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Anti tumour necrosis factor - alpha : does it rescue bone loss in inflammatory bowel disease patients?

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Anti Tumour Necrosis Factor-α: Does It Rescue Bone Loss in Inflammatory Bowel Disease Patients?

By

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A thesis submitted to

The Royal College of Surgeons in Ireland and **The National University of Ireland**
on research carried out with

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AND
Department of Gastroenterology, Adelaide & Meath Hospital, Tallaght

In fulfilment for the degree of
Doctor in Medicine (MD)
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Supervised by Dr. Jacqueline S Daly and Dr. Barbara M Ryan

DECLARATION

I declare that this thesis, which I submit to RCSI for examination in consideration of the award of a Doctor in Medicine (MD) degree, is my own personal effort. Where any of the content presented is the result of input or data from a related collaborative research programme this is duly acknowledged in the text such that it is possible to ascertain how much of the work is my own. I have not already obtained a degree in RCSI or elsewhere on the basis of this work. Furthermore, I took reasonable care to ensure that the work is original, and, to the best of my knowledge, does not breach copyright law, and has not been taken from other sources except where such work has been cited and acknowledged within the text.

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To Amma and Appa

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This dissertation, submitted for the degree of Doctor of Medicine to the Royal College of Surgeons in Ireland, describes the research work carried out in the Division of Biology, Royal College of Surgeons in Ireland and Department of Gastroenterology, Adelaide & Meath Hospital, Tallaght under the supervision of Dr Jacqueline S Daly (Jackie) and Dr Barbara M Ryan. Various individuals and organizations contributed to the successful completion of this research work. There are some whose names must be mentioned, those not included here can rest assured that they are not forgotten. First and foremost, I am extremely grateful to both my supervisors Jackie and Barbara for offering me the opportunity to undertake this MD under their supervision. I am thankful to Jackie for agreeing to take me under her supervision and her tremendous help in getting this research project kicked off. Her abundance patience to tolerate my lack of laboratory experimental skills at the beginning of this work is gratefully acknowledged. I have spent great deal of time learning from her and her generosity and encouragement have ensured that my research work in Royal College of Surgeons was enjoyable and fulfilled. I will always be very appreciative for the support, belief, and encouragement that Barbara has shown me throughout my time in Tallaght. Furthermore, the funding for this work would never been possible without her unfaltering enthusiasm and for that I am indebted to her. Her wisdom and professionalism have taught me innumerable lessons on research, mentorship, and life in general. I am honored to have had such a professional as a mentor. Needless to say, without the constant advice, encouragement, and support of my

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Dr. Sundaram Ganesan Veerappan

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LIST OF ABBREVIATIONS

25OHD	25-Hydroxy-vitamin D
ACTH	Adrenocorticotropic hormone
ALP	Alkaline phosphatase
AMNCH	Adelaide & Meath Hospital
ATCC	American Tissue Culture Collection
BALP	Bone-specific alkaline phosphatase
BMC	Bone mineral content
BMD	Bone mineral density
BMP	Bone morphogenic protein
BSP	Bone Sialoprotein
CD	Crohn's disease
CDAI	Crohn's Disease Activity Index
CRP	C-reactive protein
DXA	Dual-energy X-ray absorptiometry
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethylsulfoxide
DPD	Deoxypyridinoline
ECM	Extracelluar matrix
EDTA	Ethylenediaminetetraacteic acid
EGF	Epidermal growth factor
EIA	Enzyme-immuno assay
ELISA	Enzyme linked immunosorbent assay
FBS	Foetal Bovine Serum
FBS	Foetal Bovine Serum
FDA	Food and Drug Administration
FGF	Fibroblast growth factor
G-CSF	Granulocyte colony stimulating factor
GMCSF	Granulocyte macrophage colony stimulating factor
H_2O	Hydrogen oxide (water)
HELP	Collagen I alpha 1 helicoidal peptide
hFOB	Human foetal osteoblast
HLG	Hydroxylysine-glycosides

HPLC	High-performance liquid chromatography
HRT	Hormone Replacement Therapy
Hyp	Hydroxyproline, total and dialyzable
IBD	Inflammatory bowel disease
ICTP, CTX-MMP	Carboxyterminal cross-linked telopeptide of type I collagen
IFN	Interferon
IGF	Insulin like growth factor
IL	Interleukin
IL 1ra	Interleukin 1 receptor antagonist
IRMA	Immuno-radiometric assay
MD	Doctor in Medicine
MLO	Murine long bone osteocyte
m-RNA	Messenger ribonucleic acid
NIH	National Institute of Health
NSAID	Non-steroidal anti-inflammatory drugs
OC	Osteocalcin
OPG	Osteoprotegerin
P1CP	C-terminal propeptide of type 1 procollagen
P1NP	N-terminal propeptide of type 1 procollagen
PBS	D-Phosphate Buffer Solution
PDGF	Platelet-derived growth factor
PGE_2	Prostaglandin E_2
pNPP	p-Nitrophenyl phosphate
PTH	Parathyroid hormone
PYD	Pyridinoline
RANK	Receptor activator of NF kappa
RANKL	Receptor activator of NF kappa ligand
RANTES	Regulated on Activation, Normal T Expressed and Secreted
REDOX	Oxidation-Reduction
RIA	Radio-immuno assay
SCS	Simple Colitis Score
sCTx	Carboxyterminal cross-linked telopeptide of type I collagen
SD	Standard deviation
SJH	St. James's Hospital
sNTx	Aminoterminal cross-linked telopeptide of type I collagen
SPSS	Statistical Package for Social Sciences

TGF	Transforming growth factor
TNF	Tumour necrosis factor
TRAP	Tartrate resistant acid phosphatase
UC	Ulcerative colitis
VEGF	Vascular Endothelial Growth Factor
VGF	Vascular Growth Factor
WHO	World Health Organization

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PUBLISHED PAPERS AND PRESENTATIONS

Parts of this thesis have been published (under review) and/or presented in both national and international conference meetings as follows:

PUBLICATIONS (UNDER REVIEW)

1. **Veerappan SG**, O'Morain CA, Daly JS, Ryan BM. Review article: Effect of biologic therapy on bone metabolism in inflammatory bowel disease patients.
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2. **Veerappan SG**, Healy M, Walsh JB, O'Morain CA, Ryan BM, Daly JS. Adalimumab therapy has a beneficial effect on bone metabolism in Crohn's disease patients.
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3. **Veerappan SG**, Healy M, Walsh JB, O'Morain CA, Ryan BM, Daly JS. Long term effect of Infliximab on bone metabolism in inflammatory bowel disease patients.
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2. **Veerappan SG**, Healy M, Walsh JB, Ryan BM, Daly JS. Effect of anti-TNF alpha (infliximab) on human osteoblast cell viability in patients with active IBD: An *in vitro* study. ***Gastroenterology.*** **Vol 132, Issue 4, Supplement 1, April 2007.**
3. **Veerappan SG**, Healy M, Walsh JB, Kennedy M, Hamilton S, Keogh M, O'Morain CA, Ryan BM, Daly JS. Infliximab: Does it Rescue Bone loss in IBD patients? ***Gut.*** **Supplement III, Vol 56, 2007.**
4. **Veerappan SG**, Healy M, Walsh JB, Kennedy M, O'Morain CA, Ryan BM, Daly JS. Infliximab in inflammatory bowel disease: 'Can we heal two diseases with one drug?' ***Gastroenterology.*** **Vol 134, Issue 4, Supplement 1, April 2008, page A-513.**
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PRESENTATIONS AT NATIONAL MEETINGS – ORAL

1. **Veerappan SG**, Healy M, Walsh JB, Ryan BM, Daly JS. Effect of anti-TNF alpha (infliximab) on human osteoblast cell viability in patients with active IBD: An *in vitro* study. ***RCSI Research Day 2007. April 2007.***
2. **Veerappan SG**, Healy M, Walsh JB, Kennedy M, Breslin N, O'Morain CA, Ryan BM, Daly JS. Infliximab: Does it Rescue Bone loss in IBD patients? ***Irish Society of Gastroenterology Spring Meeting, Killarney. April 2007.***
3. **Veerappan SG**, Healy M, Walsh JB, Kennedy M, O'Morain CA, Ryan BM, Daly JS. 1 year follow up study of infliximab maintenance therapy on bone metabolism in IBD patients. ***Irish Society of Gastroenterology Winter Meeting, Dublin. November 2007.***
4. **Veerappan SG**, Healy M, Walsh JB, Kennedy M, Keogh M, O'Morain CA, Ryan BM, Daly JS. Anti TNF α: Does it Rescue Bone loss in IBD patients? ***RCSI Research Day 2008. March 2008.***
5. **Veerappan SG**, Kennedy M, O'Morain CA, Ryan BM, Daly JS. Predicting positive response to infliximab in inflammatory bowel disease using clinical and cytokine parameters. ***Irish Society of Gastroenterology Spring Meeting, Sligo. April 2008.***
6. **Veerappan SG**, Healy M, Walsh JB, Kennedy M, O'Morain CA, Ryan BM, Daly JS. Anti TNF α: Does it Rescue Bone loss in IBD patients? ***Irish Society of Gastroenterology Winter Meeting, Trim. November 2008.***

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2. **Veerappan SG**, Healy M, Walsh JB, Hamilton S, Kennedy M, Lindsay F, O'Morain CA, Ryan BM, Daly JS. Impact of Adalimumab therapy on bone metabolism in Crohn's disease patients: A 3 months follow up study. ***Irish Society of Gastroenterology Winter Meeting, Dublin. November 2007.***
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5. **Veerappan SG**, Healy M, Walsh JB, Kennedy M, O'Morain CA, Ryan BM, Daly JS. Infliximab treatment increases bone formation markers concentration in inflammatory bowel disease patients. ***Irish Society of Gastroenterology Winter Meeting, Trim. November 2008.***

PRESENTATIONS AT INTERNATIONAL MEETINGS - POSTER

1. **Veerappan SG**, Healy M, Walsh JB, Ryan BM, Daly JS. Effect of anti-TNF alpha (infliximab) on human osteoblast cell viability in patients with active IBD: An *in vitro* study. ***34th European Symposium on Calcified Tissues, Copenhagen. May 2007.***
2. **Veerappan SG**, Healy M, Walsh JB, Ryan BM, Daly JS. Effect of anti-TNF alpha (infliximab) on human osteoblast cell viability in patients with active IBD: An *in vitro* study. ***Digestive Disease Week, Washington. May 2007.***
3. **Veerappan SG**, Healy M, Walsh JB, Kennedy M, Hamilton S, Keogh M, O'Morain CA, Ryan BM, Daly JS. Infliximab: Does it Rescue Bone loss in IBD patients? ***15th United European Gastroenterology Week, Paris. October 2007.***
4. **Veerappan SG**, Healy M, Walsh JB, Kennedy M, O'Morain CA, Ryan BM, Daly JS. Infliximab in inflammatory bowel disease: 'Can we heal two diseases with one drug?' ***Digestive Disease Week, San Diego. May 2008.***
5. **Veerappan SG**, Healy M, Walsh JB, Kennedy M, O'Morain CA, Ryan BM, Daly JS. Infliximab in inflammatory bowel disease: 'Can we heal two diseases with one drug?' ***35th European Symposium on Calcified Tissues, Barcelona. May 2008.***
6. **Veerappan SG**, Healy M, Walsh JB, Kennedy M, O'Morain CA, Ryan BM, Daly JS. Impact of Adalimumab therapy on bone metabolism in Crohn's disease patients: A 3 months follow up study. ***35th European Symposium on Calcified Tissues, Barcelona. May 2008.***

CHAPTER 1 - INTRODUCTION

1.1 Inflammatory Bowel Disease

1.1.1 General Overview

Inflammatory bowel disease (IBD) is a group of disorders of the gastrointestinal tract characterized by intestinal inflammation and a chronic relapsing course. IBD has traditionally been categorized as either ulcerative colitis (UC) or Crohn's disease (CD) on the basis of clinical, radiological, endoscopic and histological criteria [1], **(Figure 1.1 and Figure 1.2)**. About 10 % of colitis cases show overlapping features of the two major forms and are designated intermediate colitis [1]. Both UC and CD are commonly characterized by a series of clinical exacerbations and remissions requiring long term use of medications, and frequently necessitating surgical interventions.

Figure 1.1: Endoscopic appearance of Ulcerative colitis [2] - *marked by diffuse, superficial inflammation of the colonic mucosa, beginning in the rectum and extending proximally to involve any contiguous length of colon.*

Figure 1.2: Endoscopic appearance of Crohn's colitis [2] – *marked by transmural inflammation of the colonic mucosa.*

Although the etiology of IBD remains to be defined, recent experimental and clinical studies suggests that the initiation and pathogenesis of these diseases are multi-factorial, involving interactions between genetic, environmental and immune factors [3].

1.1.2 Prevalence and Incidence

IBD is not evenly distributed world-wide. There is a clear tendency to a higher incidence in developed countries compared with less developed countries [4]. North America, the United Kingdom and Scandinavia have the highest rates [4]. In areas in which data are available over a number of years, the incidence of UC has remained relatively constant [5-8]. Unlike UC, the incidence of CD has risen progressively since its original description [9-13]. In a European study the reported incidence rates for UC and CD in Ireland were 14.8 and 5.9 per 100,000 populations over a two year period 1991–1993 [14]. UC and CD are most commonly diagnosed in late adolescence and early adulthood, but the diagnosis may occur at all ages.

1.1.3 Pathology and clinical presentation

CD may involve the entire gastrointestinal tract from mouth to perianal area, whereas in UC the inflammation is confined to the large bowel [1]. Furthermore, CD is characterized by transmural rather than superficial mucosal inflammation and by skip lesions rather than continuous disease [1]. The transmural inflammatory nature of CD often leads to fibrosis and to obstructive clinical presentations which are not typically seen in UC. The most common symptoms are diarrohea, crampy abdominal pain, fever, anorexia, and weight loss. UC on the other hand is characterized by recurring episodes of inflammation limited to the mucosal layer of the colon. It almost invariably involves the rectum and may extend in a proximal and continuous fashion to involve other portions of the colon. The major symptoms of UC are urgent diarrhoea, rectal bleeding, passage of mucus, and crampy abdominal pain. The severity of symptoms generally correlates with the extent of the disease.

1.1.4 Extra intestinal manifestations

Multiple other organ systems can be affected in IBD, including the bones and joints, skin, eyes, hepatobiliary system, lungs, and kidneys. Collectively, these are called extra-intestinal manifestations of IBD **(Figure 1.3)**, and they can occur prior to, in conjunction with, or subsequent to active bowel disease. The overall prevalence of any extra intestinal manifestation in IBD patients ranges from 21%-40% [15-17]. In most large studies of IBD, the prevalence of extra intestinal manifestations is higher in CD compared with UC [16-18]. There may also be racial differences in prevalence, with blacks having a higher risk for eye and joint manifestations, and Hispanics having a higher risk for skin manifestations, compared with whites [19].

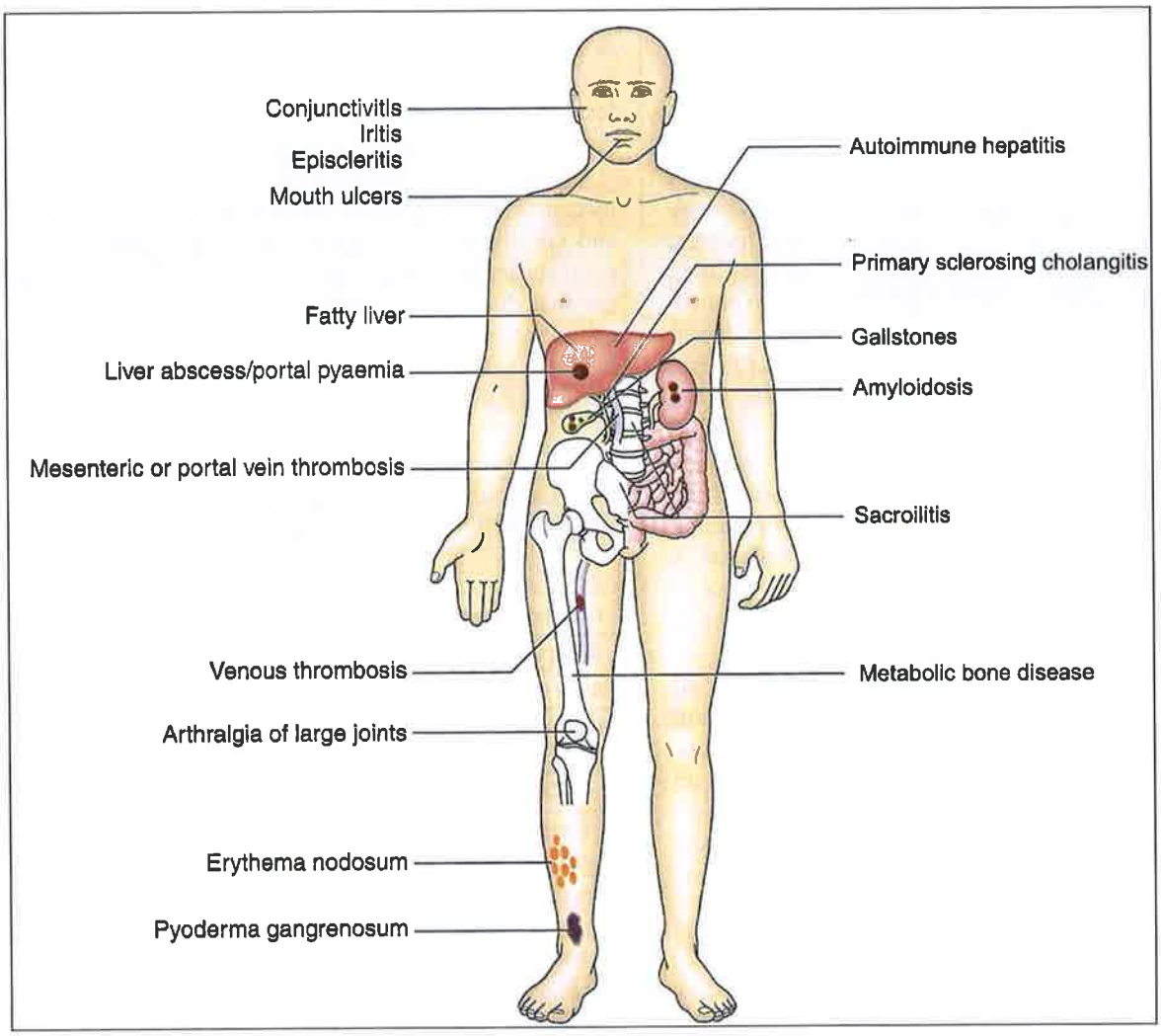


Figure 1.3: Extra-intestinal manifestations of IBD [20]

1.2 Basic aspects of bone biology

1.2.1 Definition of osteoporosis and osteopenia

The World Health Organization (WHO) Working Group defines osteoporosis according to measurements of bone mineral density (BMD) using dual-energy X-ray absorptiometry (DXA) [21]. Thus, osteoporosis is defined as a bone density T score at or below 2.5 standard deviations below normal peak values for young adults. Established or severe osteoporosis is present when there is at least one or more fragility fracture in conjunction with a T score < -2.5. Osteopenia refers to T scores between –1.0 and –2.5. Normal bone density is present if the T score is greater than –1 [21] **(Figure 1.4)**. The WHO definition of osteoporosis only takes into consideration measurement of bone density, with no component of bone quality.

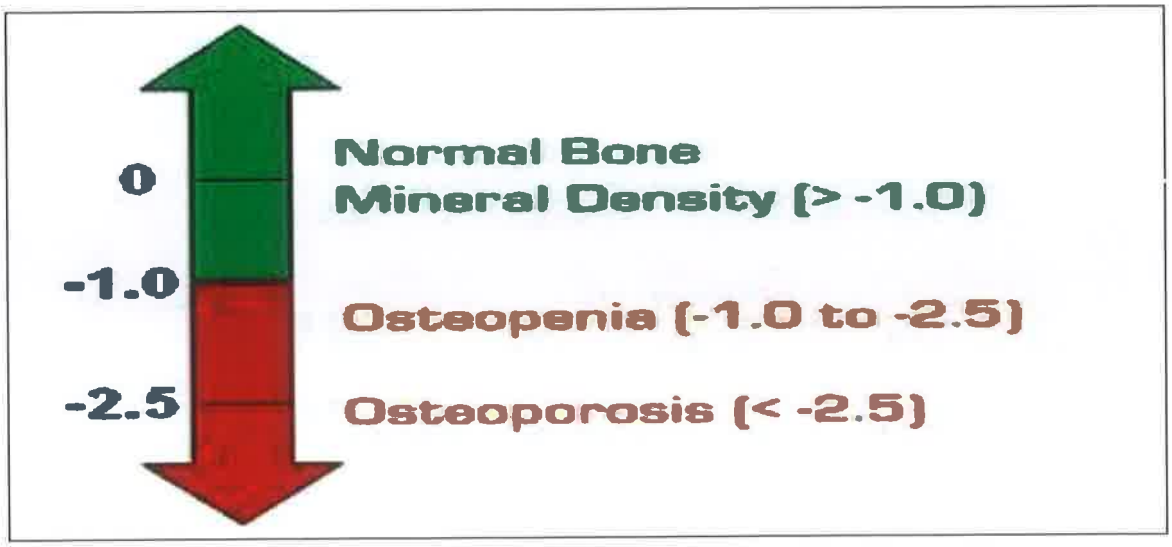


Figure 1.4: Definition of osteoporosis and osteopenia based on WHO criteria

A clinical definition of osteoporosis was developed in 2001 by the National Institute of Health (NIH) Consensus Development Panel on Osteoporosis. It stated: "Osteoporosis is defined as a skeletal disorder characterized by compromised bone strength predisposing a person to an increased risk of fracture". This definition takes into consideration that there are other factors that influence bone quality such as the micro architecture of bone.

In Caucasian populations, approximately 50% of postmenopausal women and 20% of men older than 50 years will experience at least one fragility fracture in their remaining lifetime [22]. In Caucasian populations, the lifetime risk for fragility fracture of the hip is 14% for women and 3% for men, and for fragility fracture of the spine is 28% for women and 12% for men [23]. In the majority of cases of osteoporosis, the bone loss is due to the normal ageing process, in which case it is termed primary or idiopathic osteoporosis. However, in up to 20-30% of post-menopausal women, 50% of pre-menopausal women, and more than 50% of men, osteoporosis is caused or exacerbated by a disease or treatment, in which case it is termed secondary osteoporosis [24]. Currently, there are more than 50 recognized causes of secondary osteoporosis [25]. Since very large numbers of people are affected by osteoporosis, and many of these cases of osteoporosis will be due to secondary causes, secondary osteoporosis is therefore a significant health issue.

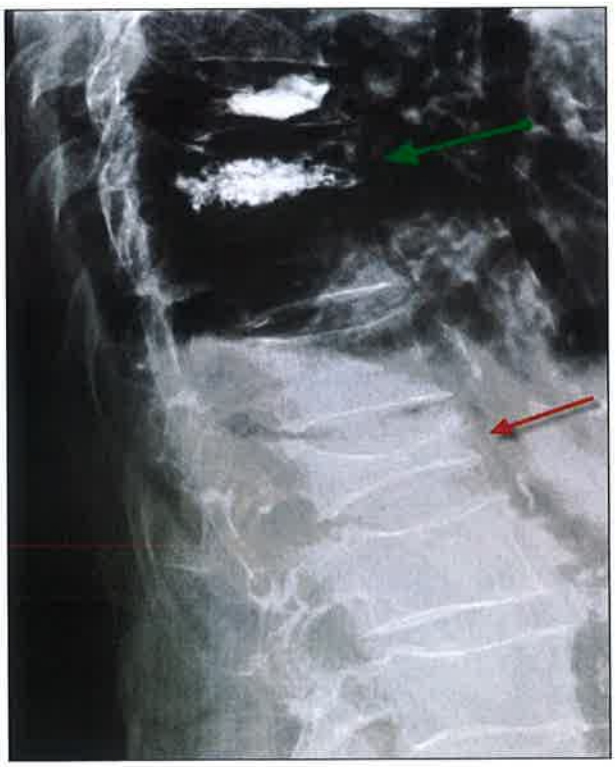


Figure 1.5: X-ray of a lumbar spine showing osteoporosis changes – *the more dense the bones, the "whiter" they appear on x –rays, these bones are fairly thin. The red arrow is a vertebrae that has been "crushed" – a compression fracture secondary to osteoporosis. The green arrow is a result of kyphoplasty – which involves a injecting a bone filler material into the bone to expand it.*

1.2.2 Introduction

Bone is a hard connective tissue that forms the skeleton within most vertebrates. It is comprised of calcium phosphate wherein bone cells are embedded in a collagenous fibre matrix [26]. The primary functions of bone include structural support, calcium and phosphate storage and production of immuno progenitor cells.

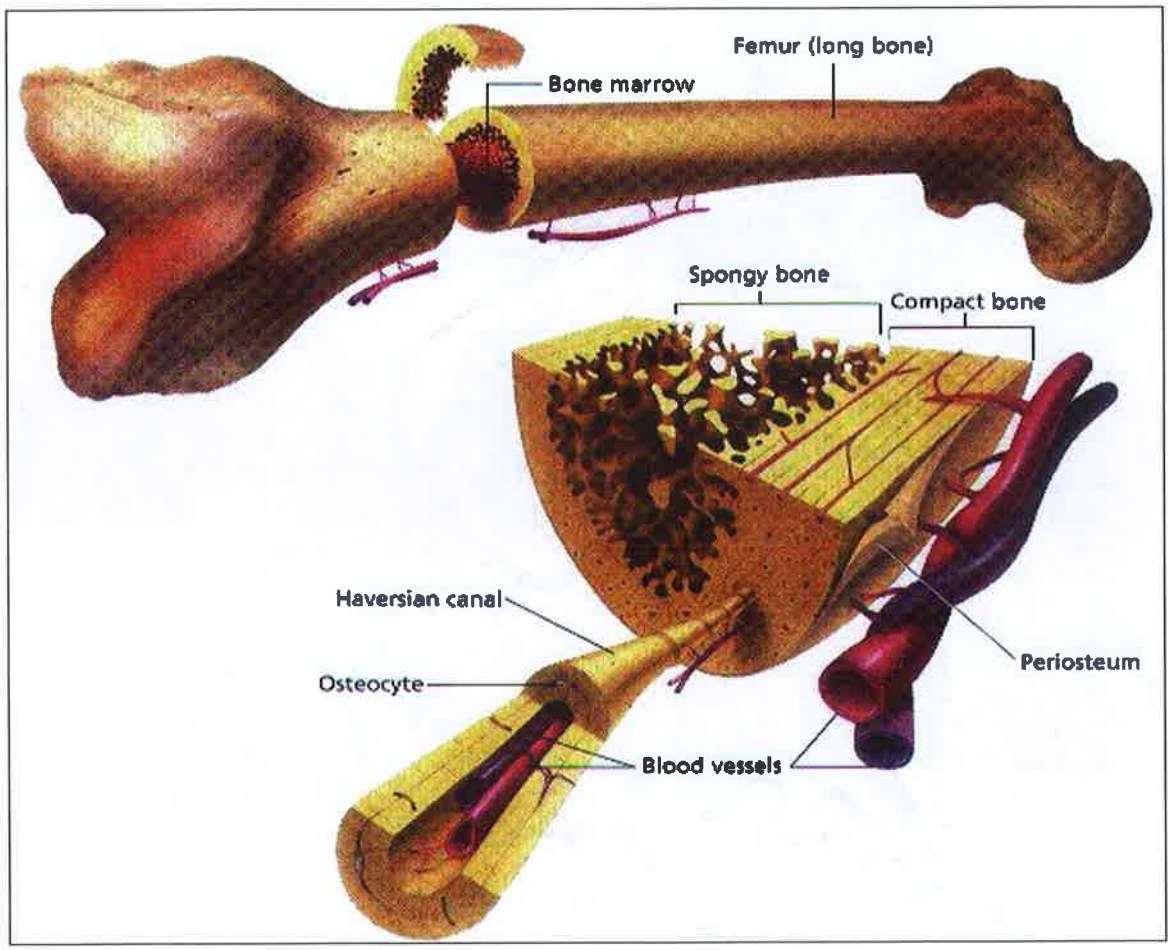


Figure1.6: Structure of human bone [27]

There are two main types of bone; compact and trabecular bone. Compact bone is always located at the surface of a bone, is quite dense and makes up about 80% of bone mass. It consists of concentric layers called *lamellae* surrounding *haversian* canals and is covered with a protective sheath called the periosteum [28]. Trabecular bone forms open networks of struts and plates known as trabeculae, within which is the marrow cavity and endosteum **(Figure 1.6)**.

1.2.3 Bone cell types

The major bone cell types are the bone-forming osteoblasts, bone resorbing osteoclasts and cartilage forming chondrocytes. Osteoprogenitors of pluripotential mesenchymal cells induce differentiation, which can give rise to a lineage of cells **(Figure 1.7)**. Mesenchymal stem cells or marrow stromal cells **(Figure 1.9)** can differentiate into osteoblasts, chondrocytes, myocytes, and adipocytes. The addition of growth factors can define the lineage of differentiation. For example, mesenchymal cells grown *in vitro* in combination with ascorbic acid, dexamethasone and a source of inorganic phosphate will differentiate into osteoblasts. For this reason mesenchymal stem cells are very beneficial for experimental research and carry the advantage of not being associated with ethical restrictions, as opposed to embryonic stem cells [29].

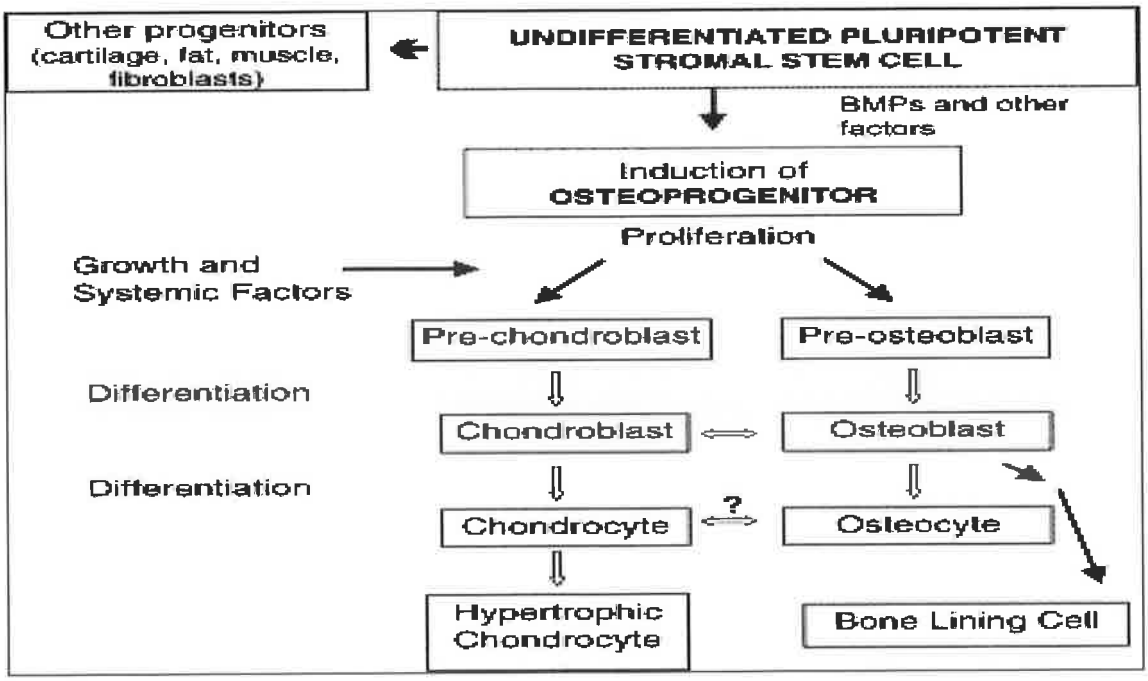
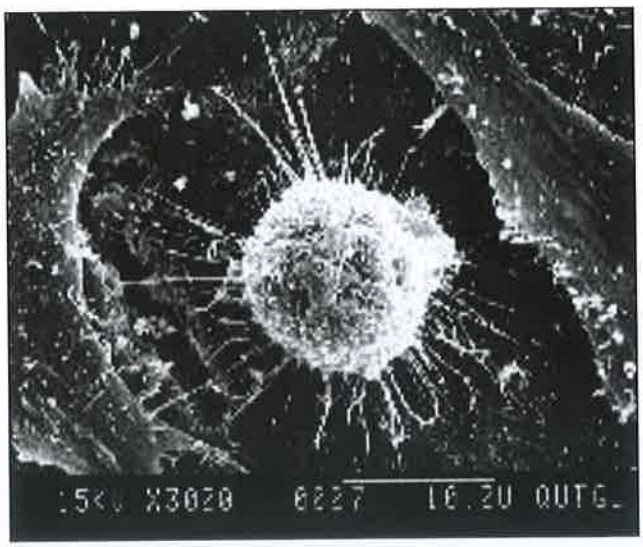


Figure 1.7: Differentiation of Pluripotent Stromal stem cells [30]

1.2.4 Osteoblasts

1.2.4a Introduction

Osteoblasts are bone forming cells that secrete collagen, osteoid, growth factors, cytokines and several enzymes to support mineralization (alkaline phosphatase). Hydroxyapatite from the extracellular matrix (ECM) crystallises around collagen fibres forming the hard bone matrix. This action seems to be regulated by certain proteins as mineralization does not occur in other connective tissue sites [29]. As the matrix forms within the osteoblasts they become isolated within the lacunae and are termed osteocytes **(Figure 1.8 and Figure 1.9)**. Osteocytes are the most common cell type in bone comprising of 80% of bone however less is known about this cell type [30].



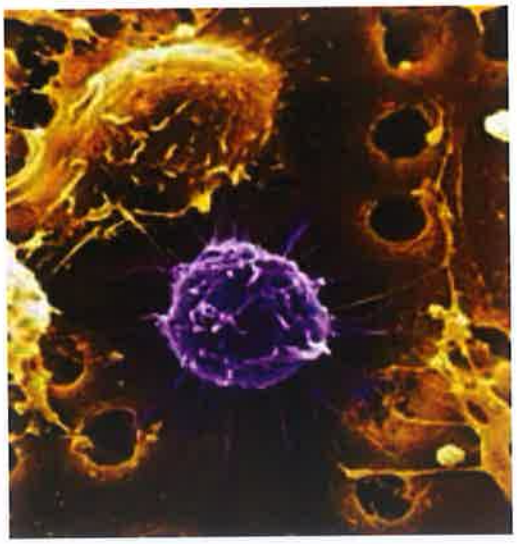


Figure 1.8: Osteocyte attached to biomaterial (left) [31]

Figure 1.9: A false-colour micrograph of a mesenchymal cell, shown in purple (right) [32]

1.2.4b Function

The main function of osteoblasts is formation of new bone matrix. Osteoblast function is under the regulatory control of various systemic and local (paracrine/ autocrine) factors. There are various systemic agents as previously discussed which influence osteoblast function; these includes endocrine hormones such as PTH and vitamin D, growth hormones, glucocorticoid hormones, gonadal steroids, insulin, and retinoids to name a few. Local agents that influence osteoblast function include paracrine factors such as parathyroid hormone related protein (PTHrP), TGF-β, FGF 1 and 2, TNF-α and -β, IL-1, and IL-6. Autocrine factors include TGF-β, FGF 1 and 2, Insulin like growth factor (IGF 1 and 11) and platelet-derived growth factor (PDGF).

1.2.4c Osteoblast cell lines

Osteoblast cell lines may be described as non-transformed or transformed. Non-transformed cell lines include: *URM-201* which was derived from neonatal rat calvaria established in 1998 by Ng *et al* and has a life span of 12 passages and represents preosteoblast cells. They have been useful for studying osteoblast differentiation by systemic hormones and local growth factors [30]. *MC3T3* is a mouse calvaria clonal primary bone cell line with phenotypic characteristics of preosteoblast cells. Variants have been produced. Transformed cells lines include: *KS-4* is a mouse calvarial clonal cell line transfected with the Harvey rat sarcoma viral oncogene homolog: c-HA-Ras -1 gene which induces an immortalized cell cycle. These cells do not have major use for bone graft studies as they typically have low Alkaline Phosphatase (osteogenic marker; ALP) activity, mRNA expression and collagen production. *hOB* (Human osteoblast) cell line is a better model system for studying osteogenesis and was produced from a 68 year

old woman. Cells were transfected with T antigens from the SV40 virus. These are well differentiated steroid responsive clonal cells with a phenotype of mature osteoblasts. *hFOB* cells also contain SV40 viral sequences. Human foetal cells from a spontaneous miscarriage were transfected with a temperature sensitive plasmid (pucsvtsAS3) one for neomycin resistance (PSV2-Neo). Clones were made and the strain hFOB 1.19 was selected as it expressed the highest levels of alkaline phosphatase. The cells have adherent growth properties and the ability to differentiate into osteoblasts expressing the normal osteoblasts phenotype. This strain proliferates best at 34°C and differentiates best at 37°C. Other transformed lines include *ROS 17/2.8* (rat osteoblastic osteo-sarcoma cells), *UMR 106, human MG-63 and SaOS-2* all derived from bone tumour cells [30]. Transformed cell lines are clonal and therefore their responses are more reproducible with less variability than primary cultures.

1.2.4d Osteoblast cells in culture

Normally cells *in vitro* are grown statically in two dimensional (2D) cultures; for example cells adhered to a tissue culture flask or a glass slide. These cells generally grow in a mono-layer. Cultures may also be grown using rotator to facilitate liquid diffusion throughout (known as dynamic culturing). Qi xin *et al* have shown that seeding cells in bone tissue cultures are comparative; dynamic seeding showed even distribution and spread of cells compared to static seeding which showed more cell aggregation. Similarly, dynamic seeding facilitated cell migration to the centre of the scaffold unlike static seeding where most cells aggregated on the surface [33]. To support cell growth modified medium such as Dulbecco's or Eagles is supplemented typically accompanied with Foetal Bovine Serum (FBS). FBS contains a range of potent growth factors and

cytokines required for *in vitro* cell growth. Typically 10% FBS is added to a culture medium. Schecroun & Delloye however examined the possibility of not using FBS as an osteoblast progenitor cell inducer *in vitro*. They found that by replacing FBS with platelet plasma (also contains chemical stimulators like PDGF & TGF-β) maintained proliferation and actually accelerated osteoblast differentiation [34].

1.2.5 Osteocytes

Osteocytes are terminally differentiated cells of the osteoblast lineage that have become embedded in mineralized matrix and no longer deposit matrix (i.e. mature bone cells). Unfortunately, their peculiar location within bone makes them the most inaccessible type of osteoblast to obtain in culture for *in vitro* study.

Bonewald *et al* have established several immortalized cell lines in culture with phenotypic characteristics of osteocytes. Bone cells were derived from transgenic mice over-expressing T-antigen driven by the osteocalcin promoter. They chose cells expressing a dendritic morphology as the initial criterion for selection and establishment of clonal cell lines. MLO-Y4 (murine long bone osteocyte Y4) was one of the immortalized clonal lines established with osteocyte-like characteristics. These cells produce extensive, complex dendritic processes, are positive for T-antigen, osteopontin, neural antigen CD44 and connexin 43. They produce large amounts of osteocalcin, have low levels of alkaline phosphatase activity, lack detectable mRNA for osteoblast-specific factor 2, and produce very small amounts of type 1 collagen [35]. The MLO-Y4 cells also support osteoclast formation and activation through the secretion of M-CSF and expression of RANKL on their surface and their dendritic processes [36]. Cells are grown

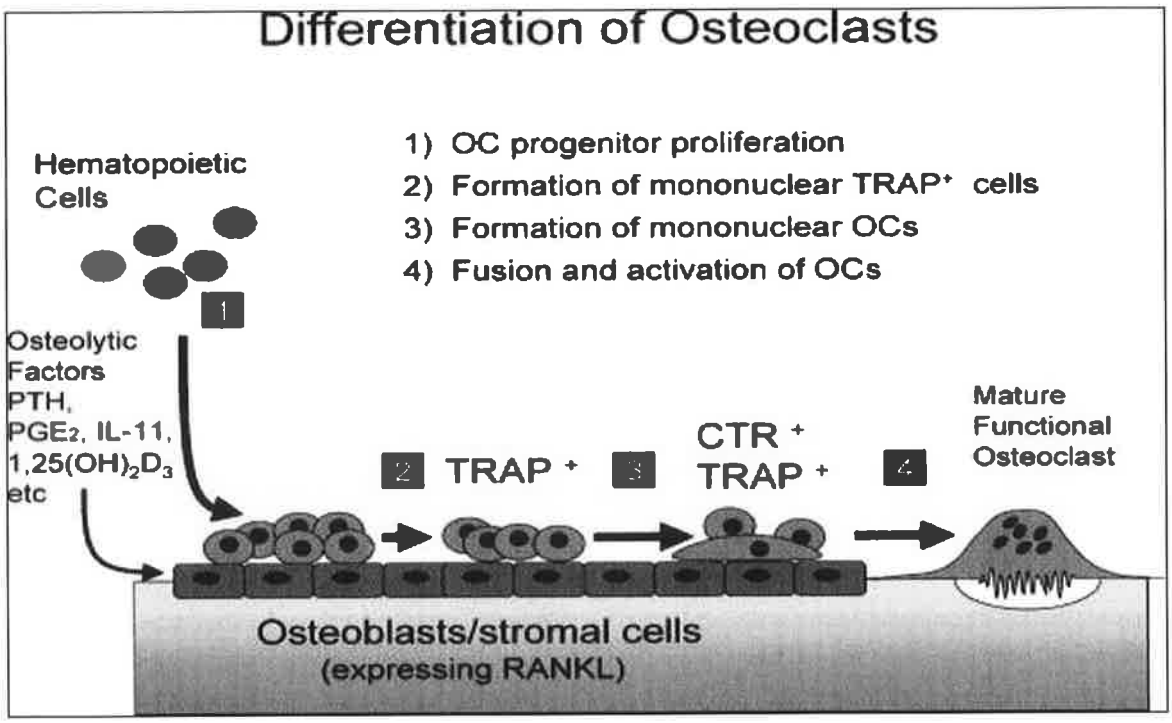


Figure 1.10: Diagrammatic representation of the formation of mature, multinucleated osteoclasts from mononuclear hematopoietic progenitors [30]

Hematopoietic osteoclast progenitors present in bone marrow come into direct contact with osteoblast or stromal cells expressing RANKL under the influence of osteolytic factors such as PTH, PGE2, IL-11 or 1,25(OH)2D3 (1). They differentiate firstly into TRAP positive mononuclear cells (2) before becoming TRAP positive and calcitonin receptor (CTR) positive mononucleate cells (3) that eventually fuses to form multinucleate, functional mature osteoclasts (4).

1.2.6b *In vitro* methods to study osteoclast formation and function

Unlike osteoblasts, osteoclasts are difficult to study *in vitro* because they are relatively scarce, terminally differentiated, adherent to mineralized surfaces and fragile. Methods have been developed to isolate these cells in vitro or to induce their formation in bone marrow cultures. The major criteria generally used to identify osteoclasts are multinuclearity, positive staining for tartrate resistant acid phosphatase (TRAP), expression of calcitonin receptors and the ability to resorb calcified matrices [40-42]. Mature, multinucleated functional osteoclasts are obtained either directly from bone as

the primary source, or else they are secondarily generated *in vitro* from hematopoietic progenitors obtained from a source of hematopoietic cells or macrophages such as bone marrow, spleen, human peripheral blood mononuclear cells and human umbilical cord blood [30]. New methods for osteoclast generation in vitro, using RANKL, M-CSF, TNF-α and IL-1 has been identified and used in variety of models [30].

1.3 Parathyroid hormone and Vitamin D

1.3.1 Parathyroid hormone

PTH is a hormone synthesized by the chief cells of the parathyroid gland that plays a central role in the regulation of calcium homeostasis [43]. There are three factors that control secretion of PTH: the extracellular calcium concentration, 1, $25(OH)_2D$, and the extracellular phosphate concentration. The most important factor is the extracellular calcium concentration, the effects of which are mediated through the calcium-sensing receptor. Elevated extracellular calcium levels lead to a decrease in PTH secretion while reduced levels lead to increased PTH secretion. 1, $25(OH)_2D$ inhibits the expression of the *PTH* gene and may directly reduce PTH secretion while phosphate stimulates the expression of the *PTH* gene.

PTH has a number of effects on its target tissues, all mediated by a G protein-coupled receptor [43]. In the kidney, PTH stimulates the resorption of calcium in the distal tubule, inhibits the resorption of phosphate in the proximal and distal tubules, and stimulates the conversion of 25OHD to $1,25(OH)_2D$, the most active form of vitamin D. $1,25(OH)_2D$ in turn, stimulates calcium absorption from the intestine, inhibits PTH secretion and stimulates bone formation. The effects of PTH on bone are complex. Acutely, PTH stimulates osteoclast mediated release of calcium and phosphate from bone, while chronic exposure to PTH leads to increased osteoclastogenesis and osteoclast activity. However, intermittent administration of exogenous PTH leads to increased osteoblast activity and bone formation.

In summary, a fall in the extracellular calcium levels produces a rapid rise in PTH secretion which, in turn, increases release of calcium from bone, increases calcium

resorption in the kidney, and increases calcium absorption from the intestine. These actions all cause an increase in the serum calcium level. Conversely, increased extracellular calcium levels produce a rapid decrease in PTH secretion, which in turn decreases calcium release from bone, decreases calcium resorption in the kidney, and decreases calcium absorption in the gut. These actions all cause a decrease in the serum calcium level. Thus PTH is an important factor in calcium homeostasis.

1.3.2 Vitamin D

Vitamin D is a hormone that is synthesized in the skin in response to ultraviolet-B radiation [44,45]. In humans, a small amount is also obtained from dietary sources. However, the predominant source is the photolysis of the steroid precursor 7-dehydrocholesterol to previtamin D by ultraviolet-B radiation. Previtamin D undergoes a membrane-enhanced, temperature-dependent, isomerisation to vitamin D. Newly formed vitamin D binds to vitamin D binding protein and is transported to the liver where it is hydroxylated to 25OHD. 25OHD is further hydroxylated in the kidney to1,25$(OH)_2$D, the most active metabolite of vitamin D.

The main function of vitamin D, in combination with PTH, is to maintain stable serum calcium levels [44,45]. Vitamin D has a number of different effects on target tissues mediated by active vitamin D metabolites binding to the vitamin D receptor in the target tissue. In the intestine, 1, 25$(OH)_2$D induces the expression of a number of calcium binding proteins that facilitate the transfer of calcium from the gut lumen to the circulation, leading to increased intestinal calcium absorption. In bone, 1, 25$(OH)_2$D promotes osteoclastogenesis leading to the release of calcium and phosphate into the serum. 1, 25$(OH)_2$D induces the expression of RANKL expression in osteoblasts.

Osteoclast precursors expressing RANK recognise RANKL through cell-to-cell interaction with osteoblasts, and differentiate into mature osteoclasts [46].

1, 25$(OH)_2$D has a number of additional effects on osteoblasts. These effects include inducing production of OPG in mature osteoblasts [47], promoting osteoblast differentiation, stimulating mineralization, inhibiting osteoblast proliferation, and altering the synthesis and production of proteins, enzymes and growth factors [48]. In the parathyroid gland, 1, 25$(OH)_2$D decreases the expression of the *PTH* gene and decreases PTH synthesis. In combination, these actions of vitamin D play a key role in calcium homeostasis. Thus, low vitamin D status leads to decreased intestinal calcium absorption, which in turn leads to low serum calcium levels. The homeostatic response to low serum calcium levels is increased 1,25$(OH)_2$D levels (mediated via increased PTH, i.e. secondary hyperparathyroidism) which in turn leads to increased intestinal absorption of calcium, the release of calcium from bone, and a rise in the serum calcium level. Conversely, vitamin D intoxication leads to high serum calcium levels. The homeostatic response to high serum calcium levels is lowered 1,25$(OH)_2$D levels (mediated via lowered PTH) which in turn leads to reduced intestinal calcium absorption, increased uptake of calcium into bone, and a lowering of the serum calcium. Vitamin D has a number of non-calcaemic actions. The vitamin D receptor is expressed in most tissues in the body, and, *in-vitro*, the binding of 1, 25$(OH)_2$D to its receptor has numerous effects including inhibition of cellular proliferation, induction of terminal differentiation of cells, stimulation of insulin production, and modulation of the immune response. It is not known what the exact role of these functions are *in-vivo*. Consistent with the multitude of effects seen *in-vitro*, vitamin D deficiency has been associated in observational studies

with a number of non-skeletal disorders including myopathy, many types of cancer, multiple sclerosis, hypertension, diabetes mellitus, ischaemic heart disease, congestive heart failure and rheumatoid arthritis [49].

In summary, low vitamin D status is associated with secondary hyperparathyroidism, increased bone turnover, low BMD and increased risk of fracture. Overt vitamin D deficiency can cause osteomalacia, and both low 25OHD levels and osteomalacia are relatively common findings in patients with hip fractures.

1.3.3 Relationship between Parathyroid hormone and Vitamin D

Vitamin D insufficiency can lead to reduced intestinal calcium absorption. Secondary hyperparathyroidism may then occur as a homeostatic response to maintain serum calcium levels. In cross-sectional studies, 25OHD levels are inversely associated with PTH levels [50-52]. Chapuy *et al* reported that in healthy adult men and women PTH levels were stable above a 25OHD level of 78 nmol/L, while PTH levels increased exponentially below this level [51]. Dawson-Hughes *et al* reported similar findings in elderly men and women but that the PTH levels increased slowly below a 25OHD level of 110 nmol/L [52]. Lips *et al* reported that in post-menopausal women a 25OHD level less than 25 nmol/L was associated with a 30% increase in PTH levels compared to women with a 25OHD level more than 50 nmol/L, while women with 25OHD levels between 25 and 50 nmol/L had a 15% increase in PTH levels [50]. Cross-sectional analyses of 25OHD and PTH levels can be flawed. Some researchers have used Pearson correlation analysis to assess the linear relationship between 25OHD and PTH, and typically such studies report a correlation co-efficient between PTH and 25OHD of -0.20

to -0.30 [50,53], however most other researchers have found non-linear relationships between 25OHD and PTH [51,52].

There are pronounced seasonal variations in 25OHD and PTH levels in countries distant from the equator [54]. The changes in PTH levels lag behind the changes in 25OHD levels by approximately one month [55], a time approximately equivalent to the serum half-life of 25OHD [56]. Cross-sectional analyses do not take this lag into account, and therefore compare values of 25OHD and PTH from different points in their seasonal cycles. A number of intervention studies have shown that vitamin D supplementation in people with low 25OHD levels leads to a fall in serum PTH levels. Malabanan *et al* reported that vitamin D supplementation with 50,000 IU weekly for eight weeks led to 35% decrease in PTH levels in people with 25OHD levels of 27.5-39.9 nmol/L, and a 26% decrease in PTH levels in people with 25OHD levels of 40-49.9 nmol/L, but no change in PTH levels in people with 25OHD levels greater than 50 nmol/L [57]. Lips *et al* reported similar results from the MORE study with 400-600 IU/day of vitamin D supplementation [50]. In summary, low 25OHD levels are consistently associated with increased PTH levels. This inverse relationship is non-linear. There is conflicting evidence as to whether there is a threshold level of 25OHD above which PTH levels do not decrease further, and, if such a threshold exists for 25OHD, at what serum level this occurs.

1.3.4 Parathyroid hormone and Vitamin D in IBD

IBD can lead to vitamin D deficiency, because patients may have decreased exposure to sunlight, decreased intake, malabsorption, and gastrointestinal loss. Regarding exposure of patients with IBD to sunlight, some investigators have found significantly decreased serum 25OHD concentration in patients with IBD during both winter and summer in the Northern Hemisphere [58], and others report a tendency toward lower sun exposure in patients with CD compared with healthy controls even in the summer [59]. Decreased oral vitamin D intake in patients with IBD compared with healthy individuals has been neither consistently documented [59] nor linked with low serum 25OHD concentrations [60,61]. Intestinal absorption of vitamin D was found to be normal in the majority of patients with IBD, regardless of the severity of disease [59,62].

Available reports on the vitamin D status of adults with CD place the prevalence of 25OHD concentration ≤15 ng/mL between 22% and 70%, depending on the study [58, 59, 61, 63-70]. Serum 25OHD concentration was found to be <10 ng/mL in 8% to 45% of adults with CD [61, 64, 65, 67]. One study reports a negative relationship among disease duration, Crohn Disease Activity Index (CDAI), ferritin, C-reactive protein (CRP), cholesterol [65], and 25OHD concentration. Other studies report that smoking [64] and small bowel resection [69,71] are also negatively correlated with 25OHD concentration, whereas sunlight exposure and nutritional status are positively correlated [68]. Fewer studies have described the vitamin D status of adults with UC compared with those with CD. Serum 25OHD concentrations have been found to be normal in some studies of patients with UC [72,73], whereas others report serum 25OHD concentrations <12ng/mL in 15% [66]. In one study [61], the serum 25OHD concentration was <10

ng/mL in 45% of adult patients with UC and was not different from that in patients with CD.

Data regarding the vitamin D status of children with IBD are limited [60, 74, 75]. Issenman *et al* [75] found normal 25OHD concentrations in children with CD. Gokhale *et al* [74] reported lower 25OHD concentrations in children with CD than in those with UC, but they were still within the reference range. Sentongo *et al* [60] found a 25OHD concentration ≤15 ng/mL in 16% of children with CD, lower concentration in patients with upper gastrointestinal tract disease and greater lifetime corticosteroid exposure, and no relationship between 25OHD concentration and vitamin D intake.

Changes in PTH levels in IBD have also shown conflicting results whilst an early study demonstrated a high prevalence of secondary hyperparathyroidism [68] amongst patients with CD, subsequent authors have generally concluded that PTH levels are normal in this group [76-81]. Ardizzone *et al* [73] did not notice any significant difference in the PTH levels between the CD and UC patients in their study.

1.4 Bone turnover markers

1.4.1 Introduction

Bone is a metabolically active tissue and undergoes continuous remodeling, a process that largely relies on the activity of osteoclasts to remove bone, and of osteoblasts to form bone. Under normal conditions, bone resorption and formation are coupled to each other, and the long-term maintenance of skeletal balance is achieved through the action of systemic hormones and local mediators. In contrast, metabolic bone diseases, states of increased or decreased mobility, and therapeutic interventions are characterized by more or less pronounced imbalances in bone turnover [82]. With the increasing awareness of disorders of bone and mineral metabolism in clinical practice, the interest in, and the need for effective measures to be used in the screening, diagnosis and follow-up of such pathologies have markedly grown. Along with clinical and imaging techniques, laboratory tests play an integral role in the assessment and differential diagnosis of metabolic bone disease. In recent years, the isolation and characterization of cellular and extracellular components of the skeletal matrix have resulted in the development of biochemical markers that specifically reflect either bone formation or bone resorption [83]. These biochemical indices have greatly enriched the spectrum of analyses used in the assessment of skeletal pathologies. They are non-invasive, comparatively inexpensive and, when applied and interpreted correctly, helpful tools in the diagnostic and therapeutic assessment of metabolic bone disease. Although the various serum and urinary markers of bone turnover include both cellular derived enzymes and non-enzymatic peptides, they are usually classified according to the metabolic process they

are considered to reflect. For clinical purposes, therefore, markers of bone formation are distinguished from indices of bone resorption **(Figure 1.11, Table 1.1, and Table 1.2)**.

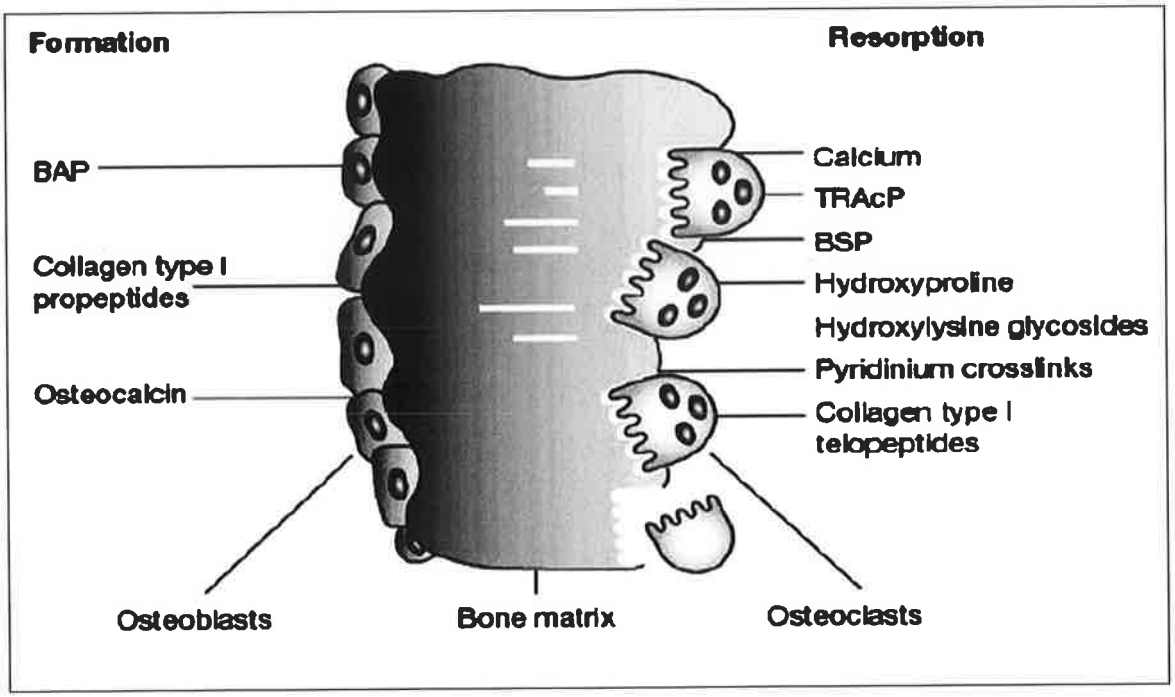


Figure 1.11: Schematic representation of the various bone markers [84]

Markers	Tissue of origin	Specimen	Analytical Method
Bone-specific alkaline phosphatase (BALP)	Bone	Serum	Electrophoresis, Precipitation, IRMA, EIA
Osteocalcin (OC)	Bone, platelets	Serum	RIA, IRMA, ELISA
C-terminal propeptide of type 1 procollagen (P1CP)	Bone, soft tissue, skin	Serum	RIA, ELISA
N-terminal propeptide of type 1 procollagen (P1NP)	Bone, soft tissue, skin	Serum	RIA, ELISA

TABLE 1.1: OVERVIEW OF BONE FORMATION MARKERS [84]

Markers – Collagen related	**Tissue of origin**	**Specimen**	**Analytical Method**
Hydroxyproline, total and dialyzable (Hyp)	Bone, cartilage, soft tissue, skin	Urine	Colorimetry HPLC
Hydroxylysine-glycosides (HLG)	Bone, soft tissue, skin, serum complement	Urine (serum)	HPLC ELISA
Pyridinoline (PYD)	Bone, cartilage, tendon, blood vessels	Urine Serum	HPLC ELISA
Deoxypyridinoline (DPD)	Bone, Dentin	Urine Serum	HPLC ELISA
Carboxyterminal cross-linked telopeptide of type I collagen (ICTP, CTX-MMP)	Bone, Skin	Serum	RIA
Carboxyterminal cross-linked telopeptide of type I collagen (CTx)	All tissues containing type I collagen	Urine (α/β) Serum (βonly)	ELISA RIA
Aminoterminal cross-linked telopeptide of type I collagen (NTx)	All tissues containing type I collagen	Urine Serum	ELISA RIA
Collagen I alpha 1 helicoidal peptide (HELP)	All tissues containing type I collagen	Serum	ELISA
Markers – Non Collagenous Proteins	**Tissue of origin**	**Specimen**	**Analytical Method**
Bone Sialoprotein (BSP)	Bone, Dentin, hypertrophic Cartilage	Serum	ELISA RIA
Osteocalcin fragments (ufOC, U-Mid-OC, U-LongOC)	Bone	Urine	ELISA
Markers – Osteoclast enzymes	**Tissue of origin**	**Specimen**	**Analytical Method**
Tartrate-resistant acid phosphatase (TRAcP)	Bone Blood	Plasma Serum	Colorimetry RIA ELISA
Cathepsins (e.g. K, L) (CathK, CathL)	K: Primarily in osteoclasts L: Macrophage, Osteoclasts	Plasma Serum	ELISA

TABLE 1.2: OVERVIEW OF BONE RESORPTION MARKERS [84]

1.4.2 Bone turnover markers in normal ageing and menopause

Once somatic growth subsides, the serum and urinary concentrations of most bone markers return to a level much below those seen during normal puberty and growth. This stabilization usually occurs during the 3rd decade and in healthy men, levels of practically all markers remain more or less unchanged until 70 years of age. After that, a slight increase is usually seen in both formation and resorption markers [85-88]. In contrast, menopause is associated with a substantial acceleration in bone turnover, mirrored by a 50–100% increase in both markers of bone formation and resorption [85, 86, 89-95]. A prospective study covering the peri-menopausal transition in healthy women suggests that changes in bone turnover occur during the late pre-menopause with a decrease in bone formation, which only later is followed by a rise in bone resorption [96]. It is now widely accepted that the accelerated rate of bone loss seen after the menopause is mainly due to an uncoupling in bone turnover and an increase in bone resorption [97,98]. Studies employing specific bone markers indicate that bone turnover continues to be increased (and to be associated with bone loss) during late menopause [99-104].

1.4.3 Bone turnover markers in osteoporosis

Osteoporosis is a heterogeneous disease. It is therefore not surprising that in untreated patients with this disorder, rates of bone turnover tend to vary over wide range. Although most cross-sectional studies show accelerated bone turnover in a certain proportion of post menopausal osteoporotic women, there is usually broad overlap between diseased and healthy populations [92-94, 105-108]. In this context, it is important to bear in mind that research studies usually include highly selective patient populations, which may not always represent the population seen in the typical clinical setting. Using a population-

based data set, and therefore avoiding this selection bias, Seibel *et al* have shown that none of the major biochemical markers of bone turnover provide sufficient diagnostic information to be useful in the screening for vertebral osteopenia or osteoporosis [94]. However, another population-based study showed that urinary levels of NTx could discriminate between older individuals with normal hip bone density, osteopenia and osteoporosis [109]. Again, this association did not hold true for men at the level of the spine. In retrospective population-based studies, Akesson *et al* [99,100,110,111] have demonstrated that previous fractures were associated with abnormal bone turnover. After adjustment for age and BMD, women with fractures occurring within six years prior to the study were characterized by lower serum levels of OC and P1CP, but normal rates of bone resorption.

Taken together, these data suggest that a long term imbalance of bone metabolism may lead to increased fragility. Together with the fact that high bone turnover may be sustained for long periods and bone loss may increase with age [111], these findings may provide a rationale for designing more effective intervention strategies. However, other factors such as medication [87,112-117], immobilization [103,118], thyroid function [119], co-morbidity [118] and the fracture itself [107,120,121] do influence bone metabolism and therefore need to be considered in the interpretation of biochemical data and their use in individual patients. Clearly, none of the biochemical markers of bone turnover has proven useful as a single diagnostic index of osteoporosis.

1.4.4 Bone turnover markers in inflammatory bowel disease

As described earlier, bone turnover markers reflect the processes of bone resorption and bone formation and several studies have been undertaken in IBD patients utilizing these markers to provide further insight into understanding the possible mechanism involved in IBD related osteopenia and osteoporosis. However, all current published studies using biochemical markers in patients with IBD have shown conflicting results. While some studies of IBD patients have reported increased levels of bone resorptive markers without a compensatory increase in formation markers [63,76,80,122-124], other studies have reported either reduced levels of markers of bone formation and no difference in resorptive markers [125], elevated levels of both type of markers [73,126], or indeed no difference in markers [127] between IBD patients and control subjects. One needs to be cautious in comparing the results of these studies because of the major differences in the various IBD patient populations which were studied, especially with regard to the type of disease (CD vs. UC), corticosteroid usage and disease activity (active disease vs. disease remission) [128]. Furthermore, there are only two studies which have compared the levels of bone turnover markers in UC patients alone compared to controls [73,126], and these have produced conflicting results. Similarly, only two studies have compared the levels of bone turnover markers in patients with quiescent CD, and not taking steroids, with those of controls [125, 127] and again these also have produced conflicting results. Whilst BMD measurements reflect the long term effects of disease on bone regulation, bone turnover markers can only represent a single time point;

therefore straightforward action-effect relationships cannot always be demonstrated. Clearly, as with primary osteoporosis, none of the biochemical markers of bone turnover has proven useful as a single diagnostic index of IBD-related osteopenia or osteoporosis.

1.5 Bone disease in Inflammatory Bowel Disease

1.5.1 Prevalence

Bone demineralization and osteoporosis in patients with IBD was first reported approximately 30 years ago [129]. According to their T-score, 54% and 78% of CD and UC patients were found to be osteopenic (T-score <-1) at the spine and at the femoral neck respectively [76], while 18-42% and 29-41% of them were osteoporotic (T-score <-2.5) at the spine and at the femoral neck respectively [130]. More importantly, an increased risk of fracture was described in CD patients in several studies with a relative risk ranging between 1.32 and 2.5 [131-134]. The largest risk was observed at the spine, particularly in women, with a 6.5-fold increase in fracture risk [131]. In a recent population based cohort study, the relative risk of hip fracture was 1.41 (0.94-2.11) for UC and 1.68 (1.01-2.78) for CD patients [135]. This study also concluded that the risk of hip fracture is increased approximately 60% in IBD patients and the majority of hip fracture risk in IBD patients cannot be attributed to steroid use [135].

1.5.2 Aetiology

The possible pathogenesis of reduced BMD in IBD is as multi-factorial and varied as that seen in the general population, relating with age, gender, oestrogen deficiency, alterations in calcium homeostasis, nutritional and dietary factors, smoking, alcohol and immobility all playing a role. Early indications are these effects and associations are weak and overshadowed by the effect of the IBD itself [61, 77, 136-139]. Support for this idea comes from a study in a selected group of IBD patients who did not have significant conventional risk factors for reduced BMD [76] and since confirmed in a relatively

unselected group of patients with IBD [63,122,140]. These studies show a consistent and characteristic change in metabolic bone marker profiles [76] in patients with IBD, distinguishing them from some other, mainly non inflammatory, diseases that are also associated with reduced BMD. Accordingly, a significant increase in resorption markers is found in these patients without compensatory increase in formation markers [63,76, 122,130]. In patients on corticosteroids, there may be a concomitant reduction in bone formation [63,77]. These bone marker profiles differ from those of post menopausal women in which is there is a reduction in formation makers [141,142] from the changes associated with smoking, where there is no changes in formation or resorption markers [143].

Some of the mechanisms which have been shown to be potentially involved in IBD patients are malabsorption in general and Vitamin D deficiency in particular [61,142], glucocorticoid treatment [136,140,144,145], hypogonadism [138], and Vitamin K deficiency leading to undercarboxylation of osteocalcin [146,147]. Considering the above and the fact that newly diagnosed patients with untreated CD have reduced BMD, there is a strong case for suggesting that demineralization in patients with IBD occurs primarily as a consequence of the intestinal inflammation. Recently, active inflammation and elevated pro-inflammatory cytokines have been implicated in the pathogenesis of bone resorption in a variety of models, including rheumatoid arthritis and postmenopausal osteoporosis [148-153]. Circulating pro-inflammatory cytokine levels are elevated in IBD patients with active inflammation [154-156] suggesting that disease activity and high cytokine levels could also play a role in IBD related bone disease. A rat model of colitis was associated with a dramatic 33 percent loss in trabecular bone and an even greater

suppression in bone formation rate [157]. Healing of colitis was associated with an increased bone formation rate and a return of bone measurements to normal levels. Serum from children with CD affects bone mineralization in an organ culture model without altering bone resorption [147]. These observations suggest that mediators produced during intestinal inflammation may alter osteoblast function and bone formation, and they are consistent with the observation that osteoporotic patients with IBD have higher serum IL-6 levels than non osteoporotic patients [130].

1.5.3 Inflammation and Bone disease

1.5.3a Introduction

Osteoclasts are known to be activated by various inflammatory cytokines, which are probably pathological mediators in systemic and regional bone loss [158,159]. This activity, is now known to be contributed to by interleukin (IL)-1α, IL-1β, IL-6, IL -11, IL-17, tumor necrosis factor (TNF)-α and -β, transforming growth factor (TGF)-α, epidermal growth factor (EGF), and prostaglandin E_2 (PGE_2) [160]. In healthy postmenopausal women, epidemiologic data show that serum IL-6 concentration is a predictor of bone loss [161]. Local or generalized bone loss has been reported in chronic infection, leukemia, autoimmune and allergic diseases, and inflammatory joint diseases, suggesting that an activated immune system can affect bone physiology. TNF-α, IL -1β and IL-6, amongst others, are potent activators of bone resorption at low concentrations *in vitro* [151,162,163]. The mechanisms of actions are multiple, including stimulation of osteoclast differentiation [164-166] and activation [167] with concomitant inhibition of osteoclast apoptosis [168]. TNF-α also inhibits osteoblast differentiation [169] and TNF-

α, IL-1β and IL-6 reduce bone formation in cultured osteoblasts [163,170]. Thus, pro-inflammatory cytokines potentially cause bone loss by both increasing bone resorption and inhibiting bone formation.

1.5.3b Pro-inflammatory cytokines

IL-1 is a multifunctional cytokine with a wide variety of activities. It is a family of two active peptides (IL-1α and IL-1β) that are encoded by two separate gene products. Both forms of IL-1 have identical activities and potencies [171]. IL-1 is the first polypeptide mediator of immune cell function that was shown to regulate bone resorption [172,173] and formation [174]. It is known for the activation, differentiation and proliferation of most cell types. IL-1 is the most potent stimulator of bone resorption yet identified [173]. In bone it is an important factor during resorption when associated with the 20-carbon unsaturated fatty acid; PGE_2. Osteoblasts exposed to IL-1 induce proliferation of osteoclast; they also produce PGE_2 thereby also controlling osteoclasts [175]. Temporary IL-1 exposure can result in bone formation however prolonged effects appear to show inhibitory effects [176]. As in high turnover osteoporosis, enhanced circulating levels of IL-1β have been reported in patients with IBD [177]. The altered production and mucosal ratio of IL-1β and interleukin 1 receptor antagonist (IL-1ra) is strongly suggested to be a pathogenic factor in IBD [178-180]. Increased production of IL-1β was implicated as a possible reason for increased bone loss in IBD [76]. Recently, the IL-1b -511 single nucleotide polymorphism which is associated with hyper secretion of IL -1β has been shown to predict lower BMD in a combined study of 36 patients with CD and 39 patients with UC [181] suggesting that genetic polymorphism attributes to bone loss in IBD patients.

IL-6 is described as an osteo-resorptive factor produced by osteoblasts. It activates glycoprotein-130-IL-6 subunit receptor important for osteoclast growth and differentiation but normally has little or no effect on osteoblasts. Franchimont *et al.* examined inconsistencies between IL-6 studies and concluded that it is not essential for normal bone resorption in homeostasis [182]. Indeed there is some evidence that IL-6, under conditions of high bone turnover, may play some role in osteoblast generation [182]. IL-6 is thought to be involved in bone remodeling and hence development of osteoporosis through stimulation of osteoclast development and regulation of bone resorption [183-185]. Higher levels of IL-6 have been found in patients with CD as previously described already [130]. Studies have also found a link between polymorphisms in the IL-6 gene and BMD in those with IBD [186,187]. Furthermore, neutralization of IL-6 present in serum of patients with CD has been shown to protect intact bone from the effects of CD serum *in vitro* [163]. In a recent study by Paganelli *et al*, serum IL-6 was found to be an important determinant factor in children with IBD in causing low BMD and a direct correlation was found between the levels of IL-6 and disease activity indexes with BMD measured [188].

Like IL-1, TNF is a family of two related polypeptides (α and β) that are products of separate genes [189,190]. TNF-α and –β have similar biologic activities and are both potent stimulators of bone resorption [173,191] and inhibitors of bone collagen synthesis [191]. Like, IL-1, TNF-stimulated induction of osteoclast like cell formation in bone marrow culture [192] and is mediated by increases in receptor activator of NF kappa ligand (RANKL) expression [193]. However, in addition to increasing RANKL expression TNF also stimulates osteoprotegerin (OPG) in an osteoblast cell model [194].

In IBD patients, elevated TNF-α have been shown even in morphologically normal intestinal biopsies [195] and recently is suspected of being an important mediator of bone loss in this group of patients [196,197]. As with IL-1β and IL-6, Lee *et al* has recently shown that TNF-α GT haplotype showed a strong association with BMD in 304 CD patients [198].

1.5.3c Anti-inflammatory cytokines

IL-4, 10, and 13 are members of a group of locally acting factors that have been termed inhibitory cytokines [199]. These proteins modulate the biosynthesis of pro-inflammatory cytokines and regulate the production of pro-inflammatory cytokine antagonists that either bind but do no activate cytokine receptors (IL-1ra) or prevent binding of active cytokines to receptors by forming complexes in solution (solution type 2 IL-1 receptor or soluble TNF receptors). IL-10 is produced by Th2 cells as well as other cell types including stimulated monocytes. In bone marrow cell cultures, which express a variety of proteins characteristic of osteoblasts including alkaline phosphatase, type 1 collagen, and osteocalcin, treatment with IL-10 suppressed the production of osteoblastic proteins and prevented the onset of mineralization [200]. IL-10 also inhibits the formation of osteoclast-like cells in bone marrow cultures without affecting macrophage formation or the resorptive activity of mature osteoclast [201]. The inhibitory effects of IL-4 on osteoclastogenesis appear to involve tyrosine phosphorylation of specific intracellular proteins [202]. *In vitro,* both IL-4 and IL-13 inhibit bone resorption by suppressing prostaglandin production in osteoblasts [203]. In addition, they have been identified as chemo attractants for osteoblasts *in vitro* [204]. Recently, Sventoraityte *et al* showed that the levels of IL-10 and IL-13 were significantly ($p<0.01$) higher in patients

with Crohn's disease than in patients with ulcerative colitis and control group before and after stimulation with phytohemagglutinin, [205] suggesting a counter-regulatory response and imbalance of cytokines in IBD patients.

1.5.3d Transforming growth factor β

There are three isomers of bone morphogenic protein (BMP) s in humans: TGF-β1, TGF-β2, and TGF-β3. TGF-βs are structurally and biologically similar to BMPs with variations in the c-terminal amino acid sequence. TGF-β is a potent multifunctional cytokine whose major effects in the body appear to be as a regulator of cell growth, a stimulator of matrix production, and an inhibitor of the immune system. TGF-βs are generally stored in bone; TGF-β1 and TGF-β2 have identical effects on bone cells, stimulating bone formation. *In vivo* studies have shown that mice injected with 1μg TGF-β daily for three days can lead to a 40% increase in the width of the calvarial bone within one month [176]. This is also the case for *in vivo* studies; however prolonged exposure inhibits differentiation and mineralization. Therefore temporary exposure of the growth factor may be most advantageous. TGF-β has also been shown to produce bone when in combination with other growth factors such as fibroblast growth factor (FGF) which enhances the expression of TGF-β in osteoblasts [176,206].

Several published studies have evaluated the role of TGF on fracture healing. Lind *et al* proved that TGF-β1 accelerates fracture healing by chemotaxis and osteoblast stimulation [207]. Also Sun *et al* proved the special role of TGF-β1 in the primary healing period [208]. Tatsuyama *et al* noted the presence of antibodies against TGF-β1 and BMP-2 as soon as the third day after the fracture of tibia in rats [209]. Si *et al.* received similar results [210]. During their research on the physiology of bone healing in

rabbits, they noted an increased expression of messenger ribonucleic acid (m-RNA) for BMP-2 in the first post-fracture period. They observed the increased levels of m-RNA for TGF-β1 in later healing periods, during chondrogenesis and the formation of callus. In their research on bone healing in rats, Matsumoto *et al* noticed increased levels of TGF-β1 between the 7th and the 14th day from the fracture [211]. Tielinen *et al* presented different findings. Based on their research on rats, they claimed that TGF-β1 does not accelerate the healing of bone defects in rats [212]. Although most experimental research on cytokine function was performed on animal models, according to Andrew *et al*, TGF-β-m-RNA in humans is similar to that in animals [213]. In experimental models of femoral fracture healing in rats, the increased levels of TGF-β, and insulin growth factor (IGF)-1 observed in the early healing period activated the production of metalloproteinases and increased their activity in the later process of fracture healing [214]. A similar relationship was noticed in a recent study performed in humans [215]. Although the methodology of those two studies differs significantly, higher levels of TGF-β1 in the first post-fracture period were noted in both of them. The level of TGF-β1 was declining in the later post trauma time, which was accompanied by a proportional increase of collagenase activity.

1.5.3e Osteoprotegerin & Receptor activator of NF-kappa ligand

The signaling system that normally maintains coupled bone remodeling has not been well defined, although it is clear that excessive osteoclastic bone resorption or defective osteoblast synthesis creates a dysequilibrium, with a net loss in bone mass. The initial step in the remodeling process involves osteoclastogenesis through a process of sequential proliferation, differentiation, and activation of mononuclear precursors. The

recent discovery of an elegant receptor-based interaction between osteoblast and osteoclast precursors appears to provide this "missing link" and simultaneously integrates this system with the immune response. Osteoblasts express a surface ligand RANKL that can bind to osteoclast precursors (the receptor activator of NF-kappa [RANK]) or an osteoblast-derived soluble decoy receptor known as OPG [216]. The binding of RANK to RANKL induces a signaling and gene expression cascade that results in differentiation and maturation of osteoclasts. OPG blocks this interaction, thereby inhibiting osteoclast formation. RANKL is also a regulator of T cell–dendritic cell interaction in the immune system and is a crucial factor in early lymphocyte development and lymph node organogenesis [217]. The central importance of this system is seen in RANKL gene–deficient mice, who are unable to support osteoclast differentiation, display severe osteopetrosis (even in the presence of bone-resorbing factors, such as vitamin D_3, dexamethasone, and PGE_2), show no evidence of bone remodeling, and simultaneously lack all lymph nodes [160]. There is emerging evidence that the RANKL–OPG system may be the final common pathway for many of the classical bone-active agents. For example, 17-beta-estradiol simultaneously increases OPG and inhibits RANKL, thereby shifting the system toward reduced osteoclast recruitment, whereas dexamethasone, parathyroid hormone (PTH), PGE_2, and 1-alpha, 25-$(OH)_2D_3$ stimulate RANKL expression but inhibit OPG production, with a corresponding increase in osteoclast function. New insights provided by these findings may lead to the development of novel approaches to osteoporosis management. Activated T cells can directly trigger osteoclastogenesis through RANKL, leading to bone loss, an effect that is blocked by OPG [160,218]. In summary, compounds that increase RANKL appear to enhance

osteoclastogenesis, whereas compounds that increase OPG inhibit osteoclastogenesis. Furthermore, this system may be critical in linking systemic or mucosal inflammation with altered bone metabolism and, ultimately, osteoporosis.

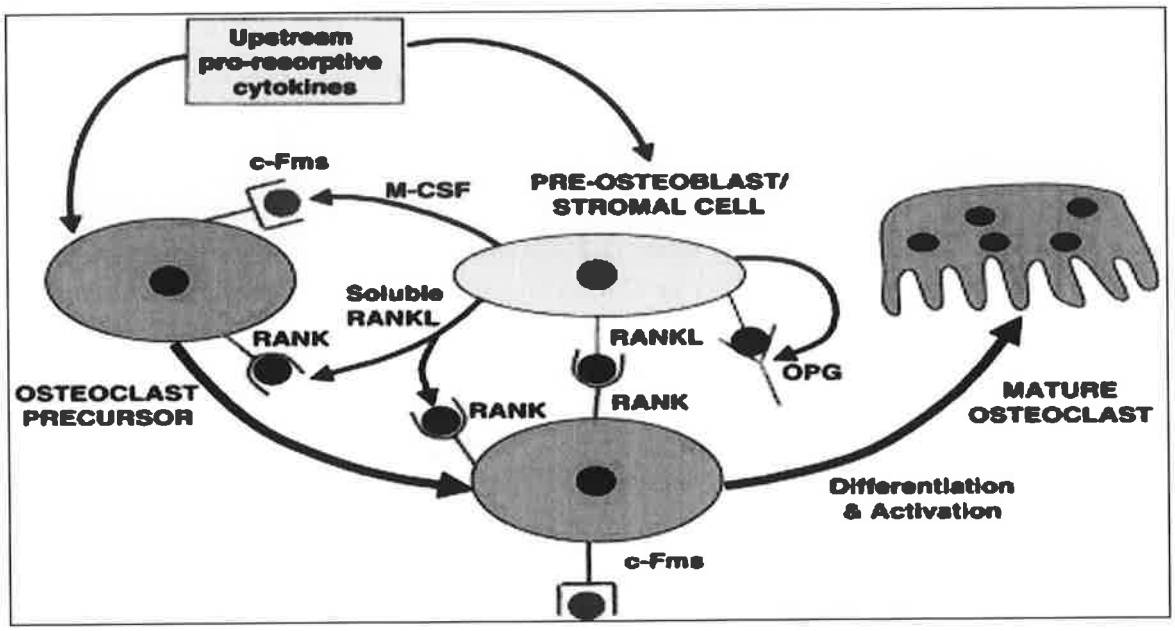


Figure 1.12: Current understanding of the osteoclastogenesis pathway [219]

RANKL, expressed on the surface of preosteoblastic/stromal cells, binds to RANK on the osteoclastic precursor cells. OPG puts a brake on the entire system by blocking the effects of RANKL.

Several studies carried out have shown that RANKL/OPG system is activated in IBD patient's and relates to the state of bone loss in this group [220-222]. Recently in a detailed study involving 180 IBD patients, Moschen *et al* [223] described alterations in the RANKL/OPG system in IBD and its relationship to decreased BMD in these patients. They demonstrated increased plasma levels of OPG as well as increased release from the inflamed colon in IBD, suggesting the colonic mucosa as a source of circulating OPG in IBD. Furthermore, OPG plasma levels were inversely correlated with BMD (that is,

osteoporotic IBD patients showed substantial elevation in circulating OPG). On the other hand, they did not observe any change in RANKL plasma levels or RANKL release from colonic explant cultures in IBD, although they noted increased numbers of RANKL positive cells, particularly in the lamina muscularis of IBD patients.

1.5.4 Therapy

1.5.4a Introduction

The clinical implications of reduced BMD in patients with IBD is that obviously, it may lead to increased risk of fractures which in turn is associated with significant morbidity and mortality. Furthermore, as IBD patients are often young, the effects of reduced BMD and resistant osteoporosis may be seen at a younger age than in patients with other demineralising conditions. Avoidance of fractures in patients with IBD may be more pressing than in some other diseases. If patients with IBD require analgesia for relief of the pain associated with fractures, they are commonly prescribed conventional non-steroidal anti-inflammatory drugs (NSAIDs). However, NSAIDs may cause severe clinical relapse of the intestinal disease and indeed very few patients can take these drugs without some increase in disease activity [224,225].

Good therapy for osteopenia and osteoporosis does not currently exist for any patient group, let alone IBD patients. Current treatment options being used in IBD patients are based on extrapolation of results from treatment trials in patients with conventional osteoporosis. General osteoporosis treatment includes the use of calcium with Vitamin D and bisphosphonate therapy. This may not be appropriate as the pathogenesis of the bone disease differs from conventional osteoporosis as has been previously described. Most of

the treatment trials for reduced BMD in IBD patients include small patient numbers. Moreover, data on therapeutic interventions to prevent bone loss in patients with IBD are also very limited. Studies looking at the effect of calcium and vitamin D supplementation in IBD patients in preventing bone loss have revealed conflicting results.

1.5.4b Calcium and Vitamin D

In a pilot study of 17 corticosteroid treated IBD patients, including both men and women, by Bernstein *et al* supplemental calcium 1000 mg/day and vitamin D 250 IU/day had no effect on bone mass after one year [226]. This study also found that patients with IBD on average ingest considerably less than the recommended daily intake of dietary calcium and vitamin D. Thus, supplementing oral and calcium and vitamin D intake may be indicated for many patients with IBD. Vogelsang *et al* in a randomized placebo-controlled study of 75 women and men with CD, reported that 1000 IU/day of vitamin D prevented bone loss in the forearm [227]. Bone density increases in the group receiving vitamin D were independent of baseline serum 25-hydroxy vitamin D level. This group reported elsewhere that their CD patients ingest only 1 μg/day of vitamin D on average [228]. This is only 20% of the recommended daily intake of vitamin D and also is much lower than the average oral vitamin D intake found in controls [226]. Von Tirptiz *et al* in a randomized controlled study of 33 patients with CD compared the effects of therapy comprising calcium 1000 mg/day plus vitamin D 1000 IU/day with this combination plus sodium fluoride 75 mg/day [229]. This study also found no impact of calcium and vitamin D alone on bone mass; however, the fluoride group exhibited a significant increase in mean spine Z score (-1.39 – -0.65, $p<0.05$). However in a larger prospective,

randomized, double-blind, parallel and placebo-controlled study, by Abitbol *et al* fluoride supplementation in osteoporotic patients with IBD treated in parallel with calcium and vitamin D failed to show any benefit on the lumbar spine BMD at 1 year [230]. Furthermore, the bone that is formed with sodium fluoride is very brittle and is also associated with toxicity, which is the reason it is currently not available in United States. A recent study in pediatric patients with IBD showed supplementation of calcium and vitamin D to have some beneficial effect on BMD [231].

1.5.4c Bisphosphonates

IBD patients and their bone density can be improved by bisphosphonates, which are analogues of pyrophosphate. In some, but not all studies, bisphosphonates have been associated with a modest increase in bone density [232-234]. Most importantly, they slow the decrease in bone density that occurs over time; they do not, however, increase bone formation. It is to be noted however that rare cases of osteonecrosis of the jaw have been reported with the use of bisphosphonates although the risk is greater with the use of intravenous forms [235]. Besides this, oral bisphosphonates also have some potential toxicity. After taking bisphosphonates orally, patients should remain upright for at least 2 hours to avoid oesophagitis. Studies evaluating the role of intravenous bisphosphonate therapy in IBD patients have also been carried out recently. Two of the recent studies have shown that patients tolerate the intravenous form well and that treatment resulted in a significant increase in BMD [236,237].

1.5.4d Conclusion

Based on the above observations, the current approach in managing osteoporosis in IBD patients is not adequate. Hormone replacement with estrogens is obviously unsuitable for the majority of patients with IBD as 50% are male and many of the females are pre-menopausal. The effect of calcium and vitamin D supplementation appears somewhat disappointing and current evidence for this treatment is scant. Although bisphosphonate treatment seems to yield some response, as a gastroenterologist it is exceedingly difficult to convince IBD patients to take yet another daily or indeed weekly oral medication, in particular when the drugs may have significant gastrointestinal side effects. There is the additional problem of patient well being not being improved in the short term. Hence, a more suitable and logical approach is to control the primary disease itself. As from previously outlined, reduced BMD in IBD is related in the main to elevated inflammatory mediators, so maintaining the disease in clinical remission with adequate treatment is the holy grail in terms of controlling bone loss. Current treatment options in IBD patients include the use of 5-aminosalicyclic acid preparations, immunomodulators such as azathioprine, 6-mercaptopurine or methotrexate and anti-TNF-α therapies such as infliximab and more recently adalimumab. Both infliximab and adalimumab have been shown to be superior in inducing and maintaining remission of the intestinal disease than the other conventional medications mentioned previously.

1.6 Anti-TNF α therapy in Inflammatory Bowel Disease

1.6.1 Introduction

Prior to the development of anti-TNF α as a modality to treatment for IBD, other medications that modulate the immune system such as 5-aminosalicylates, steroids, azathioprine, and 6-mercaptopurine were primarily used [238]. Patients with CD who developed complications, including fistulae were treated with surgery [239] and patients with UC who did not respond to conventional medications were treated with colectomy. However, basic scientific research showed that many cytokines were elevated in both CD and UC [240] and these findings has greatly improved our understanding of the pathogenesis of IBD. In CD the cytokines, are of the type 1 (*Th1*) cytokines which include TNF-α, IL-2, and interferon γ [241]. UC was less conclusively linked to the production of *Th2* cytokines [242]. TNF-α is released from a number of inflammatory cells, including monocytes, macrophages and T-cell lymphocytes in response to a number of environmental stimuli, including bacterial endotoxin, radiation and viral antigens [243]. TNF-α can then transmit signals between these immune cells leading to inflammation, thrombosis and fibrinolysis. As a potent pro-inflammatory cytokine, TNF-α must be firmly regulated, as failure to do so allows for an unregulated inflammatory response. In CD patients, TNF-α is highly localized to the intestinal mucosa and lumen. Indeed, high concentrations have been measured in the intestinal tissue and stool of both children and adults with CD [244,245]. Therefore, treatments that are targeted at inhibiting TNF-alpha may represent effective strategies for disease management. Currently, there are two anti-TNF-α therapy licensed for the use in IBD patients –

infliximab and adalimumab. Each of these will be discussed briefly in the following sections.

1.6.2 Infliximab

Infliximab is a chimeric IgG1 monoclonal antibody comprised of 75% human and 25% murine sequences, which has a high specificity for and affinity to TNF-α. Infliximab neutralizes the biologic activity of TNF-α by inhibiting binding to its receptors. However, infliximab mechanism of action most likely involves the destruction of TNF-α-bound activated effector cells through apoptosis and/or other mechanisms [246-250]. Infliximab has a half life of approximately 10 days. Infliximab was the first biological agent to be licensed for use in IBD patients. It has been licensed for use in CD patients since 1998 and in UC patients since September 2005 by the U.S. Food and Drug Administration (FDA).

Infliximab is approved for the treatment of moderate to severe CD patients who have not responded well to other conventional therapies [251,252]. In clinical studies, infliximab has been shown to decrease histologic and endoscopic disease activity and in inducing and maintaining remission in patients with active CD. Infliximab is also approved for use in fistulizing CD patients based upon the results of two randomized controlled trials [253,254]. Accumulating evidence has suggested that scheduled, maintenance therapy with infliximab has substantial clinical benefits compared with episodic treatment in CD patients who achieved remission with initial infliximab induction therapy [255]. Such therapy was shown to be associated with an increased likelihood of achieving and maintaining remission, improves quality of life, decreases corticosteroids requirements, and reduces the likelihood of developing antibodies to

infliximab and results in fewer hospitalizations. As a result of these studies, scheduled treatment is now the norm in clinical care of patients requiring infliximab.

At present infliximab is the first and only biologic approved for the treatment of moderate to severe UC which fails to respond to conventional therapies [256]. In clinical studies, infliximab was associated with reduction in signs and symptoms of, achieving clinical remission, mucosal healing and elimination of corticosteroid use. Infliximab is given as a single infusion intravenously in both CD and UC patients at 5 mg/kg body weight, initially as at week zero, two and six as an induction dose. In CD patients, if a clinical response is noted as defined by a decrease in the CDAI of 70 points or more, and there are no side effects encountered, these single infusions are continued every 8 weekly as a maintenance therapy. However, in UC patients at present, infliximab is used mainly as a rescue therapy rather than a maintenance therapy, unless a patient fails to achieve clinical remission.

1.6.3 Adalimumab

Adalimumab is a human IgG1 monoclonal antibody specific for human TNF. Unlike, infliximab which requires an intravenous infusion, adalimumab is administered by subcutaneous injection [257]. Adalimumab has been recently approved for the treatment of active CD since February 2007 by the U.S. FDA. The clinical efficacy and safety of adalimumab in patients with moderate to severe CD has been demonstrated in four pivotal studies [258-261]. All these studies have shown adalimumab to be superior to placebo for inducing and maintaining remission. Unlike infliximab, maintenance adalimumab therapy is prescribed as a subcutaneous injection every two weeks. The approved induction dosing of adalimumab in CD is 160 mg given subcutaneously

initially at week zero, 80 mg at week two, followed by a maintenance dose of 40 mg every other week beginning at week four. The drug is available in a single-use prefilled pen (HUMIRA Pen) or in a single-use, prefilled glass syringe. At present, adalimumab is only licensed for use in CD patients. Large multicentre clinical trials are currently being conducted to evaluate the use in UC patients and initial results from these trials are due in soon.

1.6.4 Anti-TNF-α therapy and bone disease

As previously described already, the discovery of the RANK ligand pathway, a transmembrane protein belonging to the TNF superfamily and its inhibition by OPG has had important implications for bone physiology as well as inflammation research. These interactions suggest that TNF blockade may have beneficial effect on bone generally. Indeed report consistent with effects of TNF blockade on BMD have begun to emerge in recent years.

The first studies to suggest that a beneficial effect was seen with TNF blockade on bone metabolism in CD patients was published in 2004. Franchimont *et al* [262] studied 71 CD patients treated for the first time with infliximab (5mg/kg) for refractory CD and observed serum concentration of the bone formation markers; BALP, OC and P1NP were lower in CD before infliximab treatment than in healthy controls while they return to normal levels after infliximab treatment at eight weeks. Serum concentration of CTx, a marker of bone resorption was significantly increased in CD at baseline but no longer different from controls after infliximab treatment at eight weeks. The authors also concluded that a relevant improvement in bone formation (defined as an increase of at least 30% in the bone formation marker) was found after infliximab treatment in 29.7%,

60.8% and 46.5% of the patients when considering BALP, OC or P1NP as marker, respectively. Relevant improvement in bone resorption (decrease of at least 30% in sCTx serum levels) was found after infliximab treatment in 38.2%.

Ryan *et al* [263] in a prospective trial studied 24 patients with active CD who were treated with infliximab (5mg/kg) for the first time. In this study, infliximab infusion led to a significant increase in both markers of bone formation, BALP and OC, but there was no control group. Levels of both bone formation markers remained significantly increased even at 4 months post treatment. No significant change in serum NTx was observed although at 4 months post treatment, levels were lower than at baseline. Of particular interest, the benefit seen on bone metabolism with infliximab treatment appeared to occur independently of the clinical response in terms of effect on CD activity; however the trend of increase in both bone formation markers and decrease in bone resorption marker was greater in responders compared with non-responders.

Following on this, Abreu *et al* [264] also reported a significant increase in BALP ($p=0.010$) whereas serum NTx, was not decreased ($p=0.801$) at week 4 in 38 CD patients treated with infliximab, but again there was no control group to compare with in this study. In this study sera were also analyzed for immunoreactive parathyroid hormone (iPTH), calcium and pro-inflammatory cytokines (IL-1α, IL-6, and TNF-α) at baseline and 4 weeks following infliximab infusion. Overall, treatment with infliximab was associated with a statistically significant decrease in PTH levels ($p=0.008$) and statistically significant increase in serum calcium ($p=0.034$) at week 4. No significant changes in serum cytokine measurements from baseline to week 4 were noted. In this study the authors also sub analyzed the data based on whether the patients were receiving

glucocorticoids at the time of their initial infliximab infusion and whether patients were responders or non-responders. 22/38 (57.9%) patients were receiving glucocorticoids at their time of initial infliximab infusion. The increase in bone formation occurred in both glucocorticoid treated individuals and those not on glucocorticoid, concluding that glucocorticoid use is not the principal reason for osteoporosis in patients with CD. Importantly, in this study the effect was also seen in both infliximab responders and non-responders, suggesting an independent effect of infliximab on bone metabolism.

Miheller *et al* [265] in a small study, studied the effect of infliximab on bone formation and resorption marker in 27 fistulizing CD patients and compared the results with 54 patients with inactive CD who acted as controls. Again, this was a short term study of just 42 days, and in treated patients, there were significant differences in β-CrossLaps (bCL), a marker for bone resorption on days 0 and 14 ($p<0.01$) and days 0 and 42 ($p<0.05$). OC levels increased significantly between day 0 and 42 ($p<0.05$) confirming the results from previous studies that infliximab has beneficial effects on bone turnover markers in CD patients.

There are currently only few limited studies that have examined the effect of infliximab on bone loss in CD patients by measuring BMD. A retrospective cohort analysis was performed by Pazianas *et al* [266] on 61 patients with CD and low BMD by serial DEXA scans. 23 patients were on infliximab and 36 patients were on bisphosphonates. Mean duration between DEXA scans was 2.2 +/- 0.99 years. After controlling for corticosteroids use, patients with concurrent infliximab and bisphosphonate treatment exhibited a greater increase in BMD compared to those on bisphosphonate alone (+6.7%/year vs. +4.46%/year, $p=0.045$); corticosteroids inhibited

this effects (p=0.025). However, infliximab alone had no effect on BMD. In this study, only lumbar spine BMD measurements were included for analysis and there was no control group.

In another retrospective study by Mauro *et al* [267], data from 15 patients with CD who had received treatment with infliximab for the first time and who underwent DEXA before and during infliximab treatment were compared with 30 CD patients who had never received treatment with infliximab and had two DEXA evaluation at least 12 months apart. Patients in this study received infliximab (5m/kg) at intervals of four to eight weeks for a mean period of 18 months. The first and second DEXA evaluations were 22.6 +/-11 months apart in the infliximab group and 20.4 +/- 8 months apart in the control group. The infliximab group had a significant increase in lumbar bone area, bone mineral content (BMC) and BMD between both evaluations compared to control (CD patients who had never received infliximab). The increase in BMC in patients who had received infliximab treatment was significant when compared with control patients who had received glucocorticoids (n=8) or had evidence of disease activity.

Bernstein *et al* [268] also found that maintenance treatment with infliximab (5mg/kg) at 6-8 weeks intervals for 1 year in 46 CD patients improved BMD after one year in the lumbar spine (2.4% increase, p=0.002), at the femoral trochanter by (2.8% increase, p=0.03), and at the femoral neck by (2.6% increase, p=0.001) and this effect was independent of concurrent administration of glucocorticoids, calcium supplementation, or changes in CRP. Also BMD gain at the lumbar spine and the left femur between the groups without and with osteopenia were not different. However, this study did not have a control group to compare differences in the changes in BMD.

Only one study has evaluated changes of osteoclastogenesis markers (OPG and RANKL) with infliximab therapy. Miheller *et al* [269] studied 29 patients who were treated with infliximab at week 0, 2 and 6 and showed that serum levels of OPG were significantly decreased and RANKL concentration was elevated at day 42 compare to day one.

1.7 Goals of the dissertation

The overall objective and primary aim of this dissertation is to investigate the effects of both currently licensed anti-TNF-α therapies - infliximab and adalimumab on bone metabolism in IBD patients using both clinical and *in vitro* models. The secondary aim of this dissertation is to increase awareness of IBD-related osteopenia and osteoporosis among IBD patients and clinicians. Existing studies which have been carried out looking at the effects of anti-TNF-α therapy on bone metabolism in IBD patients have only evaluated the role of infliximab on bone metabolism in CD patients. No studies have yet evaluated the effects of adalimumab therapy on bone metabolism in IBD patients or the effect of anti-TNF α therapy in UC patients. Furthermore, all studies published to date had a short period of follow up, with the longest being for 14 weeks [246]. Hence the long term effect of maintenance therapy with anti-TNF-α on bone metabolism in IBD patients is not yet known. Moreover, the effects of these therapies on actual bone cells have not yet been studied. Long term study is also needed to compare changes in cytokines, osteoclastogenesis markers (OPG and RANKL) and bone nutrients (PTH and vitamin D) to shed further light on the complex mechanisms involved in IBD-related bone disease. In particular, one hopes that this dissertation will improve our current understanding of the pathophysiological processes underlying bone loss in IBD and help elucidate the exact mechanism/(s) of anti-TNF α therapies on bone metabolism in these patient groups.

CHAPTER 2 – GENERAL MATERIALS & METHODS

2.1 Study design and protocol

2.1.1 Introduction

This study was a prospective, single centre open label study involving moderate to severe IBD patients who failed to response to conventional medical therapy (5-aminosalicylates, 6-mercaptopurine or azathioprine) and as a result who were started on biologic therapy, either infliximab or adalimumab. This study was reviewed and approved for human participation by the St.James's Hospital (SJH) and Adelaide & Meath incorporating National Children's Hospital (AMNCH), Tallaght Ethics Committee. This study also included a healthy control population matched for age and sex with our IBD patients. Study subjects were recruited from the Department of Gastroenterology, Adelaide & Meath Hospital, Tallaght, Dublin 24, Republic of Ireland.

2.1.2 Patient Demographics

2.1.2a Infliximab treated patients

Thirty-seven patients with IBD; 20 CD and 17 UC who received infliximab between a two year period were studied. Inclusion criteria included:

i) Infliximab naïve

ii) Normal serum calcium and phosphate levels

iii) Stable drug regime for the previous 2 months

iv) Active disease characterized by one of the following:

a) a single or multiple perianal or enterocutaneous draining fistula(e) as a complication of CD, resistant to conventional treatment for at least 3 months
b) moderate to severely active CD or UC with colitis, ileitis or ileocolitis confirmed by radiography or endoscopy
c) disease refractory to or dependent on oral (> 8mg/day prednisone equivalent) or intravenous hydrocortisone (>5 days of 100mg four times daily) and/or non-responding to immunosuppressive agents (azathioprine, mercaptopurine or methotrexate).

Patients with known confounding factors such as concomitant bone diseases (including Paget's, osteomalacia, hyperparathyroidism and other endocrinopathies with bone alterations), and chronic alcoholism were excluded from this study.

2.1.2b Adalimumab treated patients

Twenty patients with CD who received adalimumab between a one year period were studied. Inclusion criteria included:

i) Adalimumab naïve
ii) If received adalimumab administered due to loss of response to infliximab then there must have been at least 12 weeks since last dose of infliximab before commencing adalimumab.
iii) Normal serum calcium and phosphate levels
iv) Stable drug regime for the previous 2 months
v) Active disease characterized by one of the following:

a. a single or multiple perianal or enterocutaneous draining fistula(e) as a complication of CD, resistant to conventional treatment for at least 3 months
b. moderate to severely active CD with colitis, ileitis or ileocolitis confirmed by radiography or endoscopy
c. disease refractory to dependent on oral (> 8mg/day prednisone equivalent) and/or non-responding to immunosuppressive agents (azathioprine, mercaptopurine or methotrexate).

Patients with known confounding factors such as concomitant bone diseases (including Paget's, osteomalacia, hyperparathyroidism and other endocrinopathies with bone alterations), and chronic alcoholism were excluded from this study.

2.1.2c Control patients

Twenty healthy controls matched for age and gender with our IBD patients were also studied. Patients with confounding factors such as known concomitant bone diseases (including Paget's, osteomalacia, hyperparathyroidism and other endocrinopathies with bone alterations), chronic alcoholism and treatment with bone effects such as bisphosphonates, HRT, anabolic steroids, calcitonin or corticosteroids were excluded from acting as controls in this study. Patient characteristics are shown in **Table 2.1**.

	Infliximab treated (n=37)	Adalimumab treated (n=20)	Control group (n=20)
Sex, female, n (%)	15 (41)	12 (60)	10 (50)
Age (median, years)	35.1 (17-70)	35.2 (15-56)	35.7 (21-60)
Current smokers, n (%)	8 (22)	10 (50)	7 (35)
Mean body mass index, kg/m^2	25.2	24.0	25.1
IBD medications, n (%)			
Corticosteroids	22 (59)	4 (20)	N/A
5- Aminosalicylic acid	26 (70)	14 (70)	N/A
6-Mercaptopurine/Azathioprine	25 (68)	9 (45)	N/A
Disease location, n (%)			
CD - Terminal ileum	2/20 (10)	5 (25)	N/A
CD - Ileo-colonic	13/20 (65)	12 (60)	N/A
CD - Colonic	2/20 (10)	2 (10)	N/A
CD - Fistulizing disease	3/20 (15)	1 (5)	N/A
UC - Proctitis	1/17 (6)	N/A	N/A
UC - Left sided colitis	8/17 (47)	N/A	N/A
UC - Pancolitis	7/17 (41)	N/A	N/A
UC - Chronic pouchitis	1/17 (6)	N/A	N/A
Previous infliximab exposure, n (%)	N/A	13 (65)	N/A
Other concomitant medications			
Calcium/Vitamin D	15 (41)	6 (30)	N/A
Bisphosphonates	1 (3)	0 (0)	N/A

TABLE 2.1: PATIENTS CHARACTERISTICS FOR BIOLOGICAL THERAPY STUDY

2.1.3 Biological therapy regimen and follow up

Infusions with infliximab were given intravenously at a dose of 5mg/kg at 0, 2, 6 and after that at an interval of 8 weeks. Infliximab treated patients were followed up for 1 year and sera were obtained before each infusion at baseline, week 6, week 30 and week 54 for analysis. Adalimumab was given subcutaneously at a loading dose of 160mg at week 0, 80mg at week 2, and 40mg every other week thereafter. Patients receiving adalimumab were followed up for 6 months and sera were obtained prior to each injection at baseline, 1 month, 3 month, and 6 month for analysis. Control patients sera were obtained once-off for analysis.

2.1.4 Cohort size and study sample

At the end of this MD study, the numbers of samples available for analysis in each group were as below:

a) Infliximab group – 37 patients (at week 0), 37 patients (at week 6), 19 patients (at week 30) and 11 patients (at week 54)
b) Adalimumab group – 20 patients (at week 0), 20 patients (at 1 month), 20 patients (at 3 month) and 20 patients (at 6 month)
c) Control group – 20 patients

2.1.5 Disease Activity Scoring

Clinical research in IBD continues to evolve. Typically, trials focus on clinical, endoscopies, or quality of life outcomes to determine whether new therapies are more efficacious than the standard of care. A standardized and validated way to compare disease activity in IBD is thus essential. Currently, multiple scoring systems for IBD disease activity are available but only selected few are simple to administer, reproducible, valid, and responsive to change.

2.1.5a Crohn's Disease Activity Index (CDAI)

The most frequently used index for the assessment of disease activity in Crohn's disease is the CDAI **(Table 2.2)**. It has been prospectively validated and remains the most widely used scoring system for CD activity to date [270]. The outcome of the CDAI varies between 0 and 600 points. A score below 150 is associated with clinical remission, 150 to 219 with mildly active disease, 220 to 450 with moderately active disease and above 450 with very severe disease [270]. This index is used in trials to select groups of patients based on the severity of the disease activity and to evaluate the effect of treatments. In most studies complete remission is defined by a value of less than 150 points, and clinical response is characterised by a decrease in CDAI of more than 70 to 100 points [270]. The CDAI is also used to monitor patients for worsening of the disease during a trial. Relapse or reactivation of disease is defined as an increase in CDAI by more than 150 points or a score above 150 together with an increase by more than 50 to 100 points (not well defined). An increase in the index by more than 60 to 100 points or a

disease activity of more than 200 at the end of the treatment period indicates treatment failure.

Descriptions	Sum of 7 days	Factor	Subtotal
Number of liquid or soft stools	-------	2	-------
Abdominal pain[1]	-------	5	-------
General well being[2]	-------	7	-------
Number of complications (presence or absence): Arthritis or arthralgia Iritis or uveitis Anal fissure, fistula or abscess Erythema nodosum, pyoderma gangrenosum Other fistula Fever > 37.8 °C	-------[3]	20	-------
Anti-diarrhoeal medications (none=0, yes=1)	-------	30	-------
Abdominal mass (none=0, questionable=2, definite=5)	-------	10	-------
Haematocrit [males=47-Ht(%); females=42-Ht(%)]	-------	6	-------
Body weight: **(1-body weight/standard weight)x100=**	-------	1	-------

[1] *Pain score per day: 0 = none, 1 = mild, 2 = moderate, 3 = severe*
[2] *General well-being score: 0 =generally well, 1 = slightly under par, 2 = poor, 3 = very poor, 4 = terrible*
[3] *Total number of complications from the list that are present*

Table 2.2: CDAI scoring table

2.1.5b Simple Colitis Score (SCS)

This new Simple Colitis score has been shown to have a good correlation with existing more complex scoring systems and therefore could be useful in the initial assessment of patients with UC. Furthermore, high significant correlations have been shown comparing this simple scoring system with more complex Powell-Tuck Index [271].

Symptom	Score
Bowel frequency (day)	
1-3	0
4-6	1
7-9	2
>9	3
Bowel frequency (night)	
1-3	1
4-6	2
Urgency of defecation	
Hurry	1
Immediately	2
Incontinence	3
Blood in stools	
Trace	1
Occasionally frank	2
Usually frank	3
General well being	
Very well	0
Slightly below par	1
Poor	2
Very poor	3
Terrible	4
Extra-colonic manifestations	1 per manifestation

Table 2.3: Simple Colitis Score

2.2 Dual Energy X-ray absorptiometry (DXA)

All BMD measurements were obtained by DXA scan at the Department of Radiology of our institution AMNCH, Tallaght, Dublin 24, Republic of Ireland (Lunar Prodigy Advance DXA System; software version: 8.80, GE Medical System LUNAR). Hence, the same DXA machine used at baseline was used for follow-up measurement for each patient. All BMD readings were carried out by a single radiologist to reduce variability from the use of different densitometers and interpretations that could arise with using a number of radiologists. Measurement of BMD was assessed at the posterior-anterior spine (region L1-L4) and left femur (region neck) using the local reference population provided by the manufacturer of the DXA machine. Osteopenia and osteoporosis were defined according to WHO recommendations as previously been described in section 1.2.1. DXA scan was performed at baseline in all patients including controls and at 1 year in IBD treated infliximab patients who completed the study.

2.3 Sera isolation and separation

All blood samples obtained from the subjects in this research were early morning fasting blood samples. Each time, 6x 8ml of blood was drawn directly into an 8ml of Serum Separator Clot Activator tube (Greiner Bio-One GmbH, Bad Haller, Straße 32, A-4550 Kremsmünster, Austria). Samples were then let to clot for 30 minutes. The tubes were then centrifuged at 2000rpm for 10 minutes in a bench top centrifuge machine-MPW65R (Medical Supply Co. Ltd, Dublin 15, Ireland). The centrifuged tubes were then transferred to the tissue culture hood and using a sterile pasteur pipette (SARSTEDT Inc., Newton, NC, USA), serum from each tube was transferred to a 0.5ml sterile eppendorfs (SARSTEDT Inc., Newton, NC, USA). The eppendorfs were then labelled appropriately, and stored in the -80°C freezer (Kendo Lab Products, NC, USA) on a styrofoam holder (SARSTEDT Inc., Newton, NC, USA) for future use. The samples were stored in 0.5mls eppendorfs instead of larger eppendorfs/vials to limit the thaw-freeze cycle.

2.4 Bone turnover markers

2.4.1 Introduction

Total procollagen type 1 N-terminal propeptide (P1NP) and N-MID Osteocalcin (OC) were chosen as markers of bone formation and C-telopeptide of type-1 collagen (CTx) serum concentration as a marker of bone resorption. Total P1NP was measured using the Elecsys total P1NP assay (Catalogue No. 03141071, Roche Diagnostics GmbH, D-68298, Mannheim); N-MID OC was measured using the Elecsys N-MID Osteocalcin assay (Catalogue No. 12149133, Roche Diagnostics GmbH, D-68298, Mannheim) and CTx serum was measured using the Elecsys β-CrossLaps/serum assay (Catalogue No. 11972308, Roche Diagnostics GmbH, D-68298, Mannheim). These entire assays were read using the Roche Elecsys 1010 immunoassay analyzers (Roche Diagnostics GmbH, D-68298, Mannheim). All samples and standards were assayed in duplicates and average reading was obtained for analysis. This work was carried out in the Department of Biochemistry, St. James's Hospital (SJH). Each of these assays is briefly described below.

2.4.2 Total procollagen type 1 N-terminal propeptide (P1NP)

More than 90% of organic bone matrix consists of type 1 collagen, which is preferentially synthesized in bone [272]. Type 1 collagen is derived from type 1 procollagen which is synthesized by fibroblasts and osteoblasts. Type 1 procollagen contains both N-(amino) and C-(carboxy) terminal extensions. These extensions (propeptides) are removed by specific proteases during the conversion of procollagen to collagen and its subsequent incorporation into the bone matrix. The extension measured

by Elecsys P1NP assay is the amino terminal, hence P1NP-type 1 procollagen amino-terminal propeptide. This marker, P1NP is therefore a specific indicator of type 1 collagen deposition and thus may be defined as a true bone formation marker [273,274]. P1NP is released during type 1 collagen formation into the intracellular space and eventually into the blood stream. P1NP appears to be released as a trimeric structure (derived from the trimeric collagen structure) but is rapidly broken down to a monomeric form by thermal degradation effects [275,276].

The Elecsys P1NP assay detects both fractions present in blood and is therefore called total P1NP. The assay uses a sandwich principle and the total duration of the assay is 18 minutes. Briefly, 20µl of sample and a biotinylated monoclonal P1NP-specific antibody are incubated together. The reaction mixture is aspirated into the measuring cell where the microparticles are magnetically captured onto the surface of the electrode. Unbound substances are then removed with ProCell. Application of a voltage to the electrode then induces chemiluminescent emission which is measured by a photomultiplier. Results are then determined via a calibration curve which is instrument-specifically generated by 2-point calibration and a master curve provided via the reagent barcode.

2.4.3 N-MID Osteocalcin (OC)

OC, the most important non-collagen protein in bone matrix, is a bone-specific, calcium-binding protein which is dependent on vitamin K. It contains 49 amino acids and has a molecular weight of approximately 5800 daltons. It contains up to three γ-carboxyglutamic acid residues (bone-GLA-protein, BGP). During bone synthesis OC is produced by osteoblasts. Its production is dependent upon vitamin K (formation of γ-carboxyglutamic acid residues) and is stimulated by vitamin D3. After release from the

osteoblasts, OC is not only assimilated into the bone matrix but also secreted into the blood stream. Accordingly, the serum (plasma) OC level is related to the rate of bone turnover in various disorders of bone metabolism; e.g. osteoporosis in particular, but also in primary and secondary hyperparathyroidism or Paget's disease. OC is therefore termed a bone turnover marker and is used for this purpose. By means of OC measurements it is possible to monitor therapy with antiresorptive agents (bisphosphonates or hormone replacement therapy, HRT) in, for example, patients with osteoporosis or hypercalcemia. Both intact OC (amino acids 1-49) and the large N-MID fragment (amino acids 1-43) occur in blood. Intact osteocalcin is unstable due to protease cleavage between amino acids 43 and 44. The N-MID fragment resulting from cleavage is considerably more stable.

The Elecsys N-MID OC assay uses two monoclonal antibodies specifically directed against epitopes on the N-MID fragment and the N-terminal fragment. The assay hence detects the stable N-MID fragment as well as the (still) intact OC. The test is non-dependent on the unstable C-terminal fragment (amino acid 43-49) of the OC molecule and thus ensures constant measurement results under routine conditions in the laboratory. The assay uses a sandwich principle and the total duration of the assay is 18 minutes. Briefly, 20µl of sample and a biotinylated monoclonal N-MID OC-specific antibody, and a monoclonal N-MID OC-specific antibody labeled with a ruthenium complex react to form a sandwich complex. After addition of streptavidin-coated microparticles, the complex becomes bound to the solid phase via interaction of biotin and streptavidin. The reaction mixture is aspirated into the measuring cell where the microparticles are magnetically captured onto the surface of the electrode. Unbound substances are then

removed with ProCell. Application of a voltage to the electrode then induces chemiluminescent emission which is measured by a photomultiplier. Results are then determined via a calibration curve which is instrument-specifically generated by 2-point calibration and a master curve provided via the reagent barcode.

2.4.4 C-telopeptide of type-1 collagen (CTx)

As previously mentioned in section 2.4.2, more than 90% of organic bone matrix consists of type 1 collagen, which is preferentially synthesized in bone [270]. There is regulated anabolism and catabolism of the basic substance in bone. During normal bone metabolism, mature type 1 collagen is degraded and small fragments pass into the bloodstream and are excreted via kidneys. By determining a bone resorption marker, the activity of bone turnover can be found. In physiologically or pathologically elevated bone resorption (e.g. in old age or as a result of osteoporosis), type 1 collagen is degraded to an increased extent, and there is a commensurate rise in the level of collagen fragments in blood. Especially relevant collagen type 1 fragments are the C-terminal telopeptides (CTx). In the C-terminal telopeptides the α-aspartic acid present converts to the β-form of aspartic acid as the bone ages (β-CTx) [277,278]. These isomerized telopeptides are specific for the degradation of type 1 collagen dominant in bone. Elevated serum concentrations of isomerized C-terminal telopeptides of type 1 collagen have been reported for patients with increased bone resorption. The serum levels return to normal during resorption-inhibiting therapy [279-282]. Determination of the C-terminal telopeptides in serum is recommended for monitoring the efficacy of antiresorptive therapy (e.g. bisphosphonates or HRT) in osteoporosis or other bone diseases. By this means, therapy-induced changes can be demonstrated after just a few weeks [281,283].

The Elecsys β-CrossLaps/serum assay is specific for crosslinked isomerized type 1 collagen fragments, independent of the nature of crosslink (e.g. pyrrole, pyridinolines and others [284]). The assay specificity is guaranteed through the use of two monoclonal antibodies each recognizing linear β-8AA octapeptides (EKAHD-β-GGR). The Elecsys β-CrossLaps/serum assay therefore quantifies all type 1 collagen degradation fragments that contain the isomerized octapeptide β-8AA twice (β-CTx) [281,282].

The assay uses a sandwich principle and the total duration of the assay is 18 minutes. Briefly, 50µl of sample and a biotinylated monoclonal anti-β-CrossLaps antibody are incubated together; antigen in the sample is liberated from the serum components. Following addition of streptavidin-coated microparticles and a monoclonal β-CrossLaps-specific antibody labeled with a ruthenium complex, a sandwich complex is formed which becomes bound to the solid phase via interaction of biotin and streptavidin. The reaction mixture is aspirated into the measuring cell where the microparticles are magnetically captured onto the surface of the electrode. Unbound substances are then removed with ProCell. Application of a voltage to the electrode then induces chemiluminescent emission which is measured by a photomultiplier. Results are then determined via a calibration curve which is instrument-specifically generated by 2-point calibration and a master curve provided via the reagent barcode.

2.5 Cytokines

All the pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α), anti-inflammatory cytokines (IL-10 and IL-13) and osteoclastogenesis markers (OPG and sRANKL) were measured using Millipore ELISA products. All samples and standards were assayed in duplicates and average reading was obtained for analysis. IL-1β, IL-6, TNF-α, IL-10 and IL-13 were measured using the Human Cytokine Linco*plex* Kit (Catalogue number #HCYTO-60K). Briefly, this is a multiplex assay kit manufactured by LINCO Research, Inc. to be used for the simultaneous quantitative determination of the following thirty human cytokines and chemokines in any combination: EGF, Eotaxin, Fractalkine, G-CSF, GMCSF, IFN-γ, IL-1α, IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 (p40, free form), IL-12 (p70), IL-13, IL-15, IL-17, IP-10, MCP-1, MIP-1α, MIP-1β, RANTES, soluble CD40L, TGF-α, TNF-α, and VEGF.

sRANKL was measured using the Human RANKL single plex kit (Catalogue number #HBN51K1RANKL). Briefly, this single-plex assay kit manufactured by Millipore is to be used for the quantitative measurement of soluble human RANKL in cell/tissue culture supernatant samples and human serum or plasma samples (1:2 diluted).

OPG was measured using the Human Bone Panel 1A LINCOplex Kit (Catalogue number #HBN1A-51K). Briefly, this Human Bone Panel 1A multiplex assay kit manufactured by Millipore is to be used for the simultaneous quantitative measurement of the following seven Human Bone biomarkers in any combination: OPG , OC , OPN

(Osteopontin), PTH , Leptin, ACTH, and Insulin. This kit can be used for the analysis of the above bone biomarkers in 1:4 diluted human serum/plasma samples.

2.6 Analysis of bone nutrients levels

2.6.1 Parathyroid hormone

Parathyroid hormone (PTH) levels were measured in the Department of Biochemistry, SJH using the Elecsys intact PTH assay (Catalogue No. 11972103, Roche Diagnostics GmbH, D-68298, Mannheim) and were read using the Roche Elecsys 1010 immunoassay analyzers (Roche Diagnostics GmbH, D-68298, Mannheim). These assays employ a sandwich test principle in which a biotinylated monoclonal antibody reacts with the N-terminal fragment (1-37) and a monoclonal antibody labeled with a ruthenium complex reacts with the C-terminal fragment (38084). The antibodies used in this assay are reactive with epitopes in the amino acid regions 26-32 and 37-42. The total duration of the assay is 18 minutes. Briefly, 50µl of sample and a biotinylated monoclonal PTH-specific antibody and monoclonal PTH-specific antibody labeled with a ruthenium complex form a sandwich complex. Following addition of streptavidin-coated microparticles, the complex becomes bound to the solid phase via interaction of biotin and streptavidin. The reaction mixture is aspirated into the measuring cell where the microparticles are magnetically captured onto the surface of the electrode. Unbound substances are then removed with ProCell. Application of a voltage to the electrode then induces chemiluminescent emission which is measured by a photomultiplier. Results are then determined via a calibration curve which is instrument-specifically generated by 2-point calibration and a master curve provided via the reagent barcode.

2.6.2 Vitamin D

Vitamin D was measured in the Department of Biochemistry, SJH using the DiaSorin 25-hydroxycalciferol (25-OH-D) assay (Stillwater, Minnesota, 55082-0285, USA). Briefly, the DiaSorin 25-OH-D assay consists of a two-step procedure. The first procedure involves a rapid extraction of 25-OH-D and other hydroxylated metabolites from serum or plasma with acetonitrile. Following extraction, the treated sample is then assayed using an equilibrium RIA procedure. The RIA method is based on an antibody with specificity to 25-OH-D. The sample, antibody and tracer are incubated for 90 minutes at 20-25°C. Phase separation is accomplished after a 20 minute incubation at 20-25°C with a second antibody precipitating complex. A nonspecific binding/addition buffer is added after this incubation prior to centrifugation to aid in reducing non-specific binding. Reference range used was obtained from the Department of Biochemistry, SJH. A normal vitamin D level was defined as levels > 80nmol/L, vitamin D insufficiency was defined as levels between 25 nmol/L – 80nmol/L and vitamin D deficiency was defined as levels < 25 nmol/L.

2.7 Analysis of C-reactive protein (CRP) levels

CRP levels were measured using standard measure in the Department of Biochemistry, Adelaide & Meath Hospital, Tallaght, Dublin 24 as part of routine blood investigations work up in all patients. .

2.8 Osteoblast cell culture information

2.8.1 Cell source

The osteoblast cell line used during this study was the hFOB 1.19, Homo sapiens (Human) fetal osteoblast 1.19. This cell line was purchased from LGC Promochem-ATCC (American Type Culture Collection), Middlesex, UK and the catalogue number was CRL-11372. Before the arrival of frozen cells from ATCC, a complete fresh growth medium was prepared in advance.

This conditionally immortalized cell line was established with the temperature sensitive expression vector pUCSVtsA58 and the neomycin resistance expression vector pSV2-neo. Clones were selected in the presence of G418 [285]. This cell line has also been shown to be capable of osteoblastic differentiation, osteoblast physiology, and demonstrating cytokine effects on osteoblasts [286]. The mutant SV40 TAg gene (tsA58) used in the immortalization, generates a functional conformation at 34°C, while at 39°C the TAg becomes unstable and non-functional. Thus, the cells divide rapidly at 34°C (when the TAg is functional) but cease dividing at 39°C (when the TAg is inactive) and start to differentiate and produce mineralized nodules. This combination of distinguishing characteristics is unique to this human cell line, setting it apart from other osteoblastic cell models such as primary cultures derived from human adult bone, osteosarcoma cell lines, and fetal rodent primary cultures and making it an excellent cell line for this work.

2.8.2 Preparation of Complete Growth Medium

The complete growth medium recommended by ATCC is a 1:1 mixture of Ham's F12 medium and Dulbecco's modified Eagle's medium(DMEM) without phenol red with 2.5 mM L-glutamine, 0.3 mg/ml G418, 10% fetal bovine serum (FBS) and 2% Penicillin/ Streptomycin. The modified recipe was as follows: a 500ml complete growth medium contained 219.25ml Ham's F12 medium (Biosciences Ltd., Co. Dublin, Ireland), 219.25ml DMEM (Biosciences Ltd, Co. Dublin, Ireland), 1.5 ml G418, 50ml of FBS and 10ml Penicillin/Streptomycin (all from Sigma-Aldrich Ireland Ltd). The fresh FBS was slowly thawed at 4°C in a refrigerator, then mixed thoroughly and heat-inactivated in a 60°C water bath for 30 minutes (Grant Instruments Ltd., Cambridge, England). Then it was allowed to cool and aliquoted into sterile containers.

Firstly, the flow hood area and all the required items as described above were swabbed with 70% ethanol to sterilize. Then, 10ml Penicillin/Streptomycin, 1.5ml G418 and 50ml FBS were added to one of the empty sterile 500ml duran flasks. Ham's F12 and DMEM were then added into the solution and agitated gently. The entire solution was filtered using a 0.2μm filter (Sarstedt Ltd, Co. Wexford, Ireland) into a second 500ml duran flask. The complete growth medium was labeled and stored in a 4°C refrigerator.

2.8.3 Preparation of Serum Free Media

All experiments carried out using human serum were diluted to the appropriate dilution using serum free media (SFM). The recipe was as follows: a 500ml of SFM contained 244.25ml Ham's F12 medium, 244.25ml DMEM, 1.5 ml G418, and 10ml Penicillin/ Streptomycin.

2.8.4 Cell Thawing

Once the frozen cell arrived or from previous stocks stored in liquid nitrogen, they were thawed and handled as follows: Firstly, the cryogenic vials were swabbed with 70% ethanol to sterilize. Under sterile conditions of the flow hood, the cells were thawed by rolling the vial gently between hands until liquid was visible on the sides. The half-frozen cell block was then placed into a 15ml falcon tube (SARSTEDT Inc., Newton, NC, USA) which contained 10ml of fresh growth medium. This was then centrifuged at 1200rpm for 5 minutes. The supernatant was poured into a waste container and the pellet re-suspended in 10ml prewarmed medium and finally vortexed. The cells were pipetted into a labeled T25 flask.

2.8.5 Cell Re-suspending

The cells were then resuspended in 10ml fresh medium as follows: The 15ml tube was placed on a vortex machine (Biosciences Ltd., Co. Dublin, Ireland) with a vortex speed- 'medium'. A very gentle vortexing was performed until the cells were fully resolved into the medium. The cell suspension was then removed and placed into a T25 tissue culture flask (SARSTEDT Inc., Newton, NC, USA). The flask of cells was incubated overnight at 34°C in an incubator (Medical Supply Co. Ltd, Dublin 15, Ireland) with 5% CO2 atmosphere to enhance the growth of the initial passage of cells. After a two day incubation, the cells in the T25 flask were transferred into a T75 tissue culture flask (SARSTEDT Inc., Newton, NC, USA), followed by incubation in 34°C incubator. The passage number of cells in this T75 flask was counted as number one. The cells were then fed at regular intervals.

2.8.6 Cell Feeding

The cells growing in the culture flask were washed by sterile D-Phosphate Buffer Solution (PBS) (Biosciences, Ltd., Co. Dublin, Ireland) to remove dead cells, and then supplemented by complete growth medium with same volume as the removed medium (typically 13ml in a T75 and 7ml in a T25 flask). Cell feeding was performed every 2-3 days depending on how confluent the cells were.

2.8.7 Cell Sub culturing

Cell growth was observed by a light microscope – Nikon E800M microscope (Micron Optical Co. Ltd, Japan). Once the cell growth reached the plateau phase (90% confluence) the cells were washed with sterile D-PBS and further treated as follows:

2.8.7a Trypsinizing cells

The purpose of this step is to detach the cells from the flask. 3mls of pre-warmed Trypsin-EDTA (1x) which contains 0.25% Trypsin (Biosciences Ltd., Co. Dublin, Ireland) was added into the T75 flask to cover the entire surface of the flask. The flask was then placed in the 37°C incubator for 5 minutes to allow for the full detachment of the cells from the flask surface. The flask was gently shaken back and forth to help the detachment of the cells. 3mls of fresh medium was then added to the flask to neutralize the action of the trypsin.

2.8.7b Centrifuging cells

The 6 ml cell suspension was then put into a 15ml tube and centrifuged to pellet the cells together. The cells were centrifuged at 1200rpm for 5 minutes in a bench top centrifuge machine- MPW65R (Medical Supply Co. Ltd, Dublin 15, Ireland). The medium was discarded. The cell pellet was re-suspended in 8 ml of fresh medium as described in Section 2.8.5. The 8 ml cell suspension was then split into 4 tissue culture flasks (splitting ration=1:4). Extra medium was added to make 15ml in total.

After the first subculture of cells, the cells were then counted as passage number two. Recording the cell passage number is important as cells used in experiments should be all between passage numbers four to nine. Cells beyond a certain passage number will lose their characteristic phenotype resulting in lack of reliability of the experimental data [287].

2.8.8 Cell Freezing

Cells that were not used for experiments were frozen down so that there was a back up supply of the cell line. Prior to freezing cells, a freezing medium was made up according to the protocol that ATCC provided: complete growth medium, 72%; additional FBS, 20%; DMSO, 8% (Sigma-Aldrich Ltd, Dublin 24, Ireland). Cells were trypsinized as outlined in Section 2.8.7a and re-suspended in the freezing medium. The freezing medium was added to the cell pellet very gently and slowly. The cells were re-suspended at a concentration of about $2x10^6$ cells/ml. 1 ml was added into a labeled cryovial carefully. It was placed for 30 minutes in a -20°C freezer and then moved to a -80°C freezer (Kendo Lab Products, NC, USA) overnight, and finally transferred to liquid nitrogen container for long-term storage.

2.8.9 Cell Counting by Trypan Blue Exclusion Method

Cell counting in all of the experiments in the study was done by using Trypan Blue exclusion method. Trypan Blue is a stain recommended for use in estimating the proportion of viable cells in a population. Trypan Blue has been used in staining of various cell types [288]. The method of action of Trypan Blue is that it can only enter the cell if the cell membrane is damaged. For example, the stained (blue) cells are dead, whilst the unstained (white) cells are viable which prevents the dye entering them.

Measuring the percentage of Trypan Blue, can therefore assess cell viability and confirm whether the cell culture conditions are optimal. It is also useful for determining whether cells in culture conditions are optimal. It is also useful for determining whether cells in culture have escaped breakage or disruption [288] and to assess apoptosis [289]. It can therefore be used in assessing viability as well as in assessing cell mortality in response to environmental stimuli. The staining procedure with this dye is relatively simple and the determination of percentage of Trypan Blue exclusion is performed using a light microscope and a hemocytometer. Cells were first trypsinized (as described in section 2.8.7a) and re-suspended (as described in section 2.8.5) to make an even cell suspension. A 100µl of cell suspension was removed while maintaining sterile conditions. It was then mixed with 100µl of Trypan Blue (Bio-Sciences Ltd, Dun Laoghaire, Ireland) so as to avoid too much exposure of the Trypan Blue. Exposure longer than 30 minutes can possibly increase the dead cell population of cell suspension. A cover slip was mounted onto a hemocytometer (a chamber of a fixed volume with grid lines that can then be observed under the light microscope) and 10µl of the mixture of

cell suspension and trypan blue was pipetted into the notch. The number of cells was counted in the four squares (A, B, C and D).

The following formula was used to calculate the total number of cells in the cell suspension:

Cells = [Viable cells counted/Squares counted] x Dilution (2) x 10,000 x Volume (ml)

For example, if the number of cells counted in each square is 30, 25, 35, 30, and the volume of cell suspension is 10ml, then the total number of cells in the cell suspension is: $[(30+25+35+30)/4] \times 2 \times 10{,}000 \times 10 = 6 \times 10^6$.

2.8.10 Cell Viability testing – AlamarBlue Assay

There are currently various methods for measuring cell proliferation and cell viability. A new method for measuring cell proliferation and viability has recently become available. The alamarBlue assay (Bio-Source, Galway) is designed to measure quantitatively the proliferation of various human and animal cell lines, bacteria and fungi. The alamarBlue assay incorporates a fluorometric/colorimetric growth indicator based on detection of metabolic activity. This assay allows real time monitoring of viability. Specifically, the system incorporates an oxidation-reduction (REDOX) indicator that both fluoresces and changes colour in response to chemical reduction of growth medium resulting from cell growth [290].

The specific (fluorometric/calorimetric) REDOX indicator incorporated into alamarBlue has been carefully selected because of several properties. Firstly, the REDOX indicator exhibits both fluorescence and colorimetric changes in the appropriate oxidation–reduction range relating to cellular metabolic reduction. Secondly, the REDOX

indicator is demonstrated to be minimally toxic to living cells. Thirdly, the REDOX indicator produces a clear, stable distinct change which is easy to interpret.

The assay is simple to perform since the indicator is water soluble and non-toxic to the cells, thus eliminating the washing/fixing and extraction steps required in other commonly used cell viability assays. The continuous monitoring of cells in culture is therefore permitted. AlamarBlue as well does not alter the viability of the cells cultured for various time points as in the case when monitored by Trypan Blue exclusion method [291.] Proliferation and viability measurements obtained with alamarBlue have been shown to compare favorably with measurements obtained by other methods, specifically:

(a) AlamarBlue reduction, when measured spectrometrically, produces linear results over a similar range of concentrations of cells as with the MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow tetrazole] assay. AlamarBlue assay permits monitoring over a broader range of cell densities than MTT assay where reduction is monitored by colorimetry [292].

(b) In the determination of minimal number of proliferating cells, the measurement of alamarBlue reduction is a sensitive measurement compared with tritiated thymidine incorporation [293].

All these data reflect its novelty and broad application potential, and encouraged us to use it as our cell viability testing method for this research.

2.8.11 Alkaline Phosphatase Assay

A marker of early-stage differentiation by the human osteoblast cell is the expression of ALP which aids bone formation. There are a number of assays which can be used to detect the amount of ALP secreted by the osteoblast cells. One of the most commonly used assays involves adding p-Nitrophenyl Phosphate (pNPP) solution. This assay is based on the principle of ALP hydrolysis of p-Nitrophenyl Phosphate to p-Nitrophenyl. pNPP is a chromogenic substrate for most phosphatases such as alkaline phosphatases, acid phosphatases, protein tyrosine phosphatases and serine/threonine phosphatases. The reaction yields p-nitrophenol, which becomes an intense yellow soluble product under alkaline conditions and can be conveniently measured at 405 nm on a spectrophotometer.

This homogeneous "mix-and-measure" assay involves simply adding a single reagent to the phosphatase and measuring the product formation using any absorbance reader. The assay can be conveniently performed in cuvettes, tubes or multi-well plates at either room temperature or 37°C. In addition, the reagents are compatible with ELISA assays in which alkaline phosphatase conjugated secondary antibody is used. This kit is well adapted for a number of applications. For example, it can be utilized for direct characterization of enzyme activity and for assay condition optimization. It can also be applied for clinical diagnosis of diseases that are associated with increased levels of alkaline phosphatase.

2.9 In-vitro experimental procedures

2.9.1 Optimization of the alamarBlue assay as a viability assay for hFOB 1.19 cell line

In order for us to use alamarBlue assay to assess cell viability, we firstly needed to create a standard curve of hFOB cell growth using the reagent. From this one can assess the most optimal detection time point and cell density number to be used for future experiments to determine viability levels. The manufacturers of alamarBlue recommend that cells should be cultured with the reagent over a chosen period to obtain a standard growth curve. They use a cell range of $1x10^5$ and $1x10^6$ cells. For this experiment, a range of cell densities from 0.2 to$1x10^6$ cells/ml was chosen. The objective of this experiment was to create a standard curve using alamarBlue of hFOB 1.19 cell line viability and to determine the optimal detection stage and cell density for viability.

Cells were cultured until confluent (80-90%). A total cell count was carried out as described in section 2.8.9. Cells were re-suspended and concentrations from 0.2-1 x 10^6 cells/ml were prepared in tubes. 10% alamarBlue was added into each tube. A control was prepared by adding medium only. A negative control was prepared by adding medium and 10% alamarBlue only. All tubes were vortexed. A 100µl of each concentration was pipetted in triplicate onto a 96 well plate and incubated at 34°C for 10 hours. The plate was wrapped in parafilm to avoid evaporation overnight. Absorbance was read on a spectrophotometer at 540nm and 620nm over a 10 hour period.

Absorbance values were entered into an excel spread sheet, averages were calculated at

each filter type (A_{540} & A_{620}). The percentage reduction was calculated in accordance with Biosource alamarBlue documentation. With the assay, when certain filters are used one first needs to calculate a correction factor.

Firstly, the correction factor (R_o) was calculated:

$$R_o = AO_{LW}/AO_{HW}$$

Where,

AO_{LW} = absorbance of oxidized form at 540nm

AO_{HW} = absorbance of oxidized form at 620nm

The percentage reduction was then calculated as follows:

$$\% \text{ Reduction} = [A_{540}-(A_{620} \times R_o)] \times 100\%$$

An increase in percentage reduction of alamarBlue correlates to an increase in metabolic activity.

2.9.2 Viability of hFOB 1.19 cell line on exposure to human serum from study subjects

Cells were cultured until confluent (80-90%). A total cell count was carried out as described in section 2.8.9. Cells were re-suspended to obtain 0.5 x 10^6 cells/ml concentration. This concentration was chosen as it was found to be the most efficient cell density for seeding of hFOB 1.19 cell lines as described in section 2.9.1. A 100µl of 0.5 x 10^6 cells/ml concentration was pipetted in triplicate onto a 96 well plate.

3 x 96 well plates were prepared for reading the viability at Day 2, Day 7 and Day 14 incubations. Wells A1 to A9 were not seeded with cells and left blank. The 96 well plates were then wrapped in parafilm to avoid evaporation overnight and incubated at 34°C for 24 hours. After 24 hours, the waste media from each of the wells were removed. 100µl of

serum free media (described in section 2.8.3) was then added in the wells that contained cells (i.e. A10 to H12). This was done to serum deprive the cells and to also remove any excess media that may still be in the wells. The 96 well plate was then wrapped in parafilm and incubated at 34°C for another 24 hours. Following 24 hours of incubation, the serum free media (SFM) was removed from each of the wells and 10% serum from study subjects was added into the wells in triplicates.

100µl of SFM was added into wells A4 to A9. 100µl of normal growth media was added into wells A10 to A12. 10% human serum from the subjects involved in the study was then added into the appropriate wells in triplicates. 10% human serum was made from a dilution method (10% serum from Patient X = 90µl of SFM + 10µl of serum from patient X). To avoid making repeated dilutions, the required amount was made as once off, in this case we needed 900µl for the three 96 well plates, so 810µl of SFM + 90µl of serum was added and 100µl was pipetted from this stock solution into each wells.

All the three 96 well plates were wrapped in parafilm and incubated at 34°C. The day of serum exposure on the hFOB 1.19 cells were taken as day 0 of incubation. After 2 days of incubation, i.e. on Day 2, 10µl of alamarBlue was added into wells A7 to H12 in 96 well plate labeled Day 2. The plate was then incubated for 4 hours at 34°C and the absorbance was read on a spectrophotometer at 540nm and 620nm. The viability of each of the wells was calculated as described in section 2.9.1. A 4 hour incubation period with alamarBlue was chosen as described in section 5.3.1.

On Day 3 of incubation, the media was changed in the remaining two 96 well plates (labeled Day 7 and Day 14) as described above. The media was changed every 3 days for this entire experiment. On Day 7 and Day 14 of incubation respectively, the appropriate

plate's absorbance was read on a spectrophotometer at 540nm and 620nm. The viability of each of the wells was calculated as described in section 2.9.1.

2.9.3 Optimization of the p-Nitrophenyl phosphate solution (pNPP) assay as a functionality assay to detect alkaline phosphatase secretion for hFOB 1.19 cell line

In order for us to use pNPP assay to assess cell functionality, we firstly needed to create a standard curve of hFOB cell growth using the reagent. From this one can assess the most optimal detection time point and cell density number to be used for future experiments to determine functionality levels. For this experiment, a range of cell densities from 0.2 to1x10^6 cells/ml was chosen. The objective of this experiment was to create a standard curve using pNPP of hFOB 1.19 cell line functionality and to determine the optimal detection stage and cell density for the functionality experiments.

Cells were cultured until confluent (80-90%). A total cell count was carried out as described in section 2.8.9. Cells were re-suspended and concentrations from 0.2-1 x 10^6 cells/ml were prepared in tubes. A control was prepared by adding medium only. All tubes were vortexed. A 100µl of each concentration was pipetted in triplicate onto a 96 well plate and incubated at 34°C. Five 96 well plates were prepared to read the amount of ALP secreted by osteoblast cells at Day 1, Day 2, Day 4, Day 7 and Day 10.

The plates were wrapped in parafilm to avoid evaporation overnight. After 24 hours of incubation, plate labeled Day 1 was taken out from the incubator and placed in the tissue culture hood. The media was then removed from the wells that contains cells, and rinsed twice with 100µl of PBS. Following this 100µl of pNPP assay was added into each well and the plate was incubated for 30 minutes in a 37°C incubator and the absorbance was read at 405nm. Absorbance values were entered into an excel spread sheet and averages

were calculated for the each cell density and medium alone. A fresh stock solution of pNPP assay was made at each reading day. For these experiments, at each time of analysis 10mls of pNPP assay were made by adding 2mls of pNPP to 8mls of PBS.

Media was changed and replenished with new media every third day. At Day 2, Day 4, Day 7 and Day 10 incubation, the appropriate plates were read for amount of ALP secretion as described above.

2.9.4 Cell functionality of hFOB 1.19 cell line on exposure to human serum from study subjects using pNPP assay

Cells were cultured until confluent (80-90%). A total cell count was carried out as described in section 2.8.9. Cells were re-suspended to obtain 0.5 x 10^6 cells/ml concentration. This concentration was chosen as it was found to be the most efficient cell density for seeding of hFOB 1.19 cell line as described in section 2.9.3. A 100µl of 0.5 x 10^6 cells/ml concentration was pipetted in triplicate onto a 96 well plate.

3 x 96 well plates were prepared for reading the viability at Day 2, Day 7 and Day 14 incubations. Wells A1 to A9 were not seeded with cells and left blank. The 96 well plates were then wrapped in parafilm to avoid evaporation overnight and incubated at 34°C for 24 hours. After 24 hours, the waste media from each of the wells were removed. 100µl of serum free media (described in section 2.7.3) was then added to the wells that contained cells (i.e. A10 to H12). This was done to serum deprive the cells and to also remove any excess media that may still be in the wells. The 96 well plate was then wrapped in parafilm and incubated at 34°C for another 24 hours. Following 24 hours of incubation, the serum free media (SFM) was removed from each of the wells and 10% serum from study subjects was added into the wells in triplicates.

100µl of SFM was added into wells A4 to A9. 100µl of normal growth media was added into wells A10 to A12. 10% human serum from the subjects involved in the study was then added into the appropriate wells in triplicates. 10% human serum was made by dilution (10% serum from Patient X = 90µl of SFM + 10µl of serum from patient X). To avoid making repeated dilutions, the required amount was made as once off, in this case we needed 900µl for the three 96 well plates, so 810µl of SFM + 90µl of serum were added and 100µl was pipetted from this stock solution into each well.

All the three 96 well plates were wrapped in parafilm and incubated at 34°C. The day of serum exposure on the hFOB 1.19 cells were taken as day 0 of incubation. After 2 days of incubation, i.e. on Day 2, the media was removed from the wells that contained cells in Day 2 plate and rinsed twice with 100µl of PBS. Following this 100µl of pNPP assay was added into each well and the plate was incubated for 30 minutes in a 37°C incubator and the absorbance was read at 405nm as described above in section 2.9.3.

On Day 3 of incubation, the media was changed in the remaining two 96 well plates (labeled Day 7 and Day 14) as described above. The media was changed every 3 days for this entire experiment. On Day 7 and Day 14 of incubation respectively, the appropriate plate's absorbance was read as described above.

2.10 Infliximab in vitro experiments

2.10.1 Infliximab acquisition

Infliximab was obtained from the Department of Pharmacy in AMNCH. Infliximab obtained from the pharmacy in AMNCH is marketed by Centocor B.V. Einsteinweg 101, 2333 CB Leiden, Netherlands in the form of Remicade 100 mg powder. One vial of Remicade 100mg powder for concentrate solution for infusion contains 100 mg of infliximab. After reconstitution each ml contains 10 mg of infliximab. Hence each vial after reconstitution will contain 10mls of infliximab solution which is equivalent to 100mg of infliximab.

The reconstitution methods are as below: Under aseptic conditions (in a tissue culture flow hood), each of the Remicade vial were reconstituted with 10ml of water for injection, using a syringe equipped with a 21-gauge (0.8 mm) needle. The flip-top from the vial was removed and the top of the vial was wiped with a 70% ethanol swab. The syringe needle was then inserted into the vial through the centre of the rubber stopper and the stream of water for injections was directed to the glass wall of the vial. The solution was then gently swirled by rotating the vial to dissolve the lyophilized powder. The reconstituted solution was then allowed to stand for about 5 minutes. Solution was checked to be colourless-to-light yellow and opalescent. Since no preservative is present in the Remicade vial, the solutions of infliximab for use in the experiments were used as soon as possible and within 3 hours of reconstitution. When extra solution was obtained from the reconstitution, the unused portion was stored at 2°C to 8°C and used within 24 hours. Varying concentrations of infliximab were obtained for experiments through

dilution methods. For example to obtain a 2% infliximab concentration in 100µl sample for cell viability and cell functionality experiments, 2µl of reconstituted infliximab was added to 98µl of SFM.

2.10.2 Viability of hFOB 1.19 cell line over 24 hours on exposure to varying concentrations of infliximab

Infliximab solution was prepared as described in section 2.10.1. For this experiment, FBS was used as a control medium and concentration of 0.1%, 0.5%, 1%, 2.5%, 5%, 10%, 25%, 50%, 75% and 100% of infliximab was used. Notably, the range of infliximab used includes the pharmacological range of ~0.5% to ~1% of infliximab obtained in patients post infusion. Cells were cultured until confluent (80-90%). A total cell count was carried out as described in section 2.8.9. Cells were re-suspended to obtain 0.5 x 10^6 cells/ml concentration. A 100µl of 0.5 x 10^6 cells/ml concentration was pipetted in triplicate onto a 96 well plate.

Wells A1 to A9 were not seeded with cells and left blank. The 96 well plates were then wrapped in parafilm to avoid evaporation overnight and incubated at 34°C for 24 hours. After 24 hours of incubation, the media from the wells were removed. SFM was added into wells A4 to A9, a normal complete growth media (which contains 10% FBS) was added into wells A10 to A12. The following remaining wells were added with the appropriate infliximab concentration as mentioned above. As previously, described already, the appropriate infliximab concentrations were obtained by dilution. For example to obtain 0.1% infliximab; 0.1µl of infliximab was added to 99.9µl of SFM, and for 5% infliximab; 5µl of infliximab was added to 95µl of SFM and so on. Following this 10µl of alamarBlue was added into the wells from A4 to D6 and the absorbance was

subsequently read at 30, 60, 90, 120, 150, 180, 240, 300, 360, 420, 480, 540 ,600 and 1440 minutes in a spectrophotometer at 540nm and 620nm. The average readings were then calculated as described previously.

2.10.3 Viability of hFOB 1.19 cell line over 10 days on exposure to varying concentrations of infliximab

The purpose of this experiment was to assess any change in cell viability amongst different cell populations differing in infliximab concentrations. Infliximab solution was prepared as described in section 2.10.1. For this experiment, H_20 (water), PBS and 10% FBS was used as a control medium and concentration of 1%, 5%, 10%, 25%, and 50%, infliximab was used. Higher concentrations were not chosen as there was no significant difference noted in the effects of infliximab on human osteoblasts above a concentration of 50% (as shown in 5.3.3). Notably, the range of infliximab used includes the pharmacological range of 1% of infliximab obtained in patients post infusion. Cells were cultured until confluent (80-90%). A total cell count was carried out as described in section 2.8.9. Cells were re-suspended to obtain 0.5 x 10^6 cells/ml concentration. A 100µl of 0.5 x 10^6 cells/ml concentration was pipetted in triplicate onto a 96 well plate.

5 x 96 well plates were prepared for reading the viability at Day 1, Day 2, Day 4, Day 7 and Day 10 incubations. Wells A1 to A9 were not seeded with cells and left blank. The 96 well plates were then wrapped in parafilm to avoid evaporation overnight and incubated at 34°C for 24 hours. After 24 hours of incubation, the media from the wells were removed. H_20 was added into wells A10 to A12, PBS was added into wells B1 to B3, a normal complete growth media (which contains 10% FBS) was added into wells B4 to B6. The following remaining wells were added with the appropriate infliximab

concentration as mentioned above. As previously, described already, the appropriate infliximab concentrations were obtained by dilution. The day of infliximab exposure on the hFOB 1.19 cells were taken as day 0 of incubation. After 1 day of incubation, i.e. on Day 1, 10µl of alamarBlue was added into wells A7 to H12 in 96 well plate labeled Day 1. The plate was then incubated for 4 hours at 34°C and the absorbance was read on a spectrophotometer at 540nm and 620nm. The viability of each of the wells was calculated as described in section 2.9.1. A 4 hour incubation period with alamarBlue was chosen as described in section 5.3.1. Similarly, after 2 days of incubation with infliximab, the plate labeled Day 2 was subjected to the same standard alamarBlue assay.

On Day 3 of incubation, the media were changed in the remaining three 96 well plates (labeled Day 4, Day 7 and Day 10) as described above. The media was changed every 3 days for this entire experiment. On Day 4, Day 7 and Day 10 of incubation respectively, the appropriate plate's absorbance was read on a spectrophotometer at 540nm and 620nm. The viability of each of the wells was calculated as described in section 2.9.1.

2.10.4 Cell functionality of hFOB 1.19 cell line on exposure to varying concentrations of infliximab using pNPP assay

The purpose of this experiment was to assess any change in cell functionality amongst different cell populations exposed to different infliximab concentrations. Infliximab solution was prepared as described in section 2.10.1. For this experiment, H_20 (water), PBS and 10% FBS was used as a control medium and concentrations of 1%, 5%, 10%, 25%, and 50%, infliximab were used as described in section 2.10.3. This includes the pharmacological range of 1% of infliximab obtained in patients post infusion as mentioned previously. Cells were cultured until confluent (80-90%). A total cell count

was carried out as described in section 2.7.9. Cells were re-suspended to obtain 0.5 x 10^6 cells/ml concentration. A 100µl of 0.5 x 10^6 cells/ml concentration was pipetted in triplicate onto a 96 well plate.

5 x 96 well plates were prepared for reading the viability at Day 1, Day 2, Day 4, Day 7 and Day 10 incubations. Wells A1 to A9 were not seeded with cells and left blank. The 96 well plates were then wrapped in parafilm to avoid evaporation overnight and incubated at 34°C for 24 hours. After 24 hours of incubation, the media from the wells were removed. H_20 was added into wells A10 to A12, PBS was added into wells B1 to B3, a normal complete growth media (which contains 10% FBS) was added into wells B4 to B6. The following remaining wells were added with the appropriate infliximab concentration as mentioned above. As previously described, the appropriate infliximab concentrations were obtained by dilution. The day of infliximab exposure on the hFOB 1.19 cells were taken as day 0 of incubation. After 1 day of incubation, i.e. on Day 1, the media was removed from the wells that contains cells in Day 1 plate and rinsed twice with 100µl of PBS. Following this 100µl of pNPP assay was added into each well and the plate was incubated for 30 minutes in a 37°C incubator and the absorbance was read at 405nm as described in section 2.9.3. Similarly, after 2 days of incubation, the plate labeled Day 2 was read. On Day 3 of incubation, the media was changed in the remaining three 96 well plates (labeled Day 4, Day 7 and Day 10) as described above. The media was changed every 3 days for this entire experiment. On Day 4, Day 7 and Day 10 of incubation respectively, the appropriate plate's absorbance was read on a spectrophotometer at 405nm.

2.11 Statistical analysis

For statistical analysis of results obtained at baseline compared to post treatment with adalimumab and infliximab at the time intervals mentioned above (1, 3 and 6 months for adalimumab, and 6, 30 and 54 weeks for infliximab), a paired Student's t-test was used for parametric data and a Wilcoxon Signed Ranks test was used for non-parametric data. For comparison of control results with baseline results for patients treated with either adalimumab or infliximab, an independent Student's t-test was used for analysis of parametric data and a Mann-Whitney test was used for non-parametric data. A p value of < 0.05 was considered significant. The results are presented as mean +/- SD.

CHAPTER 3 – LONG TERM EFFECT OF INFLIXIMAB ON BONE METABOLISM IN INFLAMMATORY BOWEL DISEASE PATIENTS

3.1 INTRODUCTION

To date, data describing the effects of *in vivo* TNF-α inhibition on general bone metabolism in patients with IBD are still limited and restricted to infliximab treatment in CD patients [262-265]. These studies have shown that infliximab appears to have a beneficial effect on bone metabolism in CD patients. The beneficial effects appear to be primarily due to an increase in bone formation, while reduction in bone resorption seems to play a lesser role [263,264]. These effects seem to be independent of whether the patients are classified as responders or non responders to infliximab treatment based on clinical scores, and of whether the patients are on steroid therapy [262,264]. Patients who received infliximab have also been shown to have increased BMD based on DXA findings [266-268]. It's likely that several different mechanisms play a role in this positive effect of infliximab on bone metabolism.

Currently available studies looking at the effects of anti-TNF-α therapy on bone metabolism in IBD patients have only evaluated the role of infliximab on bone metabolism in CD patients. No studies have yet evaluated the effects of infliximab on bone metabolism in UC patients. Furthermore, currently available studies have only been carried out for short term, with the longest being for 14 weeks [263]. Hence the long term effect of maintenance therapy with anti-TNF-α on bone metabolism in IBD patients is not known. Moreover, the effects of these therapies on bone cells have not yet been studied. This is crucial to understanding the direct effect of anti-TNF-α on bone cells. Long term

studies are also needed to compare changes in cytokines, osteoclastogenesis markers (OPG and RANKL), bone nutrients (PTH and vitamin D) and most importantly on BMD to fully understand the mechanisms involved.

3.2 AIMS

1. To compare BMD measurements between IBD patients with that of healthy controls, and to see if infliximab therapy improves BMD at 1 year.
2. To measure serum bone turnover markers between IBD patients with that of healthy controls. In addition, to evaluate the effects infliximab therapy on bone turnover markers.
3. To measure serum circulating pro-inflammatory, anti-inflammatory, and osteoclastogenesis markers between IBD patients with that of healthy controls, and to evaluate the effects of infliximab therapy on these parameters.
4. To compare the levels of PTH and Vitamin D in IBD patients and healthy controls, and to see if plasma PTH and Vitamin D levels alter with infliximab therapy in IBD patients.
5. To evaluate and compare the effect of sera from IBD patients treated with infliximab on human osteoblasts *in vitro* model in regards to cell viability/proliferation and differentiation/functionality with that of healthy controls.

Therefore, our aim was to explore the effects of long term infliximab treatment on bone metabolism in both active CD and UC patients by measuring the effects of maintenance infliximab therapy on biochemical markers of bone turnover, bone nutrients,

pro-inflammatory cytokines, anti-inflammatory cytokines, osteoclastogenesis markers and the effect of sera of treated patients on human osteoblast compared to healthy subjects.

3.3 RESULTS

3.3.1 Effect of infliximab treatment on disease activity

As previously described in detail in section 2.1, briefly thirty-seven IBD patients; 20 CD and 17 UC who were treated with infliximab and 20 healthy controls were included in this study. Time points measured for infliximab treated IBD patients were at baseline, 6 weeks, 30 weeks and 54 weeks. Number of IBD patient's included at different time points were 37 patients at baseline, 37 patients at week 6, 19 patients at week 30 and 11 patients at week 54.

The mean disease activity in CD patients measured by CDAI score appreciably decreased from 257.95 at baseline to 126.48 after 6 weeks, 43.00 after 30 weeks and 25.88 after 54 weeks ($p < 0.001$, $p=0.003$ and $p=0.012$) respectively **(Figure 3.1)**. In UC patients, mean disease activity measured by SCS also significantly decreased over time from 9.00 at baseline to 2.00 after 6 weeks, 0.33 after 30 weeks and 0.00 after 54 weeks ($p<0.001$, $p<0.001$ and $p=0.012$) respectively **(Figure 3.2)**. To investigate any relationship between disease activity and change in bone metabolism markers, CD patients were stratified into those with a fall in CDAI of >100 points (responders, n= 11) and those with <100 points fall (non responders, n=9) at week 6. Similarly in UC cohort,

patients were stratified in those with a fall in SCS of >6 points (responders, n=13) and those with <6 points fall (non responders, n=4) at week 6.

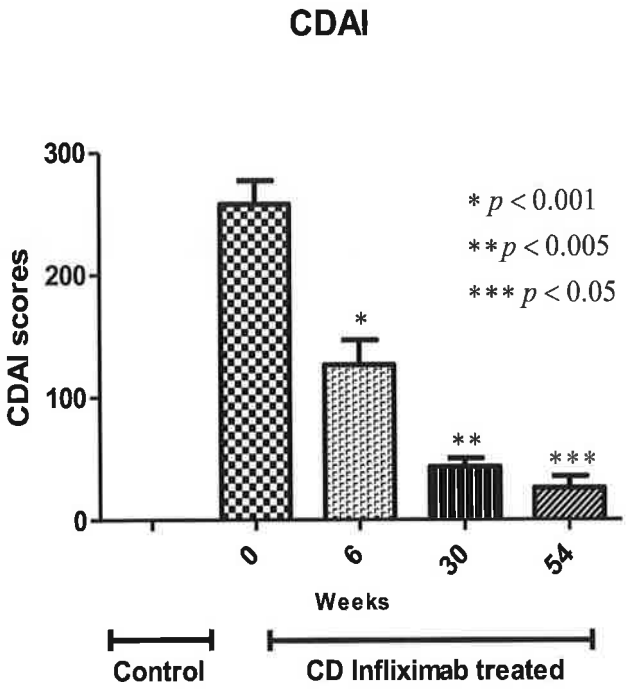


Figure 3.1: CDAI scores in control and CD patients

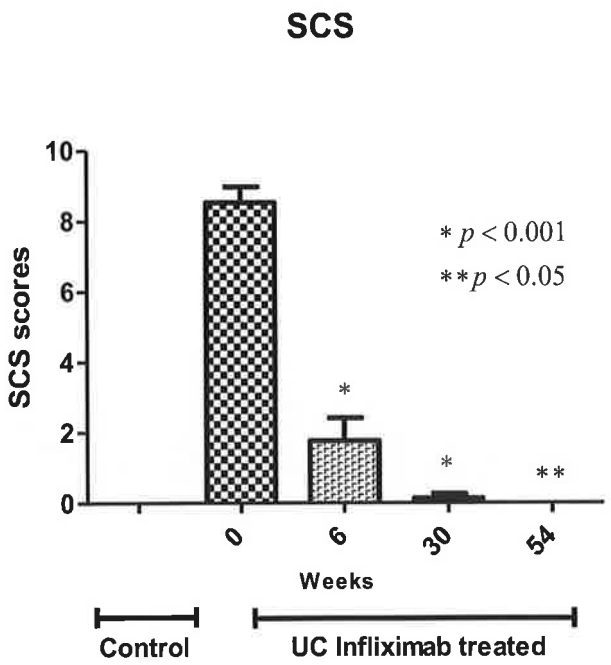


Figure 3.2: SCS scores in control and UC patients

3.3.2 Effect of infliximab treatment on C-reactive protein (CRP)

Mean CRP (mg/L) at baseline was 19.69 and fell significantly following infliximab therapy at all time points measured (p=0.006 at week 6, p=0.007 at week 30 and p=0.047 at week 54). Mean CRP was also significantly higher at baseline in IBD patients compared to controls (p=0.001) **(Figure 3.3)**. Interestingly, this significant difference was only noted in patients who were stratified as responders **(Figure 3.4)**. UC and CD patients showed similar decrease in CRP with infliximab over the time point measured.

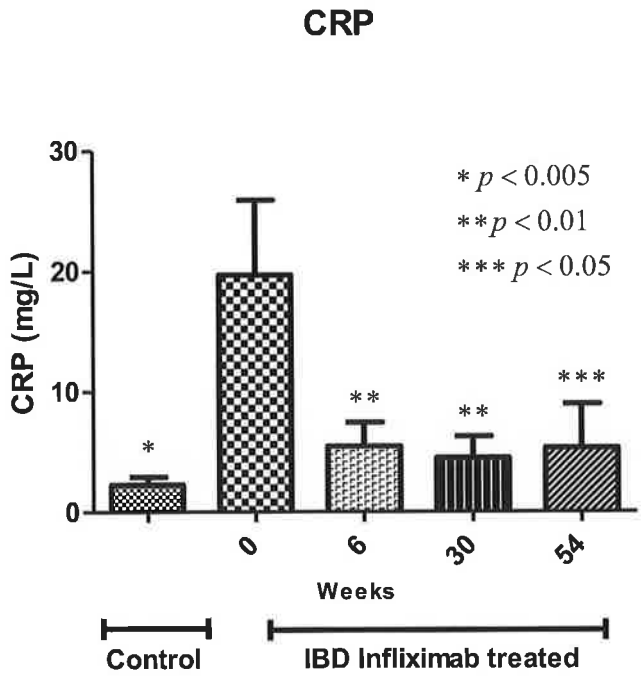


Figure 3.3: CRP levels in control and IBD patients

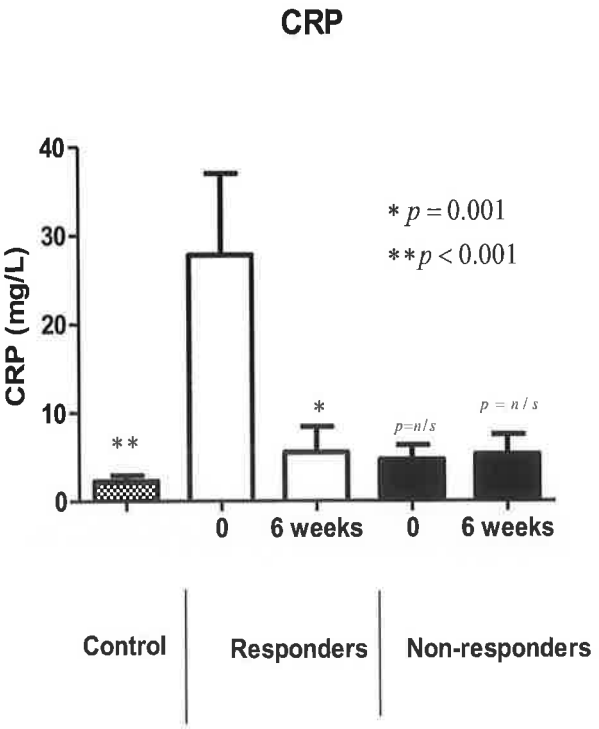


Figure 3.4: CRP levels in IBD responders and non-responders

3.3.3 Effect of infliximab treatment on corticosteroid dosage

As expected, the mean daily dosage of steroid taken by IBD patients at week 6 fell significantly ($p<0.001$) following infliximab treatment and this remained significantly lower than pre-treatment levels even at week 54 ($p=0.018$) **(Figure 3.5)**. There was no significant differences in change in the mean dosage of steroids between the groups noted (responders versus non responders and CD patients versus UC patients). All groups had significant decrease in steroid dosage following treatment with infliximab.

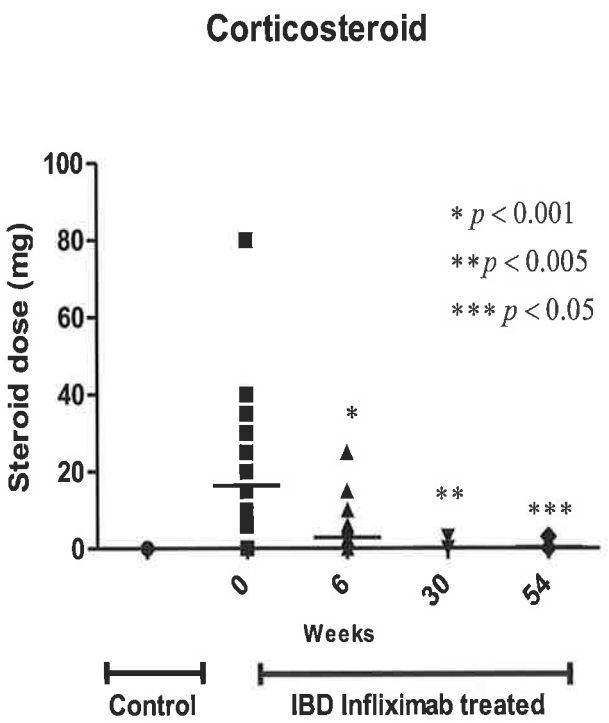
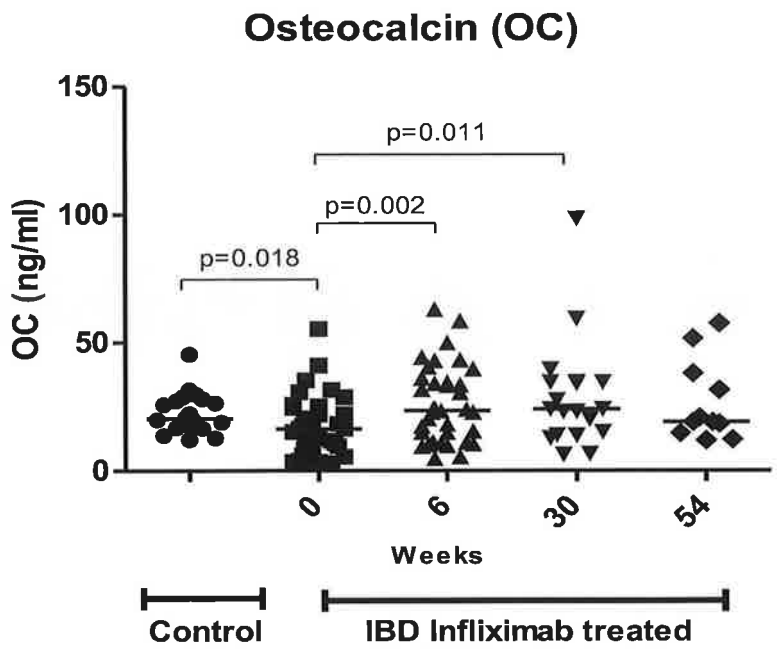


Figure 3.5: Corticosteroid dosage in control and IBD patients

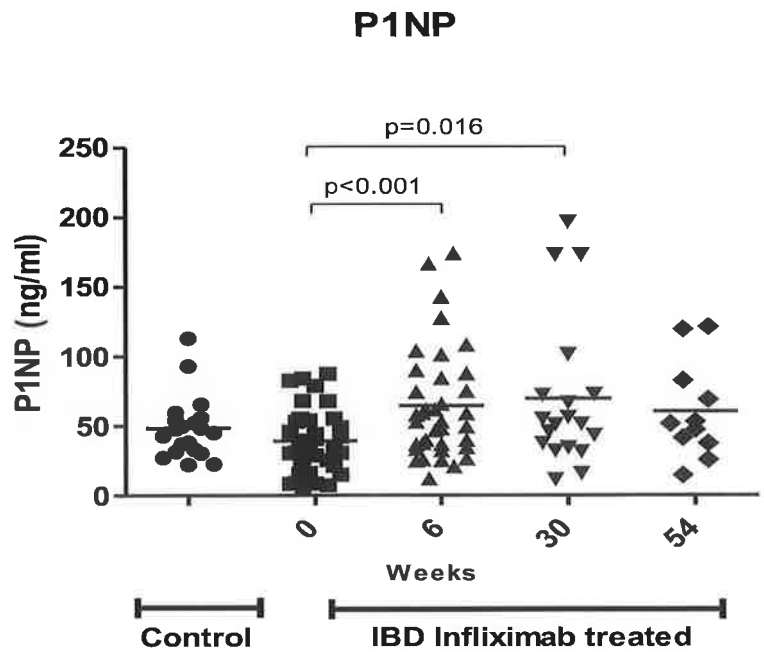
3.3.4 Effect of infliximab treatment on bone markers

Serum concentrations of OC were significantly higher in controls compared to IBD patients at baseline (p=0.018) **(Figure 3.6A)**. Although P1NP levels were higher and serum CTx levels were lower in controls compared to IBD patients at baseline, this difference did not reach statistical significances. The markers of bone formation, OC and P1NP (ng/ml) were significantly increased at week 6 (p=0.002 and p<0.001 respectively) and at week 30 (p=0.011 and p=0.016 respectively) compared to baseline, but not at week 54 **(Figure 3.6A and B)**. Fewer patients were available for analysis at 54 weeks and this might explain in part, the lack of statistical significance at this time point. The bone resorption marker, serum CTx (ng/ml) decreased from baseline (0.462) to (0.321) at 54 weeks, but this decrease was not statistically significant **(Figure 3.6C)**. Although both responders and non responders showed similar trend of increase in bone formation markers at week 6, the change was only significant in the responders **(Figure 3.7A and B)**. It would have been interesting to see whether this change remains at week 30 and 54 between responders and non responders; but patients who were classified as non responders at week 6 were not continued on with infliximab therapy. There was no significant difference in change in the bone turnover markers noted between the CD and UC patients.

A


Osteocalcin (OC)
OC (ng/ml)
150
100
50
0
p=0.011
p=0.002
p=0.018
0
6
30
54
Weeks
Control
IBD Infliximab treated

B


P1NP
P1NP (ng/ml)
250
200
150
100
50
0
p=0.016
p<0.001
0
6
30
54
Weeks
Control
IBD Infliximab treated

C

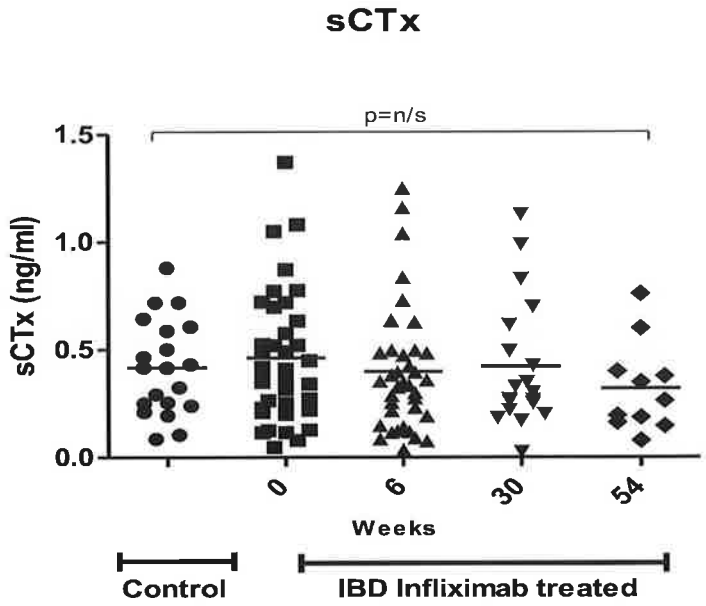
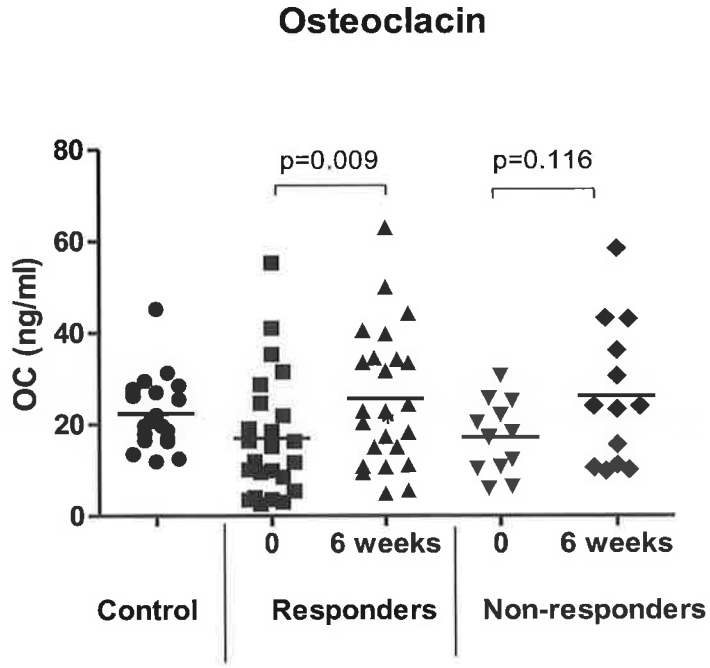


Figure 3.6 (A-C): Changes in mean OC, P1NP and CTx serum levels during treatment with infliximab.

A



B

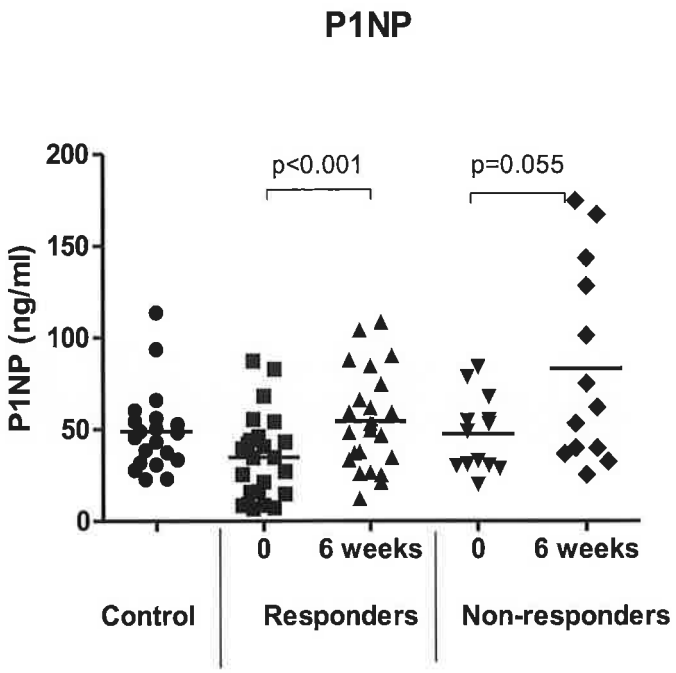
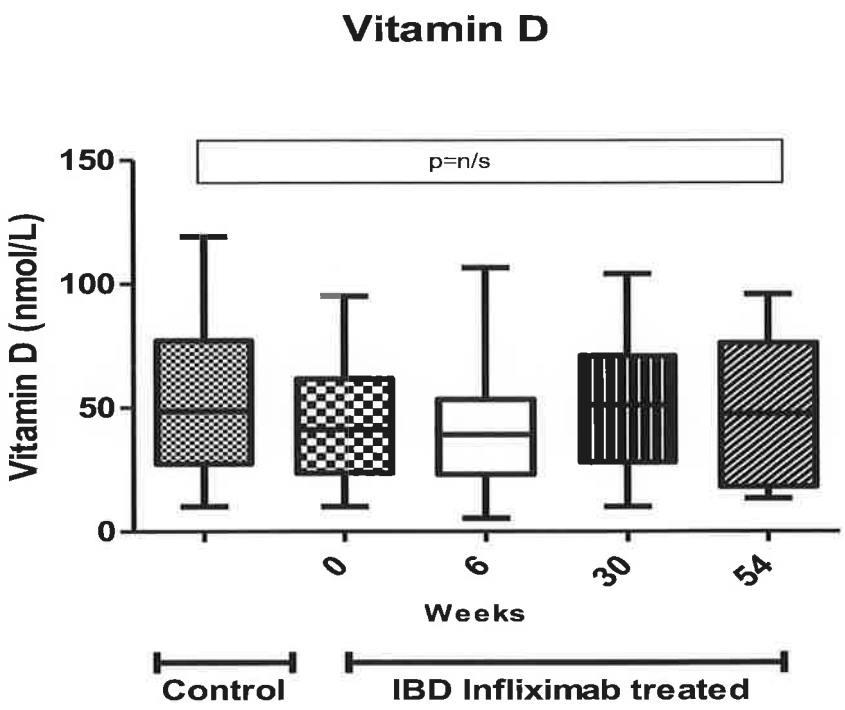


Figure 3.7 (A-B): Mean change in OC and PINP between IBD infliximab treated responders and non responders

3.3.5 Effect of infliximab treatment on serum bone nutrients levels

At baseline IBD patients had lower Vitamin D levels compared to controls (42.87 nmol/L compared to 53.45 nmol/L; p=not significant) and higher PTH levels compared to controls (38.12 pg/ml compared to 24.85 pg/ml, p=0.025). With infliximab treatment both Vitamin D and PTH levels showed no significant changes over time **(Figure 3.8A and B)**. No significant difference in response was noted for both Vitamin D and PTH between responders and non responders and between UC and CD patients.

A



B

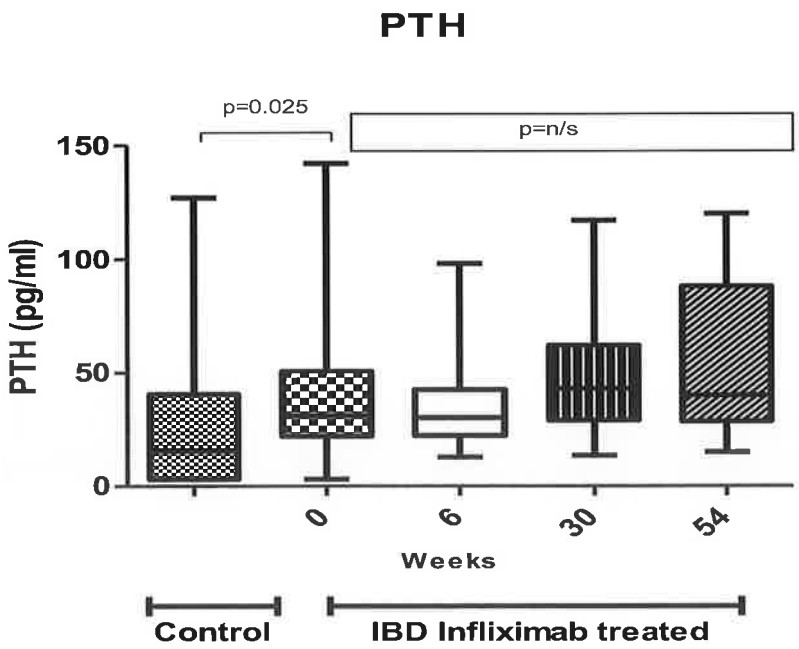
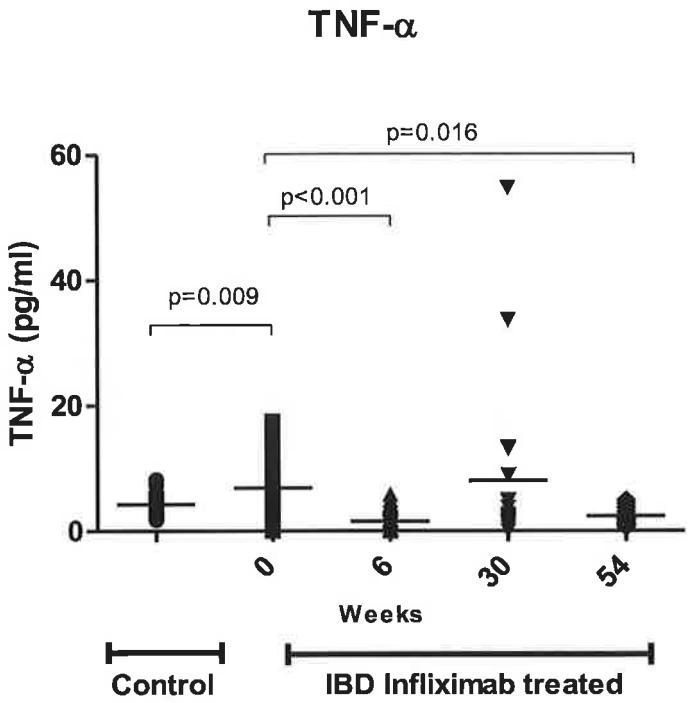


Figure 3.8 (A-B): Changes in median Vitamin D and PTH levels during treatment with infliximab

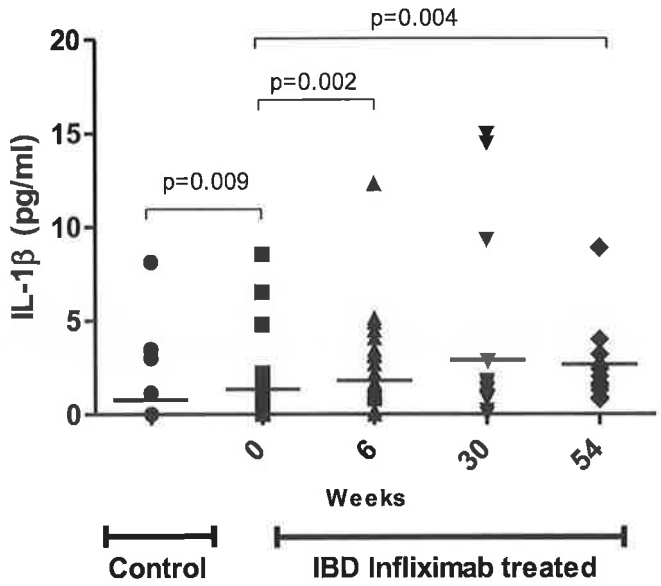
3.3.6 Effect of infliximab treatment on serum cytokines

The mean TNF-α levels in IBD patients decreased from 6.88 pg/ml at baseline to 2.39pg/ml at week 54 (p=0.016) **(Figure 3.9A)**. Pre-treatment, IBD patients had also higher TNF-α levels compared to controls (p=0.009) **(Figure 3.9A)**. Interestingly, IL-1β measured in IBD patients showed a slight but significant increase from 1.35 pg/ml at baseline to 2.66 pg/ml at week 54 (p=0.004) **(Figure 3.9B)**. Again, pre-treatment levels were higher compared to controls (p=0.009) **(Figure 3.9B)**. Mean levels of IL-10 were also noted to be significantly higher in the IBD group pre-treatment compared to controls (p<0.001) but there was no significant change noted over time with infliximab therapy **(Figure 3.9C)**. Both mean IL-6 and IL-13 showed a significant decrease in IBD treated patients with infliximab at week 6 (p=0.005 and p=0.025) respectively **(Figure 3.9D and E)**. These changes were not sustained at the later time points measured in the study **(Figure 3.9D and E)**.

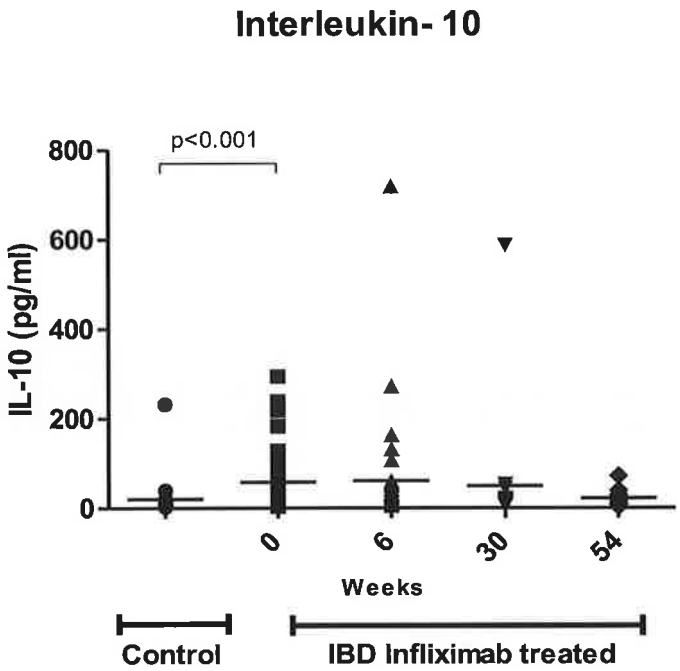
A


TNF-α
p=0.016
p<0.001
p=0.009
60
40
20
0
TNF-α (pg/ml)
0
6
30
54
Weeks
Control
IBD Infliximab treated

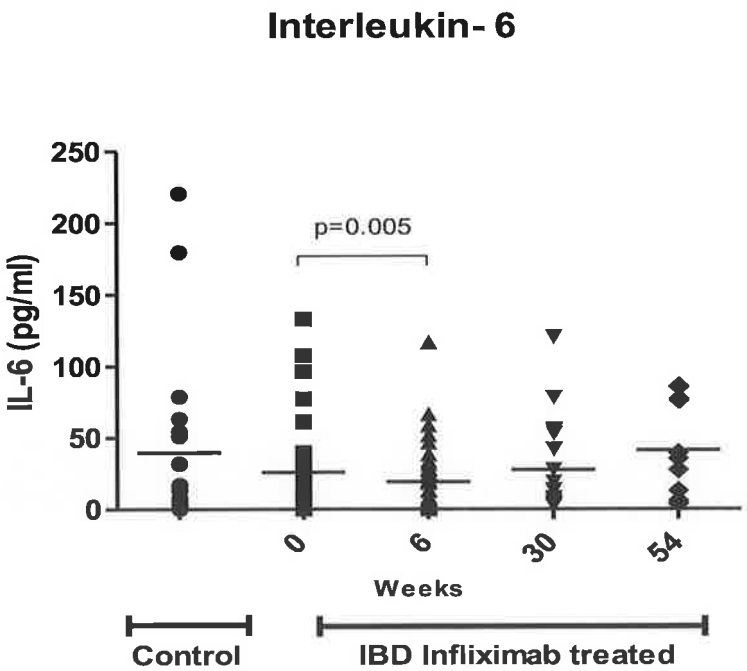
B


Interleukin- 1β
p=0.004
p=0.002
p=0.009
20
15
10
5
0
IL-1β (pg/ml)
0
6
30
54
Weeks
Control
IBD Infliximab treated

C


Interleukin- 10
p<0.001
800
600
400
200
0
IL-10 (pg/ml)
0
6
30
54
Weeks
Control
IBD Infliximab treated

D


Interleukin- 6
250
200
150
100
50
0
IL-6 (pg/ml)
p=0.005
0
6
30
54
Weeks
Control
IBD Infliximab treated

E

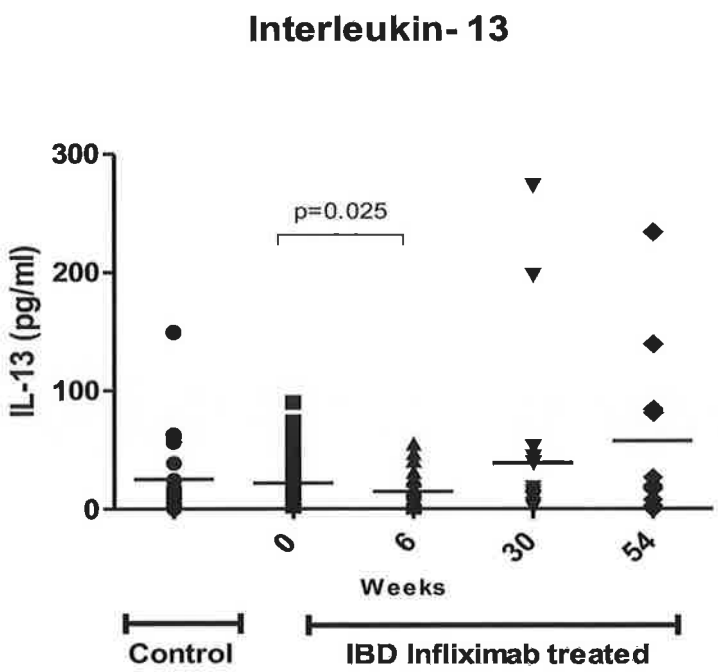
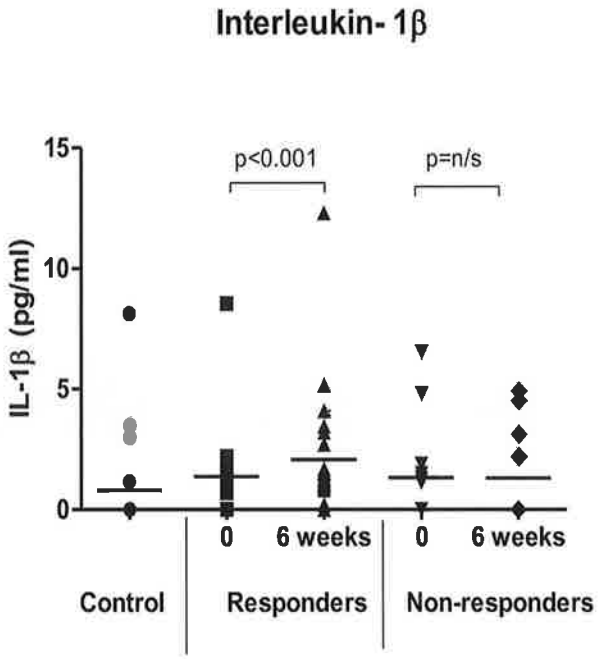


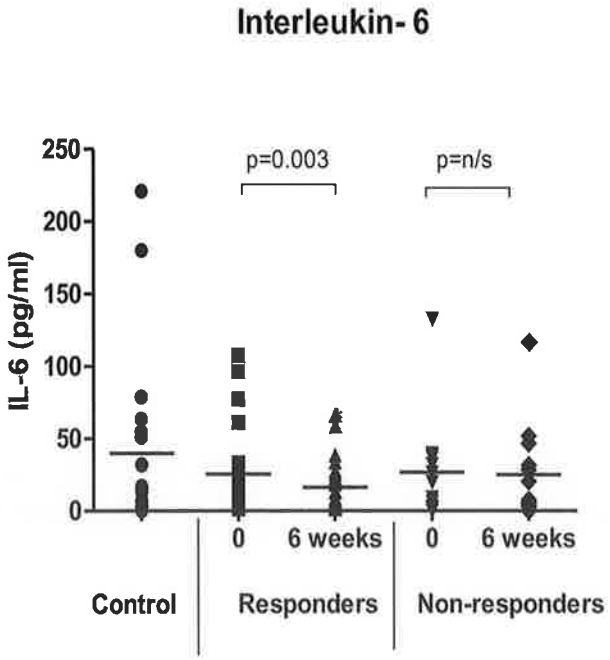
Figure 3.9 (A-E): Mean change in serum cytokines in control and IBD infliximab treated patients

Interestingly, the significant changes seen at week 6 in IL-1β, IL-6 and IL-13 were only seen in the responders **(Figure 3.10 A-C)**. There was no significant difference seen in the mean change in TNF-α between responders and non responders **(Figure 3.10 D)**. There was no significant difference in mean change in all of the cytokines measured between CD and UC patients (data not shown).

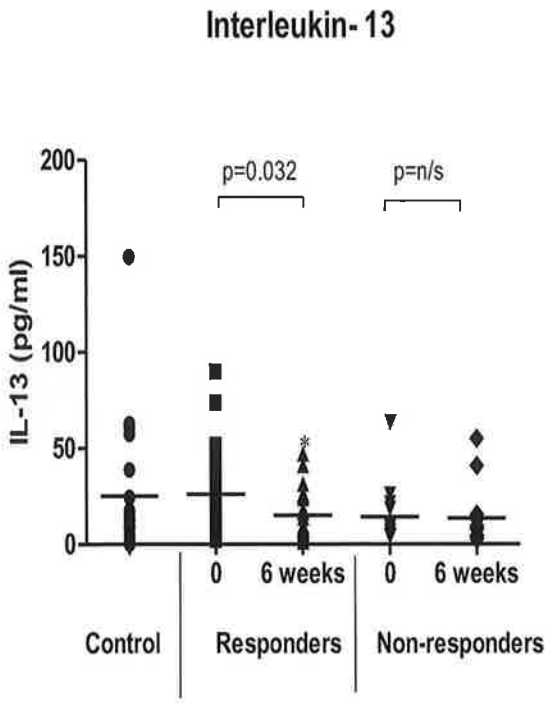
A


Interleukin- 1β
IL-1β (pg/ml)
15
10
5
0
p<0.001
p=n/s
0
6 weeks
0
6 weeks
Control
Responders
Non-responders

B


Interleukin- 6
IL-6 (pg/ml)
250
200
150
100
50
0
p=0.003
p=n/s
0
6 weeks
0
6 weeks
Control
Responders
Non-responders

C



D

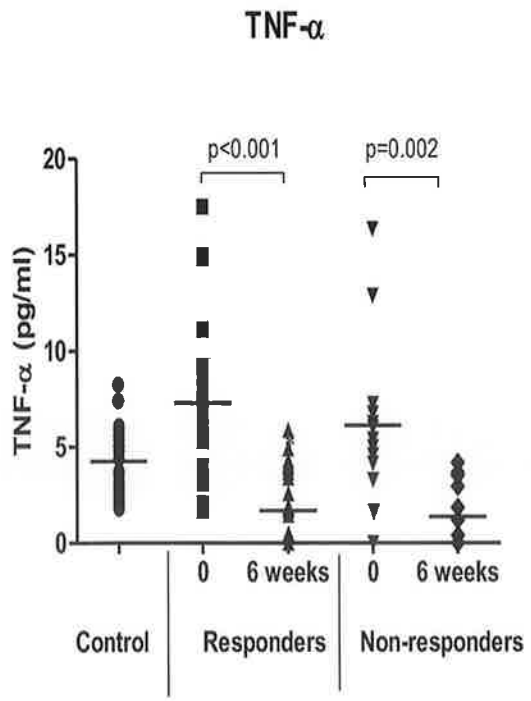
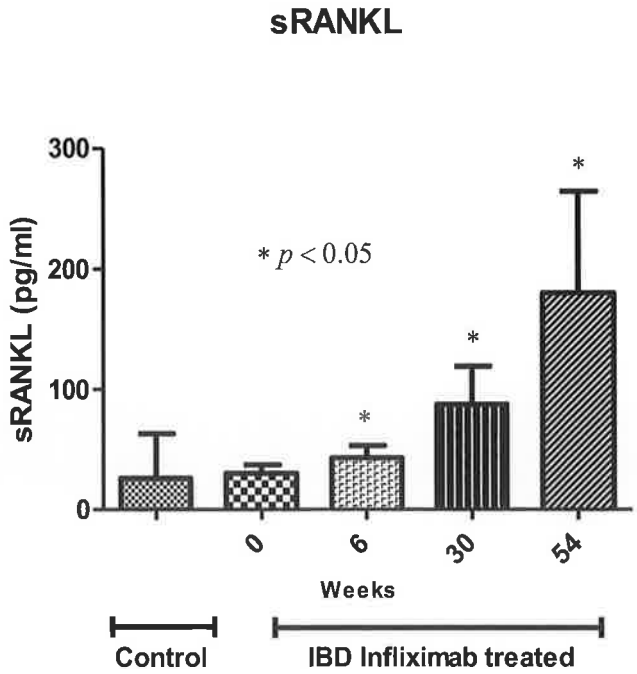


Figure 3.10 (A-D): Mean change in cytokines between responders and non responders

3.3.7 Effect of infliximab treatment on osteoclastogenesis markers

At baseline, both OPG and sRANKL levels were higher in IBD patients compared to controls, although this was not statistical significant. With infliximab therapy, interestingly there was a significant increase in sRANKL (p=0.016 at week 54) **(Figure 3.11A)** compared to baseline. Although, the mean OPG decreased with infliximab therapy to correspond with the increase seen in sRANKL, this was not statistically significant **(Figure 3.11B)**. Again, the significant change seen in sRANKL was only noted in responders **(Figure 3.11C)**. Also, interestingly the mean sRANKL changes seen in UC patients were not significant (data not shown).

A



B

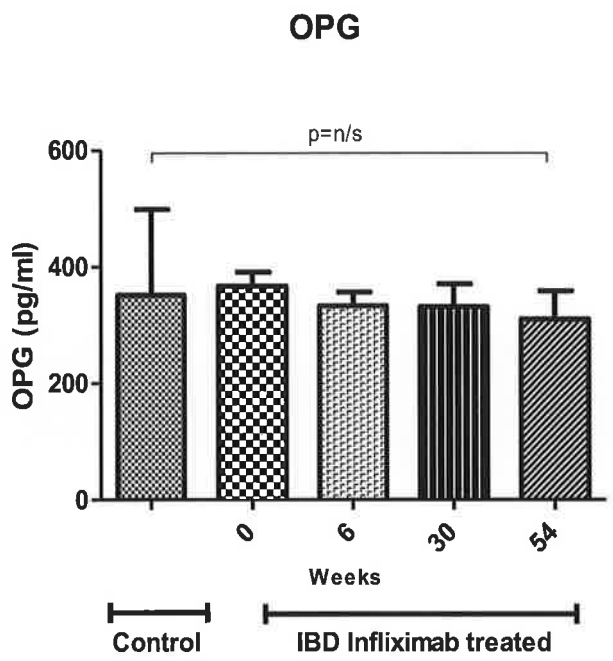


Figure 3.11 (A-B): Mean change in osteoclastogenesis markers in control and IBD infliximab treated patients

C

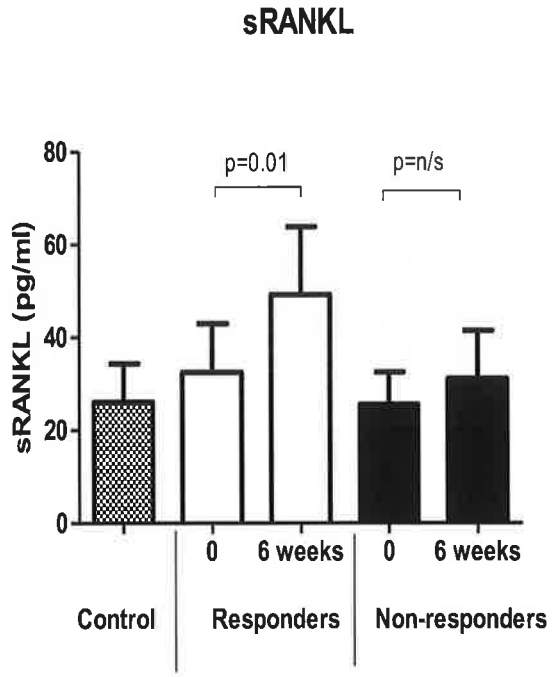


Figure 3.11C: Mean change in sRANKL between responders and non responders

3.3.8 Effect of serum from patients treated with infliximab on human osteoblast cell viability

Exposure of human FOB cells to IBD sera pre and post infliximab treatment, showed that post treatment sera reduced in vitro hFOB viability at all time points measured and this reached significance at Day 7 incubation of week 6 to week 54 ($p=0.023$, $p=0.003$ and $p=0.004$) respectively and at Day 14 of incubation of week 6 to week 54 ($p=0.014$, $p<0.001$ and $p=0.003$) respectively. Interestingly, at all time points measured pre treatment IBD sera were associated with increased viable osteoblast cell number compared to controls ($p=0.001$ at Day 2 and $p<0.001$ at Day 7 and Day 14), with infliximab treatment resulting in reduction in viability back towards control levels

(Figure 3.12). There was no significant difference in change noted in cell viability between responders and non responders and between UC and CD patients (data not shown).

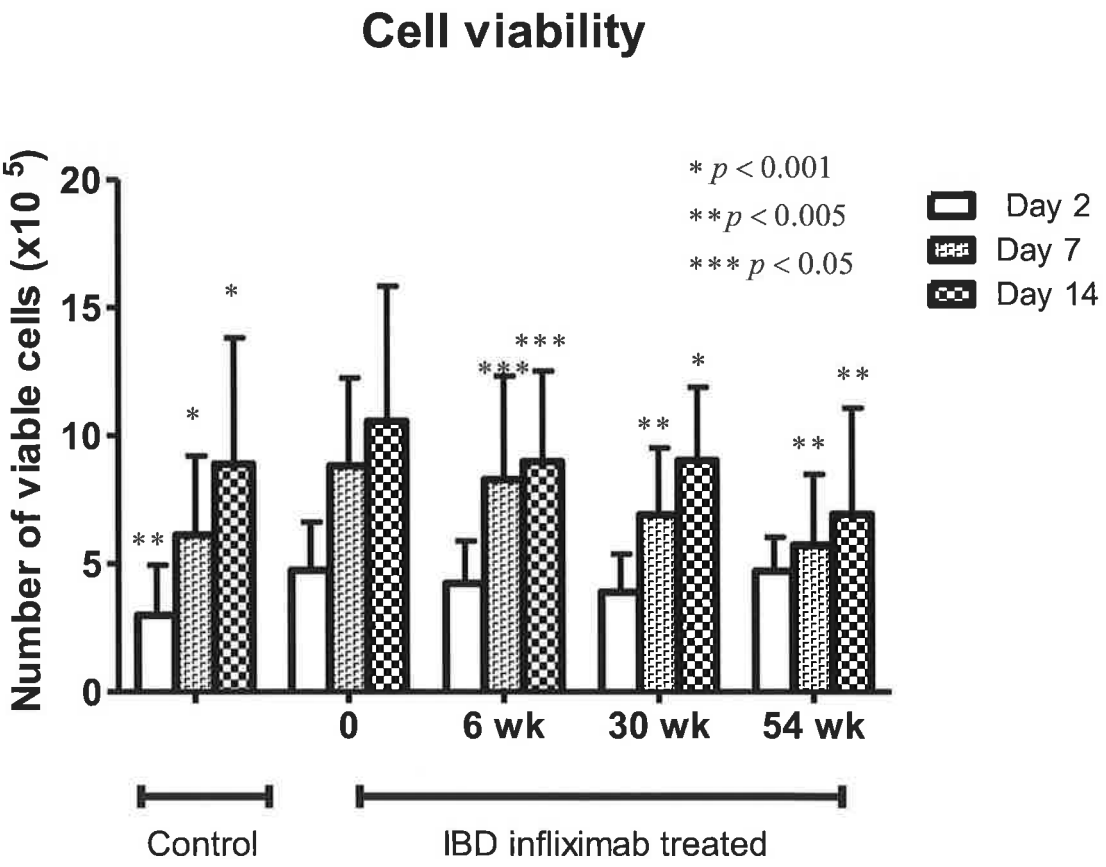


Figure 3.12: Number of viable hFOB cells on exposure to sera from infliximab IBD treated and control patients over 14 days

//*** p value in all cases were obtained by comparing the particular readings to readings at week 0*

3.3.9 Effect of serum from patients treated with infliximab on human osteoblast cell functionality

Osteoblasts exposed to control sera secreted higher levels of ALP than those exposed to pre-treatment baseline IBD sera ($p<0.001$ at Day 2 incubation) **(Figure 3.13)**. Post infliximab treatment sera resulted in higher levels of ALP secretion from osteoblasts than pre treatment baseline sera and this reached significance at week 54 of Day 2 incubation ($p=0.041$). Although ALP secreted by hFOB cells at week 54 were noted to be higher than pre treatment baseline levels on Day 7 and Day 14 incubation as well, this change was not significant **(Figure 3.13)**. There was no significant change noted in the level of ALP secreted by hFOB cells between responders and non responders and between UC and CD patients (data not shown).

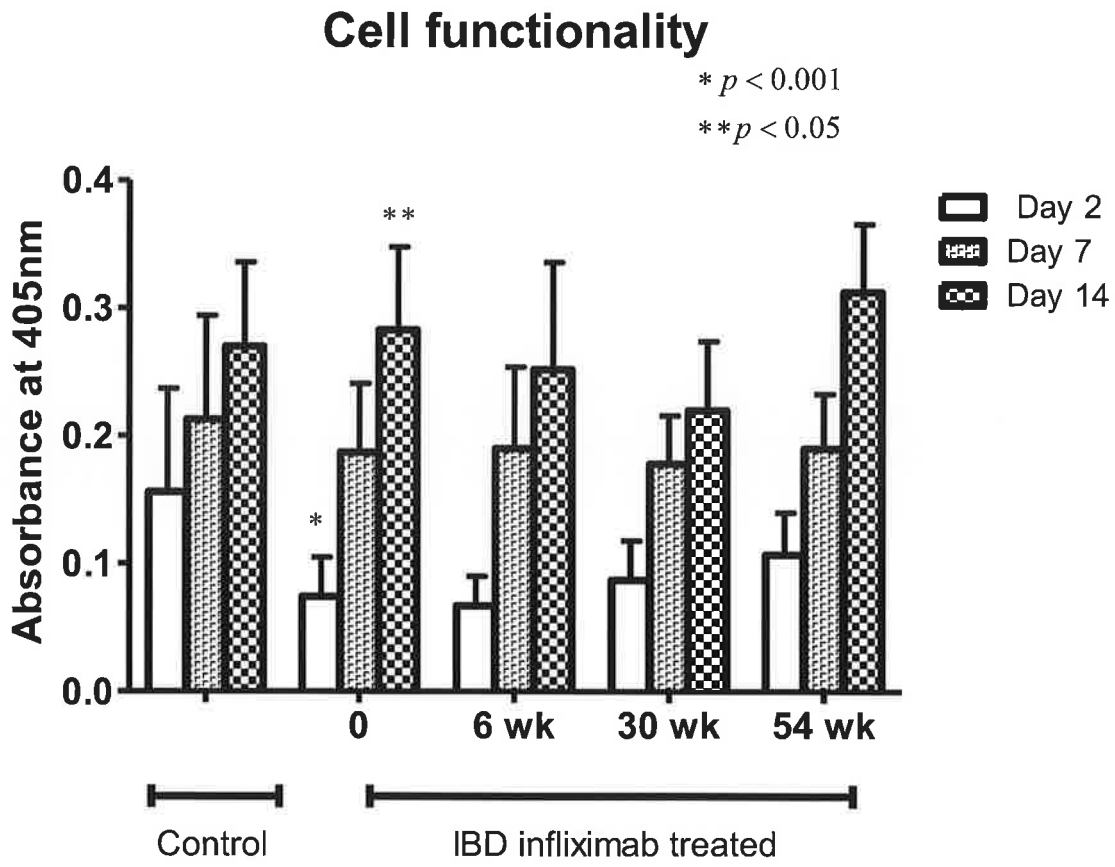


Figure 3.13: The amount of ALP secreted by hFOB cells on exposure to sera from infliximab IBD treated and control patients over 14 days

** p value denotes comparison between control and week 0 at Day 2*
*** p value denotes comparison between week 0 and week 54 at Day 2*

3.3.10 Bone mineral density in active IBD patients compared to controls

Six of 37 IBD patients had osteoporosis (T score ≤ 2.5) and a further 19 patients had osteopenia (T score -1.0 to -2.5) prior to infliximab treatment. No significant differences were noted between CD and UC patients at baseline. In control patients two of 20 patients had osteoporosis and a further 11 patients had osteopenia **(Figure 3.14)**. The two control patients who had osteoporosis were both males and were ex-smokers of 20 and 6 pack years respectively. 11 IBD patients (8 CD and 3 UC) who completed the 1 year therapy with infliximab showed various levels of improvement in their T scores compared to baseline. 4 patients (3 CD and 1 UC) had significant improvement in their T scores at 1 year, 6 patients had no change in their T scores over time and only 1 patient was noted to have documented disimprovement in his T score. However overall mean change noted just failed to reach a statistical significance (p-0.067) **(Figure 3.15)**.

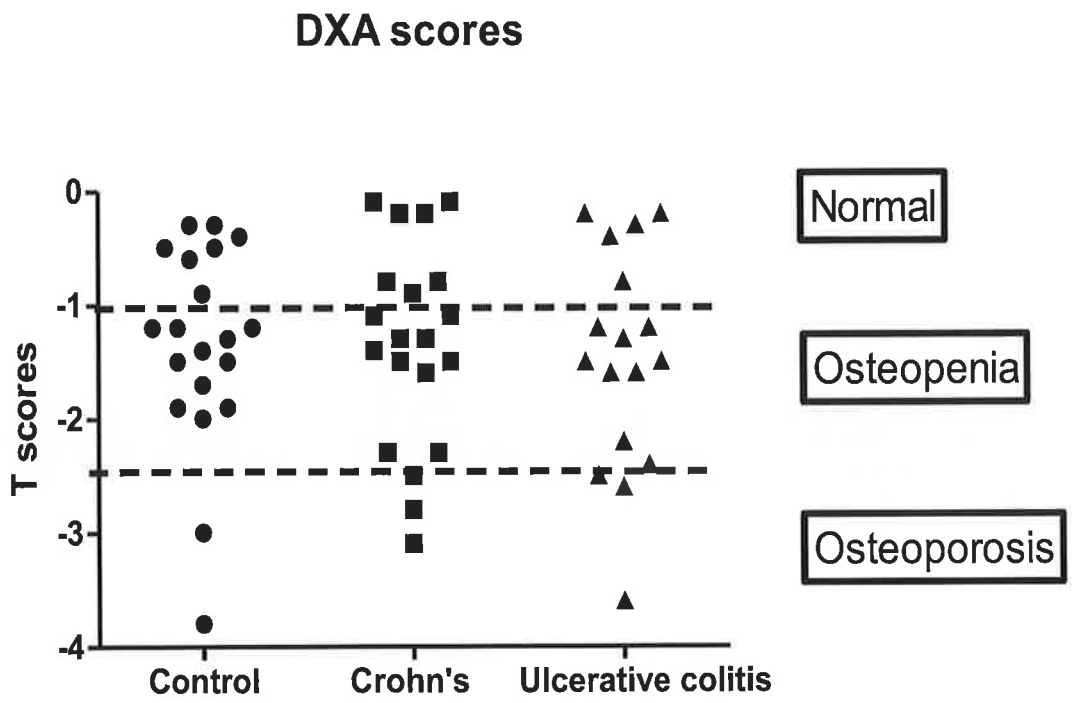


Figure 3.14: T score at baseline in control and IBD patients

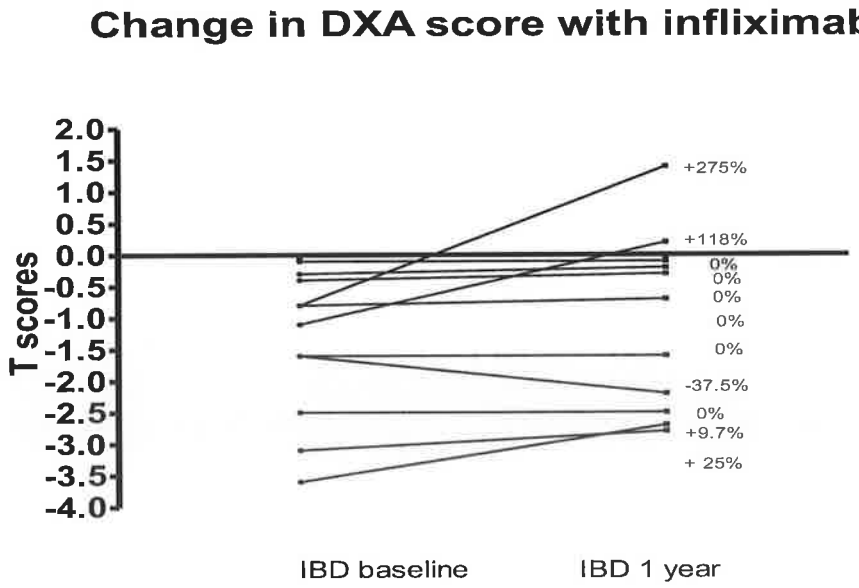


Figure 3.15: Change in T score between baseline and at 1 year following infliximab therapy in IBD patients

3.4 Discussion

In this study, we show that infliximab treatment had a favorable effect on bone metabolism in both active CD and UC patients. There was a significant and rapid increase in OC and P1NP markers of bone formation, and a decrease, albeit non significant, in serum CTx, a marker of bone resorption. Our results are comparable to those found in previous shorter term infliximab studies on bone turnover markers in active CD patients [263,264]. These favorable effects were not associated with baseline DXA findings, current steroid therapy, steroid weaning or clinical characteristics of the patients. However, a greater significant increase in OC and P1NP was observed in responders compared with non responders at week 6, suggesting that, the greater the biological response to infliximab, one can expect a higher beneficial effect on bone formation markers. Although one would have expected improvement in bone nutrients with infliximab therapy secondary to increase in absorption of the critical nutrients required for bone modeling, our study failed to show any significant effect, suggesting that the improvement seen in the bone markers are not due to increase in bone nutrients availability. Interestingly, even our control patients had a below normal Vitamin D levels (defined as $\geq$ 80nmol/L), again highlighting that Vitamin D insufficiency and deficiency is a pandemic problem.

It has been suggested that bone loss in IBD patients is a result of pro inflammatory cytokines such as IL-1, IL-6 and TNF-α released from inflamed intestine directly influencing osteoblast and osteoclast function [78,157]. In this study, there was significant association found between TNF-α, IL-1β, IL-6, and IL-13 and infliximab

therapy. Besides TNF-α, the significant changes noted in IL-1β, IL-6 and IL-13 were only seen in responders, suggesting direct relationship between mean changes in cytokine with infliximab therapy and biological response to infliximab. We found that higher levels of OPG and sRANKL in IBD patients at baseline compared to controls and these results are consistent with findings of two previous studies which reported firstly, higher concentration of OPG in active CD patients compared to controls [222] and secondly, decreased OPG levels following infliximab therapy [269]. We also found that OPG decreased and sRANKL concentration increased over time with infliximab therapy consistent with a previous study by Miheller *et al* [269].

The results of our *in vitro* studies are particularly interesting. Exposure of osteoblasts to sera from active IBD infliximab treated patients showed consistently higher levels of viable cells compared to control subjects at every time point measured. Therapy led to a decrease in viability levels towards those of controls. This shows that infliximab does in fact have an effect of osteoblast activity. Higher osteoblast cell viability observed pre treatment is most likely secondary to an inflammatory driven response. More important than osteoblast cell viability alone however, is osteoblast differentiation and functionality. Although higher osteoblast viability was found pre treatment with infliximab, the amount of ALP which was secreted was significantly lower in the pre treatment group compared to control patients. ALP is an early differentiation marker of the osteoblasts and higher levels found post infliximab treatment indicates that the osteoblasts which were present have a better functionality. This shows that infliximab treatment has a likely direct effect on the human osteoblast *in vitro* and that the increase in bone formation markers *in vivo* is a reflection of this beneficial effect.

The results of DXA findings at 1 year in IBD patients treated with infliximab showed wide variety among patients. There was no significant change noted in mean T score in 6 patients. However, 4 patients showed significant improvement in their T score with infliximab therapy at 1 year follow up. One of these patients was also on a bisphosphonate and 2 were on calcium and Vitamin D supplement during this study period, hence the improvement seen in their T scores could be partly due to known effects of bisphosphonate or calcium and Vitamin D therapy on bone density. Despite that our study together with previous studies from rheumatoid arthritis [294], spondylarthropathy [295] and CD [266-268] add to growing evidence that infliximab maintenance therapy does have a beneficial effect on BMD.

CHAPTER 4 – EFFECT OF ADALIMUMAB THERAPY ON BONE METABOLISM IN CROHN'S DISEASE PATIENTS: A 6 MONTHS STUDY

4.1 Introduction

The pathogenesis of reduced BMD in IBD is multi-factorial and, as in the general population, factors such as age, gender, oestrogen deficiency, alterations in calcium homeostasis, nutritional and dietary factors, smoking, alcohol and immobility are all likely to play a role. However existing evidence suggests that these effects and associations are weak and are overshadowed by the effect of the IBD itself [136]. Moreover, it has been shown that newly diagnosed patients with untreated CD have reduced BMD [296] suggesting that demineralization in patients with IBD may also occur as a direct consequence of intestinal inflammation. Recently, active inflammation and elevated pro-inflammatory cytokines have been implicated in the pathogenesis of bone resorption in a variety of models, including rheumatoid arthritis and postmenopausal osteoporosis [148,152]. Circulating pro-inflammatory cytokine levels are elevated in IBD patients with active inflammation [154-156] suggesting that disease activity and high cytokine levels could also play a role in IBD related bone disease. A rat model of colitis was associated with a dramatic 33% loss in trabecular bone and an even greater suppression in bone formation rate [157]. Healing of colitis was associated with an increased bone formation rate and a return of bone measurements to normal levels. Serum from children with CD affects bone mineralization in an organ culture model without altering bone resorption [147]. These observations suggest that mediators produced during intestinal inflammation may alter osteoblast function and bone

formation. Infliximab has been shown to have beneficial effects on bone metabolism in CD patients although as yet the exact mechanisms have not been fully elucidated. Adalimumab is a human IgG1 monoclonal antibody specific for human TNF and has been recently approved for the treatment of active CD. To date, there are no published data investigating the effect of adalimumab on bone metabolism in IBD patients.

4.2 Aims

1. To compare BMD measurements between CD patients with that of healthy controls.
2. To measure serum bone turnover markers between CD patients with that of healthy controls. In addition, to evaluate the effects adalimumab therapy on bone turnover markers.
3. To measure serum circulating pro-inflammatory, anti-inflammatory, and osteoclastogenesis markers between CD patients with that of healthy controls, and to evaluate the effects of adalimumab therapy on these parameters.
4. To compare the levels of PTH and Vitamin D in CD patients and healthy controls, and to see if plasma PTH and Vitamin D levels alter with adalimumab therapy in CD patients.
5. To evaluate and compare the effect of sera from CD patients treated with adalimumab on human osteoblasts *in vitro* model in regards to cell viability/proliferation and differentiation/functionality with that of healthy controls.

Therefore, our aim was to explore the effects of medium term adalimumab treatment on bone metabolism in active CD patients by measuring the effects of maintenance adalimumab therapy on biochemical markers of bone turnover, bone nutrients, pro-inflammatory cytokines, anti-inflammatory cytokines, osteoclastogenesis markers and the effect of sera of treated patients on human osteoblast compared to healthy subjects.

4.3 Results

4.3.1 Effect of adalimumab treatment on disease activity

As previously described in detail in section 2.1, briefly, 20 CD treated adalimumab patients and 20 healthy controls were included in this study. Adalimumab patients were followed for 6 months and sera were obtained at pre treatment and at 1 month, 3 months and 6 months post treatment. The mean disease activity measured by CDAI score appreciably decreased from 304.65 at baseline to 113.7 after 1 month, 61.65 after 3 months and 27.7 after 6 months ($p<0.001$ at all time points compared with baseline) **(Figure 4.1)**. To investigate any relationship between disease activity and change in bone metabolism markers, patients were stratified into those with a fall in CDAI of 70 -100 points (n=5), 100-150 points (n=4) and those with 150 points or greater (n=11) at 1 month after treatment with adalimumab. Mean CRP (mg/L) at baseline was 15.92 and fell significantly following adalimumab therapy at all time points measured ($p<0.05$) **(Figure 4.2)**. Although a trend was observed, the mean dosage of steroid that patients were on at 6 months did not fall significantly ($p=0.098$) following treatment.

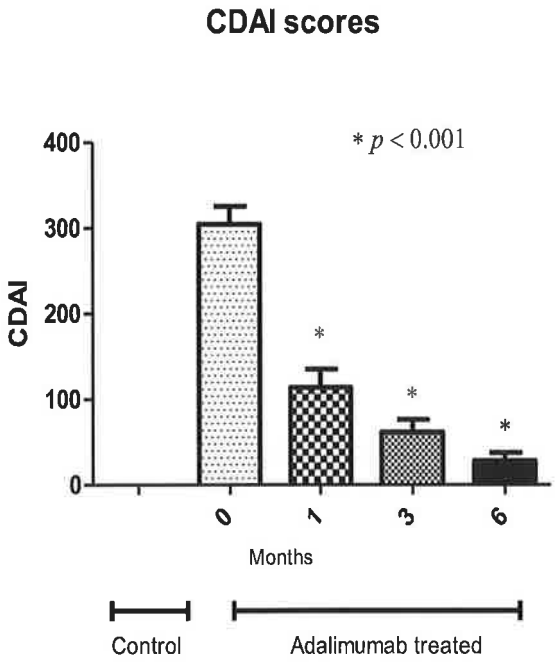


Figure 4.1: CDAI scores in control and Crohn's patients

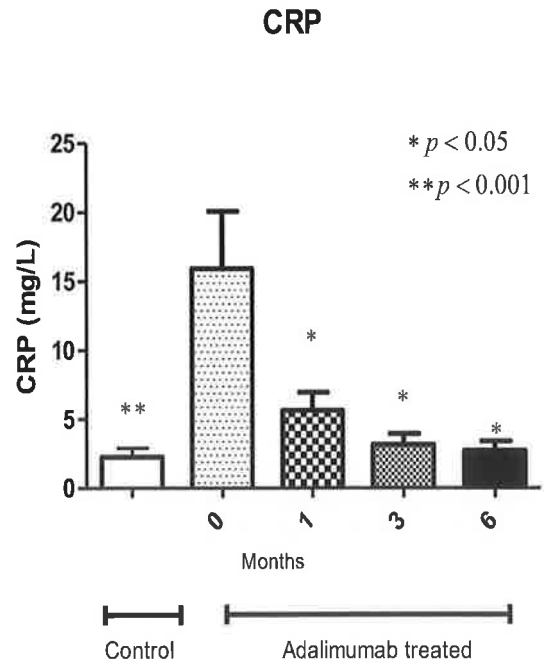


Figure 4.2: CRP levels in control and Crohn's patients

4.3.2 Bone mineral density in active CD patients compared to controls

Four of 20 CD patients had osteoporosis (T score ≤ -2.5) and a further 6 patients had osteopenia (T score of -1.0 to -2.5) prior to adalimumab treatment. In control patients two of 20 patients had osteoporosis (T score ≤ -2.5) and further 9 patients had osteopenia (T score of -1.0 to -2.5) **(Figure 4.3)**. The two control patients who had osteoporosis were both males and were ex-smokers of 20 and 6 pack years respectively.

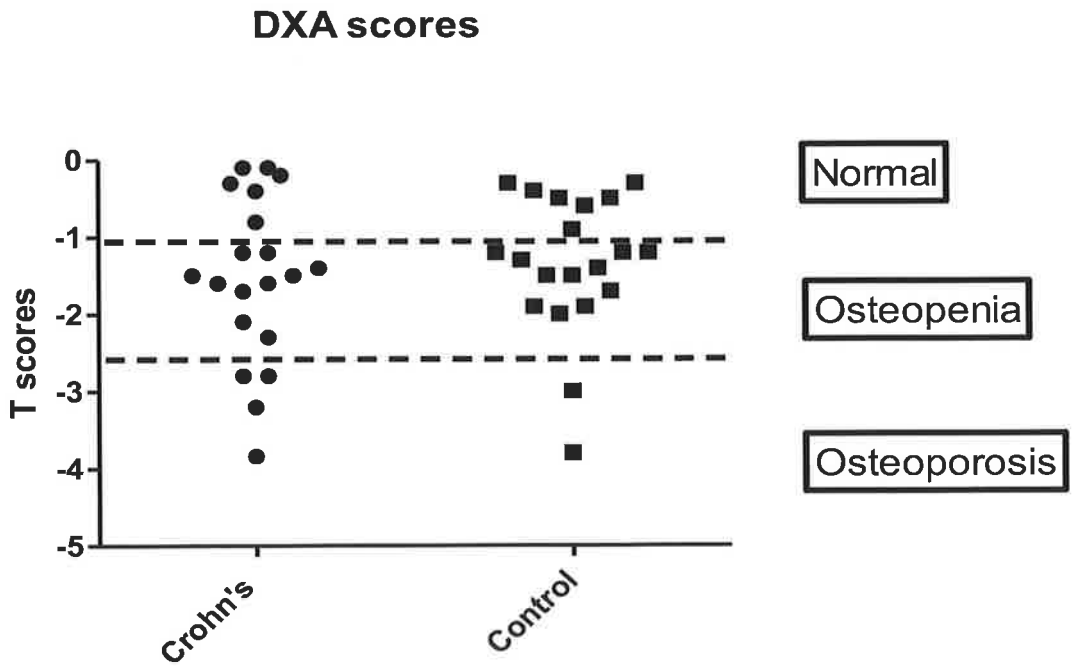
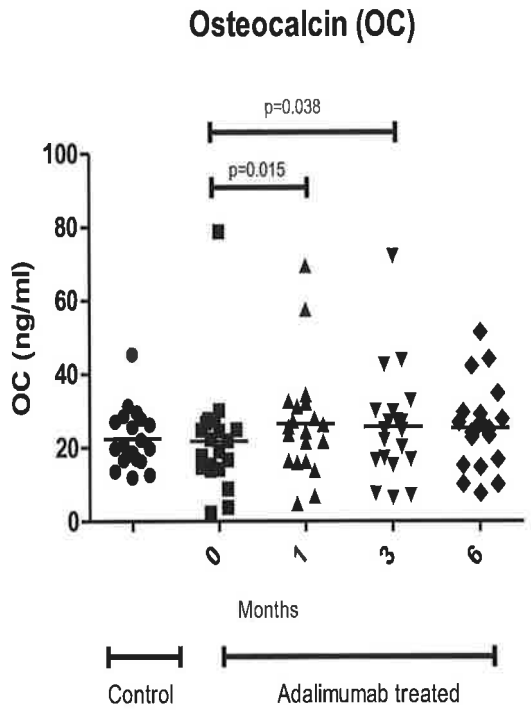


Figure 4.3: Distribution of BMD based on DXA score in control and Crohn's patients

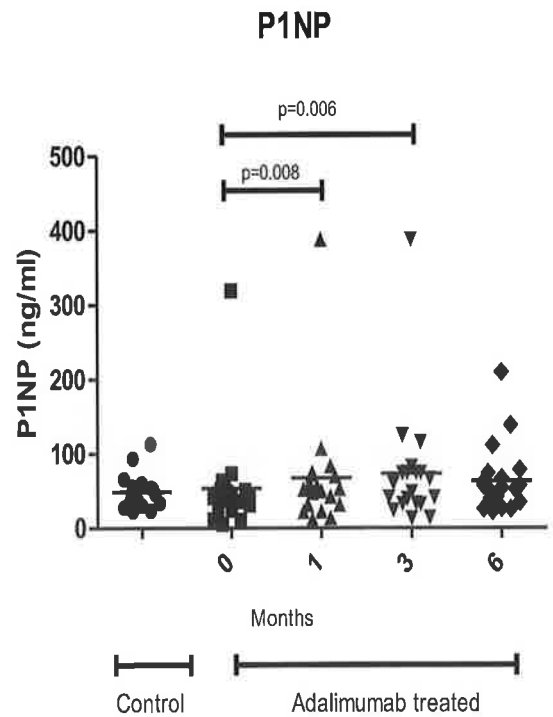
4.3.3 Effect of adalimumab treatment on bone markers

The markers of bone formation, OC and P1NP (ng/ml) were significantly increased at 1 month (p=0.015 and p=0.008 respectively) and 3 months (p=0.038 and p=0.006 respectively) compared to baseline, but not at 6 months **(Figure 4.4 A and B)**. Serum concentration of OC was lower in CD before adalimumab treatment than in controls while they return to normal levels after adalimumab treatment **(Figure 4.4A)**. The bone resorption marker, CTx serum (ng/ml), decreased from baseline (0.312) to 1 month (0.3051) and remained decreased during the rest of the 6 months (0.2734), but these decreases were not statistically significant **(Figure 4.4C)**. Interestingly, P1NP levels were lower and CTx serum levels were higher in controls compared to CD patients at baseline, although these were not statistically significant **(Figure 4.4 B and C)**.

A


Osteocalcin (OC)
p=0.038
p=0.015
OC (ng/ml)
100
80
60
40
20
0
0
1
3
6
Months
Control
Adalimumab treated

B


P1NP
p=0.006
p=0.008
P1NP (ng/ml)
500
400
300
200
100
0
0
1
3
6
Months
Control
Adalimumab treated

C

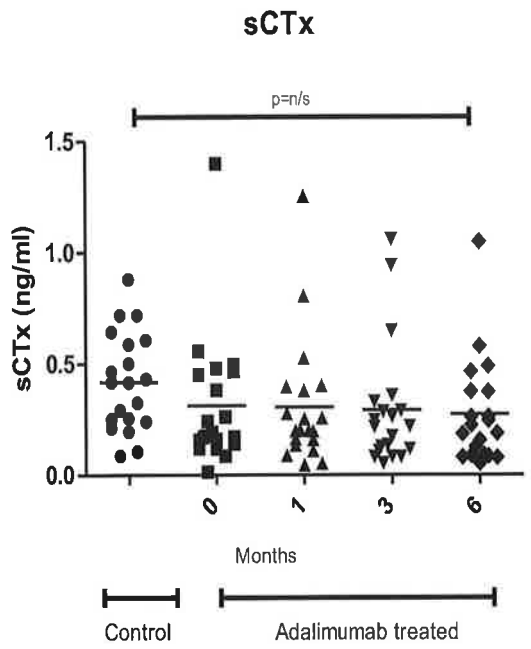
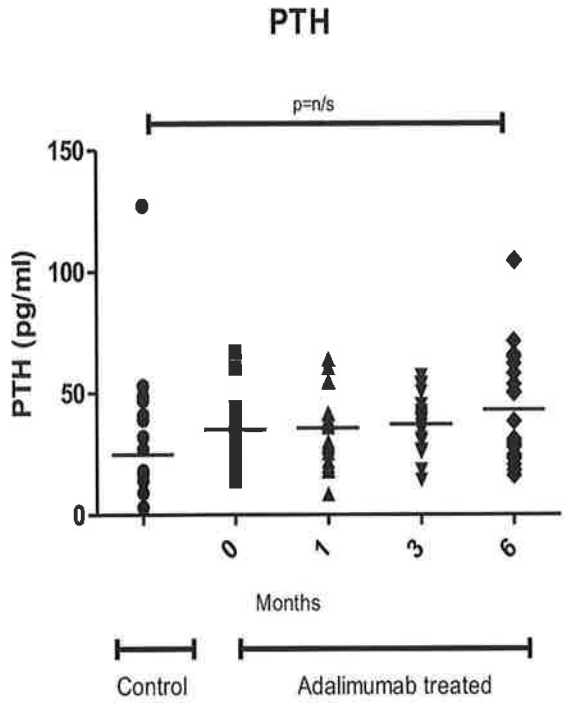


Figure 4.4 (A-C): Changes in mean Osteocalcin (OC), Pro- Collagen type 1 N propeptide (P1NP) and Carboxyterminal N-telopeptide (CTx) serum levels during treatment with adalimumab.

4.3.4 Effect of adalimumab treatment on serum bone nutrients levels

At baseline CD patients had lower Vitamin D levels and higher PTH levels compared to controls (48.02 nmol/L compared to 53.45 nmol/L and 35.08 pg/ml compared to 24.85 pg/ml respectively). This however was not statistically significant. With adalimumab treatment both PTH and Vitamin D levels showed a trend towards increase at 6 months, but these increases were not found to be significant **(Figure 4.5 A and B)**.

A



B

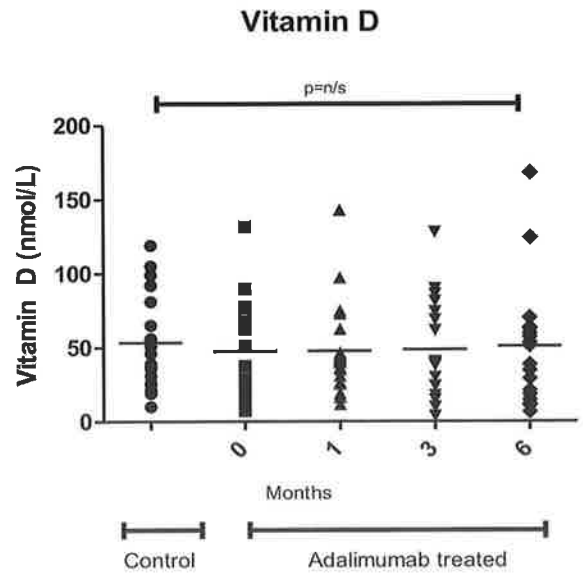
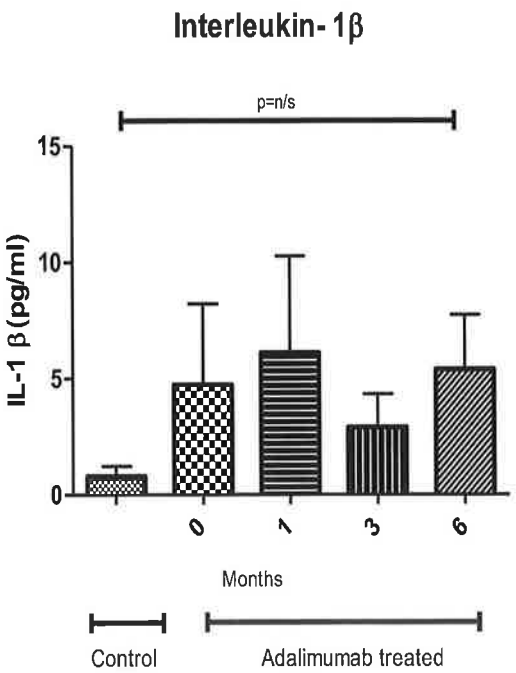


Figure 4.5 (A-B): Changes in mean Parathyroid hormone (PTH) and Vitamin D (Vit D) levels during treatment with adalimumab.

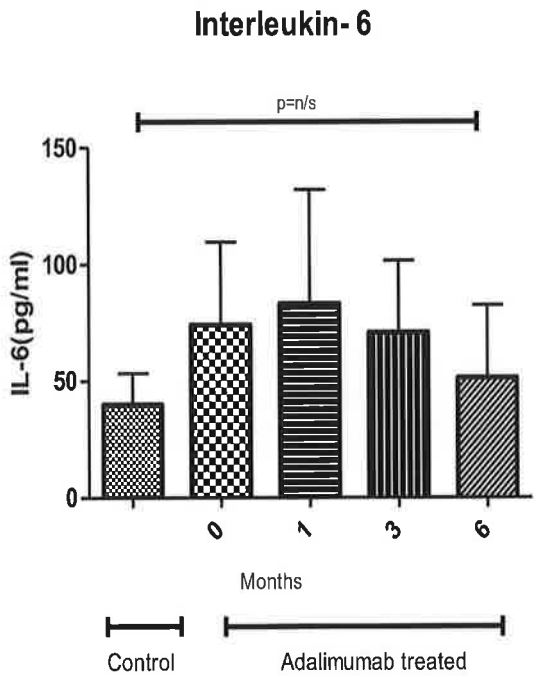
4.3.5 Effect of adalimumab treatment on serum cytokines

Not surprisingly, control patients had lower serum levels of all the cytokines measured compared to CD patients at baseline, but this was not found to be statistically significant. As shown in **Figure 4.6 (A-C)**, treatment with infliximab resulted in a trend towards decreased levels of pro-inflammatory cytokines at 3 months and 6 months, although levels were slightly increased at 1 month. None of these figures was statistically significant however. Anti-inflammatory cytokine levels were not significantly different post treatment with adalimumab as shown in **Figure 4.7 (A-B)**.

A



B



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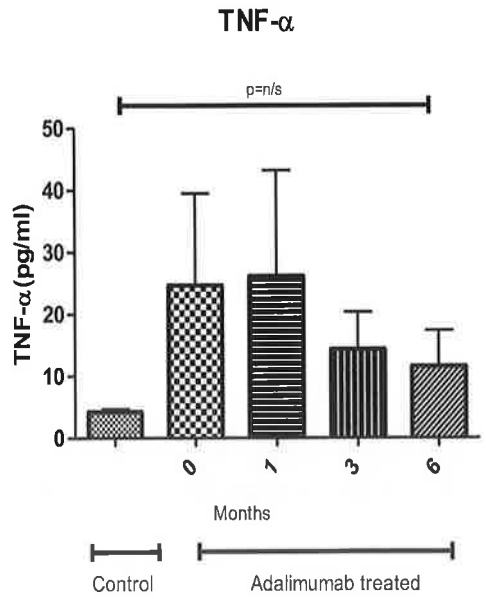
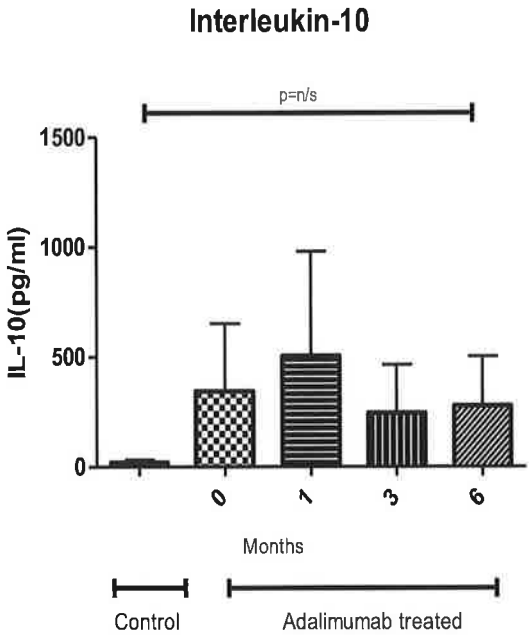


Figure 4.6 (A-C): Concentration of pro-inflammatory cytokines in control patients & pre and post adalimumab therapy

A



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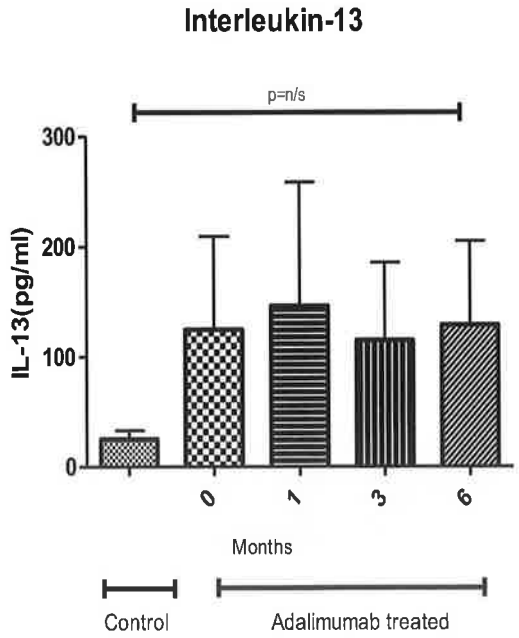
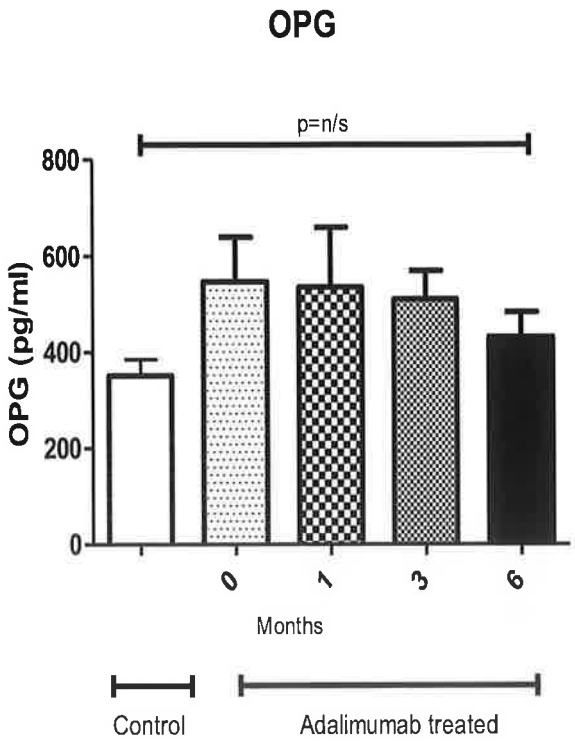


Figure 4.7 (A-B): Concentrations of anti-inflammatory cytokines in control patients & pre and post adalimumab therapy

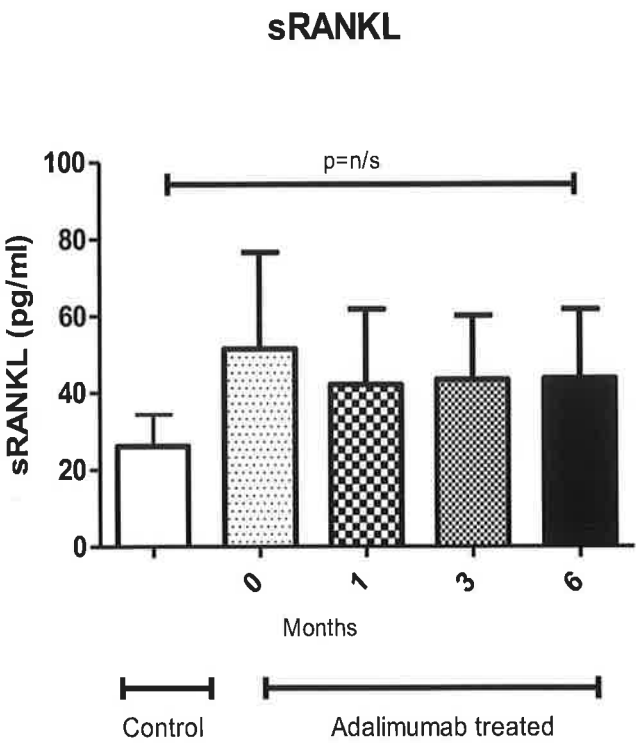
4.3.6 Effect of adalimumab treatment on osteoclastogenesis markers

Serum levels of RANKL and OPG decreased after adalimumab therapy. At baseline, both OPG and RANKL levels were higher in CD patients compared to control, and with therapy the levels decreased towards that of control, however the changes were not statistically significant **(Figure 4.8 A and B)**. OPG and sRANKL essentially work in opposite directions in osteoclast homeostasis; dynamic changes in OPG/sRANKL system are often expressed as changes in the ratio of these mediators. As shown in **Figure 4.8 C**, there were no statistically significant changes in this ratio over the time point studied.

A



B



C

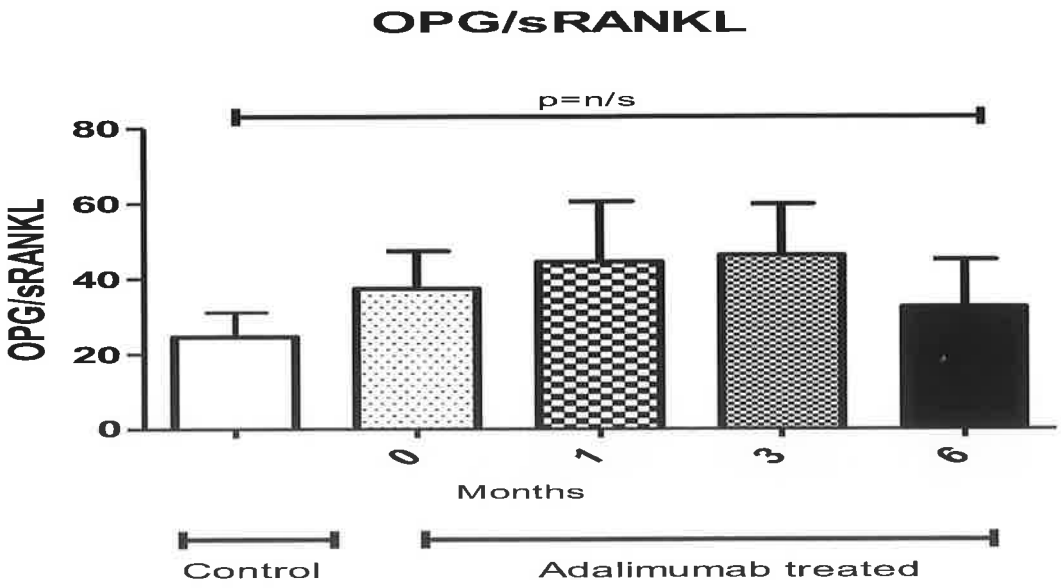


Figure 4.8: (A-C): OPG and sRANKL in control and adalimumab treated CD patients

4.3.7 Effect of serum from patients treated with adalimumab on human osteoblast cell viability

Exposure of hFOB cells to CD sera pre and post adalimumab treatment, showed that post treatment sera reduced in vitro hFOB viability at all time points measured. However the effect was significant only for 6 months post commencement of treatment at day 14 of incubation. Interestingly, pre treatment CD sera were associated with increased viable osteoblast cell number compared to controls, with adalimumab treatment resulting in a reduction in viability back towards control levels **(Figure 4.9)**.

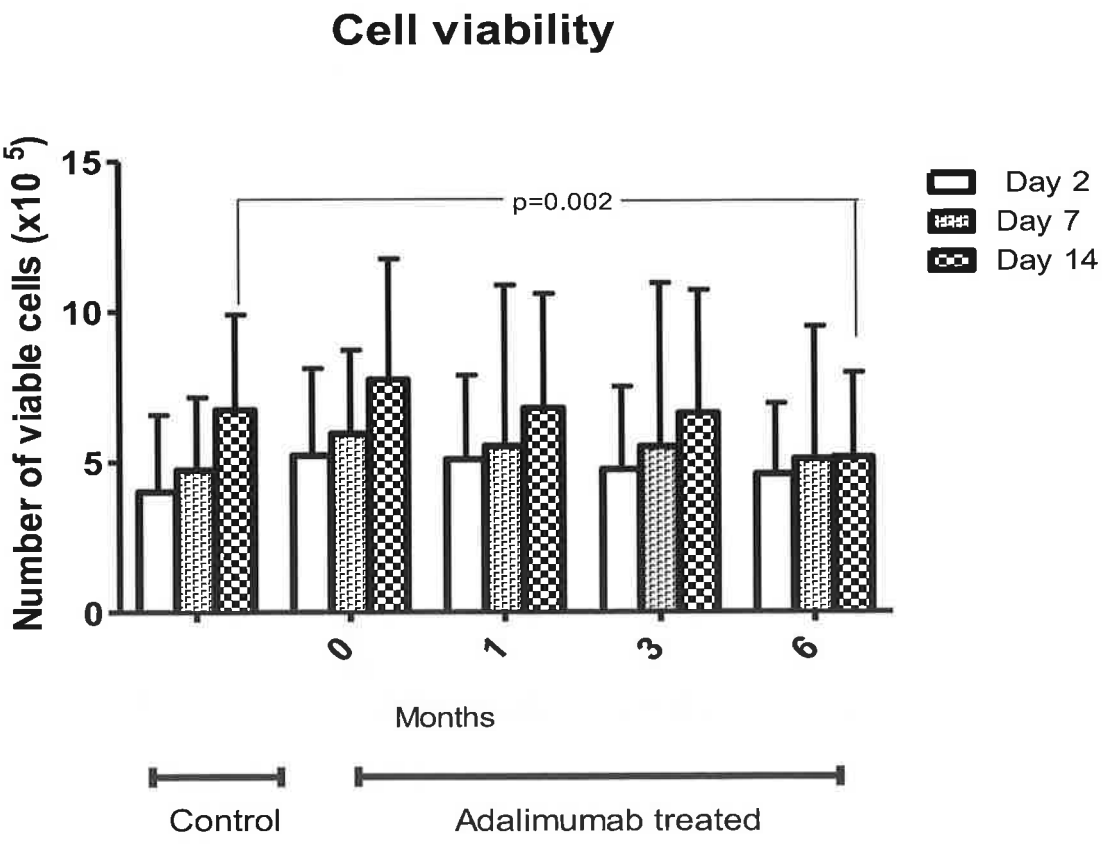


Figure 4.9: Number of viable hFOB cells on exposure to serum from adalimumab treated CD and control over 14 days

4.3.8 Effect of serum from patients treated with adalimumab on human osteoblast cell functionality

Osteoblasts exposed to control sera secreted significantly higher levels of ALP than those exposed to pre treatment, baseline CD sera ($p<0.001$ at Day 2, $p<0.001$ at Day 7 and $p=0.001$ at Day 14). Post adalimumab treatment sera resulted in higher levels of ALP secretion from osteoblasts than pre treatment baseline sera and this reached significance at 6 months of Day 14 exposure ($p=0.001$). Moreover, ALP secretion following exposure to post treatment CD sera, no longer differed from the levels secreted following exposure to control sera **(Figure 4.10)**.

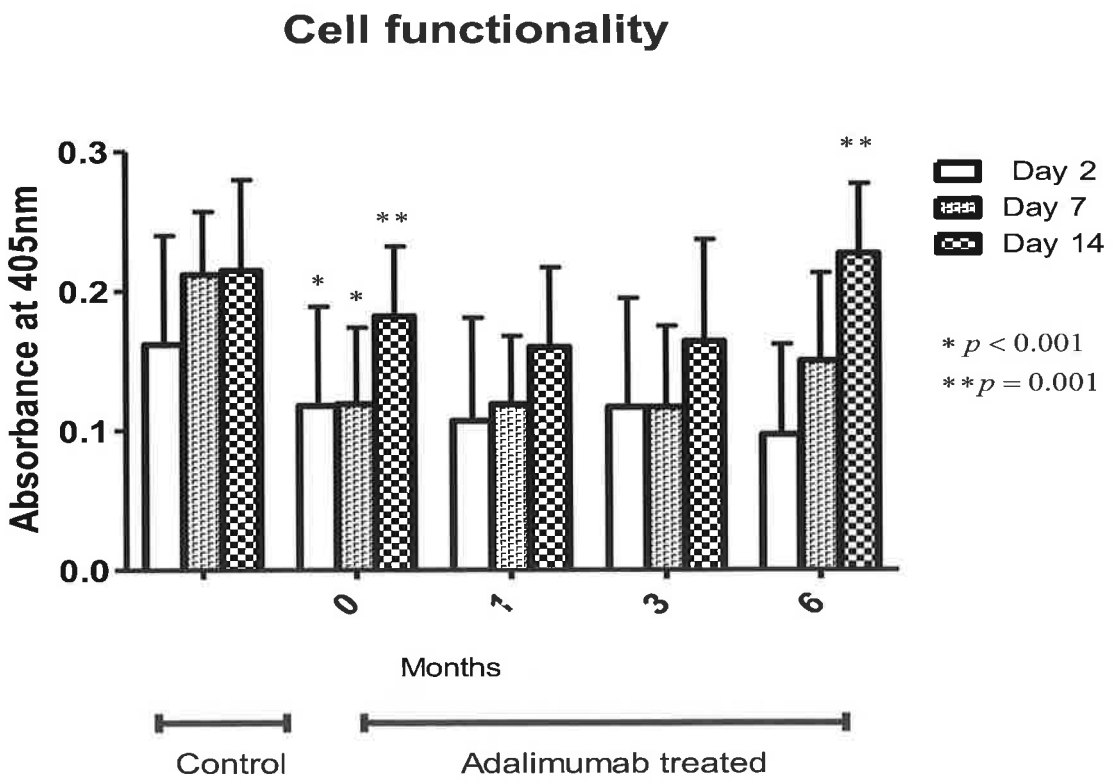


Figure 4.10: Amount of ALP secreted on exposure of serum from control and CD adalimumab treated patients over 14days

4.4 Discussion

In this study, we looked at the medium term effect of adalimumab in CD patients only. From the results obtained in this small cohort of CD patients, it is apparent that adalimumab had a beneficial effect on bone metabolism in active CD patients at the medium term. Looking at the markers of bone formation, there was rapid increase in OC and P1NP at the 3 months interval. As seen with the longer term infliximab study in Chapter 3, no significant change was seen over time with the bone resorption marker sCTx. These apparent beneficial effects were not associated with baseline CDAI's, DXA findings, previous TNF-α exposure, current steroids therapy or indeed on biological response to adalimumab. No significant change was seen with the bone nutrients and serum cytokine levels over time with the adalimumab therapy. Results of the osteoclastogenesis showed higher levels of OPG and sRANKL in CD patients at baseline compared to controls and those levels of OPG and sRANKL decreased after adalimumab therapy. These results are consistent with findings of two previous studies which reported firstly higher concentrations of OPG in active CD patients compared to healthy controls [222] and secondly, decreased OPG levels following infliximab therapy [269]. The fact that similar changes have been reported after treatment with two different anti TNF-α treatments, suggest that these are group effects of TNF-α blockade. Therefore given the fact that healthy subjects have both lower OPG and sRANKL compared to active CD at baseline, and that adalimumab therapy leads to a decrease in both parameters to approximate levels of control subjects, it would appear that the inflammatory response leads to counter-regulatory changes in bone homeostasis controls and that dampening

down the inflammatory response with biologic therapy leads to a re-setting of the controls towards normal.

Previous studies have suggested that bone loss in IBD patients is a result of pro-inflammatory cytokines (IL-1, IL-6 and TNF-α) released from inflamed intestine which in turn directly influences the osteoblast and osteoclast functions [64,78]. However, in this small study we found no such significant between the serum cytokine levels and bone turnover markers or adalimumab therapy. Our patient numbers were small, and larger studies might well find such an association. However, a previous study in patients treated with infliximab found that mucosal but not systemic measurements of pro-inflammatory cytokines mirrored response to therapy [297]. We did not measure mucosal cytokine levels in this study.

As in Chapter 3, our *in vitro* studies looking at the effect of exposure of osteoblasts to sera from active CD-adalimumab treated patients showed consistently higher levels of viable cells compared to control subjects at every time point measured, and with adalimumab therapy the viability decreased towards those of controls. This result as seen in the long term infliximab study would indicate that adalimumab also has an effect on osteoblast activity. Higher osteoblast cell viability observed pre treatment is most likely again an inflammatory driven response. Although higher osteoblast viability was found pre treatment with adalimumab, the amount of ALP which was secreted was significantly lower in pre treatment group compare to control patients. As mentioned previously ALP is an early differentiation marker of the osteoblasts and higher levels found post adalimumab treatment indicate that the osteoblasts which were present had better functionality. This shows that adalimumab treatment has a direct effect on the human

osteoblast *in vitro* and that the increase in bone formation markers *in vivo* is reflection of this beneficial effect.

There are huge similarities seen between the long term effects of infliximab on bone metabolism in both active UC and active CD patients compare to the medium term effects of adalimumab on bone metabolism in active CD patients. This likely reflects that the positive changes are class specific, i.e. secondary to the effects of anti-TNF-α itself rather than a particular drug. Given similar changes are seen with both infliximab and adalimumab, this thesis confirms that the beneficial effects seen with the biologic therapies on bone metabolism are secondary to the effect of the TNF molecule itself and not secondary to how a particular biologic is formulated (i.e. chimeric monoclonal or fully humanized) or how it is delivered (i.e. intravenous or subcutaneous). It will be interesting to evaluate other anti-TNF-α therapies available in the market such as etanercept (which is the soluble receptor for TNF) and certolizumab (Fab fragment of humanized anti-TNF-α that is attached to polyethylene glycol) to further confirm these beneficial findings, however this is beyond the scope of this thesis.

Furthermore, other smaller short term study previously carried out with infliximab [263, 264] have also shown similar changes especially with the bone markers results (significant increase in bone formation markers and albeit non-significant decrease, in bone resorption marker) and this would indicate that these findings are real observations. Longer term studies with adalimumab will be fruitful to confirm these beneficial changes seen in this chapter would continue to persist at one year as seen with our infliximab study. Given previous short term studies with infliximab [263, 264] showed similar

results to our long term infliximab results, similar changes are also likely to be seen when longer term studies on adalimumab are conducted, however this remains to be seen.

CHAPTER 5 – AN *IN VITRO* STUDY OF THE DIRECT EFFECT OF INFLIXIMAB ON HUMAN OSTEOBLASTS

5.1 Introduction

Bone is comprised of mineral salts (Calcium Phosphate derivatives), non-mineral matrix (Type I Collagen) and living cells. 3 main types of cells prevail; bone forming, osteoblasts; bone resorbing osteoclasts and bone maintaining, osteocytes [20]. All of these cells can be affected by systemic disease and the circulating cytokines and chemokines that ensue. It has been hypothesised that osteoporosis prevails in IBD due to bowel pathology reducing calcium and Vitamin D intake; however the decreased levels of these substances is unlikely to be sufficient to induce osteoporosis [263]. Of note, the markedly raised levels of certain macrophagic cytokines involved in systemic inflammation, including IL-6 and TNF-α, have been implicated in negatively effecting bone homeostasis [163]. There are many hypotheses why infliximab has a positive effect on BMD. It has been postulated that its anti-inflammatory properties simply improve bowel function and therefore improve absorption of bone formation precursors (Calcium, Vitamin D) [298]. Secondly, TNF-α is known to promote osteoclast differentiation and so infliximab may reduce the bone resorbing function of osteoclasts [263].

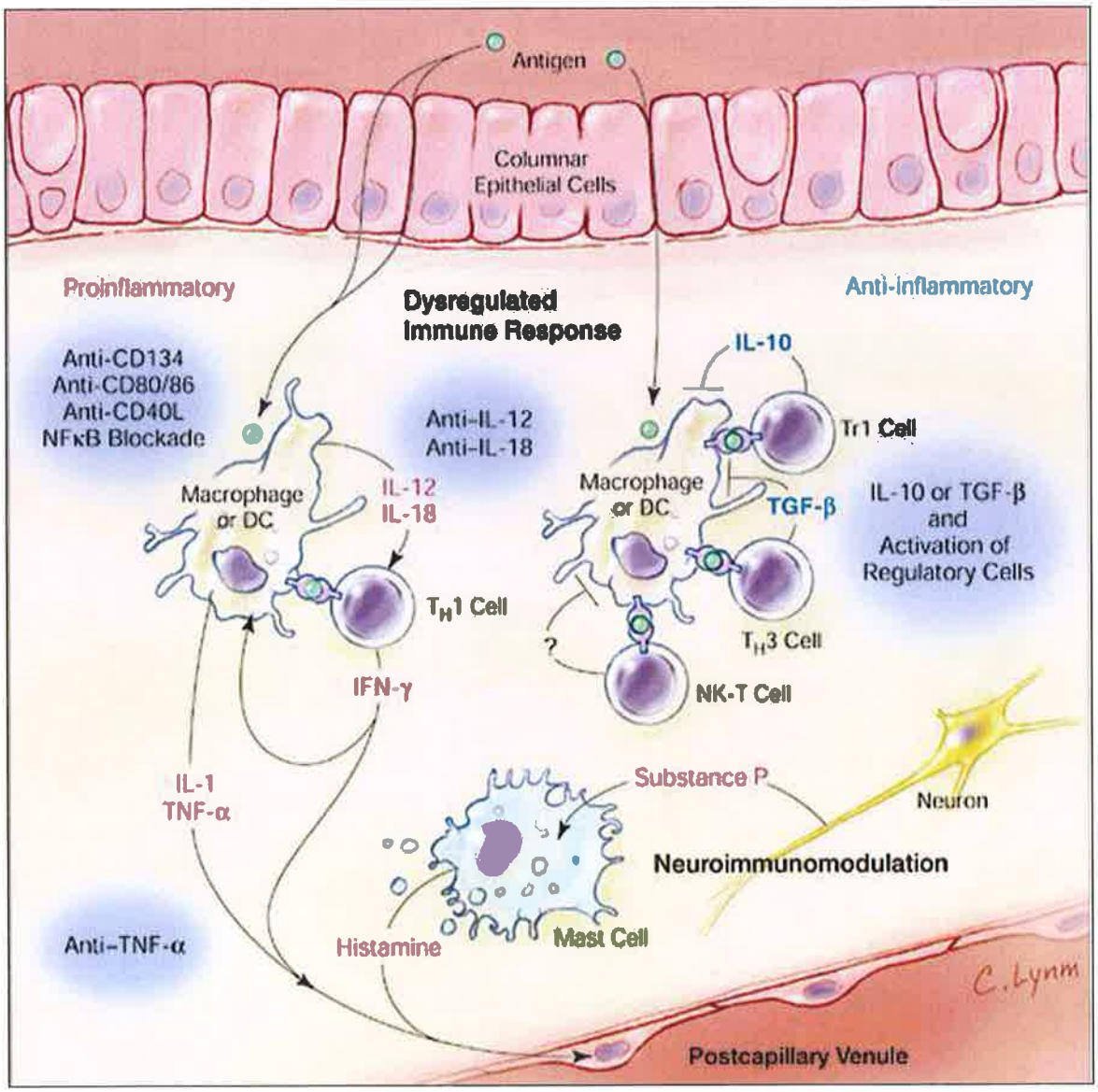


Figure 5.1: Pathogenesis of inflammatory bowel disease

The figure above **(Figure 5.1)** shows TNF-α release in response to bowel-borne antigen; TNF-α is then released into the blood stream where it then able to have systemic effects, bone being one such target [299]. Research has been carried out testing the effects of anti TNF-α therapy on the *in vivo* bone markers of resorption and formation, including sCTx and osteocalcin respectively [263]. To date there is only limited *in vitro* research on infliximab on the function of human osteoblasts.

5.2 Aims

It was decided to characterise the *in vitro* effects of Infliximab on osteoblasts in 2 key areas. Firstly, we would examine the effects of Infliximab in terms of cell viability i.e. changes in the number of living cells between cell samples with and without Infliximab. Secondly, we would look for changes in cell functionality (or differentiation) i.e. changes in the levels of the early marker of differentiation, alkaline phosphatase.

5.3 Results

5.3.1 Establishing AlamarBlue Standard Curve

As previously described in section 2.9.1 using an alamarBlue assay, it is possible to quantify the number of viable cells. In order to do this, a standard curve was produced. A standard curve is constructed by seeding cells at different densities on a plate. The densities used were $1x10^6$ cells/ml (1M), $0.8x10^6$ cells/ml (0.8M), $0.6x10^6$ cells/ml (0.6M), $0.4x10^6$ cells/ml (0.4M) and $0.2x10^6$ cells/ml (0.2M). Subsequently we carried out the alamarBlue assay by adding 10µl of alamarBlue solution into each well and reading the absorbance in a spectrophotometer at 540nm and 620nm. Following certain calculations as described by the manufacturer (section 2.9.1); it is then possible to get a reduction value (Ro). AlamarBlue is a water-soluble dye that is sensitive to the metabolites produced by viable cells. The redox reaction induces a proportional colour change and it is therefore possible to correlate reduction value with the number of cells. Absorbance was read every half hour for the first 3 hours and then every hour for the next 7 hours **(Figure 5.2)**. When looking at the separation of the points for each time-point, R

values can be calculated. The R value indicates how sensitive alamarBlue is at that particular time point. In aiming for an R value greater than 0.97, indicating strong correlation; an R value of 0.9968 was achieved for the 240 minute (4 hours) time-point as shown below **(Figure 5.3)**. Following these preliminary standardisation experiments, a 4 hour time point was chosen for all subsequent *in vitro* cell viability experiments.

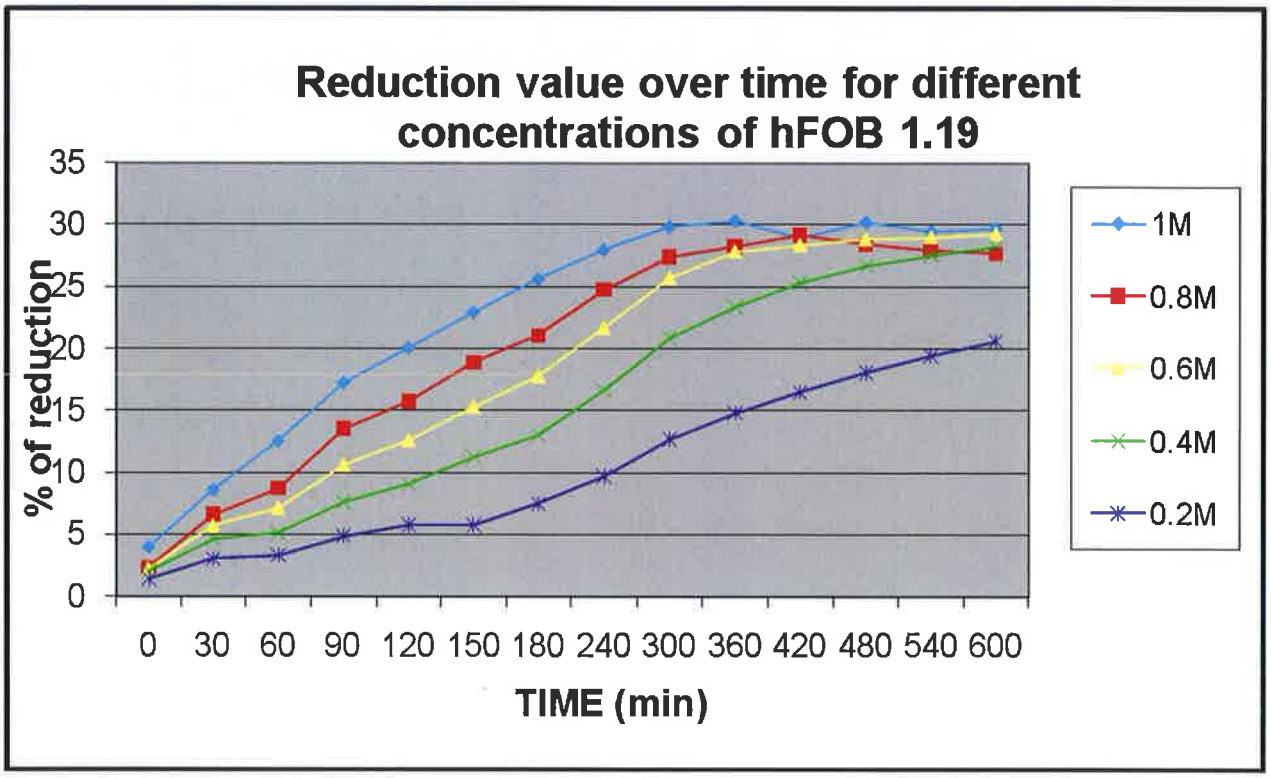


Figure 5.2: Standard curve for hFOB 1.19 cell line using AlamarBlue assay

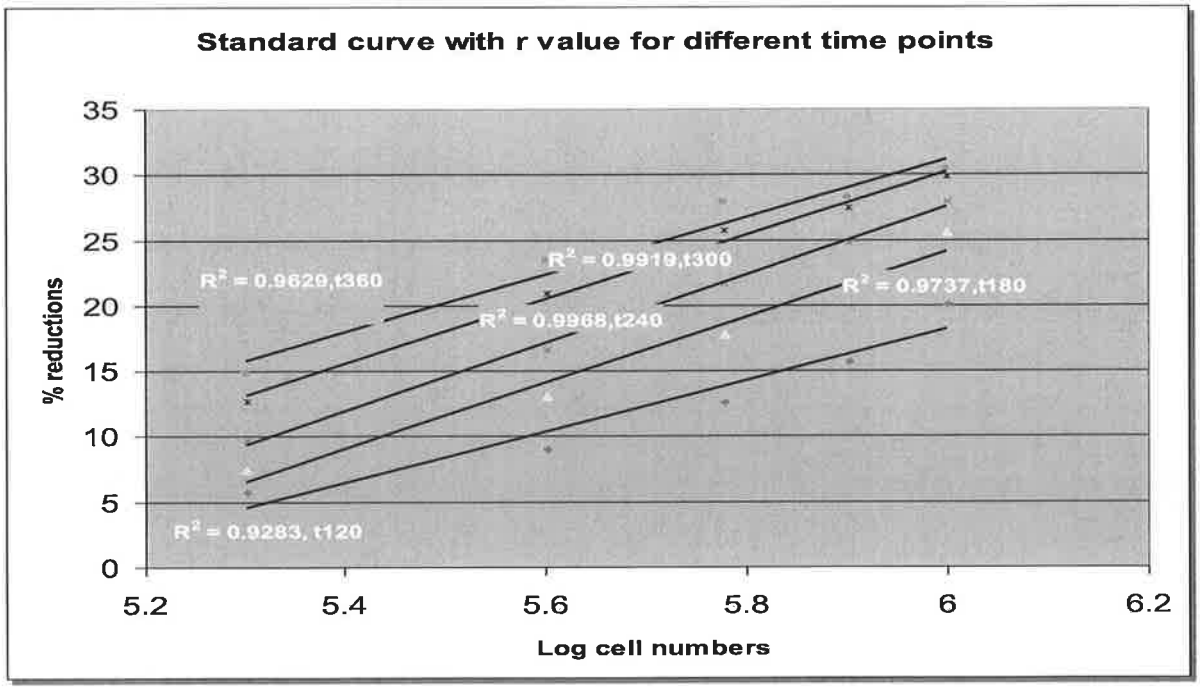


Figure 5.3: Standard curve with r value for hFOB 1.19 cell line using AlamarBlue assay

5.3.2 Establishing ALP expression of hFOB cells

This experiment was carried out to confirm that a higher number of osteoblasts equates to higher levels of ALP expression. Furthermore, it was to discover which cell density gave the best separation or sensitivity to the pNPP assay. The densities used were $1x10^6$ cells/ml (1M), $0.8x10^6$ cells/ml (0.8M), $0.6x10^6$ cells/ml (0.6M), $0.4x10^6$ cells/ml (0.4M) and $0.2x10^6$ cells/ml (0.2M). The assay involves adding pNPP solution in each well in the plate and incubating it for 30 minutes; at which point the absorbance is read in a spectrophotometer at 405nm. A plate was read at Day 1, 2, 4, 7 and 10. All readings were performed in triplicates (n=3).

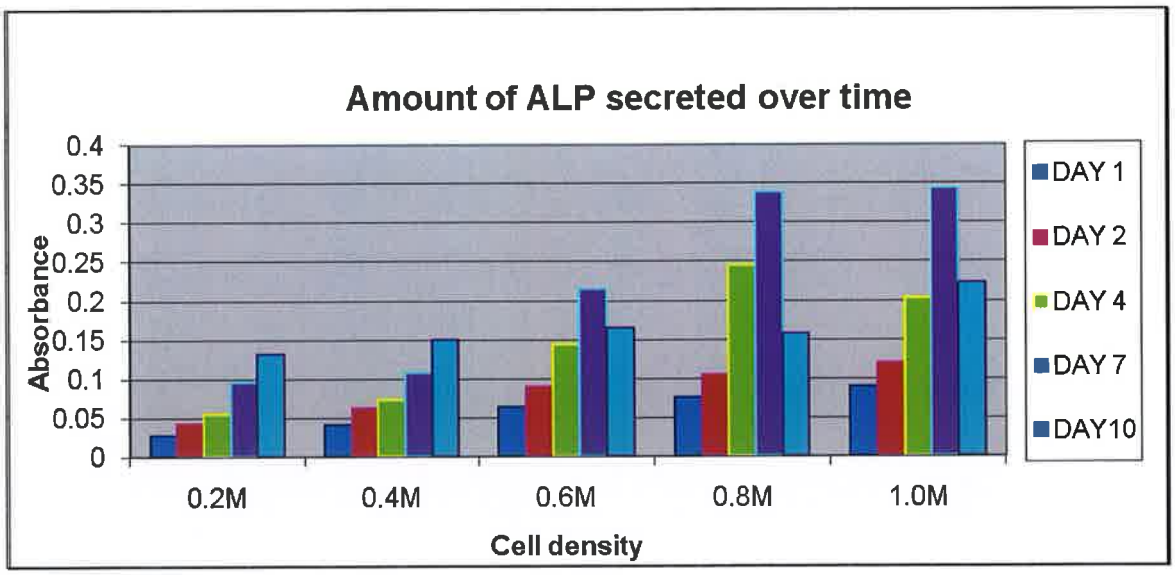


Figure 5.4: Amount of ALP secreted over time with varying cell densities

The graph above **(Figure 5.4)** shows a fairly linear increase in ALP expression as time increases. An exception to this is seen at higher cell densities at day 10 where ALP expression decreases. The graph does appear to show that higher cell densities show greater levels of ALP. From the results of this experiment, a cell density above $0.6x10^6$ cells/ml cannot be chosen as it would not give a good separation between time-points. Hence a 0.5×10^6 cells/ml was chosen for future cell functionality experiments as for the cell viability experiments. As we would be leaving the cells for 14 days, we also wanted the lowest possible amount of cells to allow for cell proliferation and at the same time give good separation between time points that will be examined.

5.3.3 Cell viability of hFOB 1.19 cell line with Infliximab

To investigate the direct effect of infliximab on osteoblast cell viability and cell functionality, we performed dose response and time course experiments using varying concentrations of infliximab. These experiments were performed to see whether the effects of post infliximab therapy sera on osteoblasts might be due to circulating infliximab in the patient's sera rather than due to changes in cytokine levels in the sera post treatments. Firstly, a cell viability experiment was performed using varying concentrations of infliximab over a 24 hour period. As described in section 2.10.2, various concentrations of infliximab from 0.1% to 100% infliximab were prepared and experiment conducted. The graph below **(Figure 5.5)** shows the reduction value against time of varying infliximab concentrations over a 24 hour exposure period. With the exception of 100% infliximab, it is apparent that all the infliximab concentrations, including those within the pharmacological range, induce a greater percentage of reduction than the FBS control. The 10% FBS is optimised for hFOB growth. There is little difference between each infliximab concentration (except for 100%).

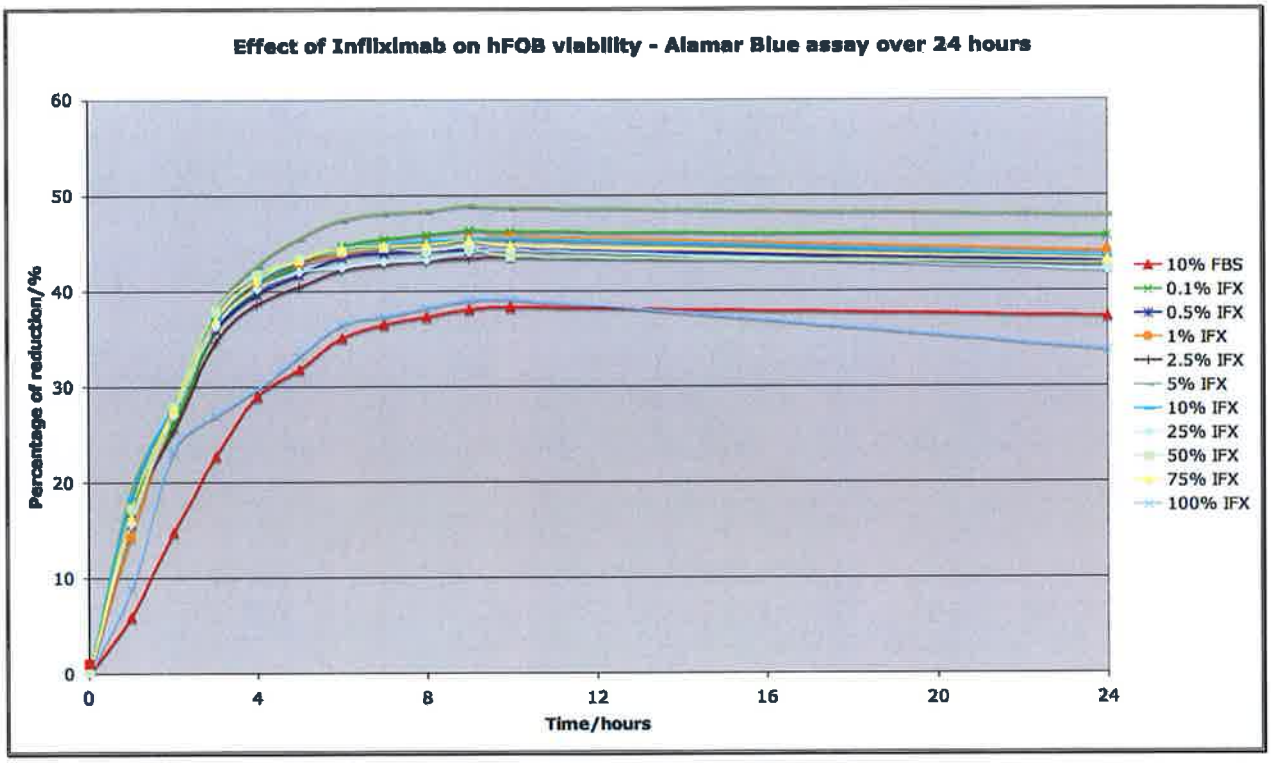


Figure 5.5: Effect of varying infliximab concentrations over 24 hours with alamarBlue assay

5.3.4 Cell viability of hFOB 1.19 cell line with Infliximab over 10 days

Following experiment above section 3.3, a time course experiment using specific infliximab concentrations was chosen to study the effect of infliximab on cell viability of *h*FOB 1.19 cell line over a 10 day period. As described in section 2.10.2, experiments were set up using a 1%, 5%, 10%, 25% and 50% infliximab concentrations. The graph below **(Figure 5.6)** shows that at Day 10, 10% infliximab gave almost 3 times as many viable cells as the control. However, above 10% the viability drops to levels comparable to 10% FBS. Notably, at the pharmacological concentration of 1%, viability is still almost twice that of the control. All readings were performed in triplicates (n=3).

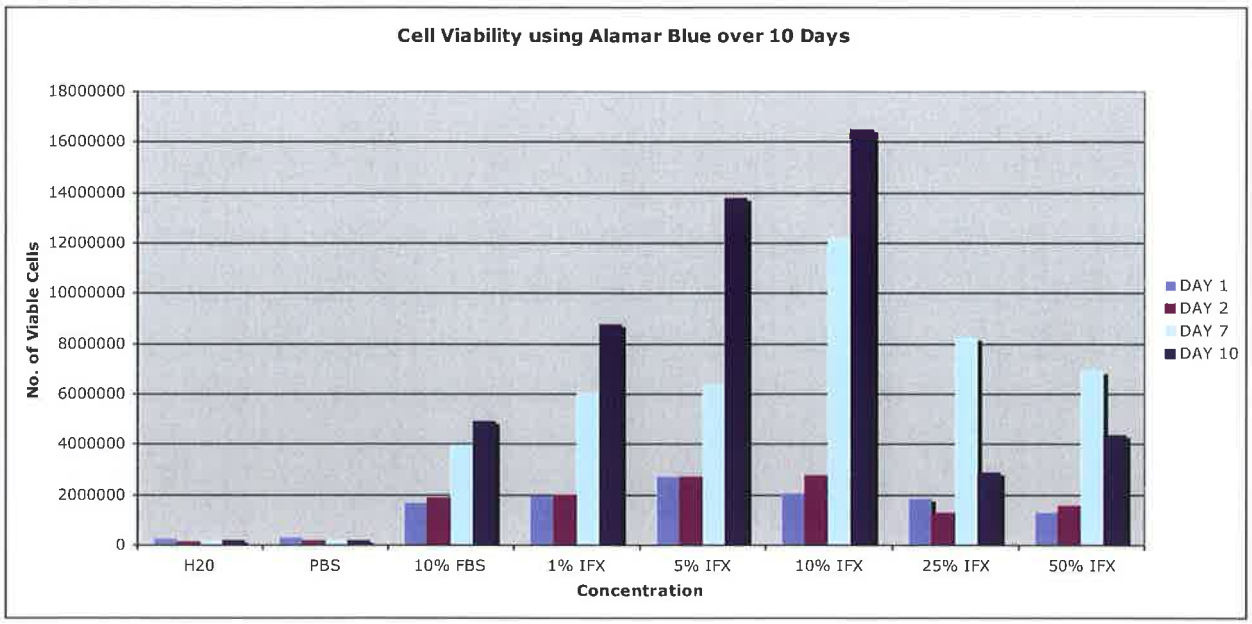


Figure 5.6: Effects of infliximab on hFOB viability with alamarBlue assay over 10days

5.3.5 Cell functionality of hFOB 1.19 cell line with infliximab over 10 days

The purpose of this experiment was to assess any change in cell functionality amongst different cell populations exposed to different infliximab concentrations. As described in section 2.10.4, the experiment was set up and carried out accordingly. From the graph below **(Figure 5.7),** that a concentration of up to 10% infliximab, it is apparent that ALP expression increases up to Day 10. Furthermore, as infliximab concentration increases, ALP expression appears to decrease. However at the pharmacological concentration of 1% infliximab, there does not appear to be much difference with the control (10% FBS).

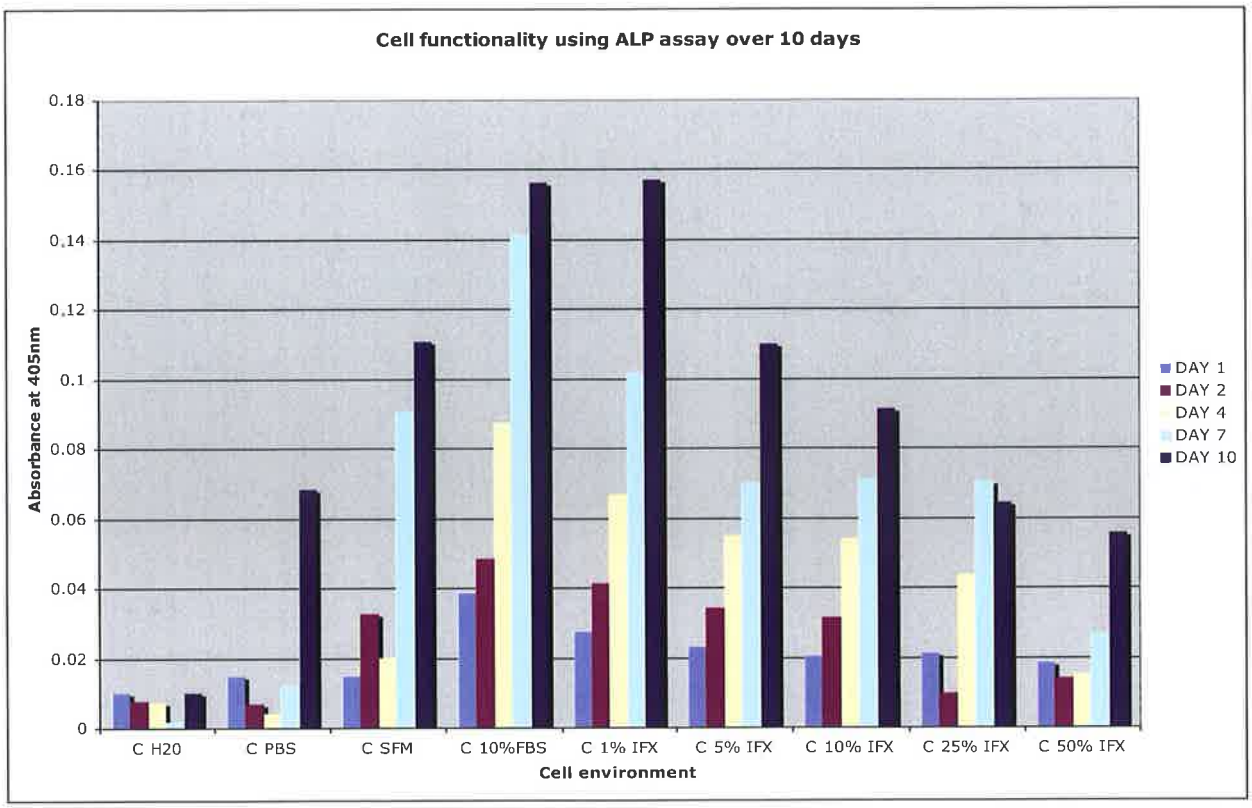


Figure 5.7: Effect of infliximab on hFOB functionality over 10 days

5.4 Discussion

In this study, we have examined how infliximab affects osteoblasts in terms of osteoblast viability and differentiation. 10% infliximab appears 3 times as effective as the control at promoting cell viability however as infliximab concentrations increase, this positive effect on viability diminishes. However, more importantly; at the physiological concentration, the number of viable cells almost doubles that of the control, after 10 days. TNF- α has pro-apoptotic properties and this could explain the increase. However, with higher concentrations of infliximab, viability falls and this implies that some TNF-α is required for optimal growth. Much more generally we can say with relative certainty is

that infliximab is not toxic to osteoblast cells at low concentration. Osteoblast differentiation assessed by the level of ALP expression, showed that as infliximab increases, the level of ALP expression decreased. However, at the physiological concentration, the ALP expression did not appear significantly altered. The idea that infliximab can increase osteoblast differentiation and viability is in line with the findings that blood ALP levels increase in patients treated with infliximab [298]. TNF-α has been shown to decrease osteoblast differentiation [300] and so an increase would be expected with infliximab and so examining other markers for differentiation may be of interest. From these preliminary results, on the whole, it appears that infliximab has a positive effect on throughout the osteoblast life cycle. Whilst a trend is apparent within our results it should be noted that this study should be thought of as preliminary work. The results must be validated by a process of repetition and by using different techniques e.g. investigation of the RNA/protein levels of markers of differentiation. Nonetheless, results from this project are suggestive that infliximab has a positive effect on the bone loss that occurs in IBD.

In conclusion, the importance of the above experiment as described in section 5.3.3, 5.3.4 and 5.3.5 is particularly important in understanding the role of infliximab directly on osteoblast cultures. On the basis of these results, the positive changes noted with infliximab on bone metabolism appear to be attributable to the direct effect on bone cultures itself as infliximab within the pharmacological usage achieved in patients caused an increase in both osteoblast viability and functionality.

CHAPTER 6 – GENERAL DISCUSSION

In this thesis we aimed to investigate the effects of two anti-TNF-α therapies; adalimumab and infliximab on bone metabolism in patients with IBD. As described previously the major differences between both these therapies are firstly, adalimumab is a fully humanized IgG1 monoclonal antibody against TNF-α that is currently licensed for the use in active CD patients whereas infliximab is a chimeric IgG1 monoclonal antibody comprising of 75% human and 25% murine sequences, which has a high specificity for and affinity to TNF-α and is licensed for the use in both active UC and CD patients. Secondly, the delivery mode of infliximab is by intravenous infusion every 8 weekly whereas adalimumab is given subcutaneously every fortnight. There are no head-to-head trials conducted to compare these two therapies, but it appears that both medications have similar results in disease treatment. However, there are a number of advantages for using adalimumab. Firstly, subcutaneous administration of adalimumab is a potential advantage to those who prefer self administration. Secondly, reported rates of anti-adalimumab antibodies are lower than with infliximab as adalimumab is less immunogenic in humans because it is a fully humanized antibody. Thirdly, adalimumab can be given to those individuals who have poor outcomes with infliximab (lose response, develop antibodies or experience infusion reactions).

To date, studies looking at the effect of these biological therapies on bone metabolism in IBD patients are limited and there are a number of huge limitations with the currently available data. Firstly, the duration of follow up are short, with longest being only for 14 weeks [263], hence the long term effect on maintenance therapy of anti-TNF-α on bone markers which most likely to correspond to a meaningful effect on bone density or even

fracture rates are not possible to be assessed. Secondly, only four studies to date [262,263,265,269] have examined the differences of bone markers changes between responders and non-responders group. The benefit seen on bone metabolism with anti-TNF therapy based on clinical or biological response in terms of effect on CD activity showed equivocal results. One study failed to show any difference between the two groups [262], another study [263], showed the trend of increase in both bone formation markers and decrease in bone resorption marker was greater in responders compared with non-responders but were not statistically significant and in two of the remaining studies [265,269] statistical significance was recorded between the two groups suggesting biological response may play a role. Hence is it simply the neutralization of TNF-α that is critical; if in fact these drugs have a role or is it that those whose disease activity improves secondary to dampening down of the inflammation by TNF-α are the ones whose bone markers improves cannot be said for certain with currently available data. This is really important as if the latter is true, then maybe the improvement in bone markers is not specific to the anti-TNF but rather is simply secondary to reducing the inflammatory cytokines and other medications such as thiopurines or methotrexate may have similar effects. Thirdly, no studies have yet evaluated the effects of adalimumab therapy on bone metabolism in CD patients or the effect of infliximab therapy in UC patients. Finally, the effects of these therapies on bone cells have not yet been studied. This is crucial to understanding the direct effect of anti-TNF-α on bone cells. Long term studies are also needed to compare changes in cytokines, osteoclastogenesis markers (OPG and RANKL), bone nutrients (PTH and vitamin D) and most importantly on BMD to fully understand the exact mechanisms involved.

In this thesis we sought to answer the above limitations that are currently present in this field. Furthermore, by using an *in vitro* model of osteoblast culture, we sought to examine the effects of sera from such patient's pre and post treatment, and control sera on osteoblast cultures in controlled, optimized conditions. The purpose of this *in vitro* model was to try to mirror/represent the possible effects that circulating cytokines within patient sera might be having on osteoblast function *in vivo*. As this cannot be readily directly measured *in vivo*, the *in vitro* model was used to examine these effects.

There were a number of similarities seen between the medium term effects of adalimumab and the long term effects of infliximab on bone metabolism in our group of IBD patients. Firstly, both treatments showed a significant improvement in treated patients disease activity as seen by a decrease in the CDAI score (in the CD patients), decrease in the SCS score (in the UC patients) and normalization of the CRP levels. These findings are in keeping with the known effects of these potent anti-inflammatory agents in achieving clinical remission as seen with larger randomized control trials [251,252,256,258-261]. Secondly, both therapies also showed reduction in the mean daily dosage of steroid use by the IBD patients with time. Although, in the adalimumab group the mean decrease in steroid use failed to reach the significant levels at the end of the study, the trend of decrease was noted. This again would correspondent with the known effects of these anti-TNF-α therapies on the elimination of steroid use as seen in larger studies [251,252,256,258-261].

Thirdly, both therapies showed a favorable effect on the bone formation markers measured in active IBD patients. In the infliximab treated patients, there was a significant and rapid increase in OC and P1NP markers of bone formation which was seen within 6

weeks of therapy and this continue to rise till week 30. Although levels of bone formation markers were higher at week 54 compare to baseline, this was not significant. Fewer patients were available for analysis at this time point and this might explain in part, the lack of statistical significance seen. In the adalimumab treated patients, again similar significant and rapid increases were seen in both OC and P1NP levels within 1 month of therapy. These results are comparable to those found in studies looking at the effect of infliximab on bone metabolism in CD patients [263, 264]. In both these studies, these favorable effects were not associated with baseline DXA findings, current steroid therapy, steroid weaning, clinical characteristics of the patients, previous TNF-α exposure (for those in the adalimumab study only) or indeed on biological response to adalimumab. However, a greater significant increase in OC and P1NP was observed in responders compared with non responders at week 6 in the infliximab treated group, suggesting that, the greater the biological response to infliximab, one can expect a higher beneficial effect on bone formation markers. This finding may suggest that the positive effects seen one bone metabolism with anti-TNF-α therapy, could be related in partly, to the decrease in the disease activity. Fourthly, both studies although did show a continuous trend of reduction in the bone resorption marker, CTx serum with time, the changes seen failed to reach a statistical significance though. As the final outcome of both the therapies resulted in a net increase in the bone formation markers, it can be concluded that both these therapies have a positive effect on the bone markers.

Although one might have expected improvement in bone nutrients with biologic therapy secondary to increase in absorption of the critical nutrients required for bone modeling, particularly in CD patients with small bowel disease, our study failed to show

any significant effect. As the findings were seen with both the anti-TNF therapies, it is unlikely the positive changes seen on bone metabolism is due to the effects of anti-TNF reducing gut inflammation, hence resulting in increase in resorption of the crucial bone nutrients required for bone formation as suggested by previous study [262]. In both studies, we found higher levels of both OPG and sRANKL in IBD patients at baseline compared to controls. These results are consistent with findings of two previous studies which reported firstly, higher concentration of OPG in active CD patients compared to controls [124] and secondly, decreased OPG levels following infliximab therapy [269].

Finally, the results of our *in vitro* studies between the two therapies resulted in similar outcome. Exposure of osteoblasts to sera from active CD adalimumab treated patients and active IBD infliximab treated patients showed consistently higher levels of viable cells compared to control subjects at every time point measured. Anti-TNF-α therapy led to a decrease in levels towards those of controls. This shows that both adalimumab and infliximab do in fact have an effect of osteoblast function which is crucial for bone formation. As previously discussed, higher osteoblast cell viability observed pre treatment is most likely an inflammatory driven response. Although higher osteoblast viability was found pre treatment with biologics, the amount of ALP which was secreted was significantly lower in pre treatment compared to control patients. With therapy, the amount of ALP secreted by these viable cells increased. This clearly shows that both adalimumab and infliximab treatment have a direct effect on the human osteoblast *in vitro* and this in turn strongly suggests that the increase in bone formation markers observed post treatment *in vivo* is a reflection of this beneficial effect.

There were some minor differences noted in our studies between adalimumab and infliximab. Firstly, with infliximab OPG decreased and sRANKL concentration increased over time consistent with a previous study [269]. However, with adalimumab therapy both OPG and sRANKL decreased over time, whether smaller number of patients in the adalimumab group or shorter term of follow up could have resulted in this outcome is not apparent and would need further analysis in a larger longer term study to explain this apparent difference noted.

Secondly, it has been suggested that bone loss in IBD patients is a result of cytokines (IL-1, IL-6 and TNF-α) released from inflamed intestine directly influencing osteoblast and osteoclast function [78, 157]. In the adalimumab study, there was no significant association found between the serum cytokine levels and bone turnover markers or adalimumab therapy. However, the patient numbers in the adalimumab study again were small, and larger studies might well find such an association. A previous study in patients treated with infliximab found that mucosal but not systemic measurements of pro-inflammatory cytokines mirrored response to therapy [297]. We did not measure mucosal cytokine levels in this study. However, there was significant association found between TNF-α, IL-1β, IL-6, and IL-13 and infliximab therapy. Besides TNF-α, the significant changes noted in IL-1β, IL-6 and IL-13 were only seen in responders, suggesting a direct relationship between mean change in cytokine with infliximab therapy and biological response to infliximab.

Finally, the major difference between the studies was based on the results of the DXA findings. A repeat DXA was only carried out in our infliximab treated patients as this was a long term study up till 1 year whereas a repeat DXA was not carried out in the medium

term adalimumab study as it was only a 6 month study. Results of DXA findings at 1 year in IBD patients treated with infliximab showed wide variety among patients. There was no significant change noted in mean T score in 6 patients. However, 4 patients showed significant improvement in their T score with infliximab therapy at 1 year follow up. One of these patients was also on a bisphosphonate and 2 were on calcium and Vitamin D supplement during this study period, hence the improvement seen in their T scores could be partly due to known effects of bisphosphonate or calcium and Vitamin D therapy on bone density. Despite that our study together with previous studies from rheumatoid arthritis [294], spondylarthropathy [295] and CD [266-268] populations add to growing evidence that infliximab maintenance therapy does have a beneficial effect on BMD. The significance of these findings, suggest infliximab may play a role in improving bone strength and lead to fracture risk reduction. However, it has to be emphasized that BMD measured by DXA is only one of many contributors to bone strength and fracture risk measurements. Other factors such as the bone quantity, which consists of density and size, and bone quality, which, in turn, consists of structure (micro and macro-architecture), material properties and turnover are all important factors that need to be considered.

Our study has a number of limitations. Firstly, this was not a randomized study and the number of patients included was small, therefore meaningful analyses of various subgroups were restricted. Secondly, markers of bone turnover, bone nutrients, osteoclastogenesis, and cytokines varied from patient to patient in this study, and this is likely to reflect the heterogeneous nature of bone loss in IBD patients. However, to counter for this, each patient acted as his/her own control. Thirdly, BMD was measured

at baseline only in the adalimumab treated patients, as the study was 6 months and it would be unlikely that DXA scans would show any meaningful changes over this time period. Previous studies looking at the effects of bisphosphonates therapy on BMD in CD patients only showed appreciable change in BMD on DXA after 1 year of therapy [232]. Repeat DXA scan at 1 year interval as done in the infliximab treated patients would be fruitful in future studies of longer duration to see whether the beneficial effects on bone formation markers and osteoblast function translate into meaningful improvements in DXA scan, and by extrapolation into reduction in fracture risk. Fourthly, the number of patients at the end of 1 year follow up in the infliximab treated group was considerably lower than at baseline, hence this could have resulted in some of the non significant results noted at week 54. Finally, it is debatable as to whether all our control patients can be considered as 'real' controls given that 2 of them had osteoporosis and 9 of them had osteopenia based on DXA scan measurement. This highlights the fact that even among presumed healthy subjects in the general population many people have undetected lower BMD and vitamin D levels. However, this sub group of control patients did not exhibit significant differences in any of the parameters measured in this study as compared to remaining control patients.

To counter for some of these limitations of our study, it is important to highlight that this research also has a number of strengths. Firstly, the period of follow up is considerably longer than for any existing studies looking at the effects of biologic therapy on bone metabolism in IBD patients. Secondly, it is the first study to attempt to comprehensively examine and correlate both the *in vivo* effects of biologic therapy on bone turnover markers, cytokines and osteoclastogenesis markers with detailed *in vitro*

osteoblast studies. In this context, as discussed above, this body of work has given *in vitro* explanations for the *in vivo* changes in bone markers found in studies of biologics on bone metabolism. Thirdly, no existing studies have looked at the effects of biologics on bone metabolism in UC patients. Our study included a group of UC patients treated with infliximab, and the results found were comparable to those of CD patients. Fourthly, this is the first study to examine the effect of adalimumab on bone metabolism. The results found were comparable, if not identical to those found with infliximab treatment, suggesting that the effects of anti-TNF-α biologic agents on bone metabolism represent a group effect rather than an effect specific to a particular agent. We have shown the beneficial effect seen with the biologic therapies does seem to be heterogeneous in aetiology and to vary from patient to patient. Primarily, the main beneficial effect appears to be related to an increase in bone formation although a decrease in bone resorption also appears to play a role in some patients. The effects seem to be dependent of whether the response and non-response to treatment based on clinical score at least in the infliximab treated patients. Furthermore, the beneficial effects on bone metabolism appear to be independent of steroid therapy for both infliximab and adalimumab group. This is also, the first study that has examined the direct effect of the infliximab at physiological concentration on human osteoblasts and we have shown that infliximab has a direct effect on bone cultures which would correspondent to the positive changes noted with infliximab on bone metabolism in our *in vivo* work.

Finally, we believe that this dissertation has succeeded in fulfilling one of our primary aims of this study, which was to improve our understanding of the mechanisms involved in biologic related changes in bone metabolism. These mechanisms are clearly very

complex, and in this regards, some of the findings raise further questions and further study. For example, why do we observe a fall in OPG and rise in sRANKL associated with biologic therapy, and yet we observe a concomitant increase in markers of bone formation *in vivo*, a reduction albeit non significant in the markers of bone resorption *in vivo*, and increased osteoblast functionality *in vitro* when exposed to relevant sera. These findings are similar to those found in another, more limited study [269], suggesting that these are very real observations.

CHAPTER 7 – CONCLUSION AND FUTURE WORK

Bone metabolism is a dynamic, complex balance of bone formation and resorption, and disruptions to this homeostasis can result in bone loss, as seen in patients with IBD and other inflammatory conditions. The mechanisms involved are most likely various and heterogeneous but our results show that both adalimumab and infliximab therapy have a direct effect on the osteoblast and a beneficial effects on markers of bone metabolism. We did not look at osteoclast function *in vitro* as a suitable model was not available at the time of this study. However, future studies involving a human *in vitro* osteoclast model would be helpful to further our understanding of the complex mechanism involved. Both adalimumab and infliximab treatments were found to have similar, if not identical effects on bone metabolism. This is the first study to examine the effects of adalimumab in this context but a larger study is needed. These results suggest that the effects of anti-TNF-α biologic agents on bone metabolism represent a group effect rather than an effect specific to a particular agent. As with most research, this body of work raises further questions for further research. For example, it would be important to examine the longer term effects of the biologic therapies on bone metabolism and bone health after a number of years. Do apparent benefits in markers of bone metabolism translate into long term benefits in BMD as measured by DXA? What happens to bone metabolism and BMD when biologic therapies are withdrawn as they often are due to loss of efficacy or immune-type reactions? These important questions are beyond the scope of this current body of work, but we would hope to continue follow up of these patients in the longer term to address some of these issues.

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