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Mechanism of bone loss in rheumatic diseases

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To my mother

Contents

Acknowledgements **1**
Papers relating to this work **3**
Papers in preparation **3**
Conference presentations relating to this work **4**
Abbreviations **5**
Abstract **8**
Lay abstract **10**
Chapter 1: General introduction **11**
1.1 Bone 12
1.2 Bone Cells 15
1.2.1 Osteoclasts 15
1.2.1.1 Osteoclast signalling 15
1.2.1.2 Osteoclast induced bone resoption 18
1.2.2 Osteoblasts 20
1.2.3 Osteocytes 23
1.2.4 Chondrocytes 23
1.3 Osteogenesis 24
1.3.1 Endochondral ossification 24
1.3.2 Intramembranous ossification 25
1.3.3 Mineralization 26
1.4 Bone Remodeling Unit 27
1.4.1 Bone resorption 27
1.4.2 Cessation of resorption and reversal phase 27
1.4.3 Bone formation 28
1.5 OPG/RANKL/RANK system 31
1.5.1 RANKL 31

1.5.2 RANK ... 32
1.5.3 Osteoprotegerin ... 32
1.5.4 Osteoprotegerin autoantibodies ... 34
1.6 Osteoporosis ... 36
1.6.1 Definition ... 36
1.6.2 Pathogenesis of Osteoporosis ... 38
1.6.3 Diagnosis of osteoporosis and fracture assessment ... 41
1.6.3.1 FRAX ... 41
1.6.3.2 QFracture ... 42
1.6.3.3 Other fracture risk tools ... 43
1.6.4 Bone turnover markers ... 44
1.7 Mechanism of bone loss in inflammatory arthritis ... 46
1.7.1 Introduction ... 46
1.7.2 Rheumatoid Arthritis ... 47
1.7.2.1 Osteoporosis risk ... 47
1.7.2.2 Fracture risk ... 48
1.7.2.3 Pathogenesis ... 49
1.7.2.4 Mediators of bone loss in RA ... 52
1.7.3 Ankylosing Spondylitis ... 67
1.7.3.1 Osteoporosis risk ... 67
1.7.3.2 Fracture risk ... 68
1.7.3.3 Pathologenesis of bone involvement in AS ... 69
1.7.3.4 Mediators ... 71
1.7.4 Systemic Lupus Erythematosus ... 74
1.7.4.1 Osteoporosis in SLE ... 74
1.7.4.2 Fracture risk ... 75
1.7.4.3 Pathologenesis of bone involvement in SLE ... 75
1.7.4.4 Mediators ... 76
1.7.5 Psoriatic Arthritis ... 78
1.7.6 Other rheumatic diseases ... 83
1.7.6.1 Scleroderma ... 83
1.7.6.2 Primary Sjoegren Syndrome ... 84

1.7.6.3 Dermatomyositis 84
Chapter 2: Material and Methods 87
2.1 Laboratory Methods 87
2.1.1 Osteoprotegerin autoantibody ELISA 87
2.1.2 OPG ab ELISA – updated 90
2.1.3 RANKL and OPG measurements 93
2.1.3.1 Free serum RANKL measurements 93
2.1.3.2 Total serum RANKL measurements 94
2.1.3.3 Serum Osteoprotegerin measurements 95
2.1.4 Serum CTX measurements 95
2.2 Immunoglobulin purification 96
2.2.1 Melon Gel Immunoglobulin G (IgG) purification 96
2.2.1.1 Buffer Exchange Procedure 96
2.2.1.2 Spin-column Procedure for IgG Antibody Purification 97
2.2.2 IgG purification with Protein G Spin columns 97
2.2.2.1 Procedure for Antibody Purification 97
2.2.3 HEK-293 NF-KB reporter assay 99
Chapter 3: Prevalence and predictors of Osteoporosis in Rheumatoid Arthritis 100
3.1 Abstract 100
3.2 Introduction 101
3.3 Patients and Methods 102
3.3.1 Rheumatoid Arthritis cohort 102
3.3.2 ORCADES cohort 103
3.3.3 Joint erosions 104
3.3.4 Measurements of BMD measurements and fracture risk assessment 104
3.3.5 Statistical analysis 104
3.4 Results 106
3.4.1 Patient Characteristics 106
3.4.2 Risk factors for osteoporosis 108
3.4.3 Prevalence of osteoporosis in RA population versus controls 110

3.4.4 Development of a diagnostic algorithm 111
3.4.5 Risk factors for fractures 116
3.4.6 Fracture prediction with OPRA 118
3.5 Discussion 119
Chapter 4: Osteoprotegerin antibodies and Rheumatoid Arthritis 122
4.1 Abstract 122
4.2 Introduction: 123
4.3 Patients and Methods: 124
4.3.1 Patients Rheumatoid Arthritis cohort for OPG ab study 124
4.3.2 Healthy control cohort 124
4.3.3 DXA measurement 124
4.3.4 Serum measurements 125
4.3.5 Statistical methods 125
4.3.6 Ethics 125
4.4 Results: 126
4.4.1 Clinical characteristics of the RA cohort and controls 126
4.4.2 Antibodies to OPG in cases and controls 129
4.4.3 Association between OPG ab and clinical characteristics in RA 130
4.4.4 Functional analysis of OPG antibodies 133
4.5 Discussion 135
Chapter 5: Osteoprotegerin autoantibodies and Ankylosing Spondylitis 137
5.1 Abstract 137
5.2 Introduction 139
5.3 Methods 140
5.3.1 Patients 140
5.3.2 BMD assessment 141
5.3.3 Osteoprotegerin autoantibody measurement 141
5.3.4 Statistics 141
5.3.5 Ethics 142
5.4 Results 143

5.4.1 Demographics and clinical characteristics 143
5.4.2 Association between OPG ab positivity and disease characteristics ... 143
5.4.3 Difference in BMD between OPG ab status 145
5.4.4 OPG ab positivity and clinical associations 147
5.4.5 Correlation between OPG ab and bone mineral density 148
5.4.6 Association between OPG ab status and difference between spine and hip BMD 149
5.4.7 Association between BMD and fractures and OPG ab status 150
5.5 Discussion 151

Chapter 6: Correlation of RANKL, OPG and OPG antibodies in Rheumatic Diseases 154

6.1 Abstract 154
6.2 Introduction 155
6.3 Methods 156
6.3.1 Study subjects 156
6.3.2 Serum measurements 156
6.3.3 Free RANKL ELISA with recombinant RANKL standard curve 156
6.3.4 Statistical methods 157
6.4 Results 158
6.4.1 Demographics and measurements of disease groups 158
6.4.2 Osteoprotegerin concentration in Rheumatic Diseases 159
6.4.3 Correlation between Osteoprotegerin and Osteoprotegerin autoantibody concentrations 160
6.4.4 Correlation between OPG ab and free RANKL concentrations 161
6.4.5 Total sRANKL concentrations 163
6.4.6 Effect of OPG ab on free RANKL detection 165
6.5 Discussion 168

Chapter 7: Discussion and Conclusion 170

References 175

Appendices 214

A1 Materials and Reagents 214
A2 Apparatus 218

A3 Software 219
A4 Solutions.. 220

Declaration

I hereby declare that this work has been carried out by me, except where specifically acknowledged, and has not been submitted for any other degree or professional qualification. The thesis has been composed by myself and the information obtained from other sources rather than this study has been specifically acknowledged by proper use of quotes and references.

Barbara Hauser

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Papers relating to this work

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Hauser B, Riches PL, Wilson JF, Horne AE, Ralston SH. Prevalence and clinical prediction of osteoporosis in a contemporary cohort of patients with rheumatoid arthritis. **Rheumatology** 2014 Oct;53(10):1759-66.

Real A, Gilbert N, **Hauser B**, Kennedy N, Shand A, Gillett H, et al. Characterisation of Osteoprotegerin Autoantibodies in Coeliac Disease. **Calcified Tissue International** 2015 Aug;97(2):125-33

Papers in preparation

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Hauser B*, Riches P, Ralston S. Prevalence and predictors of osteoporosis in Rheumatoid Arthritis **Scottish Society of Rheumatology 2013 Dundee,** Oral Presentation

Hauser B*, P. Riches, S. Ralston, Predictors of Osteoporosis in Rheumatoid Arthritis: Retrospective cohort study **ECTS 2012 Stockholm**, Poster Presentation

Lip S*, Morrison E., Ralston SH, **Hauser B**
Current Practice of Bone Mineral Density Assessment in Patients with Rheumatoid Arthritis and Evaluation of OPRA: A New Osteoporosis Prediction Tool, **BSR 2015 Manchester**, Poster Presentation

Zhao S* & **Hauser B**, Visconti M, Riches PL, Ralston SH, Goodson N. Autoantibodies to osteoprotegerin are independently associated with low hip bone mineral density and increased fractures in axial spondyloarthritis **American College of Rheumatology, San Francisco 2015,** Poster Presentation

**presenting author*

Abbreviations

ALP	Alkaline phosphatase
AP-1	Activator protein 1
AS	Ankylosing Spondylitis
BMD	Bone mineral density
BMI	Body mass index
BMP	Bone morphogenic protein
BSA	Bovine serum albumin
CT	Computed tomography
CTX	Carboxy-terminal collagen crosslinks
DAP12	DNAX activation protein of 12kDa
DEXA	Dual energy X-ray absorptiometry
DKK	Dickkopf
DMP 1	Dentin matrix protein 1
DPD	Deoxypyridinolene
ELISA	Enzyme linked immunosorbent assay
FCR-γ	Fc receptor common γ chain
FCS	Fetal calf serum
FGF	Fibroblast growth factor
FZD	Frizzled
HLA	Human leukocyte antigen
HRP	Horse radish peroxidase
IFN β	Interferon beta
IFN μ	Interferon gamma
IgG	Immunoglobulin G

IgM	Immunoglobulin M
IκB	Inhibitor of kappa B
IL	Interleukin
ITAM	Immunoreceptor tyrosine based activation motif
M-CSF	Macrophage colony stimulating factor
mg	Milligram
ml	Millilitre
MMP 9	Matrix metallopeptidase 9
MWU	Mann-Whitney U test
NFATc1	Nuclear factor of activated T-cells, cytoplasmic 1
NFκB	Nuclear factor kappa B
NICE	National Institute for Health and Care Excellence
NOGG	National osteoporosis guidelines group
OP	Osteoporosis
OPG	Osteoprotegerin
ORCADES	Orkney Complex Diseases Study
OSCAR	Osteoclast associated receptor
PBS	Phosphate buffered saline
PsA	Psoriatic Arthritis
PTH	Parathyroid hormone
RA	Rheumatoid Arthritis
RANK	Receptor Activator of Nuclear Factor kappa B beta
RANKL	RANK Ligand
ROC	Receiver operating characteristic
RPM	Revolutions per minute
Runx2	Runt related transcription factor 2

SIGN	Scottish Intercollegiate Guidelines Network
SLE	Systemic Lupus Erythematosus
SOST	Sclerostin
SpA	Spondyloarthropathy
TBST	Tris buffered saline with Tween 20
TGF	Transforming growth factor
TMB	Tetramethylbenzidine
TNF	Tumour necrosis factor
TRAP	Tartrate resistant acid phosphatase
VEGF	Vascular endothelial growth factor
Wnt	Wingless type MMTV integration site family member
μg	Microgram
μl	Microlitre

Abstract

Osteoporosis and fragility fractures are recognized complications of inflammatory rheumatic diseases. This is thought to result from the effects of chronic inflammation, relative immobility and corticosteroid use. A rare syndrome of osteoporosis in a patient with coeliac disease has been described which results from production of neutralizing antibodies to the bone protective protein osteoprotegerin (OPG). The aim of my thesis is to evaluate prevalence and clinical predictors of osteoporosis in a contemporary cohort of patients with rheumatoid arthritis (RA) and to investigate the role of OPG autoantibodies in the pathogenesis of osteoporosis in rheumatic diseases.

In a retrospective cohort study, I found that the overall prevalence of osteoporosis in patients with RA was 29.9% which is in keeping with older reports that recorded a prevalence rate between 17% and 36%. In our contemporary cohort osteoporosis was significantly more common than in a gender and age matched control cohort (17.4%). Further analysis showed that only age and BMI were independent predictors of osteoporosis in RA. A predictive tool based on age and BMI was developed which had 91.4% sensitivity for the detection of osteoporosis in an independent RA population.

I went on to screen for the presence of autoantibodies to OPG in patients with various rheumatic diseases. In a study of 75 patients with rheumatoid arthritis and 199 healthy controls OPG autoantibodies were detected in two controls (1%) compared with seven patients with RA (9.3%). The RA patients with detectable OPG antibodies had a longer disease duration, higher DAS28 scores and higher levels of the bone resorption marker CTX than RA patients who did not have autoantibodies. Purified IgG from patients with high levels of OPG antibodies blocked the ability of recombinant OPG to inhibit RANKL

induced NFκB activation in a HEK293 cell based assay indicating that they were functional.

In a further study of 134 patients with ankylosing spondylitis (AS), 16 patients (11.9%) had detectable OPG antibodies. The presence of OPG-Ab was independently associated with reduced hip bone mineral density and an increased risk of fractures in this population. In patients with a longer disease duration we have also observed that there was a higher discrepancy between spinal and hip BMD in OPG-Ab positive patients compared with OPG ab negative patients (p=0.003).

In order to investigate if OPG antibodies affected measurement of serum RANKL concentrations as detected by ELISA using OPG as the capture reagent, I measured OPG ab and free RANKL concentrations in 55 rheumatic disease patients. Surprisingly there was a significant positive correlation between free RANKL and OPG Ab concentrations (r=0.430, p=0.001) which was the opposite to what I had expected. These findings reject the hypothesis that OPG ab block binding of synthetic OPG to RANKL in the ELISA.

In conclusion, I have shown that osteoporosis is a common complication in RA and I have developed a new risk prediction tool for the use in clinical practice. I have also found that OPG antibodies are produced more commonly in patients with RA and AS than in healthy controls and that antibody levels correlate with bone resorption markers in RA and bone mineral density in AS patients. In vitro studies have shown that some OPG antibodies have functional effects on RANKL signalling. These findings raise the possibility that OPG antibodies may contribute to the pathogenesis of local and systemic bone loss in rheumatic diseases and signal the need to study the relationship between these antibodies and bone disease in large-scale longitudinal studies.

Lay abstract

Rheumatic diseases, such as Rheumatoid Arthritis, Ankylosing Spondylitis and Systemic Lupus Erythematosus are chronic, frequently debilitating autoimmune diseases, which primarily affect joints, spine and skin but can also involve other organs. Patients with rheumatic diseases frequently suffer from osteoporosis (thinning of the bones) and are therefore more likely to sustain a fracture. The main aim of this thesis was to investigate why patients with rheumatic diseases are at increased risk of developing osteoporosis and I was particularly interested to see if the immune system, which is usually responsible for combating infections in the body, is partly responsible for the osteoporosis.

I have found that in a small proportion of patients the immune system has produced auto-antibodies (types of protein that attack parts of your own body) that block a bone protective protein called osteoprotegerin (OPG). Patients with Rheumatoid Arthritis who have these antibodies seem to lose bone more rapidly and patients with Ankylosing Spondylitis who have been shown to have increased levels of OPG antibodies have lower bone mineral density than patients without these antibodies. However, we do not know yet when and why these antibodies occur and what the best treatment for OPG antibody associated osteoporosis is. Further studies are required to answer these questions and to find out if testing patients for OPG antibodies routinely will help to predict the risk of osteoporosis in some patients or inform us about what is the best osteoporosis treatment to use.

Chapter 1: General introduction

In this chapter I will outline current understanding of bone pathology and introduce the cells and regulatory mediators involved in bone turnover. In the second part of the introduction, I will summarize up-to-date knowledge and recent findings of the mechanism of bone loss in Rheumatic Diseases with a particular focus on the concepts of autoimmune mediated bone loss. This should give an overview of current understanding of bone homeostasis in rheumatic diseases, outline gaps of current knowledge and introduce the research questions and hypotheses of my thesis.

1.1 Bone

Bone is a specialized connective tissue which together with cartilage forms the skeletal system. The skeleton has a role as supporting organ to which muscles, tendons and ligaments are attached. Additionally bone acts as calcium and phosphate reservoir and it harbours bone marrow where blood cells form and mature before being released into the blood stream (Colledge NR et al. 2010;Rosen CJ 2013).

The skeleton consists of two types of bone, cortical and trabecular bone as shown in Figure 1.1.

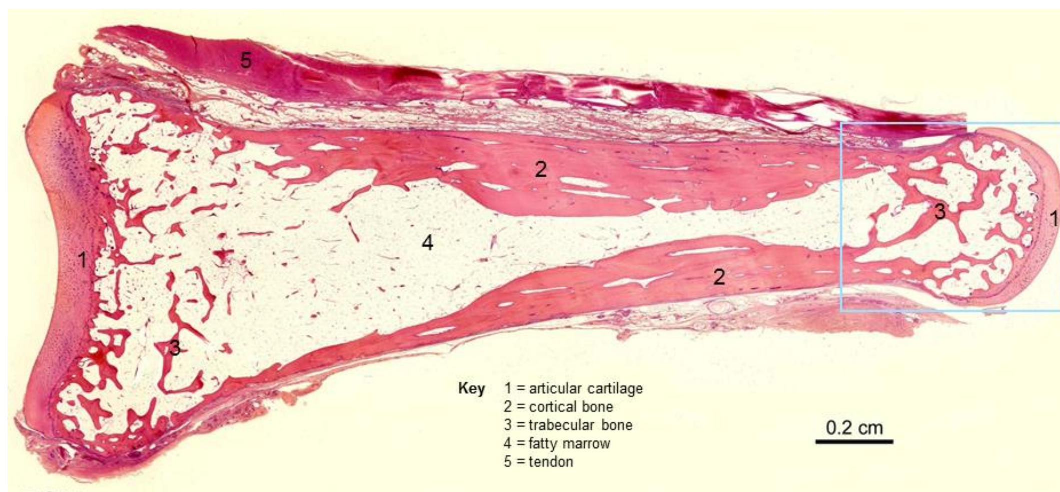

Figure 1.1 Crosssection of human phalanx *(adapted from Bone Research Society Teaching slides, provided by Prof Tim Arnett)*

Cortical bone is highly mineralized compact bone that constitutes 85% of the skeleton and undergoes little bone remodelling. Cortical bone is found in the diaphysis of long bones and the outer layer of flat bones.

Osteons are the basic structural unit of both cortical and trabecular bone. Cortical osteons (Haversian system) are cylindrical structures which contain a central (Haversian) canal with blood vessels and nerve fibres (Fig.1.2). Concentric layers of bone matrix (lamellae) surround the central canal. The

outer layer of cortical bone is the periosteum, which contains blood vessels and nerve fibres, and is connected with the Haversian system through perforating perpendicular canals (Volksman's canal).

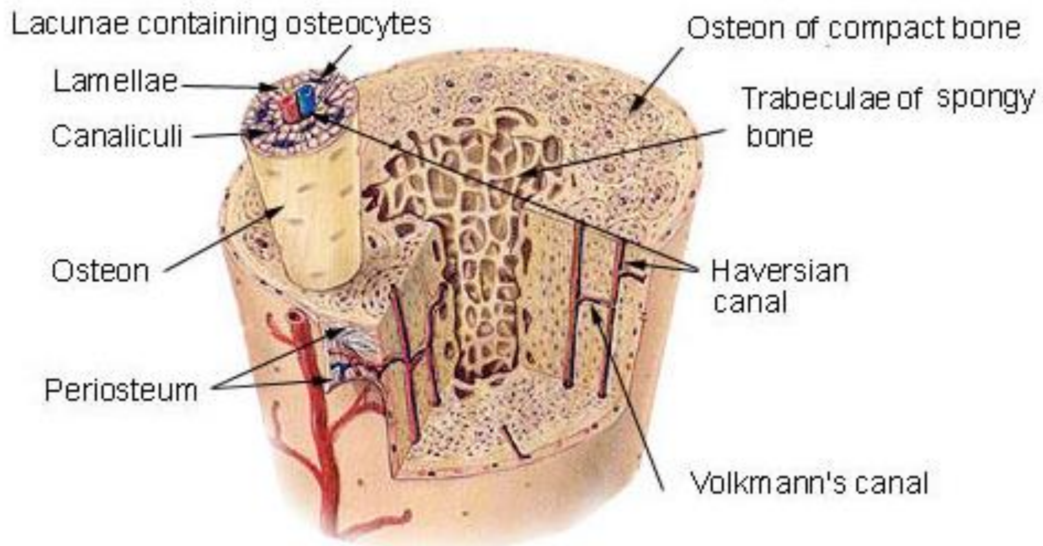

Figure 1.2 Structure of cortical bone (Haversian system)
See text for details. Adapted from https://embryology.med.unsw.edu.au/embryology /images/a/a6/Bone_structure_cartoon.jpg

Trabecular bone is mainly found in flat bones, epiphysis and metaphysis of long bones. It fills the centre of bones and contains a network of trabeculae, which provides structural strength and is surrounded and filled by bone marrow. Trabecular bone is, in contrast to compact bone, metabolically active and is therefore more affected by disorders of bone remodelling than cortical bone. Trabecular osteons also comprise bony lamellae which run, in contrast to the Haversian system, parallel to the bone surface.

Medullary cavity is filled with bone marrow where mesenchymal and haemapoetic blood lineage cells differentiate into skeletal tissue, blood vessels and mature blood cells (Lorenzo J et al. 2011).

Bone matrix consists of organic and inorganic components. Type I collagen is the most abundant component of bone matrix and accounts for approximately 90% of total protein of bone. Type I collagen is formed by two α1 peptide chains and one α2 chain which are wound into a triple helix. After intracellular processing, Type I collagen is laid down in extracellular matrix, where single fibrils are 'cross-linked' to one another in order to increase bone strength. When bone resorption occurs these cross-links are released into bloodstream and act as marker for bone resorption, like N-telopeptide (NTX) and C-telopeptide (CTX). The other organic components which mediate cell adhesion include osteocalcin, fibronectin, vitronectin, osteopontin and osteonectin.

The inorganic component of bone is mineral. Mineralized matrix consists of calcium and phosphate crystals which are present in between collagen fibrils in form of hydroxyapatite $[Ca_{10}(PO_4)_6(OH)_2]$.This component is of vital importance for the bone's rigidity and strength. However over-mineralisation as in osteogenesis imperfecta can lead to increased bone fragility (Colledge NR, Walker BR, & Ralston SH 2010;Rosen CJ 2013).

1.2 Bone Cells

1.2.1 Osteoclasts

Osteoclasts are multinucleated, highly specialized cells that are formed by fusion of multiple mononuclear osteoclast precursor cells. The principal action of a mature osteoclast is bone resorption. Macrophage colony stimulating factor (M-CSF) and Receptor Activator of NfκB Ligand (RANKL) are essential for osteoclast differentiation, maturation and survival (Boyle et al. 2003;Jones et al. 2002). Osteoclasts differentiate from haematopoetic stem cells initially to colony forming units for granulocytes (CFU-GM). M-CSF binds to its membrane receptor c-Fsm on precursors and mature osteoclasts. Binding of M-CSF induces the expression of receptor activator of nuclear factor κB (RANK) and on cells which are committed to become osteoclasts (Rosen CJ 2013;Teitelbaum 2000). Receptor activator of nuclear factor-κB ligand (RANKL), a cytokine expressed by osteocytes, osteoblasts, T-cells and synoviocytes binds to RANK. RANKL binds to the RANK receptor and induces the activation of various signaling pathways including NFκB and Activation Protein-1 (AP-1) which promotes the fusion of mononuclear cells into multinucleated osteoclasts (Takayanagi 2005;Teitelbaum 2000). The sequential stages of osteoclast differentiation are shown in Figure 1.3.

1.2.1.1 Osteoclast signalling

Beside the essential signals of M-CSF and RANKL for differentiation of osteoclasts there are a multitude of signalling pathways which allow fine tuning of osteoclast maturation and activity (Fig. 1.4). The central pathway is RANK signalling which results in strong NFκB activation and lineage commitment of osteoclasts. The binding of RANKL activates TNF-receptor associated factors (TRAF), in particular TRAF-6 which is essential for osteoclast differentiation and activation (Lomaga et al. 1999). Downstream of TRAF6, several kinases including mitogen activated protein kinases (MAPK) get activated which subsequently induce the transcription of osteoclast-specific genes via the AP1 family of proteins.

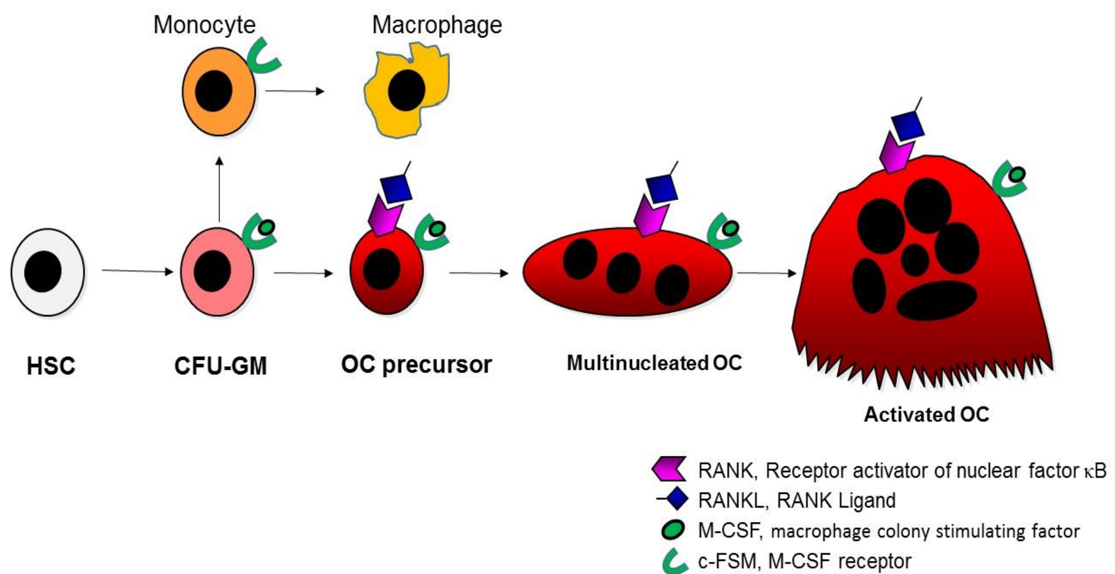

Figure 1.3 Diagram of Osteoclast differentiation

Haematopoetic stem cells (HSC) differentiate initially into colony forming units for granulocytes (CFU-GM) then to Osteoclast (OC) precursors. M-CSF and RANKL promotes fusion into multinucleated OC and maturation into active OC.

Activation of the RANK receptor can also lead to the formation and subsequent activation of the IKK α/IKKβ/IKKγ complex. The catalytic IKK subunits (IKKα and IKKβ) and non-catalytic IKKγ mediate NFκB activation. Tumour necrosis factor-α (TNFα) boosts osteoclast differentiation through potent NFκB activation via IKK pathways and various Kinases including p-38 and extracellular signal-regulated kinase (ERK) (Hayden and Ghosh 2004). Interleukin 1 enhances osteoclastogenesis via p38/ERK pathway. Beside above mentioned inflammatory cytokines Interleukin 6 (IL6) influences osteoclastogenesis positively via MAPK and/or Janus kinases (JAK) and signal transducers and activators of transcription STAT signaling pathway

(Redlich and Smolen 2012). On the other hand Interferon α (IFN-α), Interferon β (IFN-β) and mainly Interferon γ attenuate osteoclast proliferation through negative crosstalk to RANK signaling and inhibition of activator protein 1 (AP1) (Takayanagi et al. 2000).

Osteoclasts derive from the monocyte/macrophage lineage and therefore express an Fc receptor common gamma subunit (FcRγ) and DNAX-activating protein (DAP12) receptor. Both are immunoreceptor tyrosine based activation motif (ITAM) harbouring molecules, which interact with spleen tyrosine kinase (SYK), which allows activation of the essential osteoclast transcription factor NFATc1 via Phosphoinositide-specific phospholipase C (PLC) and Calcineurin. NFATc1 regulates multiple osteoclast specific genes such as Cathepsin K and tartrate resistant acid phosphatase (TRAP), together with other transcription factors. ITAM signals are not able to stimulate or enhance osteoclastogenesis without RANK stimulation and are therefore called co-stimulatory signals (Takayanagi 2005). However Koga et al (Koga et al. 2004) demonstrated that mice deficient of FcRγ and DAP 12 develop severe osteopetrosis and mice only lacking FcRγ develop a milder form of osteopetrosis. FcRγ gets activated by immune complexes and therefore may play a particular important role in osteoclast activation in autoimmune diseases with known autoantibodies such as Rheumatoid Factor and ACPA in Rheumatoid Arthritis. The function and importance of FcRγ in osteoclasts has been for a long time unknown but recent findings demonstrate its important role in osteoclast regulation. This pathway may therefore become of interest as potential therapeutic target. (Harre et al. 2012;Negishi-Koga et al. 2015).

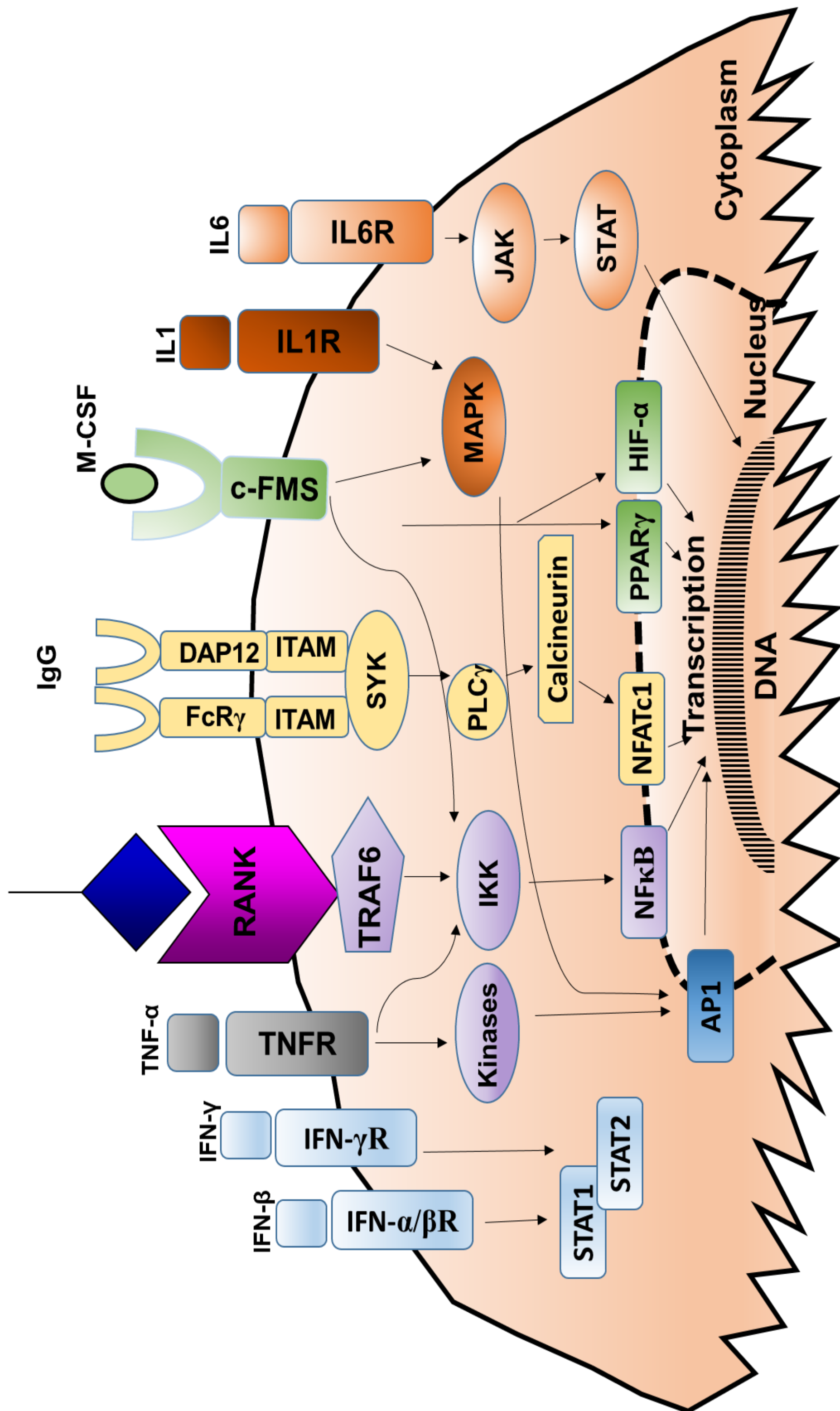

Figure 1.4 Schematic representation of signalling pathways involved in osteoclast differentiation and function. *See text for more details. Abbreviations IFN Interferon, TNF Tumour Necrosis factor, RANK Receptor Activator of NfκB (nuclear factor κB), STAT Signal transducer and activator of transcription, TRAF TNF receptor associated factor, IKK =IκB kinase, NFAT = nuclear factor of activated T-cells, PLC= Phosphoinositide-specific phospholipase C, SYK= spleen tyrosine kinase, ITAM=immunoreceptor tyrosine based activation motif, DAP12= DNAX-activating protein, FcRγ= Fc receptor common gamma chain, M-CSF= Macrophage colony stimulating factor, c-FMS= receptor of M-CSF, AKT= PPARγ =peroxisome proliferator activated receptor γ, HIF-α= hypoxia inducible factor 1α, MAPK= mitogen activated protein kinases, IL1 and IL6= Interleukin 1 and 6, JAK=Janus Kinases*

1.2.1.2 Osteoclast induced bone resoption

The resorption process starts by the adhesion of osteoclasts onto bone with help of actin –rich structures called podosomes.

The attached osteoclasts form a “sealing zone” which separates the resorption area from extracellular space. Activated osteoclasts use a complex folded membrane structure, “the ruffled border”, as secretory site and induce bone degradation by secretion of proteolytic enzymes such as Cathepsin K, Matrix Metaolprotease 2, chlorid ions and acid. Resorption and degradation of bone matrix induces differentiation of the bone forming cells, the osteoblasts. This cross-talk between osteoclasts and osteoblasts is called coupling (Rosen CJ 2013). Overstimulation of osteoclasts leads to bone disease such as Osteoporosis, Paget’s Disease of Bone and bone erosions in Rheumatoid Arthritis (Ralston 2013;Sambrook and Cooper 2006a;Schett and Gravallese 2012). The majority of available treatment options for osteoporosis are targeting osteoclast differentiation (Cummings et al. 2009), function or survival (Reid et al. 2009;Sambrook & Cooper 2006a).

1.2.2 Osteoblasts

Osteoblasts derive from mesenchymal stem cells and mature into highly specialized bone-forming cells Figure 1.5.

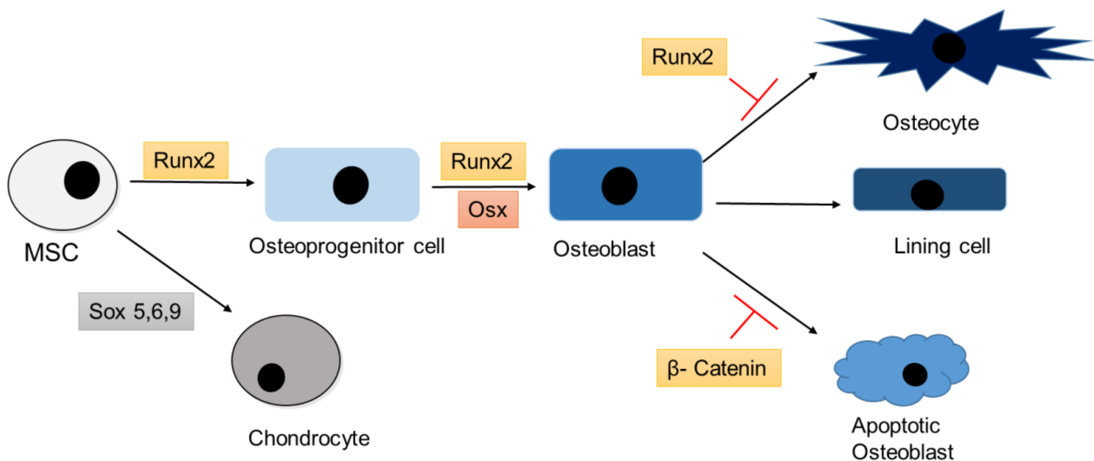

Figure 1.5 Diagram showing differentiation from mesenchymal stem cells to mature osteoblasts *MSC (Mesenchymal stem cells) differentiate under influence of RUNX2 (runt related transcription factor)into osteoprogenitor cells which differentiate with help of the transcription factors RUNX2 and Osx (Osterix) into mature osteoblasts. Osteoblasts undergo either apoptosis or terminal differentiation in either osteocytes or bone lining cells. MSC can also differentiate to Chondrocytes under influence of Sox (sex determining region Y (SRY)-box) 5,6 and 9 proteins.*

Osteoblast differentiation and activation requires multiple signaling pathways. Some important signaling pathways are depicted in Figure 1.5. RUNX2 (runt related transcription factor) is “the master regulator of osteoblastogenesis as its activation allows the differentiation from mesenchymal progenitor cells into preosteoblasts and mature bone forming cells(Redlich & Smolen 2012). RUNX2 can be activated by SMAD proteins, which are phosphorylated by bone morphogenic proteins with their receptors, growth factors such as

transforming growth factor-β and fibroblast growth factor and the parathyroid hormone (PTH)(Rosen CJ 2013). Mice lacking Runx2 were found to have defective bone formation due to inhibited maturation of osteoblasts (Komori et al. 1997;Otto et al. 1997).
The zinc finger transcription factor Osterix (Osx), acting downstream of Runx2 (Fig 1.5), was also found to be essential for the development of osteoblasts (Lecka-Czernik et al. 1999) as osterix deficient mice are unable to form bone (Koga et al. 2005;Nakashima et al. 2002). Another important signaling pathway is Wnt-Frizzeled-β catenin pathway. WNT glycoproteins bind to a receptor complex which consists of the Frizzeled receptor alongside associated low-density lipoprotein receptor related proteins (LRP) such as LRP 4, LRP5 and LRP6(van Amerongen and Nusse 2009). Once activated the Frizzled receptor signals via β catenin. Inhibitors of the WNT pathway are important as they efficiently reduce osteoblast function and as they recently became central to drug development in the treatment of osteoporosis. Sclerostin, a cytokine secreted by osteocytes, binds to LRPs and inhibits binding of WNT to the complex(Rosen CJ 2013).

Dickkopf 1 (DKK1) inhibits osteoblastogenesis in a similar way to Sclerostin but requires an additional co-receptor called Kremen in order to bind and eliminate LRP(Diarra et al. 2007).Parathyroid Hormone (PTH) also uses the β -catenin pathway for osteoblast activation.

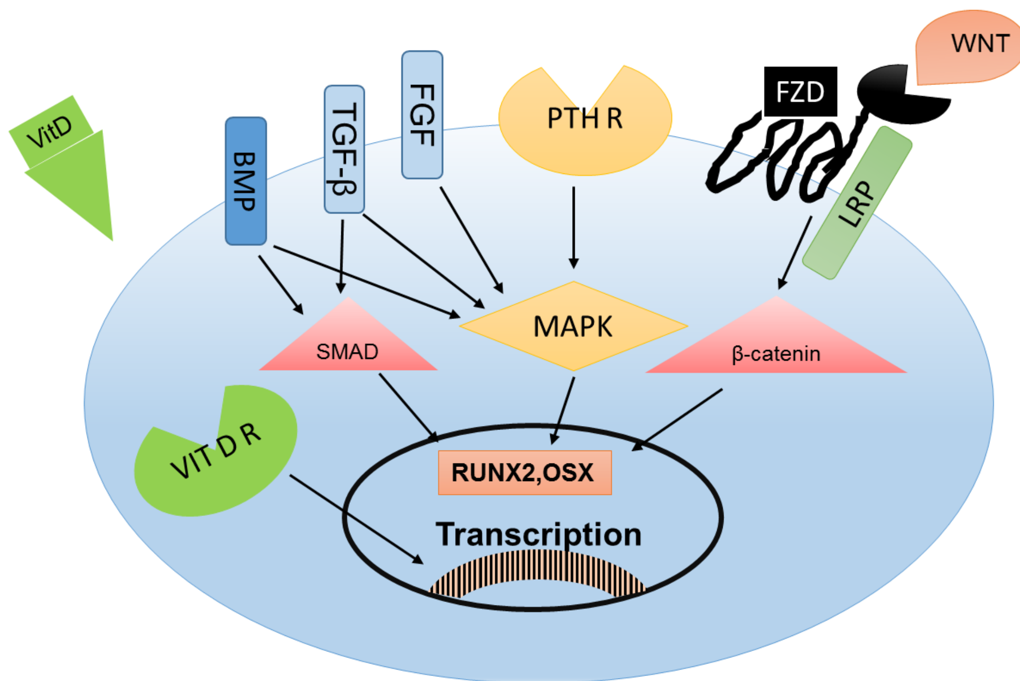

Figure 1.6 Key signalling pathways in Osteoblast differentiation. *The runt related transcription factor 2 (Runx2) together with Osterix (Osx) are essential factors for osteoblast differentiation. Pathways activating these factors involve: The stimulation of SMAD proteins by bone morphogenetic protein (BMP) and transforming growth factor β (TGF-β); activation of mitogen activated protein kinases (MAPK) through pathways shown above including fibroblast growth factor (FGF) and Parathyroid hormone (PTH); through WNT-Frizzled pathway which uses and associated low-density lipoprotein receptor related proteins (LRP) which uses β catenin signaling*

The main action of osteoblasts is the production of extracellular matrix. This includes Type 1 collagen, alkaline phosphatase and osteocalcin which, laid into resorption pits, is the starting point for newly formed bone (Katagiri and Takahashi 2002). Beside their anabolic role Osteoblasts also stimulate and regulate bone resorption through secretion of m-CSF, RANKL and OPG (Lorenzo et al. 2011).

1.2.3 Osteocytes

Osteocytes derive from the Osteoblast cell lineage and are the most abundant cells of bone. After a bone remodeling cycle osteoblasts transform either into bone-lining cells or osteocytes, encased in bone(Lorenzo et al. 2011) (Fig. 1.5).

For a long time osteocytes were thought to be physiologically inert. However more and more evidence shows the importance of osteocytes in bone metabolism. These cells not only monitor for mechanical stimulation, pressure changes and bone quality, they also orchestrate bone remodeling through secretion of crucial cytokines and proteins such as RANKL and Sclerostin. Further products are Fibroblast growth factor 23 (FGF23) and Dentin matrix acidic phosphoprotein 1 (DMP1), which are regulators of phosphate and mineralization metabolism. DMP1 also serves as marker of osteocyte viability and activity (Bonewald 2011;Lorenzo et al 2011).

1.2.4 Chondrocytes

Chondrocytes are the first skeletal cells to appear during embryogenesis. Chondrocytes are derived from mesenchymal stem cells and mature into cells able to secrete extracellular matrix. Chondrocytes produce cartilage which mainly consists of type II and type X collagen and proteins such as elastin and aggrecan (Karsenty and Wagner 2002). The crucial transcription factor for chondrocyte maturation is sex determining region Y (SRY)-box 9 (Sox9) (Bi et al. 1999). Sox9 promotes chondrocyte differentiation and expression of aggrecan and Type II collagen. Sox 5 and Sox6, also belonging to the HMG box class DNA binding proteins, bind to Sox 9 and enhance its function. All three Sox proteins also inhibit chondrocyte hypertrophy, which is the final stage of a chondrocyte life. In contrast, RUNX2, the crucial transcription factor for osteoblastogenesis, stimulates chondrocyte hypertrophy. These hypertrophied cells are able to express Vascular Endothelial Growth factor (VEGF), promoting blood vessel

development and hence attracting haematopetic and osteoprogenitor cells for bone formation and development (Karsenty & Wagner 2002).

1.3 Osteogenesis

Skeletal morphogenesis consists of two types of bone development, endochondral ossification and intramembranous ossification.

1.3.1 Endochondral ossification

Endochondral ossification is the differentiation of osteochondral progenitor cells into chondrocytes, which form a cartilage template that transforms into bone. A schematic illustration of endochondral ossification is shown in Figure 1.7. During embryonic development the initial stage for axial skeleton and long bones is hyaline cartilage. Mesenchymal stem cells differentiate into chondrocytes and bone formation initially occurs at the primary ossification centre where chondrocytes become hypertrophic and secrete matrix molecules that help recruiting bone cell precursors and angiogenic factors such as VEGF (Vascular Endothelial Growth Factor). These factors promote the invasion of blood vessels, bone cells and the apoptosis of chondrocytes. Recruited osteoclasts resorb cartilage matrix and osteoblasts initially form osteoid and woven bone, which undergoes repeated remodelling. Subsequently secondary ossification centres are formed which are separated from primary ossification sites by growth plates (epiphyseal plates). Growth plates consist of longitudinal columns of chondrocytes which are organized in distinct zones, separated according to their different functional state. Chondrocytes in growth plates undergo rapid proliferation and drive the expansion of primary and secondary ossification centres which allows bone to lengthen. Once bone growth is completed epiphyseal and metaphyseal bone fuse and transforms into finalized adult bone. Remaining chondrocytes at both ends of long bones form the articular cartilage (Mackie et al. 2008;Rosen CJ 2013).

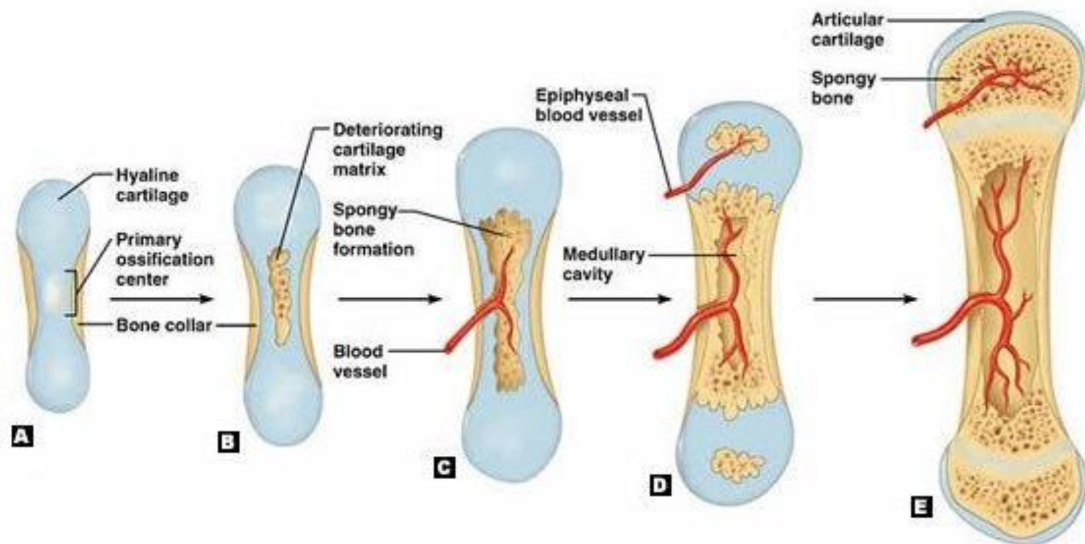

Figure 1.7 Schematic illustration of different stages of endochondral ossification *(adapted from: https://classconnection.s3.amazonaws.com/274/flashcards/1202274/jpg/2807224_dyn1329428793213.jpg)*

1.3.2 Intramembranous ossification

Flat bones which mainly derive from the neural crest are primarily formed by intramembranous ossification. Mesenchymal cells which are grouped in nodules differentiate in either osteoblasts or endothelial cell precursors. The presence of endothelial cells allows the formation of blood vessels whereas mature osteoblasts produce osteoid which gets mineralized into first bone. The bone formation occurs in a centrifugal way, which means ossification radiates out from a central point, leaving the proliferating and most immature cells at the periphery. Various bone spicules eventually fuse and form a disorganised mesh of bone matrix, which appears 'woven' under the light microscope. Woven bone undergoes repeated remodelling to finally transform into lamellar bone (Rosen CJ 2013).

1.3.3 Mineralization

Mineralization of the matrix strengthens the collagen composite, increases mechanical resistance and acts as source of calcium, phosphate and mineral homeostasis. In order to achieve a solid phase of hydroxyapatite, complex biochemical processes are involved. As the concentration of calcium and phosphate is too low in extracellular fluids to form apatite crystals, matrix vesicles are formed out of plasma membranes from chondrocytes and osteoblasts. Calcium ion channels and calcium binding lipids facilitate calcium influx into matrix vesicles, resulting in a high intra-vesicular Calcium concentration. Additionally various phosphatases cleave phosphoester substrates to increase inorganic phosphate (Pi) concentration within and outside matrix vesicles. The first stage of mineralization is driven by high concentrations of calcium and inorganic phosphate in matrix vesicles which are stored as hydroxyapatite. Hydroxyapatite crystals are then secreted into extracellular space and to cluster around matrix vesicles which initiates the second stage of mineralization. Various mineralization points fill space between collagen fibrils and amalgamate until the bone matrix is fully mineralized. The mineralization process is regulated by various factors including calcium and inorganic phosphate concentrations and mineralization inhibitors such as pyrophosphate, matrix gla protein and osteopontin. Various osteoblast derived matrix vesicle proteins regulate mineralization by influencing extracellular inorganic phosphate concentrations including , tissue non-specific alkaline phosphatase (TNAP), a nucleotide pyrophosphatase/phosphodiesterase isozyme (NPP1) and ANK gene product(Houston et al. 2004;Rosen CJ 2013). PHOSPHO-1,a phosphatase which cleaves phosphoethanolamine (PEA) and phosphocholine in order to generate inorganic phosphate has been shown to play an important role in initiating matrix mineralization. PHOSPHO-1 increases inorganic phosphate concentrations inside matrix vesicles where the first steps of mineralization occur. Inhibition of PHOSPHO-1 leads to significant decrease of calcification

of calvaria in mouse models (Houston, Stewart, & Farquharson 2004;Roberts et al. 2007).

1.4 Bone Remodeling Unit

Bone is metabolically active and is frequently renewed through a combination of bone resorption and bone formation. It has been estimated that every year 5-10% of bone in an adult skeleton is replaced by new bone. Bone remodeling occurs continuously in trabecular and cortical bone at microscopic small remodeling foci called basic multicellular units (BMU).The remodeling process allows adequate response to micro damage and different mechanical loading (Lorenzo et al. 2011). Bone Remodeling consists of bone resorption, reversal and formation as shown in Figure 1.8 and discussed below.

1.4.1 Bone resorption

Bone resorption is initiated by the digestion of a thin layer of non-mineralized matrix by enzymes (collagenase, MMPs and gelatinase) secreted by osteoblasts and bone lining cells (Suda et al. 1997). By-products from mineralized bone matrix such as TGF-β, type-I collagen and Osteocalcin are thought to attract quiescent osteoclast precursors (QuOPs) or mature osteoclasts to the site of micro-damage or mechanical stress (Rosen CJ 2013;Suda et al. 1997). Osteoclasts get activated in the presence of RANKL and M-CSF and bind to the bone surface through
adhesion receptors, such as vitronectin, fibronectin and type I collagen which are found on the exposed mineralised bone (Lakkakorpi et al. 1991). The resorption process by osteoclasts is described previously in detail in 1.2.1.2 subsection.

1.4.2 Cessation of resorption and reversal phase

The resorptive process has to be terminated in order to allow the formation of new bone. It is not entirely clear why osteoclasts stop resorbing bone and

what drives them into programmed cell death. I would like to mention a few theories about the initiation of apoptosis in Osteoclasts. Firstly we know that Osteoclasts phagocytise Osteocytes which have been shown to be the main producer of RANKL, the essential cytokine for Osteoclast activity and survival (Elmardi et al. 1990;Nakashima et al. 2011). Secondly, the amount on circulating Transforming growth factor- beta (TGF-β) increases with resorption of bone matrix and the cytokine gets activated by acidic PH. TGF-β has been shown to promote osteoclast apoptosis (Hughes and Boyce 1997).Lastly osteoclasts seem to auto-regulate themselves. It has been shown that RANKL induces the Interferon β (IFN β) gene in osteoclast precursors which introduces a negative feedback loop. Increased levels of IFN β further inhibit osteoclast differentiation by blocking RANKL induced c-Fos expression which is an essential transcription factor for osteoclastogenesis (Takayanagi et al. 2002).

After osteoclast apoptosis and termination of bone resorption mononuclear cells, mostly macrophage like cells, populate the resorption pits and remove organic residues. A thin layer of non-collagenous mineralized extracellular matrix- the cement line forms the boundary at which bone resorption terminates and new bone deposition starts (Everts et al. 2002;Lorenzo et al. 2011).

1.4.3 Bone formation

Normally bone resorption and bone formation are orchestrated in such way that there is a balance between the amount of bone removed during resorption and that replaced by formation. This coordinated process is called coupling. In order to initiate bone formation osteobalsts and their precursors have to be recruited to the site. Bone resorption releases chemo tactic factors such as Insulin like growth factor-I (IGF I) and TGF β which attract bone forming cells to the resorption pit. Subsequent differentiation and activation of Osteoblasts is again mediated by factors released through bone resorption such as IGF I and II, FGF, TGF β, BMP and Platelet derived growth factor (PDGF) (Baylink et al. 1993;Rosen CJ 2013).

Once Osteoblasts have arrived at the resorption lacuna and have differentiated into mature cells they start to build bone. Initially mature osteoblasts secrete type I collagen, Osteocalcin and Alkaline Phosphatase (ALP), which altogether forms unmineralized bone matrix or osteoid (Long 2012). ALP promotes matrix maturation and mineralisation by breaking down pyrophosphate,an inhibitor of the mineralisation. Furthermore Osteoblasts assist this process by secreting matrix vesicles containing Calcium, Phosphate and phospholipids which allows hydroxyapatite formation and hence mineralisation of osteoid. I've described the mineralization process in more detail in 1.3.3 subsection. The resulting end product is mineralized rigid bone (Katagiri & Takahashi 2002).

Bone formation is terminated by a multitude of mechanism. The secretion of Sclerostin (SOST) by Osteocytes certainly plays a crucial role. SOST acts on two maturation points of osteoblast formation. It inhibits the transition from pre-osteoblast into osteoblasts and promotes the differentiation from mature osteoblasts into bone lining cells (Sutherland et al. 2004). Beside bone lining cells, at the terminal stage a subset of osteoblasts gets encased in bone matrix and become osteocytes and the remaining cells will undergo apoptosis.

The completion of the bone formation leads to the resting phase with bone covered by quiescent bone lining cells until the next bone remodelling cycle starts (Long 2012).

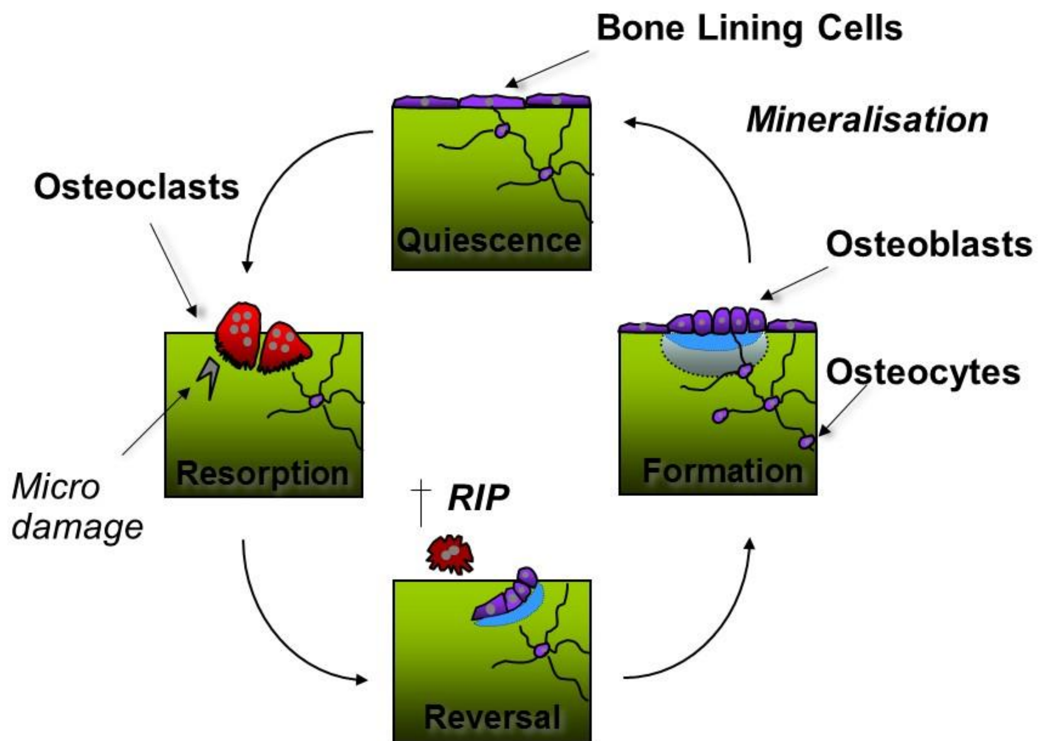

Figure 1.8. Bone Remodeling Unit *Microdamage induces Osteoclast activation in order to resorb the damaged bone. Osteoclasts are driven into apoptosis and osteoblasts are activated to initiate new bone formation. Osteocytes regulate and coordinate bone remodelling through regulation of osteoblasts and osteoclasts. Newly formed osteoid undergoes mineralisation in order to form rigid bone, which then will be covered by inactivated osteoblasts- the bone lining cells.*

1.5 OPG/RANKL/RANK system

1.5.1 RANKL

Observations during the early 80s noted that the presence of osteoblast or osteoblast-conditioned medium are essential for osteoclast differentiation. Fifteen years later Receptor activator of nuclear factor–κB ligand (RANKL) was identified as the key mediator for osteoclast proliferation and differentiation. It was first described as OPG ligand or osteoclast differentiation factor (ODF) (Lacey et al. 1998) and it was quickly established that this cytokine is identical to TRANCE (TNF-related activation induced cytokine) (Lacey et al. 2012;Wong et al. 1997) which we now know as RANKL.

RANKL belongs to the TNF-alpha receptor superfamily and occurs in two forms: the 40-45kDa cellular membrane bound which is expressed by osteocytes, preosteoblasts and stromal cells and the 31kDa soluble cytokine. Recent studies demonstrated that RANKL is expressed to a large extent by osteocytes which are chief regulators of osteoclast differentiation (Nakashima et al. 2011). However multiple other cells like osteoblasts, endothelial cells, mesenchymal cells, chondrocytes (Bonewald 2011) and importantly also activated T lymphocytes and immature CD4/CD8 have been found to produce RANKL (Kong et al. 1999a;Suda et al. 1997). RANKL activates the signaling receptor RANK and stimulates together with M-CSF osteoclast precursors into differentiation of mature osteoclasts with bone resorbing activity (Lorenzo et al. 2011). RANKL also acts on T-cells and activates c-Jun (JNK kinase) on T-cells, promotes t-cell proliferation and plays a key role in lymphocyte development and lymph node organogenesis (Kong et al. 1999b;Lorenzo et al. 2011). RANKL knock out mice have severe osteopetrosis, retardation of T- and B cell development, impaired tooth eruption and lymphogenesis and underdeveloped mammary glands (Dougall et al. 1999;Fata et al. 2000;Kong et al. 1999b).

Inflammatory mediators such as TNF α and IL1 can stimulate osteoclastogenesis either directly through a enhanced response of osteoclasts to RANKL and indirect by increased expression of RANKL on dendritic cells and T-cells (Lorenzo et al. 2011). More detailed explanation about the interaction of inflammatory cytokines and RANKL/OPG pathway is given in the section 1.7.

1.5.2 RANK

The receptor activator of nuclear factor-κB (RANK) is a 616-amino acid signal peptide with a N-terminal extracellular, short transmembrane and a large C-terminal cytoplasmic domain. RANK is a signalling receptor expressed on the surface of osteoclast progenitors, mature osteoclasts, chondrocytes, dendritic cells, endothelial cells, smooth muscle cells and T and B lymphocytes and various malignant cells (Hsu et al. 1999;Lacey, Boyle, Simonet et al. 2012; Min et al. 2003). In vivo studies confirm the crucial importance of RANK in bone homeostasis as RANK knock out mice develop severe osteopetrosis due to an absence of osteoclasts (Dougall et al. 1999).

Mutations in the signal peptide region of RANK protein can lead to increase of RANK translation products leading to increased RANK signaling. Clinical consequences of these mutations are seen in familial expansile osteolysis which is an autosomal dominant disorder leading to increased fracture risk secondary to focal areas of enhanced bone resorption and familial Paget's Disease of Bone which is characterized by areas of increased bone turnover which cause bone pain and increased fracture risk (Hocking et al. 2000) (Khosla 2001).

1.5.3 Osteoprotegerin

Osteoprotegerin (OPG) was the first protein from the TNF receptor superfamily to be identified as crucial player in osteoclastogenesis. Interestingly OPG was discovered almost simultaneously by two research groups, Amgen (Simonet et al. 1997) and the investigators at the Snow Brand Milk products who called their novel protein OCIF (Osteclast inhibitory

factor) (Yasuda et al. 1998). In contrast to most members of the TNF receptor superfamily OPG exists only in soluble form. The protein consists of 401 amino acids, one end of OPG contains a cysteine rich domain which is the ligand binding site, the other end has a carboxy terminal domain. The structure of OPG reminds of the "structure of decoy receptors which are encoded by certain virulence genes of poxviruses" (Khosla 2001;Lacey et al. 2012). OPG is expressed by osteoblasts and acts as soluble decoy receptor for RANKL. The high affinity binding between RANKL and OPG prevents the stimulation of the RANK receptor and therefore inhibits osteoclast differentiation. Hence OPG is a potent inhibitor of osteoclast maturation and activation (Simonet et al. 1997;Yasuda et al. 1998). OPG mRNA was found in multiple tissues including lungs, kidney heart, thyroid and brain though its role in these tissues remains largely unknown (Khosla 2001). OPG knock out mice develop early onset osteoporosis (Bucay et al. 1998) but also marked vascular calcification, which demonstrated functional activity of OPG outside bone. Transgenic mice overexpressing OPG develop severe osteopetrosis (Yasuda et al. 1998) .

In physiological conditions the ratio between RANKL and OPG is such that bone resorption and formation is balanced. Increased secretion or expression of RANKL leads to excessive bone resorption as seen in oestrogen deficiency (Eghbali-Fatourechi et al. 2003), through prolonged glucocorticoid administration (von Tirpitz et al. 2003); in malignancies such as multiple myeloma and bone metastases (Michigami et al. 2001); and in various inflammatory autoimmune diseases (Lorenzo et al. 2011). Further details about the role of OPG/RANKL system in rheumatic diseases are given below under the section "Mechanism of bone loss in rheumatic diseases".

The clinical importance of OPG/RANKL/RANK triad is demonstrated by the successful introduction of Denosumab a monoclonal Ab against RANKL as a treatment for osteoporosis (McClung et al. 2006). Denosumab is a licensed

Osteoporosis treatment in women with postmenopausal osteoporosis and in patients with metastatic cancer (Cummings et al. 2009;Lacey et al. 2012).

1.5.4 Osteoprotegerin autoantibodies

In 2009 Riches and colleagues (Riches et al. 2009) described a 40-year-old who presented with a low trauma fractures and severe osteoporosis (lumbar spine T score of -6.6). The patient was diagnosed with coeliac disease, hypothyroidism and severe high bone turnover osteoporosis. He was found to have markedly elevated bone turnover markers (Urinary deoxypyridinoline, Alkaline phosphatase) but serum osteoprotegerin and RANKL levels within normal range.

A bone biopsy (Fig 1.9) showed an increased number of osteoclasts and osteoblasts indicating high bone turnover. Despite the commencement of gluten free diet he continued to lose bone and sustained further low fragility fractures. The patient was investigated further and serum was obtained for experimental studies. Immunoprecipitation studies with the patient's serum found the precipitation of osteoprotegerin at 55 kD indicating the existence of antibodies to osteoprotegerin.

These newly identified antibodies were tested further in functional studies and they were shown to block the inhibiting effect of OPG on RANKL. These results strongly point towards the existence of functional OPG antibodies which allowed excessive bone resorption and caused severe Osteoporosis.

As biochemical and histological findings indicated high resorptive state the patient was treated with Zoledronic acid – a potent bisphosphonate inhibiting osteoclasts, which successfully restored his bone mineral density.

Further detectable autoantibodies to OPG were shown in three out of 15 patients with Coeliac Disease and low bone mineral density but none were detected in 14 patients with autoimmune hypothyroidism or 10 healthy controls.

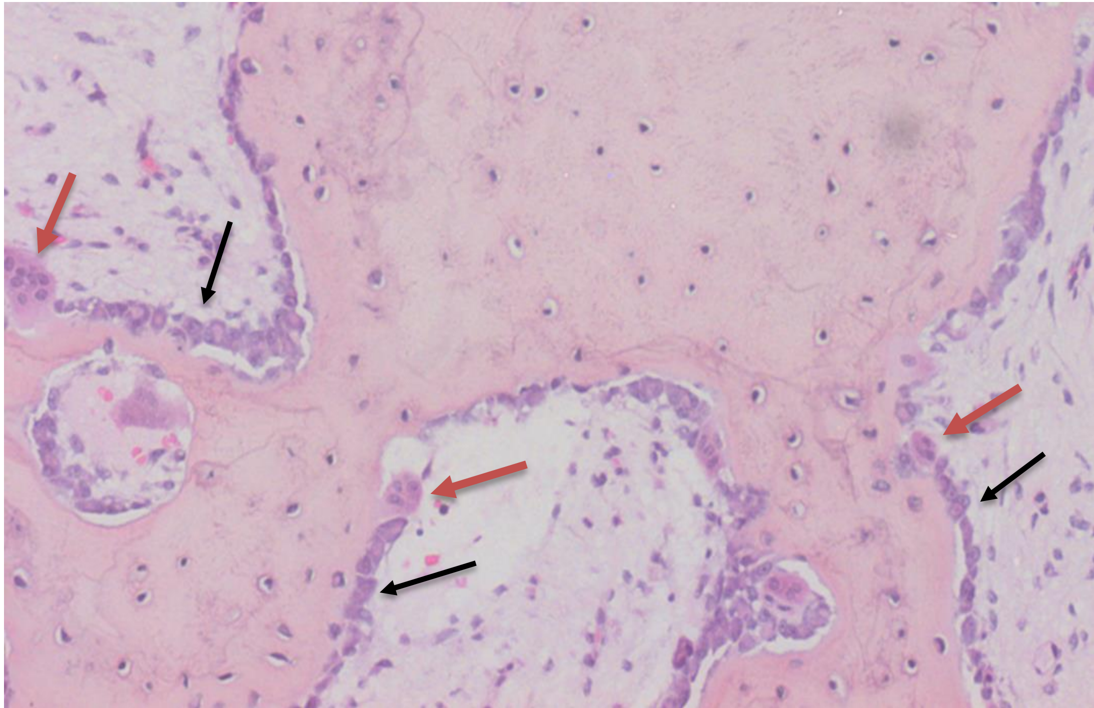

Figure 1.9 *(adapted from Riches et al Brief Report(Riches, McRorie, Fraser, Determann, van't Hof, & Ralston 2009a)) A photomicrograph of the bone-biopsy specimen, hematoxylin and eosin stained,shows an increase in the numbers of osteoclasts (red arrows) and osteoblasts (black arrows).*

A subsequent pilot study using the same immunoprecipitation assay as mentioned above was performed in order to detect OPG antibodies in other patients with autoimmune disease. The test population included 12 patients with celiac disease; 6 patients with RA and 5 patients with SLE .The study detected the presence of OPG autoantibodies in 3/15 patients with coeliac disease (20%) in 1/6 (16%) of patients with RA and 1/5 (20%) of patients with SLE (data not published). An independent Italian research group tested 30 patients with Coeliac Disease for the presence of OPG antibodies using the immunoprecipitation assay described by Riches et al.(Riches et al. 2009). None of the patients were found to have detectable antibodies and the authors concluded that OPG antibodies may be a rare phenomenon and not be of crucial importance in the pathogenesis of Osteoporosis (Larussa et al.

2012). This data, though derived from a preliminary underpowered study, demonstrated the clear need for further large-scale investigations.

1.6 Osteoporosis

1.6.1 Definition

The word Osteoporosis (osteo=bone poros=brittle, porous) derives from Greek and means "porous" bone. Osteoporosis is defined by low bone mass, deterioration of microarchitectural bone structure and cortical porosity (Fig 1.10) leading to increased risk of fragility fractures particularly at hip, spine and lower forearm (Peck et al. 1993).

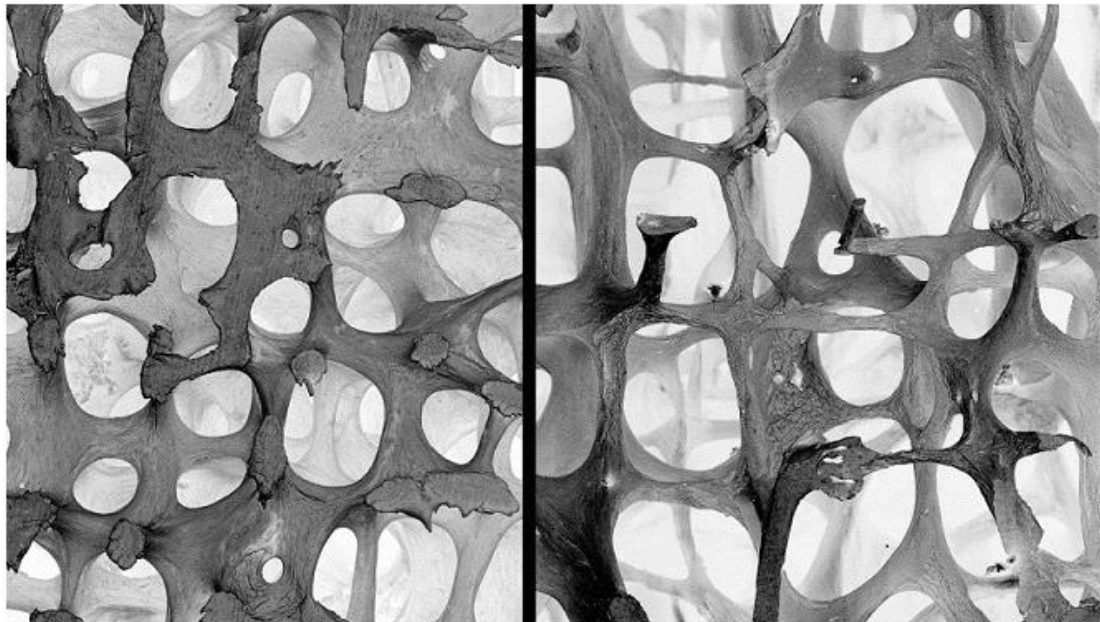

Figure 1.10 Image courtesy of Tim Arnett, University College London
The figure shows microscopic view of the 3rd vertebrae. The left panel shows normal trabecular bone , the panel on the right shows osteoporotic bone with thinning of the trabecular structure and pitting of the bone surface as a result of increased resorptive activity

Patients who sustain fractures, frequently require hospital admissions, surgical intervention and in some cases prolonged periods of rehabilitation. Fractures reduce quality of life and can cause serious disability. Hip fractures are particularly devastating as one fifth of elderly individuals who sustain a hip fracture will die the following year (Center et al. 1999) and 50% will not regain their previous level of mobility (Osnes et al. 2004). Individuals who sustain a hip fracture are at higher risk to sustain another fracture, have decreased quality of life including physical and mental health and are twice more likely to need residential care than individuals without fractures (Brenneman et al. 2006;Osnes et al. 2004;Sambrook & Cooper 2006).

In contrast to hip fractures, vertebral fractures remain frequently undiagnosed as only about a third of patients who sustain a vertebral fracture will seek medical attention (Cooper et al. 1991;Cooper et al. 1992). The majority of vertebral fractures occur by simple bending or lifting up light objects and only a quarter of vertebral fractures are a result from a fall. Nevertheless vertebral fractures can have a severe impact on quality of life due to pain, decreased mobility and increased thoracic kyphosis(Sambrook & Cooper 2006).

Having sustained a vertebral fracture increases the risk of a further vertebral fracture by 10 times (Lindsay et al. 2001;Melton et al. 1999) and non-vertebral fractures (Black et al. 1999;Melton et al. 1999) significantly. Most patients who have suffered vertebral fractures are therefore considered to have osteoporosis and treatment of osteoporosis, even in the absence of osteoporotic BMD as defined by DXA, should be considered.(Scottish Intercollegiate Guidelines Network (SIGN) publication no.142 2015).

Beside mortality and morbidity osteoporosis related fractures have a huge economic impact with an estimated cost of £4.3 billion per year in the UK (Holroyd et al. 2008;Svedbom et al. 2013) .

1.6.2 Pathogenesis of Osteoporosis

Pathogenesis of Osteoporosis is multifactorial. Increased skeletal fragility is frequently caused through a combination of the following factors: a) reduced bone formation and growth through childhood and adolescence which results in a skeleton with reduced peak bone mass (Sambrook & Cooper 2006) b)increased bone resorption leading to accelerated bone loss and c)inadequate bone formation in response to bone resorption which at a cellularlevel means imbalance between osteoclast and osteoblast activity (Raisz 2005). Bone mass usually peaks at the age between 20 and 45 and is influenced by gender, genetic factors, exercise, Calcium and Vitamin D intake, and environmental factors during intrauterine life(Sambrook & Cooper 2006).

The most frequent form of Osteoporosis is postmenopausal Osteoporosis which is to a large extent is caused by oestrogen deficiency after the menopause. A significant drop of oestrogen levels during the menopause, increases the secretion of pro-inflammatory cytokines such as IL1 and TNF, which subsequently promote RANKL production and enhance osteoclastogenesis. Oestrogen deficiency also drives osteoblast apoptosis and hence reduces bone formation. (Eghbali-Fatourechi, Khosla et al. 2003;Sambrook & Cooper 2006). Oestrogen has also been shown to be important in maintaining bone health in men as reduced oestrogen levels are associated with accelerated bone loss in men and animal models demonstrate that male mice lacking oestrogen receptor alpha (ERα) loose cortical bone excessively (Almeida et al. 2013;Khosla et al. 2011).

Another important factor for development of osteoporosis is old age. Age related bone loss starts gradually after the bone mass has peaked at 20-30 years of age. The rate of bone loss increases exponentially at around 65 years as bone resorption surpasses bone formation. Besides its effect on bone, age is the strongest risk factor for low fragility fractures and that is largely independent of changes in BMD (Delaet et al. 1997;Khosla, Melton, &

Riggs 2011). Other factors attributable to old age contribute to fracture risk such as reduced muscle strength and proprioception and reduced visual acuity. Other important factor in the pathogenesis of osteoporosis, which were traditionally classified as causes for secondary osteoporosis are summarised in Table 1.1.

For most of these conditions the current SIGN recommendation is a DXA assessment for patients over 50 in particular for patients with additional risk factors (Scottish Intercollegiate Guidelines Network (SIGN) publication no.142 2015).

Secondary causes of osteoporosis	
Rheumatological	Rheumatoic Diseases as outlined in chapter 1.7
Gastrointestinal	Chron's Disease, Ulcerative Colitis, Celiac Disease, steatorrhoea, blind loop syndrome
Drugs	Glucocorticoids, Anticonvulsants, Antidepressants, GnRH agonists, Medroxyprogesteron acetate
Endocrine Disorders	Hyperthyroidism, Hyperparathyroidism, Cushing's Syndrome, Type I +II Diabetes, Hypercalciuria, use of HRT
Respiratory	COPD, Asthma
Collagen defects	Marfan Syndrome and Ehlers Danlos, Osteogenesis imperfecta
Metabolic diseases	Chronic renal disease, ckronic liver disease, Homocystinuria, Gaucher's Disease and hereditary Haemochromatosis
Infections	Chronic Osteomyelitis, HIV infection
Other	Care or nursing home resident, Alcoholism, Vitamin D and Calcium deficiency

Table 1.1 Secondary causes of osteoporosis in premenopausal women and men *(adapted from uptodate website (www.uptodate.com) and Sign 142 guidelines: Management of Osteoporosis and prevention of fragility fractures(Scottish Intercollegiate Guidelines Network (SIGN) publication no.142 2015))*

1.6.3 Diagnosis of osteoporosis and fracture assessment

The most commonly used method to diagnose osteoporosis is the assessment of bone mineral density (BMD) with dual-emission Xray absorptiometry (DEXA).The results are interpreted according to the World Health Organisation (WHO) guidelines. Osteoporosis is defined as a BMD more than 2.5 Standard Deviation (SD) below the mean of a young adult reference and BMD more than 1 SD below the young adult mean has been classified as Osteopenia(Sambrook & Cooper 2006a) (Kanis et al. 1994). BMD is an important information for fracture risk assessment although there are several BMD independent risk factors, which need to be considered when diagnosing osteoporosis and decisions about treatment being made. Age is by far the strongest risk factor for fractures. Recently a Danish study(Rubin et al. 2013) showed that age alone is as good as other tools such as OST(Richy et al. 2004), FRAX (Kanis et al. 2008), and ORAI score (Cadarette et al. 2000) in predicting fracture risk.

1.6.3.1 FRAX

The World Health Organisation developed FRAX, a fracture prediction tool (Kanis et al.1994;Kanis et al. 2008) which is a freely available web-based tool (www.shef.ac.uk/FRAX/index.aspx), shown in Figure 1.11.

FRAX encompasses risk factors such as low body mass index (BMI), history of fracture, glucocorticoid use, family history of fracture, cigarette smoking, excessive alcohol intake and the presence of conditions, which predispose to secondary osteoporosis. FRAX calculates through multivariate analysis a 10-year hip and major osteoporotic fracture probability. In contrast to other tools FRAX gives the option to include Femoral Neck BMD into the fracture risk calculation which is undoubtedly valuable information to add to fracture risk prediction. Furthermore FRAX gives also treatment threshold recommendations based on the fracture probability and health economic calculations (Kanis et al. 2008;Sambrook & Cooper 2006). This approach results in relative underestimation of fractures compared to other tools such as QFracture and Garvan tool. Other points of criticism are the limited option

of dichotomous data entry for various risk factors and the secrecy around the algorithm used which limits external validation attempts (Scottish Intercollegiate Guidelines Network (SIGN) publication no.142 2015).

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Figure 1.11 Screenshot of FRAX tool (www.shef.ac.uk/FRAX)

1.6.3.2 QFracture

Another frequently used fracture risk prediction tool in the UK is QFracture (http://www.qfracture.org/index.php). QFracture is a tool developed on data from GP databases from England and Wales. The initial online risk scoring tool was based on 2.2 million of patients and underwent external validation by the use of data from another 2.2 million individuals. Q fracture allows prediction of hip and other major osteoporotic fractures, which include fractures of spine, wrist, hip and shoulders. A comparison of the two most frequently used fracture risk assessment tools (FRAX and QFracture) is

shown in Table 1.2(Scottish Intercollegiate Guidelines Network (SIGN) publication no.142 2015).

The benefits of QFracture over FRAX are the fact that QFracture has been extensively validated in the UK, it can be applied to a wider age group (from 30-100 years), it allows risk prediction from 1-10 years, and the algorithm used is freely available so that further validation studies can easily be done.Clearly the weakness is that it doesn't take BMD in account which is one of the single strongest fracture risk predictors. Therefore QFracture assessment should be performed prior to DXA scan assessment and may help to decide if DXA assessment is necessary or not(Scottish Intercollegiate Guidelines Network (SIGN) publication no.142 2015). A comparison of included risk factors considered by FRAX and Q-fracture is shown in Table1.2.

1.6.3.3 Other fracture risk tools

Garvan fracture risk prediction tool (http://www.garvan.org.au/promotions/bone-fracture-risk/calculator/) is a simpler tool taking only age, gender, fall and fracture history and BMD into account. Another tool based on age sex and femoral neck BMD is CAROC (Canadian Association of Radiologists and Osteoporosis Canada). A few studies have outlined that simpler tools such as Garvan have similar discriminative ability for hip and osteoporotic fractures compared to more complex tools such as FRAX (Bolland et al. 2011) and QFracture (Bolland et al. 2013). In summary, there is no consensus as to which fracture risk prediction tool to use in every day practice. These tools depend on availability of data, evidence of regional validation and individual preference.

1.6.4 Bone turnover markers

There are also several biochemical markers of bone turnover available, mostly useful for prediction and monitoring treatment response and investigating pathogenetic mechanisms of osteoporosis. To some extent these markers are also useful in fracture prediction (Sambrook & Cooper 2006).The bone resorption markers, urinary C-telopeptide, cross-links of collagen type I (CTX) and free urinary deoxypyridinoline have been shown to predict fractures independently of BMD as has the bone formation marker undercarboxylated osteocalcin (Garnero et al. 1996;Szulc et al. 1993).

Risk factor	**FRAX**	**QFracture**
Age	√	√
Sex	√	√
Weight	√	√
Height	√	√
Ethnicity	x	√
Previous fracture	√	√
Parental history of hip fracture	√	√
Smoking	√	√
Alcohol	√	√
Menopausal symptoms	x	√
Epilepsy (or use of anticonvulsants)	x	√
Cardiovascular disease	x	√
History of falls	x	√
Use of glucocorticoids	√	√
Use of antidepressants	x	√
Bone mineral density (femoral neck T-score)	√ (optional)	
Secondary osteoporosis	Binary yes/no choice	Divided into multiple secondary causes of osteoporosis: *Endocrine* *Gastrointestinal* *Metabolic* *Neurological* *Oncological* *Respiratory* *Rheumatological* *Other*

Table 1.2 Risk factors included in FRAX and QFracture algorithms (adapted from 142 SIGN guidelines: *Management of Osteoporosis and prevention of fragility fractures(Scottish Intercollegiate Guidelines Network (SIGN) publication no.142 2015)*)

1.7 Mechanism of bone loss in inflammatory arthritis

1.7.1 Introduction

As mentioned previously bone undergoes continuous remodeling and restructuring in order to maintain its strength and function. In healthy individuals, a precisely coordinated process of bone resorption and formation allows the repair of damaged bone and the rejuvenation of old bone. In patients with inflammatory rheumatic diseases the balance between bone resorption and bone formation gets disrupted. Rheumatoid Arthritis (RA), Ankylosing Spondylitis (AS), Psoriatic Arthritis (PsA) and Systemic Lupus Erythematosus (SLE) present clinically distinctively different. Rheumatoid Arthritis is characterized by chronic inflammation with a predilection for the small joints of hands and feet which frequently leads to localized bone loss in form of bone erosions and cartilage and joint destruction. Ankylosing Spondylitis is a Spondyloarthropathy and is characterized by chronic inflammation of the spine presenting in form of spinal pain and stiffness. The inflammatory process in AS promotes localized increased bone turnover resulting in extra bone formation in form of syndesmophytes, which leads to fusion of vertebral bodies and increased rigidity of the spine. Psoriatic Arthritis also belongs to the seronegative spondyloarthropathies and one of its manifestations (arthritis mutilans) can lead to localized osteolysis and severe localized bone destruction. Systemic bone loss in Psoriatic Arthritis however does not seem to differ greatly from the general population, although there is too little data available to draw definite conclusions. Although localized bone involvement in Systemic lupus erythematosus is not prominent, generalized bone loss in form of osteoporosis and increased fracture risk is a substantial burden of SLE. In view of the variety of clinical manifestations and bone involvement I would like to summarize the mechanisms of bone pathology in rheumatic diseases and highlight the

differences and common features of RA, AS, PsA, SLE and other rarer Rheumatic Diseases

1.7.2 Rheumatoid Arthritis

Rheumatoid Arthritis (RA) is a common inflammatory arthritis affecting about 1% of the population. RA is characterised by chronic synovial inflammation of small and midsized joints, which is associated with chronic pain, stiffness and functional impairment. The natural history of the disease is progressive articular damage and functional disability. Systemic complications such as extra-articular manifestations of the cardiopulmonary and nervous system reduce life expectancy of RA sufferers by 7 years.
The cause of RA remains uncertain. The aetiology is thought to be an interplay between genetic predisposition (HLA-DR allele subtypes), immunological dysregulation and environmental factors (Burmester G et al. 2012).

1.7.2.1 Osteoporosis risk

Amongst rheumatic diseases, the largest pool of evidence for accelerated systemic bone loss lies with Rheumatoid Arthritis (Roux 2011). Postmenopausal women with RA are at twofold risk of developing osteoporosis compared to healthy controls (Haugeberg et al. 2000a;Hauser et al. 2014) and men over 45 with RA have significantly reduced Hip BMD compared to healthy controls (Haugeberg et al. 2000b;Hauser et al 2014). Studies that analysed the data from Oslo registrar more than a decade ago detected that the overall prevalence of hip osteoporosis in pre- and postmenopausal women with RA was around 15%. Interestingly this figure has not changed greatly over the last 15 years despite the changes in RA assessment and treatment (Haugeberg et al 2000a;Hauser et al. 2014;Smolen et al. 2010). Disease-specific characteristics which were identified most commonly as risk factors are immobility and high Health Assessment Questionnaire (HAQ) score – a functional disability score,

inflammation, glucocorticoid use and Rheumatoid Factor (RF) seropositivity (Haugeberg et al. 2000a;Roux 2011). The impact and mechanism of these risk factors are discussed below.

Although generalized bone loss is a well-recognized complication of RA, there are currently no guidelines or recommendations as to when RA patients should be screened for osteoporosis. In order to better target DXA screening of patients with RA prediction tools have been developed (Haugeberg et al. 2002a;LEMS and DIJKMANS 1998). These tools are using largely arbitrarily chosen variables including age, weight, inflammation, immobility and steroid use. Our group has recently developed a simple tool (Osteoporosis Prediction in Rheumatoid Arthritis- OPRA) based on age, gender and BMI. It detects most patients at risk of osteoporosis (91.4% sensitivity) but at a cost of relatively low specificity (38.2%).The development of this osteoporosis risk prediction tool is shown in chapter 3.

1.7.2.2 Fracture risk

Large case control studies have shown that RA patients have an increased fracture risk, which is exacerbated by the use of corticosteroids (van Staa et al. 2006). A recent study showed that particularly young women with RA have an elevated fracture risk (OR 4.3 (95 CI %, 2.4–7.8) when compared to healthy controls. This is unlikely a reflection of low BMD as for individuals under 45 years of age there is little difference between BMD in RA and the healthy population.(Hauser et al 2014). Rather, this points more towards other risk factors of fractures such as decreased bone strength due to decreased bone mineral content, altered bone geometry or mass distribution, sarcopenia and functional disability.

Amongst the fracture risk assessment tools FRAX (http://www.shef.ac.uk/FRAX) and Q-fracture (http://www.qfracture.org/index.php) have incorporated RA as general risk factor. FRAX has several limitations as it doesn't take into account vertebral fracture history, cumulative glucocorticoid dose or functional disability (Hoes

et al. 2015). Q-fracture on the other hand includes history of falls and vertebral fractures but does not take into account BMD, a measure that has a strong association with fracture risk. Although we know that the impact of reduced BMD changes significantly with age, ultimately the diagnosis and treatment of OP are largely based on BMD result (Sambrook & Cooper 2006). There is currently no consensus as to which fracture risk assessment tool is the most appropriate for patients with Rheumatoid Arthritis. Further external validation of different tools is required.

1.7.2.3 Pathogenesis

RA is characterized by joint swelling in form of synovial inflammation and cartilage and bone destruction leading to erosions, joint deformity and generalised osteopenia. A summary of cell types and mediators involved in the inflammatory process and their interaction with osteoclasts is shown in Figure 1.12.

Inflammation of the synovium promotes localized production of pro-inflammatory cytokines, attracting lymphocytes and macrophages to the synovial lining and stimulating endothelial cells in synovial microvessels. The inflammatory infiltrate stimulates fibroblast activity leading to synovial hypertrophy and pannus formation (Braun and Sieper 2007;McGonagle et al. 2007;McInnes and Schett 2011). The presence of myeloid cells and plasmacytoid dendritic cells that express HLA class II molecules, pro-inflammatory cytokines and their interaction with T cell and B cell propagate chronic inflammation. It also promotes the differentiation of myeloid cells into macrophages and osteoclasts which have the potential to destroy tissue through the release of matrix degrading enzymes.

Amongst the T cell subsets Th1 and Th2 cells have been shown to suppress osteoclastogenesis through the expression of cytokines which inhibit osteoclastogenesis (IFN-γ and IL-4) (Takayanagi et al. 2000;Takayanagi 2007). On the other hand T-helper 17 (Th17) cells were found to be of crucial importance in the pathogenesis of inflammatory arthritis (McInnes & Schett 2011) as they were found to be the exclusive T-cell subset (Sato et al.

2006), which promotes osteoclast activation and differentiation. TH17 cells promote osteoclastogenesis indirectly through IL17 production, which stimulates the secretion of pro-osteoclastogenic cytokines as described below (Sato et al. 2006). Experiments in murine models showed that blockade of IL17A results in reduced localized and systemic bone loss (Zwerina et al. 2012). Surprisingly clinical studies did not find an increase of TH17 circulating cells in RA patients compared to controls (Yamada et al. 2008).

Further T cell subset of interest are regulatory T cells (T reg) expressing cytotoxic T-lymphocyte antigen– 4 (CTLA-4). Despite their capacity to express RANKL the presence of T reg is associated with potent osteoclast suppression. Zaiss et al. (Zaiss et al. 2007) recognized that the suppression is dependent on cell to cell contact and is largely a result of interaction of CTLA-4 with CD80/CD86 on osteoclast precursors and is independent of RANKL/OPG pathway. CTLA-4 was also shown to supress RANKL – and TNF mediated osteoclastogenesis independently of T cells (Axmann et al. 2008). Recent findings provided more insight into the molecular mechanism of CTLA-4 influencing bone resorbing cells. The engagement of CD80 and 86 activates the enzyme indoleamine 2,3- dioxygenase which triggers tryptophan degradation promoting osteoclast precursor apoptosis(Bozec et al. 2014).Furthermore Komatsu et al found that Forkhead box protein P3 (FOXP3) is a transcription factor of T reg cells and is essential for their osteoclast suppressive function . In mouse models with inflammatory arthritis, T reg cells were found to have reduced or absent FoxP3 expression which allowed a transformation of T reg cells into Th17 cells, a process which exacerbates inflammation and boosts osteoclastogenesis (Komatsu et al. 2014).

Abatacept (Orencia; Bristol-Myers Squibb), a cytotoxic t-lymphocyte protein-4 Immunoglobulin (CTLA4–Ig) is a chimeric protein that acts as T-cell co-stimulation modulator. Abatacept is a licensed biologic therapeutic in treatment resistant RA. Clearly, the outcome of clinical studies with

Abatacept is of great interest to the bone field. Abatacept was shown to suppress the development of erosions and structural damage in RA potently (Genant et al. 2008;Westhovens et al. 2009) and to reduce bone loss in a murine model of hyperparathyroidism (Bedi et al. 2010). However to date there's no data of the effect of Abatacept on systemic bone loss in humans but a study looking into Abatacept and its effect on BMD is ongoing (Coy et al. 2014).

The clinical success of B-cell depletion in RA in preventing erosions and joint destruction provides strong evidence for the important role of B-cells in RA pathophysiology (Tak et al. 2011). In the field of osteoimmunology it is occasionally difficult to distinguish the immunosuppressive effects from bone protective properties of a drug. However B lymphocytes express RANKL and it has been shown that ovariectomy in mice induces the proliferation of RANKL expressing B cells (Kanematsu et al. 2000). Onal et al. (Onal et al. 2012) showed that ovariectomized mice lacking B cell mediated RANKL expression had reduced bone loss in comparison to T-cell dependent RANKL knock out mice with unchanged bone loss. Another mechanism why anti-CD20 therapy successfully inhibits bone damage might be the reduction of Immunoglobulin producing plasma cells. Immunoglobulins have recently been shown to directly interact with bone cells in specific with osteoclasts (Harre et al. 2012;Hauser et al. 2015) and I will elaborate on autoantibody – mediated bone loss in following section. Interestingly activated B cells have also been shown to produce Osteoprotegerin and µMT heavy chain-deficient mice which lack mature B-cells have been found to be osteoporotic (cortical bone) and deficient in OPG. These findings could be reversed by B –cell reconstitution (Li et al. 2007).

1.7.2.4 Mediators of bone loss in RA

1.7.2.4.1 RANKL

RANKL is together with M-CSF an essential cytokine for osteoclastogenesis and has been found in abundance in the hypertrophied synovium. The ability of synovial fibroblasts to produce RANKL is thought to be one of the crucial factors in the localized increase of osteoclastogenesis (Lorenzo et al.2011). Although Osteocytes were recently identified as the prime source of RANKL in the bone microenvironment (Nakashima et al. 2011) many other cell types have the ability to produce RANKL. In rheumatic diseases inflammatory cells (Kong et al. 1999a) in particular TH 17 cells (Sato et al.2006) are further sources for RANKL production. Inflammatory cytokines such as IL1, TNF-alpha and PGE2 upregulate RANKL production in osteoblasts and synovial fibroblasts and promote differentiation of OC precursor cells (Lorenzo et al. 2011). The crucial role of RANKL or OPGL for the development of osteopenia and bone destruction was demonstrated by Kong et al. (Kong et al. 1999a) who showed that RANKL knock out in a T cell dependent arthritis model in rats prevents bone erosions and systemic bone loss but does not impact on inflammation. The same group also showed that the destructive effect of T-cell mediated osteoclastogenesis can be attenuated by RANKL blockade through OPG. Further murine models with T cell independent arthritis and RANKL knock out demonstrated that animals lacking RANKL do not form osteoclasts and have a severe osteopetrotic phenotype. Similar findings were made in humans who lack RANKL due to a mutation at TNFSF11, the gene encoding RANKL. These individuals develop severe osteoporosis due to lack of osteoclasts (Sobacchi et al. 2007) (Pettit et al. 2001). Cohen et al showed that blockade of RANKL with Denosumab not only reduces systemic bone loss but also attenuates the formation of peri-articular erosions, which is in line with previous findings of RANKL blockade in animal models (Cohen et al. 2008;Romas et al. 2002).

1.7.2.4.2 Pro-inflammatory cytokines

The inverse relationship between inflammatory markers and bone mineral density (Gough et al. 1994b) and bone resorption markers (Gough et al. 1994b) in patients with RA shows that inflammation is a crucial driver of accelerated bone loss and even in a general population a small increase in CRP is associated with significantly increased fracture risk (Schett et al. 2006).

Early aggressive control of inflammation and immunosuppression in patients with RA does not only prevent bone erosions but is also thought to improve general bone health as demonstrated by reduced bone resorption markers and stable hip and spine bone density measures in RA patients who were tightly controlled and treated with anti-TNF early on in the disease(Guler-Yuksel et al. 2009).

IL1

The IL1 superfamily includes IL1α, IL1β, IL18 and IL33. In RA IL1α and IL1β are expressed in abundance in the synovial membrane by synoviocytes, monocytes and macrophages, B cells and chondrocytes (McInnes and Schett 2007). IL1 promotes osteoclastogenesis as it induces RANKL and RANK expression in mesenchymal cells and promotes the differentiation of pre-osteoclasts into mature osteoclasts. Although IL1 has been shown to be a crucial cytokine for arthritis related bone loss in animal models IL1 receptor blockade in humans with Rheumatoid Arthritis has been disappointing and is currently not used as RA treatment (Schett & Gravallese 2012).

IL6

In contrast to IL1, IL6 blockade has been shown to be an effective strategy to counteract inflammation and the development of bone erosions in patients with RA (Smolen et al. 2008). IL6 is over-expressed in the inflamed synovium of RA patients and increased concentrations of IL6 are found in serum and

synovial fluid of these patients. Many inflammatory cells including macrophages, osteoblasts and T-cells have the ability to express IL6 (Holt et al. 1996;Schett & Gravallese 2012). There is also data demonstrating that osteoclast like cell cultures which derive from pagetic bone produce substantial quantities of IL6, attributing an autocrine function to IL6 (Hoyland et al. 1994) . However we don't know about the role of osteoclasts in IL6 production in rheumatic diseases. Interestingly the addition of IL6 to murine and human osteoclast cell cultures inhibits osteoclastogenesis (Duplomb et al. 2008) however in the setting of inflammatory arthritis IL6 is thought to have a pro-osteoclastogenic effect mediated by increased RANKL production by osteoblasts and by direct stimulation of osteoclast precursors through gp130 signalling (Schett & Gravallese 2012). The use of Tocilizumab over a 1 year period in a RA cohort was also associated with a mild increase of BMD in patients with underlying osteopenia (Kume et al. 2014).

TNF

TNF is expressed by a variety of cells involved in the inflammatory cascade in RA including monocytes, macrophages and mast cells, T and B cells, synovial fibroblasts and osteoblasts (McInnes & Schett 2007).

TNF also enhances osteoclastogenesis by promoting RANKL expression and by inducing expression of receptors on the surface of monocytes and osteoclast precursors that are important co-stimulatory signals. Herman et al found on peripheral blood monocytes of RA patients increased expression of OSCAR, which is a member of the leucocyte receptor complex and is associated to ITAM harbouring molecules FcyR and DAP12. Stimulation of OSCAR is an important co-stimulatory signal cytokine independent of OC differentiation. TNF-alpha was found to increase the expression of OSCAR in CD14 + RA monocytes markedly. (Herman et al. 2008). TNF also suppresses bone formation by increasing Dickkopf-1 and Sclerostin expression, which are potent inhibitors of the Wnt signalling pathways and hence suppress osteoblast formation(Diarra et al.2007;Vincent et al. 2009)

The introduction of biological treatment which targets TNF has revolutionized the field of Rheumatology not only because of its rapid and potent suppression of inflammation but also its ability to suppress localized and systemic bone loss efficiently. Biologically engineered antibodies blocking TNF-α (Haugeberg et al. 2009;Lipsky et al. 2000;Vis et al. 2006) were shown to reduce accelerated bone loss and the formation of bone erosions, bringing joint damage to almost complete halt which boosts optimism to be able "to cure RA" in future.

IL17

An example for indirect stimulation of osteoclastogenesis is the lymphokine IL17, a product of mast cells and Th17 cells, which are found in abundance in the inflamed synovium. IL17 potently induces RANKL expression in synovial fibroblasts and osteoblasts and stimulates innate immune cells to express IL1 and TNF, as described above (Takayanagi 2009). Anti IL17 blockade not only suppress inflammation and the expression of pro-inflammatory cytokines but also shift the T- cell balance towards Th1 and Th2 cells which express anti-osteoclastogenic factors, such as IL4 and IFN-γ (Schett & Gravallese 2012). Despite IL17 being a good theoretical target in RA a randomized placebo controlled phase II trial showed that IL17 receptor blockade in RA patients does not improve disease activity significantly compared to placebo (Pavelka et al. 2015). Similarly Secukinumab, a fully human monoclonal anti IL17A antibody (Secukinumab) failed to reach the primary endpoint of ACR20 disease activity reduction (Genovese et al. 2013). Because of the disappointing results in reducing disease activity in RA long term effects of IL17 inhibition on bone in RA may not become available.

Dkk-1

Dickkopf related protein-1 (Dkk-1) is expressed in the synovium in patients with RA and its expression is enhanced by TNF. Dkk-1 is a potent suppressor of Wnt pathway and inhibits bone formation and therefore maybe also the repair of bone damage i.e. erosions. Although the idea of bone

repair is very interesting and important to date it is unclear whether antagonizing wnt path inhibitors would assist the repair of bone erosions (Schett & Gravallese 2012).

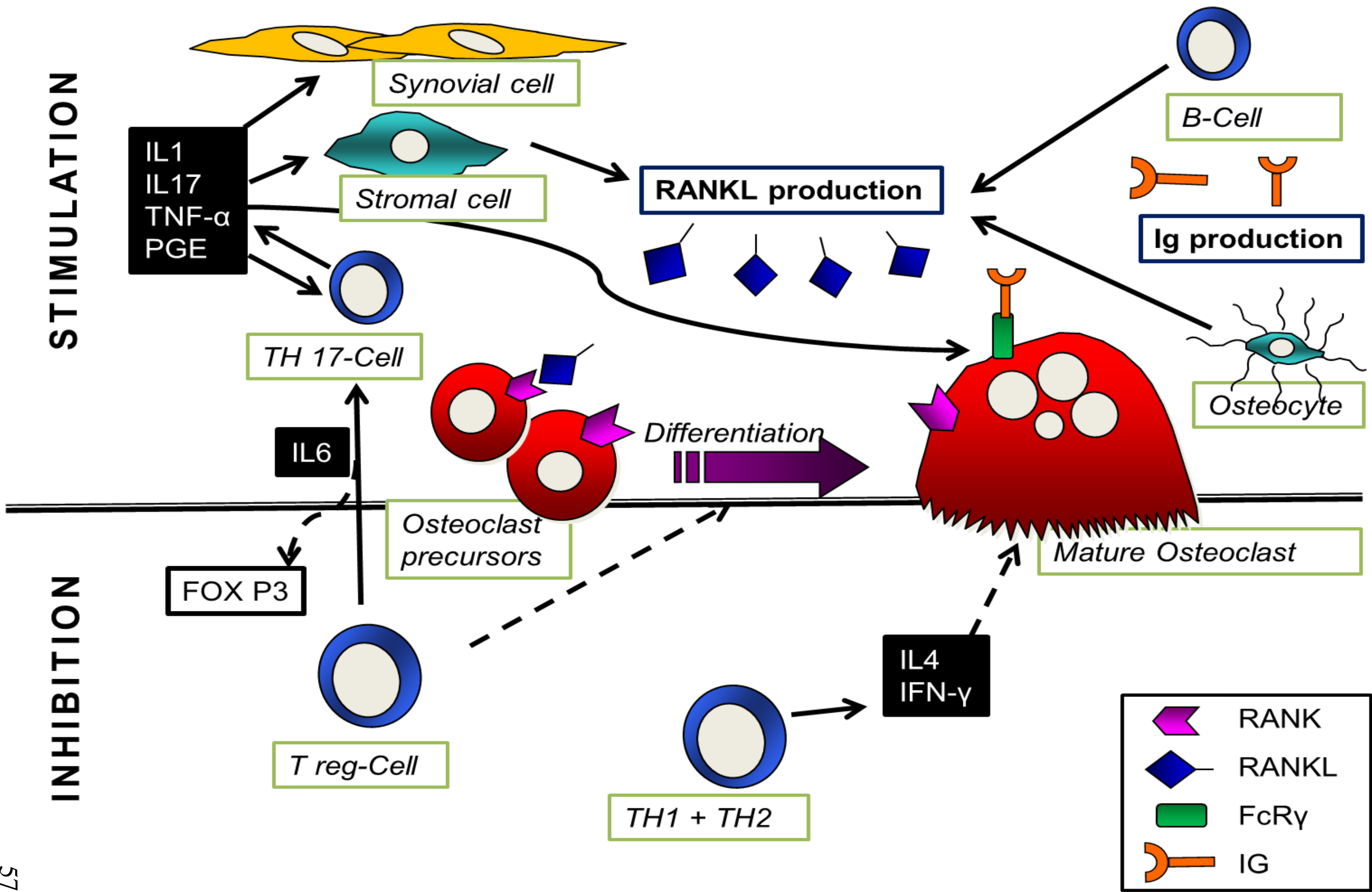

Figure 1.12 Impact of Inflammation on Osteoclastogenesis, for explanation refer to text; IG=Immunoglobulin; RANK=Recepto Activator of NFκB; FcγR=Fc gamma receptor; RANKL = RANK Ligand; FOXP3= Forkhead Box Protein P3

1.7.2.4.3 Immunoglobulins

About two thirds of patients with Rheumatoid Arthritis have antibodies against Immunoglobulins (these are termed Rheumatoid factors (RF)) or against citrullinated proteins (ACPA).The presence of these antibodies is strongly linked to severity of disease and accumulative joint and bone damage (Miriovsky et al. 2010a;van Gaalen et al. 2004a). Although RF are non-specific, ACPA have been found to be highly specific for RA and are detectable in some individuals 10 years prior to disease onset (van der Woude et al. 2010). Several recent studies have shown functional roles of ACPA antibodies, which correlate with increased bone loss and erosive disease. The impact of immunoglobulins on bone can be divided into direct effects on bone cells through interaction between Immunoglobulins on osteoclasts or osteoclast regulating cytokines, and indirect impacts through interaction with immune cells and subsequent alteration of the osteoclastogenic milieu.

Indirect effects

Circulating antibodies have the ability to bind with low affinity with their Fc portion to Fc receptors on the surface of immune cells as shown in Figure 1.13 (Nimmerjahn and Ravetch 2008). Monocytes and macrophages express various types of FcyR on their surfaces. Clavel et al (Clavel et al. 2008) showed that ACPA containing immune complexes induced TNF-alpha secretion in macrophages via engagement FcyRII. Further experiments demonstrated that both monocytes and macrophages were responsive to ACPA containing immune-complexes but antibody stimulated macrophages produced 9 times more TNF-alpha than stimulated monocytes. The TNF-alpha boosting effect of ACPA containing immune-complexes was observed in macrophages derived from both blood and synovial fluid (Laurent et al. 2011).

Interestingly established therapeutics for RA such as MTX and glucocorticoids have been shown to potently suppress FcyR expression on

monocytes which might help to explain the effectiveness of these drugs in suppressing inflammation and preventing structural damage (Torsteinsdottir et al. 1999;Wijngaarden et al. 2005).

Direct effect

Seeling et al. have shown that FcγRIV expressed on OC precursors play a crucial role in bone destruction in inflammatory arthritis in mice. FcγRIV knock out mice were found to have a reduced number of osteoclasts and less bone erosions at the site of inflammation compared to litter mates with K/BxN serum transfer arthritis (Seeling et al. 2013). Further remarkable studies investigating Immunoglobulin mediated osteoclastogenesis were published earlier this year. A research group led by Takayanagi have demonstrated that under physiological conditions activation of some Fcγ receptors expressed on osteoclast precursors, such as FcγRIIB and FcγRIII inhibit osteoclastogenesis, whereas stimulation of FcγRI and IV stimulates maturation and differentiation of osteoclasts. In the presence of hypergammaglobulinaemia, FcγRIIB is downregulated and the osteoclast activating effect of IgG immune complexes (IC) is prevailing (Negishi-Koga et al. 2015). Another group led by Georg Schett identified that the level of IgG glycosylation impacts on the binding affinity of IgG to FcγR. Desialylated IC were shown in in-vitro and in-vivo experiments to potently enhance osteoclastogenesis. Mice treated with sialyic acid precursor, N-actylmannosamine (ManNac), which sialytes IC, are protected from inflammation induced local and systemic bone loss (Harre et al. 2015).

Anticitrullinated peptide antibodies (ACPA)

Harre et al. have shown that citrullinated vimentin can be found on the surface of osteoclast precursors. Isolated antibodies against mutated citrullinated vimentin (MCV) were shown to bind to the surface of osteoclast precursor and to stimulate osteoclastogenesis through TNF-alpha increase. The transfer of anti citrullinated peptide antibodies (ACPA) into mice caused increased osteoclastogenesis and resulted in accelerated bone loss (Harre et al. 2012).

In clinical practice Kleyer and colleagues have found that ACPA positive healthy individuals without symptoms or signs of inflammatory arthritis have reduced BMD and reduced cortical thickness in comparison to ACPA negative individuals (Kleyer et al. 2014).

Osteoprotegerin autoantibodies

We previously reported a case which illustrated a new mechanism of antibody mediated osteoporosis. A patient with autoimmune thyroid disease and coeliac disease was found to have severe osteoporosis in association with neutralizing autoantibodies to osteoprotegerin (OPG). The binding of OPG which is a decoy receptor to RANKL results in to enhanced RANKL mediated osteoclastogenesis and accelerated bone resorption(Riches et al. 2009). A subsequent study, described in this thesis (Chapter 4) demonstrated the presence of detectable OPG antibodies in patients with RA and an association between autoantibody levels and the bone resorption marker CTX. Functional studies showed that purified IgG from patients who were tested positive for OPG antibodies inhibited the ability of OPG to block RANKL induced NFκB activation in vitro. This suggests that in some RA patients functional autoantibodies to OPG are present with the potential to enhance osteoclastic bone resorption. A simplified diagram about the mechanism of autoantibody induced osteoclast activation is shown in Figure 1.13

Above findings, suggest inflammation independent mechanisms of bone loss in Rheumatoid Arthritis. The emerging importance of immune complexes in the pathogenesis of bone disease will potentially open new avenues for therapy.

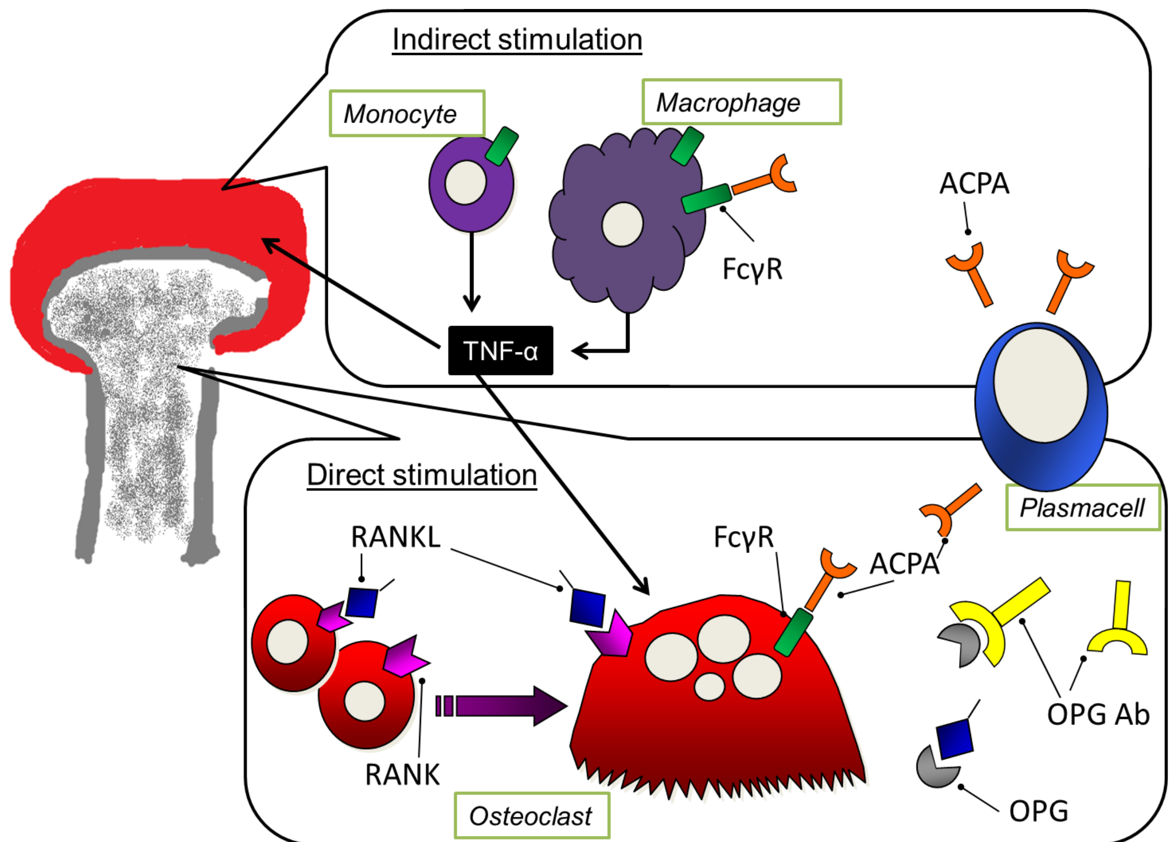

Figure 1.13 Auto-antibody mediated Osteoclastogenesis. *Indirect stimulation of osteoclastogenesis via stimulation of FcγR on macrophages and monocytes by IC including ACPA leads to enhancement of inflammatory cascade. Direct stimulation of FcγRI,III, IV on OC by IgG including ACPA promotes osteoclastogenesis. OPG ab block OPG, a decoy receptor to RANKL,which leads to increased RANKL mediated osteoclastogenesis.*

1.7.2.4.4 Relative immobilisation

Patients with RA are less active than their healthy peers due to pain, weakness, fatigue and disability (Mancuso et al. 2011;Pollard et al. 2006;Sokka et al. 2008). A prolonged period of decreased physical activity and chronic inflammation leads to bone loss in addition to an accumulation of visceral fat and sarcopenia (Lilleby et al. 2007;Santos et al. 2011;Zerwekh et al. 1998). Ekdahl et al have shown that longstanding active Rheumatoid Arthritis leads to reduced isokinetic and isometric muscle strength and reduced aerobic capacity (Ekdahl and Broman 1992). Change of body composition in form of muscle loss and addition of visceral fat associated with glucocorticoid use not only increase the risk of osteoporosis but also increases the risk of sustaining low fragility fractures (Sayer et al. 2006;Wickham et al. 1989). These changes in body habitus also promote the development of other comorbidities such as atherosclerosis and insulin resistance and might lead to a vicious cycle as it has been postulated that central obesity and inactivity may exacerbate chronic inflammation and worsen inflammatory symptoms (Benatti and Pedersen 2015).

At a cellular level, unloading of the human skeleton stimulates a quick and sustained increase of bone resorption and to a lesser extent a decrease of bone formation (Zerwekh et al 1998). The underlying mechanisms include the upregulation of Sclerostin (SOST), a cytokine secreted by osteocytes, which suppresses Wnt/beta catenin signalling and leads to reduced osteoblast differentiation and function. Lin et al (Lin et al. 2009) showed that SOST(-/-) mice, when mechanically unloaded, preserve their bone mass in contrast to their wild type littermates implying that SOST is responsible at least in part for bone loss due to skeletal unloading. Skeletal unloading also increases bone resorption through upregulation of PDK4 (Pyruvate dehydrogenase kinase 4) which promotes osteoclastogenesis (Wang et al.).

A longitudinal study including 70 RA patients allocated to either strength and endurance training or low intensity exercise over a 2 year period, showed

that strength and endurance training not only improved maximum strength of all muscle groups (grip strength, knee and trunk extensors) and maximal walking speed but also maintained normal femoral neck BMD in patients with recent –onset RA (Hakkinen et al. 2001). A further randomized controlled trial showed that a 2 year high intensity weight-bearing exercise program slows down hip BMD decrease compared to usual care (de Jong et al. 2004). Additionally the beneficial effects of exercise on endothelial function were shown by a recently published study which found that RA patients in the exercise group had improved micro- and macrovascular endothelial function compared to patients who received usual care (Metsios et al. 2014). Contrary to previous beliefs when patients with RA frequently were treated with bed rest and immobilisation (Alexander et al. 1983), the above findings emphasize the importance of exercise and maintenance of normal body weight in the prevention and treatment of systemic bone loss in rheumatic diseases.

1.7.2.4.5 Glucocorticoids

Patients with Rheumatoid Arthritis who are treated with glucocorticoids have double the risk of hip fractures compared with sex matched community controls (Cooper et al. 1995). Even low dose Glucocorticoid use in Rheumatoid Arthritis has been associated with increased risk of fractures besides other side effects such as GI complications and serious infections (Saag et al. 1994). The pathophysiology of corticosteroid induced bone loss is complex. Glucocorticoids (GC) have a direct impact on bone remodelling by reduction of bone formation and bone turnover. Weinstein et al (Weinstein et al. 1998) have shown that chronic glucocorticoid treatment in mice decreases proliferation of osteoblast precursors and stimulates osteoblast and osteocyte apoptosis, which together leads to a reduction of bone formation.. These findings were confirmed on biopsies of patients with glucocorticoid induced osteoporosis (Hofbauer and Rauner 2009). Long-term glucocorticoid exposure increases the expression of the transcription factor peroxisome proliferator-activated receptor (PPAR) which promotes the

differentiation of mesenchymal cells to adipocytes as opposed to osteoblasts. At the same time Runx2, a pivotal transcription factor for osteoblastogenesis is repressed by GC. Therefore long-term corticosteroid use leads to bone loss and fatty transformation of bone marrow (Hofbauer & Rauner 2009;Vande Berg et al. 1999).

Glucocorticoid treatment has also a significant impact on bone resorption. Corticosteroids suppress Osteoprotegerin (OPG) production by decreasing levels of the phospho-c-Jun protein and by interfering with the canonical Wnt –pathway by decreasing β-catenin protein expression (Kondo et al. 2008). This leads to an increase in RANKL/OPG ratio and subsequent stimulation of osteoclast proliferation (Hofbauer & Rauner 2009;Kondo et al. 2008). That RANKL plays an important role in the development of corticosteroid induced osteoporosis demonstrates the effect of administration of fully human monoclonal antibodies against RANKL (Denosumab) to hRANKL knock-in mice. Hofbauer and colleagues (Hofbauer et al. 2009) showed that a short course of corticosteroid treatment in hRANKL knock in mice induces vertebral and femoral bone loss. Concomitant administration of Denosumab to these mice prevented the glucocorticoid induced bone loss largely by its antiresorptive effect There are no definitive studies of Denosumab's effectiveness in glucocorticoid induced osteoporosis in man but it has been shown that it is an effective treatment in preventing osteoporosis in patients with Rheumatoid Arthritis of whom , a third were treated with glucocorticoids at the time (Cohen et al 2008). Additionally to reduced OPG production glucocorticoids prolong the lifespan of osteoclasts which further contributes to the imbalance of bone formation and resorption in favour of bone resorption and hence to net bone loss (Jia et al. 2006;Weinstein, Jilka, Parfitt, & Manolagas 1998).

Furthermore, extra skeletal actions of glucocorticoids on organs such as muscles, kidney and endocrine system contribute to accelerated bone loss and increased fracture risk in rheumatic diseases. A summary of these actions is shown in Figure 1.14. Glucocorticoids decrease calcium absorption

in the gastrointestinal tract (Huybers et al. 2007) and possibly reduce tubular calcium reabsorption in the kidneys (Reid and Ibbertson 1987), which leads to secondary hyperparathyroidism which is a strong driver of osteoclastogenesis and hence increased bone resorption. Glucocorticoids also decrease the production of sex steroids such as LH, FSH or Testosterone and growth hormone that puts a halt on bone turnover (Canalis et al. 2007). Steroid associated muscle loss not only leads to reduced skeletal loading and hence increased bone loss, sarcopenia also leads to postural instability, which is an important risk factors for falls (de Rekeneire et al. 2003).

It is important to note that glucocorticoids used as treatment in inflammatory arthritis are not always thought to have a negative impact on bone homeostasis. The strong anti-inflammatory effect of glucocorticoids reduces the amount of circulating pro- inflammatory cytokines such as IL1, IL6, TNF which in turn may reduce osteoclast differentiation and activity. For example patients with early Rheumatoid Arthritis treated with the COBRA regime, which includes the simultaneous introduction of Prednisolone, Methotrexate and Sulfasalazine had similar changes in bone turnover markers and in BMD of hip as the control group, which was treated with Sulfasalazine monotherapy only (Verhoeven et al. 2001). Furthermore, van Everdingen showed that low dose glucocorticoid therapy (10mg Prednisolone/day) in RA prevented bone erosions and joint space narrowing when compared to early RA patients who were untreated (van Everdingen et al. 2002). These findings might be explained through the anti-inflammatory effects of glucocorticoids, which at the initial stage of Rheumatoid Arthritis counteract the negative impact on bone (Boers et al. 1997).

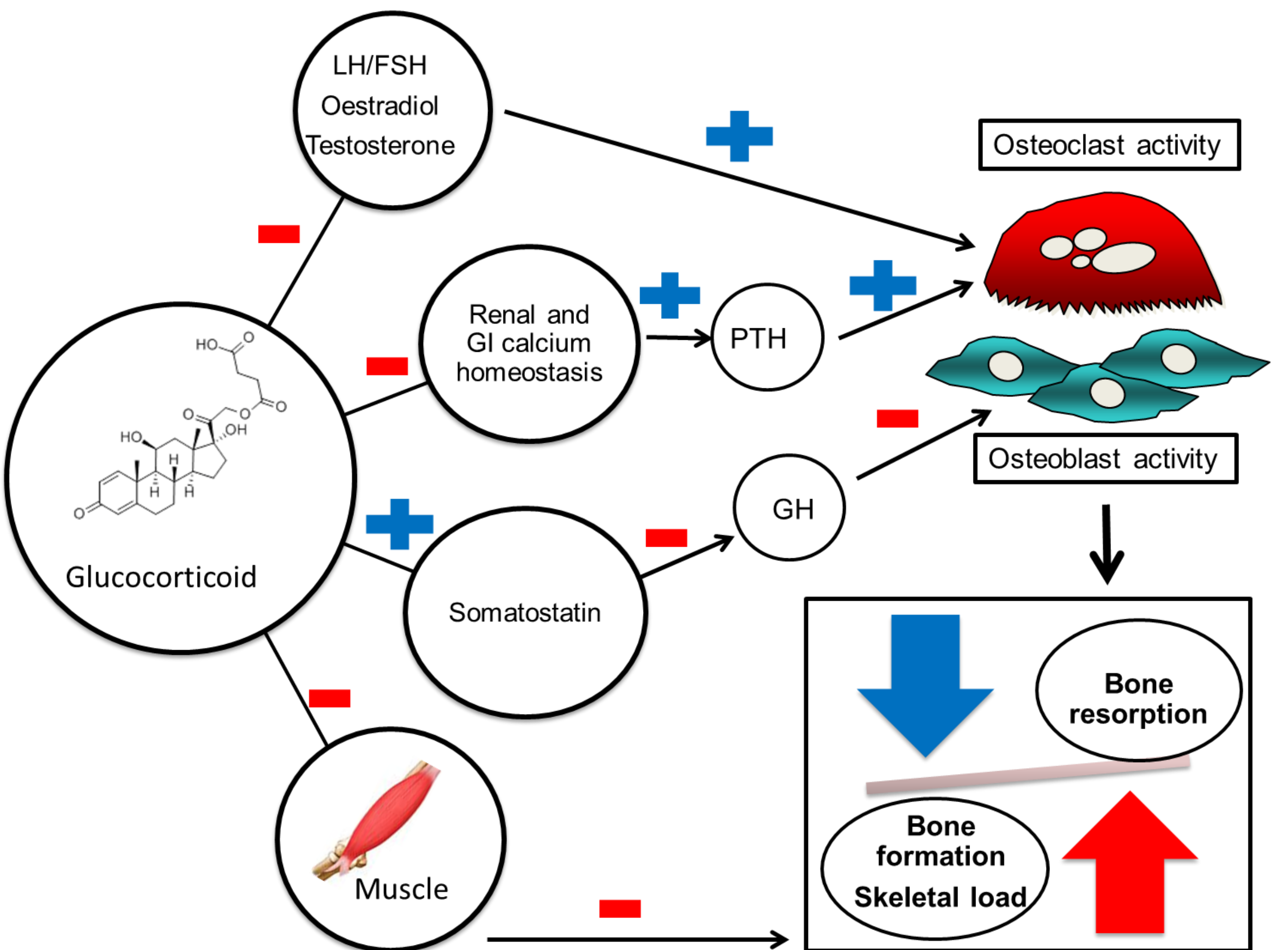

Figure 1.14 Summary of extraskeletal mechanisms of glucocorticoid induced bone loss. *For further explanation please refer to text. GI= Gastrointestinal, PTH= Parathyroid Hormone; GH=Growth Hormone, LH= Luteinizing Hormone FSH= Follicle Stimulating Hormone*

1.7.3 Ankylosing Spondylitis

Ankylosing Spondylitis (AS) is a chronic inflammatory disease that is characterized by inflammation, predominantly targeting the axial skeleton resulting into back pain and stiffness. The inflammation mostly affects entheses (tendon insertion sites) at spine and other sites. Frequently peripheral large joints such as hips, knees and ankles may be affected. The natural course of the disease leads to increased bone formation and sclerosis, which promotes the fusion of SI joints, and the production of bonyspurs called syndesmophytes (67). Ankylosing Spondylitis belongs to the family of seronegative Spondyloarthropathies, which are genetically linked and associated with carriage of the MHC class I variant HLA-B27. The prevalence ranges from 0.1-1.4% and occurs more frequently in regions and tribes with higher HLA-B27 prevalence such as in Scandinavian countries and Inuit populations. In contrast to other rheumatic diseases the age of onset is predominantly under the age of 30 years and it affects men more commonly than women with a ratio of 2:1 (Braun & Sieper 2007).

1.7.3.1 Osteoporosis risk

Despite localized increases in bone formation, it is well established that patients with AS are at increased risk of generalized bone loss (Karberg et al. 2005;Lee et al. 1997) and vertebral fractures (Ralston et al. 1990). A recent Swedish study that assessed BMD at spine, hip and forearm and bone michroarchitecture of the limbs with HRpQCT in 69 AS patients showed that lumbar osteoporosis and vertebral fractures were associated to lower volumetric BMD and decreased microarchitecture in peripheral bone (Klingberg et al. 2012) suggesting that bone loss in AS is generalized. This study also showed that increased syndesmophyte presence was associated with lower lumbar spine BMD. Interestingly this correlation was found in several studies that pre-dated this one (Karberg et al. 2005;Lange et al. 2005;van der Weijden et al. 2011). In contrast to other rheumatic diseases such as RA and SLE, men are at higher risk of developing Osteoporosis

(Sambrook & Cooper 2006;van der Weijden et al. 2011). Amongst men with AS above 50 years of age around 20% have Osteoporosis at either spine or hip. Amongst younger men with recent onset of AS with a mean age of 38 years 38% were found to have osteopenia of hip or spine (van der Weijden et al. 2011). Accelerated bone loss occurs frequently early on in the disease when patients are commonly treated conservatively with NSAID treatment only (van der Weijden et al.2011) . It is worth noting that the interpretation of spinal BMD in AS is difficult as the presence of syndesmophytes and calcified ligaments can lead to overestimation of spinal BMD (Braun & Sieper 2007).

1.7.3.2 Fracture risk

Patients with axial spondyloarthropathy are at increased risk of fractures at a relatively young age, frequently under 40 years of age, which limits the value of conventional fracture risk assessment tools such as FRAX (Ralston et al. 1990;van der Weijden et al. 2012;Vosse et al. 2006;Vosse et al. 2009).

In established disease vertebral fractures occur 3 times more commonly in patients with AS than healthy controls which is likely due to reduced BMD and secondly due to biomechanical properties of an encased spine with decreased elasticity and reduced spinal mobility (Vosse et al. 2009). We currently don’t know if AS patients are at increased risk of hip or wrist fractures as to date there is no data available to show an increased non-vertebral fracture risk in AS (Bultink et al. 2012) except in cases with co-existing inflammatory bowel disease (Vosse et al. 2009). To reduce fracture risk in AS not only low BMD should be targeted but also localized bone formation, which encases the spine and increases its rigidity. To date it is unclear if bone formation is closely linked to inflammation and if fracture risk can be altered by anti-TNF treatment (Maksymowych et al. 2009;Schett et al. 2009).

1.7.3.3 Pathologenesis of bone involvement in AS

The pathology of AS and the underlying mechanism of systemic or localized bone loss and formation are not as well understood as in RA. The inflammatory process in AS presents itself predominantly as enthesitis at the zygopophyseal joints, hip, sacroiliac (SI) joints and peripheral tendon insertion sites. Clinically the most common site of inflammation in AS are the vertebrae. The schematic picture (Fig 1.15) below shows the sequelae of pathological changes. Histological samples from zygapophyseal and SI joints from AS patients have shown inflammatory cell infiltrates with predominantly T-cells, macrophages and osteoclasts.

Bone erosions and destruction is followed by excessive bone formation which is similar to the endochondral bone forming process seen in embryonic development. Chondrocytes and osteoblasts are the cells responsible for extra bone formation.

All three stages (inflammation, bone destruction and formation) do not necessarily occur in sequential order and localized increased bone turnover frequently occurs independently from inflammation (Tam et al. 2010).

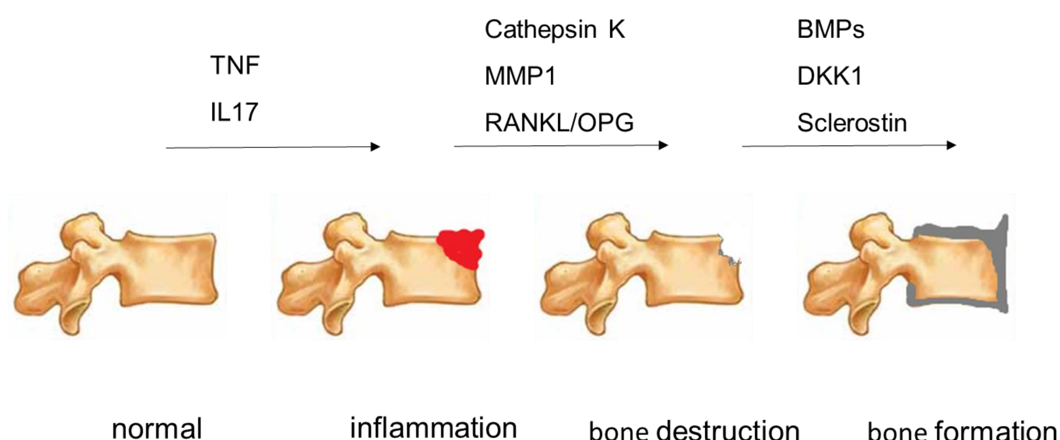

Figure 1.15. Pathological changes of enthesial site of vertebra in Ankylosing Spondylitis *(adapted from Tam et al. Nat Rev Rheumatol. 6, 399-405 (2010).Pro-inflammatory cytokines (TNF, IL17) promote inflammation at the entheses of vertebrae. RANKL promotes proliferation and activation of osteoclasts which degrade organic matrix through expression of Cathepsin K. Matrix Metalloproteinase 1 is a collagen degrading enzyme and mostly expressed by chondrocytes. Bone destruction is followed by excessive bone formation which is initially driven by bone morphogenis proteins (BMPs) and at later stage by Wnt pathway antagonists (DKK-1, Dickkopf 1 and Sclerostin).*

1.7.3.4 Mediators

TNF

TNF is expressed by a variety of cells of the innate and adaptive immune system as previously discussed. Cells in the SI joints of AS patients have been shown to express high levels of TNF (Braun et al. 1995). TNF stimulates osteoclastogenesis directly via increased OSCAR expression and inhibits bone formation through increased expression of Dkk-1 and Sclerostin. Clinically the introduction of TNF blocker for the treatment of AS has been a great success. Anti- TNF treatment suppresses pain and stiffness, reduces peripheral synovitis, acute phase reactants and the degree of bone marrow oedema on MRI (Tam, Gu, & Yu 2010). It may inhibit generalized bone resorption since after 6 months of regular Infliximab administration a marginal increase of BMD spine and hip was noted in AS patients (Allali et al. 2003). However the role of TNF in the development of bone erosions and syndesmophytes is less clear as there is no convincing evidence that TNF blockade has an impact on syndesmophyte formation. This indicates that there are potentially different pathways of localized bone destruction with associated bone formation and systemic bone loss in AS (Baraliakos et al. 2005;Schett et al. 2009).

IL17-IL23

IL23R has been identified and validated by a genome wide association study to be one of the significant loci associated with AS (Reveille et al. 2010). IL23R is expressed on Th17 cells which are responsible for IL17 production. In contrast to RA patients, seronegative Spondyloarthropathy patients were found to have increased IL17 levels and increased numbers of TH17 cells (Jandus et al. 2008;Wendling et al. 2007). New therapeutic agents have been designed to target TH17 cells and its cytokines in the IL17-23 axis. Secukinumab is a monoclonal antibody targeting IL17A which has been tested in various autoimmune diseases such as RA, PsA, Psoriasis, AS, Multiple Sclerosis and Uveitis (Sanford and McKeage 2015). To date

Secukinumab has only been approved by the FDA for the treatment of Psoriasis but the drug is currently undergoing further phase II and III trials in PsA and AS (Baeten et al. 2013;Genovese et al. 2013;McInnes et al. 2014). The study programme for the seronegative arthritides are promising but long-term data and data about the impact on localized and systemic bone loss are outstanding (Sanford & McKeage 2015).

Cathepsin K

Cathepsin K is highly expressed by osteoclasts and acts as cysteine protease that degrades organic matrix such as type1 collagen (Costa et al. 2011). Immunohistochemistry analysis of bone tissue from AS patients and patients with degenerative disc disease showed a marked increase of mono- and multinuclear cells which are most likely osteoclasts or osteoclast progenitors expressing Cathepsin K (Neidhart et al. 2009).Cathepsin K inhibitors (Odanacatib) have been developed as medication for generalized osteoporosis and once on the market, it maybe of future interest to examine the impact of Odanacatib on bone disease in AS (Costa et al. 2011).

RANKL/OPG

RANKL levels were found to be significantly increased in serum of AS patients compared to healthy controls (Kim et al. 2006). This can be easily explained by the activation of the inflammatory cascade and the increase of pro-inflammatory cytokines, which boost RANKL production as explained above. Less plausible is the finding by Franck et al who showed that bone loss in AS is, amongst other factors, associated with low OPG serum levels (Franck et al. 2004). Our group has recently found that autoantibodies to OPG are strongly associated with low hip BMD in patients with AS (Chapter 5) and hypothesize that these antibodies may play a role in the accelerated bone loss in AS.

These findings involving the RANKL/OPG pathway may help to explain the inflammation independent bone changes in AS.

DKK-1

It is unknown what exactly triggers excessive bone formation in AS. Dickkopf-related protein (DKK-1), a glycoprotein belonging to the Wnt family, which inhibits bone formation by interfering with Wnt/beta-catenin signaling in osteoblasts. Decreased levels of DKK-1 are usually associated with increased bone formation and in animal models DKK-1 blockade induces fusion of sacroiliac joints (Tam, Gu, & Yu 2010;Uderhardt et al. 2010).
Interestingly two studies have found higher serum DKK-1 levels in AS patients than in healthy controls (Daoussis et al. 2010;Nocturne et al. 2015). One study went on to assess Dkk-1 function in vitro and demonstrated that DKK-1 in AS is dysfunctional as it doesn't inhibit bone formation in the same way as it does in RA. Daoussis et al also showed that TNF blockade in patients with AS increases DKK-1 concentrations substantially which may suggest that anti-TNF treatment could inhibit the syndesmophyte formation and bone fusion although this has not been verified in clinical practice yet (Baraliakos et al. 2005;Schett et al. 2009;Daoussis et al. 2010).

Sclerostin

Sclerostin is expressed by osteocytes and is another endogenous inhibitor of Wnt signaling. A study looking into Sclerostin concentration in osteocytes in AS patients found decreased skeletal expression and lower circulating levels of Sclerostin in AS patients compared to healthy controls (Appel et al. 2009).
Recent analysis of an early AS cohort also showed that Sclerostin serum concentration were significantly lower in AS patients than in healthy controls (Nocturne et al. 2015). As Sclerostin inhibitors may soon become available as monoclonal antibodies for the treatment of osteoporosis, its use in Spondyloarthropathies may need to be evaluated as there's a theoretical risk of worsening accelerated bone formation.

1.7.4 Systemic Lupus Erythematosus

Systemic lupus erythematosus is a multisystem autoimmune disease, which affects most commonly women of childbearing age. The prevalence ranges from 20- 150 cases per 100000 population and it is more common in individuals of African, Asian or Hispanic ancestry. In order to make a diagnosis of SLE 4 out of 11 criteria need to be fulfilled. The diagnostic criteria include mucocutaneous manifestations such as malar rash, discoid rash, photosensitivity and oral ulcers, systemic involvement in form of arthritis, serositis, renal involvement, neurological disorder, blood disorder such as anaemia, leucopenia or thrombocytopenia and autoantibody positivity (anti-nuclear antibody, anti-ds-DNA, anti-Smith antibody, anti-phospholipid Ab). Treatment of SLE frequently includes corticosteroids and in severe cases Cyclophosphamide, both of which can have detrimental impact on bone (Tsokos 2011).

1.7.4.1 Osteoporosis in SLE

There is huge variability in the epidemiological data of SLE associated bone loss and the osteoporosis prevalence rate ranges from 1.6 to 60% (Bultink, Vis, Horst-Bruinsma, & LEMS 2012). The wide variations in prevalence are likely a result of different study designs and criteria but may also reflect a true difference in incidence according to latitude or ethnicity. A UK study (Yee et al. 2005) which included 242 SLE patients with a median age of 40 years showed that about 10% of patients had osteoporosis and 9% had a history of fragility fractures. The study also showed that the non-Afro-Caribbean race is at higher risk of reduced BMD than Afro-Caribbeans which is in keeping with findings in the general population (Melton et al. 2002). In contrast, a study, which included 298 women with SLE from Chicago and Pittsburgh, showed that Afro American women had a lower BMD of lumbar spine than white patients. This might be explained by the fact that Afro Americans develop

more severe disease as shown by the increased SDI index (Systemic Lupus International Collaborating Clinics/American College of Rheumatology Damage Index) and get treated with an average higher corticosteroid dose (Lee et al. 2007).

Beside race as a possible risk factor for osteoporosis the usual traditional risk factors, such as age, postmenopausal status and low body mass index have been found to be independent predictors of reduced BMD in SLE. Additionally disease specific risk factors such as Vitamin D deficiency (Bultink et al. 2005), glucocorticoid intake (Jardinet et al. 2000) and renal failure (Almehed et al. 2007) were also shown to be associated with low BMD. Clearly above markers are also indicating severe disease, which might be a risk factor of osteoporosis in SLE per se.

1.7.4.2 Fracture risk

SLE patients are at increased risk of fractures. A large population based cohort study in the US found that SLE patients are at fivefold risk of fractures compared to healthy controls. The risk factors identified from this study were age and length of corticosteroid use (Ramsey-Goldman et al. 1999). A Swedish study showed in SLE patients an increased risk of combined vertebral and hip fractures with an odds ratio of 2.9 compared to age and sex-matched healthy controls (Weiss et al. 2010) . A more recent study also showed ongoing high prevalence (20%) of asymptomatic vertebral fractures in a Chinese SLE cohort (Li et al. 2009).

1.7.4.3 Pathologenesis of bone involvement in SLE

Chronic systemic inflammation can affect most organs and accelerate disease related bone loss. SLE is characterized by an increase of Th1 and Th2 T-cells, hyperactivity of T- and B-cells and an increase of circulating plasma cells with amplified production of autoantibodies .The stimulation of the innate immune system leads to increased TNF and IL17 concentration and subsequent increase in osteoclastogenesis and decrease of IL2 which leads to reduced activity of cytotoxic T cells.

1.7.4.4 Mediators

Pro-inflammatory cytokines

SLE patients with active disease have increased levels of TNF, IL17 and IFNα. As mentioned above TNF and IL17 are potent osteoclast stimulating factors. However in contrast to the rheumatic diseases discussed previously TNF inhibition has not demonstrated significant benefit in the treatment of SLE. IL-17 remains an interesting target as TH17 cells are found in abundance in peripheral blood and in affected kidneys in SLE patients (Tsokos 2011). Type I interferons, particularly Interferon α (IFNα) is usually involved in viral defence and is secreted mostly by dendritic cells through activation of Toll Like Receptor (TLR) signalling (TLR 7 and TLR 9). IFNα is an important cytokine in the pathogenesis of SLE as it has the ability to break self-tolerance by activating antigen presenting cells after uptake of autoantigens (Tsokos 2011).Interferon signalling has also been shown to be an important mechanism in the interplay between immune system and bone metabolism. Interferon γ is a potent osteoclast inhibitor (Takayanagi) and RANKL induced IFN β (type I interferon) expression has a negative auto-regulatory effect on osteoclasts by interfering with c-Fos expression (Takayanagi Nature 2002). However, the effect of increased IFNα on bone metabolism in SLE is unknown (Coelho et al. 2005). Similarly no study to date has found an association between disease activity and low BMD however, several studies have shown that accumulative organ damage, which is a result of chronic inflammation, is associated with low BMD(Bultink et al. 2012).

Vitamin D

Vitamin D is vitally important for maintenance of normal bone homeostasis and muscle strength. Vitamin D regulates intestinal Calcium and Phosphate absorption and acts on osteoclasts, osteoblast and osteocytes directly via Vitamin D receptor. Vitamin D deficiency causes secondary hyperparathyroidism with subsequent osteoclast activation and increased

bone resorption (Bikle 2012;Holick 2007). Low 1,25- Hydroxyvitamin D (1,25 [OH]D) can be caused by a variety of mechanism in SLE. Firstly sun avoidance due to photosensitivity reduces 25 [OH]D production in the skin . Secondly renal impairment will impact on the ability to hydroxylate 25 [OH]D into 1,25 [OH]D. Thirdly the most commonly used DMARD in SLE, hydroxychloroquine, supposedly inhibits 1α hydroxylase, which allows the enzymatic conversion into the active Vitamin D form (Bultink et al. 2012).

Cyclophosphamide

Intravenous pulses of Cyclophosphamide are frequently used in lupus nephritis. The use of Cyclophosphamide is associated with a number of potential serious side effects such as bone marrow suppression, infections and premature ovarian failure causing early menopause (McDermott and Powell 1996). Since SLE most commonly occurs in women of childbearing age the use of Cyclophosphamide has serious impact on fertility and bone health. Gonadal failure is characterized by a sharp drop of oestrogens, which play an important role in the maintenance of normal bone homeostasis. Oestrogens suppress osteoclast function by reducing RANK expression, increasing OPG production and decreasing lysosomal enzyme secretion (Kremer et al. 1995). Oestrogen also drives osteoblast differentiation and activation by stimulation of wnt signalling (Manolagas et al. 2013).Hence oestrogen deficiency induced by premature menopause increases bone resorption and decreases bone formation, resulting in bone loss.

Glucocorticoids

Glucocorticoids are frequently used in patients with active SLE. The mechanism of glucocorticoids on bone cells has been described before. The use of glucocorticoids in SLE is in the majority of studies associated with increased bone loss. Jardinet et al showed in a longitudinal study including 35 premenopausal women that daily use of at least 7.5mg Prednisolone is associated with an average lumbar spine bone loss of 2.1% per year

(Jardinet et al. 2000). Similar to RA, the use of glucocorticoids increases the risk of fractures significantly and in the Hopkins cohort study the cumulative glucocorticoid dose has been identified as independent predictor of fractures (Bultink et al. 2012).

1.7.5 Psoriatic Arthritis

Psoriatic Arthritis is an autoimmune inflammatory arthritis that is usually associated with Psoriasis. The prevalence of Psoriatic Arthritis ranges from 0.3- 1% in the general population. About 10-30% of patients with Psoriasis will develop inflammatory arthritis. Psoriatic Arthritis can present in various forms but the most common presentation is an oligoarticular arthritis affecting predominantly distal joints. In contrast to RA, joint involvement occurs more commonly in asymmetrical distribution and frequently affects more joints of one digit, including distal interphalangeal joints. Additionally inflammation can occur around tendons or tendon insertion sites and cause dactylitis, enthesitis and inflammatory back pain (Gladman et al. 2005).

1.7.5.1 Pathology

About 20% of all Psoriatic Arthritis patients will develop severe polyarticular disease with joint space loss, eccentric bone erosions and ultimately joint destruction and deformation. Arthritis mutilans (Fig 1.15) is characterized by bone lysis which can result in severe destruction of the joint with telescoping of digits and pencil in cup appearance on x-rays. In addition, patients with Psoriatic Arthritis frequently developed excessive bone formation in form of bone spurs at sites of enthesitis or in form of ankyloses at the spine. These bone changes suggest a disorganised pattern of bone remodelling (Mease 2011). Histological examinations of hip, knee and foot joints of Psoriatic

Arthritis patients with erosive disease revealed large numbers of osteoclasts in particular in resorption pits and bone pannus junction and in subchondral bone.

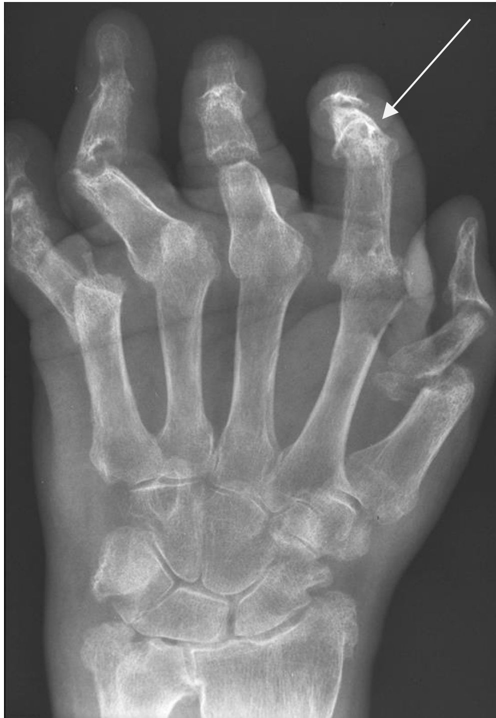

Figure 1.15 X-ray of left hand taken from a patient with Psoriatic Arthritis with arthritis mutilans *(solid arrow points towards large erosion, pencil in cup deformity)*

1.7.5.2 Mediators

Psoriatic Arthritis belongs to the group of seronegative Spondyloarthropathies. Hence the genetics, pathophysiology and osteoimmunolgy aspects are in many ways similar to the one presented in AS.

RANKL

Ritchlin et al demonstrated that PBMCs isolated from Psoriatic Arthritis patients contain an increased number of osteoclast precursors compared to PBMC form healthy controls and immunohistochemistry and RT-PCR of synovial membranes showed increased RANK and RANKL expression in samples from Psoriatic Arthritis patients (Ritchlin et al. 2003). The same research group also showed that the addition of OPG in vitro inhibits osteoclastogenesis in the setting of Psoriatic Arthritis.

TNF

Similar to AS, anti-TNF has been shown to substantially reduce inflammation. The use of Golimumab and Adalimumab have shown significant reduction in PSarc score, significant inhibition of structural damage and generally positive effects on skin. On a molecular level Ritchlin et al (2003) has demonstrated that the addition of anti-TNF to in vitro cultured PBMCs from Psoriatic Arthritis patients significantly reduce the production of osteoclast precursors. A comparison with healthy control PBMCs suggests that PBMCs from Psoriatic Arthritis patients secrete significantly greater quantities of TNF α than PBMCs from healthy controls. Again all the favourable effects of anti-TNF seem to involve bone resorption. Although anti TNF significantly reduces the extent of enthesitis (Sterry et al. 2010) and the development of erosions (Kavanaugh et al. 2010;Mease et al. 2009)in Psoriatic Arthritis , no therapeutic agents have been shown to alter bone formation at enthesial sites or at the spine.

IL17-IL23

Levels of circulating TH17 cells are higher in PsA than in RA (Jandus et al. 2008). Moreover, IL17RA and IL17 producing CD4+ T cells are increased in synovial fluid and psoriatic plaques (Raychaudhuri et al. 2012). Polymorphisms in IL23R are associated with Spondyloathropathies including Psoriatic Arthritis (Rahman et al. 2009). Scherlock et al (Sherlock et al. 2012) published interesting results highlighting the importance of IL23 and its receptor in the development of enthesitis. The research group identified new IL23 receptors on CD3+ $CD4^{-}CD8^{-}$ T cells. This cell population has been found to reside at entheseal interface between tendon and bone. These resident T cells have the ability to produce IL22 which increases STAT3 signalling and hence osteoblast driven new bone formation. Animal models confirm above findings as increasing expression of IL23 in transgenic mice promotes the development of enthesitis and enthesial new bone formation (Sherlock et al. 2012) . Ustekinumab, a monoclonal antibody against IL12/23p40 initially licenced for Psoriasis is now also licensed for the use of Psoriatic Arthritis. The PSUMMIT trials have shown that Ustekinumab significantly reduces radiographic progression after 24 and 52 weeks compared to placebo (Kavanaugh et al. 2014). To date there is no data on the impact of Ustekinumab on bone mineral density or bone formation in Psoriatic Arthritis or Ankylosing Spondylitis.

BMP

Inhibition of bone morphogenic protein in spondyloarthritis animal models has shown changes in ankylosing enthesitis and peripheral arthritis. Noggin is an extracellular BMP antagonist and Lories et al have shown that mice with induced spondyloarthritis and a noggin gene transfer are less likely to develop ankylosing enthesitis than their litter mates (Lories et al. 2005). On the quest for therapeutics which inhibit bone formation and structural damage in Spondyloarthropathies BMP signalling appears to be an attractive target however to date there are no licensed products available.

Osteoporosis risk

The degree of systemic bone loss in PsA is controversial. Frediani et al (Frediani et al. 2001) found that 2/3 of patients with PsA had reduced bone mineral density compared to healthy controls. Interestingly this study showed also a high prevalence of osteopenia (56%) and osteoporosis (11%) in premenopausal women. Relatively high prevalence rates of OP in men (29%) and postmenopausal women (47%). However subsequent studies looking into systemic bone loss of PsA did not find a significant difference between bone mineral density of Psoriatic Arthritis patients when compared to a reference range (Busquets et al. 2014) or to age and sex-matched healthy controls (Pedreira et al. 2011). The latter study hypothesised that BMD in PsA might be preserved secondly to increased BMI and due to an element of excess bone formation.

Fracture risk

Pedreira et al (Pedreira, Pinheiro, & Szejnfeld 2011)found that PsA patients had an increased risk of fractures compared to healthy controls despite there being no difference in BMD between these groups. The increased facture risk was found to be associated to disease duration and a history of falls. This study has also demonstrated that PsA patients are more likely to become sarcopenic and obese and are at higher risk of developing metabolic syndrome

1.7.6 Other rheumatic diseases

1.7.6.1 Scleroderma

Scleroderma is an autoimmune disease characterized by microvascular damage, dysregulation of the innate and adaptive immunity with production of autoantibodies against various intracellular antigens and generalized fibrosis of skin and internal organs. There are two subtypes categorized according to skin involvement. Limited cutaneous Scleroderma usually affects the skin of hands, forearms and face and is associated with preceding Raynaud syndrome, Pulmonary Hypertension and the presence of anti-centromere antibodies. Diffuse cutaneous scleroderma is characterized by extensive progressive skin involvement and by inner organ, in particular renal, lung and cardiac involvement. Autoantibodies associated to the diffuse cutaneous form are anti-topoisomerase I, anti-RNA polymerase and anti-Fibrillarin (U3RNP). Scleroderma occurs relatively infrequently with a prevalence rate of <150 per million, affecting more commonly women with peak onset around 50 years of age. Several studies have demonstrated that patients with SS are at increased risk of OP and osteoporotic fractures (Avouac et al. 2012) (Lai et al. 2015). A comparative study with RA revealed that prevalence rates of OP and OP fractures in SS, (30% and 35% respectively) are similar to the high prevalence rates in RA (32% and 33%) and significantly higher compared to healthy controls (Avouac et al. 2012). A systematic review (Omair et al. 2013) summarized multiple risk factors for the development of OP, including family history of OP, age, postmenopausal state, low vitamin D levels, diffuse scleroderma subtype, presence of internal organ involvement and calcinosis. However the authors emphasize that the evidence of these risk factors is conflicting and further definitive studies are necessary. The largest study investigating osteoporotic fractures in Systemic Sclerosis was a Taiwanese cohort study. Lai et al analyzed the National Health Insurance database and identified 1712 SS patients who were followed up over a median period of 5.2 years. When compared to controls they identified that SS patients are at significantly increased risk of vertebral

and hip fractures with a incident rate ratio of 1.78 and 1.89 respectively. The identified risk factors for fractures were age, being female, daily Prednisolone dose above 7.5 mg and bowel dysmotility treated with intravenous Metoclopramide administration.

1.7.6.2 Primary Sjoegren Syndrome

Sjoegren syndrome is an autoimmune disease characterized by impairment of exocrine gland function resulting in oral and ocular dryness. Systemic manifestations of Sjoegrens include non erosive polyarthralgia, renal disease in form of distal renal tubular acidosis and glomerulonephritis, lung involvement in form of interstitial lung disease, peripheral polyneuropathy and autoimmune thyroiditis. There is very little published data about the impact of Sjoegren Syndrome on bone. Several case reports have noted the occurrence of osteomalacia as a result of distal renal tubular acidosis (Fulop and Mackay 2004;Saoud et al. 2005). Distal renal tubular acidosis (dRTA) is frequently caused by a defect of the collecting tubule anion exchanger, or of hydrogen ATP pump. Bone biopsies of patients with distal renal tubular acidosis have revealed reduced bone formation with impaired bone mineralization. These findings were confirmed by in vitro studies, which demonstrated that acidosis impairs osteoblast differentiation and reduces ALP mRNA and its enzyme activity (Disthabanchong et al. 2007). A recent study by Garvani et al (Gravani et al. 2015) found no difference in BMD between pSS patients and controls however pSS patients with high urine pH had a significantly lower BMD than controls proposing a possible impact of dRTA in Primary Sjoegren's on bone turnover.

1.7.6.3 Dermatomyositis

Dermatomyositis is an autoimmune inflammatory myopathy causing proximal muscle weakness. Typical skin involvement in the form of heliotrope rash and oedema of the upper eyelids, an erythematous rash of the face, upper trunk

and over the knuckles which is accompanied by a raised, violaceous scaly eruption (Gottron's papules) (Robinson and Reed 2011). Active disease requires usually prolonged courses of high dose glucocorticoids. Single studies have shown that both juvenile (Stewart et al. 2003) and adult onset of Dermatomyositis (de Andrade et al. 2012) have been associated with increased risk of Osteopenia and Osteoporosis. De Andrade et al have also demonstrated that around a quarter of patients with Dermato-or Polymyositis had a history of low fragility fractures which was significantly higher compared to age-sex and BMI matched controls. The risk factors for Osteoporosis identified in this study were similar to those found in general population such as age, weight and menopausal state (de Andrade et al. 2012). Treatment or prevention of Osteoporosis in juvenile DM is a challenging therapeutic area as the individuals affected are frequently in pre-pubertal and early pubertal stages when they often haven’t reached peak bone mass yet. Furthermore the use of anti-resorptives in young women of childbearing age is tricky as the longterm effects of previous use of Bisphosphonate on the foetus is still unknown (Stewart et al. 2003).

In summary

Patients with Rheumatic Diseases are at high risk of OP and osteoporotic fractures. Many therapeutics target mediators of bone loss but long-term data on the use of biologic drugs and their impact on bone is largely outstanding. Recent insights into osteoimmunological mechanism and new discoveries of autoimmune mediated bone loss will help to shape future research with the aim of early detection, efficient treatment and ultimately prevention of inflammatory arthritis related bone disease.

Chapter 2: Material and Methods

2.1 Laboratory Methods

2.1.1 Osteoprotegerin autoantibody ELISA

(used for the analysis of osteoprotegerin autoantibodies in RA, chapter 4)

Levels of OPG antibodies were measured by a direct enzyme-linked immunosorbent assay (ELISA) which was developed in-house. The principles of the OPG ab ELISA are shown in Figure 2.1.

Recombinant OPG (R&D Systems) was reconstituted in order to prepare an OPG stock solution of 100µg/ml by adding 250µl of 0.2um filtered PBS to 25ug lyophilised powder. The reconstituted OPG was stored at 4°C.

The OPG stock (100 ng/µl) was diluted to 0.3ng/µl using 16µl stock + 5319µl of coating buffer per plate. A coating buffer (Sigma) was prepared by dissolving 1 capsule in 100ml distilled water. Microtiter plates were coated (50µl/well) with 0.3 µg/ml recombinant OPG (R&D Systems, 185-OS-025,100 µg/ml) in the coating buffer (Sigma, 0.05M carbonate- bicarbonate buffer, pH 9.6). The 96 well plate was sealed and incubated at 4°C for 24 ± 2 hours and then washed 7 times with 300 µl TBST (0.05 % Tween 20, 1 x TBS, pH 7.4) per well. Subsequently a 1% BSA blocking buffer (1 g BSA in 100 ml TBST) was added and the plate was incubated at 23°C for 2 hours followed by 7 washes again with TBST. To create a standard curve, a polyclonal non-biotinylated anti-OPG antibody (Abcam, ab9986) was diluted in a 1% BSA blocking buffer in serial dilutions (from 5 to 500 µg/ml) and added to the appropriate wells (50µl/well). At this stage serum samples were diluted 1:2000 in a blocking buffer (1% BSA) and added (50µl/well) in duplicates to the wells. Serum from the previously described index patient (Riches et al 2009) was diluted 1:5000 in a blocking buffer and used as a positive control.

Negative control serum, obtained from a healthy individual with normal bone mineral density which tested negative in the previously described immunoprecipitation assay for OPG antibodies (Riches et al.2009) , was diluted 1:2000 in blocking buffer and added to the wells. The well contents were mixed by gently tapping the plate for approx. 1 min and then the plate was re-covered and incubated at 37°C for 1 hour. After incubation, the plate was washed 8 times with TBST and dried thoroughly. At this stage the secondary peroxidase conjugated, goat anti-human detection antibody detecting heavy and light chains of IgG and IgM (Jackson Immuno Research Labs. Inc., 109-035-127) was diluted 1: 40.000 and added to each test well. This step was followed by another incubation at 23°C for 1½ hours. Following this incubation, the plate was washed 8 times as done previously and dried.

For visualisation, 100µl of 3,3′, 5,5′-Tetramethylbenzidine (TMB) substrate solution (Sigma T0440) was added to each test well. The plates were incubated in the dark at 23°C for approximately 5 min followed by the addition of 50 µl of sulphuric acid stop solution (0.16 M) to each well. Within 30 min of adding the stop solution absorbance was measured at 450 nm with the microplate reader (BioTek, Synergy HT). Measurement of the optical density and a comparison to the standard curve allowed the computerised calculation (Gen5 Software) of the resulting antibody concentration in each sample. A typical standard curve is shown below in Figure 2.2.

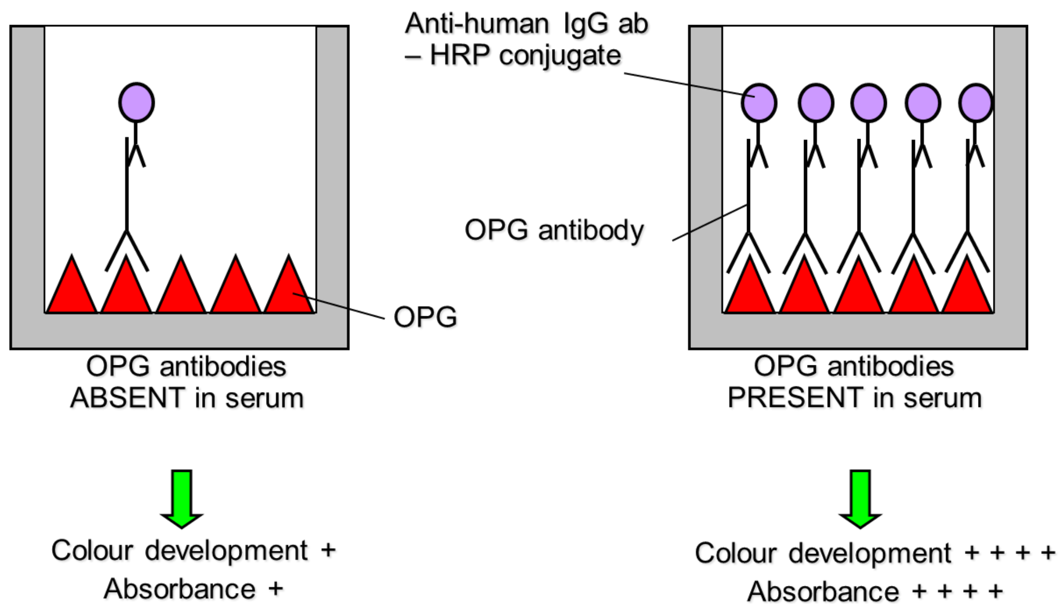

Figure 2.1 Schematic depiction of principles of OPG ab ELISA. *A 96 well plate is coated with human recombinant OPG, used as a capture antigen. The OPGab present in the serum will bind to the OPG in proportion to their concentration in the serum. HRP conjugated secondary antibodies attach to the bound OPG ab and can be visualized by the addition of TMB substrate. The colour development is proportional to the serum concentration of the OPG antibodies.*

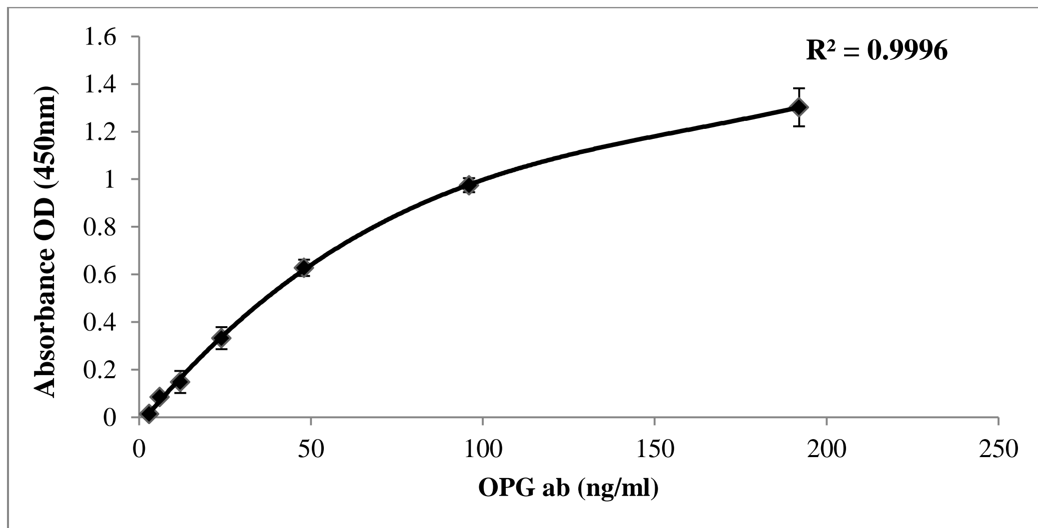

Figure 2.2 Typical standard curve of ELISA measuring circulating antibodies against OPG. *The antibody concentration is calculated against a 4 parameter logistic equation, r^2=0.9996.*

2.1.2 OPG ab ELISA – updated

(used for the analysis of OPG autoantibodies in Ankylosing Spondylitis, chapter 5)

This second version of the original ELISA was developed in order to avoid the usage of a murine polyclonal antibody (produced in a rabbit) for the creation of the standard curve. We recognized that secondary anti-human IgG antibodies may bind with different affinity to the antibodies from a human serum and to the primary antibody used for the standard curve, which is produced in a rabbit. The difference in affinity may introduce an error into the antibody quantification through ELISA. The protocol of the updated ELISA looks similar to the original one with the exception of the standard curve and the omission of a drying step after coating the plates with OPG. Human recombinant OPG (R&D Systems) was reconstituted as before to an OPG stock solution of 100µg/ml and stored at 4°C for later use.

The OPG stock (100ng/µl) was diluted to 1ng/µl using 99µl stock + 9801µl of coating buffer per plate. The coating buffer (Sigma) was prepared by dissolving 1 capsule in 100ml distilled water.

A Costar high-binding micro-titre plate (Corning) is used for the ELISA. 100µl of diluted OPG is added to all wells resulting in a total of 100ng OPG per well. After the plate was sealed with a plate sealer it was incubated at 4°C for approximately 24 hours (±2 hour). After the incubation process, the wells are washed twice with 200µl/well of phosphate-buffered saline (PBS) containing 0.05% Tween-20 (washing buffer) wash buffer and blocked with a blocking solution (PBS with 3% non-fat dry milk) for 1 hour at room temperature.

The standard curve was now prepared using serum from the index patient.

The serum is initially diluted to 1 in 100 in binding buffer (PBS-3% bovine serum albumin (BSA) and is then further diluted to 1/2500, 1/5000, 1/10000, 1/20000, 1/25000 and 1/50000. Neat positive was given an arbitrary value of 100 units, so that the standard curve ranges from 0.002 to 0.04 units. All samples that measured below the lower limit of the standard curve were assigned 0. All serum samples from patients that needed to be analyzed were diluted to 1/1000 (typically 2µl of the sample was added to 198µl of the binding buffer, with a second dilution step of 30µl added to 270µl of the binding buffer) and added to the wells. After 1 h of incubation at room temperature, the plates were washed five times with wash buffer. Now the secondary antibody that was a peroxidase conjugated goat anti-human detection antibody (Jackson ImmunoResearch) was diluted to 1:20,000 and added at a volume of 100ul per well. The plate was incubated again at room temperature for 1 hour and subsequently washed five times. For the final step of colour development 100µl of TMB substrate solution (Sigma) was added to each well and the plate was incubated at 24°C for 30 minutes to permit for colour development. In order to avoid interference from UV light the plate was covered with foil during this incubation step. Finally 100µl of TMB stop solution (KPL) was added to each well of the plate and the optical density for all samples was measured at 450nm with a reference wavelength

of 640nm. Sample concentrations were calculated by an automated four-parameter analysis by the plate reader's software.

2.1.3 RANKL and OPG measurements

2.1.3.1 Free serum RANKL measurements

Free soluble RANKL levels were measured using an Enzyme-Linked Immunosorbent Assay (ELISA) (BI-20452, Biomedica Gruppe Vienna, Austria) that detects soluble, uncomplexed human RANKL by using recombinant OPG as the capture antigen. All samples were defrosted from minus 70°C and brought to room temperature prior to assay. Wash buffer, standards and controls were reconstituted according to the manufacturer's protocol and added to the respective wells. 100 µl of undiluted serum sample was added to the respective wells. The duplicate samples were diluted to 1:10 by adding 10ul of the corresponding serum sample to 90 µl of the Wash buffer which had already been added to the wells. The assay was performed according to the manufacturer's protocol. To each well 100 µl of biotinylated anti sRANKL antibody was added and the plates were incubated overnight (20 hours) at room temperature while being vigorously shaken by a plate shaker. After incubation, the wells were aspirated and washed 5 times with 300 µl wash buffer. Into each well 200 µl conjugate was added and the plates were incubated at room temperature for 1 hour. After incubation the plates were washed again 5 times. First 100 µl of Amplifier A and subsequently 100 µl of Amplifier B were added, followed by incubation at room temperature. After 20 min the absorbance was read with a micro plate reader periodically at 490nm every 5 minutes in order to establish the correct time point for the addition of the stop solution. After 30 minutes the highest standard reading had an optical density (OD) value of 2.0, the blank a value of less than 0.2 and the reaction was stopped by the addition of 50 µl STOP solution to each well. The absorbance at 490nm with a reference at 630nm was measured with a micro plate reader. The manufacturer indicates that the intra- and interassay coefficients of variation (CV) for free sRANKL are 3–5% and 6–9%, respectively. When using the results of an undiluted sample (according

to the protocol) and the same sample 1:10 diluted as duplicate the intraassay CV rose to 78.92% which made the second diluted sample reading invalid.

2.1.3.2 Total serum RANKL measurements

Total RANKL levels (free RANKL and RANKL complexed to OPG) were measured using a commercially available ELISA (Cat.No.: RD193004200R, BioVendor, R&D) which uses polyclonal RANKL antibodies as capture antigens. The assay was performed according to the manufacturer's protocol. Serum samples were brought from minus 70°C to room temperature and diluted to 1:100 by adding 2.5 µl of serum to 247.5 µl of dilution buffer (provided by a commercial kit). The standards, controls and wash solutions were reconstituted with dilution buffer using the volumes stated in the Certificate of Analysis just prior to the assay. 100 µl of standards, controls, and samples were pipetted (in duplicate) into the appropriate wells. The plate was incubated at 2-8°C overnight with shaking at ~300 rpm. After incubation the plates were washed 5 times and dried, 100 µl of biotin labelled antibody was added followed by 1 hour incubation at room temperature. All the wells were washed again 5 times and 100 µl of Streptavidin-HRP Conjugate was added to each well. The plates were incubated at room temperature for 1 hour whilst being shaken vigorously. After another wash cycle (5 times) 100 µl of substrate solution was added to each well. The plate was covered with foil to protect it from light and incubated for 25 min at room temperature. The colour development was stopped by the addition of 100 µl of stop solution and the absorbance was determined by a microplate reader at 450nm with a reference of 630nm. The intraassay CV according to the manufacturer is 7% and inter-assay CV 11-13% for this method. We obtained an intraassay CV of 9.3% when analyzing 38 samples.

2.1.3.3 Serum Osteoprotegerin measurements

The Osteoprotegerin (OPG) concentrations in the serum samples were measured with a commercially available ELISA kit (Human Osteoprotegerin ELISA, Cat.No.: RD194003200, BioVendor, R&D). The assay was performed according to the manufacturer's protocol. Serum samples were brought from minus 70°C to room temperature. Standards, controls were reconstituted with the dilution buffer using the volume stated on the Certificate of Analysis. The prepared standards and the reconstituted controls were further diluted 3 times with the dilution buffer (100 µl standard + 200 µl of dilution buffer). 100 µl of the diluted standards, controls, dilution buffer (Blank) and samples were pipetted in duplicates into the appropriate wells. The plate was incubated at room temperature for 1 hour with shaking at ~300 rpm. Subsequently, the wells were washed 3 times with 350 µl of the prepared wash solution and dried by inverting the plate onto paper towels. 100 µl of the biotin labelled antibody was added to each well and the plates were incubated at room temperature for 1 hour with shaking (300rpm). The wells were washed again 3 times followed by the addition of 100 µl of Streptavidin-HRP conjugate with 30 minutes incubation at room temperature with shaking. After the wells had been washed again 3 times 100 µl substrate solution was added to each well. The plates were covered with foil to protect from light and incubated for 10 min at room temperature. Colour development was then stopped by the addition of 100 µl of stop solution and the absorbance at 450nm (with a reference of 630 nm) was determined. The intra and inter-assay CV are, according manufacturer, 3-5% and 2-9% respectively. We obtained an intraassay CV of 4.1% when analyzing 39 samples.

2.1.4 Serum CTX measurements

We measured C-terminal telopeptides of Type I collagen (CTX) concentrations in serum according to the manufacturer's instructions using an automated chemiluminescence assay (CTX-1 IDS-iSYS, CLIA, Immunodiagnostic Systems, Tyne and Wear, UK). The manufacturer's

reported inter-assay coefficient of variation was 5.2 and 2.5%. The limit of quantification (LoQ) was 0.033 ng/ml and the reported range of the CTX-I assay was 0.05 to 6.0 ng/ml(Seres et al., 2010).

2.2 Immunoglobulin purification

Immunoglobulins (IgGs) were purified from serum in order to investigate the effect of IgGs on free RANKL measurements. Furthermore purified IgGs were used in the functional assay and in order to investigate if purified IgGs containing high concentrations of OPG ab have the ability to neutralize OPG.

2.2.1 Melon Gel Immunoglobulin G (IgG) purification

This type of IgG purification enables the purification of antibodies from serum. The method is based on direct filtration of IgGs as the Melon Gel Resin contains a proprietary ligand which binds most proteins but is unable to bind IgGs.

2.2.1.1 Buffer Exchange Procedure

To avoid the need of diluting the serum sample when performing the Melon Gel Igg Spin purification we performed a buffer exchange of the serum sample using a Zeba™ Desalt Spin Column (Thermo Scientific Pierce, 89882). This allowed the preparation of the serum sample with the purification buffer which was immediately used for IgG purification. The buffer exchange was performed according manufacturer's protocol.

The spin columns were placed in 2.0 ml collection tubes. The tubes were centrifuged at 1,500 × *g* for 1 minute to remove storage solution. 300 µl of Purification buffer was added on top of the resin bed and the tubes were centrifuged at 1,500 × *g* for 1 minute to remove the buffer. The previous step was repeated three additional times and the buffer was discarded from the collection tube. Now the column was placed in a new collection tube, the cap removed and 130 µl of serum sample was applied to the top of the compact

resin bed. The collection tube containing the serum sample was centrifuged at 1,500 × *g* for 2 minutes and the buffer exchanged sample was collected. For each serum sample this procedure was performed twice with two separate columns in order to obtain 260 µl of buffer exchanged serum.

2.2.1.2 Spin-column Procedure for IgG Antibody Purification

Melon™ Gel IgG Spin Purification Kit (Thermo Scientific 45206) was used for purification of IgG from serum. The purification was performed according to the manufacturer's protocol. Melon Gel IgG Purification Support was brought to room temperature. 500 µl of gel slurry was dispensed into a spin column which had been placed in a micro-centrifuge tube. The uncapped column/tube assembly was centrifuged for 1 min at 5,000 × *g*, then the spin column was removed and the flow-through discarded. At this point 300 µl of purification buffer was added to the column, briefly centrifuged (10 s) and the flow-through was discarded. After two washes the bottom cap was placed onto the column. The undiluted serum samples (260 µl of buffer exchanged serum) were added to the column. The columns were capped and incubated for 5 min at room temperature with end-over-end mixing. Subsequently the bottom cap was removed from the columns and top cap loosened. The spin columns were re-inserted in the collection tube and centrifuged for 1min at 5,000 × *g* in order to obtain purified antibodies.

2.2.2 IgG purification with Protein G Spin columns

2.2.2.1 Procedure for Antibody Purification

In order to purify Immunoglobulin (IgG) from 200 µL of serum we have used IgG purification with NAb™ Protein G Spin columns, 0.2mL for Antibody Purification (Thermo Scientific). To start columns, buffers and patient sera were equilibrated to room temperature. The storage solution in the spin columns were centrifuged for 1 minute (5000 g) and the flow through (storage solution) discarded. The column was prepared by adding 400µL of Binding Buffer ((100mM phosphate, 150mM sodium chloride; pH 7.2 when dissolved

in 500mL of ultrapure water) which was mixed briefly and discarded through centrifugation (5000 g). This step was repeated once. The spin column was then capped and 250 µL of antibody-containing serum sample was added. Column and serum were mixed at room temperature (24°C) by end-over-end mixing for 10 minutes. The spin column was placed in a collection tube and centrifuged for 1 minute. The resulting flow through contained the nonbound sample components whereas the IgG were captured by Protein G coated spin column. Now the columns were washed three times by adding 400µL of Binding Buffer, brief mixing of buffer and resin and centrifugation of buffer. Before collecting the purified immunoglobulins, 40µL of Neutralization Buffer (1M Tris•HCl, pH 8.5) was put into the collection tubes. The spin column were put into one of the collection tubes and 400µL of IgG Elution Buffer (pH 2.8) was added to the spin column, mixed gently and centrifuged for 1 minute. The same step was repeated twice but the spin column was placed each time into different prepared collection tubes resulting in three different elution fractions. In order to determine which fraction(s) contain the purified antibody the protein content was quantified using Nanodrop technology with the Spectrophotometer Nanodrop reader 1000 3.7.0 (Thermo Scientific) by measuring the relative absorbance of each fraction at 280nm. The elution fractions were either used immediately for the functional assay or stored at -20°C.

In order to re-use the columns, the columns were washed three times with 400µL of Elution Buffer and then three times with 400 µL of Storage Solution (0.02% sodium azide in phosphate-buffered saline (PBS)). The regenerated columns were stored at 4°C and could have been used again up to ten times.

2.2.3 HEK-293 NF-KB reporter assay

We studied the functional effects of OPG antibodies on RANKL induced nuclear factor kappa B (NFκB) activation in the human-Embryonic-Kidney cell line, HEK-293 (Promega, E8520). The HEK-293 cells were cultivated in 75T flasks in growth medium (90% DMEM, 10% FCS, 1% Penicillin/Streptomycin (Pen/Strep, 50ug/ml Hygromycin B) and were maintained between 10% and 90% confluence in a 37°C, 5% CO2 tissue culture incubator. Growth medium was changed every 2 to 3 days and the cells required passaging every 4 days. 2 days before the assay the medium was changed to Hygromycin free growth media (90% DMEM, 10% FCS, 1% Penicillin/Streptomycin). One day before the assay 200 µl cell suspension was aliquoted into a 96 well plate at a density of 5.5x 10^5 cells/ml. The cells were incubated overnight and 2 hours prior to the assay the medium was changed to a serum free medium (97% DMEM, 2% TCH, 1% Pen/Strep) in order to serum-starve the cells. The cells were stimulated with 20 µl of 50 and 100 µg/ml RANKL , 100 µg/ml OPG ,in the presence or absence of purified IgG from the index patient known to have functional OPG antibodies as a positive control (Riches et al., 2009)or purified IgG from RA patients that tested positive or negative for OPG antibodies in this study. The IgG samples were diluted at 1:20 and 1:40. Following addition of these reagents, the cells were incubated for 3 hours. Subsequently, 150µl of the cell suspension was removed and discarded and the final analysis was performed on the remaining 50 µl of cell suspension. Then, 50 µl of Alamar Blue was added to each well in order to assess cell viability. Alamar Blue was measured with an excitation wavelength of 560 nm and an emission wavelength of 590 nm. Finally 50µl of one-Glo reagent (Promega E6120) were added to all 96 wells and the resulting luminescence was read after 3 minutes, 4 hours and 6 hours with the microplate reader (BioTek,Synergy HT). The Glo-response luminescence results were divided by Alamar Blue readings providing final luminescence per cell results in arbitrary units.

Chapter 3: Prevalence and predictors of Osteoporosis in Rheumatoid Arthritis

3.1 Abstract

Osteoporosis has previously been reported to be twice as common in patients with rheumatoid arthritis (RA) as compared with controls but these studies predate the introduction of aggressive management of RA. The aim was to evaluate prevalence and clinical predictors of osteoporosis in RA in a contemporary cohort and to develop a clinical tool for the identification of patients at risk. The prevalence of osteoporosis was related to clinical and demographic variables in 304 consecutive RA patients undergoing DEXA at a single centre between 2009 and 2010 and compared to the frequency of osteoporosis in a population based cohort of 903 subjects. The results of this study showed that the RA cohort were predominantly female (81.9%), with an average (±SD) age of 63.5±11.8 years and a disease duration of 9.6±10.2 years. Osteoporosis was present in 91(29.9%) patients at either the spine or total hip compared with 157/903(17.4%) of age and gender matched controls. In RA patients, osteoporosis was associated with female gender ($p=0.002$), age ($p<0.001$), time since menopause ($p<0.001$), BMI ($p<0.001$), ESR ($p=0.006$), Larsen score ($p=0.011$) and co-morbidities ($p=0.020$), but logistic regression analysis showed that only age and BMI were independent predictors. A predictive tool based on age and BMI was developed which had 91.4% sensitivity for detection of osteoporosis in an independent RA population. In conclusion the prevalence of osteoporosis in RA remains high in the modern era despite aggressive management and the use of biologic therapy. Most RA patients with osteoporosis can be identified by a simple algorithm taking age and BMI into account.

3.2 Introduction

Rheumatoid arthritis (RA) is associated with reduced bone density (Haugeberg et al., 2000a, Sinigaglia et al., 2000, Haugeberg et al., 2000b) and an increased risk of fragility fractures (van Staa et al., 2006). The pathophysiology of osteoporosis is complex with contributions from general risk factors such as age, low body weight and postmenopausal status, along with disease-specific risk factors such as decreased mobility, corticosteroid use and production of pro-inflammatory cytokines (Gough et al. 1994b;Haugeberg et al. 2000a;Laan et al. 1992;Sinigaglia et al. 2000). The importance of inflammatory disease as a pathogenic factor is emphasised by the fact that there is a positive correlation between disease activity and biochemical markers of bone resorption in RA (Gough et al. 1994b). Analysis of data from the Oslo registrar over a decade ago indicated that about 15% of RA patients had osteoporosis at the femoral neck as defined by dual energy x-ray absorptiometry (DEXA) (Haugeberg et al., 2000a). By comparing BMD values in the RA population with those from the manufacturer's reference range it was estimated that the risk of osteoporosis was increased by two-fold as compared with the general population (Haugeberg et al., 2000a). Since this time there have been major changes in the management of RA with earlier and more aggressive use of synthetic disease modifying antirheumatic drugs (DMARD) and deployment of biologic agents for patients who do not respond adequately (Smolen et al 2010). Since there is evidence that control of inflammation with TNF alpha inhibitors in RA may protect against systemic osteoporosis as well as preventing local bone erosions (Vis et al., 2006) we have evaluated the prevalence and risk factors for the development of osteoporosis in a contemporary population of patients with RA presenting to a UK referral centre. We also developed an algorithm for the investigation of osteoporosis based on demographic and clinical risk

factors for the disease within this population with the aim of better targeting DEXA to patients at highest risk of having osteoporosis.

3.3 Patients and Methods

3.3.1 Rheumatoid Arthritis cohort

All patients attending a DEXA scan at the Western General Hospital are audited with the acquisition of basic demographic data, detailed information on osteoporosis and fracture risk factors, lifestyle factors and calcium intake. Patients with Rheumatoid Arthritis were identified by the selection of referring physician and review of case notes. Additional clinical data was collected through review of digital and paper case notes.

The study cohort comprised all patients with rheumatoid arthritis who had been consecutively referred for DEXA examination over a 24-month period between January 2009 and December 2010. In the resulting group of 304 patients, information was collected on gender, age, weight, height, alcohol intake, smoking, age at menopause, self-reported non-vertebral fractures and dietary calcium intake (assessed by food frequency questionnaire) at the time of the DEXA scan according to routine practice. Details of disease duration, presence of rheumatoid factor and anti-CCP antibodies, erosions, inflammatory markers, and anti-rheumatic and anti -osteoporosis drug treatment were obtained through case note review. Inflammatory activity around the time of DEXA examination was assessed by taking the average of 3 measurements of ESR and/or CRP performed within a 1 year window before the time of DEXA. For statistical analysis we classified anti-rheumatic and anti-osteoporosis treatments into categories of current use, past use (more than 6 month before the DEXA assessment) and ever use. We also collected information on the following co-morbidities: cardiovascular disease, respiratory disease, cerebrovascular diseases, neurological disease, chronic

renal and liver impairment, gastrointestinal disease, diabetes, thyroid disease and cancer. The frequency of osteoporosis in the RA population was compared with that in a population based cohort of 903 subjects (709 women and 194 men) of average (±SD) age of 62.7±10.3 years who underwent DEXA scanning as part of the ORCADES study. Details of this study have previously been described (Estrada et al. 2012). Relevant clinical and demographic information was collected in the controls as described for the cases and patients with RA excluded. The prediction tool for osteoporosis described in this paper was evaluated in an independent cohort of 171 RA patients (130 women and 41 men) with average age of 60.8±12.3 years and average BMI of 28.0±5.8 kg/m^2 who underwent DEXA scanning at our centre between 2010 and 2013. Information about the RA population was obtained through an audit of data that had been collected during routine clinical practice hence ethical approval was not required.

3.3.2 ORCADES cohort

The Orkney Complex Disease (ORCADES) study is a population-based study for health research, designed to identify genetic risk factors for complex diseases (Orkney Research Ethics Committee approval - 27/02/2004). Out of a total population of 12000 Orcadians 2000 healthy volunteers have been recruited into this cross-sectional study. Most of the patients (1578) had a bone mineral density assessment by DEXA and beside basic demographic measures, data on fracture risk factors, family history and medical and surgical history had been collected. Dr James Wilson has kindly allowed access to plasma and serum of 200 healthy controls for the measurement of OPG ab and subsequent calculation of a normal range for OPG ab. Furthermore I've been given access to the BMD data of 2000 healthy controls for the use as a control cohort which was used to compare BMD and osteoporosis prevalence between Rheumatoid Arthritis patients and healthy controls. I've not recruited any patients but I've assisted in the transfer of

samples from the Orcades to the Edinburgh Clinical Trials Unit. Recruitment and characteristics of this cohort was described before (Estrada et al. 2012).

3.3.3 Joint erosions

Hand radiographs were reviewed and scored for the presence of erosions and joint damage using the modified Larsen score as previously described (Larsen, 1995). In total 140 sets of radiographs were available for analysis in the study cohort.

3.3.4 Measurements of BMD measurements and fracture risk assessment

Measurements of BMD in all study populations were made by dual-energy X-ray absorptiometry (DEXA) at the lumbar spine (L1-L4) and femoral neck using a Hologic QDR 4500 osteodensitometer. Vertebral fractures were initially ascertained on lateral spine images from T4 to L4 using vertebral fracture assessment (VFA) software of the DEXA device. All vertebral fractures were subsequently confirmed on review of lateral x-rays of the thoracolumbar spine by radiologists according to normal clinical practice. Vertebral morphometry was not performed. Data on non-vertebral fractures was obtained by questionnaire and validated by analysis of case records and/or radiology reports.

3.3.5 Statistical analysis

Statistical analyses were performed using SPSS version 16.0 (SPSS, Chicago, IL, USA). Two tailed independent student t-test was used for between-group comparisons of continuous data. If the variables were not normally distributed, Mann Whitney U test was applied. The chi-square test was used to evaluate for differences between categorical variables. Binary stepwise logistic regression was used to identify independent predictors of osteoporosis and fractures with inclusion of variables that were significantly ($p \leq 0.05$) associated with osteoporosis or fractures with in the case-control

analysis. The 95% confidence intervals (CI) were calculated for differences between means and odds ratios (OR) for paired data.

Sensitivity and specificity were calculated according to standard methods and receiver operating characteristics (ROC) curves were constructed to identify models with the highest AUC (Area under the curve) and the simplest algorithm.

3.4 Results

3.4.1 Patient Characteristics

Relevant clinical and demographic characteristics of the RA cohort are shown in Table 1 broken down by gender. The mean age of the study population was 64 years and 81.9% were female. There was no significant difference between genders for age, BMI, current smoking status, seropositivity, disease duration, Larsen scores or fractures. As expected, men were taller and heavier than women, had significantly higher BMD values (p=<0.001), higher alcohol consumption (p=0.016), had previously smoked more often (p=0.034) and had higher CRP levels (p=0.005). Nearly all of the patients (96.8%) had been treated with a disease modifying drug (DMARD), 53.6% had received glucocorticoids in the past and 33% were on either oral glucocorticoids or received regular intramuscular glucocorticoid injections at the time of the DEXA scan. For patients on glucocorticoids, the average dose was 7.4±10 mg prednisolone daily with men receiving a significantly higher dose than women (p=0.009) (Table 3.1). About one quarter of patients (22.7%) had previously received anti-TNF treatment and 17.1% were receiving anti-TNF treatment at the time of the DXA scan. About one fifth of patients (20.7%) were receiving a bisphosphonate and 26.6% calcium and vitamin D supplements.

Table 3.1 Patient demographics and disease characteristics of RA cohort

	Women (n=249)	Men (n=55)	p-value
Demographics			
Age, yrs,	63.8 ± 11.8	62.3 ± 11.5	0.398
BMI (kg/m^2)	27.5 ± 6.1	26.9 ± 4.4	0.527
Post-menopausal	210 (84.3)	-	
Age at menopause (yr)	46.9 ± 6.5	-	
Current Smoker	64 (25.7)	19 (34.6)	0.183
Cigarettes per day	15.3 ± 7.3	15.8 ± 5.1	0.808
Drinks alcohol	70 (28.5)	23(41.8)	0.053
Alcohol units/week	6.41 ± 5.0	14.1 ± 20.7	0.016
Calcium intake, (mg/day)	818.0 ± 388	833 ± 38	0.643
Disease characteristics			
Disease duration (months) (n=279)	129.1 ± 129.1	152 ± 139	0.280
Time from diagnosis to DEXA (n=220)	109.1 ± 117.8	140.4 ± 141.6	0.135
RF or CCP positive	143 (70.5)	41 (80.4)	0.200
CRP mg/l (n=199)	27.4 ± 38.3	39.2 ± 43.0	0.005
ESR mm/h (n=252)	32.8 ± 23.5	33.3 ± 22.5	0.814
Erosions (n=221)	83 (47.2)	19 (45.2)	0.823
Larsen score (n=140)	50.52 ± 25.49	46.48 ± 26.39	0.454
Number of co-morbidities	0.92 ± 0.92	0.91 ± 0.87	0.945
Current DMARD therapy (Y/N)	202 (81.1)	46 (83.6)	0.664
Current Biologic therapy (Y/N)	56 (22.5)	9 (16.4)	0.316
Current steroid therapy (Y/N)	76 (30.8)	23 (41.8)	0.114
Current Prednisolone dose mg (n=89)	6.0 ± 6.5	12.4 ± 17.3	0.009
Spine BMD (g/cm^2)	0.89 ± 0.15	0.99 ± 0.13	<0.001
Total hip BMD (g/cm^2)	0.78 ± 0.16	0.88 ± 0.14	<0.001
Osteoporosis of Spine or Hip	84 (33.7)	7 (12.7)	0.002
Vertebral fracture	25 (10.0)	7 (12.7)	0.557
Non-vertebral fractures	83 (33.3)	9 (16.4)	0.013
Hip fracture	13 (5.2)	1 (1.8)	0.276

Values are number (%) or mean (standard deviation). The number of observations available is shown in brackets for variables with >1% of missing data. BMI=Body Mass Index, RF=Rheumatoid Factor, ACCP = Anti-cyclic Citrullinated Peptide, CRP = C reactive protein ESR= Erythrocyte Sedimentation Rate, Rad.= Radiographic erosions on hand or feet x-rays, BMD=Bone Mineral Density

3.4.2 Risk factors for osteoporosis

Relevant clinical and demographic variables in patients with and without osteoporosis as defined by a BMD T-score of -2.5 or less on DEXA at the spine or femoral neck are summarised in Table 3.2. Osteoporosis was associated with female gender, age, BMI, years since menopause, number of co-morbidities, Larsen Score, CRP and ESR. There was no association between seropositivity for rheumatoid factor or CCP, previous or current glucocorticoid intake, anti-TNF treatment or DMARD therapy. As expected vertebral and non-vertebral fractures were more common in patients with osteoporosis ($p=0.002$ and $p<0.001$).

Table 3.2 Clinical and demographic characteristics of patients from the study cohort with and without osteoporosis

	Normal BMD	Osteoporosis	p-value
Number	213 (70.1)	91 (29.9)	
Female gender	165 (77.5)	84 (92.3)	0.002
Age (yrs)	60.5±11.2	70.6±9.8	< 0.001
BMI (kg/m^2)	28.8±5.7	24.1±4.6	< 0.001
Age at Menopause (n=196)	47.2±6.2	46.5±7.1	0.901
Years since Menopause	16.4±10.1	24.6±10.9	<0.001
Calcium intake (mg) (n=304)	834±430	790±430	0.949
Ex-Smoker	89 (41.8)	38 (41.8)	0.997
Current Smoker	59 (27.7)	24 (26.4)	0.812
Alcohol drinker (n)(n=305)	69 (32.9)	24 (26.4)	0.264
Alcohol units/week	8.18±12.86	7.88±5.48	0.912
Duration of RA (months) (n=283)	133±127	134±141	0.938
Seropositive (n=255)	133 (73.9)	51 (71.8)	0.740
Erosions (n=221)	76 (47.8)	26 (44.1)	0.624
Larsen score (n=140)	47.07± 26.38	56.59± 22.42	0.011
ESR (mm/hr) (n=252)	30±22	40±26	0.006
CRP mg/l (n=199)	26±37	38±44	0.023
Ever received glucocorticoids	119 (56.4)	43 (47.3)	0.144
Current glucocorticoid	73 (34.6)	26 (28.6)	0.306
Current Prednisolone dose (mg) (n=89)	8.3±11.4	4.8±3.8	0.162
Ever received anti-TNF therapy	53 (24.9)	16 (17.6)	0.164
Number of co-morbidities	0.85±0.9	1.1±0.9	0.020
Vertebral fracture	15 (7.1)	17 (18.7)	0.002
Hip fracture	2 (0.9)	12 (13.2)	0.001
Non-vertebral fractures	48 (22.5)	44 (48.4)	0.001

Values are numbers (%) or mean ± SD. P-values refer to the difference between the groups assessed by t-test for continuous variables and chi-square test for categorical variables. Numbers in parenthesis show the number of patients in which data was available for variables where data was missing in more than 1% of subjects; BMI=Body Mass Index,RF=Rheumatoid Factor, ACCP = Anti-cyclic Citrullinated Peptide, ESR= Erythrocyte Sedimentation Rate

Multivariable logistic regression was used to identify the independent predictors of osteoporosis. For this analysis factors that were associated with osteoporosis in the case control study at a p-value of 0.05 or less were entered into the model, except CRP which was excluded due to a large number of missing values. This revealed that the only independent predictors of osteoporosis were age (OR=1.08, 95% CI 1.03, 1.14) and BMI (OR=0.82, 95% CI 0.74, 0.91). The variables ESR (OR=1.01 CI 0.99, 1.03), number of co-morbidities (OR=1.05 CI 0.57, 1.96), gender (OR=0.44 CI 0.14, 1.42), Larsen score (OR =1.01 CI 0.99, 1.03) and years since menopause (OR=1.00 CI 0.91,1.10) failed to reach statistical significance. Overall the regression model explained 44 % of variance (Nagelkerke R^2=0.44). Further logistic regression analysis of the RA study group in which patients receiving bisphosphonates were excluded revealed very similar results to those mentioned above (data not shown).

3.4.3 Prevalence of osteoporosis in RA population versus controls

Levels of BMD at the lumbar spine and total hip in RA cases and controls are illustrated in Figure 3.1, broken down by gender. Levels of BMD were significantly lower in female RA cases as compared with female controls from the age of 45 years onwards (Panels A and B). In males, total hip BMD was also significantly lower from the age of 45 onward. There was also a trend for lower spine BMD in men but this was not significant at any individual time point (Panels C and D). However overall spine BMD in men was significantly lower in the RA population than in healthy controls (-0.7±1.6 *vs.* -1.3±1.3, p<0.001). The overall prevalence of osteoporosis at spine and total hip (as defined by a T-score less than -2.5) in the cases and controls is summarised in Table 3.3

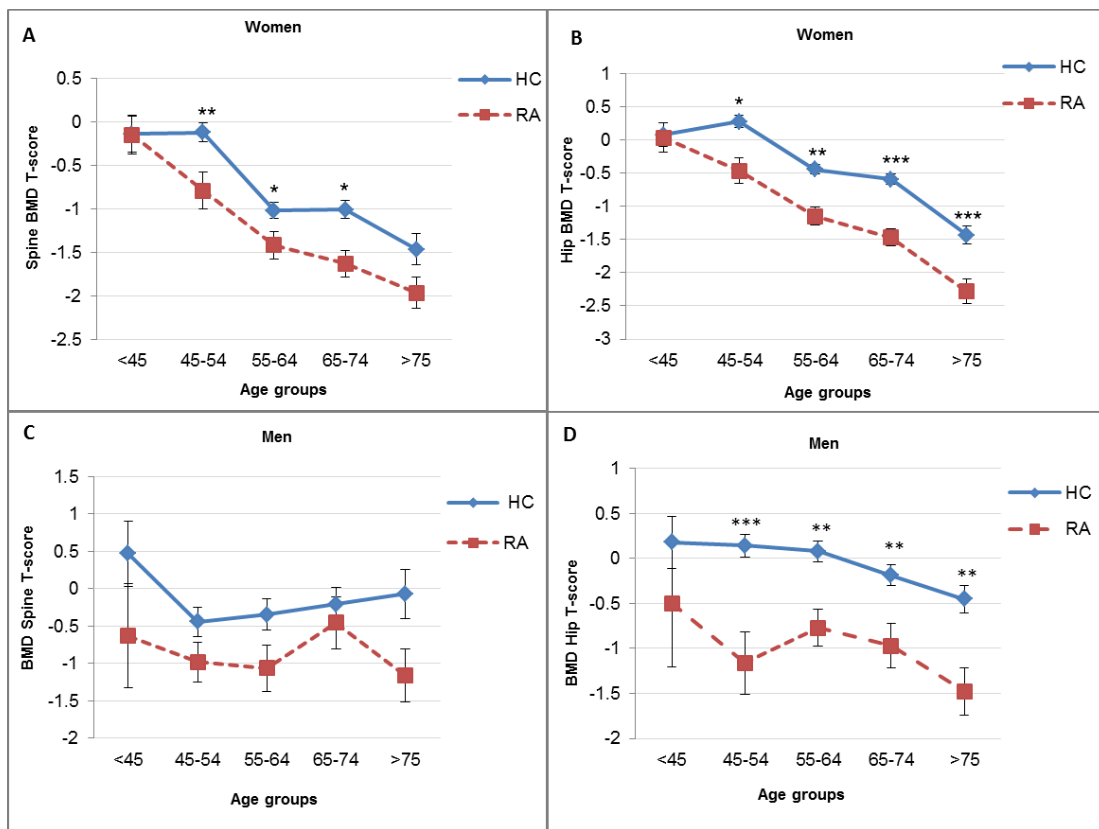

Figure 3.1 Spine and Hip BMD of RA patients and healthy controls (HC)

*BMD= Bone Mineral Density *p< 0.05 **p<0.01 ***p<0.001*

3.4.4 Development of a diagnostic algorithm

Based on the logistic regression analysis we developed an algorithm with which to predict the presence of osteoporosis in RA considering men and women separately. A receiver-operator curve (ROC) analyses showed that age and BMI alone were significant predictors of osteoporosis with an area under the curve (AUC) of 0.848 (CI 0.798-0.891). Addition of other variables such as ESR, sex and number of co-morbidities into the model did not improve the ROC (AUC = 0.861 [95% CI 0.812-0.910]) curve significantly (p=0.74) . Based upon this, we divided subjects into four BMI categories (<20, 20-24.9, 25-29.9 and >30) and four gender specific age categories (<44.9, 45-54.9, 55-59.9 and > 60 for women and <49.9, 50-59.9, 60-69.9,>70 for men). By allocating 1 point for each stratum we developed a risk stratification

score based on age and BMI alone with which to identify patients at low, medium or high risk of having osteoporosis. The prevalence of osteoporosis (at either spine or femoral neck) in RA patients according to these age and BMI strata are shown in Figure 3.2. Using these stratification variables we developed an algorithm which we named the osteoporosis prediction in RA (OPRA) tool. The OPRA risk assessment tool is freely accessible under http://www.opradxa.org.uk/opratool.htm .

Table 3.3 Prevalence of osteoporosis in RA patients (study cohort) and controls

	Spine OP			**Total Hip OP**		
Age groups	Cases	Controls	p-value	Cases	Controls	p-value
<45	0 (0%)	1 (2.4%)	1.000	0 (0%)	0 (0%)	1.000
45-54	4 (7.8%)	4 (2.5%)	0.103	2 (4.3%)	1 (0.6%)	0.133
55-64	16 (18.0%)	41 (14.1%)	0.397	8 (9.5%)	6 (2.1%)	0.002
65-74	20 (25.3%)	51 (17.5%)	0.146	10 (13.7%)	17 (6.0%)	0.028
75+	17 (28.8%)	31 (25.2%)	0.596	24 (41.4%)	16 (13.4%)	<0.001
All	57 (19.3%)	128 (14.2%)	0.036	44 (15.8%)	40 (4.5%)	<0.001

OP= Osteoporosis, Values are individuals with osteoporosis (%) in each age group

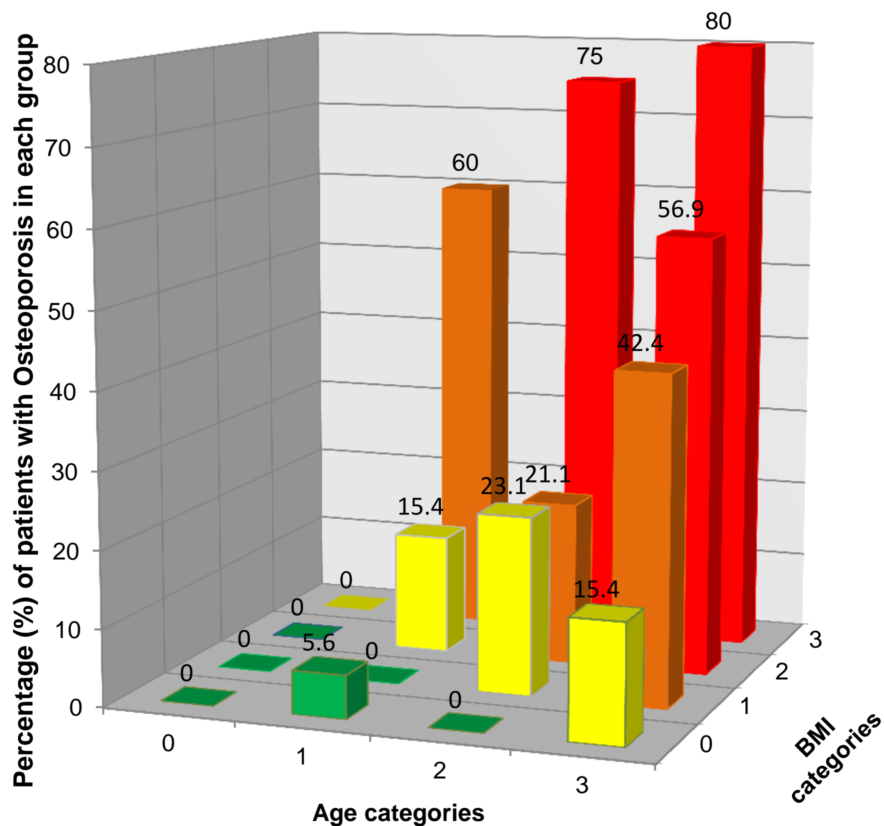

***Figure 3.2 The prevalence of osteoporosis in the study cohort, divided in different subgroups of individuals according to age category and body mass** index, is shown. The BMI categories were 0 (>30 kg/m^2); 1 (25-29.9 kg/m^2); 2 (20-24.9 kg/m^2) and 3 (<20 kg/m^2). The age categories were 0 (<44.9 women, <49.9 men); 1 (45-54.9 women; 50-59.9 men); 2 (55-59.9 women, 60-69.9 men) and 3 (>60 women and >70 men). OPRA= Osteoporosis prediction in rheumatoid arthritis is based on point allocation according to age and BMI. BMI=Body Mass Index*

The performance of OPRA was evaluated in an independent cohort of 171 RA patients (41 men and 130 women) who underwent DEXA scanning at our unit between 2010 and 2013. The tool had a sensitivity of 93.5% in women and 75% in men for the prediction of osteoporosis with a specificity of 34.3% and 48.6% respectively. The positive predictive value was 30.9% in women and 13.6% in men and negative predictive value 94.4% and 94.7% respectively. We compared the performance of OPRA with other tools that have been used for the prediction of osteoporosis including OST (Richy et al., 2004), ORAI (Cadarette et al., 2000), and OSIRIS (Sedrine et al., 2002). The results are shown in Table 3.4. The OPRA tool was more sensitive than the other tools in predicting osteoporosis in both men and women but had lower specificity when compared with the other risk assessment tools. The only other prediction tool which has been tested in men (OST) was a poor predictor of osteoporosis in males (25% sensitivity).

Table 3.4 Performance of the osteoporosis risk assessment tool developed in this study (OPRA) with other risk assessment tools in the replication cohort.

		OPRA	OST	ORAI	OSIRIS
Sensitivity (%)					
All		91.4	73.5		
Women		93.5	80.00	83.87	80.00
Men		75	25.00	-	-
Specificity (%)					
All		*38.2*	61.90		
Women		34.3	58.16	46.46	59.77
Men		48.6	72.22	-	-
PPV (%)					
All		27.6	32.90		
Women		30.9	36.92	32.91	36.36
Men		13.6	89.66	-	-
NPV (%)					
All		94.5	90.20		
Women		94.4	90.48	90.20	91.23
Men		94.7	9.09	-	-

For OPRA, the values shown are for subjects with a score of three or more. The values shown refer to prediction of osteoporosis at either the spine or femoral neck. OST= Osteoporosis Self-Assessment Tool (Richy et al., 2004), ORAI= Osteoporosis Risk Assessment Instrument (Cadarette et al., 2000), OSIRIS=Osteoporosis Index of Risk (Sedrine et al., 2002), PPV= Positive Predictive Value, NPV= Negative Predictive Value

3.4.5 Risk factors for fractures

Age, years since menopause, spine BMD and total hip BMD were significantly associated with the presence of vertebral fractures in a case-control analysis (Table 3.4). When we performed logistic regression analysis entering these factors into the model we found that only age (OR 1.06 CI [1.01- 1.12]) and total hip BMD (OR 0.04 CI [0.01- 0.89) were independent predictors. Non-vertebral fractures were significantly associated with female gender, Larsen score, spine BMD and total hip BMD in a case control analysis (Table 3.4). Subsequent logistic regression analysis identified only spine (OR 0.07 CI [0.01-0.89]) and hip BMD (OR 0.03 CI[0.01-0.05]) as independent predictors of non-vertebral fractures.

	Control *	Vertebral fractures		Control*	Non-vertebral fractures
Number	272 (89.5)	32 (10.5)		212 (69.7)	92 (30.3)
Female gender	224 (82.4)	25 (78.1)		166 (78.3)	83 (90.2)*
Age (yrs)	62.6±11.7	71.6±8.4***		62.9±11.8	65.1±11.5
BMI (kg/m^2)	27.4±5.7	27.1±6.9		27.7±5.5	26.6±6.5
Age at Menopause (n=196)	47.0±6.4	46.0±7.6		47.1±6.3	46.4±6.9
Years since Menopause	18.7±10.9	27.0±10.8		18.6±10.7	21.7±11.8
Calcium intake (mg) (n=304)	818±420	841±497		843±436	770±408
Ex-Smoker	109 (40.1)	18 (56.3)		90 (42.5)	37 (40.2)
Current Smoker	72 (26.5)	11 (34.4)		60 (28.3)	23 (25.0)
Alcohol drinker (n)(n=305)	87 (32.3)	6 (18.8)		66 (31.6)	27 (29.3)
Alcohol units/week	8.3±11.8	7.6±5.0		8.5±13.0	7.8±6.6
Duration of RA (months) (n=283)	131±127	157±165		125±127	152±140
Seropositive (n=255)	166 (73.5)	18 (72.0)		135 (75.0)	49 (69.0)
Erosions (n=221)	93 (47.9)	9 (37.5)		72 (45.9)	30 (49.2)
Larsen score (n=140)	49.1±26.4	55.6±15.9		47.3±25.6	56.6±24.8*
ESR (mm/hr) (n=252)	32±23	41±24		34.4±24.2	29.4±20.6
CRP mg/l (n=199)	29±36	36±31		31.9±43.6	24.4±26.6
Ever received glucocorticoids	145 (53.7)	17 (53.1)		113 (53.8)	49 (53.3)
Current glucocorticoid	91 (33.7)	8 (25.0)		91 (33.7)	8 (25.0)
Prednisolone dose (mg) (n=89)	7.2±10.3	6.6±5.6		7.8±11.4	5.6±3.4
Ever received anti-TNF	65 (23.9)	4 (12.5)		46 (21.7)	23 (25.0)
Number of co-morbidities	0.9±0.9	1.2±0.8		0.9±0.9	0.9±0.9
Spine BMD	0.92±0.15	0.83±0.18**		0.94±0.15	0.84±0.14***
Total Hip BMD	0.82±0.15	0.69±0.19***		0.83±0.15	0.73±0.17***

Table 3.4 ***Comparison of demographic and clinical characteristics of RA study group with and without vertebral or non-vertebral fractures.*** *Values are numbers (%) or mean ± SD. * p<0.05; ** p<0.01 and *** p<0.001, Numbers in parenthesis show the number of patients in which data was available for variables where data was missing in more than 1% of subjects; BMI=Body Mass Index, ESR= Erythrocyte Sedimentation Rate, BMD= Bone Mineral Density.*

3.4.6 Fracture prediction with OPRA

We evaluated the ability of the OPRA tool predicting fractures in the study cohort. This showed that 97% of patients with vertebral fractures and 85% of patients with non-vertebral fractures would have been correctly identified by OPRA . The specificity when using our model for fracture prediction was 27.4% and 26.1% respectively. Further details are listed in Table 3.5.

Table 3.5 Sensitivity and Specificity of OPRA model in fracture prediction in the study cohort

	Non- vertebral fractures(%)*	Vertebral fractures(%)*
Sensitivity		
All	84.8	96.9
Women	88	100
Men	55.6	85.7
Specificity		
All	27.4	26.1
Women	24.7	22.8
Men	37.0	41.7
PPV		
All	33.6	13.4
Women	36.9	12.6
Men	14.7	17.6
NPV		
All	80.6	98.6
Women	80.4	100
Men	81.0	95.2

*Sensitivity, specificity and predictive values of proposed model for fracture prediction. The inclusion criteria are patients with 3 points or more according to the OPRA scale, based on BMI and age (see Figure 3). OP= Osteoporosis of Hip or Spine PPV= Positive Predictive Value, NPV= Negative Predictive Value *Fracture prediction analysis in study cohort*

3.5 Discussion

This study of patients with RA has shown that the overall prevalence of osteoporosis was 26.5% which is significantly higher than the prevalence of osteoporosis in a gender and age matched control cohort. These observations are in keeping with previous reports which recorded a prevalence of osteoporosis ranging between 17% and 32% at the spine and 15 to 36% at the hip (Haugeberg et al. 2000a;Sinigaglia et al.2000). As in previous studies (Gough et al. 1994a;Lodder et al. 2004;Orstavik et al. 2004;Sambrook et al. 1987) the presence of osteoporosis in our cohort was associated with female gender, increasing age, years since menopause, and low body mass index. Disease activity has been identified previously as a risk factor for bone loss in RA (Gough et al. 1994a;Laan et al. 1993;Lodder et al. 2004;Roldan et al. 2006) and in keeping with this we observed a significant association between the levels of inflammatory markers and osteoporosis. Neither of these variables emerged as independent predictors of bone density in the logistic regression analysis however. Unfortunately DAS28 measurements were not recorded systematically in the study cohort and in view of this we were unable to evaluate possible associations between this measure of disease activity and osteoporosis. Although Larsen scores were associated with osteoporosis in the case control study they did not emerge as an independent predictor of osteoporosis in the logistic regression analysis. The relatively high prevalence of osteoporosis observed in this study, which is comparable with that observed in studies performed almost 15 years ago (Haugeberg et al. 2000a;Sinigaglia et al. 2000) indicates that the more aggressive approach to management of RA that has been widely adopted over recent years has not been accompanied by a major reduction in the proportion of RA patients who develop osteoporosis. This is in keeping with the fact that Haugeberg did not identify DMARD use as a determinant of systemic bone loss in RA patients followed over a two year period

(Haugeberg et al. 2002b). I would like to acknowledge that the patients for this study had an average disease duration of 10 years which means many patients of this cohort were diagnosed and treated before the change of treatment paradigm advocating early aggressive treatment. Furthermore this study was unable to check whether early aggressive treatment and treat to target approach have been adopted correctly by all clinicians and health care professionals treating early RA patients.

Although there is evidence that anti-TNF therapy can arrest systemic bone loss in RA over a 12 month treatment period (Vis et al. 2006) only one quarter of our patients had received anti TNF therapy. The observations here therefore suggest that in routine clinical practice, classical risk factors such as age, low BMI and gender play a predominant role in the pathogenesis of systemic osteoporosis in patients with RA (Haugeberg et al 2000a;Sambrook & Cooper 2006). Although we did not observe an association between corticosteroid use and osteoporosis, this may be explained by the fact that the average dose of prednisolone was less than 10mg daily and that about half (48.4%) of the patients on steroid therapy were also on bone protective treatments (data not shown).

We found a positive association between the number of other co morbidities and osteoporosis in the RA cohort. The likely explanation for this is that these co morbidities are strongly related to age (Michaud et al. 2011) which could account for the fact that co-morbidity did not emerge as independent predictor of osteoporosis in the logistic regression.

An interesting finding to emerge from this study was that there was a 10 year delay between the diagnosis of RA being made and the first request for DEXA scanning. This indicates that, in routine clinical practice, DEXA is not a priority investigation in patients with RA, especially in the first few years after diagnosis.

In an attempt to better target DEXA screening of patients with RA previous efforts have been made to develop prediction tools based on clinical risk factor analysis (Haugeberg et al. 2002a;Lems and Dijkmans 1998). In these studies, the risk criteria and cut off values were chosen arbitrarily which differs from the approach used here where the OPRA risk assessment tool was based on the outcome of logistic regression analysis. The negative predictive value and positive predictive value of the OPRA tool in our population (94.4% and 30.4% respectively) did not differ significantly from existing tools that have been used for the prediction of osteoporosis in women, including the osteoporosis self-assessment tool (OST) (Richy et al. 2004) the osteoporosis risk assessment instrument (ORAI) (Cadarette et al. 2000) and the osteoporosis index of risk (OSIRIS) (Sedrine et al. 2002). However OPRA was clearly superior to the only other available tool (OST) in the prediction of osteoporosis in men (Table 4). Further studies would be of interest to establish the performance of OPRA in comparison with existing tools in other cohorts. We were unable to evaluate the Lems-Dijkman (Lems & Dijkmans 1998) and Haugeberg models (Haugeberg et al. 2002a) in our population since HAQ data were not collected in our study but a previous analysis by Nolla reported a sensitivity of 86% for the Lems-Dijkman tool and 82% for the Haugeberg tool in predicting osteoporosis in RA. (Nolla et al. 2001).

Chapter 4: Osteoprotegerin antibodies and Rheumatoid Arthritis

4.1 Abstract

Osteoporosis and fragility fractures are recognized complications of rheumatoid arthritis which are thought to result from the effects of chronic inflammation, relative immobility and corticosteroid use. A rare syndrome of osteoporosis has previously been described in a patient with autoimmune disease caused by production of neutralizing antibodies to the bone protective protein osteoprotegerin (OPG). Here we screened for the presence of OPG autoantibodies in patients with rheumatoid arthritis and evaluated their clinical significance.

In order to measure OPG antibodies we developed an ELISA to detect OPG autoantibodies in serum and measured antibody levels in 75 patients with RA and 199 healthy controls. The functional effects of the antibodies were investigated by their ability to block the inhibitory effects of OPG in a RANKL induced NFκB reporter assay. Autoantibodies to OPG were detected in 2 controls (1%) compared with 7 patients with RA (9.3%; $\chi 2= 19.2$, $p=0.001$). The RA patients with detectable OPG antibodies had a longer disease duration ($p=0.01$), higher DAS28 scores ($p=0.02$) and higher levels of the bone resorption marker CTX ($p=0.01$) than RA patients who did not have autoantibodies. Purified IgG from patients with high levels of OPG antibodies blocked the ability of recombinant OPG to inhibit RANKL induced NFκB activation in a HEK293 cell based assay ($p<0.01$).

In conclusion, autoantibodies to OPG were present in about 10% of this series of patients with RA and were associated with disease activity, disease duration and markers of bone resorption.

4.2 Introduction:

Rheumatoid Arthritis (RA) is a chronic inflammatory disease principally targeting the synovium which is characterized by periarticular bone erosions and cartilage destruction which if untreated can lead to progressive joint damage and deformity. Patients with RA also have an increased risk of generalized osteoporosis (Haugeberg et al. 2000b). This is thought to be multifactorial in origin and caused by decreased mobility, corticosteroid use, vitamin D deficiency and the production of proinflammatory cytokines such as IL1, IL6, TNF (Redlich et al. 2002) which stimulate bone resorption through activation of the receptor activator of NFκB Ligand (RANK) signaling pathway (Kong et al. 1999a;Romas et al. 2002). It has more recently been demonstrated that bone loss can occur very early during the course of RA (Kleyer et al. 2014) (Guler-Yuksel et al. 2009;Machold et al. 2007) due to the osteoclast-activating effects of antibodies directed against citrullinated proteins (ACPA) (Harre et al. 2012). We previously reported the occurrence of severe osteoporosis in a patient with autoimmune thyroid disease and coeliac disease in association with neutralizing autoantibodies to osteoprotegerin (OPG), an inhibitor of RANK signaling (Riches et al. 2009). The aim of the present study was to determine if autoantibodies to OPG also occur in RA and to relate the presence of these antibodies to clinical characteristics of the disease.

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4.3 Patients and Methods:

4.3.1 Patients Rheumatoid Arthritis cohort for OPG ab study

Patient recruitment took place at the outpatient department of the Western General Hospital in Edinburgh between January 2011 and December 2012. We included 75 consecutive patients with a clinical diagnosis of Rheumatoid Arthritis who attended the outpatient clinic for a routine review. We collected information on patient's demographics, disease characteristics, co-morbidities, medication history and risk factors of osteoporosis through a one to one interview and undertook a retrospective case note analysis for disease duration, medication history and ACPA or Rheumatoid Factor status. Clinical examination and disease activity assessment (DAS 28) were performed on the day of recruitment. Each patient donated a sample of serum and plasma for the study when undergoing routine blood test for clinical purposes. The samples were stored frozen at -80°c until analysis.

Measurements of BMD were made by dual-energy X-ray absorptiometry (DEXA) using a Hologic QDR 4500 osteodensitometer at the lumbar spine (L1-L4) and the femoral neck. Patients with rheumatic diseases had a DXA scan performed in a time frame of 5 ± 15 months from the time of recruitment into the study.

4.3.2 Healthy control cohort

For the healthy control data serum and plasma were obtained from a population based cohort with normal bone mineral density. Recruitment and characteristics of this cohort was described before (Estrada et al. 2012).

4.3.3 DXA measurement

Measurements of BMD were made by dual-energy X-ray absorptiometry (DEXA) using a Hologic QDR 4500 osteodensitometer at the lumbar spine (L1-L4) and the femoral neck. For healthy controls the DXA scan was done at the time of sample collection. Patients with rheumatic diseases had a DXA

scan performed in a time frame of 5 ± 15 months from the time of recruitment into the study.

4.3.4 Serum measurements

Serum and plasma samples were stored frozen at -80°c until analysis.
OPG ab serum concentrations were analyzed as described in Chapter 2.1.1.
CTX measurements are described in Chapter 2.1.4. Details of immunoglobulin purification are outlined in Chapter 2.2.2 and the functional assay in Chapter 2.2.3.

4.3.5 Statistical methods

Analyses were performed using SPSS version 16.0 (SPSS, Chicago, IL, USA). Two tailed independent student t-test was used for between-group comparisons of continuous data. If the variables were not normally distributed Mann Whitney U test was applied. The results are shown as the mean ± SD. Chi-square tests were used to test for differences between categorical variables and the results are shown as total numbers and percentages. Correlations between OPG antibodies levels, serum CTX and DAS28 were performed using Spearman's rank correlation test.

4.3.6 Ethics

Approval for the study was sought from NHS Lothian Tissue Governance Committee. All subjects provided informed consent prior to participation of the study. The R&D ID number is 2011/BRP/05, RTB: South East of Scotland NRS (formerly SAHSC) BioResource, RTB REC No: 10/S1402/33.

4.4 Results:

4.4.1 Clinical characteristics of the RA cohort and controls

Clinical characteristics of the Rheumatoid Arthritis patients and 200 healthy controls are shown in Table 4.1 and Table 4.2. RA patients were slightly older than the controls (p=0.01) but there was no significant difference in gender distribution. The average spine and hip BMD t-score was higher in the healthy control group than in the RA cohort (p=0.01 and p=0.01). Women with RA were significantly younger than healthy controls when going through the menopause. Most of the RA patients (80%) were Cyclic citrullinated peptide (CCP) antibody or rheumatoid factor (RF) positive, but 13 (17%) were CCP or RF negative and in 2 (3%) patients the serological status was unknown. The RA patients had on average high disease activity with a mean DAS28ESR score of 5.43± 1.31. Amongst the RA cohort 13 patients were treatment naïve and had symptoms for less than one year which was defined as early disease.

Table 4.1: Demographics and clinical characteristics of study population

	RA (75)	Controls (199)	p value
Demographics			
Age (years)	61.52± 13.10	55.74 ±13.89	0.002
Women, n(%)	54 (72%)	126 (70%)	0.177
Height (cm)	162.69 ± 9.45	166.44 ± 8.78	0.004
Weight (kg)	70.15 ± 14.54	79.04 ±16.31	0.001
BMI (kg/ m^2)	26.45 ± 4.68	28.44 ± 4.85	0.005
Age at menopause	44.86 ± 6.42	50.16 ± 3.96	0.001
Bone mineral density			
Spine BMD (g/cm^2)	0.91 ± 0.17	1.08 ± 0.09	0.001
Spine BMD T-score	-1.37 ± 1.51	0.15 ± 0.79	0.001
Spine Osteoporosis,	17 (28.3%)	0 (0%)	0.001
Hip BMD (g/cm^2)	0.84 ± 0.17	1.00 ± 0.07	0.001
Hip BMD T-score	-0.95 ± 1.28	0.21 ± 0.34	0.001
Hip Osteoporosis	9 (14.8%)	0 (0%)	0.001

Values are mean ± standard deviation or number (%).

BMI=Body Mass Index, BMD=Bone Mineral Density. Spine BMD measurements were available in 61 RA patients and hip BMD on 60 patients.

Table 4.2 Clinical characteristics of RA cohort

Disease Duration (Years)*	7.18 ± 8.56
Early disease[1]	13 (17.3%)
ESR (mm/hr)	30.74 ± 19.96
DAS 28	5.43 ± 1.32
Medications	
Ever DMARD	57 (76%)
Synthetic DMARD	35 (46.7%)
Current anti-TNF	8 (10.7%)
Ever anti TNF	23 (30.7%)
Current Glucocorticoid	23 (30.7%)
Current Glucocorticoid dose*	5.05 ± 4.94
Osteoporosis treatment	16 (21.3%)

*Values are mean ± and number (%). [1]Early RA was defined as a disease duration <1 year and not currently on DMARD treatment. *Disease duration and current glucocorticoid dose were not recorded in 2 patients and DAS28 was not recorded in 5 patients.*

4.4.2 Antibodies to OPG in cases and controls

Circulating levels of OPG antibodies in cases and controls are shown in Figure 4.1. Subjects were considered to have tested positive for OPG antibodies if the levels were higher than 3 standard deviations above the mean in the healthy controls. According to this definition 2 (1%) out of 199 healthy controls were positive for OPG antibodies compared with 7 out of 75 patients (9.3%) of the RA cohort (p=0.01). The mean levels of OPG antibodies were also significantly higher in the RA cohort (mean ± SD, p<0.0001).

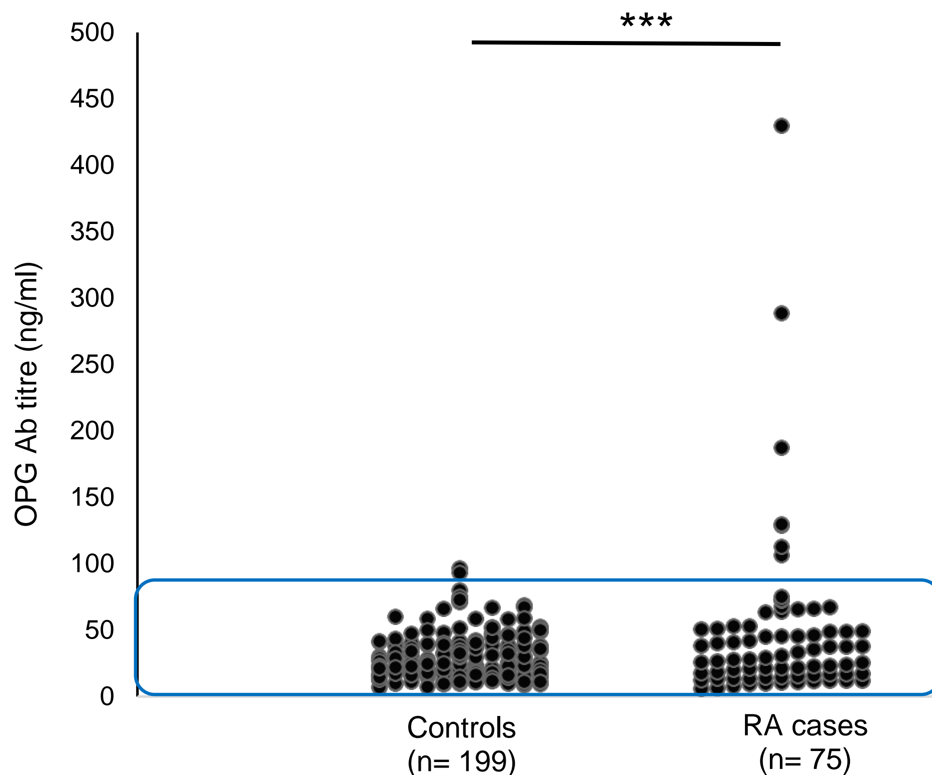

Figure 4.1 *Osteoprotegerin antibody concentrations in healthy controls (n= 199) and patients with Rheumatoid Arthritis (n=75) . The demarcated area (blue line) depicts the normal range (mean ± 3SD of healthy controls). *** = p=0.001 (difference between number of cases with OPG ab concentrations above normal range*

4.4.3 Association between OPG ab and clinical characteristics in RA

Associations between OPG antibody positivity and the clinical characteristics of RA are shown in Table 4.3. Patients with RA who tested positive for OPG antibodies were associated with duration of the disease, high disease activity (DAS28 ESR) and increased levels of the bone resorption marker CTX. Despite this there was no association with bone mineral density at either the spine or hip. Of interest was the fact that all patients who tested positive for OPG antibodies were female and were seropositive for anti CCP antibodies or Rheumatoid factor, although this was not statistically significant.

Table 4.3: Relation between OPG autoantibodies and selected clinical variables

Characteristics	Positive (n=7)	Negative (n=68)	p value
Age	63.3 ± 11.8	61.5±13.2	p=0.64
Female Gender	7/7 (100%)	47/68 (70%)	p=0.18
Disease Duration (years)	16.0±12.3	6.4±7.8	p=0.01
ACPA or RF positive*	7/7 (100%)	53/66 (80%)	p=0.34
DAS 28	6.1±0.6	5.4±1.4	p=0.02
Serum CTX (ng/ml)	0.30±0.08	0.16±0.10	p=0.01
Hip BMD (g/cm^2)	0.86±0.26	0.84±0.16	p=0.82
Spine BMD (g/cm^2)	0.94±0.23	0.91±0.16	p=0.67

*Data are shown as mean ± SD or numbers (%). *Data on CCP and RF status was unavailable for 2 patients. The comparison of CTX levels was performed after exclusion of patients on bisphosphonate therapy.*

There was a positive correlation between OPG antibodies and disease activity as assessed by the DAS28 score (Fig 4.2A). Further analysis of each DAS 28 component shows that there was a positive association between OPG antibody levels and the number of swollen ($r = .341$ $p=0.006$) and tender joints ($r = .343$, $p=0.006$) but not with global disease activity (VAS) ($r = .039$ $p=0.761$) or ESR ($r=.157$ $p=0.182$). There was a positive association between OPG antibody levels and levels of the bone resorption marker CTX in RA patients who were not receiving anti-resorptive treatment (Fig 4.2B).

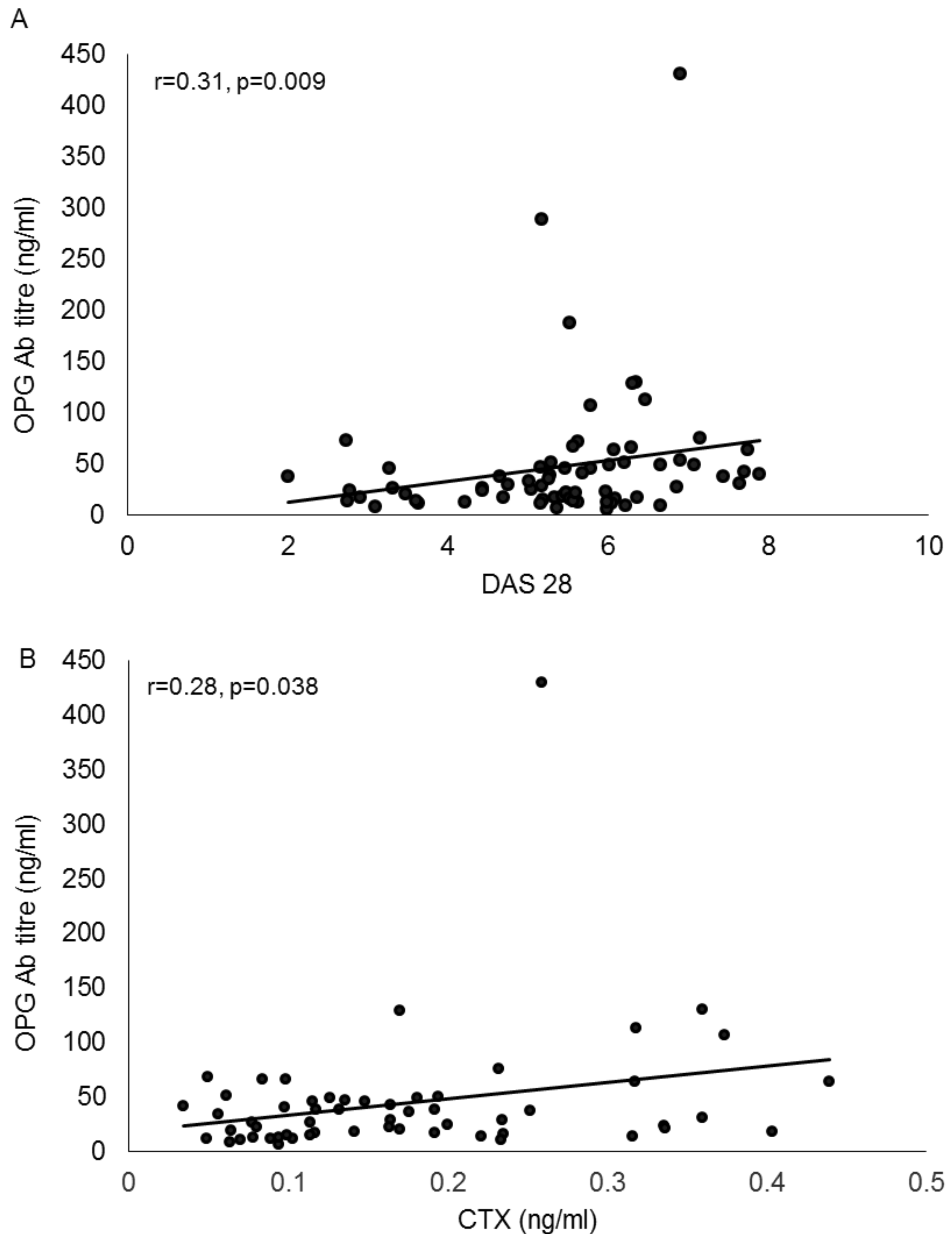

***Figure 4.2**. Correlation of OPG ab concentrations and DAS 28 ESR (disease activity score).* *B: Correlation between OPG ab concentrations and bone resorption markers CTX (patients who received antiresoptive treatment at time of sample collection were excluded). r_s=correlation coefficient (Spearman's Rho) p=significance level*

4.4.4 Functional analysis of OPG antibodies

In order to determine if the OPG antibodies we detected in patients with RA had functional effects on RANKL induced NFκB signaling we investigated IgG samples from patients who tested positive or negative for OPG autoantibodies in cell based NFκB reporter assay. Isolated Immunoglobulin concentrations from OPG ab positive patients (2.76± 0.93 µg/µl) did not differ significantly from IgG concentrations from patients tested negative for OPG ab (3.20± 1.70 µg/µl, $p = 0.57$). The cell assay showed that the addition of Immunoglobulin from four of six OPG antibody-positive patients partially (n=1) or significantly (n=3) reversed the inhibitory effect of OPG on RANKL-induced NFκB activation compared with zero of seven negative patients ($p=0.02$, Fisher's exact test). A representative experiment is shown in Figure 4.3. Amongst the OPG antibody negative samples there was no difference between Rheumatoid Factor (RF) positive and RF negative sera.

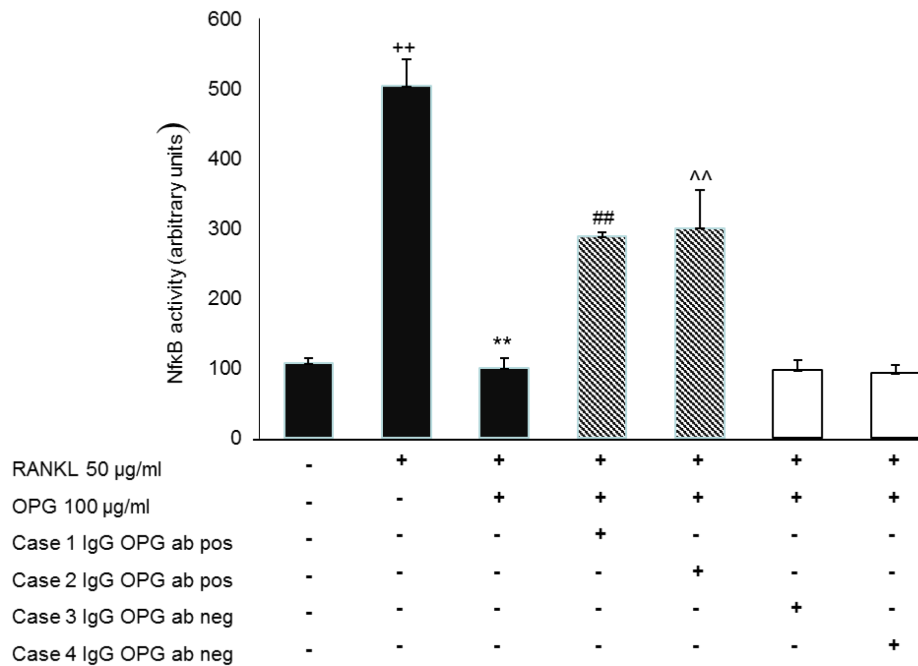

Figure 4.3 *shows the level of NfκB activation of human embryonic-kidney (HEK-293) cells, stably transfected with NF-kB luciferase reporter vector in the presence of various combinations of treatments. The column without stimulation depicts the negative control. Stimulation of cells with RANKL (50ug/ml) increase NF-kB response significantly (++, p<0.001). Addition of OPG (100 µg/ml) attenuates stimulation significantly (**, p<0.001). The addition of 20µl of purified IgG from OPG ab positive patients neutralize OPG action and increase NfkB activity significantly (##,p<0.001 and ^^, p=0.004 respectively).The addition of IgG from patients tested negative for OPG ab (2 white columns) did not abrogate the inhibitory OPG effect on RANKL(p= 0.867 and p= 0.590).*

4.5 Discussion

This study showed that autoantibodies to OPG can be detected in a proportion of patients with RA and are more commonly present than in healthy controls. We also found an association between levels of autoantibodies to OPG and the bone resorption marker CTX and found that purified IgG from patients who tested positive for OPG antibodies inhibited the ability of OPG to block RANKL induced NFκB activation in vitro. This suggests that in some RA patients functional autoantibodies to OPG are produced which have the potential to enhance osteoclastic bone resorption.

Previous studies have shown that autoantibodies to citrullinated peptides contribute to bone loss in RA by interacting with the Fc receptor on osteoclasts (Harre et al. 2012). Although we cannot exclude the possibility that the autoantibodies to OPG detected here might also affect osteoclastogenesis by binding Fc receptors, the observation that samples from patients who tested positive were able to block the inhibitory effect of OPG on RANKL induced NFκB activation suggests that they exert a specific effect on the RANK pathway.

The correlation between high disease activity and OPG antibody levels may be an epiphenomenon as we know that patients with autoantibodies against citrullinated proteins develop more aggressive disease than seronegative patients (Miriovsky et al. 2010;van Gaalen et al. 2004). It is therefore possible that patients with a propensity to develop ACPA may also be more likely to develop antibodies against OPG. At the present time we have no information with regard to the time course of OPG antibody development in RA. Although patients with OPG antibodies tended to have a longer disease duration than those that tested negative and it would be of interest to test for the presence of OPG antibodies in population based cohorts and cohorts of patients with early arthritis.

Whilst we found a positive association between OPG antibodies and the bone resorption marker CTX, there was no association with BMD. We think that this may be explained by the fact that this was a relatively small study with limited power to detect such an effect given that a large number of

variables influence BMD including age, gender, BMI and other genetic and environmental factors (Sambrook and Cooper 2006). Future studies of larger cohorts would be needed to adequately address this issue. Similarly, our study was too small to detect possible associations between OPG antibody levels and bone erosions. This however would be of interest given the crucial role that OPG/RANKL system plays in the pathogenesis of localized bone loss and the development of bone erosions (Kong et al. 1999;Romas et al. 2002).

While further work will need to be done to fully explore the clinical significance of the findings reported here, the present study shows that OPG antibodies are produced more commonly in RA patients as compared with controls and that some have functional effects on RANKL signaling. This raises the possibility that OPG antibodies may contribute to the pathogenesis of local and systemic bone loss in RA and signal the need to study the relationship between these antibodies and bone disease in large-scale longitudinal studies.

Chapter 5: Osteoprotegerin autoantibodies and Ankylosing Spondylitis

5.1 Abstract

Osteoporosis and vertebral fractures are recognised complications of axial spondyloarthritis (axSpA) but the underlying causes are incompletely understood. Osteoprotegerin (OPG) is a bone protective protein that acts as a decoy receptor for RANK-L and inhibits osteoclastogenesis. Previous studies have demonstrated that antibodies to OPG (OPG-Ab) may develop in patients with autoimmune diseases and be associated with osteoporosis and increased bone turnover. The aim of this study was to determine whether OPG-Abs were detectable in axSpA patients and whether they were associated with bone health.

Patients with a clinical diagnosis of axSpA were recruited from routine outpatient clinics at two centres in the United Kingdom between 2011-2015. Patient demographics and disease characteristics as well as history of fractures were recorded. All had BMD assessment using antero-posterior dual-energy X-ray absorptiometry (AP-DXA). Serum levels of OPG-Ab were measured for each patient using an in-house ELISA. Patients were considered to be positive for OPG-Ab if values were ≥13units (3 standard deviations above mean in healthy controls). Associations between OPG-Ab and BMD and fractures were assessed using logistic regression, adjusted for age, gender, duration since diagnosis, BMI and study centre.

We studied 134 patients, of whom 75% were male. The mean age was 47 (SD±15) years and median disease duration from diagnosis was 6.5 years. 16 patients were tested positive for OPG-Ab (11.9%). The presence of OPG-Ab was associated with lower hip BMD, and an increased number of fractures. There was no association between OPG-Ab and patient demographics, disease characteristics or activity. In patients with a longer disease duration (>6.5 years) there was a higher discrepancy between spinal

and hip BMD in OPG-Ab positive patients compared with OPG ab negative patients (p=0.003). Logistic regression revealed an association between OPG-Ab and disease duration (OR 1.04; 95%CI 1.00, 1.07; P=0.045). Strong independent association were seen with hip T-score (OR_{adj} 0.43; 95%CI 0.22, 0.85; P=0.015) and history of fractures (OR_{adj} 4.78; 95%CI 1.37, 16.7; P=0.014).

In conclusion this cross-sectional study demonstrates that OPG-Ab were present in 11.9% of axSpA patients. OPG-Ab was strongly and independently associated with hip BMD and history of fractures. This raises the possibility that OPG-Ab may play an important role in accelerated bone loss and increased fracture risk in axSpA.

5.2 Introduction

Ankylosing spondylitis (AS) is an autoinflammatory condition which is predominantly characterized by localized inflammation at entheses which causes back pain and stiffness and localized new bone formation in form of syndesmophytes. Despite osteoproliferation being a cardinal problem in axial SpA, AS patients are at increased risk of systemic bone loss and of developing generalized Osteoporosis (Braun & Sieper 2007;Donnelly et al. 1994;Karberg et al 2005). Accelerated bone loss and the presence of osteoporosis is particularly problematic in a fused and rigid spine, thus vertebral fractures is not an uncommon complication in AS (Ralston et al. 1990;Vosse et al. 2009), even in early disease(van der Weijden et al. 2012).

The main driver for systemic bone loss in AS are thought to be immobility due to functional incapacity (van der Weijden et al.2011) and systemic inflammation characterized by increased inflammatory cytokines (anti-TNF, IL6 and IL17) which promote osteoclast proliferation and bone resorption (Franck, Meurer, & Hofbauer 2004;Gratacos et al. 1999;Wendling et al. 2007). However there are many unanswered questions regarding the pathogenesis, prevention and treatment of OP in Spondyloarthropathies. In contrast to general population men are at higher risk of developing OP (Sambrook & Cooper 2006;van der Weijden et al. 2011) and fractures (Vosse et al. 2006;Vosse et al.2009) at a younger age which limits the value of conventional fracture risk assessment tools such as FRAX. We know that treatment with anti-TNF reduces BMD loss (Allali et al. 2003) but it is unclear if bone formation is closely linked to inflammation and if fracture risk can be altered by anti-TNF treatment (Maksymowych,et al. 2009;Schett et al. 2009). These uncertainties highlight the need to investigate other mechanism which may impact on AS related bone loss.

Autoantibodies have recently come into spotlight in the pathogenesis of localized and systemic bone loss in RA (Harre et al. 2012;Hauser et al. 2013;Hauser et al. 2015;Kleyer et al. 2014). In specific OPG ab, which neutralize the bone protective effect of OPG were found to correlate with increased bone resorption markers in RA. In contrast to seropositive

arthritidis, autoantibodies are not thought to have a significant role in axSpA. However a few SpA patients were found to be positive for OPG-Ab in an early pilot study (Hauser et al. 2013).
The aim of this study was to determine whether OPG-Ab were detectable in AS patients and if they were associated with bone health in axSpA.

5.3 Methods

5.3.1 Patients

The Ankylosing Spondylitis cohort consists of patients recruited from routine outpatient clinics at the Western General Hospital in Edinburgh and Aintree University Hospital in Liverpool. The cohort included patients recruited from January 2011 to February 2015. I've recruited all AS patients belonging to the Edinburgh cohort. Inclusion was by clinical diagnosis based on the modified New York criteria for AS and/or the ASAS criteria for axSpA. Patients attending a general Rheumatology clinic were invited to give serum and plasma samples. BMD measurements were performed as necessary for routine clinical care. At the time of the clinical assessment I've collected data on demographics, disease characteristics, medication history, risk factors of osteoporosis and fracture history. Disease activity and functional status were assessed using BASDAI, spinal pain VAS and BASFI, along with ESR and CRP at the time of obtaining samples for this study.During DEXA scanning additional detailed information on risk factors for osteoporosis was recorded including patient demographics, BMI and calcium intake. Duration of disease since diagnosis was divided by the median into long (≥6.5years) and short (<6.5years). The difference in BMD (g/cm2) between spine and hip was calculated and compared between OPG-Ab status and disease duration. Serum was separated by centrifugation and stored at -80°C.

5.3.2 BMD assessment

BMD was assessed by dual-energy X-ray absorptiometry (DEXA) of the lumbar spine (L1-L4) and the femoral neck using Hologic QDR4500 at Edinburgh and Lunar iDXA (GE Healthcare) at Liverpool. DXA scans were performed within a median of 11 months from study recruitment.

5.3.3 Osteoprotegerin autoantibody measurement

This was performed as described in Chapter 2.1.2. Each sample was run in triplicates and the assay repeated at least once on a different day. Coefficient of variation for the ELISA was 10.4%.An arbitrary unit for OPG-Ab titre was generated from standard curve created using serum from the index patient previously described (Riches et al. 2009). Previous studies of healthy controls with normal BMD was used to define the limits for positivity as three standard deviations above the mean. Positivity was defined as greater or equal to 13 units.

5.3.4 Statistics

Statistical analysis was performed using SPSS 19. Variables were assessed for normality of distribution using skewness-kurtosis test. Mann-Whitney U test was used to compare non-parametric, Student's t-test for parametric and Chi-squared or Fisher's exact tests for categorical variables. Non-parametric data was transformed prior to linear regression. Association between OPG-Ab and disease characteristics assessed with univariate logistic regression. Association with BMD and fractures were assessed by linear and logistic regression, respectively (adjusted for age, gender, duration since diagnosis, BMI and study centre).

5.3.5 Ethics

This study received ethical approval from both the Edinburgh (Lothian Research Ethics Committee, RTB REC No: 10/S1402/33. and Liverpool sites (REC reference 14/NW/1320). All patients provided informed consent.

5.4 Results

5.4.1 Demographics and clinical characteristics

The axSpA cohort consisted of 134 patients with complete DXA data; 53 from Edinburgh and 81 from Liverpool. There were no statistically significant differences in age, gender, duration of disease, BMI or BMD between the two study sites (data not shown). The median OPG-Ab titre was 3 (range 0, 30.8; interquartile range [IQR] 1.4, 4.3). OPG-Ab was positive in 16 patients (11.9%). Patient demographics and disease characteristics are shown in Table 5.1. The cohort was predominantly male (75%) with a mean age of 47 years (SD±15) and medium disease duration of 6.5 years [IQR 1.9, 17.8]. Most patients reported a moderate to high disease activity as measured by BASDAI (median 5.4). Peripheral joint involvement was reported in 35% of patients and 11% had associated inflammatory bowel disease (IBD). A third of patients received TNFi at the time of recruitment.

5.4.2 Association between OPG ab positivity and disease characteristics

Differences in patient and disease characteristics between those positive and negative for OPG-Ab are shown in Table 5.1. There were non-significant trends for older age and longer disease duration in the OPG-Ab positive group. Measures of axSpA disease activity, spinal pain and functional impairment were similar in both groups. A greater proportion of patients positive for OPG-Ab were taking bisphosphonates (25% vs 6%, P=0.027).

Table 5.1 Patient and disease characteristics by OPG-Ab status.

		Total	Positive (n=16)	Negative (n=118)	P-value
Demographics	Age (years)	47.0 ± 15.0	53.1 ± 14.1	46.2 ± 14.9	0.086
	Males (%)	100 (75%)	11 (69%)	89 (75%)	0.551
	HLA-B27 (n=58)	42 (71%)	5 (63%)	37 (73%)	0.678
	Age diagnosed	41.0 ± 14.5	42.5 ± 12.9	40.8 ± 14.8	0.663
	Median years disease duration [IQR]	6.5 [1.9, 17.8]	13.7 [1, 27.3]	5.6 [2, 17.5]	0.194
	Ever smokers	45 (34%)	7 (44%)	38 (33%)	0.398
Disease activity	Median BASDAI	5.4 [3.2, 7.5]	5.1 [2.8, 5.8]	5.4 [3.2, 7.7]	0.390
	Median spVAS	5.5 [2.5, 8]	6 [2, 6]	5.3 [2.5, 8]	0.756
	Median BASFI	5.3 [3.4, 7.4]	4.7 [3.9, 8.3]	5.3 [3.1, 7.2]	0.682
	Median ESR (mm/hr)	8 [5, 19]	15 [5, 31]	8 [5, 17]	0.207
	Median CRP (md/L)	4 [1, 9]	4 [1, 8]	4 [1, 9]	0.511
Extra-axial involvement	Peripheral joint involvement	46 (35%)	5 (31%)	41 (35%)	1.000
	Psoriasis	20 (15%)	3 (19%)	17 (15%)	0.709
	Uveitis	46 (35%)	5 (31%)	41 (35%)	1.000
	IBD	15 (11%)	1 (6%)	14 (12%)	0.694
Medication	TNFi	45 (34%)	8 (50%)	37 (31%)	0.138
	Synthetic DMARD	15 (11%)	0	15 (13%)	0.132
	NSAIDs	83 (64%)	9 (56%)	74 (65%)	0.470
	Glucocorticoid	3 (2%)	1 (6%)	2 (2%)	0.319
	Bisphosphonates	11 (8%)	4 (25%)	7 (6%)	0.027
	Calcium and vitamin D	64 (48%)	9 (56%)	55 (47%)	0.469

BASDAI, Bath Ankylosing Spondylitis Disease Activity Index; spVAS, spinal pain visual analogue scale; BASFI, Bath AS Functional Index; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; IBD, inflammatory bowel disease; TNFi, TNF inhibitor; DMARD, disease modifying antirheumatic drug

5.4.3 Difference in BMD between OPG ab status

Differences in BMD between patients positive and negative for OPG-Ab are shown in Table 5.2. Both BMD g/cm^2 (0.882 ± 0.137g/cm^2 vs 1.004 ± 0.173g/cm^2, P=0.013) and T-scores (-1.26 ± 1.08 vs -0.33 ± 1.15, P=0.006) were significantly lower at the hip in the OPG-Ab positive patients. No significant differences in bone density were observed at the spine. Only four patients (3%) of the cohort were osteoporotic. However there was a higher prevalence of osteopenia in OPG-Ab positive patients (63% vs 22% in OPG-Ab negative, P=0.002). OPG-Ab positive patients had a higher prevalence of history of fracture than OPG-Ab negative patients (50% vs 19%, P=0.007). There was trend toward shorter height in OPG-Ab positive patients (166 ± 8cm vs 171 ± 10.0cm, P=0.064) although BMI was similar between the groups.

Table 5.2 Difference in bone mineral density (BMD) between OPG-Ab positive and negative patients.

	Total	Positive (n=16)	Negative (n=118)	P-value
Height (cm) n=119	171 ± 9.8	166 ± 7.6	171 ± 10.0	0.064
Weight (kg) n=122	81.8 ± 17.3	82.6 ± 15.2	81.7 ± 17.6	0.861
BMI (kg/m^2) n=119	28.0 ± 5.4	29.9 ± 5.4	27.7 ± 5.3	0.130
Spine BMD (g/cm^2) n=114	1.161 ± 0.224	1.244 ± 0.230	1.149 ± 0.221	0.125
Spine T-score n=113	0.027 ± 1.684	0.6 ± 1.650	-0.060 ± 1.681	0.158
Spine Z-score n=70	0.333 ± 1.692	1.018 ± 1.550	0.205 ± 1.698	0.145
Hip BMD (g/cm^2) n=106	0.988 ± 0.173	0.890 ± 0.144	1.004 ± 0.173	**0.018**
Hip T-score n=105	-0.449 ± 1.174	-1.167 ± 1.095	-0.329 ± 1.149	**0.010**
Hip Z-score n=71	-0.132 ± 1.106	-0.909 ± 0.903	0.01 ± 1.087	**0.010**
Hip osteopenia	36 (34%)	10 (67%)	26 (29%)	**0.007**
Hip osteoporosis	4 (4%)	2 (13%)	2 (2%)	0.097
History of previous fracture	31 (23%)	8 (50%)	23 (19%)	**0.007**

BMI, body mass index; BMD, bone mineral density.

5.4.4 OPG ab positivity and clinical associations

OPG-Ab positivity was shown to be associated with disease duration since diagnosis (OR 1.04; 95%CI 1.00, 1.07) and a trend was seen towards association with age (table 5.3). Markers of disease activity, spinal pain and functional impairment were not associated with OPG-Ab.

Table 5.3 Univariate logistic regression for association with OPG-Ab positivity

Table 3 Univariate logistic regression for association with OPG-Ab positivity.			
	Odds ratio	95% confidence interval	P-value
Age (years)	1.03	1.00, 1.07	0.090
Gender	0.72	0.23, 2.24	0.566
Duration (years)	1.04	1.00, 1.07	**0.045**
HLA-B27	0.63	0.13, 2.99	0.562
BMI (kg/m^2)	1.08	0.98, 1.19	0.134
BASDAI	0.92	0.74, 1.14	0.421
spVAS	1.03	0.86, 1.24	0.726
BASFI	1.06	0.84, 1.22	0.644
Vit D (nmol/L)	1.00	0.98, 1.01	0.932
ESR (mm/hr)	1.01	0.99, 1.04	0.329
CRP (mg/L)	1.00	0.97, 1.03	0.823
TNFi	2.19	0.76, 6.28	0.145

BMI, body mass index; BASDAI, Bath Ankylosing Spondylitis Disease Activity Index; spVAS, spinal pain visual analogue scale; BASFI, Bath AS Functional Index; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; IBD, inflammatory bowel disease; TNFi, TNF inhibitor.

5.4.5 Correlation between OPG ab and bone mineral density

An inverse correlation was observed between increasing OPG-Ab titre and hip T-score (Fig. 5.1A). Similar correlations were observed for hip BMD (r_s = -.20 p=0.042 and Z-score r_s= -.26 p=0.026). No correlation was observed between OPG-Ab titre and BMD spine (r_s =.11 p=0.254), Spine BMD Z-score (r_s=.13 p=0.264) or spine T-score, shown in Figure 5.1B.

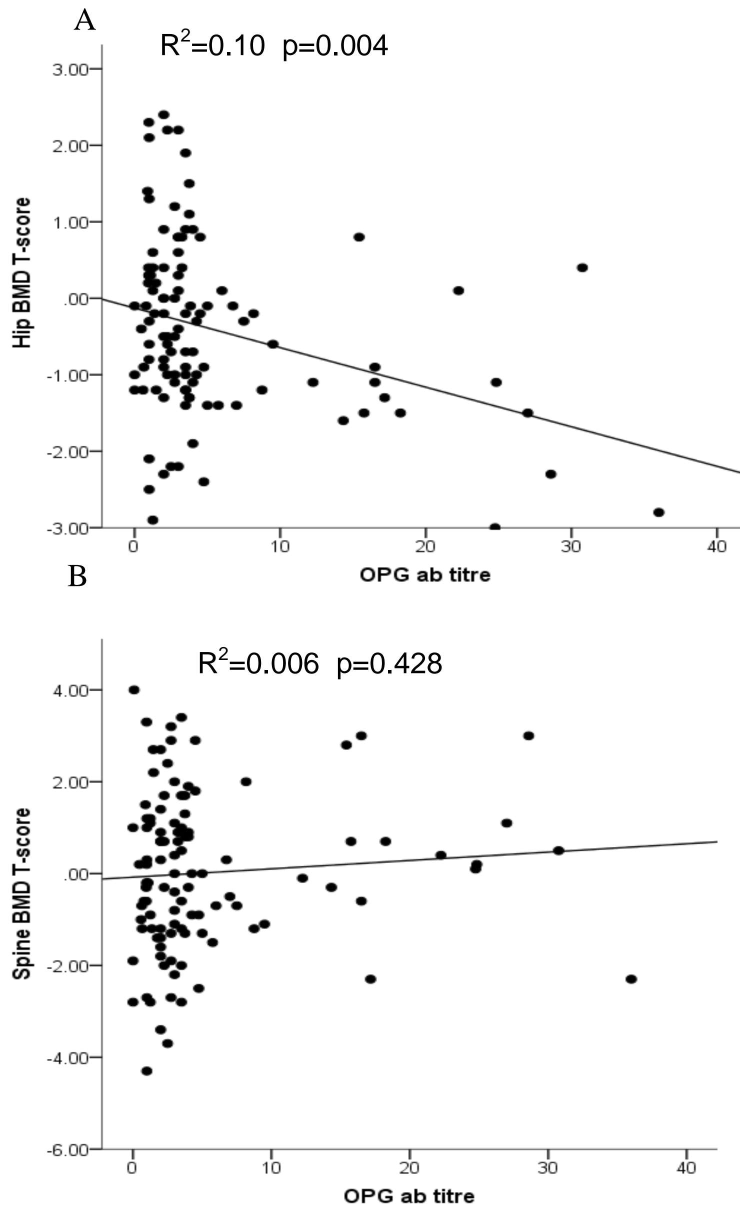

Figure 5.1 Scatterplot of OPG ab titres against (A) Hip BMD T-score and (B) Spine BMD T-score. *Black lines shows line of best fit.*

5.4.6 Association between OPG ab status and difference between spine and hip BMD

In patients with longer disease duration (>6.5 years), 100% of OPG-Ab positive patients had higher spinal BMD compared with 65% of negative patients (p=0.026) (table 5.4). This was not significant in those with shorter disease duration. The difference between spinal and hip BMD increased with disease duration; and in those with longer than 6.5years' duration, OPG-Ab positivity was associated with greater difference (table 5.5).

Table 5.4 Association between OPG ab status and spine –hip BMD discrepancy

	OPG-Ab	Bone mineral density g/cm^2		P-value
		Spine ≤ hip	Spine > hip	
Long duration n=68	positive	0	11 (100%)	0.026
	negative	20 (35%)	37 (65%)	
Short duration n=66	positive	1 (20%)	4 (80%)	0.645
	negative	23 (38%)	38 (62%)	

Long duration = disease duration >6.5 years, Short duration= disease duration >6.5 years

Table 5.5 OPG ab status and BMD difference between spine and hip

	OPG-Ab	Mean (sd)	P-value
Long duration n=68	positive	0.424 (0.182)	0.003
	negative	0.227 (0.189)	
Short duration n=66	positive	0.158 (0.152)	0.275
	negative	0.091 (0.115)	

Long duration = disease duration >6.5 years, Short duration= disease duration >6.5 years

5.4.7 Association between BMD and fractures and OPG ab status

Multivariable logistic regression (adjusted for age, gender, duration since diagnosis, BMI, study site) demonstrated that OPG-Ab positivity was significantly and independently associated with lower BMD at the hip for T-score, Z-score and density g/cm^2 (table 4). Multivariate logistic regression revealed that OPG-Ab positivity was strongly associated with both hip osteopenia (OR 6.78; 95%CI 1.69, 27.2) and history of prior fracture (OR 4.78; 95%CI 1.37, 16.7). No significant associations were observed with osteopenia at the spine.

Table 5.6 Associations between BMD and fractures and OPG -Ab status (adjusted for age, gender, duration since diagnosis, BMI, study site)

	Odds Ratio	95% confidence interval	P-value
Spine T-score	1.13	0.78, 1.65	0.519
Spine Z-score	1.44	0.89, 2.34	0.142
Spine BMD (g/cm^2)	2.89	0.12, 69.8	0.513
Hip T-score	0.43	0.22, 0.85	**0.015**
Hip Z-score	0.35	0.14, 0.88	**0.025**
Hip BMD (g/cm^2)	0.00	0.00, 0.32	**0.016**
Hip osteopenia (T-score <-1)	6.78	1.69, 27.2	**0.007**
Hip osteoporosis (T-score ≤-2.5)	10.0	0.80, 125.5	0.074
History of previous fracture	4.78	1.37, 16.7	**0.014**

BMD, bone mineral density.

5.5 Discussion

This cross-sectional study demonstrated the presence of anti-OPG antibodies (OPG-Ab) in 11.9% of patients with clinical diagnosis of axSpA, which is similar to previous prevalence rates reported in RA (9.3%) in Chapter 4 and Coeliac Disease (9.8%)(Real et al. 2015) but significantly higher than the prevalence rates we have reported previously in healthy control populations (1 -1.4%) in Chapter 4.4.2 and in a study looking into OPG ab prevalence in Coeliac Disease (Real et al. 2015). This is a somewhat unexpected finding as the pathophysiology of axSpA is usually not associated with the production of specific autoantibodies.

Although previous studies have shown an association between OPG ab and increased bone resorption this is the first study to demonstrate OPG ab to be independently associated with reduced hip BMD and fractures. The lack of association with spinal BMD will likely reflect the difficulty in accurately assessing vertebral BMD using AP-DXA, where syndesmophytes lead to overestimation of BMD(Braun & Sieper 2007).

Our results are compatible with previous molecular findings (Riches et al. 2009) (Chapter 4.3.4) that showed that OPG ab interfere with the OPG/RANKL homeostasis by blocking OPG which acts as decoy receptor to RANKL, an essential cytokine for osteoclast activation and proliferation. Therefore the development of OPG-Abs may contribute to systemic reduction in BMD observed in axSpA.

Of interest is the finding that in patients with longer disease duration, those positive for OPG ab seemed to have higher spinal than hip BMD. This may be a result of increased loss of BMD at the hip with increasing disease duration. Another explanation could be that OPG Ab are associated with dysregulatjon of bone remodelling in axSpA given the BMD loss at hip and increase at spine, which might possibly be due to redistribution of bone from the vertebral bodies to syndesmophytes. Our findings fit with previous

observations that systemic bone loss in axSpA is associated with increased syndesmophyte burden (Karberg et al. 2005;Klingberg et al. 2012).

The study has also found that OPG ab positive patients are more commonly receiving Bisphosphonate treatment than patients without detectable OPG ab, which is in keeping with the finding of lower BMD in antibody positive patients. A trend towards lower height in OPG ab patients may also be attributable to loss of vertebral height due to fractures as noted previously (Vosse et al. 2006).

We have also found an association between OPG ab positivity and longer disease duration, a phenomenon which was also seen in our previous studies examining OPG ab associations in RA (Chapter 4.4.3) and a trend was noted in the Coeliac cohort (Real et al. 2015) . The reason for this association remains unexplained and I hypothesize that a perpetuate cycle of chronic inflammation, activation and proliferation of B and T-cells and chronically increased RANKL/OPG concentrations may increase the risk of breaking tolerance and promotes the production of autoantibodies. Interestingly there seems to be a different immunological mechanism to other functional antibodies seen in rheumatic diseases such as anti-CCP antibodies, which frequently precede the disease and symptom onset (Kleyer et al.2014;Real et al. 2015). Longitudinal studies are required to explore the timing or triggering factors of OPG ab development during the course of disease.

Our data did not demonstrate any association between OPG-ab and disease activity or inflammatory markers which suggests that development of OPG ab may be an inflammation-independent mechanism for accelerated bone loss in ax-SpA. This links to the theory that bone formation in AS is at least partly independent of inflammation and thus less amenable to prevention using anti-TNF treatment (Schett et al. 2009).

There are a few limitations in this study due to its observational character. We know that men with axSpA are more commonly affected by accelerated

bone loss (van der Weijden et al. 2011) and vertebral fractures (Vosse et al. 2006;Vosse et al. 2009) but due to the small number of female patients we are unable to draw conclusions on the impact of gender on OPGab presence. There is also a time lag between patient recruitment and DXA scan however the usual small change (0.5 -1%) of BMD per year (Warming et al. 2002) should make this of minimal clinical relevance (Sambrook & Cooper 2006). Most patients had established disease which means that we can't extrapolate our data to newly diagnosed younger patients which we know are at higher risk of osteoporotic fractures(van der Weijden et al. 2012).

This small cross-sectional cohort had variable exposures to DMARDs, NSAIDs and biologic therapies and whilst no association between these drug exposures were observed, the study was not adequate to identify whether these therapies influenced the development of OPG-Abs. Lastly the independent association to history of fractures is an interesting finding but we are unable to specify fracture location as the study is underpowered and we don't have systematic spine radiograph assessments for the identification of subclinical vertebral fractures.

Previous studies have shown the efficacy of bisphosphonates in reducing accelerated bone resorption in AS (Cairns et al. 2004) and one study also showed a reduction of disease activity and functional decline (Maksymowych et al. 2002). Our data certainly reinforces that at least a subset of patients suffer of OP due to increased bone resorption and thus antiresoptive treatments are the right choice in treatment. The jury is out as to whether generalized suppression of osteoclasts with bisphosphonates or targeted suppression of RANKL induced Osteoclast activation with Denosumab (Lacey et al. 2012) is superior in treating OPG ab associated osteoporosis. Clearly further functional and longitudinal studies are needed to establish and quantify the impact of OPG ab in axSpA associated bone loss and to explore the potential of early detection and prevention of OPG ab associated osteoporosis.

Chapter 6: Correlation of RANKL, OPG and OPG antibodies in Rheumatic Diseases

6.1 Abstract

RANKL and OPG play a key role in the pathogenesis of osteoporosis in rheumatic diseases. Our aim was to examine the relation between OPG ab concentration and RANKL and OPG levels and to investigate if OPG antibodies interfere with free RANKL ELISA which uses recombinant OPG as capture antigen.

We measured circulating soluble RANKL, total soluble RANKL and OPG in sera from patients with rheumatic diseases. In order to investigate the effect of OPG antibodies on free RANKL ELISA purified IgG from 6 patients sera was added to different RANKL concentrations (200 and 800pg/ml) in order to be analysed by free RANKL ELISA.

Free RANKL concentrations were measured in 55 patients and were undetectably low in 23 patients. Median OPG concentration in 44 patients was 4.66 pmol/l (2.26-11.81pmol/l) and there was no association to OPG ab found. However a significant correlation between free sRANKL and OPG Ab concentrations ($r=0.273$, $p=0.001$) was found. The addition of purified IgG reduced free RANKL detection through ELISA significantly ($p<0.001$) however there was no difference between IgG with high and low OPG ab levels .

In conclusion there is no correlation between circulating OPG and OPG ab concentration. Purified Immunoglobulins but not OPG ab interfere with the measurement of free soluble RANKL. Surprisingly we found that high OPG ab concentration correlate with increased free RANKL concentration.

6.2 Introduction

The RANKL/RANK/OPG system is an essential factor in the regulation of bone resorption. As shown in chapter 1.6 chronic inflammation increases RANKL and OPG concentrations through activation of synovial cells, lymphocytes and osteoblasts. Various rheumatic diseases including Rheumatoid Arthritis(van Tuyl et al. 2010), Ankylosing Spondylitis(Crotti et al. 2002), Psoriatic Arthritis (Chandran et al. 2010) and SLE (Carmona-Fernandes et al. 2011) have been found to have increased circulating RANKL or OPG concentrations or both. RANK is expressed on membranes of mesenchymal cells such as pre-osteoblasts and osteocytes as type II membrane protein. The membrane bound form can be cleaved by a TNF-α converting enzyme like protease to the extracellular soluble form of RANKL (sRANKL)(Hofbauer and Heufelder 2001;Nakashima et al. 2000). Total soluble RANKL consists of free and OPG-bound soluble RANKL(described Chapter 2.1.3.2) . The ELISA measurement for this form is based on anti-RANKL antibody as capture antigen. Free sRANKL is the unbound soluble form which can be measured using an ELISA with OPG as capture antigen (described Chapter 2.1.3.1) (Hofbauer et al. 2004).

We hypothesized that circulating OPG ab might interfere with free sRANKL detection by competitive binding to OPG, expecting an inverse relationship between OPG ab and free sRANKL measurements. We further wanted to explore if increased OPG concentration drives OPG ab production.

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6.3 Methods

6.3.1 Study subjects

Patient recruitment and ethical approval as per Chapter 4.3. We included 55 patients with different rheumatic diseases including 27 RA, 8 PsA, 7 AS and 14 SLE patient. Each patient donated a serum and plasma sample.

6.3.2 Serum measurements

Serum and plasma samples were stored frozen at -80°c until analysis.
OPG ab serum concentrations were analyzed as described in Chapter 2.1.1. Free sRANKL, total sRANKL and OPG measurements using commercial available ELISA kits are described in Chapters 2.1.3.1, 2.1.3.2 and 2.1.3.3 respectively.Immunoglobulins were purified from 6 patient' samples as described in Chapter 2.2.1.

6.3.3 Free RANKL ELISA with recombinant RANKL standard curve

All reagents and samples were brought to room temperature before use. Recombinant RANKL (100ug/ml) was diluted with washbuffer (provided by Biomedica) to a concentration of 1000pg/ml. Further dilutions with washbuffer resulted in RANKL concentrations of 800pg/ml, 400pg/ml, 200pg/ml and 100 pg/ml. 50µl of various RANKL concentrations were mixed with 50µl of samples or washbuffer in a 96 well plate beforehand. Samples, standards, controls and various RANKL concentrations were added to a 96 well plate. Subsequent steps were as described for the free sRANKL ELISA.

6.3.4 Statistical methods

Analyses were performed using SPSS version 16.0 (SPSS, Chicago, IL, USA). Two tailed independent student t-test or one-way Anova testing was used for between-group comparisons of continuous data. For non parametric data the Mann Whitney U test was applied. The results are shown as the mean ± SD or median and interquartile range. Correlations between OPG antibodies levels, serum free RANKL, total RANKL and OPG were performed using Spearman's rank correlation test.

6.4 Results

6.4.1 Demographics and measurements of disease groups

Basic demographic data, disease characteristics and free and total RANKL, OPG and OPG ab levels are shown in Table 6.1.

Table 6.1 Demographic data and RANKL/OPG/OPG ab measurements of analysed samples

	RA	SpA (7 AS)	SLE
Number	27	15	14
Age	64 ± 11	49 ±16	50 ±16
Female	22 (82%)	7 (47%)	13 (93%)
Disease Activity	5.8 ± 1.4 (DAS 28)	4.3 ± 1.3 (BASDAI)	
ESR	28.4 ± 19.2	26.8 ± 25.6	18.5 ±9.9
Total RANKL (pmol/L)	911 ± 1008	313 ± 99	446 ± 318
free RANKL (pmol/L)	0.06 ±0.13	0.13 ± 0.15	0.08 ± 0.14
OPG (pmol/L)	5.3 ± 1.7	5.3 ± 3.2	4.8 ± 1.2
OPG ab (ng/µl)	75.1 ±95.7	85.1 ±58.0	62.7 ± 59.4

6.4.2 Osteoprotegerin concentration in Rheumatic Diseases

Circulating OPG concentrations were measured in sera of 23 RA, 10 SLE and 11 patients with Spondyloarthropathy. Median OPG concentration in 44 patients was 4.66 pmol/l (2.26-11.81pmol/l). There was no difference of mean OPG concentrations between the disease groups (p=0.819).There was a non significant trend of increased OPG concentration in older patients as shown in Figure 6.1. When split into groups mean OPG levels were significantly higher in patients above 67 years (6.31 ± 2.50 pmol/L) than in patients under 67 years of age (4.75 ± 1.70pmol/L) (p=0.022).

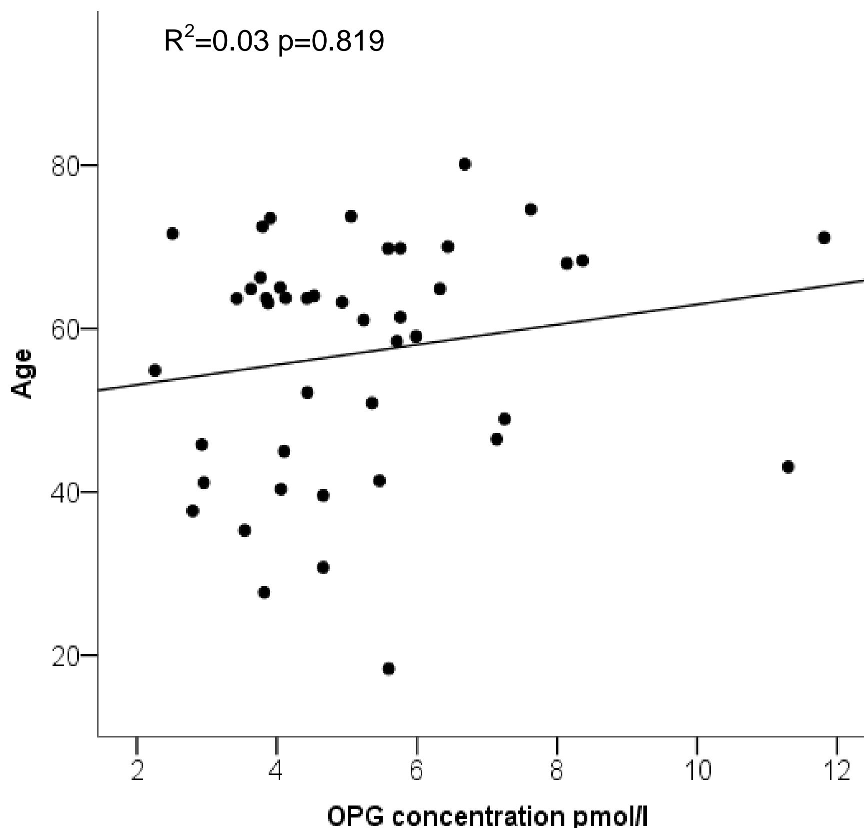

Figure 6.1 Correlation of circulating OPG level in serum of patients with rheumatic diseases and age

6.4.3 Correlation between Osteoprotegerin and Osteoprotegerin autoantibody concentrations

In order to investigate if high circulating OPG concentrations correlate to increased OPG ab concentrations, OPG concentration was measured in 44 patients with known OPG ab concentrations. Median OPG concentration in 44 patients was 4.66 pmol/l (2.26-11.81pmol/l). There was no correlation seen between circulating OPG concentrations and OPG ab concentrations (r_s=-.18 p=0.235), as shown in Figure 6.2. Mean OPG concentrations in patients with high OPG ab titre as defined in chapter 4.3.2 are not statistically different from mean OPG concentrations in patients with low OPG ab levels (4.71 ± 1.67 vs 5.33 ± 2.15 pmol/L, p=0.331).

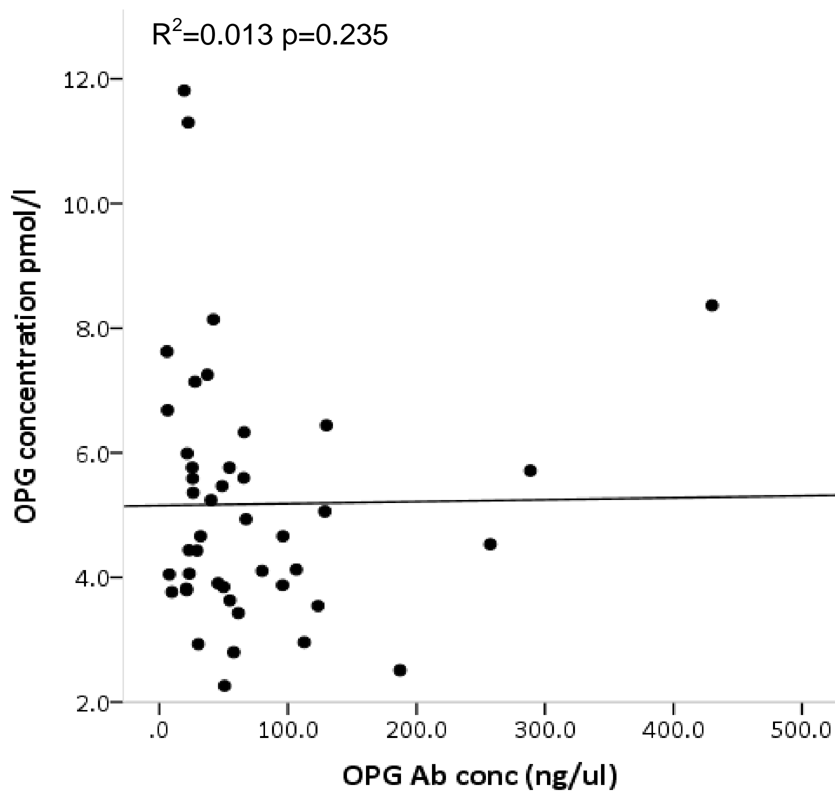

Figure 6.2 Correlation between OPG antibody levels measured by ELISA with circulating OPG level in serum of patients with rheumatic diseases

6.4.4 Correlation between OPG ab and free RANKL concentrations

We further analyzed 55 patient's sera with known OPG ab concentrations for the presence of free soluble RANKL. Amongst the patients were 26 patients with RA, 15 patients with Spondyloarthropathy and 14 patients with SLE. There was no difference of mean free sRANKL concentrations between patient groups (p=0.256). In almost half of the patients (n=23, 41.8 %) free

sRANKL concentration was undetectable. The median free RANKL concentration was 0.022 pmol (0.00- 0.524 pmol/l). There was a significant positive correlation between free RANKL and OPG Ab concentrations (Figure 6.3).

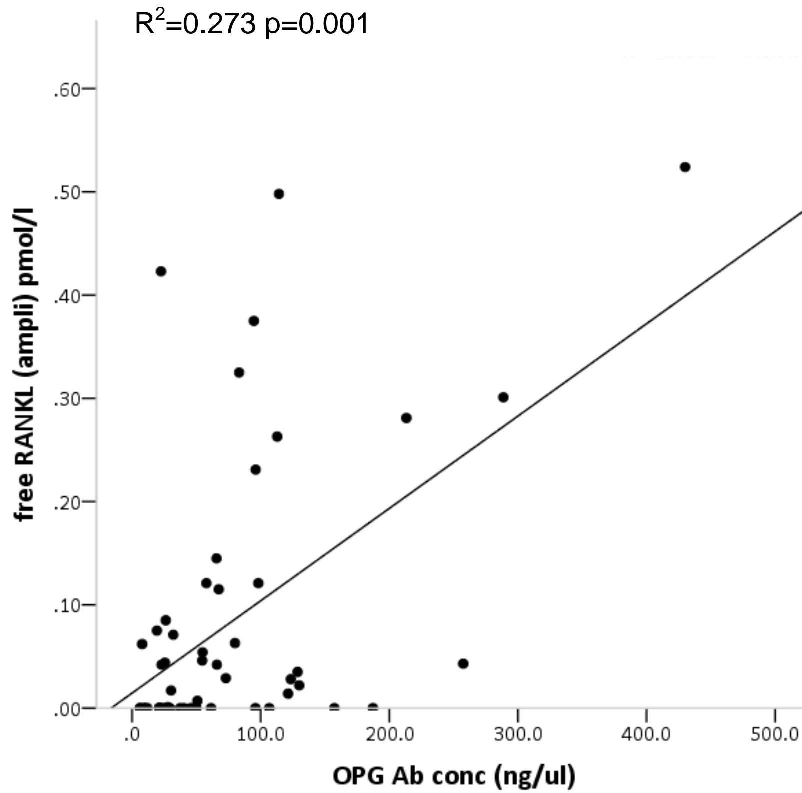

Figure 6.3 Correlation between free RANKL and OPG Ab concentrations

6.4.5 Total sRANKL concentrations

Total mean sRANKL concentration (n=43) was 376.56 pmol/l (72.95pmol/l to 3200 pmol/l) with significantly higher total sRANKL concentrations in patients with RA (910.79 ± 1007.27) than in patients with Spondyloarthropathies (313.01±98.58) (p=0.036), as shown in Figure 6.4. There was no significant correlation between total RANKL levels and OPG, OPG ab or free RANKL concentrations (Table 6.2).

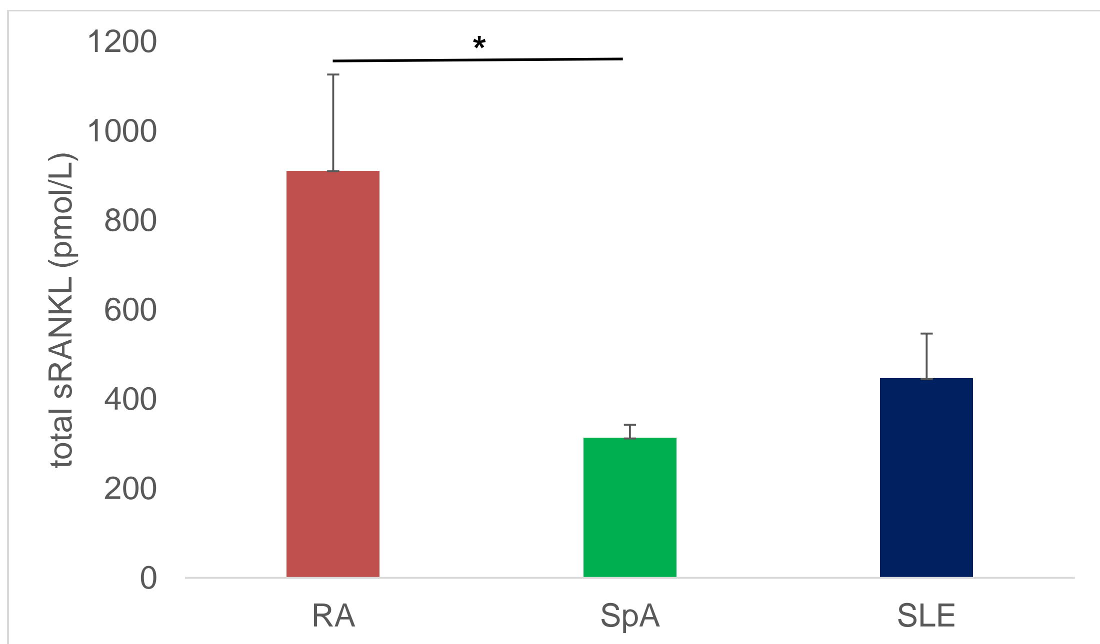

***Figure 6.4 Total sRANKL concentrations in different rheumatic diseases**, RA= Rheumatoid Arthritis, SpA= Spondyloarthropathy, SLE=Systemic Luous Erythematosus*

Table 6.2 Correlation between circulating OPG, total and free RANKL and OPG ab in rheumatic diseases

		Total RANKL (pmol/L)	OPG (pmol/L)	free RANKL (pmol/L)	OPG Ab (ng/ml)
Total RANKL	r	1	0.082	0.089	0.275
(n=43)	p-value		0.600	0.571	0.075
OPG	r	0.082	1	0.192	-0.183
(n=44)	p-value	0.600	.	0.217	0.235
free RANKL	r	0.089	0.192	1	**0.430****
(n=55)	p-value	0.571	0.217	.	0.001
OPG Ab	r	0.275	-0.183	**0.430****	1
(n=55)	p-value	0.075	0.235	0.001	.

***Correlation is significant at the 0.01 level (2-tailed)*
Spearman's rho r= Correlation coefficient

6.4.6 Effect of OPG ab on free RANKL detection

With the use of various concentrations of recombinant RANKL I have produced a RANKL standard curve on free RANKL ELISA, shown in Figure 6.5. Purified IgG of 4 patients with high OPG ab titre and purified IgG of 2 patients with low OPG ab levels were added to a simplified RANKL standard curve with three RANKL concentrations (0, 200 pg/ml and 800pg/ml) . Figure 6.6.A shows the effect of added IgG purified from sera from AS patients with low OPG concentrations. The addition of IgG reduces RANKL detection by 31% when 1:15 diluted IgG added and by 44% when undiluted IgG are added to a RANKL concentration of 800 pg/ml. A similar effect is seen with the addition of purified IgG from the index patient with high OPG ab titre (Riches et al. 2009), which reduces RANKL detection by 25%. Figure 6.6B shows the effect of added IgG with known low OPG ab titre.

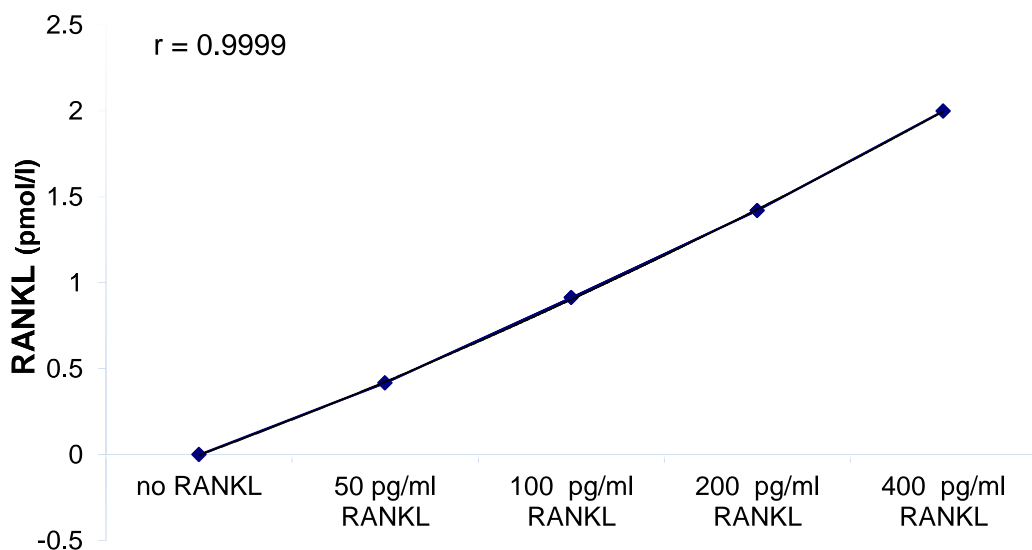

Figure 6.5 Indicative standard curve for free RANKL ELISA by addition of various recombinant RANKL concentrations

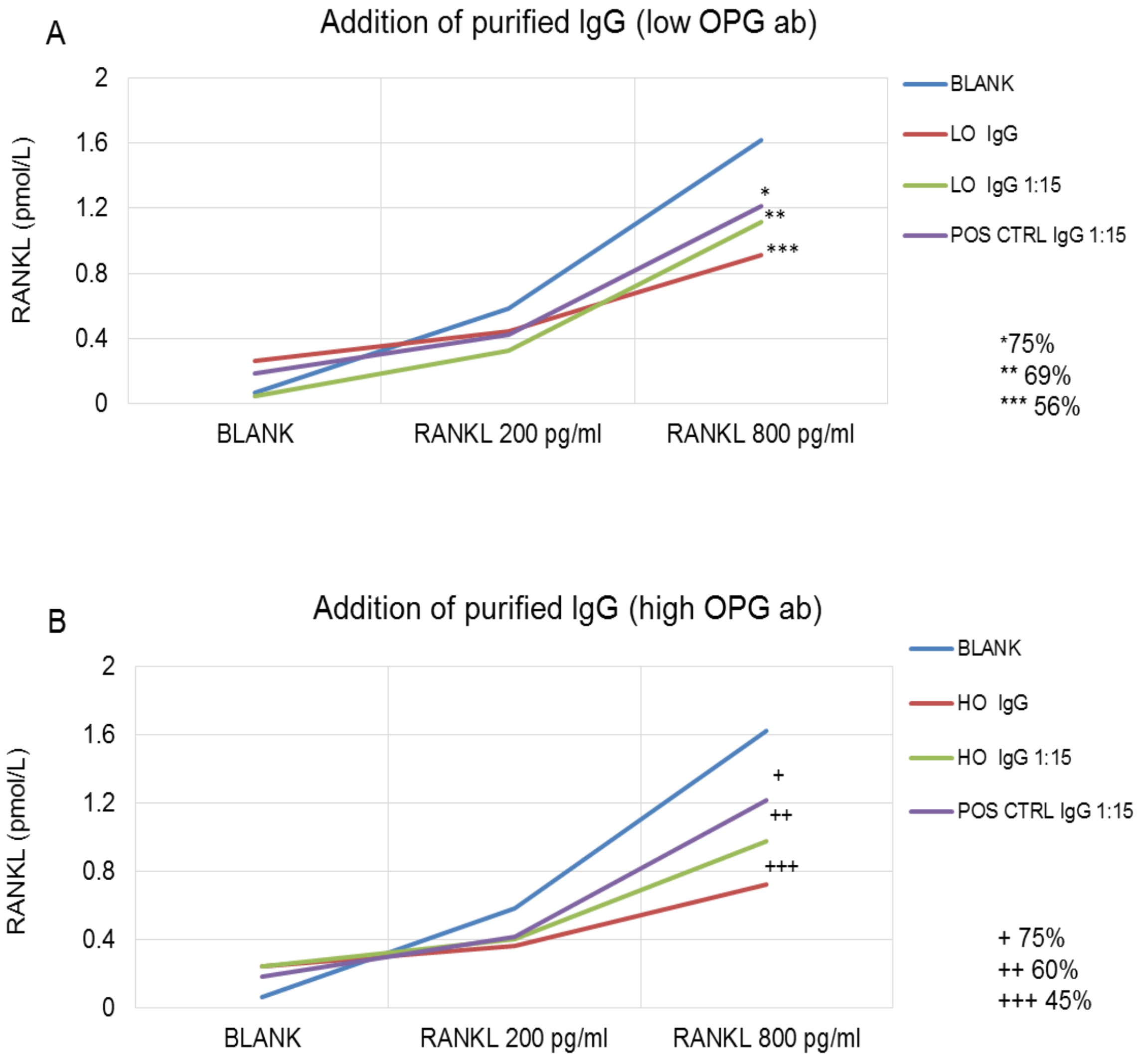

Figure 6.6 Effect on RANKL detection by addition of IgG

Addition of IgG with high OPG ab concentrations result in a significant reduction to 75%(p=0.007) of RANKL detection when IgG added from pos Ctrl, 60% (p=0.005) with addition of 1:15 diluted IgG with high OPG ab and 45%(p=0.017) with addition of undiluted IgG with high OPG ab concentrations. Addition of IgG with low OPG ab concentrations result in a reduction to 69% (p=0.064) reduction of RANKL detection when 1:15 diluted IgG added with low OPG ab and a significant reduction when to 56% (p=0.017) when undiluted IgG with low OPG ab levels added LO= IgG with low OPG ab, HO=IgG with high OPG ab titre

As shown in Figure 6.7 the addition of purified IgG with high OPG ab titre showed a mean reduction to 58.8 ± 12.6% RANKL detection compared to the impact of IgG with low OPGab addition which reduces RANKL detection to 61.4 ± 2.4%. The addition of purified IgG reduces free RANKL detection through ELISA significantly ($p<0.001$) but there is no significant difference between the addition of IgG with high or low OPG ab titre ($p= 0.799$).

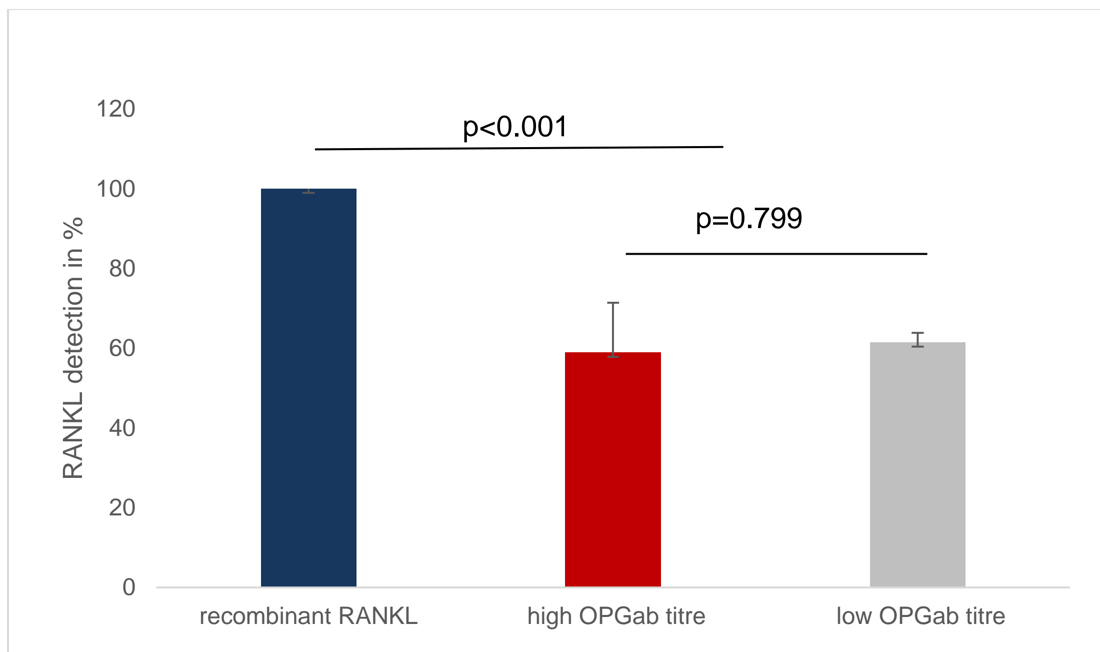

Figure 6.7 Mean reduction in RANKL detection by addition of purified IgG from serum samples with known high or low OPG ab titre

6.5 Discussion

I have shown that circulating OPG levels are not associated with OPG ab concentrations. As reported previously (Szulc et al. 2001) our findings demonstrated that elevated OPG levels were more frequently found in the elderly (patients above 67 years of age) but levels were not associated with OPG ab concentration. We know that OPG levels are increased in individuals with rheumatic diseases (Crotti et al. 2002) but we could not detect a difference in OPG concentrations between the disease groups possibly due to the small sample numbers in each group. Interestingly in previous studies looking into associations between OPG ab and disease characteristics in both RA (Chapter 4.4.3) and AS (Chapter 5.4.4),disease duration was found to be significantly associated with OPG ab concentrations. It would therefore be interesting to investigate, instead of single time point measurements of OPG, if serial measurements and the calculation of total OPG concentration over time is associated to OPG ab production.

Unsurprisingly total circulating RANKL concentrations are found to be significantly higher in patients with RA than in patients with Spondyloarthropathy. As described in Chapter 1.6 high RANKL/OPG ratio in RA lead to increased osteoclast proliferation and activation and the development of erosive disease. However free RANKL concentrations did not differ in various disease groups and there was no association between total and free RANKL levels. This might be due to increased compensatory OPG production in RA which binds to free circulation RANKL as it was shown before (Hein et al. 2008).

In order to investigate if OPG antibodies bind to recombinant OPG, which is used as capture antigen in free RANKL ELISA we initially compared OPG ab and free RANKL concentrations. Surprisingly we have found a significant positive correlation between free RANKL and OPG Ab concentrations (r=0.430, p=0.001) which was the opposite to what we had expected.. In

order to investigate this further we added purified Immunoglobulins with variable OPG antibody concentrations to a RANKL standard curve in the free RANKL ELISA. We found that the addition of immunoglobulin significantly decreases free RANKL detection but this effect was independent of OPG ab levels. These findings reject the hypothesis that OPG ab block binding of synthetic OPG to RANKL in the ELISA. However, OPG ab in serum may increase free circulating RANKL concentrations by stopping OPG binding it, hence the positive correlation between OPG ab and free RANKL concentrations.

At this stage, we are unable to explain as to why purified Immunoglobulins interfere with free RANKL ELISA but this might be a reason for frequently undetectable free RANKL concentrations as seen in previous studies (Hofbauer et al. 2004). It is unlikely that OPG is the factor that decreases free RANKL detection as the sera underwent IgG purification. In view of the existence of OPG antibodies, which are antibodies against TNF receptor superfamily, we speculate that there might be other antibodies against TNF receptors, such as RANKL antibodies which might explain above findings.

In conclusion OPG/RANK/RANKL system as crucial regulator of osteoclastogenesis in rheumatic diseases is complex and incompletely understood. Many factors such as circulating OPG, free RANKL, OPG antibodies and their relation to each other in rheumatic disease requires further experiments and studies.

Chapter 7: Discussion and Conclusion

Patients with rheumatic diseases are at increased risk of development of localized and systemic bone loss. Although chronic inflammation is thought to be the overarching key driver for accelerated bone loss in rheumatic diseases there are distinct differences in mechanism and mediators of systemic and localized bone loss in RA, seronegative Spondyloarthropathies, SLE and other rheumatic diseases as summarized in Chapter 1.6. Recent findings of autoimmune related bone pathophysiology highlight inflammation independent pathways, which may contribute to some extent to localized and systemic bone loss in autoimmune diseases. (Finzel et al. 2012;Harre et al.2012;Harre et al. 2015;Negishi-Koga et al. 2015;Riches et al. 2009).

In order to investigate if prevalence and risk factors of osteoporosis in RA has changed since the introduction of a more aggressive treatment approach and biologic therapeutics, I have re-evaluated the frequency and predictors of osteoporosis in a contemporary Rheumatoid Arthritis cohort. In comparison to a gender and age matched control cohort bone mineral density was significantly lower in men and women with RA above the age of 45. I have found that about 30% of the RA cohort had Osteoporosis, which is in keeping with older reports which recorded a prevalence rate between 17% and 36%. Amongst the RA patients diagnosed with Osteoporosis there was a long delay between the diagnosis of Rheumatoid Arthritis and the referral for bone mineral density assessment which might represent lack of awareness of Osteoporosis risk in Rheumatoid Arthritis. In order to raise awareness and to better target DEXA screening of patients with RA I developed a osteoporosis prediction model for patients with RA which can be used in everyday clinical practice. However, the study was limited by its retrospective nature, incomplete data in particular that of disease activity and corticosteroid use and the inclusion of patients with long disease duration. The question, if a change in treatment paradigm and the frequent use of biologic treatment has

made a difference to osteoporosis and fracture prevalence, was not completely answered. A large prospective case control studies with systematic data collection and regular DEXA scanning would allow a better judgement if and how RA treatment alters BMD loss.

Apart from understanding clinical risk factors for Osteoporosis one of the key question of this thesis was to explore the potential role of newly identified auto-antibodies against osteoprotegerin in the development of Osteoporosis in rheumatic diseases. Our research team in Edinburgh have previously described a young man who sustained multiple fractures and who was found to have severe osteoporosis due to autoantibodies, which have eliminated the bone protecting effect of osteoprotegerin (OPG). Our main question was if these antibodies do occur in patients with rheumatic diseases and if they are related to increased bone loss. We have found that antibodies against OPG can be detected in about 10% of patients with Rheumatoid Arthritis and Ankylosing Spondylitis. The detection of OPG ab in these rheumatic diseases is significantly higher than in control populations. We have also shown that there is a relation between high OPG antibody levels and bone resorption markers in RA and bone mineral density in AS. Additional functional studies have shown that OPG ab block OPG in a HEK-293 reporter assay. These findings are in keeping with our hypothesis that Osteoporosis may in some cases be caused by an immune 'attack'. A summary of the proposed mechanism of OPG ab and their effect on osteoclastogenesis is shown in Figure 7.1. The fact that not all detectable OPG ab may be functional might be related to different epitope recognition as recently shown in the study by Real et al (2015). These findings may also help to explain why OPG ab are also detected in a small proportion (approx. 1%) of healthy controls with normal BMD (T-score between -1 and 1 SD) as shown in Chapter 4.4.2. However in order to explore the question about antibody functionality further we would need to test detected OPG ab in further in-vitro or preferably in-vivo functional experiments.

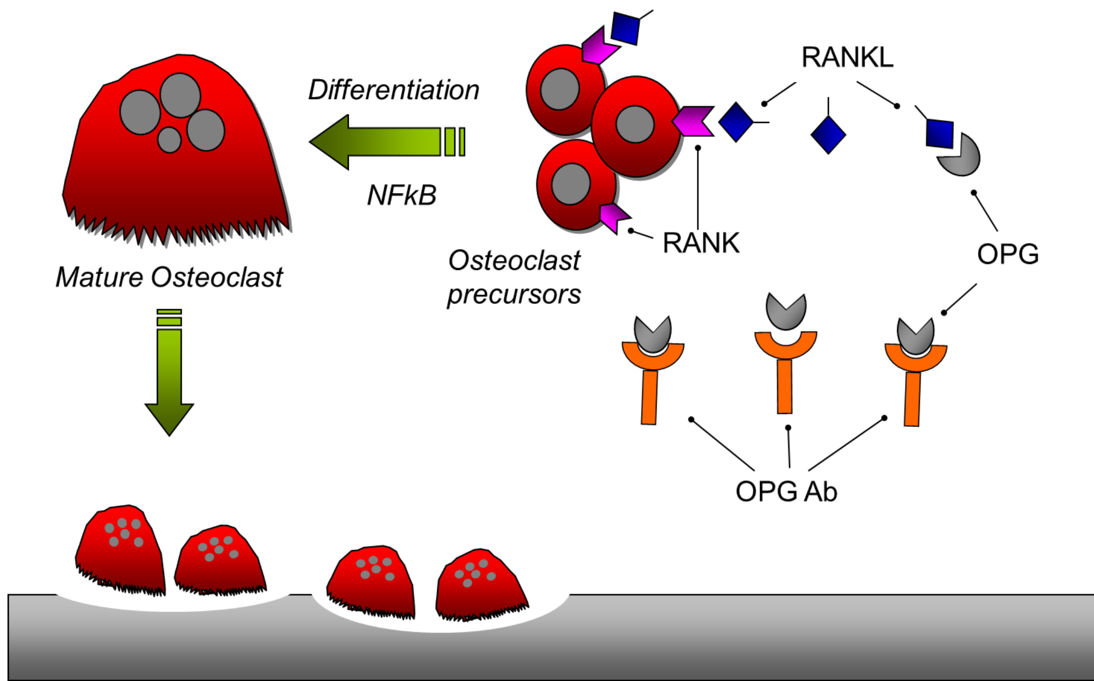

Figure 7.1 Schematic diagram of proposed mechanism of OPG antibodies on osteoclastogenesis *RANK (Receptor Activator of NfκB) is a receptor expressed on pre-osteoclasts and osteoclasts. RANKL (RANK- Ligand) binds to RANK and promotes osteoclast differentiation and activation. OPG (Osteoprotegerin) acts as decoy receptor to RANKL. Osteoclastogenesis is largely regulated by RANKL/OPG ratio. OPG antibodies (ab) bind and neutralize OPG and allow RANKL induced increased osteoclastogenesis*

It is currently unknown if OPG ab play also a role in other rheumatic diseases such as Psoriatic Arthritis, SLE or Scleroderma. Our research group has identified increased prevalence of OPG ab in patients with coeliac disease and showed that OPG ab presence was independently associated with decreased hip bone mineral density Z-score.

It would be of interest to study OPG ab presence and role in Psoriatic Arthritis as in contrast to other rheumatic diseases systemic bone loss does not seem to be a cardinal problem, however localized bone loss in form of aggressive erosive disease and rapid osteolysis, can lead to a destructive, debilitating form of Psoriatic Arthritis.

Another disease of interest would be SLE where the pathophysiology is strongly linked to the formation and presence of auto-antibodies and where disease mechanism have been shown to involve functional antibodies.

The results of chapter 6 have highlighted that the interplay between OPG, free and total RANKL and OPG antibodies are complex. It has also demonstrated that the measurement of one or more of these cytokines in clinical practice may at times not be reliable or useful and requires careful interpretation as suggested previously for OPG and RANKL measurements (Hofbauer et al 2001).

In order to answer the question about clinical relevance of OPG ab further large scale and longitudinal studies are necessary. However if these studies confirm our findings that OPG ab do play a role in the development of osteoporosis in a subgroup of patients, the presence of OPG ab may guide future osteoporosis treatment. Clearly antiresoprtive treatment will be the preferred choice however the jury is out if RANKL blockade through administration of Denosumab or generalised Bisphosphonate induced osteoclast inhibition will be more effective in OPG ab associated osteoporosis.

Additionally longitudinal studies may help to answer the question what drives OPG ab production, specially since longer disease duration was found to be associated to OPG ab presence in RA and AS. The positive correlation between disease activity and OPG ab in RA is thought to be more likely an epiphenomenon as CCP antibody and Rheumatoid Factor positivity are associated to more severe disease. We could not find an association between disease activity (BASDAI) or inflammatory markers and OPG ab in AS.

Further areas of interest would be to investigate the role of OPG ab in the development of bone erosions in RA and PsA. As shown in chapter 1.6 Rheumatoid Arthritis is an autoimmune condition which is characterized by chronic inflammation and the development of articular and periarticular bone loss. Erosions can occur in the early stages of the disease and in some cases even precede disease onset. The development of erosions is in many ways similar to the pathogenesis of osteoporosis with the exception that the inflammatory pannus is in immediate vicinity to bone and cartilage. Erosions are the result of increased osteoclast activity and blunted bone formation. Therefore it would be of interest to investigate if OPG antibodies in early RA influence the development of bone erosions and if OPG ab levels are affected by RA therapy in particular by B cell inhibition (Rituximab).

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Appendices

A1 Materials and Reagents

All **materials** and **reagents** are listed in the table below in an alphabetical order.

Materials and reagents	**Supplier**
A Minimum Essential Medium (α MEM)	Sigma Aldrich, Dorset, UK
alamarBlue^{TM} reagent	Invitrogen, Paisley, UK
Bovine serum albumin (A7906)	Sigma Aldrich, Dorset, UK
Centrifuge tubes (15 and 50ml)	Fisher Scientific, Leicestershire, UK
Coating buffer (C3041)	Sigma Aldrich, Dorset, Kent
Costar ELISA plate (3369 EIA/RIA)	Fisher Scientific, Leicestershire, UK
D Minimum Essential Medium (dMEM)	Sigma Aldrich, Dorset, UK
DL-Dithiothreitol (DTT)	Sigma Aldrich, Dorset, UK
DMSO	Sigma Aldrich, Dorset, UK
EDTA	Sigma Aldrich, Dorset, UK
Ethanol absolute	Fisher Scientific, Leicestershire, UK
Fetal calf serum (FCS)	Fisher Scientific, Leicestershire, UK
HEK 293 luciferase reporter cell line	eBioscience Ltd,Hatfield, UK

(RC0014)	
Human recombinant RANKL	Gift from Dr. Patrick Mollat (Proskelia SASU)
IgG elution buffer (Cat 21004)	Fisher Scientific, Leicestershire, UK
Jackson ImmunoResearch Anti-rabbit secondary antibody	Stratech Scientific Unit, Newmarket Suffolk, UK
L-Glutamine	Invitrogen, Paisley, UK
LPS (IMG-2204)	Imgenex,2B scientific, Oxford, UK
Magic Marker	Invitrogen, Paisley, UK
Melon™ Gel IgG Spin Purification Kit Thermo Scientific 45206	Fisher Scientific, Leicestershire, UK
Methanol	Fisher Scientific, Leicestershire, UK
Micro titre plate (Sterilin, 611F96)	Fisher Scientific, Leicestershire, UK
Neubauer haemocytometer	*Hawksley*, Lancing, UK
Osteoprotegerin (Human recombinant Cat 185-05)	R&D Systems, Abingdon, UK
Osteoprotegerin ELISA Biomedica, BI-20403	Oxford Biosystems, Oxford, UK
PBS tablets	Invitrogen, Paisley, UK
Penicillin/Streptomycin	Invitrogen, Paisley, UK
Pierce Protein G IgG Binding Buffer	Fisher Scientific, Leicestershire, UK

21019	
Pierce SuperSignal® West Dura Extended Duration Substrate	Fisher Scientific, Leicestershire, UK
Pierce Protein G spin columns	Fisher Scientific, Leicestershire, UK
Primary mouse monoclonal antibody to OPG	AbCam, Cambridge, UK
(Cat Ab1194 – clone number 98A1071)	
Primary rabbit polyclonal antibody to OPG (Cat Ab 9986)	AbCam, Cambridge, UK
Primary rabbit polyclonal antibody to-OPG biotin conjugated (Cat Ab18068)	AbCam, Cambridge, UK
Protein G IgG Binding Buffer (Cat 21019)	Fisher Scientific, Leicestershire, UK
RANKL ELISA Total (Biovendor RD193004200R)	Oxford Biosystems, Oxford, UK
RANKL ELISA Free (Biomedica BI-20452)	Oxford Biosystems, Oxford, UK
Secondary anti-human peroxidase conjugated antibody (109-035-088)	Stratech Scientific Unit, Newmarket Suffolk, UK
Secondary anti- mouse secondary antibody	Stratech Scientific Unit, Newmarket Suffolk, UK
(Cat 715-035-151)	
Sodium dodecyl sulphate (SDS)	Bio-Rad Laboratories, Hertfordshire, UK

Steady-Glo reagent	Promega, Southampton, UK,
Stripettes (5, 10, 25 and 50ml)	Sarstedt Ltd, Leicester, UK
Syngene BIO imaging system	Fisher Scientific, Leicestershire, UK
Syringes (all sizes)	Becton Dickinson, Berkshire, UK
TBS 10x (T5912)	Sigma Aldrich, Dorset, UK
TBE buffer 10X	Invitrogen, Paisley, UK
2%TCH (serum replacement)	MP-Biomedicals, Cambridge, UK
Tissue culture 75cm^2 flasks	Fisher Scientific, Leicestershire, UK
Tissue culture microplates (6– 96well plates)	Fisher Scientific, Leicestershire, UK
TMB Stop solution (50-85-06)	KPL, Maryland, USA
Tris	Bio-Rad Laboratories, Hertfordshire, UK
Tris-EDTA buffer	Sigma Aldrich, Dorset, UK
Trizol reagent	Invitrogen, Paisley, UK
Trizol® Reagent	Invitrogen, Paisley, UK
Trypsin/EDTA	Sigma Aldrich, Dorset, UK
Tween-20 (27,434-8)	Sigma Aldrich, Dorset, UK
UV 96 well plates for plate reader	Fisher Scientific, Leicestershire, UK

A2 Apparatus

All **apparatus** are listed in the table below in alphabetical order.

Apparatus	Supplier
Balancer Fisherbrand	Fisher Scientific, Leicestershire, UK
Bench-top centrifuge	SciQuip, Shropshire, UK
Benchtop Eppendorf centrifuge	Fisher Scientific, Leicestershire, UK
Bio-Tek Synergy HT plate reader	Fisher Scientific, Leicestershire, UK
Envair Bio2 safety cabinets	H&V Commissioning Services Ltd., Ayrshire, UK
Grant OLS 200 water bath	Thistle Scientific, Glasgow, UK
Nichiryo America Inc. Pipettes (2, 10, 100, 200 and 1000µl)	Thistle Scientific, Glasgow, UK
NoAir Class II Biological safety cabinet	TripleRed Ltd., Buckinghamshire, UK
PowerPac basic™	Bio-Rad Laboratories, Hertfordshire, UK
Syngene GeneGenius Gel Bio-Imaging system	Fisher Scientific, Leicestershire, UK
SynSyngene GeneGnome Bio-Imaging system for chemiluminescence	Fisher Scientific, Leicestershire, UK
Vertical Criterion™ gel tanks	Bio-Rad Laboratories, Hertfordshire, UK

A3 Software

All **software** are listed in the table below in alphabetical order.

Software	**Supplier**
Bio-Tek Gen5^{TM} plate reader software	Fisher Scientific, Leicestershire, UK
Minitab Release 16.2.4	Minitab Inc., Pennsylvania, US
SPSS statistics 19	SPSS Inc
Syngene GeneSnap software	Fisher Scientific, Leicestershire, UK
Syngene GeneTool software	Fisher Scientific, Leicestershire, UK

A4 Solutions

Appendix 3.3 Solution for ELISA
TBST wash buffer 0.05 % (v/v) Tween 20 in TBS (pH 7.4) made up in dH2O. *Coating buffer* Carbonate/bicarbonate buffer 0.2 M, pH 9.6, prepared commercially with deionised water from sigma and buffer capsules C3041-100cap.