism is critical for determining the severity of metabolic bone disease in RA; it may be a reliable indicator of future disease course and potentially will give clinicians valuable information about individual patient’s response to treatment and gives insights into the pathophysiology of osteoporosis.

2.0 STUDY RATIONALE

This multicenter, open-label, single treatment arm, prospective clinical trial will investigate bone density and biochemical markers of bone turnover in RA patients treated with rituximab, a B-cell depleting monoclonal antibody. We have recently demonstrated in a retrospective study that rituximab-treatment results in a reduction of biochemical markers of bone resorption, and now want to confirm and extend these results in a new, prospective cohort of patients. It is postulated that the presumed bone-protective effects of rituximab on bone density and turnover may be either due to a direct effect of B cell depletion on osteoclastogenesis, or due to reduction of disease activity, modulation of autoreactivity or all of the above.

3.0 STUDY OBJECTIVES

3.1 Primary Goal

To investigate the effects of rituximab treatment on bone turnover (biochemical markers of bone resorption and formation and bone mineral density (BMD) measurements in RA patients.

3.2 Secondary Goals

To correlate changes in bone density with changes in biochemical markers of bone turnover, changes in biomarkers of inflammation and of autoreactivity, and changes in circulating Bcells and B cell subsets in RA patients treated with rituximab.

4.0 PATIENT ELIGIBILITY

4.1 Inclusion criteria

1. Minimum age 18 years.

2. Diagnosis of rheumatoid arthritis according to ACR classification criteria.
3. Eligibility for treatment with rituximab according to NICE-guidelines
4. First treatment course of rituximab within 6 weeks after screening
5. Written informed consent

4.2 Exclusion criteria
1. Use of bisphosphonates
2. Previous B-cell depleting therapy
3. Poor compliance of the patient as assessed by the referring physicians.

5.0 STUDY DESIGN
Multicenter, open-label, single treatment arm, prospective clinical trial in patients with rheumatoid arthritis who are starting treatment with rituximab.

6.0 STUDY ENDPOINTS

6.1 Primary endpoint
The primary endpoint is change in BMD of the lumbar spine as measured by DXA.

6.2 Secondary endpoints
• Changes in BMD of forearms and hips by DXA
• Changes in biochemical markers of bone turnover (TNFQ, OPG, sRANKL, BAP, TRAP5b, P1NP, CTX)
• Changes in biomarkers of inflammation (ESR, CRP) and autoreactivity (blood T cell responses against autoantigens)
• Changes in clinical parameters of disease activity (DAS28), functional ability (HAQ-DI)
• Number of new fractures
• Duration of B cell depletion in blood and changes in absolute and relative numbers of circulating CD19+ B cells, including subsets (CD27+, CD24+, CD38+) by FACS
• Changes in osteoclast differentiation (osteoclastogenesis assays on blood mononuclear cells)

7.0 CONDUCT OF THE STUDY

7.1 Patient Registration
All patients who fulfill the eligibility criteria will be registered by fax to the study administration
office (SAO) – full contact details are on the cover of the protocol.

7.2 Baseline Evaluation

The following investigations will be completed and documented at baseline:

- comprehensive patient’s history with particular attention to RA specifics, treatment history
- detailed physical examination
- DAS28 and HAQ-DI
- X-rays of hands and feet if not done in past year
- DXA lumbar spine, hips, forearms according to local protocol
- laboratory examinations as part of usual care (except for those marked by *)


Immunology FACS of peripheral blood CD19+ cells incl subsets*; immunoglobulins incl IgG, IgA, IgM; ANA, anti-CCP, IgM rheumatoid factor.

Serum* (15 mL) and heparinised blood* (40 mL) to be cryopreserved for biomarker studies. Because of the effects of food intake and the diurnal variation of markers of bone turnover, fasting blood samples are collected (6,7).

7.3 Outline of Study

The duration of the study is one year for each patient, which includes 3-monthly study visits at baseline, 3, 6, 9 and 12 months after enrolment. Patients will be treated with 2x 1,000 mg rituximab i.v. after a bolus of i.v. methylprednisolone 100 mg in accordance with NICE-guidelines. Patients who responded to the first course will receive a second course at 6 months unless they have attained a state of low disease activity, in accordance with clinical practice. Retreatment will be delayed in patients with low disease activity at 6 months until a state of moderate disease activity has been documented at 2 consecutive visits one month apart.

The dose of NSAIDs may be adjusted during the study if deemed necessary, eg because of a flare, but the dose of DMARDs and corticosteroids should remain stable. The aim of treatment is
attainment of a state of low disease activity. Assessment of DAS28 and HAQ will be done by an independent research nurse.

Blood samples for biomarkers will be stored at -80C. The following biochemical markers will be quantified in human serum by a manual solid phase, monoclonal antibody immunoenzymatic assay:

OPG, BAP, TRAP5b and sRANKL, while P1NP and CTX will be quantified in human serum by an electrochemiluminescence immunoassay on an automated analyser, Roche - Elecsys 2010.

BMD measurements of forearms, hips and spine will be performed by Dual Energy X-ray absorptiometry (DXA) according to local protocol. A number of studies have reported that the greatest reduction in bone density occurs at the foreram sites and that forearm bone density correlates with clinical features of disease activity and markers of bone turnover (8-12).

To investigate the effects of B-cell depletion on osteoclastogenesis, cryopreserved mononuclear cell preparations will be cultured in vitro, and osteoclast numbers determined by morphology, TRAP staining, and immunochemical localisation of surface integrins. The in vitro osteoclast activity will be assessed using the Osteoclast Activity Assay Substrate (OAASTM) where both number and size of the resorption bits will be determined.

7.4 Toxicity

Rituximab is a B-cell depleting chimeric monoclonal antibody. It will be used within its licensed indication, and no unexpected toxicities other than reported in the SmPCs are therefore expected.

7.5 Clinical Evaluations, Laboratory Tests and Follow-up

The following assessments will be done at 3, 6, 9, 12 months after registration:

- Patient’s history, concomitant medication, detailed physical examination
- Adverse event recording according to RCTC vs 2.0
- DAS28, HAQ-DI
- Laboratory tests for hematology (ESR, Hb, HCT, WBC and differential, thrombocytes), chemistry (electrolytes, renal and liver function tests, proteins), urinanalysis, FACS
- serum and blood for biochemical markers of bone turnover and osteoclastogenesis assay (as baseline sample)

DXA of lumbar spine, forearms, hips (total hip and femoral neck) will be repeated at 12 months.

8.0 RECORDING AND REPORTING OF ADVERSE EVENTS

8.1 Definitions of Adverse Events

Adverse Event (AE)
This is defined as any untoward medical occurrence or effect in a patient treated on a study protocol which does not necessarily have a causal relationship with the study treatment. An AE is therefore described as any unfavourable and unintended sign (including an abnormal laboratory finding), symptom or disease temporally associated with the use of the study treatment, whether or not related to the study treatment.

Adverse Reaction (AR)
This is defined as all untoward and unintended responses to a study treatment related to any dose administered. A causal relationship between the study treatment and an adverse event is at least a reasonable possibility, i.e. the relationship cannot be ruled out.

Serious Adverse Event (SAE)
This is defined as any untoward medical occurrence or effect in a patient treated on a study protocol which does not necessarily have a causal relationship with the study treatment, that also, at any dose:

• Results in death
• Is life threatening
• Results in persistent or significant or disability/incapacity
• Requires in-patient hospitalisation or prolongs existing hospitalisation
• Results in a congenital anomaly or birth defect
• Is otherwise medically significant (i.e. withdrawal reactions, all accidental or intentional overdoses whether they result in an adverse event or not, or any event which the investigator considers significant but which is not covered by the above.)
Suspected Serious Adverse Reaction (SSAR)
This is defined as an adverse reaction, the nature or severity of which is consistent with the known study treatment information (e.g. Summary of Medicinal Product Characteristics (SmPC), Investigator Brochure (IB) or Investigator Medicinal Product Dossier (IMPD)).

Suspected Unexpected Serious Adverse Reaction (SUSAR)
This is defined as an adverse reaction, the nature or severity of which is not consistent with the known study treatment information.

A serious event or reaction is not defined as a SUSAR when:
• it is serious but expected
• it does not fit the definition of an SAE, whether expected or not

8.2 Procedures for Adverse Event Reporting

All adverse events

Adverse events and adverse reactions thought to be related to rituximab but not listed in its SmPC will be recorded. All serious adverse events that occur between the first study-related procedure (i.e. screening) and 30 days post the last study treatment (or after this date if the investigator feels the event is related to the study treatment) will be recorded and reported using the Serious Adverse Event Report form.

Investigators must record in the CRF and the patient notes their opinion concerning details of nature, onset, duration, severity and any relationship to the investigational product. Medical terminology should always be used to describe any event. Investigators should avoid vague terms such as “sick”.

Severity

Severity for each adverse event, including any lab abnormality, will be determined by using the WHO Common Toxicity Parameters, wherever possible (appendix B). In those cases where these criteria do not apply, severity should be defined according to the following criteria:
• Mild: Awareness of sign or symptom, but easily tolerated
• Moderate: Discomfort enough to cause interference with normal daily activities
• Severe: Inability to perform normal daily activities
• Life threatening: Immediate risk of death from the reaction as it occurred.

Causality

Relationship to study treatment will be determined as follows:

• None: No relationship between the experience and the administration of the study treatment; related to other etiologies such as concomitant medications or patient’s clinical state.
• Unlikely: The current state of knowledge indicates that the relationship is unlikely
• Possible: A reaction that follows a plausible temporal sequence from administration of the study treatment and follows a known response pattern to the suspected treatment. The reaction might also have been produced by the patient’s clinical state or other modes of therapy administered to the patient.
• Probable: A reaction that follows a plausible temporal sequence from administration of the study treatment and follows a known response pattern to the suspected treatment. The reaction cannot be reasonably explained by the known characteristics of the patient’s clinical state or other modes of therapy administered to the patient.
• Definitely: An adverse event, which is listed as a possible adverse reaction and cannot be reasonably explained by an alternative explanation, e.g., concomitant drug(s), concomitant disease(s).

Expectedness

An expectedness assessment needs to be conducted for all Serious Adverse Events and recorded appropriately on the Serious Adverse Event Report form. Expectedness of the event to the medicinal product will be determined as follows:

• Expected: The event is listed in the SmPC/IB or the study protocol as expected.
• Unexpected: The event is not listed in the SmPC/IB or in the study protocol, or the severity of the event is greater than that listed in the SmPC/IB or the study protocol (e.g. mild nausea is listed as expected in the SmPC/IB/study protocol but the event is moderate or severe nausea).
8.3 Reporting of Serious Adverse Events (SAE)

The Sponsor is responsible for pharmacovigilance. Events defined as serious must be reported by fax to the sponsor, using the Serious Adverse Event Report form, within 24 hours of observing or learning of the event. Should you need a copy of the Serious Adverse Event Report form, contact the study co-ordinator.

The following attributes must be assigned when reporting:

• Detailed description of the event
• Adverse event term (see Adverse Event Term section for details)
• Date of onset and date of resolution
• Severity of the event (see Severity section for details)
• Assessment of relatedness to the protocol treatment (see Causality section for definitions) and action taken
• Assessment of expectedness for the protocol treatment
• Other suspect drugs/devices
• Outcome

All Serious adverse will be followed up until resolution. The investigator will be asked to provide interim and follow-up reports, as necessary, if the SAE has not resolved at the time of initial report.

All deaths occurring on study must be reported as an SAE and on the CRFs to the SAO. For all deaths, available autopsy reports should be sent with the notification.

Investigators may receive an Investigator Notification from the SAO at any time for any serious unexpected adverse reactions, which the sponsor for the study deem necessary. These should be processed according to the local regulations.

8.4 Expedited Reporting of SUSAR

As of May 1st 2004, the sponsor of a clinical trial conducted in the EU must ensure that all relevant information regarding suspected unexpected serious adverse reactions (SUSARs) are recorded and reported in an expedited fashion. It is a legal requirement of the sponsor to report
fatal or life threatening SUSARs within 7 calendar days to the relevant Regulatory Authorities after receiving first notification of the event. Non-fatal and non life-threatening SUSARs must be reported to the Regulatory Authorities within 15 calendar days. As from 01/09/10 all SUSAR reporting must be carried out electronically using the MHRA eSUSAR website.

9.0 STATISTICAL METHODS

9.1 Determination of the Sample Size

The determination of the sample size is based on the comparison of the BMD at baseline and 12 months. Assuming the true change in BMD of the lumbar spine is $T0.01 \, \text{g/cm}^2$ (based on previous unpublished data from Dr S Tuck) and that the DXA scan is reproducible with a standard deviation of $0.02 \, \text{g/cm}^2$ (based on data from the manufacturer); then the study will have 80% power to detect a statistically significant difference (at the 5% confidence level) if we have 33 patients in the final analysis. A 1% increase in bone density will show that the study drug is effective based on a metaanalysis on antiresorptive agents in osteoporosis which concluded that a 1% gain in bone density of the spine was associated with a statistically significant reduction of 8% in nonvertebral fractures and a study in a large population cohort showing decline of bone density over time in RA patients not treated with antiresorptive agentsa (13,14). To allow for 20% dropout (based on the treatment criteria of NICE which mean some patients will discontinue therapy early due to treatment failure or complete disease resolution) 42 patients are needed based on a one sample T-test.

9.2 Statistical Analyses

A full statistical analysis plan has been completed for this study (available as a separate document). In brief descriptive statistics will be used to quantitatively summarise the distribution, central tendency and dispersion of the data set. The primary endpoint; change in BMD of the lumbar spine and secondary endpoints; changes in BMD of the hips (total hip and femoral neck) and forearms; bone turnover markers; inflammatory markers and disease activity between baseline and 12 months will be investigated using a one sample T-test.

Pearson’s correlation coefficient will be used to correlate the changes in BMD with changes in
biochemical markers of bone turnover, in changes in biomarkers of inflammation and in changes in circulating B-cells.

P values $U0.05$ will be considered statistically significant, and a trend towards significance will be defined as $p < 0.10$.

10.0 ETHICAL AND TRIAL ADMINISTRATIVE CONSIDERATIONS

10.1 Study administration office

All study administrative procedures are coordinated and conducted by the study administration office (SAO). These include: registration of patients, collection of registration forms, dispatch of case report forms, treatment allocation, data collection, data management.

All questions concerning the data processing aspects of this study should be addressed to the study administration office. For contact details see cover page of protocol.

All other questions should be addressed to the study chairperson (JM van Laar). See Protocol Synopsis for contact details.

10.2 Finances

The study is supported financially by Roche UK to cover trial administrative costs.

There will be no contribution to medical costs, as the study concerns a study with a licensed drug.

The study protocol will be submitted to UKCRN for adoption on the NIHR-portfolio.

10.3 Publication Procedures

No partial or complete written publication of the results will be made by the study coordinator without a previous information and agreement of the participating members selected as co-authors on the basis of accrual of patients evaluable and eligible. The publications will include as co-authors the study coordinator and co-investigator from each participating centre.

10.4 Trial Duration

The trial launch is scheduled for 1 November 2010. Enrolment is expected to be completed in 2011. Analyses of outcome parameters will commence once the last patient has completed one year follow-up.
10.5 Discontinuation of the Trial, a Centre or a Patient. Planned termination of the whole trial. It is planned that the study will be terminated as soon as the last patient has completed the study, in principle 12 months after registration.

Early termination of the whole trial

The trial may be prematurely terminated, if in the opinion of the Sponsor and the Coordinating Investigator there is sufficient reasonable cause. Written notification documenting the reason for study termination will be provided to the investigators by the terminating party.

Circumstances that may warrant termination include, but are not limited to:

• Failure to recruit patients at an acceptable rate
• Insufficient adherence to protocol requirements
• Insufficient complete and/or evaluable data

Termination of a study centre by an investigator

The investigator may terminate participation in the study. If this occurs they should provide a written statement of the reasons for terminating participation, and should provide the data coordinating office with all available and up-to-date study data.

The Sponsor may also decide to terminate participation of an investigator or study centre for the following reasons:

• Breach of agreement
• Serious non-compliance to ICH-GCP standards
• Insufficient patient recruitment

If a participating centre closes, or is closed, prior to termination of the whole trial, the sponsor expects that data from patients already entered into the study will be reported as per protocol.

Early withdrawal of patient

Any patient enrolled into the protocol who is withdrawn at any time for any reason before the last follow up visit (at one year) is considered an early withdrawal and will not be replaced. A complete set of data including the reason for withdrawal should be collected on every patient up to the time of withdrawal from the study.
The investigator should withdraw a patient from the study treatment whenever continued participation is no longer in the patient’s best interests. Reasons for discontinuing treatment may include the occurrence of a serious adverse event or an intercurrent illness, a patient’s request to end treatment, or significant uncertainty on the part of the investigator that continued treatment would be prudent.

Possible reasons for a patient to go off study

1. Death whatever the cause
2. Noncompliance of the patient
3. Major protocol violations
4. Loss to follow-up
5. Adverse events to go off the study

10.6 Good Clinical Practice (GCP)

The study will be conducted in accordance with the International Conference on Harmonization for Good Clinical Practice (ICH-GCP) and the appropriate regulatory requirement(s). The investigator will be thoroughly familiar with the appropriate use of the study drug as described in the protocol and Investigator’s Brochure. Essential clinical documents will be maintained to demonstrate the validity of the study and the integrity of the data collected. A trial master file should be established at the beginning of the study, maintained for the duration of the study and retained according to the appropriate regulations.

10.7 Ethical considerations

The study will be conducted in accordance with ethical principles founded in the Declaration of Helsinki. The ethics committees will review all appropriate study documentation in order to safeguard the rights, safety and well-being of the patients. The study will only be conducted at sites where ethics approval has been obtained. The investigator will provide the relevant ethics committee(s) with the final version of the protocol, patient information and consent forms, any other written information given to patients, safety updates, annual progress reports, and any revisions to the study protocol or any other trial documentation.
No amendments are allowed to the protocol unless agreed to by the Sponsor and the Coordinating Investigator. Substantial amendments must be submitted for regulatory and ethical approval and the amended protocol will not be activated until such approval is in place.

10.8 Informed Consent

A “Patient and Donor Information Leaflet and Informed Consent form” will be provided as separate document. After the study has been fully explained, written informed consent will be obtained from either the patient and/or his/her guardian or legal representative prior to study participation. The method of obtaining and documenting the informed consent and the contents of the consent will comply with ICH-GCP and all applicable regulatory requirement(s):

• A properly signed and personally dated informed consent form is required for each subject before any trial specific procedure. Subject should be given ample time to read the consent forms and ask questions.

• The informed consent process should be recorded in source documents (date of information and consent, parties present).

• The investigator is responsible for checking entries made by the patient on the consent form, and to request correction immediately in case of missing, illegible or incorrect dates. The person taking the patient consent should sign and date both consent forms to confirm he/she provided information to the subject.

• All entries on the consent forms must be permanent (in ink, no pencil).

• The Informed Consent form will be updated by the Investigator-Sponsor whenever important new information becomes available that may be relevant to subject’s consent. This may be a result of amendments to the protocol, new information regarding the trial medication alternative treatments. Revised versions must be approved by the relevant ethics committee(s).

• The revised Consent Form must be signed by subjects who are entered in the trial and not yet completed, if these changes are relevant to the subject’s willingness to continue participation. In particular, if the Consent Form is updated with new safety information, a new version of the
informed Consent Form must be provided to all subjects in a timely manner as soon as written ethics approval is obtained.

- Patient withdrawal of consent from the study should be explicitly documented in the source documents.

10.9 Patient Confidentiality

In order to maintain patient privacy, all data capture records, study drug accountability records, study reports and communications will identify the patient by initials and the assigned patient number. The full patient name should never be used in any correspondence with the Sponsor or on the CRF.

The investigator will grant monitor(s) and auditor(s) and/or Regulatory Authority(ies) direct access to the patient’s original medical records for verification of data gathered on the data capture records and to audit the data collection process. Direct access includes examining, analysing, verifying, and reproducing any records and reports that are important to the evaluation of the monitoring. The investigator is obliged to inform the patient that his/her trial-related records will be viewed without violating their confidentiality and that the collected information will only be made publicly available to the extent permitted by the applicable laws and regulations.

10.10 Protocol Compliance

The investigator will conduct the study in compliance with the protocol given approval/favorable opinion by the ethics committee(s) and the appropriate Regulatory Authority. Any changes to the protocol will be made by the Coordinating Investigator in liaison with the Sponsor and will require approval and written ethics committee approval/favorable opinion prior to implementation, except when the modification is needed to eliminate an immediate hazard(s) to patients. The investigators will submit any protocol amendments, if applicable, to the ethics committee and the Regulatory Authority in accordance with the governing regulations. Version control of the protocol is the responsibility of the Sponsor in collaboration with the Coordinating Investigator. All protocol distribution is via the sponsor to ensure adherence to strict version
control. Any departures from the protocol, at a participating site must be fully documented in the source documents. Any such protocol violations will be reviewed by the Coordinating Investigator and remedial action taken if required.

10.11 Quality Assurance & Monitoring

Monitoring of trial data

Reports of severe adverse events are dealt with in the appropriate manner, as described in section 8 of the protocol.

The study administration office collects the CRF paper-copies and the data is entered by trial specific trained person(s). Commonly made mistakes and misinterpretations of the CRFs questions should be picked up. Consistent reporting errors should lead to amendment and clarification of the CRFs. All data relating to the trial objectives/endpoints will be audited prior to publication.

Monitoring frequency

In adherence to CGP and EU Law, each study site will be initiated as well as monitored during the study. A close out visit to participating study sites will be conducted at the end of the study before final data analysis.

Monitoring of participating study centres:

Study centres will be initiated via a telephone conference, guided by PowerPoint presentation of the protocol and study procedures. At a minimum the principal investigator and coordinator/study nurse/data manager at the centre must participate, subsequently they may initiate any other relevant staff at the institution.

On-site monitoring will be carried out, to focus primarily on safety data as all end point data will be verified through quality assurance procedures. Each site will be visited once during the study, and once for a close/out visit/ end point review.

The scope of work for on-site monitoring will include the following activities:

• Verify for each subject that Informed Consent was obtained prior to the subjects' participation in the study and that the correct version of the PIL/IC was used
• Ensure all approval documents are in the site file
• Source Document verification will be carried out as specified below:
  a) Verify 100% of SAEs
  b) Verify 25% of protocol treatment information
• Ensure that the process of reporting SAE is followed as outlined in the adverse events section
• Document all activities and discussions with investigational staff in a written report and sent this to the trial data coordinating office.
• Arrange for appropriate follow-up of all action items in the monitoring report.

The Investigator is obliged to provide source documentation, CRFs of subjects and the trial related documents to the monitor during all monitoring visits. Investigators should have ample time to discuss problems and make corrections identified by the monitor.

In addition remote monitoring will be done to ensure adherence to the protocol and ICH-GCP and relevant EU Directive(s). Monitoring of clinical and safety data will be carried out by statistical analysis of data submitted on the CRFs. As the most frequent cause of SAE reports is the hospitalisation of a patient from whatever cause, monitoring of SAE reporting will be carried out by checking the Adverse Event (AE) Data in the CRFs and cross referencing the number of AEs rated as serious with the number of SAEs submitted. Should such analysis identify anomalies or discrepancies then this will trigger a query and/or on site visit to the relevant Institution.

Quality Assurance of Study Endpoints
Each participating Investigator is responsible for reviewing the end point data of each patient at their site to:
• cross reference the source documentation against the submitted CRFs
• check that the evaluation of efficacy data points are correct

This should be carried out as each patient completes the study treatment or as clinically dictated.

The data review will be documented and any changes required to CRFs made and any amended CRFs submitted. The Coordinating Investigator will review all reviewed data from each site
prior to the planned analysis of the study.

10.12 Audit and Inspection

In accordance with applicable laws participating centres will allow direct access for monitoring, audit and inspection. Access will be granted to all trial related documentation and sites (including trial master file, patient files, clinics, laboratories and pharmacy).

The sponsor maintains the right to perform audits during the active phase of the study and/or after the study has been completed. There are two types of audits:

1) Routine audit: A systematic examination of study-related activities and documents to assure compliance with the protocol, and national/local regulations. The principal investigator of a selected centre will be given a 3 months notice period prior to the proposed audit visit. The aim of a routine audit is to:
   • inspect the study facilities
   • ensure that the study is conducted in accordance with ICH-GCP guidelines, the principles in the Declaration of Helsinki and relevant regulatory requirements
   • ensure that the site staff adhere to the protocol and SOP’s
   • review the informed consent process
   • review the SAE identification and reporting process
   • aid in identifying and correcting problem areas and provide suggestions to improve performance, if required

The principal investigator will receive an audit report within 4 weeks of the site visit.

2) “For-cause” audit: Where there is evidence or suspicion of non-compliance with important aspect(s) of the trial requirements. The notification period is dependent on the reason(s) triggering the audit.

In case of a for cause audit a letter is sent to the Principal Investigator outlining:
   • the reason(s) for the for-cause audit
   • any hold that has been placed on the protocol related research
   • all of the documents that are to be reviewed
• interviews that will be conducted
• planned inspections of the facilities (primarily how data is stored, drug storage, accountability, destruction etc.)
• who will come and perform the audit
• when the audit will likely occur

The principal investigator will receive an audit report within 2 weeks of the site visit.

10.13 Drug Accountability

Accountability for the study drug at the study site is the responsibility of the principal investigator at each study center. The principal investigator may delegate this responsibility to the local pharmacist, or other appropriate personnel. The responsible person will ensure that the study drug is used only in accordance with this protocol.

Drug accountability records, maintained by the clinical site, should indicate:
• use by each patient (including Lot numbers)
• disposal of unused drug or destruction of used vials (date and Lot numbers)

10.14 Protocol Amendments

No amendments to the protocol may be made without the explicit agreement of the Sponsor and Coordinating Investigator. Changes to any of the study related documentation and practices should always be made according to GCP. Prior to implementing the changes, significant amendments to the documentation must also be approved by the appropriate Ethics Committee(s) of participating centers.

10.15 Storage and Archiving of Study Material

It is the responsibility of the principal investigator at the study centre to keep all essential documents relating to the study for at least 5 years (or according to national legislation) after the completion or premature termination of the study. Essential documents are those which enable both the conduct of the study and the quality of the data produced to be evaluated and show whether the institution complied with the principles and guidelines of good clinical practice.
The medical files of patients enrolled into the study must be kept in accordance with national legislation and for the maximum period of time permitted by the institution. The sponsor or subsequent owner is required to keep all other documentation for the life of the product studied. The archived data can be kept in electronic form, provided that a back-up copy is kept and that a paper copy can be provided if necessary.

The protocol, ethical and government approvals, together with all other documents concerning the study, including any audit and inspection certificates are all to be kept as part of the trial master reference file. All data about serious adverse events also need to be kept in this trial master file.

All data should be available for inspection by the appropriate authorities on demand.

10.16 Liability Insurance

All centres collaborating in this multicentre study have NHS liability insurance cover, the terms of which include standard risks associated with biological therapy such as administered in this trial.

11.0 REFERENCES


5. Garcia-Perez MA, Noguera I, Hermenegildo C, Martinez-Romero A, Tarin JJ. Alterations in the phenotype and function of immune cells in ovariectomy-induced osteopenic mice. Human


ABBREVIATIONS

ESR erythrocyte sedimentation rate
Hb haemoglobin
HCT haematocrit
i.v. intravenous
HAQ health assessment questionnaire
DAS disease activity score
CTX crosslaps
OPG osteoprotegerin
P1NP procollagen type 1 amino-terminal propeptide
sRANKL soluble receptor activator of nuclear factor kappaB ligand

Centre Number:
Study Number:
Patient Identification Number for this trial:

CONSENT FORM

Title of Project: Bone study

Name of Researcher:
Please initial box

1. I confirm that I have read and understand the information sheet dated 1st February 2013 (version 4.0) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.

2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected.

3. I understand that relevant sections of my medical notes and data collected during the study may be looked at by individuals from regulatory authorities or from the NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records.

4. I agree to my GP being informed of my participation in the study.

5. I agree with my blood samples being taken for research studies as outlined in the patient information sheet dated 1st February 2013 (version 4.0).

6. I agree to take part in the above study.

Name of Patient Date Signature

Name of Person Date Signature
taking consent

When completed: 1 for participant; 1 for researcher site file; 1 (original) to be kept in medical notes.
Flow cytometric analysis of patient 0101 (Sample of all visits)

Baseline visit
Flow cytometric analysis of patient 0101 using FlowJo software

3 Months visit
6 Months visit
9 months visit
12 Months visit
A) **Standard operation procedures (SOPs):**

- Patients fulfilling the eligibility criteria were invited to join the trial. If the patient showed interest in joining the trial, the patient information leaflet was provided to the patient. The patient is allowed few weeks to discuss the trial’s information leaflet with the treating physician and his/her family.

- Eligible patients who wanted to join the trial were then explained all the procedures of the trial and then consented.

- All patients were anonymised by a four-digit study ID. The first two digits represent the centre’s ID and the second two digits represent the patient’s number on the corresponding site. No patient identifiable data are being stored in the trial master file or the regional site file according to the ethics committee and MHRA guidelines.

- The DXA scans and the first rituximab infusion were usually done within a week prior to the baseline visit. Follow up visits were appointed 3 months from the baseline visit date. However, in some patients this was not possible due to patient or staff reasons.

- Patients who responded to the first course received a second course at 6 months unless they attained a state of low disease activity, in accordance with clinical practice. Retreatment was delayed in patients with low disease activity (DAS score less than 3.2) at 6 months until a state of moderate disease activity was documented.

- All samples are collected fasting and whenever possible in the morning as bone markers tend to change dramatically during the day and are markedly affected by food intake.

- Bone biomarkers samples are stored in -80°C while flow cytometry is done on fresh samples on the same day of being taken. FACS samples are analyzed using the medical school FACSCanto II (1). Data are analyzed using FLOWJO software.

- **Data management:**
Case record forms are regularly collected from the 10 participating centres. Data are then completed including HAQ scores, DXA results calculation if not supplied by the reports. All data are being entered on our database after undergoing extensive quality control checks using the CRFs data and supplied copies of laboratory and radiology reports. Quality control is also being done to all DXA reports to check the proper positioning of the part scanned and the validity of the results provided.

**B) FACS protocol**

1. Pipette the pre-titrated volume recommended for each antibody into the TruCount tube

2. Avoid resuspending the bead by pipetting the volume on top of the mesh located at the bottom of the tube, which serves to hold the TruCount bead in place

3. Once all the antibodies have been added, add 100µl of whole blood via reverse pipetting to the same location where the antibodies were added

4. Tap the bottom of the tube to thoroughly mix the blood with the antibodies, and incubate in the dark for 20 minutes at room temperature

5. After 20 minutes, add 1000µl of the 1:10 BD Pharm Lyse lysis buffer to the blood / antibody mix to lyse all non-nucleated cells

6. Mix thoroughly by tapping the bottom of the tube and incubate in the dark for 20 minutes at room temperature

7. Analyse the sample on FACSCANTO 1

**FlowJo analysis protocol:**

1. Locate the .fcs files that you exported to the shared drive immediately after acquisition.

2. Back up these raw files, in case of unforeseen circumstances
3. Keep a copy of said files on the analysis computer you are using otherwise FlowJo will get confused!!

4. Open FlowJo via the Application folder or taskbar, use one FlowJo workspace per patient, to allow the presence of all timepoints

5. To add the .fcs files to the workspace, either drag and drop the files, or add tubes via the tube icon at the top of the worksheet

6. Click upon the layout editor icon, to generate a page layout for each timepoint whereby you can place copies of the plots upon

7. Double-click on the tube of interest, which will open the forward vs. sideward scatter dot plot

8. To isolate the lymphocytes, change the x-axis from forward scatter (FSC) to CD45.

9. Gate around the lymphocytes which are CD45+, sideward scatter (SSC) dim, whilst avoiding the debris located at the dimmest point of the sideward scatter

10. To ensure purity of population, double click on the gate, or the label, and revert the x-axis back to forward scatter and see that the cells / events are in the correct Lymphocyte location

11. If there is some debris contamination then, gate around the lymphocytes within this gate.
12. Working within the lymphocyte gate, change the x axis to CD19 and gate around the CD19+ cells

13. Now generate graphs plotting CD19 as the x-axis, against CD24, CD27 and finally CD38 as the y-axis, and drag a copy of each to the layout editor

14. Now we need to create a plot with only CD19+ B lymphocytes in. And have the x-axis as CD38 and the y-axis as CD24

15. The resultant plot will show 3 CD19+ populations

   i) CD19+ CD24hi CD38hi
      Largely comprised of CD27- negative Immature B cells (approx. 90%)

   ii) CD19+ CD24+ CD38-
      Mainly memory B cells

   iii) CD19+ CD24int CD38int

Which are primarily mature B cells

16. To confirm the naïve status of all the above subsets. For instance the CD19+ CD24hi CD38hi, open a plot with just these cells and split them between CD27 positive and negative. In theory, the majority of them should be negative, confirming their naïve status.

17. Open the original ungated dot plot and by comparing different surface markers with each other, try to locate the TruCount beads, whereby you should gate upon them.

18. Note the number of events of each population of interest, and the number of beads acquired. Also have to hand the exact number of TruCount beads present in the tubes used (according to the sachet of the batch). Use these numbers in the below calculation. Test volume includes antibodies lysis buffer and blood volume.
19. In the instance of dead cells, gate around said cells and label as such

20. Highlight the original ungated graph and generate a Boolean Gate where by you ask FlowJo to use all lymphocytes “AND NOT” the dead cells which you have gated on

21. The resultant Boolean gate will therefore be your lymphocytes, without the dead cells

22. Now print off all the pages containing your multiple timepoint layouts, as well as a layout summarising all the timepoints. Also print off the table containing the event numbers of each gate etc

23. Input all the relevant numbers into the spreadsheet and do the necessary calculations

**C) DKK1 assay protocol:**

Day 1:

1- All contents must be at room temperature

2- Mark the Blank/Standard/Sample/Control sheet

3- Samples:

Dilute as 1:4 with assay buffer (25 µl sample + 75 µl assay buffer).

4- Standards (STD) & Control (CTRL):

Dissolve each in 400 µl deionised water for 10 mins at room temperature. Then mix it well manually then leave it for 5 mins then mix it well. (Total of 15 mins)

5- Antibody (AB):

- Reconstitute the 2 antibody vials.

- Each vial is reconstituted in 11 ml assay buffer for 15 mins at room temperature.
6- Add 50 µl of STD/Sample/CTRL into wells except blank
7- Add 200 µl of AB into all wells except blank
8- Swirl gently

Incubation 1:
Cover tightly and incubate for 18-24 hours at room temperature in the dark.

Day 2:

1- Prepare the plate reader to measure absorbance at 450 nm with reference 620 nm.
2- Wash buffer (WASHBUF):
   Dilute as 1:20 (50 ml WASHBUF + 950 ml distilled water at room temperature.
3- Wash 1:
   - Fill 5-6 containers with diluted wash buffer
   - Wash 5x using multi-pipette with 300 µl wash buffer.
   - Hit the plate against paper towel after last wash.

4- Add 200 µl Conjugate (CONJ) into all wells.

5- Wash 2:
   - Fill 5-6 containers with diluted wash buffer
   - Wash 5x using multi-pipette with 300 µl wash buffer.
   - Hit the plate against paper towel after last wash.

6- Add 200 µl substrate into all wells.
**Incubation 3:**
Incubate for 30 mins at room temperature in the dark without cover.

7- Add 50 µl STOP solution into all wells.
8- Measure absorbance immediately at 450 nm with reference 620 nm.

Reference data:

```
Mean = 52.6 pmol/l
Median = 45.7 pmol/l
SD = 20 pmol/l
```

Calculation of the results:
1- Subtract the blank from all other values.
2- The assay is evaluated using 4PL algorithm.
3- The dilution factor 1:4

**D) Sclerostin assay protocol**

Day 1:
- All contents must be at room temperature
- Mark the Blank/Standard/Sample/Control sheet
- Standards (STD) & Control (CTRL):
  Dissolve each in 400 µl deionised water for 10 mins at room temperature. Then mix it well manually then leave it for 5 mins then mix it well. (Total of 15 mins)
- Add 150 µl of assay buffer (ASYBUF with red cap) into all wells.
- Add 20 µl of STD/Sample/CTRL into wells except blank
- Add 50 µl of AB (Green cap) into all wells except blank
- Swirl gently

**Incubation 1:**
Cover tightly and incubate for 18-24 hours at room temperature in the dark.

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**Day 2:**

- Prepare the plate reader to measure absorbance at 450 nm with reference 630 nm.

- Wash buffer (WASHBUF):
Dilute as 1:20 (50 ml WASHBUF + 950 ml distilled water at room temperature.

- **Wash 1:**
  - Fill 5-6 containers with diluted wash buffer
  - Wash 5x using multi-pipette with 300 µl wash buffer.
  - Hit the plate against paper towel after last wash.

- Add 200 µl CONJ (CONJUGATE, AMBER CAP) into all wells.

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9- **Wash 2:**

  - Fill 5-6 containers with diluted wash buffer
  - Wash 5X using multi-pipette with 300 µl wash buffer.
  - Hit the plate against paper towel after last wash.

- Add 200 µl Substrate (BLUE CAP) into all wells.
Add 50 µl STOP solution (WHITE CAP) into all wells.

Measure absorbance immediately at 450 nm with reference 620 nm.

Reference data:

Mean = 19.3 pmol/l
Minimum = 10.9 pmol/l
Maximum = 28.7 pmol/l

Calculation of results

1- Subtract the blank from all wells.

2- The assay is evaluated with 4PL algorithm