The role of adipose secreted cytokines in driving the pathological changes in Osteoarthritis

by

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Abstract

Adipose secreted cytokines are thought to contribute to pro-inflammatory state seen commonly in obese individuals, providing a potential metabolic link between obesity and osteoarthritis. The aim of this study is to further our understanding of the role of adipokines within OA by examining the serum and joint fluid adipokine expression profiles in relation to disease severity, BMI, and joint tissue turnover markers. The result of this study show that subchondral bone from overweight/obese hip OA patients exhibited reduced trabecular thickness, increased bone surface/bone volume ratio and an increase in the type I collagen α1/α2 ratio, compared to normal-weight hip OA patients. The serum concentration of resistin was higher in overweight/obese OA patients, compared to normal-weight OA patients (12740 vs 9818pg/mL respectively; $p<0.05$). Stimulation of normal-weight bone explant with recombinant resistin resulted in a 2.4 fold increase in type I collagen α1/α2 ratio (1.6:1 vs 3.8:1, $p<0.01$). Stimulation of primary OA osteoblasts with resistin increased Wnt signalling activation, osteoblast metabolic activity, and bone nodule formation. In addition, visfatin was elevated in the synovial fluid and in isolated synovial fibroblasts of obese hip OA patients compared to normal-weight patients. In cartilage, visfatin increased the production of 15 pro-inflammatory cytokines and chemokines, with significant increases in IL-6, CCL4, MCP-1 and in CCL20 compared to the media only control (>7 fold, 20-fold, 4-fold and 7-fold respectively). Visfatin significantly increased in catabolic proteases including MMP-1 (4-fold), MMP-2 (3-fold), MMP-3 (3-fold), MMP-7 (2.2-fold), MMP-8 (1.3-fold), MMP-9 (1.2-fold), MMP-10 (1.5-fold), and MMP-13 (5-fold) and localised to areas of cartilage damage.

Targeted inhibition of adipokine signalling could therefore be a rewarding strategy for developing a novel therapeutic.

Dedication

To my dear husband Andy and daughter Olivia.

“Piglet noticed that even though he had a Very Small Heart, it could hold a rather large amount of Gratitude.”

— **A.A. Milne**, **Winnie-the-Pooh**

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°C	Degree centigrade
ADAMTS	A Disintegrin and Metalloproteinase with Thrombospondin motifs
ALP	Alkaline Phopsphatase
AMPK	AMP- activated protein kinase
ANOVA	Analysis of variance
ATP6V1G3	ATPase H+ Transporting V1 Subunit G3
AXIN2	Axis Inhibition protein 2
BCA	bicinchoninic acid assay
BDKRB1	Bradykinin receptor B1
BDKRB2	Bradykinin receptor B2
BGLAP	Bone Gamma-Carboxyglutamate Protein
BMD	Bone mineral density
BMI	Body mass index
BMP	Bone Morphogenetic Protein
BOD1	Biorientation of chromosomes in cell division 1
BS/BV	Bone surface/Bone volume
Ca^{2+}	Calcium
CBL	Casitas B-lineage lymphoma
CCL	CC Chemokine ligands
cDNA	Complementary DNA
CHSY1	Chondroitin Sulfate Synthase 1
CILP	Cartilage intermediate layer protein
CO_2	Carbon Dioxide
COL1A1	Collagen type I Alpha I

COL1A2	Collagen type I Alpha 2
COMP	Cartilage oligomeric matrix protein
CTSB	Cathepsin B
CTSL2	Cathepsin L2
CTX-II	C-terminal crosslinked telopeptide type II collagen
CXCL	C-X-C motif ligand
DAP12	DNAX-activating protein 12
Dkk1	Dickkopf-related protein 1
DMOADS	Disease modifying osteoarthritis drugs
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
eVisfatin	Extracellular visfatin
F7	Coagulation factor VII
FABP4	Fatty acid binding protein 4
FADS2	Fatty acid desaturase 2
FASLG	Fas ligand
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
FOSL1	FOS Like Antigen 1
FRZB	Frizzled-Related Protein
FZD4	Frizzled Class receptor 4
GABBR1	Gamma-Aminobutyric Acid Type B Receptor Subunit 1
GAD2	Glutamate Decarboxylase 2

gp130	Glycoprotein 130
GPR61	G Protein-Coupled Receptor 61
GPRC5B	G Protein-Coupled Receptor Class C Group 5 Member B
GSK3β	Glycogen Synthase Kinase 3 Beta
HANES I	National Health and Nutrition examination Survey I
HCRTR2	Hypocretin Receptor 2
HNRNPU	Heterogeneous nuclear ribonucleoprotein U
HSC	Hematopoietic stem cells
IL	Interleukin
IP10	C-X-C motif chemokine 10
IPFP	Infrapatellar fat pad
iVisfatin	Intracellular visfatin
K and L grade	Kellgren and Lawrence grade
KCNJ11	Potassium Voltage-Gated Channel Subfamily J Member 11
KCNJ12	Potassium Voltage-Gated Channel Subfamily J Member 12
KCNK9	Potassium Two Pore Domain Channel Subfamily K Member 9
KD	Knockdown
KO	Knockout
LFNG	Lunatic Fringe protein
LIF	Leukemia inhibitory factor
LN_2	Liquid nitrogen
LRP5	Low-density lipoprotein receptor-related protein
MAP	Mean arterial pressure
MC3T3-E1	Osteoblast precursor cell line
MCP	Monocyte chemoattractant protein

M-CSF	Macrophage colony-stimulating factor
MIP1α	Macrophage Inflammatory Proteins 1 alpha
MIP1β	Macrophage Inflammatory Proteins 1 beta
MIP3α	Macrophage Inflammatory Proteins 3 alpha
MMP	Matrix mettaloproteinase
MYC	Myelocytomatosis viral oncogene homolog
NAD	Nicotinamide adenine dinucleotide
NC	Negative Control
NFAT1c	Nuclear factor of activated T-cells 1c
NFATC1	Nuclear Factor of Activated T-Cells
NMN	Nicotinamide mononucleotide
NOF#	Neck of femur fracture
NSAID	Non-steroidal anti-inflammatory drugs
NW	Normal weight
OA	Osteoarthritis
OARSI	Osteoarthritis Research Society International
OB	Obese
OPG	Osteoprotegerin
OSN	Osteonectin
OW	Over weight
PEAR1	Platelet endothelium aggregation receptor 1
PI3K/AKT	Phosphoinositide 3-kinase/Protein kinase B
PIIANP	Type IIA procollagen amino terminal propeptide
PINP	Procollagen I Intact N-Terminal
PITX2	Paired like Homeodomain 2

PLA2G3	Phospholipase A2 Group III
PODXL2	Podocalyxin Like 2
P-P plot	Probability–probability plot
PRELP	Proline/arginine rich end leucine rich repeat protein
PRG-4	Proteoglycan-4
PTH	Parathyroid hormone
QSOX1	Quiescin Q6 Sulfhydryl Oxidase 1
RANKL	Receptor activator of nuclear factor kappa-B ligand
RFU	Relative fluorescence units
RNA	Ribonucleic acid
RT	Room temperature
RT-PCR	Reverse transcription polymerase chain reaction
RunX2	Runt-related transcription factor 2
SDC2	Syndecan 2
SEM	Standard error of the mean
sGAG	Sulfated glycosaminoglycan
siRNA	Small interfering RNA
SLC2A11	Solute Carrier Family 2 Member 11
SLC7A8	Solute Carrier Family 7 Member 8
STAT3	Signal transducer and activator of transcription 3
Tb. N	Trabecular number
Tb.Sp	Trabecular separation
Tb.Th	Trabecular thickness
TCF/LEF	T-cell factor/lymphoid enhancer factor
TCF7	Transcription Factor 7

TGF-β	Transforming growth factor beta
TIMP	Tissue inhibitor of matrix metalloproteinase
TNFα	Tumour Necrosis Factor
TRAcP	Tartrate-resistant acid phosphatase
TSPAN9	Tetraspanin 9
VIF	Variance inflation factor
W:H	Waist:Hip ratio
WAT	White adipose tissue
WNT2B	Wnt Family Member 2B
WNT5A	Wnt Family Member 5A
WNT7B	Wnt Family Member 7B
WT	Wildtype
YLD	Years lived with disability

1.1 General Introduction

The World Health Organization has characterized obesity as reaching epidemic proportions globally, with over 1 billion individuals being overweight, and 300 million of those clinically obese [1]. In England alone, obesity has increased from 14.9% of the population in 1993, to 25.6% in 2014 [2]. Should this trend continue, this figure is set to rise to 55% of the population affected by obesity by 2050 [3]. Excessive weight is associated with a number of other health detriments including hypertension, stroke, diabetes, and arthritis. Increases in these co-morbidities in ageing populations will result in a substantial burden on the economic costs to the healthcare system, in an era of already exponential medical expenditure.

With improved sanitation, socio-economic developments and medical advances, life expectancy has steadily increased. In fact, from 2015 to 2020, the number of individuals over the age of 65, 85, and 100 is set to rise by 12%, 18% and 40% respectively. This is in comparison to the 3% increase in the general population [4]. Yet despite the improvement in living conditions and subsequent lifespan, demands on a healthcare system supported by a relatively smaller tax paying population may prove to be the most challenging of this century.

While life expectancy continues to increase, healthy life expectancy has had little improvement, with only an additional 0.3 and 0.6 years gained since 2000-2011 in healthy life expectancy in males and females respectively. From 2005 to 2012, UK women have gained 1.3 years in life expectancy, yet only 0.1 years additional healthy life years [5]. This suggests that the extra years of life gained are spent in ill health and disability.

In the Global Burden of Disease Study 2010, osteoarthritis (OA) accounted for 2.2% of years lived with disability (YLD). Critically, OA was found to be the fastest growing major health concern with an increase of 64% between 1990-2010 (Figure 1.1) [6], not only contributing to burden on the healthcare system, but also to personal burden experienced by individuals with symptomatic OA. For example, OA pain significantly impacts the lives of OA patients, through the inability to perform everyday tasks [7], reduced independence [8], insomnia [9], and personal stress [10]. The aim of an OA therapeutic is therefore to provide pain relief as well as to prevent disease progression and restore joint function. However, the development of such therapeutics has proved highly challenging, in part due to the heterogeneity of OA [11], and due to the source of OA pain remaining elusive [12, 13]. Thus many patients with OA will take over-the-counter analgesic medications, including topical creams, paracetamol (acetaminophen) and non-steroidal anti-inflammatory drugs (NSAIDs), such as Ibuprofen. These medications often provide only limited pain relief, particularly for patients with advanced disease, and critically, they do not prevent disease progression. Furthermore, toxicity has been demonstrated with prolonged high doses of paracetamol, and therefore despite being the primary therapeutic subscribed by clinicians, it is prescribed with caution [14].

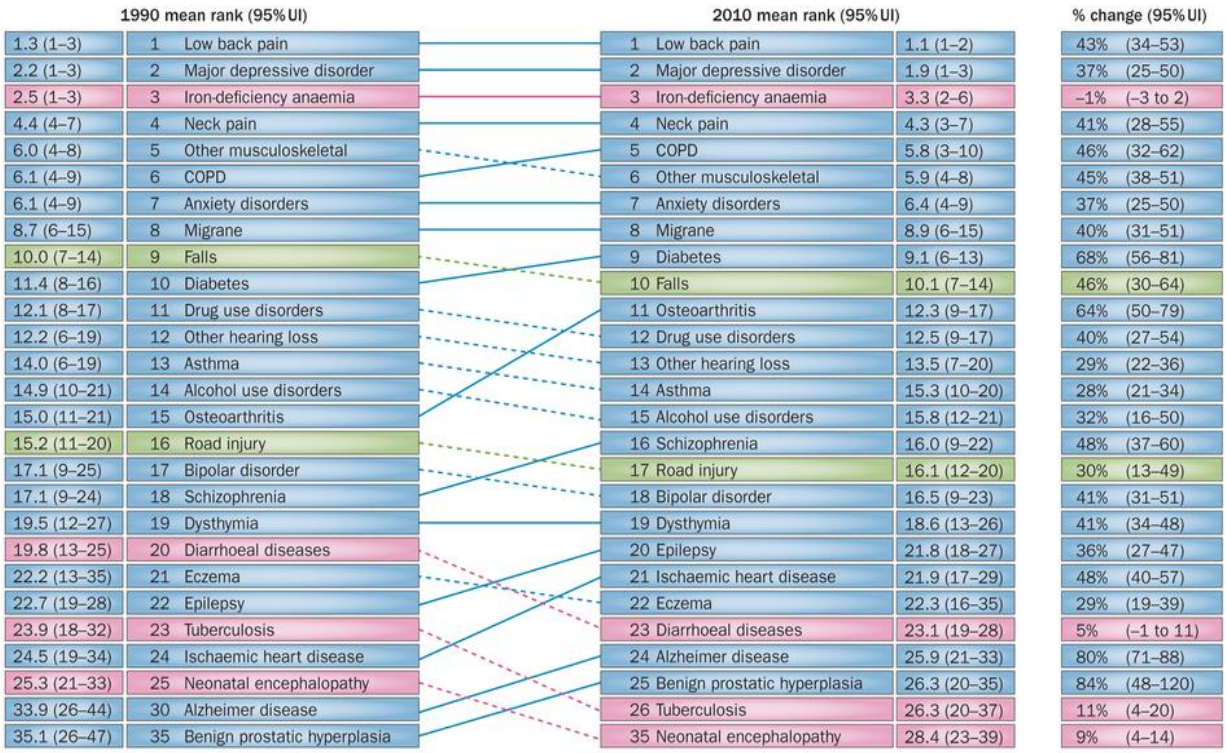


Figure 1.1 Ranking of chronic disorders associated with YLD between 1990 and 2010, demonstrating the increased burden of osteoarthritis in healthy life expectancy.

Pink boxes = communicable, maternal, neonatal, and nutritional disorders; blue boxes = Non communicable disorders; and green boxes = Injury.

Some NSAIDs, such as selective COX2 inhibitors, have demonstrated promising disease modifying capabilities in clinical studies. However the side effects are less desirable, for example heart failure, persistent headaches, stomach ulcers and anaemia [15]. Therefore a great unmet clinical need remains for more effective OA therapeutics, particularly those that can prevent disease progression. Without such medications, the recent OARSI guidelines [16] suggest a more holistic approach to

OA treatment, including acupuncture, weight loss, and increased patient/doctor communication as treatment for patients with symptomatic OA [16].

Arguably one of the most important, and most modifiable risk factors associated with OA is obesity. Adipose tissue expansion with obesity can result from both hypertrophy (increased adipocyte size), and hyperplasia (increase in adipocyte number), both of which are impacted by genetics and dietary intake. Adipocyte hypertrophy is defined as an adaptive response to nutrient excess in adulthood, thus protecting other organs from lipotoxicity [17]. In lean humans, the increase in adipocyte size is essential to maintain a homeostatic response to over-feeding [18], however in obesity it is understood that a hypertrophic threshold may be surpassed, adipocyte buffering capacity is exceeded, and lipid is deposited in peripheral tissues [19]. Further in obese, adipocyte hypertrophy can result in inadequate oxygenation of the adipose tissue due to a number of factors including ineffective O_2 diffusion [17], increased O2 consumption of adipose tissue [20] and decreased capillary density [21]. This hypoxic state may result in altered adipose tissue function including reduced adipogenic differentiation [22], altered glucose homeostasis and inflammatory cytokine [23, 24]

Historically, adipocyte hypertrophy was regarded as the only route whereby fat mass increased in obese adults however recent evidence has demonstrated a role for adipocyte hyperplasia in regulating fat mass in adult obesity. Adipocyte hyperplasia consists of two stages, namely; pre-adipocyte proliferation and pre-adipocyte differentiation. Contrary to the role of adipocyte hypertrophy, adipocyte hyperplasia is thought to have a protective role over the development of metabolic disorders associated with adipocyte hypertrophy. Using ^{14}C tracer analysis, average adipocyte half-life was determined as 8.3 years, with over 10% of adipocytes renewed annually

in adulthood [25]. Furthermore, the total cell number remains stable throughout different BMI categories [25]. This infers that propagation of precursor cells into mature lipid filled adipocytes occurs at the same rate of adipocyte apoptosis. This is particularly relevant to obesity where a higher rate of apoptotic cell death results in increase adipocyte cell turnover [26] therefore a greater number of small adipocytes exist to offset the increasing lipid content in these individuals.

As adipose tissue expansion occurs with progressive obesity, so too does the release of a plethora of bioactive molecules and cytokines referred to as 'adipokines' into the systemic circulation. Accordingly, this adipokine release has been mechanistically linked to metabolic complications and the metabolic syndrome [27].

Adipokines are pleiotropic molecules that contribute to the low-level pro-inflammatory state seen commonly in obese individuals [28, 29]. Several hundred adipokines have so far been identified, including adiponectin, leptin, resistin, and visfatin [30]. Being secreted into the circulation (although not always exclusively derived from adipose tissue), it is not surprising that the circulatory concentrations of several adipokines correlate with body fat mass, and are often associated with anthropometric measures such as BMI and W:H ratio. Furthermore, studies have demonstrated a "normalization" of adipokine profiles following weight loss which is associated with the normalization of metabolic indices, suggestive of adipokines being key mediators of metabolic health [31-33].

In addition to fat mass percentage, adipose tissue distribution can also play a significant role in metabolic complications and inflammation. There are number of fat depots in the human body including; intra-abdominal (omental or visceral fat), lower body (gluteal, subcutaneous and intramuscular fat), and upper body (subcutaneous

fat) [34]. The amount of visceral fat in relation to peripheral tissue obesity can have a significant impact on the development of metabolic disorders, with an increase in atherosclerosis, diabetes and hypertension disease risk with an increasing ratio of central to peripheral adiposity [35, 36]*et al.,* . This association between fat depots and metabolic dysfunction may be due to the fat depot specific expression of adipokines. For example, IL6 has previously been shown to have increased expression in visceral fat tissue [37] and omental fat tissue [38] compared with subcutaneous adipose tissue. Adiponectin expression has been shown to be increased in omental tissue compared with subcutaneous [39], however leptin expression shows the opposite with higher expression identified in subcutaneous tissue compared to omental [40].

In the last 10 years, adipokines have received much interest in relation to OA joint pathophysiology, demonstrating important roles in maintaining cartilage and bone health [28]. Adipokine biology is now considered to be a key biological effect area for identifying and developing new OA drugs. Therefore, examining the expression and function of adipokines in cartilage, bone, and adipose tissue from patients with OA, and understanding the key signalling mechanisms, may identify new targets for therapeutic modification, which mediate adipokine signalling pathways and modulate pathological processes within the OA joint.

The overarching aims of this thesis are two-fold: firstly to determine the relationship between adipokine profiles and markers of joint pathology and OA disease severity across different patient populations with OA. Secondly is to further our understanding of the role of adipokines in OA by determining the functional effect of adipokine stimulation on cartilage and bone pathology. Ultimately, such studies will aid in the

identification of novel targets for therapeutic modulation to prevent OA disease development.

1.2 Anatomy of the synovial joint

In this study, we have focused on the hip and knee joints; therefore we shall focus upon the anatomic structure of these two synovial joints herein. The coxofemoral (hip) joint is a ball-and-socket type articulation of the femur and the acetabulum cup of the pelvic bone [41], and joins the upper and lower parts of the body. The femoral head is a spherical structure with a small depression at the site of the Ligamentum Teres attachment. In contrast, the knee joint is a modified hinge joint comprising of the tibia, femur and patella, allowing flexion and extension [42]. Unlike the hip joint, it allows only a minimal amount of medial and lateral rotation. The strength and stability of the knee joint depend almost entirely upon the ligaments and muscles surrounding the joint, and fibrocartilaginous crescents known as menisci serve as shock absorbers to joint loading [43]

Synovial joints articulate within a fluid-filled cavity, which lubricates the joint and allows for a wide range of movement with reduced friction. Synovial joints consist of distinguishable features including; articular cartilage, a synovial cavity, an articular capsule, synovial fluid and fatty pads. The hip and knee joint anatomy have many similarities. Each have an outer articular capsule important to passive and active joint stability *via* its proprioceptive nerve endings [44] and a synovium responsible for joint fluid production, cartilage nutrition, and modulation of immune cells in response to bacteria or fragment material [45]. It is able to adapt to environmental stress through thickening, vascularisation, villi formation and inflammatory cell infiltration

[45]. Furthermore, to allow for smooth articulation, both the knee and hip joint articulating bones are covered by layers of articular cartilage tissue.

The hip and the knee anatomy are thought to differ in their intracapsular fat pads termed the fossa fat pad (hip) and infrapatellar fat pad (IPFP, knee). The IPFP has received a lot of attention with regards to its role in knee disease pathologies [46], and has been defined as pro-inflammatory, releasing TNFα, FGF and IL6 [47], and adipokines adiponectin, leptin and chemerin [29, 48]. However total IPFP area in patients with OA has been reported to be negatively associated with osteophytes, bone marrow lesions, knee pain and JSN, suggesting it may have a protective role in the development of OA [49]. Contrary to the IPFP, the fossa fat pad has received very little attention in terms of its role in the pathogenesis of hip disorders, perhaps because of its much smaller size and relative inactivity compared to the IPFP [50].

1.2.1 Zonal articular cartilage organization

Articular cartilage is a highly specialized tissue, which covers the epiphyseal ends of diarthrodial (synovial) joints. It is principally designed to allow for smooth joint movement across the range of motion. Morphologically, articular cartilage can be separated into distinct zones, each with a different function and cellular organisation. Namely, the superficial zone, the transitional zone and the deep zone (Figure 1.2).

According to Fox *et al.,* (2009), the superficial zone, situated immediately under the articular surface, contains parallel collagen fibres and phenotypically flattened individual chondrocyte cells. These chondrocyte cells are embedded within a matrix of high collagen and low proteoglycan concentration, and in culture secrete less collagen and proteoglycans compared to chondrocytes cultured from the other

cartilage zones [52]. It is thought the dense, parallel collagen fibres are responsible for the greater tensile strength and resistance of shear stress generated during articulation [53-55]. Maintenance of the superficial layer is believed to be integral to cartilage health [53], since disruption precedes any gross structural damage in the cartilage matrix.

In the transitional zone the collagen fibres exist obliquely, proteoglycan content increases, and the chondrocytes exhibit a more rounded morphology. In comparison to the superficial zone, water and collagen content are both reduced. This zone contributes to over 40% of the total cartilage volume, and provides resistance to compressive forces [53].

The deep zone of cartilage extends through to the calcified cartilage interface (termed the tidemark), with collagen fibres formed perpendicular to the articular surface extending into the tidemark, and chondrocytes arranged in a columnar structure. Due to the arrangement of the collagen fibres, the deep zone provides the greatest resistance to compressive forces. It also has the highest proteoglycan content, along with the lowest water content [51].

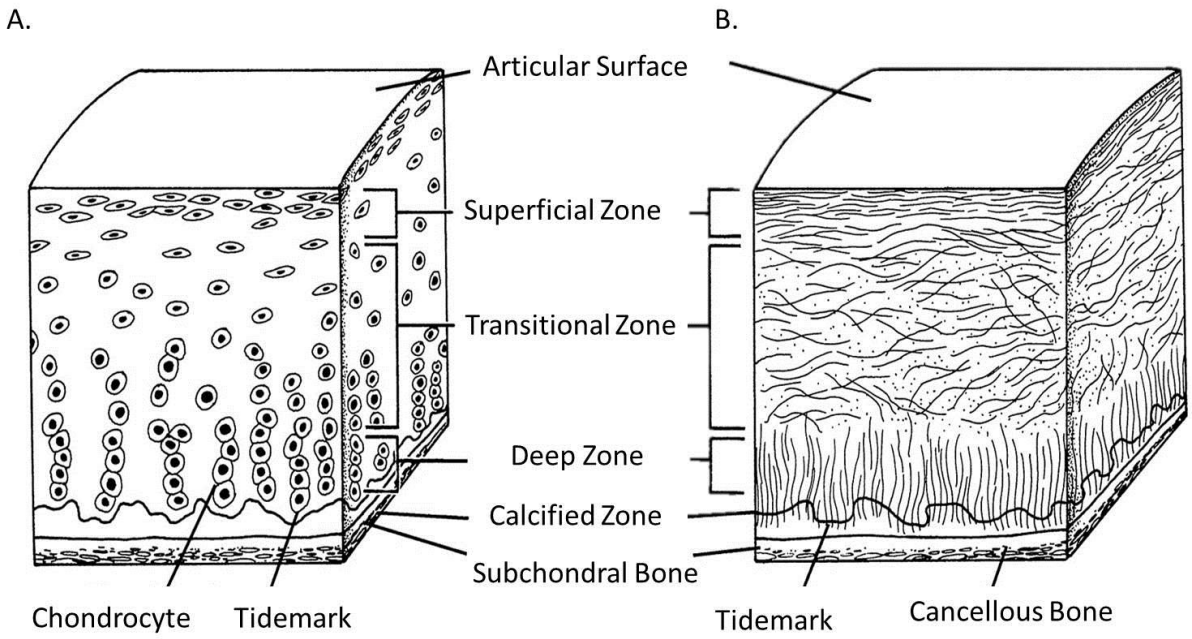


Figure 1.2. Zonal articular cartilage organization adapted from the American Academy of Orthopaedic Surgeons [56].

A. A cross-sectional diagram of the cellular organisation of healthy articular cartilage zones. B. A cross-sectional diagram of the collagen fibre architecture throughout healthy articular cartilage zones.

1.2.2 Regional organisation of extracellular matrix

In addition to the zonal organization of articular cartilage, the extracellular matrix (ECM) surrounding chondrocytes is divided into three distinct regions; the pericellular, territorial, and interterritorial regions. The pericelluar region refers to the narrow layer of matrix that immediately surrounds the chondrocyte cell [51, 57, 58] which together is referred to as a chondron [59]. This region is abundant with fibronectin, prostaglandins [60-64] and collagen Type II, but is distinguished by its expression of collagen type VI surrounding the chondrocyte cell [65]. The role of the

pericellular region is not fully established, however recent evidence suggests it may play a significant role in signal transduction during load bearing [59, 66, 67].

The territorial region is a mesh of fine collagen fibrils, which surround and adhere to the pericellular matrix [68-70] in a 'basket-like' formation. This formation of collagen fibrils is thought to provide structural support and protection for the chondrocytes during compressive loads. With increasing space from the chondrocyte cell, the collagen fibrils begin to widen in diameter and orientate into a more parallel fibril alignment, marking the beginning of the inter-territorial matrix region [52].

The inter-territorial matrix region forms the majority of the ECM, and consists of wide-diameter collagen fibrils arranged randomly according to their location within the articular cartilage zones. This zone contains an abundance of proteoglycans, collagen oligomeric matrix protein (COMP), and type II collagen, and maintains the tensile stiffness and strength of the ECM [52, 59, 71].

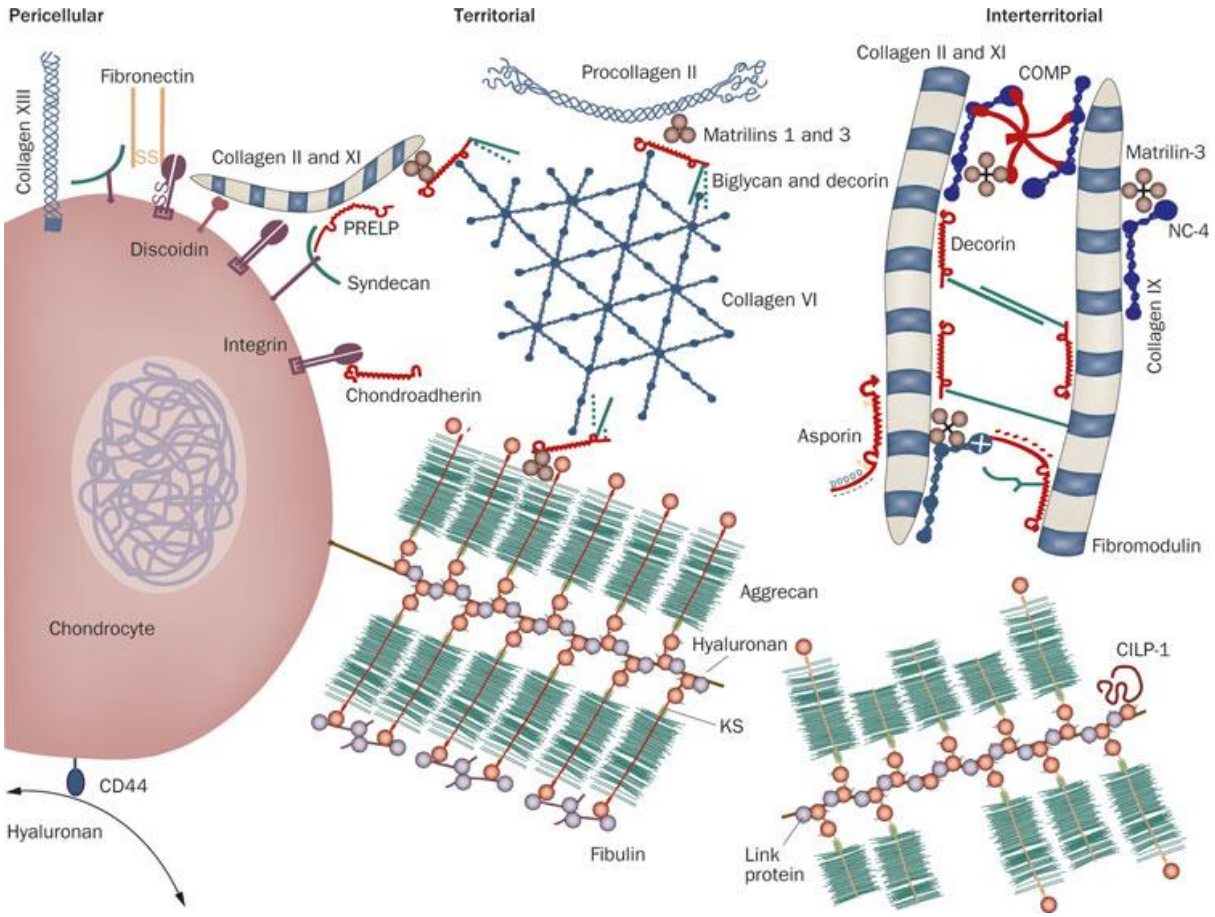


Figure 1.3. Protein components of the extracellular matrix of articular cartilage [72].

Healthy articular cartilage matrix is arranged according to the distance from the cell, with collagens and collagen proteins differing in each zone. Abbreviations: CILP-1, cartilage intermediate layer protein 1; COMP, cartilage oligomeric matrix protein; CS, chondroitin sulfate; KS, keratin sulfate; PRELP, proline-arginine-rich end leucine-rich repeat protein.

1.2.3 The chondrocyte

Chondrocytes are the only cell found in articular cartilage [73]. Despite contributing to a mere 5-10% of total cartilage volume, chondrocyte cells play an integral role in

epiphyseal growth and the maintenance of cartilage ECM integrity [74]. A chondrocyte's life cycle consists of proliferation, differentiation, maturation, and ultimately apoptosis; any biochemical or genetic deregulation of these processes can alter the form and integrity of the surrounding cartilage tissue [75]. During foetal development, chondrocytes are metabolically active proliferative cells, providing the template for the development of several tissue structures. However in adult life, the chondrocytes are restrained to a fixed distribution, and their function alter from that of supporting growth to providing strength and structural support.

Though often described as quiescent cells, they are intracellularly active, producing and organising the key structural components required to maintain a state of dynamic equilibrium with the matrix volume, including; proteoglycans such as aggrecan and decorin, collagen, elastin, and fibronectin (Table 1.1) [76].

Table 1.1. Extracellular matrix components of articular cartilage.

Proteoglycans	Structural Proteins	Glycosaminoglycans	Collagens
Aggrecan	COMP	Chondroitin sulfate	Collagen II
Versican	Thrombospondin -1	Keratin sulfate	Collagen III
Link Protein	Thrombospondin -3	Heparin sulfate	Collagen VI
Biglycan	Matrilin -1	Hyaluronan	Collagen IX
Decorin	Matrilin -3		Collagen X
Epiphycan	CILP		Collagen XI
Fibromodulin	C-type Lectin		Collagen XII
Lumican	Fibronectin		Collagen XIV
Perlecan	PRELP		
Lubricin	Chondroadherin		
	Tenascin C		
	Fibrillin		
	Elastin		

Prostaglandins and proteins of the articular cartilage extracellular matrix [77, 78]
Abbreviations: CILP, cartilage intermediate layer protein; COMP, cartilage oligomeric matrix protein; PRELP, proline- and arginine-rich end leucine-rich repeat protein.

The location of chondrocytes within articular cartilage largely determines their function and pathology. Unlike chondrocytes in other zones, these superficial zone chondrocytes produce proteoglycan-4 (PRG-4), which complexes with hyaluronic acid and aids joint lubrication [79]. However, they also synthesise less aggrecan proteoglycan and collagen compared to chondrocytes from other cartilage zones. This arrangement of cells and matrix and specific chondrocyte phenotype results in this zone exhibiting a high water content and resistance to shear and compressive stresses [51].

The transitional and radial zone chondrocytes adopt a more spherical morphology, and exhibit a much higher concentration of endoplasmic reticulum and Golgi membranes than chondrocytes from other cartilage zones. In the deep zone, the chondrocytes align to form columns, perpendicular to the articular cartilage surface. This alignment, combined with the higher concentration of proteoglycan and larger-diameter collagen fibrils make the radial zone more suitable for resisting compressive forces (Figure 1.2).

1.2.4 Extracellular Matrix

The ECM consists primarily of collagen type II fibres, and proteoglycans including aggrecan, which are linked through collagen binding proteins such as COMP and chondroadherin [80].

1.2.4.1 Collagen

Collagen accounts for approximately 60% of cartilage dry weight, 90% of which is solely collagen type II [51]. The remaining 10% is composed of collagens I, IV, V, VI, IX and XI [51]. Structurally, collagen consists of three parallel polypeptide strands, which form a tightly packed helical conformation resulting in a glycine at every third residue [81]. The most abundant triplet sequence within collagen consists of a proline, glycine and a hydroxyproline which allows for hydrogen bonds to stabilise the length of the molecule [82]. It is this structure which provides mechanical strength and stability to the ECM [51].

1.2.4.2 Proteoglycans

Proteoglycans are highly glycosylated proteins which are abundantly expressed within cartilage ECM. Proteoglycan structure consists of a core protein, to which one or more glycosaminoglycan chains are covalently bound [83]. There are a number of proteoglycans found in articular cartilage, including decorin, biglycan, and the most abundant, aggrecan [51]. Aggrecan is a unique proteoglycan consisting of over 100 keratin and chondroitin sulfate chains, which form large proteoglycan aggregates through the interaction with link proteins and hyaluronan [83]. Conversely, non-aggregating proteoglycans interact with surrounding collagen proteins. Proteoglycans bind water molecules and repel other molecules of a negative charge. Through binding water, the proteoglycans maintain the osmotic properties of articular cartilage and confer resistance to compressive loads [51].

1.2.5 Bone

Adult human bones are divided into four categories; long (such as the femur, tibiae and fibulae bones), short (such as the carpals and tarsals), flat (such as the skull, mandible and sternum) and irregular (such as the coccyx, hyoid and sacrum bone) [84, 85]. As well as permitting movement via the muscular system, bones protect vital organs, serve as a reservoir for cytokine and growth factors, and maintain mineral and acid base balance [85, 86].

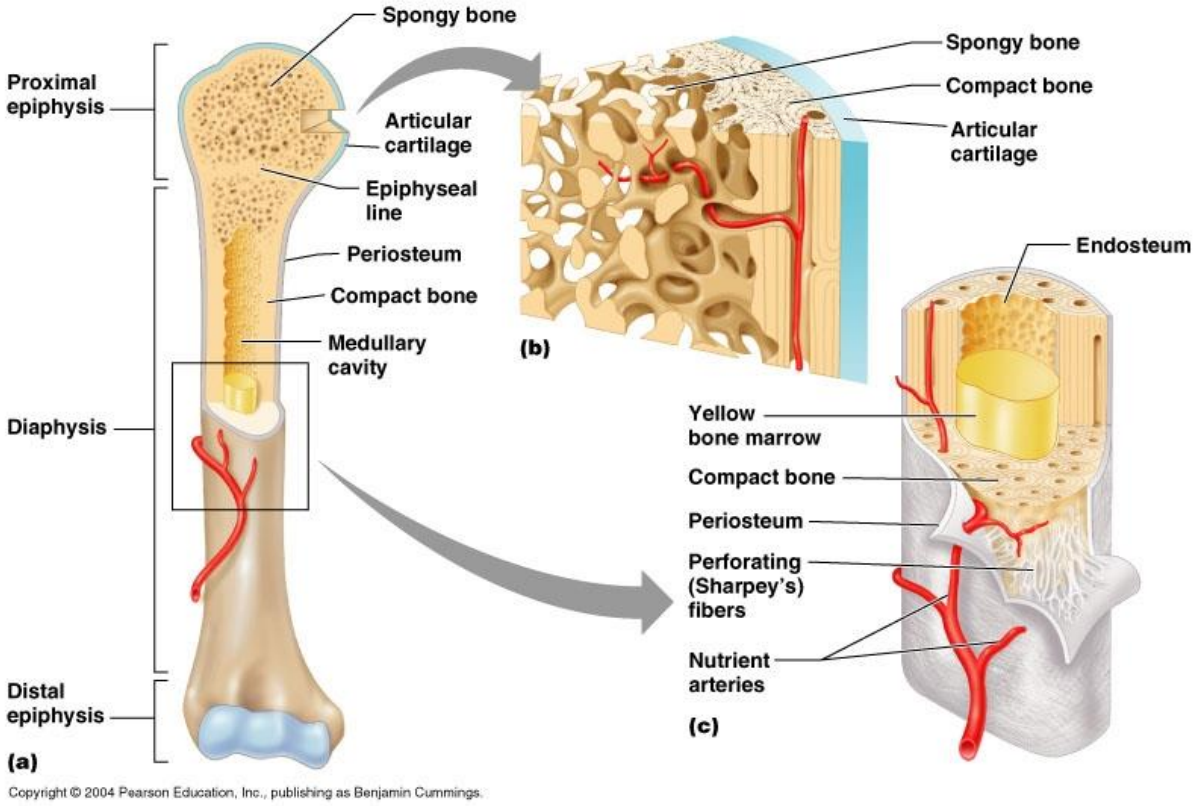


Figure 1.4. The anatomic structure of long bone.

A. Gross structure of long bone detailing epiphysis and diaphysis areas of long bone. B. Spongy bone with vascularisation. C. Compact bone with yellow bone marrow core. (Copyright© 2004 Pearson Education Inc. publishing as Benjamin Cummings).

Bone is a highly vascularized tissue (Figure 1.4), and is best described as having a hierarchical structural organization. This structure consists of five components spanning the macrostructure to the sub-nanostructure; cortical and trabecular bone, osteons, lamellae, collagen fibre, and bone mineral crystals and collagen molecules.

In bone macrostructure there are two distinct morphological types of bone; dense cortical bone and “spongy” trabecular bone. Overall, the human skeleton is

composed of 80% cortical bone to 20% trabecular bone, but the proportion varies depending on the bone and the skeletal site [87]. The femur has a 50:50 ratio of cortical to trabecular bone, and consists of both an epiphysis (an end), and a diaphysis (a shaft) (Figure 1.4A). The diaphysis of the femur is dense cortical bone, surrounding a yellow bone marrow core interspersed with honeycomb-like trabecular rods and plates (Figure 1.4C).

At the nanostructure and sub-nanostructure scale, collagenous fibres and molecules are predominant (85-90% of bone proteins) with collagen type I being the main form present within mature bone, and collagen III, V and X expressed to a lower degree. Non-collagenous proteins such as serum albumin, glycosaminoglycans and glycoproteins contribute to the remaining 10-15% of total bone protein. Finally, bone is composed of approximately 50-70% mineral, most of which is hydroxyapatite crystals [88]. Mineral provides structural rigidity and compressive strength to bone, and non-collagenase proteins such as osteonectin, osteocalcin and osteopontin may play a significant role in regulating the size and orientation of the hydroxyapatite crystals within the bone. Therefore, modulation of any of these proteins may play a significant role in altering bone structure and strength at a sub-nanostructure scale, which could ultimately result in pathological macrostructural and mechanical bone alterations.

1.2.6 Osteoblasts and osteoclasts

Maintaining bone strength and stiffness is determined by bone material composition and the dynamic relationship between bone forming and bone resorption cells; known as osteoblasts and osteoclasts respectively. Located along the bone surface, osteoblasts contribute to approximately 5% of total bone cells [89], and exhibit

characteristics typical to high protein content producing cells, including an abundance of Golgi apparatus and endoplasmic reticulum, and a high concentration of secretory vesicles [90].

Osteoblasts are derived from mesenchyme stem cells (MSCs) [91], which follow a temporal stepwise expression of genes specific to early osteoblastogenesis, including bone morphogenetic proteins (BMPs) and Wingless pathway (Wnt) members [92]. The expression of these genes drive MSCs to a chondro/osteoprogenitor cell type, defined by the expression of Runt-related transcription factor (Runx2), distal-less homeobox 5 (dlx5) and osterix (osx) [92, 93], all of which are crucial for osteoblast differentiation. Importantly, Runx2 is a master regulator of osteoblast differentiation, as demonstrated in Runx2 null mice who exhibit arrested osteoblast differentiation [92-94], and is essential to the expression of osteoblast-related genes including; COL1A1, BGLAP, OSN and ALP [95]. Following differentiation, osteoprogenitors undergo a proliferative state, demonstrated by an increase in alkaline phosphatase (ALP) activity, reflective of the increased biosynthetic activity of pre-osteoblasts [90]. Upon maturation of pre-osteoblasts to osteoblasts, the morphology changes to become cuboidal in shape, and they actively secrete bone matrix proteins, including collagen type I, bone sialoprotein and osteocalcin [93, 96-98]. These mature osteoblasts deposit organic matrix through the secretion of collagenous and non-collagenous proteins and proteoglycans, and subsequently mineralize the bone matrix (osteoid), through the synthesis of hydroxyapatite crystals [99].

Prior to osteoid mineralization, a proportion of mature osteoblasts (20%) become embedded within their secreted osteoid matrix. These cells are referred to as osteoid osteocytes or pre-osteocytes [100]. As the osteoid is mineralized, the cells become

permanently embedded within the bone matrix, resulting in cellular aggregation and the development of cellular processes to allow communication of osteocytes deep within the bone matrix with cells located on the bone matrix surface [100]. Osteocytes are referred to as mechanosensors, because of their ability to sense bone stresses and communicate with osteoblasts and osteoclasts on the bone surface to modulate their activity and alter bone remodelling. Recent evidence would suggest that this communication between osteocytes with osteoblasts and osteoclasts is through the release of signalling factors including RANKL, sclerostin, NO and IGF-1 [101-103].

Osteoclasts are predominantly derived from mononuclear monocyte-macrophage precursor cells from bone marrow [85]. Osteoclastogenesis is reliant upon the secretion of two cytokines; macrophage colony-stimulating factor (M-CSF) and Receptor activator of nuclear factor kappa-B ligand (RANKL), from bone marrow stromal cells and osteoblasts [104]. Importantly, the production of osteoclasts is dependent on the activity of osteoblasts via the osteoblast expression of osteoprotegerin (OPG), which serves as a decoy to RANKL, thus limiting the binding of RANKL to its receptor RANK [105]. Therefore, the ratio of RANKL and OPG is critical in determining the proliferation and differentiation of osteoclast precursor cells, and ultimately regulates bone remodelling [106]. Furthermore, inflammatory mediators such as TNFα, TGF-β and IL6 [107], vitamin D3 [108] and parathyroid hormone (PTH) have also been shown to increase osteoclast formation [109, 110]. Osteoclast formation via RANKL and the co-stimulation of DNAX-activating protein (DAP12) and Fc receptor γ chain (FcRγ) leads to the activation of nuclear factor of activated T cells cytoplasmic 1 (NFAT1c) and nuclear factor kappa B activator protein 1 [111], which in turn activate genes essential for pre-osteoclast differentiation

including; tartrate-resistant acid phosphatase (TRAcP), cathepsin-K, β3-integrin and matrix metalloproteinase (MMP) 9 [112].

1.2.7 Bone remodelling

Bone remodelling relies on the dynamic equilibrium of osteoblast and osteoclast cell activity to maintain a constant bone mass, while also repairing microfractured bone and maintaining calcium homeostasis [113]. Bone remodelling consists of four phases; activation, resorption, reverse and formation. In the activation stage, stimuli such as micro-fractures, factors released into the microenvironment (including IGF-1, TNF-α, and IL6), and alterations in bone loading as sensed by the osteocytes activate the quiescent osteoblasts lining the bone matrix surface. These cells subsequently increase their RANKL expression, and activate the RANK receptor on pre-osteoclasts, causing differentiation towards a multinucleated osteoclast formation. Upon differentiation, osteoclasts become polarized and adhere to the bone surface to begin the resorption phase. During the resorption phase, osteoclasts secrete hydrogen ions [114] and lysosomal enzymes (including TRAP, cathepsin K and MMP-9) [115] resulting in acidification and digestion of the bone mineral matrix and the formation of resorption pits [85]. To conclude the resorption phase, osteoclasts undergo apoptosis to prevent excess bone resorption (Rucci, 2008). The reversal phase begins the transition from bone resorption to bone formation, through generating an osteogenic environment at sites of resorption, though the signalling mechanism surrounding this phase remain unclear. One theory suggests macrophage-like reversal cells remove matrix debris from the site of resorption [116], Following the reversal phase, osteoblasts are recruited to the resorption pit through the secretion of growth factors including BMPs, FGFs and TGFs. Once recruited, osteoblasts begin to produce new, osteoid bone matrix, which is subsequently mineralized, therefore completing the bone remodelling process.

1.3 Osteoarthritis

OA is a leading cause of joint degeneration, pain and disability in the world, with over 8.75 million individuals in the UK over the age of 45 years seeking treatment. Typically defined as a disease of articular cartilage degeneration and joint space narrowing, many patients experience limited movement, and are unable to perform everyday tasks [76]. Furthermore, with OA disease prevalence increasing with age, these figures are set to worsen given the ageing population. Within a clinical setting, the diagnosis of OA relies on radiographic images and a scoring system originally developed by Kellgren and Lawrence (1957) which defines radiographic indices of OA (such as joint space narrowing and osteophytosis) into a scale of increasing severity (Table 1.2) [117].

Currently, approved therapeutics for OA patients primarily provide pain relief and as such can also help to improve joint mobility in patients with painful joints. For example, non-steroidal anti-inflammatory drugs (NSAIDs) such as Paracetamol/Acetaminophen are often prescribed as a first line of treatment by clinicians. However, in part due to the heterogeneity of OA, determining the source of pain is difficult [118] and therefore these analgesic medications are often largely ineffective and of limited therapeutic benefit for patients. Some NSAIDs such as COX2 inhibitors, have demonstrated analgesic efficacy and also promising disease modifying capabilities in the clinic. However the side effects reported are less desirable, including increased risk of heart failure, persistent headaches, stomach ulcers and anaemia [15], leading to the termination of these clinical studies. Indeed, toxicity has even been reported with long-term use of paracetamol and is thus is prescribed with caution [14]. Therefore, despite the high prevalence of OA within the

UK alone, there remains a high unmet clinical need to develop a clinically safe Disease Modifying OA Drug (DMOAD).

To identify potentially novel targets with disease modifying efficacy requires a better understanding of the complex multi-pathophysiology of the OA joint and the key molecular signalling pathways that underpin it. In addition, given the heterogeneity of OA, it also requires an understanding of how this differs across different OA patient cohorts. Thus, this research project, which aims to determine the expression of adipokines across different OA patient cohorts and to determine the functional role of adipokines in joint tissue, is highly pertinent.

Table 1.2. Kellgren and Lawrence Grading Scale

Grade	Description
0	No observed pathological indices of OA.
1	Doubtful narrowing of joint space and possible early development of osteophytes,
2	Osteophytes and joint space narrowing.
3	Moderate osteophytes and definite narrowing of joint space. Some subchondral bone sclerosis and deformity.
4	Large osteophytes, marked joint space narrowing, severe sclerosis and bone contour deformity.

1.3.1 OA Pathology

Importantly, OA is now widely considered to be a heterogeneous disorder, that affects all tissues that encompass the joint, including the articular cartilage, subchondral bone, synovium and skeletal muscle [119](Figure 1.5).

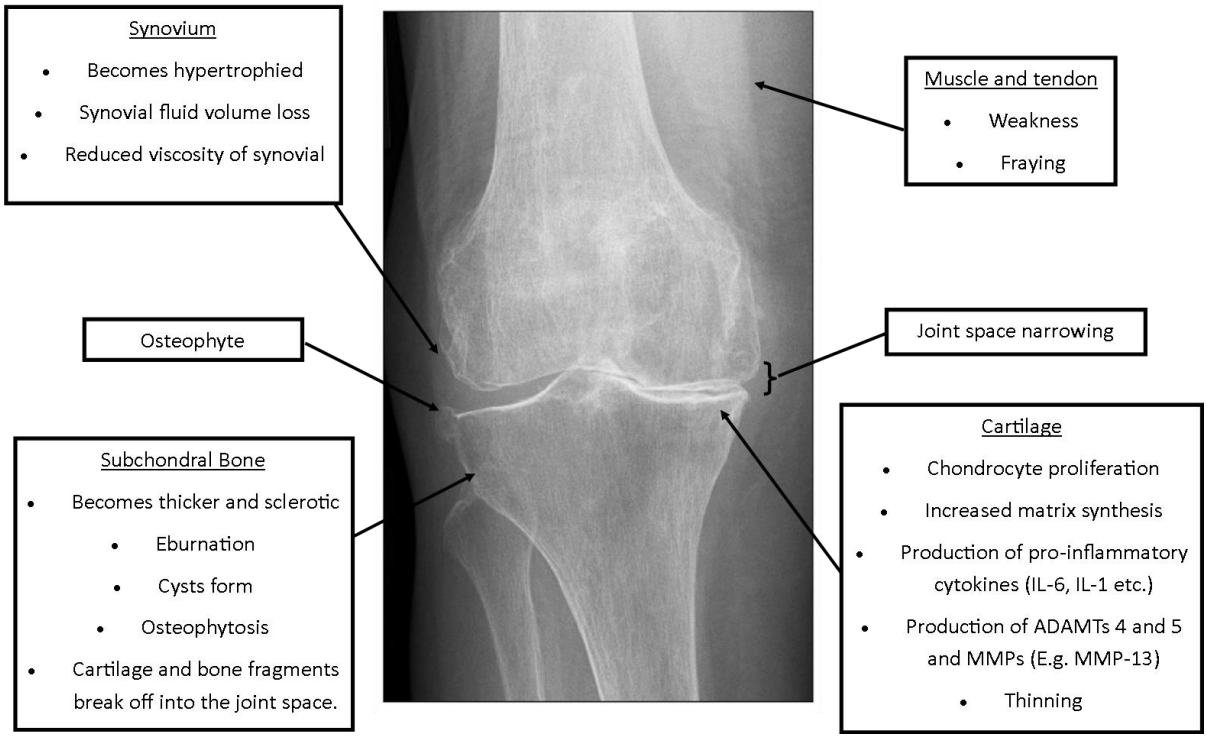


Figure 1.5. The heterogeneity of OA.

OA is a disease encompassing the whole joint including the articular cartilage, synovium, subchondral bone and muscle, and is heterogeneous in nature.

1.3.1.1 OA and Cartilage Degradation

In a steady state, chondrocytes are quiescent and maintain the ECM which comprises of collagen proteins (including collagen type II, IV, IX, and XI) and interlaced proteoglycans, including aggrecans. However, under mechanical or biochemical stress, chondrocytes can undergo a phenotypic alteration, involving cellular proliferation, overt hypertrophy (marked by the up-regulation of collagen X)

and the production of matrix degrading enzymes and matrix remodelling proteins. In addition, calcification of chondrocytes results in an advanced tidemark and vascular intrusion from the subchondral bone [120].

Cartilage matrix degrading enzymes include the MMP family and the disintegrin and metalloproteinase with thrombospondin motifs aggrecanases (ADAMTS) family of proteins. Specifically, MMP-1, MMP-3 and MMP-13 have been implicated in OA disease progression since they specifically degrade collagen type II and activate aggrecanases such as ADAMTS4 and ADAMTS5, which degrade aggrecan proteoglycan. According to Goldring (2012), collagen is normally protected from MMP1 and MMP-13-induced degradation by the 'proteoglycan coating'. The removal of the proteoglycan coating and subsequent collagen network degradation is believed therefore to mark the beginning of irreversible cartilage degradation. Furthermore, with the degradation of cartilage matrix proteins, protein fragments are produced which may interact with receptors of inflammatory cytokines, matrix-degrading proteinases, and chemokines to augment further matrix destruction [121].

1.3.1.2 OA and subchondral bone degeneration

Traditionally seen as a disease of cartilage degeneration, recent research has demonstrated that remodelling to the subchondral bone tissue occurs in OA, and these changes precede and may drive cartilage degeneration [122, 123]. Specifically, in the Dunkin Hartley guinea pig, an outbred Pirbright strain of guinea pig derived from the short haired English guinea pig, widely utilised as a model of spontaneous OA development, histological modifications were detected in subchondral bone at 3 months of age despite no cartilage histological alterations [124].

In OA, subchondral bone undergoes a number of pathological modulations, including bone marrow lesion (BML) formation, increased subchondral bone volume despite reduced bone mineral density (Figure 1.6), sclerotic bone formation, osteophyte formation and cortical bone thickening [125-129]. It is believed that these alterations are likely to result in weaker bones, with a reduced capacity to absorb and reduce forces transmitted through the joint during daily activities [130].

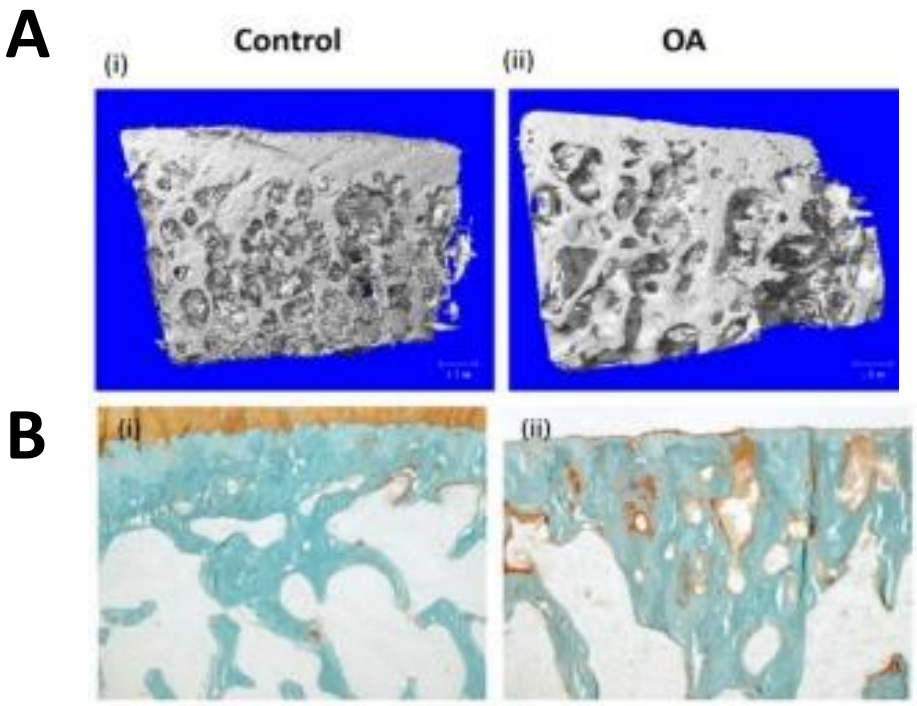


Figure 1.6. Pathological alterations of subchondral bone in OA.

A. Micro CT scan indicates a higher bone volume and thickened trabecular in OA. B. Goldner stain (orange stain) shows areas of under-mineralised bone in OA subchondral bone when compared to the control [131].

The notion that subchondral bone alterations precede cartilage degeneration has led to research aimed at better understanding the role subchondral bone in OA. In OA, subchondral bone has been shown to exhibit increased metabolism of collagen type

I, altered fibril cross-linking and subsequently hypomineralisation [132]. Furthermore, there is a reported phenotypic shift in the ratio of collagen type I homo/heterotrimer in OA subchondral bone, suggestive of a change in osteoblast phenotype [133]. Typically, collagen type I is a heterotrimer which consists of two α1 chains to every α2 chain (Figure 1.7), however it is the α2 chain which provides the structural integrity of the collagen molecule [134]. In a brittle bone disease mouse model, represented by an Col1a2 (oim) mutation and therefore a replacement of the α2 chain with an α1 chain to form a homotrimer, there was shown to be an increase in water content and a subsequent loss of the lateral packing of the collagen fibrils [135]. Such changes in the structural components of bone have been purported to affect bone biomechanics, and be a key contributory factor to anatomical and gait alterations within the joint.

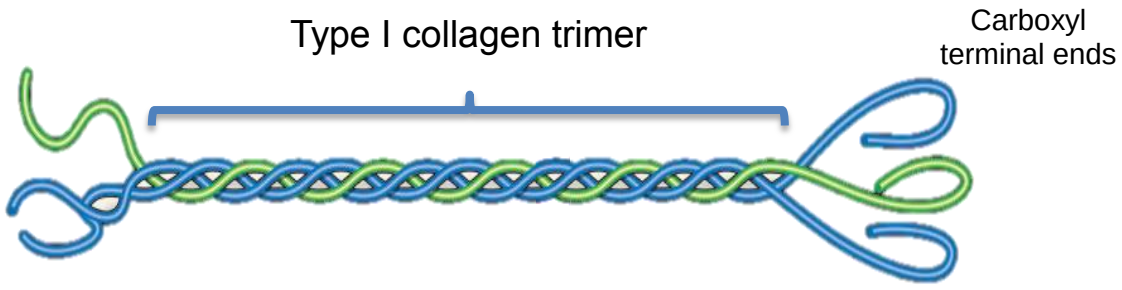


Figure 1.7. Collagen type I is a heterotrimer, composed of two identicalα1 protein chains (blue) and one α2 chain (green).

The carboxyl terminal ends direct the assembly of the collagen type I heterotrimer structure [136].

Alterations in the structural integrity of subchondral bone during OA has a significant impact on cartilage degradation [129]. For example, the thickening of the subchondral plate during OA is associated with a thinning of the cartilage layer [137, 138], and bone cysts and bone marrow lesions are directly associated with areas of cartilage deformation [139, 140].

1.4 Synovitis and Osteoarthritis

Synovitis is defined as inflammation of the synovial joint lining. Synovitis has been demonstrated to precede radiographic indices of OA disease including osteophyte formation and bone sclerosis [141]. Indeed, histological studies indicate that synovitis is present in 50% of patients in the early stages of OA, and in nearly all patients in the later stages of OA [142]. Despite this, OA has been largely overlooked as an inflammatory disease due to the relatively non-inflammatory OA joint phenotype compared to that of rheumatoid arthritis (RA) joints. While there is no definitive hypothesis for the cause of synovitis, the most widely accepted hypothesis is that cartilage breakdown fragments serve as foreign bodies within the joint space, thus stimulating the release of inflammatory mediators into the synovial fluid. However, a more recent hypothesis suggests that the synovial tissue can initiate an OA disease process through an increased cellular infiltrate of macrophages and T-cells [143-145]. This hypothesis is supported by MRI, immunohistochemical and ultrasonography studies, which have all demonstrated synovitis in early OA, Furthermore, Benito *et al.,* (2005) found an increased mononuclear cell infiltration and over expression of inflammatory mediators (TNFα and IL1β) in early OA synovial tissue samples [146].

In 2013, Sokolove and Lepus identified a stepwise increase in the levels of inflammatory mediators from normal plasma, to OA serum, to OA synovial fluid, to RA serum, through to RA synovial fluid (Figure 1.8) [147]. Although RA synovial fluid and plasma samples display a significant inflammatory profile, the heat map also reveals a greater inflammatory profile in the OA samples compared with the normal plasma. The presence of these pro-inflammatory mediators can initiate a vicious cycle, through the activation of MMP production from articular cartilage resulting in further degradation of cartilage, which stimulates the inflammatory response from synovial cells. Further, inflammatory mediators are able to stimulate further synthesis of inflammatory cytokines and MMPs from the synovial cells [148].

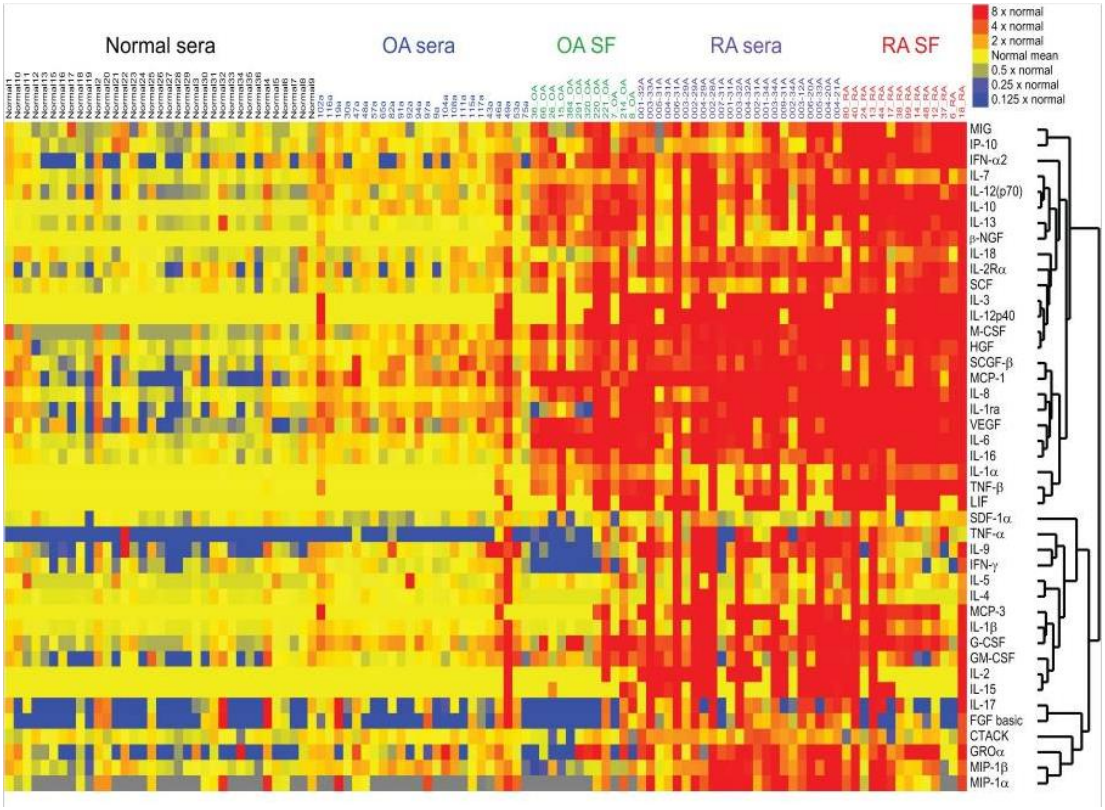


Figure 1.8. The inflammatory profile of osteoarthritis and rheumatoid arthritis serum and synovial fluid compared with healthy sera [147].

Samples from individual patients are listed along the top of the heatmap, with cytokines identified to the right of the heatmap. Cytokine concentrations were determined using a multiplex bead based immunoassay.

1.4.1 Cytokines and OA

There are a number of inflammatory mediators which have been identified as being modulated in an OA population. Among the pro-inflammatory cytokines found to be elevated in OA, perhaps most notable is IL1β, which has been purported to be a driver of OA pathology [149] by mediating damage to articular cartilage tissue [150-

152]. Formed as a precursor protein in the cytosol (pro–IL1β), intracellular proteolysis by Caspase I converts pro-IL1β into its active form, which is then released into the extracellular space. IL1β can be synthesised by chondrocytes, osteoblasts, synovial fibroblasts and mononuclear cells [153-157]. In OA patients, IL1β is found in its active form in cartilage, synovial fluid, subchondral bone and synovium, and has been shown to blunt chondrocyte synthesis pathways and stimulate further production of pro-inflammatory cytokines, MMPs and prostaglandins. Increased amounts of IL1β have been reported in synovial fluid of both knee OA and ACL patients, compared to healthy individuals [158-161], and in the synovial membrane its expression has been found to correlate with OA grade [162]. IL1β, along with IL1α and IL1Ra, binds to the IL1R1 receptor [163], which is significantly increased in cartilage surface chondrocytes and synovial fibroblasts in patients with OA [164, 165]. Upon binding with the receptor, IL1R1 forms a heterodimer complex with IL1 receptor accessory protein, leading to subsequent activation of the NFκB [166], p38 MAPK, and c-Jun N-terminal kinase (JNK) pathways [166, 167]. The activation of these pathways results in the synthesis of cytokines and chemokines including TNFα, IL6, IL8, and CCL5 [168-171]. Furthermore, in terms of cartilage health, IL1β inhibits the ability of chondrocytes to synthesise key structural components of articular cartilage matrix, including collagen type II and aggrecan [172, 173]. Finally, IL1β has been demonstrated to increase expression and secretion of MMP-1, 3, and 13 [174, 175] while also upregulating aggrecanase ADAMTS4 [176].

Through inhibiting the cartilage restorative capacity of chondrocytes, and up-regulating ECM degradation enzymes, IL1β clearly demonstrates a significant role in the pathological progression of inflammation-mediated OA.

Another cytokine thought to be significant in the pathological progression of OA disease is TNFα. TNFα binds to receptors TNFR1 and TNFR2, with TNFR1 seemingly having the greatest impact in cartilage degradation due its ability to bind both the membrane and soluble form of TNFα [177], and the differing downstream signalling pathways associated with each receptor. Binding of TNFα to either receptor results in activation of the NFκB [178, 179] and MAPK [180-182] pathway, thus initiating the transcription of a number of target genes associated with OA development.

TNFα has been reported to be elevated in the synovial membrane, synovial fluid, subchondral bone and cartilage, in OA compared to non-OA individuals [162, 183-187]. Stimulation of porcine metacarpal articular cartilage with TNFα has been shown to reduce proteoglycan synthesis and collagen type II synthesis [188, 189]. In rodent models of traumatic joint injury, expression of TNFα is induced [190] and correlates to joint space narrowing (JSN) [191]. Furthermore, overproduction of TNFα induces NO production [192, 193], aggrecanase ADAMTS4 [194], and the expression of MMPs -1, -3 and -13 [195], whilst TNFα receptor antagonists block NO production in *ex vivo* human cartilage tissue [196]. Elevated secretion of IL1β and TNFα in OA chondrocytes, osteoblasts and adipocytes is known to promote an increase in IL6 expression and secretion [169, 197-199].

IL6 signalling requires binding to either the membrane-bound or soluble form of IL receptor (mIL6-R and sIL6-R respectively), and complexed with either the membrane-bound or soluble receptor β-subunit gp130 (mgp130 and sgp130 respectively) [200, 201]. Binding of the IL6 ligand to IL6R complexed with the sgp130 form inhibits the signalling of the IL6 pathway, however binding of the IL6 ligand to IL6R complexed with mgp130 leads to signal transduction to the cell and

subsequent STAT3 activation, MAPK phosphorylation, and PI3K/AKT pathway activation [202, 203].

Both IL6 and s-IL6R have been shown to be up-regulated in synovial fluid and serum from OA patients, compared to individuals without OA [204]. However, the role of IL6 in OA has remained controversial in current literature. While some literature suggests that IL6 plays a synergistic role to IL1β and TNFα through reduced collagen synthesis and increased matrix degradation enzyme expression [205, 206], other studies have demonstrated a protective role of IL6 in cartilage health. In particular, IL6 KO mice demonstrated advanced degenerative joint disease when compared to their WT littermates [207].

1.5 Metzincins and OA

The metzincin family consists of several groups of calcium and zinc-dependent, enzymatic proteins responsible for regulating cellular and extracellular matrix interactions. The most notable groups of enzymes secreted by cells within the joint and implicated in OA disease progression are the matrix metalloproteinases (MMPs) and the disintegrin metalloproteinase with thrombospondin type I like repeats (ADAMTSs) [208].

1.5.1 MMPs and OA

MMPs are a group of enzymes able to cleave proteins encompassed within the extracellular matrix. These proteases were first discovered by Gross and Lapiere

(1962) where they demonstrated secreted collagenase activity from metamorphosing tadpole tail cultures [209].

MMPs are classified into either secreted, or membrane anchored MMPs and further sub-classified into substrate specificities including collagenases (MMP-1, -3, -13 and -18), gelatinases (MMP-2 and MMP-9), matrilysins (MMP-7 and MMP-26), metalloelastase (MMP-12), stromelysins (MMP-3, -10, and -11) and membrane anchored (MMP-14, -15, -16, -17, -24, and -25), with further unclassified MMPs [210].

MMPs have been identified as a major contributor to the catalytic breakdown of cartilage in OA [211, 212]. In particular, MMP-1, -3, -9 and -13 mRNA have been detected in human OA cartilage [212, 213], and a subsequent increase in type II collagen breakdown in areas of increased collagenase protein expression [214]. Recently, evidence has implicated chondrocytes, and their dynamic response to pro-inflammatory cytokines, in mediating MMP expression [215-217] however little is known with regards to how adipokines may alter MMP expression, and which MMPs will be modulated.

1.5.2 ADAMTSs and OA

Proteoglycans are highly glycosylated proteins which provide the viscoelastic properties to cartilage through their highly negative charge and thus the ability to hold large amounts of water molecules within the cartilage tissue [176]. Aggrecan is one of the major proteoglycan molecules found in cartilage [218], and is one of the first proteins in cartilage to be modulated and degraded prior to cartilage volume loss [219].

ADAMTSs are the main enzyme family responsible for cleavage of the aggrecan molecule during early onset of OA. In OA cartilage and synovial fluid, cleavage fragments of aggrecan and its associated core protein have been detected [220]. While there are 19 members of the ADAMTS family, 5 have been demonstrated to play a functional role in cartilage degradation [221-223]. However, of these 5, ADAMTS4 and ADAMTS5 have received the most attention. For example, it has been previously reported that ADAMTS4 and ADAMTS5 siRNA-mediated gene silencing in human cartilage tissue was shown to ablate cytokine stimulated aggrecan loss [224]. Furthermore, deletion of ADAMTS4 and 5 expression in animal models have demonstrated a reduction in proteoglycan degradation and OA severity [225-227].

1.6 OA risk factors

Risk factors associated with the progression of OA can be divided into genetic, systemic, or mechanical factors. The genetic risk of OA development is quite complex as it does not follow the pattern of Mendelian inheritance [228] however systemic factors such as age and obesity, and mechanical factors including acute injury, are much more established. Historically, increased mechanical stress was considered the fundamental cause of higher prevalence of OA in obese individuals, due to chondrocytes responding to mechanical loads with increased MMP and aggrecanase expression. However, more recently this view has been challenged, due in part to the recognition that OA is a disease of the whole joint, but also because of our increasing understanding of adipose tissue as an endocrine organ and the distal inflammatory effects attributed to adipose-secreted cytokines and associated “metabolic syndrome” disorders.

1.6.1 Gender and OA

Gender is identified as a significant risk factor of OA. Indeed, the prevalence of hip and knee OA in the female population is higher than that of males (13% vs. 8%, and 20% vs 17% respectively) [229]. Furthermore, the prevalence of erosive and symptomatic hand OA is much higher in females compared to their age matched male counterparts (9.9% vs. 3.3% and 15.9% vs. 8.2%) [230].

Notably, there are several previous studies that are indicative of sexual dimorphism in hip OA. In particular, Maillefert and colleagues (2003) performed a longitudinal prospective follow-up study in a large cohort of patients, and found that females exhibited a greater incidence of poly-articular OA, and had more rapid structural progression with a more severe symptomatic disease [231]. Currently, the mechanism for the sexual dimorphism apparent in OA is not established. However, importantly, the higher incidence of multi-joint OA in females with hip OA suggests a potential systemic driver of OA which may not be present (to the same degree) in males [231]. Thus hormonal factors have been suggested to play a role in the sexual dimorphism seen in hip OA. For example, in 2006, researchers demonstrated a lower prevalence of hip OA in females on hormone replacement therapy (HRT) [232]. However, research into this area remains limited, and other researchers have found HRT ineffective in preventing OA associated knee pain [233].

Another factor that may explain the sexual dimorphism in OA is obesity, which is a known risk factor for OA (Figure 1.9). Indeed, it has been reported that the association between central adiposity and hip OA is stronger in females than in males [234], indicative of a gender difference in the metabolic/obesity-related risk of OA.

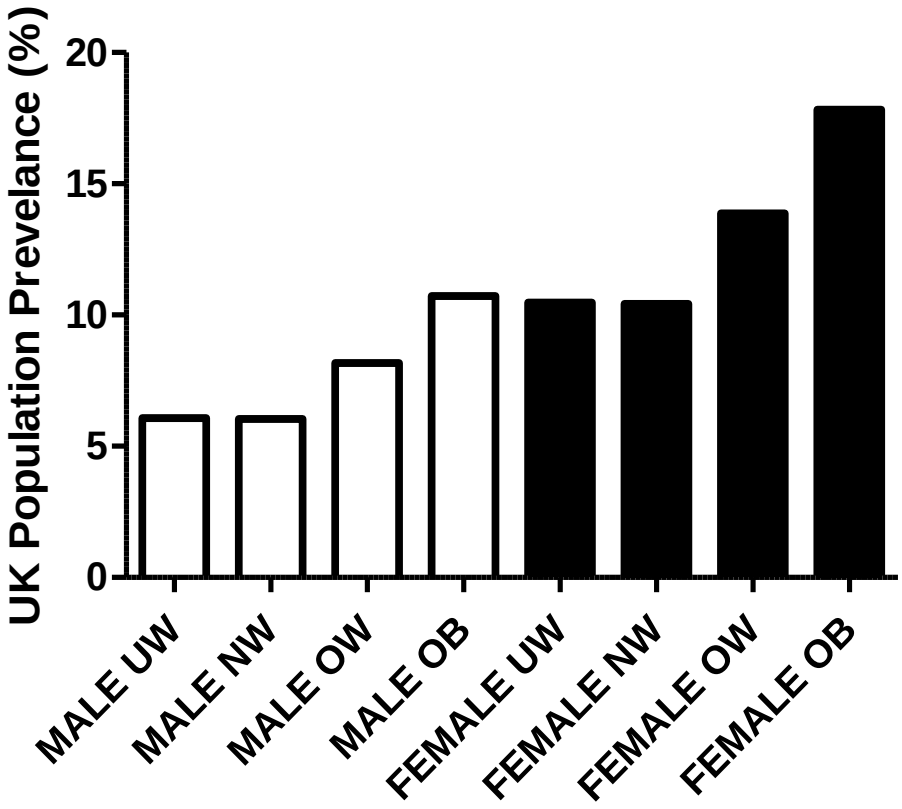


Figure 1.9. UK percentage prevalence of hip OA (total) in males and females of different BMIs.

UW=underweight; NW=normal weight; OW=overweight; OB=obese [229].

1.6.2 Obesity and OA

For many years, obesity (BMI>30kg/m^2) has been recognised as a significant risk factor of OA. In fact, in 1988 the National Health and Nutrition Examination Survey (HANES I) concluded that obese females were nearly four times more likely to experience OA symptoms than non-obese females, and male obese individuals were nearly five times more likely to develop OA symptoms than their normal weight counterparts [235].

Intuitively, early reports investigating how obesity correlates with the prevalence of OA focused upon the increased mechanical loading on the joint, and thus the destruction of the extracellular matrix of articular cartilage [236]. However, there has since been significant evidence to suggest a systemic inflammatory effect of obesity, for example the increased prevalence of hand OA [237], a non-weight bearing joint and thus not subjected to increased mechanical load. In addition, loss of adiposity, as opposed to loss of body weight, is associated with the relief of OA symptoms [238], concluding that metabolic dysfunction and inflammation require further investigation.

Importantly, white adipose tissue (WAT) is no longer considered merely an energy storage tissue, but instead is defined as a dynamic endocrine organ that maintains energy, and inflammatory and insulin-sensitivity homeostasis. Ouchi and colleagues confirmed that adipose tissue secreted cytokines termed ‘adipokines’ which contribute to the low-level pro-inflammatory state seen commonly in obese individuals [28, 29, 239].

Secreted into the circulation (although not always exclusively derived from adipose tissue), it is not surprising that adipokine serum profiles generally correlate with body fat mass, and are often associated with anthropometric measures such as BMI and W:H ratio. Furthermore, studies have demonstrated a “normalization” of adipokine profiles following weight loss which is associated with the normalization of metabolic indices, suggestive of adipokines being key mediators of metabolic health [31-33]. Thus, adipokines have been mechanistically linked to metabolic syndrome disorders [27], and have provided a metabolic link between obesity and osteoarthritis.

1.7 Adipokines and osteoarthritis

Several hundred adipokines have so far been identified, [30], and in the last 10 years, their role in relation to OA joint pathophysiology has received much attention [28]. A number of adipokines have been implicated in connecting obesity, osteoarthritis and inflammation. This thesis will detail the key adipokines that have been investigated in joint degradation, namely adiponectin, leptin, visfatin, and resistin.

1.7.1 Adiponectin and Osteoarthritis

Adiponectin, or AdipoQ, constitutes approximately 0.01% of the total circulating plasma proteins [240]. It is synthesised by adipose tissue, and exerts its mechanism by binding to two known receptors: AdipoR1 and AdipoR2. Adiponectin exhibits insulin sensitizing properties while also regulating glucose and fatty acid metabolism via the activation of AMPK, Ca^{2+}, and PPAR-α transduction pathways [241, 242].

It is known that serum adiponectin concentrations are inversely correlated with body weight. However, the specific role of adiponectin in OA disease progression remains unclear. For example, Chen *et al.,* suggested that adiponectin plays a protective role in maintaining cartilage integrity due to a marked increase in Tissue Inhibitor of Metalloproteinase-2 (TIMP-2), and a down-regulation of the pro-degradative MMP-13 at both the mRNA and protein level [243]. Conversely, Lago and colleagues (2008) demonstrated an increase in pro-inflammatory cytokine and MMP production (namely IL6, MMP-3, MMP-13 and MCP-1) following the stimulation of chondrocytes with adiponectin [244], and Kang *et al.,* (2010) reported an increase in collagen type II neopeptide following stimulation of cartilage explants [245]. Moreover, Filkova *et al.,*

(2009) found higher adiponectin expression levels in erosive hand OA when compared with non-erosive hand OA, which the authors suggest could indicate an ECM degradative role of adiponectin [246].

De Boer et al., (2012) found that circulatory levels of adiponectin were markedly increased in end-stage OA patients, when compared with a control population without signs of OA [247]. Furthermore, adiponectin was negatively associated with BMI in female patients, and showed a trend towards correlating with synovial joint inflammation. The authors therefore suggested that adiponectin may play an important role in the development of inflammation within the OA joint.

1.7.2 Leptin and Osteoarthritis

Originally discovered in white adipocytes, leptin is a 16kDa protein product of the *ob* gene, which is known to regulate energy metabolism and appetite suppression [248]. In obese individuals, circulating levels of leptin and its soluble receptor (s-Ob-R) are known to be increased [249]. In OA, higher circulatory concentrations of leptin have been observed in individuals with OA, when compared to non-OA controls [250-252]. However, the role of leptin and its receptor requires further elucidation, since both protective and disease-promoting effects of leptin have been reported. For example, exogenous injections of leptin into the knee joint of rats increased the expression of the insulin-like growth factor (IGF-1), and transforming growth factor-β (TGF-β) [253], suggesting that leptin performs a protective role in OA disease progression. In addition, Dumond *et al.,* (2003) demonstrated an increase in chondrocyte proliferation, and increased proteoglycan and collagen synthesis in the presence of leptin [253].

In contrast, using isolated primary human chondrocytes, Hui *et al.,* (2013) discovered that leptin alone, and in synergy with IL1β, induced the expression of MMP-1 and MMP-13 with activation of p38, ERK, PI3K and Akt pathways [254]. Furthermore, through the use of selective inhibitors of the aforementioned transduction pathways, Hui *et al.,* demonstrated a decrease in cartilage collagen release, typically induced by leptin. Leptin has also been demonstrated to increase the production of multiple inflammatory mediators including IL1β, IL6, IL8 and prostaglandin E_2 [255], a major contributor to inflammatory pain. In rats, Bao *et al.,* (2009) demonstrated an increase in gene expression of ADAMTS-4 and ADAMTS-5 aggrecanases following exogenous leptin treatment [256].

It has been proposed that leptin *per se* is detrimental to cartilage; however recent evidence suggests the effect of leptin may be more complex. Berry *et al.,* found sOB-Rb to be associated with a reduction in the cartilage synthesis biomarker PIIANP, an increased cartilage defect score, and cartilage volume loss [257]. This suggests a biphasic response whereby at lower concentrations, leptin assumes a protective role over cartilage degradation, and excess levels have a detrimental effect. In short, leptin may both synthesise and degrade articular cartilage, thus further understanding into the mechanism of leptin in OA disease progression is warranted.

1.7.3 Resistin and Osteoarthritis

As its name alludes to, the dimeric protein resistin was first recognised as inducing murine insulin resistance [258] and belongs to the FIZZ (or Found in inflammatory zone) family of proteins. In recent years, resistin has received growing interest, particularly for its role in the inflammatory response in tissues including the heart,

lungs, neutrophils and synovium. In reference to OA, Bokarewa and colleagues [259] demonstrated that intra-articular administration of recombinant resistin into mouse joints, resulted in degeneration of cartilage and the induction of pro-inflammatory cytokine release (IL1, IL6 and TNFα). Furthermore, resistin could be detected in the local environment of inflamed joints.

Ex-vivo experiments have also found recombinant resistin exhibits pro-inflammatory qualities. Lee *et al.,* (2009) discovered that stimulation of human cartilage explants with recombinant resistin inhibited proteoglycan synthesis [260]. In addition, mouse femoral head cultures had increased PGE_2 production and proteoglycan degradation following treatment with recombinant resistin [260]. However, these experiments were conducted using an injury-induced OA model, which may induce alternate signalling mechanisms as those differentially regulated in obesity-induced OA.

Several studies on the association between the incidence of OA and circulatory levels of resistin have been reported. However these studies are not in agreement, with some authors reporting elevation of resistin with OA, whilst other studies have either shown no association or an inverse relationship [261-264]. Furthermore, a study by Gomez *et al.,* (2009) found no association between resistin serum levels and cartilage volume loss [265].

1.7.4 Visfatin/NAMPT and Osteoarthritis

Visfatin is a highly conserved protein which is expressed throughout many tissue types. Originally defined as a pre-B cell colony enhancing factor [266], visfatin has received great attention in recent years for numerous potential roles, including; catalysing the conversion of nicotinamide and phosphoribosyl-pyrophosphates to nicotinamide mononucleotide [267], an insulin mimetic [268] and growth factor [269],

and as an inflammatory cytokine able to induce TNFα, IL6 and IL1β [270]. Interestingly, the original paper defining visfatin and an insulin mimetic through binding and activating the insulin receptor and lowering plasma glucose levels in mice [268] has since been retracted due to the inability of other laboratories to repeat this finding.

Visfatin is termed an ‘adipokine-enzyme’ due to its enzymatic nature when in a homodimeric conformation [271], and is secreted independently from the golgi apparatus and the endoplasmic reticulum from visceral adipose tissue [272].

As a secreted protein, visfatin exists in an intracellular (iVisfatin) and extracellular (eVisfatin) form. eVisfatin has received a lot of attention in the current literature due to elevated expression and its potential contribution to a number of pathological conditions associated with ageing including diabetes [273] and obesity [274-277]. Although the biosynthesis of nicotinamide adenine dinucleotide is well established as one of the functional roles of iVisfatin, the pathophysiological relevance and the functional consequence of elevated levels of eVisfatin in disease states remains unknown. This is further impacted by the current limited understanding of the signal transduction mechanism for eVisfatin, since a visfatin receptor has not yet been identified (Figure 1.10).

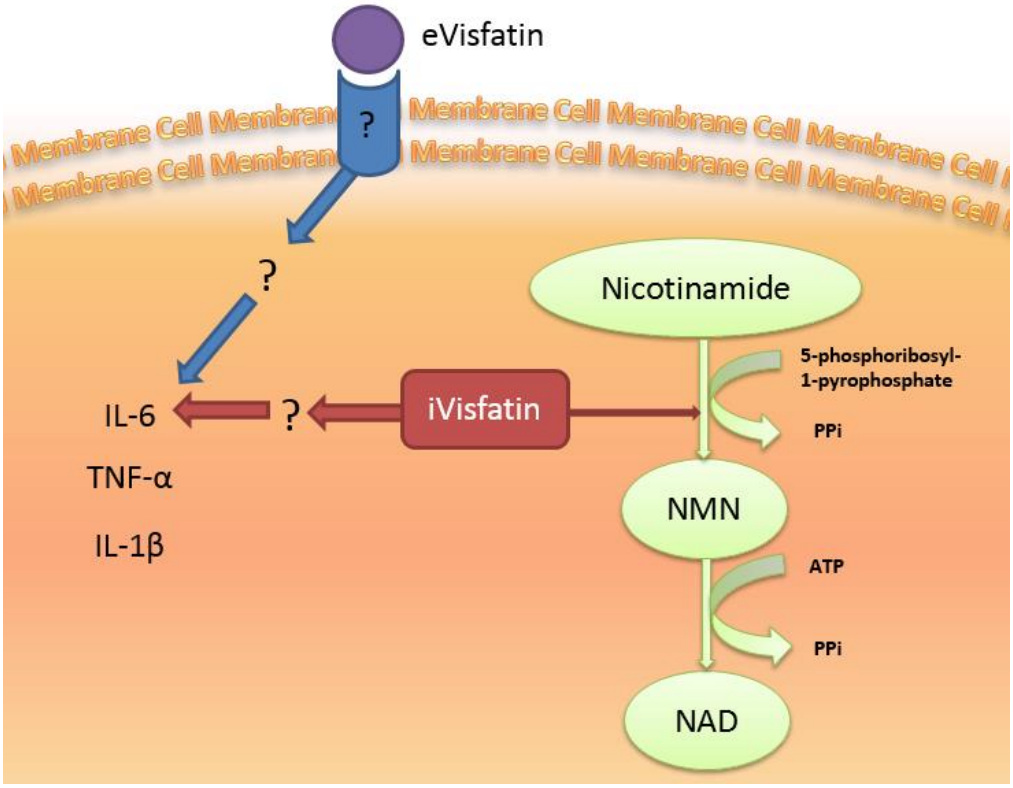


Figure 1.10. Potential roles of iVisfatin and eVisfatin in mammalian cells. As a secreted protein, visfatin exists in an intracellular (iVisfatin) and extracellular (eVisfatin) form.

iVisfatin, as shown in red, is integral to NAD biosynthesis however there is some suggestion that iVisfatin may also stimulate proinflammatory cytokine production, though the mechanisms are so far unknown. Alternatively, eVisfatin may drive proinflammation in articular cartilage, however the cellular entry and signal transduction of visfatin is so far unknown.

Studies by Gosset *et al.,* (2008) suggest that visfatin plays a role in mediating human articular cartilage degradation (Figure 1.11) [278]. Firstly, visfatin expression was increased in human chondrocytes upon stimulation with IL1β. Visfatin was also deemed by the authors to be a potent inducer of PGE_2 production, through increased mPGES-1 and decreased 15-PGDH synthesis. Furthermore, treatment of human

chondrocytes with recombinant visfatin led to an increased expression of several matrix proteases including MMP-3, MMP-13, ADAMTS-4, and ADAMTS-5, and decreased aggrecan mRNA expression.

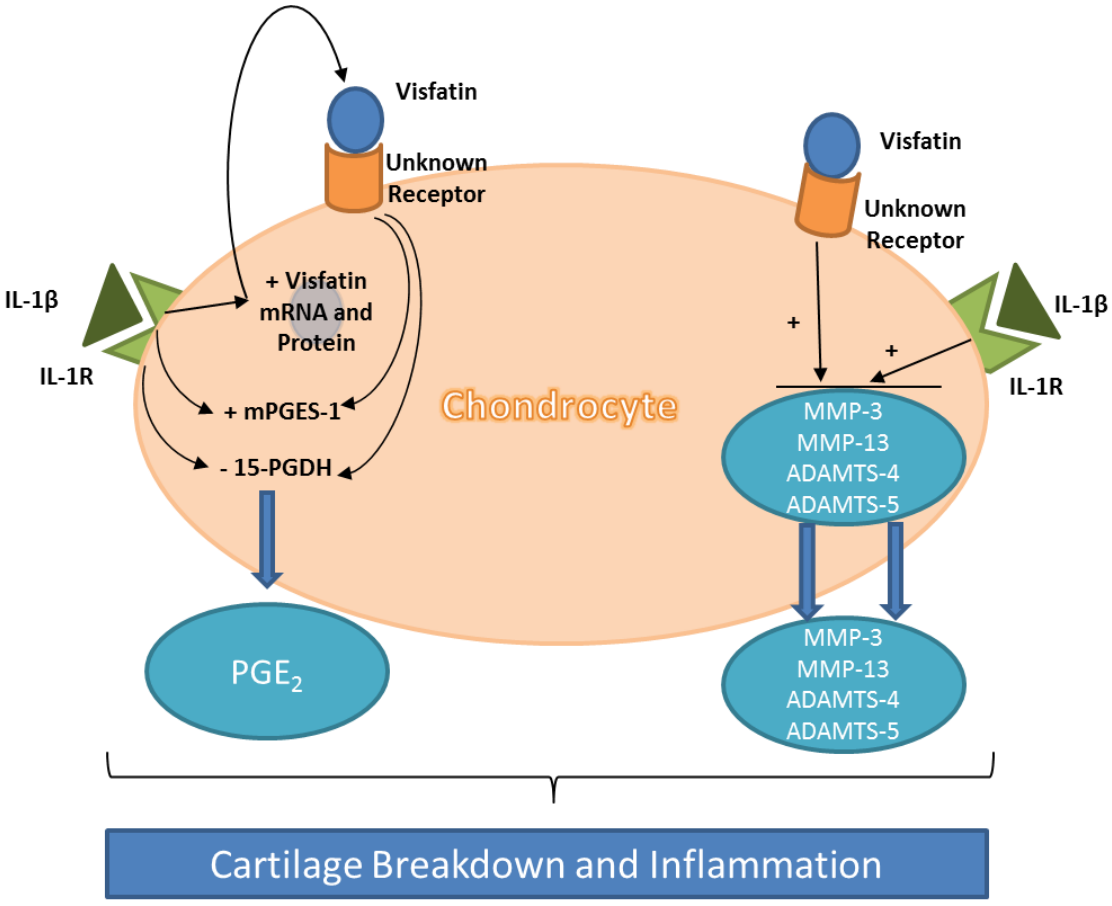


Figure 1.11 Gosset *et al.,* (2008) demonstrate the hypothetical role of visfatin in driving the pro-inflammatory and catabolic pathogenesis of OA though the synthesis of MMPs aggrecanases, and PGE2.

Gosset *et al.,* (2008) noted an increase in visfatin expression in human chondrocytes upon stimulation with IL1β which subsequently induced PGE_2 production and several matrix proteases including MMP-3, MMP-13, ADAMTS-4, and ADAMTS-5.

The notion that visfatin may be a catabolic mediator of OA disease progression was further supported by Laiguillon *et al.,* (2014) who examined human tissular visfatin expression and activity in relation to OA. Of note, all tissues within the OA joint (synovium, subchondral bone, and cartilage) expressed and secreted visfatin in its enzymatically active form, with significantly higher quantities from the synovium [271]. Following recombinant visfatin stimulation, OA chondrocytes and osteoblasts significantly increased expression of IL6, MCP-1, and keratinocyte chemoattractant. This effect however was blocked with pre-treatment with FK866, a pharmacologic NAMPT competitive inhibitor which binds to the active site of a visfatin homodimer. However, neither the expression of visfatin across different OA patient cohorts (of varying BMI) nor its functional effect on human articular cartilage has been reported.

1.8 Hypothesis

Growing evidence would suggest that adipokines provide a systemic functional link between obesity and the increased prevalence of multi-joint OA. However, functional studies using intact human hip cartilage and bone tissue to determine the role of adipokines in mediating OA pathology are currently lacking. In particular, no studies have reported the functional role of adipokines on cartilage and bone tissue from patients with hip OA. Furthermore, no studies have reported on the joint tissue expression and serum profile of adipokines in patients with hip OA, or how this differs with gender and varying adiposity.

1.9 Aims of this thesis

The aims of this thesis are therefore;

(1) To determine how the expression profile of adipokines in the serum and joint tissues in patients with hip OA varies with joint damage severity and the degree of adiposity.

(2) To determine the relationship between adipokine expression and markers of joint remodelling and severity in patients with hip OA.

(3) To determine the functional roles of various adipokines in modulating hip cartilage and bone OA pathology using human ex vivo tissue and primary cells.

(4) To understand the signalling mechanisms surrounding adipokine stimulation and cartilage and bone deformations in human OA tissue.

Ultimately, determining the functional role of adipokines in hip OA pathology, and profiling their expression in the serum and joint tissue across different hip OA patient populations could lead to the identification of a patient group amenable to an adipokine-targeted therapeutic.

Chapter 2. Material and Methods

2.1 Ethical Approval

Ethical approval was granted by the University of Birmingham, UK Research Ethics committee (NRES RG_13-148; NRES ES-14-1044). Participants were recruited on a volunteer basis, after being fully-informed of the study requirements by the clinical research staff of collaborating hospitals. Hip OA and knee OA samples were obtained from patients with age 45-80 years undergoing elective total hip joint replacement surgery (K and L grade 3-4) at The Royal Orthopaedic Hospital, Birmingham (UK) or the Russell Halls Hospital, Dudley (UK). Non-OA samples were obtained from neck of femur fracture (NOF#) patients who were also undergoing total hip replacement or hemiarthroplasty. All patients provided informed consent prior to sample collection. Patients who exhibited secondary causes of OA were excluded from this study. Secondary causes for patients with hip OA include, but are not limited to; developmental dysplasia, avascular necrosis, Perthes disease, slipped upper femoral epiphysis, and previous acetabular or femoral neck fractures. Secondary causes of knee OA include malalignment due to a previous knee, tibia, or femur fracture, significant ligament injury and avascular necrosis. Secondary causes of OA were determined through the use of radiographic x-rays taken during the pre-operative consultation. NOF# patients were excluded from this study if they exhibited any of the following: Presence of inflammatory arthritis, evidence of hand osteoarthritis, or evidence of knee or hip OA. All patients receiving/received immunosuppressive therapy for inflammatory conditions or cancer, oral steroid treatment, and patients who have received an intra-articular steroid injection within 6 months were also excluded from this study.

2.2 Pre-operative consultation and blood sample collection

During the pre-operative consultation a history of the patient's previous and current occupations and injuries were recorded to control for any mechanical stress that could have contributed to the development of OA. Participant's hands were inspected for evidence of hand OA and blood pressure was determined.

Patients removed heavy clothing and footwear for anthropometric data collection. Weight and body composition were recorded using Tanita Bioimpedance scales (BC-420MA, Amsterdam, NL) to the nearest 0.1kg, and height was recorded to the nearest 1.0cm using a stadiometer. BMIs were calculated by dividing weight (kg) by height (m) squared. As a marker of central adiposity, waist:hip ratios (WHR) were calculated by measuring the circumference of the narrowest part of the torso as viewed from the anterior aspect, and dividing by the circumference of the widest part of the buttocks above the gluteal fold, using a tape measure.

Fasted blood samples were collected in a vacutainer containing a clot activator by a qualified phlebotomist. Blood samples were centrifuged at 3000xg for 10 min, and serum samples aliquoted into cryovials and stored in a -80°C freezer.

2.3 Primary Cell Culture

For the isolation of human primary hip chondrocytes, articular cartilage was excised from the femoral head subchondral bone using a sterile scalpel blade. Excised cartilage slices were digested in 2mg/mL, sterile filtered collagenase Clostridium Histolyticum, Type 1A (Sigma Aldrich, USA), for 4 hrs at 37°C on a rotator. The cartilage-collagenase solution was strained using a 70µm cell strainer and

centrifuged at 400xG for 5 min. The cell pellet was washed in primary chondrocyte growth media (section 2.3.1), plated, and incubated in a humidified atmosphere of 37°C and 5% CO_2 in chondrocyte growth media.

For human primary hip osteoblast cultures, subchondral bone chips were cut from the femoral head using a Friedman Rongeur, and washed three times in DMEM (section 2.3.1) containing 100U/mL penicillin streptomycin to remove excess fat, blood, marrow, and connective tissue. Small bone chips (~<3mm^3) were placed into a 25cm^2 vented flask with osteoblast differentiation media (section 2.3.1). Bone chips were cultured in a humidified atmosphere of 37°C and 5% CO_2 and left for 5 days before the initial media change. Following 5 days, differentiation media was changed every 3 days, and bone chips were removed once primary osteoblast cell coverage reached approximately 30% confluency.

For human synovial fibroblasts, synovium tissue was washed and diced into approximately 2mm squares. Three pieces of synovium tissue were added to a 25cm^2 vented flask and incubated in synovial fibroblast media in a humidified atmosphere of 37°C and 5% CO_2. Media was changed every 3 days, and synovium samples were removed from the flasks when cell outgrowth reached approximately 40% confluency.

To maintain the phenotype of human primary cells as much as possible, cultures were maintained for a maximum of 5 passages. To passage, adherent cells were washed with sterile phosphate buffered saline (PBS), coated with Trypsin-EDTA (0.25%, phenol red) and incubated in humidified atmosphere of 37°C and 5% CO_2 for 5-15 min until cells had detached. Trypsin was then inhibited with the addition of growth media, and cells were passaged, cryogenically stored, or counted using a

Countess II (ThermoFisher, USA) and plated in a known cell density for experimental use.

To cryogenically store primary chondrocytes and primary osteoblasts, trypsinized cells were centrifuged at 400xG for 5 min. Cells were resuspended in 1mL/cryovial of Cryo-SFM (Promo-Cell, Germany), placed in a cell freezing container (Nalgene®, Mr Frosty, Thermo Scientific, USA) and stored in a -80°C freezer for 24 hours to achieve the -1°C cooling rate. For long-term storage, cells were transferred to liquid nitrogen (LN_2).

2.3.1 Cell Culture Media

Primary chondrocyte growth media

Dulbecco's Modified Eagle Medium (DMEM) – High Glucose (D6546, Sigma, USA)

10% Fetal Bovine Serum (FBS), qualified E.U. –approved, South America origin (10270106, GIBCO, UK)

100 Units/mL Penicillin/streptomycin (15070-063, GIBCO, UK).

2mM L-Glutamine, G7513 (Sigma, USA)

1% Minimum Essential Medium (MEM) Non-essential amino acids, (11140050, GIBCO, UK).

Primary chondrocytes stimulation media

Dulbecco's Modified Eagle Medium (DMEM) – High Glucose (D6546, Sigma, USA)

0.1% Fetal Bovine Serum (FBS), qualified E.U. –approved, South America origin (10270106, GIBCO, UK)

100 Units/mL Penicillin/streptomycin (15070-063, GIBCO, UK).

2mM L-Glutamine, G7513 (Sigma, USA)

1% Minimum Essential Medium (MEM) Non-essential amino acids, (11140050, GIBCO, UK).

Primary Osteoblast Differentiation Media

Dulbecco's Modified Eagle Medium (DMEM) – High Glucose (D6546, Sigma, USA)

10% Fetal Bovine Serum (FBS), qualified E.U. –approved, South America origin (10270106, GIBCO, UK)

100 Units/mL Penicillin/streptomycin (15070-063, GIBCO, UK).

2mM L-Glutamine, G7513 (Sigma, USA)

1% Minimum Essential Medium (MEM) Non-essential amino acids, (11140050, GIBCO, UK).

β-glycerophosphate disodium salt hydrate (2mM), (G9422, Sigma, USA).

L-Ascorbic acid (50µg/ml), (A4403, Sigma, USA).

Dexamethasone (10nM), (D4902, Sigma, USA)

Primary osteoblast stimulation media

Dulbecco's Modified Eagle Medium (DMEM) – High Glucose (D6546, Sigma, USA)

0.1% Fetal Bovine Serum (FBS), qualified E.U. –approved, South America origin (10270106, GIBCO, UK)

100 Units/mL Penicillin/streptomycin (15070-063, GIBCO, UK).

2mM L-Glutamine, (G7513, Sigma, USA).

1% Minimum Essential Medium (MEM) Non-essential amino acids, (11140050, GIBCO, UK).

β-glycerophosphate disodium salt hydrate (2mM), (G9422, Sigma, USA).

L-Ascorbic acid (50µg/ml) (A4403, Sigma, USA).

Dexamethasone (10nM) (D4902, Sigma, USA)

Synovial fibroblast growth media

Roswell Park Memorial Institute-1640 (RPMI-1640) medium (R0883, Sigma, USA).

10% Fetal Bovine Serum (FBS), qualified E.U. –approved, South America origin (10270106, GIBCO, UK)

100 Units/mL Penicillin/streptomycin (15070-063, GIBCO, UK).

2mM L-Glutamine, G7513 (Sigma, USA)

1% Minimum Essential Medium (MEM) Non-essential amino acids, (11140050, GIBCO, UK).

1% Sodium Pyruvate (100nM, S8636, Sigma, USA).

2.4 Explants

Full-thickness hip articular cartilage was excised from the femoral head using a sterile scalpel. Explants of 3mm diameter were cut using a cork-borer from the articular cartilage, and placed into a 96 well tissue culture plate containing chondrocyte growth media (section 2.3.1). Explants were cultured for at least 1 week prior to their experimental use to prevent bias from cutting, and media was changed every 3 days. Human femoral head subchondral bone chips were also cultured in 24-well plates for adipokine stimulation, with each well containing a matched bone weight.

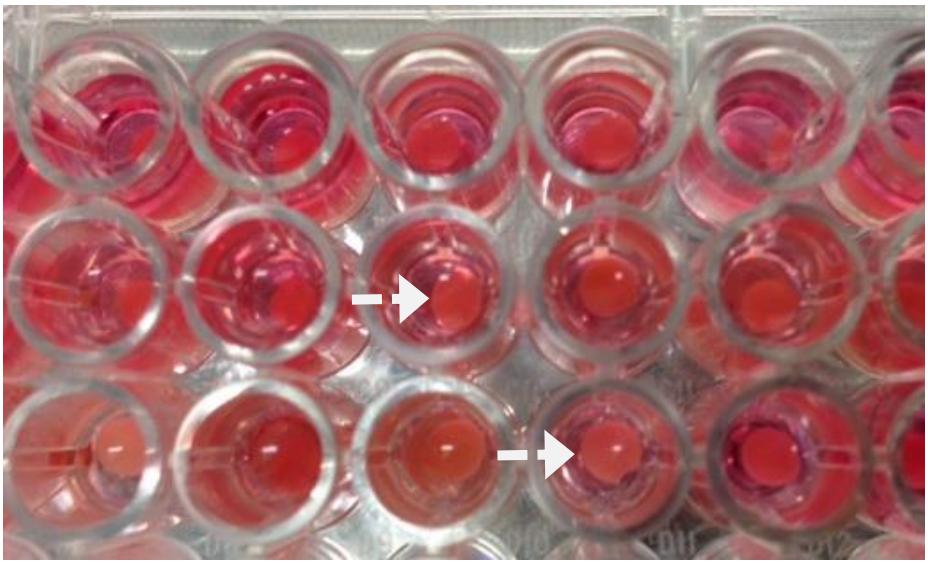


Figure 2.1: Explants of articular cartilage cut using a cork borer and cultured in a 96 well plate in chondrocyte growth media.

The white dashed arrows show the location of the cartilage explants within the well.

2.5 Tissue protein preps

Joint tissue including cartilage, synovium, and subchondral bone was isolated from the femoral heads, diced, and frozen in LN_2 for long-term storage. For protein preparation, joint tissue was powdered using a Spex Sample Prep 6770 Freezer mill (Stanmore, UK) following a protocol of 1min pre-cooling and a 50 sec cycle of 15 counts per second. Frozen powdered cartilage and synovium tissue was re-suspended in RIPA buffer (150 mM NaCl, 1.0% IGEPAL® CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0) containing protease inhibitor cocktail (PIC) and homogenized on ice for 1 min using a TissueRuptor (QIAGEN). Frozen powdered bone tissue was resuspended in Urea lysis buffer (8M Urea, 2M Thiorurea, 5% SDS, 50mM Tris pH 7) containing PIC and homogenized for 1 min on ice, and

then sonicated for 1 min in 10 sec pulses at 70% magnitude using a Vibracell sonicator (Sonics, USA).

Following homogenization, samples were centrifuged for 20 min at 16900xg at 4°C. The supernatant was aliquoted and stored at -80°C. Samples resuspended in RIPA buffer were quantified using the standard bicinchoninic acid assay (BCA) assay protocol (Pierce, UK). Samples resuspended in Urea lysis buffer were quantified using the standard QUBIT protein assay kit (Thermo Scientific, UK).

To denature proteins for western blotting, samples were diluted in sterile H_2O and 4x laemmli sample buffer (8% SDS, 20% β-mercaptoethanol, 40% glycerol, 0.008% bromophenol blue, 0.25M Tris HCl, pH6.8) and heated at 100°C for 5 min.

2.6 Western Blotting

Following protein sample preparation, samples were then loaded on to a 16% (Resistin) or 12% (all other proteins) SDS PAGE gel, along with a molecular weight marker (Biorad, UK). The gels were run at 150 volts for between 1.5 to 2 hr, and then transferred to methanol activated 0.45µ polyvinylidene difluoride (PVDF, Biorad, UK) membrane for 1.5 hr. Following the transfer, specific binding was blocked through the incubation of the PVDF membrane in 3% bovine serum albumin (BSA)/TBS-T (Tris-buffered saline+0.1% tween 20 (Sigma USA)) for 1 hr at RT on the orbital shaker. Primary antibody incubation was performed overnight in 3% BSA/TBS-T at 4°C. The primary antibodies used within this study are listed in Table 2.1. Following primary antibody incubation, the PVDF membrane was washed 3x5 min washes in TBS-T before incubation with the secondary antibody for 1 hr at RT. The secondary antibodies used for western blotting within this study are stated in Table 2.2. Following 3x5 min washes in TBST, blots were incubated in enhanced

chemiluminescence (ECL) solution (ECL Prime, Amersham, USA) for 3 min, and visualised using the BioRad ChemiDoc technology (BioRad).

Table 2.1. Primary antibodies

Target	Species	Dilution	Clone/ reference	Purification	Company
α-Leptin	Rabbit	1:1000	PA1-052	Affinity Purified	Pierce, UK
α-Adiponectin	Rabbit	1:1000	PA1-054	Affinity Purified	Pierce, UK
α-Visfatin	Rabbit	1:5000	PA1-1045	Affinity Purified	Pierce, UK
α-Leptin Receptor	Rabbit	1:1000	PA1-053	Affinity Purified	Pierce, UK
α-Resistin	Rabbit	1:4000	PA1-1049	Affinity Purified	Pierce, UK
α-Nucleobindin-2	Rabbit	1:1000	PA5-34526	Affinity Purified	Pierce, UK
α-Chemerin	Mouse	1:1000	Ab72965	Affinity Purified	Abcam, UK
α-Actin	Mouse	1:2000	AC-40	Ascites fluid	Sigma, USA

Table 2.2. Secondary antibodies

Target	Species	Dilution	Clone/ reference	Purification	Type	Company
α-mouse IgG	Sheep	1:10000	NA931V	Purified	Horse radish peroxidase (HRP)	NEB (USA)
α-Rabbit IgG	Sheep	1:10000	NA9340V	Purified	HRP	NEB (USA)

To confirm equal loading of proteins, PVDF membranes were stripped with a harsh stripping buffer (0.8% 2-mercaptoethanol, 0.5% SDS, 62.5mM Tris HCl pH6.8). Membranes were incubated in harsh stripping buffer at 50°C for 30 min with gentle agitation, and then washed in 3x5min TBS-T. Following the washes, the membrane was blocked in 3% BSA/TBS-T for 1 hr at RT, and primary antibody incubation was repeated with α-actin.

2.7 Recombinant protein

Unless otherwise stated, the properties of the recombinant proteins are stated in Table 2.3. Where possible, the same batch of recombinant protein was aliquoted and utilised for all experiments detailed in this thesis (leptin and EGFR). When it was not possible to use the same batch throughout all the experiments, the activity of each batch was compared by stimulating human primary chondrocyte cells and measuring IL6 secretion (resistin, visfatin, and IL1β). Prior to stimulation, cells and explants were cultured in primary chondrocyte or primary osteoblast stimulation media for 1hr in a humidified atmosphere of 37°C in 5% CO_2. Recombinant proteins were diluted in primary chondrocyte or primary osteoblast stimulation media and added to cells or explants for 24 hr unless otherwise stated. Following the 24 hr stimulation, media supernatant was removed and aliquoted for subsequent cytokine/chemokine analysis, and explants were snap frozen. Cells were then either harvested for subsequent RNA or protein analysis.

Table 2.3 Recombinant proteins used in this study

rProtein	Source	Concentration	Sequence	Company	Catalogue Number
Resistin	E.Coli	500ng/mL	Full length (aa 15-110)	Cambridge Bioscience	GFH107-25
Visfatin	E.Coli	500ng/mL	Full length (aa 1-491) and N-term His-Tag	Cambridge Bioscience	4907-50
Leptin	E.Coli	100ng/mL	Full length (aa 1-146)	Cambridge Bioscience	GFH37-1000
IL1β	E.Coli	1ng/mL	Unknown (153 aa residues)	Sigma	I9401-5UG
EGFR	E.Coli	2.5ug/mL	Unknown (53 aa residues)	Cambridge Bioscience	228-10360-2

2.8 Adipokine, cytokine and chemokine Luminex

To determine cytokine and chemokine concentrations in serum and synovial fluids, multiplex technology (Luminex® Screening Assay, R&D Systems) was performed. In the case of synovial fluid samples, in order to improve intra-assay variation, samples were treated with 2mg/mL hyaluronidase as previously described by Jaydev *et al.,* (2012). Thus, synovial fluid samples were diluted 1:1 in 4mg/mL hyaluronidase for 1hr at RT on a shaker. Following incubation, samples were centrifuged at 1000 g for 5 min, and the resulting hyaluronidase-treated supernatant was used for the assay.

Multi-plex analysis was performed according to the manufacturer's instructions. In brief, 50 µl of a 1x antibody magnetic bead stock ((Adipokine; limit of detection (LOD), intra-assay variation (%)) Adiponectin; 148pg/mL, 10.32%, Serpin E1; 0.67pg/mL, 17.2%, Aggrecan; 249pg/mL, 5.12%, Amphiregulin; 131pg/mL, 11.62%, CCL11; 14.6pg/mL, 5.98%, CCL2; 9.9pg/mL, 9.6%, CCL3; 16.2pg/mL, 9.33%, CCL20; 3.39pg/mL, 10.01%, Chemerin; 69pg/mL, 9.68%, CXCL10; 1.18pg/mL, 6.99%, Dkk1; 50.9pg/mL, 5.78%, Galectin-1; 632pg/mL, 6%, gp130; 11.2pg/mL, 13.34%, IL1β; 0.8pg/mL, 7.5%, IL10; 1.6pg/mL, 11.53%, IL15; 1.01pg/mL, 12.76%, IL7; 0.41pg/mL, 14.42%, Visfatin; 2243pg/mL, 4.24%, TNFα; 1.2pg/mL, 8.04%, Galectin-3; 1.68pg/mL, 12.4%, IL6; 1.6pg/mL, 5.66%, CCL4; 5.8pg/mL, 9.43%, FABP4; 95.7pg/mL, 9.08%, LIF; 9.31pg/mL, 14.14%, Leptin; 10.2pg/mL, 11.94%, Resistin; 3.04pg/mL, 8.25%, and MMP-1; 35pg/mL, 8.7%, -2; 450pg/mL, 7.9%, -3; 116pg/mL, 6.0%, -7; 5.4pg/mL, 7.0%, -8; 1.5pg/mL, 6.1%, -9; 24pg/mL, 5.0%, -10; 1.6pg/mL, 6.1%, -12; 1.0pg/mL, 7.9%, -13; 4.9pg/mL, 5.0%) was added to each well of a 96 well plate. 50 µl of standard solution or pre-diluted serum or hyaluronidase-treated synovial fluid was then added to relevant wells and the plate was sealed with foil and incubated for 2 hr at RT on an orbital shaker (speed 800±50 rpm). Post incubation, the plate was washed 3x with wash buffer and 50 µl of a biotinylated antibody added to all wells. Following a 1 hr incubation at RT on an orbital shaker (speed 800±50 rpm), the plate was washed 3x and 50 µl of diluted streptavidin-PE added to all wells. After a further 30 min incubation in the dark on an orbital shaker (speed 800±50 rpm), the plate was washed 3x and 100 µl of wash solution added to all wells. Cytokine and chemokine concentrations were then analysed using a Luminex® 200™ instrument (Luminex® Corporation, Austin, Texas, USA).

2.9 Proseek cytokine analysis of cartilage explant supernatants

The concentration of cytokine and chemokines secreted from stimulated explants was determined using the Proseek Multiplex Inflammation Kit (Olink, Sweden). Proseek Multiplex technology provides rapid, high throughput screening of human inflammatory protein biomarkers, yet requires only a small amount of sample (1uL) per panel. Due to our limited sample availability, this method proved to be the most effective. The Proseek technology utilizes Proximity Extension Assay (PEA) technology (Figure 2.2). In this proximity assay, target proteins are recognized by antibody pairs with DNA oligonucleotide conjugates. Once bound to the target protein, the antibody-oligonucleotide conjugates anneal and extend to form an amplifiable reporter DNA template via real-time PCR.

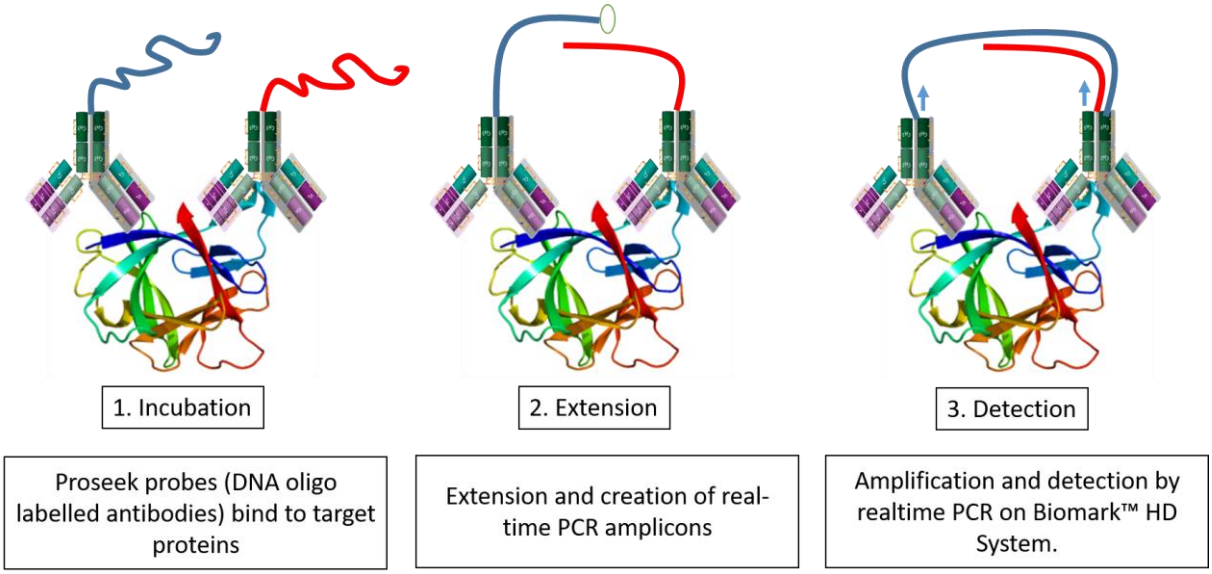


Figure 2.2. Proseek PEA technology procedure.

In this proximity assay, target proteins are recognized by antibody pairs with DNA oligonucleotide conjugates. Once bound to the target protein, the antibody-oligonucleotide conjugates anneal and extend to form an amplifiable reporter DNA template via real-time PCR.

The Proseek Multiplex inflammation kit was performed as per manufacturer's instructions. Briefly, 3uL incubation mix containing probe set A and set B were incubated with 1uL of cartilage explant tissue culture supernatants and plate controls overnight at 4°C. After 24 hr, 96uL of extension mix (High Purity Water, PEA solution, PEA enzyme, PCR polymerase) was added to the *Incubation Plate* and run in the thermal cycler using manufacturer's PEA program (50°C 20 min, 95°C 5 min, (95°C 30 s, 54°C 1 min, 60°C 1 min) x 17, 10°C hold). 7.2uL of Detection Mix (Detection solution, high purity water, detection enzyme, PCR polymerase) was added to the *Sample plate* and 2.8uL was removed from the *Incubation plate* before being combined with the detection mix in the *Sample plate.* Finally 5uL from the *Primer plate* was combined with 5uL from the *Sample plate* into the primed Dynamic Array IFC. The chip was then loaded in the Fluidigm IFC Controller HX as per the manufacturer's instructions (50°C 120 s, 70°C 1800s, 25°C 600s, 95°C 300s, 95°C 15s, 60°C 60s). LOD ranged from 0.7 and 2.4 NPX across the protein biomarker panel and intra-assay variability was an average of 12.6% CV. Data was converted from NPX (Normalized protein expression) values to linear values using the excel software provided by Olink.

2.10 ELISA

ELISAs were performed using pre-validated kits from commercial sources. Cartilage formation was assessed by measuring serum N-terminal type IIA collagen N-propeptide (PIIANP, LOD 1.1ng/mL, CV 6.60%) (Millipore, USA). Fragments of cartilage oligomeric matrix protein (COMP) were also measured by ELISA (LOD

0.2ng/mL, CV 4.8%, RnD Systems, USA). Bone resorption was determined using an ELISA to cross linked C-telopeptide of Type 1 collagen (CTX-1, 0.1ng/mL, CV 6%, Elabscience®, CN). Procollagen 1 C-terminal Propeptide (PICP) ELISA was also used to investigate the extent of bone formation (0.04ng/mL, CV 10%, Elabscience®, CN). The protocol for the ELISA was carried out according to manufacturer's instructions. Recombinant protein standards were assayed in duplicate, and sample concentrations were determined using the standard curve generated. All ELISAs were quantified on a Synergy-2 microplate reader (BioTek, Bedfordshire, UK) immediately following the addition of stop solution (2N H_2SO_4) and extrapolated using GraphPad Prism® software v5 (GraphPad software, La Jolla, USA)

2.11 DMMB assay of explant tissue culture supernatants

Sulfated glycosaminoglycan (sGAG) from cartilage explant supernatants was measured as an indicator of aggrecan degradation via a dimethylmethylene blue (DMMB) assay [280]. The DMMB assay was originally developed by Farndale and colleagues in 1986, followed by modification by Riley in 1994. To quantify the amount of sGAG in tissue culture supernatants, a standard curve of shark chondroitin sulphate C (C4384, Sigma) was used ranging from 0-50ug/mL in chondrocyte tissue culture media. 40uL of standards and samples were added to a clean 96 well plate and combined with 250uL DMMB assay reagent (50uM 1,19 Dimethyl methylene blue (DMMB, 341088, Sigma), 40mM NaCl, 40mM Glycine, 1.2N HCl). The absorbance was read at 540nm on a 96 well plate reader (Biotek Elx808) (Intra-assay CV 4.68%).

2.12 Immunohistochemistry

The femoral heads were decalcified in 5% formic acid at room temperature and embedded into paraffin. Slides were dewaxed and rehydrated in xylenes and ethanol series, and washed in PBS. Samples were heated as free-floating sections in 10 mM sodium citrate (pH 8.5, 60 min, 80°C) with gentle agitation prior to staining. Free floating sections were blocked in 10% v/v goat serum in phosphate buffer and 0.3% Triton-X 100 and incubated overnight at 4°C in primary antibodies. All primary antibodies were used at their optimal concentrations, which were determined empirically, and are detailed in Table 2.4. Sections were then incubated in Alexa-conjugated secondary antibodies and DAPI (4083, CST, USA) for 1 hr. and mounted with Prolong® Gold Antifade mountant (Life Technologies, UK). Images were obtained using a Zeiss Axiovert UV confocal microscope and Zeiss Zen 2010 software. H and E staining was performed on de-paraffinized and rehydrated sections. Sections were stained in Mayer Hematoxylin (Sigma, UK) for 8 min before standard washing procedure. Sections were then counterstained in eosin Y (Sigma, UK) solution for 45 s and washed via standard washing procedures.

Table 2.4. Primary and Secondary antibodies used in IHC staining of human OA femoral heads.

Antibody	Species	Dilution	Clone/Reference	Purification	Company
α-Visfatin	Rabbit	1:2500	PA1-1045	Affinity Purified	Pierce, UK
α-NFkB	Mouse	1:500	SC-8008	Affinity purified	Santa Cruz, USA
α-MMP-13	Mouse	1:1000	11365013	Affinity purified	Thermo Scientific, USA
α-mouse IgG1 Alexa Fluor® 488	Goat	1:1000	A-21121	Affinity Purified	Thermo Scientific UK
α-Rabbit H+L Alexa Fluor® 555	Goat	1:1000	A21428	Affinity Purified	Pierce, UK

2.13 Immunocytochemistry of β-Catenin in osteoblasts

Osteoblasts were plated in a 24 well plate for 24 hr prior to resistin stimulation (as previously stated). Following stimulation, media was aspirated and cells were washed 2x with ice cold PBS. Ice cold 4% paraformaldehyde was added to each well and incubated for 20 min with gentle rocking. Cells were then washed 3x with PBS before incubation for 1 hr in vehicle (10% goat serum in PBS and 0.1% Triton X-100). Cells were then incubated in vehicle containing primary β-Catenin antibody (AB6302, Abcam, UK) overnight. Following primary antibody incubation, cells were

washed 3x in PBS and incubated in vehicle and secondary antibody (α-Rabbit H+L, Alexa Fluor ® 555, Pierce, UK) for 1 hr with the addition of DAPI (4083, CST, USA). Cells were washed 3x with PBS before mounting with Prolong® Diamond Antifade Mountant (Thermofisher) before being visualised on a Leica DM6000 microscope.

2.14 Collagen Type I quantification

Collagen type I quantification was performed as previously described by Bailey *et al.,* [281]. Briefly, 150mg of powdered bone was washed in PBS (Sigma, UK) and centrifuged for 5 min at 1620xg. Bone powder was then washed 3x with acetone to remove bone fat, and air dried. The pellets were then resuspended in decalcifying buffer (10% (w/v) EDTA, 30mM TRIZMA base and 4M guanidine hydrochloride at pH 7.5) and placed on a rotator at 4°C. After one week, the decalcifying buffer was refreshed and the samples were placed back on the rotator in 4°C for a second week to remove mineral and non-collagenase proteins from the powdered bone sample. Decalcified bone samples were centrifuged at 4500xg for 10 min, with the insoluble fraction being retained. The powder pellet was washed 3x in ddH_2O, and resuspended in pepsin solution (0.5M acetic acid and 0.5% w/w pepsin (based upon original bone weight), P6887, Sigma) and rotated at 4°C for 24 hr. After 24 hr, the remaining pellet was combined with fresh pepsin solution and incubated at 4°C for a further 24 hr. Following pepsin incubation, the centrifuged pellet was discarded and pooled supernatants were freeze dried and analysed by SDS PAGE gel electrophoresis. Freeze-dried samples were resolved in urea lysis buffer (previously described in section 2.5), to a concentration of 2.5 µg/µl. A total of 12.5 µg protein (5 µl) was loaded into each well on a 7.5% polyacrylamide gel and bands were detected

using coomassie blue stain (0.1% w/v Coomassie Brilliant Blue (Biorad), 50% methanol, 10% glacial acetic acid) and visualized using Biorad Chemidoc Technology.

2.15 Alizarin red staining

Primary human hip osteoblasts were seeded at $6x10^3$ cells per well in a 24 well plate and treated with or without adipokine stimulation as described previously. After 14 days, cells were stained with alizarin red solution in order to quantify the degree of mineralisation following the formation of bone nodules. Briefly, cells were incubated in alizarin red staining solution (0.5% Alizarin Red (Sigma, UK) in 1% ammonia solution at pH 4.5) for 10min at RT and washed with PBS to remove excess stain. Cells were then incubated in 10% cetyl pyridinium chloride (Sigma, UK) for 10 min at RT. The supernatant was collected from each well and diluted 1:10 with the 10% cetyl pyridinium chloride and read at OD_{550nm} on a microplate Reader (Biotek, Elx808).

2.16 Alkaline Phosphatase activity

Alkaline phosphatase catalyses p-Nitrophenyl phosphate (pNPP) + H_2O to form p-nitrophenol + Pi, resulting in colorimetric reaction, which can be read at 405nm optical density. Stock Alkaline Phosphatase (ALP) (Human placenta, P3895, Sigma) was diluted to 100Units/mL in 1mM $MgCl_2$ (P2670, Sigma) and stored at -20°C. For standard preparation, ALP was diluted to 0.3unit/mL in 1mM $MgCl_2$ and serially diluted 1:2 in RIPA diluent (0.2x RIPA buffer, 1mM $MgCl_2$). Human osteoblasts were seeded at $6x10^3$ cells per well in a 24 well plate and treated with or without adipokine

stimulation as described previously. Osteoblasts were lysed in RIPA buffer diluent and diluted 1:5 with 1mM $MgCl_2$. From the diluted osteoblast lysates, 10uL was combined with 100uL of ALP substrate (solution containing pNPP, P7998, Sigma) and incubated at 37°C for 15 min. The reaction was stopped with the addition of 0.1N NaOH and read immediately at 405nm (Biotek, ELx808). ALP concentrations calculated from the standard curve were corrected for dilution factor (LOD 0.004 units/mL, CV 7.5%).

2.17 NAD activity assay

Primary human hip chondrocytes ($6x10^3$ cells per well) were plated in an opaque white-walled 96 well tissue culture plate (Corning® 3917) and treated with NAMPT small molecule inhibitors SB57 or SB58 for 1 hr (1uM, 10nM and 0.1nM) prior to co-incubation with recombinant visfatin (500ng/mL) for 24 hr in a humidified atmosphere of 37°C and 5% CO_2. NAD/NADH glo assay™ (Promega Corporation, USA) detects oxidised and reduced nicotinamide adenine dinucleotides. The assay was used as per the manufacturer's instructions. Briefly, 25uL of NAD/NADH glo™ detection reagent (Luciferin Detection Reagent, Reductase, Reductase Substrate, NAD cycling Enzyme, NAD Cycling Substrate) was added to each well containing 25uL of chondrocyte growth media. The plate was agitated for 5 min and incubated at RT for a further 60 min. Luminescence was recorded at 30 and 60 min using a luminometer (Centro LB 960, Berthold Technologies, Germany).

2.18 Gene Expression Analysis

2.18.1 RNA Extraction

RNA was isolated from powdered tissue samples and primary cells using a standard TRIzol® Reagent extraction method as detailed by the manufacturer. Briefly, 1mL of TRIzol® Reagent was added to 50-100mg of powdered tissue, or 10cm^2 of culture dish surface area, and the sample was sonicated for 6x10s bursts at 70% amplitude. Following sonication, samples were centrifuged at 12000g for 5 min to remove the extracellular matrix containing pellet. Supernatants were combined with chloroform and centrifuged at 12,000g for 10 min for phase separation. The RNA-containing aqueous phase was then diluted in 5 vol of isopropanol and incubated overnight at -20°C to precipitate the RNA. The precipitated RNA was then pelleted by centrifugation at 12,000g for 10 min. Finally the pellet was washed in 75% ethanol, air dried, resuspended in RNAse free water, and stored at -80°C until required. RNA samples were quantified using a Nanodrop (NanoDrop 2000, ThermoScientific, U.S.A) and 260/280 ratios were considered acceptable for subsequent qRT-PCR analysis when >1.7.

2.18.2 qRT-PCR

Forward and reverse custom primers were generated (Primer Design, UK) for qRT-PCR using SYBR green detection. Primers used within this thesis are detailed in Table 2.6. All assays were performed on a 384 white opaque plates. The 5uL reaction was set up as follows; 2uL RNA quantified at 5ng/uL, 2.5uL 2x Precision OneStepPLUS qRT-PCR MasterMix (Primerdesign, UK), 0.25uL forward and reverse primer mix, 0.25uL RNAse free H_2O. The one-step amplification protocol was 40

cycles as follows; reverse transcription for 10min at 55°C, enzyme activation for 2min at 95°C, denaturation for 10s at 95°C, data collection for 60s at 60°C (Lightcycler® 480 system, Roche, DE). ΔΔCt was calculated using the following calculation; ratio = $2^{\Delta Ct \text{ target (control – treated)}} / 2^{\Delta Ct \text{ reference (control – treated)}}$ [282]. To calculate percentage of gene expression knockdown, the following formula was used; %KD= (1-ΔΔCt) x100.

For WNT pathway gene expression analysis, a RT2 profiler WNT signalling PCR array was used (Qiagen, Manchester, UK). Each reaction was 10uL and the mastermix was set up as follows; 102uL RNA quantified at 5ng/uL, 650uL 2x Precision OneStepPLUS qRT-PCR MasterMix (Primerdesign, UK), 548uL RNAse free H_2O. The protocol for amplification was the same as stated above, as were the methods of analysis. All data was normalised to the expression of the housekeeping genes GAPDH, ACTB and RPLP.

Table 2.5 Custom Primers for RT-PCR assays

Gene Symbol	Sequence	Tm	GC%
BDKRB1	5'-TGCCAACATTTATCATCTCCATCT-3'	57.2	37.5
	3'-GCCCAAGACAAACACCAGAT-5'	56.4	50
BDKRB2	5'-CACACTGCGGACCTCCAT -3'	56.7	61.1
	3'-CCCTCAATCCTTACACAAATTCAC -5'	57	41.7
GAPDH	UNKNOWN	UNKNOWN	UNKNOWN
ACTIN	UNKNOWN	UNKNOWN	UNKNOWN

BDKRB1 and BDKRB2 primers were chosen by PrimerDesign Ltd, UK based on their specificity and selectivity to the BDKRB receptor sequence. Primer melting temperatures were within the optimal range (56.4-57.2°C) to avoid secondary annealing and GC content ranged from 37.5-61.1%. Prior to sending, analysis of primer/primer interactions and folding analysis is performed. Laboratory tests such as melt analysis are performed to ensure a single product is produced. As such Primer Design Ltd. UK guarantees a minimum 95% PCR efficiency.

2.19 IPA analysis

Pathway and gene network analysis was performed using the pathway analysis software application "Ingenuity Pathway Analysis" (www.ingenuity.com). Individual protein nodal networks were developed using the "grow" tool to identify direct relationships. Each protein was "grown" to a maximum of 10 nodes. Path explorer was then used to identify direct and indirect pathway links between protein nodal networks and the pathology networks. These novel "protein/pathology network"

pathway maps were then overlaid with gene data in order to evaluate the degree of likely network modulation in an established *in vitro* model of OA.

2.20 Proliferation Assay

Proliferation was determined using the CellTiter 96® Aqueous One Solution Cell Proliferation Assay kit (Promega, USA), as per the manufacturer's instructions. MTS tetrazolium compound is reduced by cells to formazan which allows a colorimetric quantitation of the tissue culture supernatant. The quantity of formazan is directly proportional to the number of cells/well. Briefly, media was aspirated from each well of a 96 well plate, and fresh media was added (100uL). CellTiter 96® Aqueous One Solution Reagent (20uL) was added to each well, and the cells were incubated for 4 hr in a humidified atmosphere of 37°C and 5% CO_2. Following the 4 hr incubation, absorbance was recorded at 490nm (Biotek, ELx808).

2.21 Determining cellular metabolic respiration

Osteoblasts were seeded at a density of $6x10^3$ cells in XFe24-well cell culture microplates (Seahorse Bioscience, North Billerica, MA). Cells were then incubated in a humidified atmosphere of 37°C and 5% CO_2 for 24 h prior to adipokine stimulation. Following 24 h, cells were treated with recombinant adipokines (see Table 2.4) for either 24 h or 2 weeks. Prior to the assay, cells were placed in 600 µl of Seahorse XF Base Medium (pH 7.4, 10% FBS, 4.6g/L glucose, 2mM β-glycerophosphate disodium salt hydrate, 10mM Dexamethasone) pre-warmed to 37°C. The plate was then transferred to a non-CO_2 incubator for 1 hr. Following calibration, oxygen consumption rate (OCR), extracellular acidification rate (ECAR) and proton

production measurements were performed for basal respiration. Upon completion of the assay, cells were collected in lysis buffer (RIPA buffer; 0.4% protease inhibitor cocktail) and centrifuged for 10 min at 8,000 *g* and the supernatant was removed for protein determination. Protein concentration was determined using the BCA protein assay (Pierce, UK). OCR was reported relative to protein content (pmol/min/µg).

2.22 Micro-CT sample preparation

Femoral heads were cut into approximately 1cm^3 cubes by the Royal Orthopaedic Hospital pathology service (University of Birmingham). Cubes were cut from the most medial aspect of the femoral head, as depicted in Figure 2.2. Bone cubes were washed 3x in acetone to de-fat the bone sample before being allowed to air dry prior to micro-CT scanning.

2.22.1 Micro-CT scanning

The scanner used to determine the gross structural parameters of bone tissue was a Bruker Sky scan 1172 (Bruker Skyscan 1172, e2v technologies plc, Chelmsford, UK). Samples were placed on a 68mm sample holder, with the axis of the femur perpendicular to the scanning plane. Sample resolution size was set to 9.87um, with an exposure time of 200ms and a rotation step of 0.4°, and no filter was applied. The flat field correction was determined for the first sample, and then maintained throughout all subsequent samples in order to reduce variations due to camera pixel sensitivity. Approximately 800 scan slices were collected with 60% beam hardening correction and a ring artefact correction of 5, and reconstructed using the NRecon software version 1.6.2 (SkyScan, e2v technologies plc, Chelmsford, UK).

2.22.2 Micro-CT reconstruction and analysis

Post alignment of each image was minor at 0±1. The reconstruction settings were maintained for all samples (Smoothing = 2, smoothing kernel = 0, reconstruction duration per slice 0.18s). One hundred slices immediately below the cortical bone layer were isolated and assayed for quantitative analysis. Regions of interest were drawn within the bone area (as shown in Figure 2.3) for each sample. Adaptive thresholding was performed (Settings; round kernel, radius 4, constant 0, background dark, pre-threshold on, lower grey threshold 69, upper grey threshold 255), and white speckles were removed (<20 voxels). Finally a despeckle sweep of the 3D space was used to correct for image irregularities and images were analysed using CTVol software (SkyScan, e2v technologies plc, Chelmsford, UK).

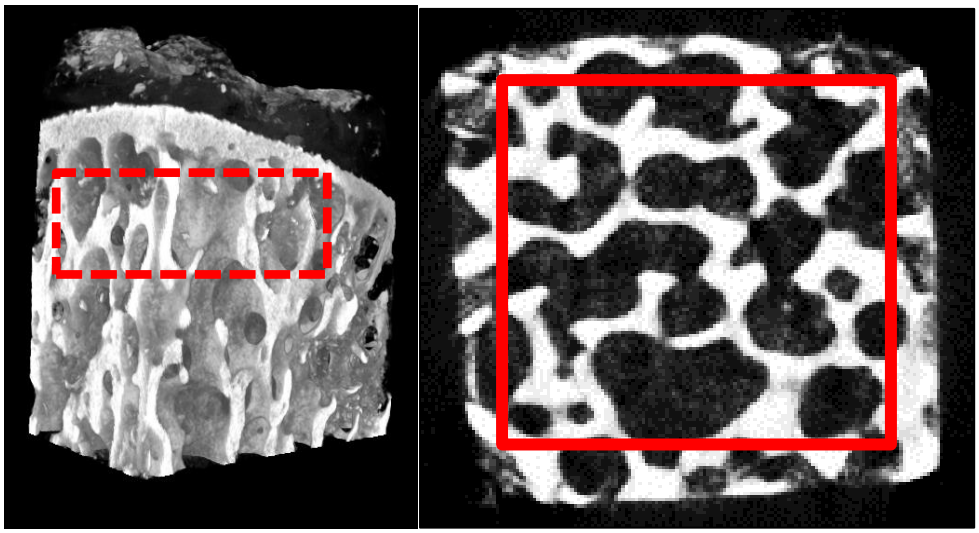


Figure 2.3. Representation of the region of interest selected from the trabecular bone area for micro-CT analysis.

2.23 Receptor identification screen

In order to identify candidate receptors for visfatin, a binding screen of His-tagged recombinant visfatin against >2000 human membrane proteins (representing approximately 65% of the total known) was performed using the Retrogenix Cell Microarray platform (Retrogenix, Macclesfield, UK). In brief, binding conditions were optimised for binding of His-tagged recombinant proteins, based on a prior Retrogenix screen using a His-tagged EGF protein. Expression vectors encoding each of the human membrane proteins were spotted onto glass slides. A HEK293 cell monolayer was cultured over the glass slide resulting in overexpression of each of the human membrane proteins via reverse transfection. In the primary screen, slides were incubated with 2.5 ug/ml His-tagged visfatin or His-tagged visfatin labelled beads, and interactions were detected using a mouse anti-His antibody (Millipore) followed by an Alexa Fluor 647 anti-mouse antibody (Life Technologies). Protein 'hits' were identified by visual inspection using Image Quant software (GE). Following the primary screen, vectors encoding each of the positive hits were sequenced and confirmation/specificity screen was done, with each of the positive hits re-spotted and re-probed His-tagged visfatin at 2.5ug/mL, His-tagged EGF at 2.5ug/mL, or an anti His antibody alone, to confirm specificity.

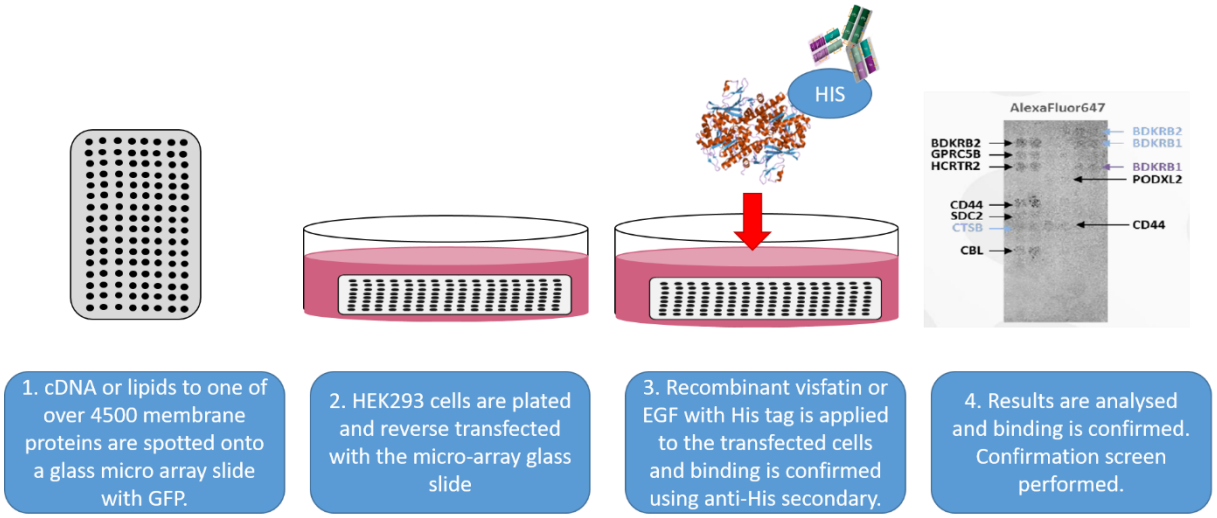


Figure 2.4. Retrogenix procedures.

As stated above, cDNA of potential plasma membrane receptors are spotted onto a glass slide for overexpression in HEK293 cells. His-tagged recombinant protein is added to the cultured cells and binding is confirmed through the use of anti-His antibodies and Alexa Fluor 647 antibodies. Specificity is confirmed through the absence of binding in secondary only staining or following His tagged EGF binding.

2.24 Transfection of human primary chondrocytes

Human primary hip chondrocytes were cultured in a 75cm^2 flask until cells were at 80% confluency. Cells were then trypsinized in Trypsin-EDTA (0.25%, phenol red) and incubated in humidified atmosphere of 37°C and 5% CO_2 for 5-15 min until adherent cells had detached. Trypsin was then inhibited with the addition of chondrocyte growth media. A small aliquot of media containing cells (10uL) was diluted 1:2 with trypan blue and counted using a Countess II (ThermoFisher, USA). Cells were centrifuged at 400xg for 5 min and resuspended in 18uL of P3 4D-Nucleofector™ solution (Lonza) per 2.5x10^5 cells/transfection at RT. siRNA was diluted in P3 4D-Nucleofector™ solution, combined with the cell suspension at a final

concentration of 100uM, and then placed into the Nucleocuvette™ vessels (Lonza). Cells were then electroporated using the P3 human primary chondrocyte pre-set program (Amaxa 4D Nucleofector, Lonza). Following electroporation, cells were combined with chondrocyte growth media and plated into 96 well plates. Cells were then cultured for 24 hr prior to RNA isolation.

2.25 Data handling and statistical analysis

All statistical calculations were performed using Graphpad Prism® software (La Jolla, U.S). Descriptive statistics were tabulated to detail patient characteristics (Mean ± SD). Gaussian distribution was confirmed using Kolmogorov-Smirnov test. Data that followed a normal distribution was analysed using a paired or unpaired Student's T-test to compare between two matched paired or two independent groups respectively. A one-way ANOVA with Tukey Post-Hoc was used to determine differences between three or more groups with a repeated measure ANOVA with a Tukey post was used to compare matched observations between three or more groups. When there was more than one categorical independent variable, a two-way ANOVA was used with a Bonferroni post-hoc.

To analyse the differences in patient serum profiles, a linear regression analysis was used. In order to perform a linear regression analysis, controlling for age, mean arterial pressure (MAP) and BMI, our data was analysed to confirm that it met with the assumptions required of regression analysis. These assumptions included independence of observations as determined by a Durbin-Watson score from 1.2-2.5, non-multicollinearity as determined with VIF values of 1-10, and normal distribution of residuals via P-P plot.

To analyse gene expression, and address the skewed linear scale, all samples were log transformed prior to statistical analysis. Data presented in graphs represent the mean ± standard error of the mean (SEM) and statistical significance was stated with a minimum confidence level of 95% ($p<0.05$).

Chapter 3: Profiling adipokine expression and systemic inflammation in patients with OA

3.1 Introduction

The role of inflammation in OA is a controversial subject, as many researchers still consider OA to be a non-inflammatory disease. However more recent evidence suggests that inflammation is a key driver of joint pain and tissue damage in OA [147, 283]. Unfortunately, the use of OA tissues as controls for RA inflammation studies has reinforced the notion that OA is a non-inflammatory disease, however studies as early as 1959 have demonstrated a heightened concentration of inflammatory proteins in the serum and synovial fluid of OA patients when compared to a non-arthritic population [284].

OA is a multi-risk factor disorder including age, joint trauma, gender and obesity [285]. Obesity is considered a major and preventable risk factor associated with OA progression. However, the mechanistic relationship between adiposity and joint destruction and risk of OA progression is not fully established. For many years, joint damage was considered a consequence of increased weight-bearing on the hips and the knees of obese individuals. However, the two-fold increase in prevalence of hand OA in obese individuals indicates not only a biomechanical factor associated with OA but also a potential systemic driver, affecting distal non-weight bearing joints such as the hands [286].

Importantly, adipose tissue is no longer considered an inert, inactive tissue [287], but a metabolically active endocrine organ secreting a plethora of factors including chemokines, cytokines and adipokines. Inflammatory protein release has been mechanistically linked to metabolic complications and the metabolic syndrome [27, 33] by contributing to the low-level pro-inflammatory state seen commonly in obese individuals [28]. Critically, whether these inflammation-associated proteins are central

regulators of OA disease pathology or purely biomarkers of an inflammatory tissue is an important question.

In this chapter, the aim was to characterise the serum and synovial fluid adipokine profiles of hip OA patients and to determine the relationship to adiposity (BMI, W:H ratio, fat mass %) and to radiographic indices of joint severity (JSN, KL grade and osteophytes). Furthermore, to compare serum adipokine profiles in patients with or without hip OA.

3.2 Results

3.2.1 Population characteristics

In total, the study included 150 serum samples from patients with end-stage hip OA of varying adiposity. In addition, the study included 24 serum samples from individuals without OA (NOF# patients). The absence of OA was confirmed through radiograph scoring by orthopaedic surgeons and visual inspection of the femoral head following surgery. The patient was also observed for evidence of hand OA and injury and surgery history was recorded for each patient. Table 3.1 details the main demographics of each group. Our OA population had significantly higher BMI (29.4±5.8 vs. 24.1±3.8 kg/m^2 respectively) and mean arterial pressure (MAP) (98.8±9.6 vs. 91.2±8.7 mmHg respectively) compared to our non-OA population. Furthermore, the OA population was significantly younger than our non-OA control patient population (68 ± 8 vs. 75 ± 6 years respectively), all of which are important confounders that must be considered for data analysis. Unfortunately W:H ratio and body fat % could only be obtained from our OA cohort due to the delicate nature of our non-OA patient cohort (neck of femur fracture) at the time of sample collection.

Table 3.1 General population demographics

	Non OA	OA	P
Gender (M/F)	7/17	76/74	-
Age (yr.)	75±6	68±8	***<0.0001***
Height (cm)	164.6±7.6	166.8±9.5	0.203
Weight (kg)	65.1±10.4	81.9±17.3	***<0.0001***
BMI (kg/m^2)	24.1±3.8	29.4±5.8	***<0.0001***
W:H ratio	N/A	0.9±0.09	-
Body Fat (%)	N/A	33.1±12.05	-
MAP (mmHg)	91.2±8.7	98.8±9.6	***0.0008***

3.2.2 Comparing the inflammatory profile of non OA and OA patient cohorts.

In order to determine the profile of inflammatory cytokines and adipokines in the serum of patients with hip OA compared to a non-OA population, and identify potential systemic drivers of OA, we performed a 25-plex cytokine analysis, using the Luminex platform. Table 3.2 illustrates the mean data obtained from the luminex assay.

The adipokines visfatin, resistin, chemerin and adiponectin were all found to be significantly higher in the OA cohort, compared to the non-OA cohort. Furthermore, the concentrations of the cytokines IL7, IL10 and IL15 and the IL6 trans-signalling inhibitor, soluble gp130, were all significantly higher in the OA cohort. In addition, the OA serum samples exhibited increased concentrations of the chemokine eotaxin-1 and WNT inhibitor Dkk1, and significantly lower concentrations of FABP4, aggrecan, amphiregulin, and galectin 1, compared to the non-OA NOF# serum samples.

Perhaps surprisingly, the serum concentration of the inflammatory cytokines TNFα and IL6 were significantly lower in the OA cohort compared to the non-OA cohort. However, this could reflect a potentially heightened systemic inflammatory state due to fracture trauma in the NOF# patients at the time of sample collection. Specifically, IL6 has been shown to be integral to early fracture healing, with a significant reduction in osteoclastogenesis and callus strength in IL6 KO mice at 2 weeks post-surgery [288]. In addition, TNFα has also been shown to play an important role in fracture healing through the recruitment and osteogenic differentiation of mesenchymal stem cells (MSCs) [289]. This was further supported by higher serum concentrations of the chemokines MIP1α, MCP-1, and MIP1β, which are known osteoclast chemoattractants in the NOF# patients compared to the non-OA cohort.

Table 3.2. The inflammatory profile of OA and non-OA patient serum

(pg/mL)	Non-OA	OA	P
TNF	5.5±1.9	4.6±2.1	***0.02***
IL10	4.3±2.3	5.3±11.55	***0.03***
IL1β	16±23.7	18.7±19.2	0.213
Dkk1	1141±1292	3140±1640	***<0.0001***
MIP1α	744.8±473.1	325.8±263.6	***<0.0001***
Galectin 1	106238±66655	45579±30997	***0.0005***
Chemerin	3878±3835	7125±4971	***0.001***
Eotaxin	88.6±102.9	158.5±162.9	***0.002***
gp130	38399±46711	88071±34617	***<0.0001***
IP10	123.4±254.7	34.32±57.73	0.807
MCP1	2692±3056	524.1±1236	***<0.0001***
IL7	2.22±1.04	3.54±2	***0.0002***
MIP3α	257.2±577.1	81.29±392.8	0.068
Amphiregulin	622.7±97.65	594.7±213	0.241
IL15	2.43±1.3	4.15±4.67	***0.0006***
Aggrecan	243.1±68.75	225.2±301.7	***0.0004***
Resistin	7818±8715	11836±8683	***<0.0001***
SerpinE1	157125±57327	142708±64655	0.305
Adiponectin	$1.60x10^7$±$1.03x10^7$	$1.10x10^7$±$6.33x10^6$	***0.013***
IL6	23.41±17.58	11.03±62.65	***<0.0001***
LIF	-	-	-
Leptin	16839±17712	25579±33803	0.1197
FABP4	88567±103164	29754±49008	***<0.0001***
MIP1β	211.1±125.4	141.5±71.89	***0.0045***

3.2.3 Age, BMI and MAP contributions to the inflammatory profile of non OA and OA patient cohorts.

As represented in table 3.3, it is important to control for the anthropometric differences identified in our cohort populations. In order to perform a linear regression analysis, controlling for age, MAP and BMI, our data was analysed to confirm that it met with the assumptions required of regression analysis. These assumptions included independence of observations as determined by a Durbin-Watson score from 1.2-2.5, non-multicollinearity as determined with VIF values of 1-10, and normal distribution of residuals via P-P plot. Table 3.3 details the regression coefficients (B values) following correction with age, MAP and BMI.

Table 3.3. The contributions of disease, age, MAP and BMI on serum profiles of OA and non-OA patient cohorts.

(pg/mL)	Disease	p value	Age	p value	MAP	p value	BMI	p value
TNF	-0.884	0.06	0.01	0.69	-0.01	0.71	0.03	0.37
IL10	0.25	0.94	-0.53	0.68	0.21	0.06	-0.13	0.53
lL1β	2.82	0.60	0.17	0.44	-0.88	0.65	0.47	0.15
Dkk1	1723.6	***<0.001***	-3.26	0.84	12.88	0.36	21.23	0.36
MIP1α	-375.8	***<0.001***	-4.41	0.18	-1.79	0.53	-7.34	0.13
Galectin 1	-52255	***<0.001***	454.1	0.26	-568.0	0.09	-199.2	0.72
Chemerin	2658.9	***0.018***	-12.70	0.78	-31.92	0.42	121.7	0.06
Eotaxin	64.21	0.08	-1.59	0.30	-0.11	0.93	-2.01	0.36
gp130	31374.8	***0.003***	-391.2	0.36	160.73	0.66	667.4	0.28
IP10	-85.32	***0.004***	-0.50	0.68	-1.31	0.21	0.75	0.67
MCP1	-2141.1	***<0.001***	-7.37	0.67	-2.4	0.87	-9.65	0.70
IL7	1.12	***0.01***	-0.07	0.71	0.02	0.15	0.02	0.35
MIP3α	-173.71	0.13	0.93	0.84	3.33	0.41	-2.2	0.75
Amphiregulin	-44.8	0.45	2.99	0.21	-0.94	0.65	8.68	***0.01***
IL15	1.44	0.15	-0.01	0.83	0.04	0.27	-0.01	0.94
Aggrecan	-28.4	0.58	-0.09	0.97	-0.17	0.92	-2.73	0.35
Resistin	-1432.6	0.50	74.06	0.39	-124.5	0.09	31.95	0.79
SerpinE1	-21247	0.18	405.6	0.54	-950.8	0.09	2885.4	***0.003***
Adiponectin	$-4.2x10^6$	***0.02***	$1.3x10^5$	0.07	-2784	0.66	$2.1x10^4$	0.84
IL6	-23.8	0.07	-0.31	0.56	0.98	***0.03***	-0.90	0.91
LIF	-	-	-	-	-	-	-	-
Leptin	890.8	0.90	264.4	0.39	-448.4	0.09	2209.8	***<0.0001***
FABP4	-34069	***0.001***	228.3	0.56	-1071	***0.002***	1567.9	***0.01***
MIP1β	-87.54	***<0.001***	-0.72	0.46	0.31	0.71	1.02	0.47

Table 3.3 demonstrates a significant disease effect on the serum concentrations of Dkk1, MIP1α, galectin 1, chemerin, gp130, IP10, MCP-1, IL7, adiponectin, and MIP1β. Age had no significant impact on the serum concentration of any of the cytokines or adipokines measured in our patient cohorts, whereas MAP significantly increases IL6 and FABP4 serum concentration. Finally, BMI was positively associated with an increase in leptin and FABP4 expression, and a decrease in serpinE1 expression.

3.2.4 Serum adipokine and cytokine expression in relation to BMI

Given the association between adiposity and OA it is important to determine the contribution of BMI within the OA patient cohort on the serum adipokine profile. Therefore, the 150 patients with hip OA previously referred to in section 3.2.1 who were assigned to a BMI category of either NW (<25kg/m^2) or OW/OB (>25kg/m^2) were further analysed Table 3.4 details the main demographics of each patient cohort, when classified as either NW or OW/OB.

Table 3.4 Patient demographics for OA patient cohort.

	NW	OW/OB	P
Gender (M/F)	16/22	60/52	-
Age (yr.)	67±9	69±8	0.385
Joint (K/H)	7/31	52/61	-
Hand OA (y/n)	21/11	81/30	-
Height (cm)	167.5	166.5	0.621
Weight (kg)	65.5±9.7	87.5±15.6	***<0.0001***
BMI (kg/m^2)	23.2±1.1	31.5±5.1	***<0.0001***
W:H ratio	0.87±0.08	0.93±0.08	***0.005***
Body Fat (%)	24.6±10.1	34.8±9.2	***<0.0001***
MAP (mmHg)	96.8±9.9	99.4±9.4	0.191

Data was non-parametric; therefore a Mann Whitney-U test was used to compare the inflammatory adipokine serum profiles of the two OA patient cohorts. OW/OB individuals had significantly higher concentrations of TNFα (p0.04), galectin 1 (p=0.04), resistin (p=0.03), serpinE1 (p=0.002), leptin (p<0.0001) and FABP4 (p=0.003) as shown in Table 3.5. This data is depicted in graph form in Figure 3.1 A-F.

Table 3.5. The effect of BMI on the serum inflammatory profile in OA individuals.

(pg/mL)	NW	OW/OB	P
TNFα	4.10±1.67	4.85±2.13	***0.04***
IL10	4.28±1.61	4.25±1.74	0.57
IL1β	12.32±6.47	20.73±21.60	0.22
Dkk1	3195±1841	3120±1565	0.71
MIP1α	386.1±306.1	304.9±245.4	0.28
Galectin 1	39324±32563	47161±29915	***0.04***
Chemerin	7036±5563	7156±4780	0.60
Eotaxin	178.2±196.2	151.8±150.4	0.75
gp130	83546±40193	89649±32509	0.49
IP10	28.97±30.85	27.75±18.01	0.34
MCP1	441.6±685.1	551.1±1371	0.90
IL7	3.49±1.94	3.56±1.98	1.00
MIP3α	67.77±182.2	85.92±443.1	0.98
Amphiregulin	563.6±181.4	585.7±244.3	0.77
IL15	4.25±5.32	4.48±5.486	0.60
Aggrecan	231.8±250.4	215.3±317	0.73
Resistin	9818±7325	13041±9013	***0.03***
SerpinE1	114472±67611	152462±60926	***0.002***
Adiponectin	$9.48x10^6$±$6.39x10^6$	$1.12x10^7$±$5.70x10^6$	0.20
IL6	2.07±1.45	5.33±10.83	0.26
LIF	-	-	-
Leptin	8685±6671	31578±37391	***<0.0001***
FABP4	18487±15514	33718±55772	***0.003***
MIP1β	130.4±48.68	145.2±77.98	0.59

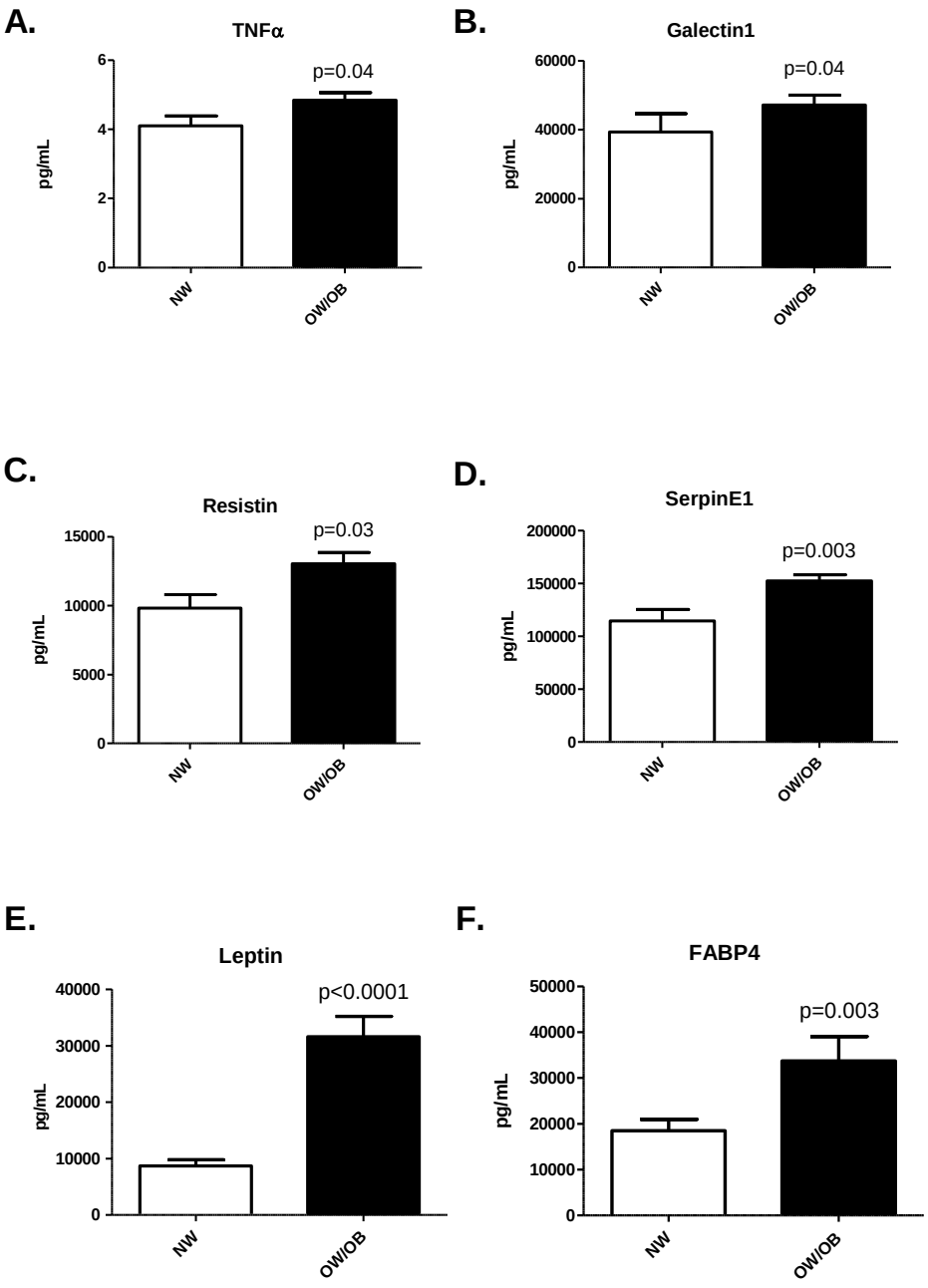


Figure 3.1. Differential serum adipokine and inflammatory profiles in OW/OB OA patients compared to NW OA patients.

Cytokines were measured by Luminex multiplex technology and expressed as pg/mL. OW/OB = overweight/obese; NW = normal weight.

Simplicity and ignorance to body composition has led to inaccuracies and bias often reported with BMI measures [290]. Therefore, data was also collected using bioelectrical impedance scales (Tanita, BC-420MA, Amsterdam, NL) to measure fat mass as a proportion of body composition. In contrast to BMI, when the serum inflammatory markers were compared with fat mass, expressed as a percentage of total body mass, only serum leptin ($p<0.0001$) and FABP4 ($p=0.001$) demonstrated a significant correlation, as shown in Figure 3.2.

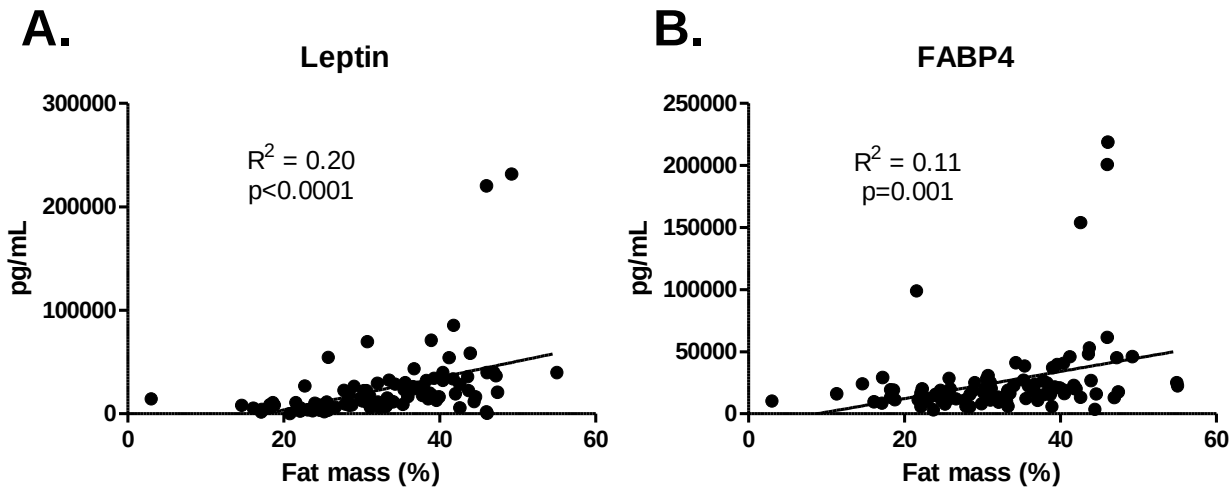


Figure 3.2. Correlation of systemic Leptin (A) and FABP4 (B) to fat mass percentage (%) as measured by bioelectrical impedance.

Serum cytokines were measured by Luminex multiplex technology.

Recent publications have highlighted the importance of central adiposity on the risk of developing obesity-associated disorders [291, 292] and systemic inflammation [293]. Therefore, W:H ratio data was collected as a measure of adiposity distribution.

Interestingly, serum concentrations of adiponectin and FABP4 were significantly negatively associated with W:H ratio, suggesting lower expression levels with increasing central adiposity (Figure 3.3). All other inflammatory markers measured had no significant relationship with W:H ratio.

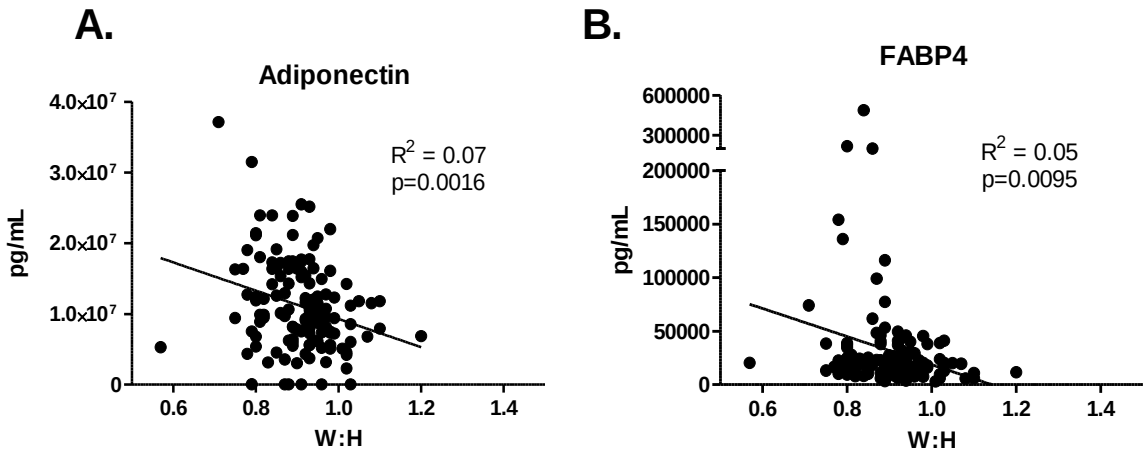


Figure 3.3. Correlation of systemic A. Adiponectin and B. FABP4 to W:H ratio.

Serum cytokines were measured by Luminex multiplex technology. W:H ratio; waist:hip ratio.

3.2.5 Serum adipokines and cytokines in relation to joint disease severity.

During the pre-operative consultations, weight-bearing radiographic images were collected in order to determine OA disease severity. From these radiographic images it was possible to ascertain the K and L grade (see Table 1.2) and the joint space as markers of disease severity. Furthermore, multi-joint OA as evident by the presence of hand OA was measured in each patient to determine if which cytokines or adipokines may be potential systemic drivers of joint degeneration.

There were no correlations identified between any of the serum concentrations of the cytokines or adipokines with K and L grade, or with the total amount of joint space. There was also no association with any of the cytokines or adipokines measured with hand OA presence (Appendix Table 3) and no association of cytokine or adipokine levels when K and L grade was separated by ≤ Grade 3 compared to Grade 4. Unfortunately, due to nature of the samples collected (i.e. end-stage OA), it was not possible to compare less severe K and L grades with systemic cytokine and adipokine expressions (Appendix Table 1). Interestingly, there was a significant increase in resistin (11113 ± 6759 vs 15256 ± 9148 pg/mL) and amphiregulin (529.3 ± 162 vs 618.9 ± 225) in joints with <1.5mm joint space compared to joints with ≥1.5mm joint space (Figure 3.4). All data is detailed in Appendix Table 2.

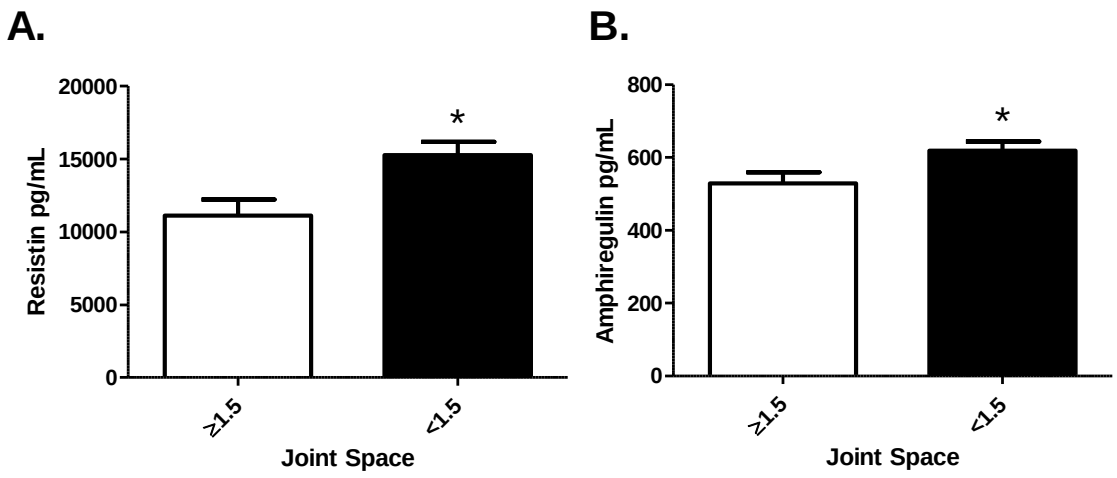


Figure 3.4. Systemic concentrations of adipokines and cytokines in relation to joint space narrowing.

A. Resistin pg/mL; B. Amphiregulin pg/mL. ≥1.5 = greater than or equal to 1.5mm joint space; <1.5 = less than 1.5mm. Serum cytokines were measured by Luminex multiplex technology.

Table 3.6 summarises the serum cytokines or adipokines that were found to be significantly different between OA and non-OA individuals, as well as those that were significantly different within the OA patient cohort dependent on BMI, fat mass %, W:H ratio and joint space. The cytokines and adipokines found to be significantly different in disease were determined in patients where age, MAP, height and BMI were controlled. For cytokines and adipokines differentially expressed according to BMI, fat mass %, W:H and joint space, patients were controlled for age, height and MAP only.

Table 3.6. Summary of serum adipokine and cytokines differentially altered in OA and NOF# patient populations.

OA vs. NOF#	OA only			
Disease	BMI	Fat Mass %	W:H	Joint Space
Dkk1	TNFα	Leptin	Adiponectin	Resistin
MIP1α	Galectin 1	FABP4	FABP4	Amphiregulin
Galectin 1	Resistin			
Chemerin	SerpinE1			
gp130	Leptin			
IP10	FABP4			
MCP1				
IL7				
Adiponectin				
FABP4				
MIP1β				

Bone vascularisation is highly important to maintain biological homeostasis and to allow the bone to perform its many functions including locomotion, calcium and phosphate metabolism, and endocrine molecule synthesis [294]. Blood flow, and its subsequent nutrient delivery, is tightly correlated with bone metabolism therefore modulating this nutrient delivery can directly impact bone resorption and formation [294].

3.2.6 Synovial fluid adipokines and cytokines expression in relation to BMI.

Unlike bone, cartilage is an avascular tissue and therefore is not directly impacted by the concentrations of nutrients of cytokines in the systemic circulation. However, local cytokine concentrations found in the synovial fluid are likely to impact cartilage turnover because nutrients are typically transported to chondrocyte cells via diffusion through the matrix from the synovial fluid [295]. Therefore while serum adipokine or cytokine levels may impact bone turnover, it is important when considering OA cartilage tissue to investigate proteins within the synovial fluid which may be impacted by BMI or central adiposity. Table 3.7 shows a significant increase in TNFα, IL7, and leptin, with a significant decrease seen in IL6, and adiponectin in synovial fluid from OW/OB individuals compared to their NW counterparts.

Table 3.7. The synovial fluid concentrations of adipokines and cytokine in NW or OW/OB OA patients at the time of elective joint replacement surgery.

(pg/mL)	NW (n=20)	OW/OB (n=52)	P
TNFα	4.34±2.92	5.13±7.87	***0.03***
IL10	16.40±9.76	20.67±10.02	0.11
IL1β	19.22±14.33	23.85±14.11	0.23
Dkk1	545.7±727.6	608.0±551.4	0.73
MIP1α	310.5±141.0	291.6±174.9	0.65
Galectin 1	115791±26364	102113±26375	0.06
Chemerin	2679±1339	3091±1220	0.24
Eotaxin	33.91±38.24	33.84±56.38	0.99
gp130	69361±14308	67543±70860	0.62
IP10	153.4±171.1	177.7±602.8	0.61
MCP1	389.3±489.5	602.8±1064	0.25
IL7	3.34±1.13	4.29±1.47	***0.004***
MIP3α	36.62±19.90	29.67±48.26	0.39
Amphiregulin	1210±611	1324±493	0.46
IL15	25.09±12.82	31.19±10.38	0.06
Aggrecan	-	-	-
Resistin	5614±7664	4806±9589	0.51
SerpinE1	33720±40188	22806±21045	0.27
Adiponectin	$4.87x10^6$±$3.53x10^6$	$2.74x10^6$±$1.71x10^6$	***0.01***
IL6	762.8±920.3	255.8±362	***0.03***
LIF	-	-	-
Leptin	12461±7312	46736±40238	***<.0.001***
FABP4	26346±38056	121662±625780	0.28
MIP1β	114.8±42.2	93.16±44.01	0.10

3.3 Discussion.

The aim of this chapter was to define the serum and synovial fluid inflammatory profile of OA disease sufferers and identify candidate cytokines or adipokines that may be integral to the progression of joint destruction. A second aim was to identify adipokines in patients with hip OA that are differentially expressed in relation to anthropometric measures including BMI, W:H ratio, and fat mass %. Identifying those inflammatory adipokines and cytokines that are associated with OA disease and disease severity, and determining differences in inflammatory profiles between OA patients of varying body composition could in the future help to stratify patient populations for adipokine-targeted therapeutics.

In this study, 11 inflammatory adipokines/cytokines were differentially expressed in OA patients compared to NOF# patients. These were Dkk1, MIP1α, Galectin 1, chemerin, gp130, IP10, MCP1, IL7, adiponectin, FABP4 and MIP1β. As known risk factors (age, BMI, MAP) were not even across both patient groups, these variables were controlled for using generalized linear regression.

The serum FABP4 data obtained from this study was intriguing. Although concentrations were lower in OA samples compared to NOF# samples (B value = -34069, $p<0.001$), there was a significant increase in FABP4 in the OA patient cohort with obesity (18487 ± 15514 pg/mL vs. 33718 ± 55772 in NW vs OW/OB respectively, $p = 0.003$), and a positive correlation with fat mass% ($R^2 = 0.11$, $p<0.0001$). Furthermore, FABP4 was negatively associated with W:H ratio ($R^2 = 0.05$, $p=0.0095$). FABP4 is primarily expressed in adipocytes and macrophages, and has a number of roles in metabolic regulation including fatty acid uptake, transportation and oxidation [296]. Further, FABP4 activates BMP signalling through the downregulation of the leptin like cytokine GDF3 [297]. Levine and Brivanlou

(2006), in accordance with others [298, 299], showed increased FABP4 levels are associated with BMP-induced bone formation. The data in this study demonstrates a significant increase in circulatory FABP4 levels with obesity in patients with hip OA. This association between adipokines and BMP signalling has previously suggested by Witthuhn and Bernlohr (2001) who noted that GDF3 is antagonized with obesity, resulting in BMP activation and bone formation [300]. With this, it is possible to deduce that adiposity is protective against bone pathologies characterised by low bone formation, such as osteoporosis. To add to the current literature, this study showed FABP4 correlated negatively with W:H ratio, suggesting that adiposity location is a significant determinant of serum FABP4 expression.

In terms of OA disease, an increased FABP4 concentration in NOF# patients compared to OA patients seems counterintuitive. While hip fractures could be a consequence of extra-skeletal factors such as poor eyesight and overall frailty, osteoporosis is also a significant risk factor to hip fractures with over 50% of fractures in women associated with osteoporosis [301]. This must be considered in our control population. Lower serum FABP4 in OA patients may also serve as an attempt to limit the well-described increase in bone turnover in patients with OA [132, 302, 303]. With little published research, this theory requires further study.

Serum resistin expression was significantly increased in OW/OB patients with OA compared to their normal weight counterparts (9818 ± 7325pg/mL and 13041 ± 9013pg/mL, in NW vs OW/OB respectively). Furthermore, resistin was significantly increased in joints of minimal joint space (11113 ± 6759 pg/mL in ≥1.5mm vs 15256 ± 9148 pg/mL in <1.5mm) and showed a trend towards increased expression in association with K and L grade ($p=0.09$).

Resistin is a dimeric adipokine secreted from adipocytes, macrophages and abundantly expressed in bone marrow cells [259], and was first identified as being down-regulated in response to the use anti-diabetic thiazolidinediones in mice [258]. Furthermore, in support of the data in this study, serum resistin was previously shown to increase in diet-induced obese mice [258].

In OA, resistin has remained poorly researched in comparison to the popular adipokines such as adiponectin and leptin, and most research is directed towards the effect of resistin on cartilage damage. For example, resistin injected into the joint space of healthy mice induced cartilage degeneration and synovial inflammation [304], in addition to upregulating catabolic cartilage mediators including MMPs, TNFα and IL6 [305]. The role of resistin in mediating cartilage damage was further supported recently by Song *et al.,* (2015) who identified a significant association between synovial fluid resistin expression and the Noyes score (a scoring system based upon four variables: cartilage surface integrity, depth of involvement, lesion location, and lesion diameter), K and L grade and WOMAC scores [306].

While we found no significant difference in resistin levels between NOF# patients and OA patient serum samples, there is an association with BMI in OA patient serum and joint space, therefore further investigating the role of resistin in modulating the pathological progression of OA in obese patient bone is highly relevant.

In addition to resistin, the adipokines adiponectin and leptin also exhibited interesting data in this study. The role of adiponectin in OA is still debatable, with many research studies citing a pro-inflammatory or anti-inflammatory role. Adiponectin represents the highest proportion of adipokines within the circulatory system, and in this study there was a significantly decreased expression in OA serum compared with NOF# patient serum (B value = -4.2×10^6, p=0.002), and it was negatively associated

with W:H ratio (R^2 = 0.07, p=0.0016). This data indirectly supports previous work by Honsawek and Chayanupatkul (2010) who found a negative association of adiponectin and joint damage severity, which infers a protective role of adiponectin in the pathogenesis of OA [307]. The authors suggested that with reduced circulating adiponectin there may be an increased inflammation and joint destruction. Therefore, as we have seen in our data, those of a lower serum adiponectin expression may be at risk of OA disease.

Conversely to our data, De Boer et al., (2012) found that serum adiponectin was markedly increased in end-stage OA patients, when compared with a control population without signs of OA [247]. Furthermore, adiponectin was specifically associated with BMI in female patients, and showed a trend towards correlating with synovial joint inflammation. The authors therefore suggested that adiponectin may play an important role in the development of inflammation within the OA joint. In comparison to our own study, the OA population within the De Boer *et al.,* study was solely those with knee OA, and control patients had recent pain and/or stiffness of the knee or hip associated with early OA, but no radiographical signs of OA. While these variables may account for the discrepancy between ours and De Boers adiponectin data, particularly as pain in both the hip and knee joint may be a result of different bone pathologies, it is possible the assays used to determine adiponectin concentrations also exhibit differing affinities for the adiponectin multimeric complexes. This requires further study.

In addition to serum cytokine levels, this study investigated local cytokine concentrations found in the synovial fluid of NW and OW/OB OA patients. There were some limitations encountered within this study including a high standard deviation in synovial fluid cytokine samples, typically seen in OA. While we

attempted to limit assay variability through hyaluronidase treatment of samples, increasing subject numbers may decrease the standard deviation and allow for more subgroup analysis. Furthermore, due to the difficulties with obtaining synovial fluid in end-stage OA, we received synovial fluid from a subset of the patients recruited for this study which may be unintentionally skewing the data towards a certain population of OA. Finally, for all patients we received a medical history and current medication list at the time of surgery. We do not consider the time it takes for some medications to clear the system and therefore could still be having an effect on the serum and synovial fluid levels of cytokines and disease severity. For example, bisphosphonates which are normally used to treat osteoporosis and therefore may be a medication used in our NOF# population, can remain in the system for up to 200 days following treatment [308].

In this study, there was a significant increase in synovial fluid TNFα, IL7, and leptin, with a significant decrease seen in IL6 and adiponectin in NW versus OW/OB patients with hip OA. Perhaps the most notable cytokines increased in synovial fluid were IL7 and TNFα. In RA, IL7 has been shown to increase pro-inflammatory cytokine production via the intra-articular CD4+ T cells and antigen-presenting cells. In particular, IL7 stimulation of synovial fluid mononuclear cells and synovial fluid CD4+ T cells resulted in a significant increase in TNFα production [309]. Despite OA being considerably less inflammatory compared with RA, histological analysis, ultrasound and MRI imaging have shown evidence of synovitis in OA joints [145] with increased cellular infiltration of activated B cells and T lymphocytes. This is further supported by Shen *et al.,* (2011) who demonstrated a role for CD4+ T cells in the initiation of anterior cruciate ligament transection induced-OA in a CD4 T cell knockout mouse model [310]. In relation to this study data, it is possible that with the

increased immune cell infiltration with OA, there is an increase in localised IL7 cytokine production and subsequent TNFα secretion into the synovial fluid. This ultimately leads to a pro-inflammatory and pro-catabolic state and subsequent tissue damage.

Chapter 4: The role of resistin in altering the biochemical composition of Collagen Type I in obese patients with OA

4.1 Introduction

To date, research into understanding the role of adipokines in OA joint pathology has predominantly focussed on the cartilage tissue by determining the effect of adipokines on chondrocyte phenotype. For example, using isolated primary human chondrocytes, Hui *et al.,* (2012) discovered that leptin alone, and in synergy with IL1β, induced the expression of catabolic factors MMP-1 and MMP-13 with activation of p38, ERK, PI3K and Akt pathways [254]. Leptin has also been demonstrated to increase the production of inflammatory mediators including IL1β, IL6, IL8 and prostaglandin E_2 [255]. However, cartilage tissue is avascular and thus is unlikely to be directly affected by systemic increases in pathological levels of adipokines.

In contrast, subchondral bone is a highly vascularised tissue, and thus would be expected to be influenced by differential concentrations of circulatory adipokines reported in over-weight and obese individuals [31, 247, 311]. Despite this, very little is currently understood with regards to the effect of obesity on subchondral bone composition in patients with OA, or the functional role of adipokines in mediating sclerotic OA bone pathology or modulating osteoblast phenotype. It has previously been reported that there is a temporal relationship between serum levels of adipokines and biomarkers of bone remodelling in females with knee OA [312], and more recently studies have shown that found that adipokines modulate osteoblast proliferation [313-315] and differentiation, supporting the concept that adipokines and obesity may impact on OA bone pathology.

Importantly, OA subchondral bone has been referred to as “sclerotic”, with reports of irregular trabecular architecture [316] abnormal collagen type I composition [281],

and dysregulated mineralisation [317]. Therefore, an important part of this chapter was to determine whether adiposity affected the architecture and collagen type I composition of subchondral bone in patients with OA, and secondly to examine the effect of candidate pathological adipokines on the composition of OA bone and on the phenotype of primary OA osteoblasts.

4.2 Results

4.2.1 Profiling the structural composition of OA bone samples from patients with differing BMIs

We first aimed to determine if varying BMI was associated with different molecular composition of collagen type I human femoral head bone samples by quantifying the relative ratio of α1 and α2 collagen type I chains. Collagen isolation was confirmed by electrophoretic mobility against a molecular weight marker. There was a significant increase in the α1 and α2 ratios in OW/OB patient bone only, with barely a detectable increase in normal weight OA bone compared with non-OA fracture neck of femur bone samples (Figure 4.1A). We investigated if gross structural parameters were different between NW and OW/OB OA patient bone samples using micro-CT technology. These data revealed that trabecular bone thickness (TbTh.) was significantly reduced in OW/OB bone samples compared with NW (0.29 ± 0.02 vs 0.34 ± 0.03 mm respectively), whereas bone surface/bone volume ratio was significantly increased (6.85 ± 0.5 vs 5.91 ± 0.5 1/mm) (Figure 4.1C-F). Other parameters measured using micro-CT demonstrated no significant alterations as detailed in Table 4.1.

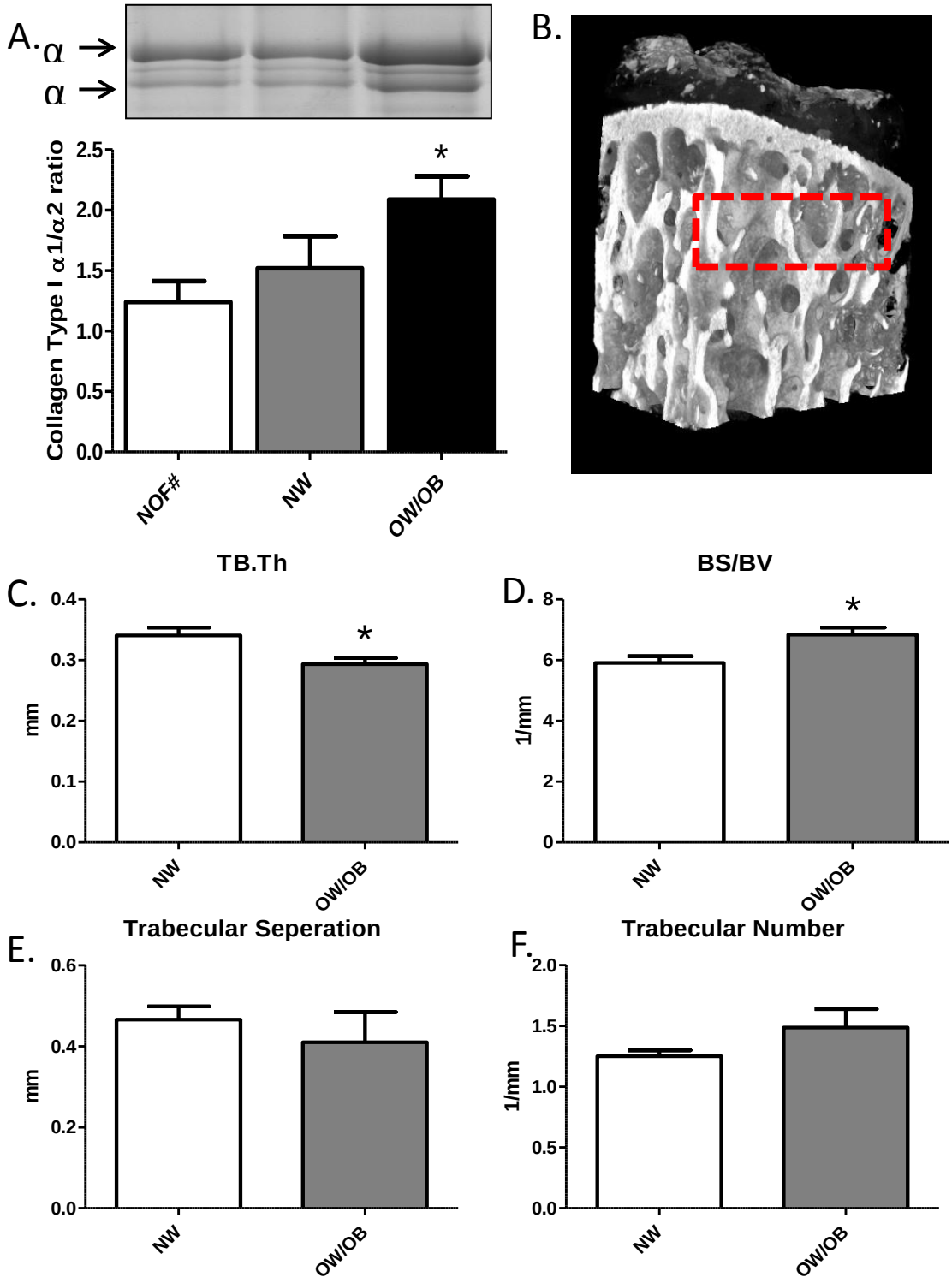


Figure 4.1. Comparison of the structural composition of femoral head subchondral bone in patients of different BMI cohorts.

A. A typical coomassie gel of subchondral bone collagen demonstrating the ratios of α1/α2 quantities. Below the coomassie image are the quantified mean α1/α2 ratios

for OA patients in different BMI groups. NOF# = fracture neck (n=6), NW = normal weight (n=7), OW/OB = overweight/obese (n=13) (*=p<0.05). **B.** Representation of the area from the trabecular bone area for micro-CT analysis. **C-F.** Micro-CT analysis of OA femoral head subchondral bone samples from NW (n=6) and OW/OB patient cohorts (n=6). Patient samples were excluded if a bone cyst was present (*=p<0.05). Tb.Th = Trabecular thickness; BS/BV = Bone surface/Bone volume.

Table 4.1. Micro CT of human OA femoral head subchondral bone.

PARAMETER	NW (Mean±SD)	OB (Mean±SD)	p value
Tb.Th	0.34 ± 0.03	0.27 ± 0.02	0.02
Tb.N	1.25 ± 0.12	1.66 ± 0.34	0.14
Tb.Sp	0.47 ± 0.08	0.41 ± 0.17	0.47
BS/BV	5.91 ± 0.54	7.54 ± 0.52	0.02

Micro-CT analysis of OA femoral head subchondral bone samples from NW (n=6) and OW/OB patient cohorts (n=6). Patient samples were excluded if a bone cyst was present (*=p<0.05). Tb.Th = Trabecular thickness; BS/BV = Bone surface/Bone volume; Tb.Sp. = trabecular separation, TB.N = trabecular number. Data expressed as mean ± SD.

4.2.2 Adipokines alter the collagen composition of OA bone

Following identification of differential collagen formation in OW/OB patients, we examined whether adipokines may provide a systemic link to altering bone pathology. In serum, we identified a significant increase in leptin and resistin

concentrations in the OW/OB population compared to the NW OA population (31578 vs 8685pg/mL and 12740 vs 9818pg/mL respectively). There was no difference in circulating visfatin concentrations in OW/OB compared to NW (4410 vs 4467pg/mL).

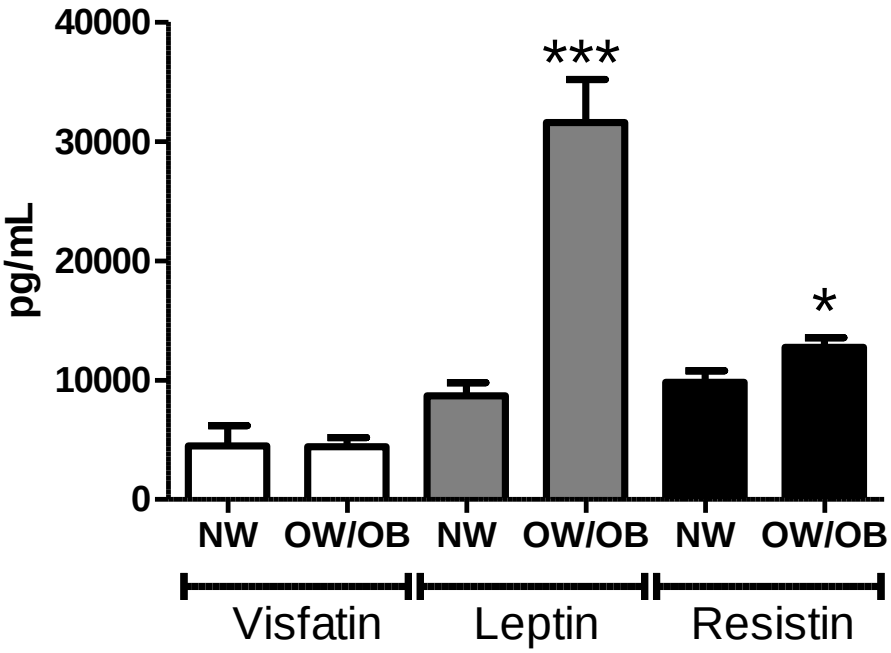


Figure 4.2. Serum adipokine expression in NW and OW/OB patients with OA. Adipokines were measured by Luminex multiplex technology. ***=p<0.0001, *=p<0.05.

We then examined the effect of chronic adipokine stimulation on the collagen composition phenotype of NW OA bone. Bone samples were cultured for 4 weeks in adipokine-containing media and collagen Type I was isolated. Resistin was shown to be the only adipokine that affected collagen homotrimer formation with a 2.4 fold increase (3.8 compared to 1.6 in resistin versus media control culture bone) (Figure 4.3A). This was further confirmed with an increase in COL1A1 gene expression

($p<0.005$) compared to no significant change in COL1A2 gene expression (Figure 4.3B).

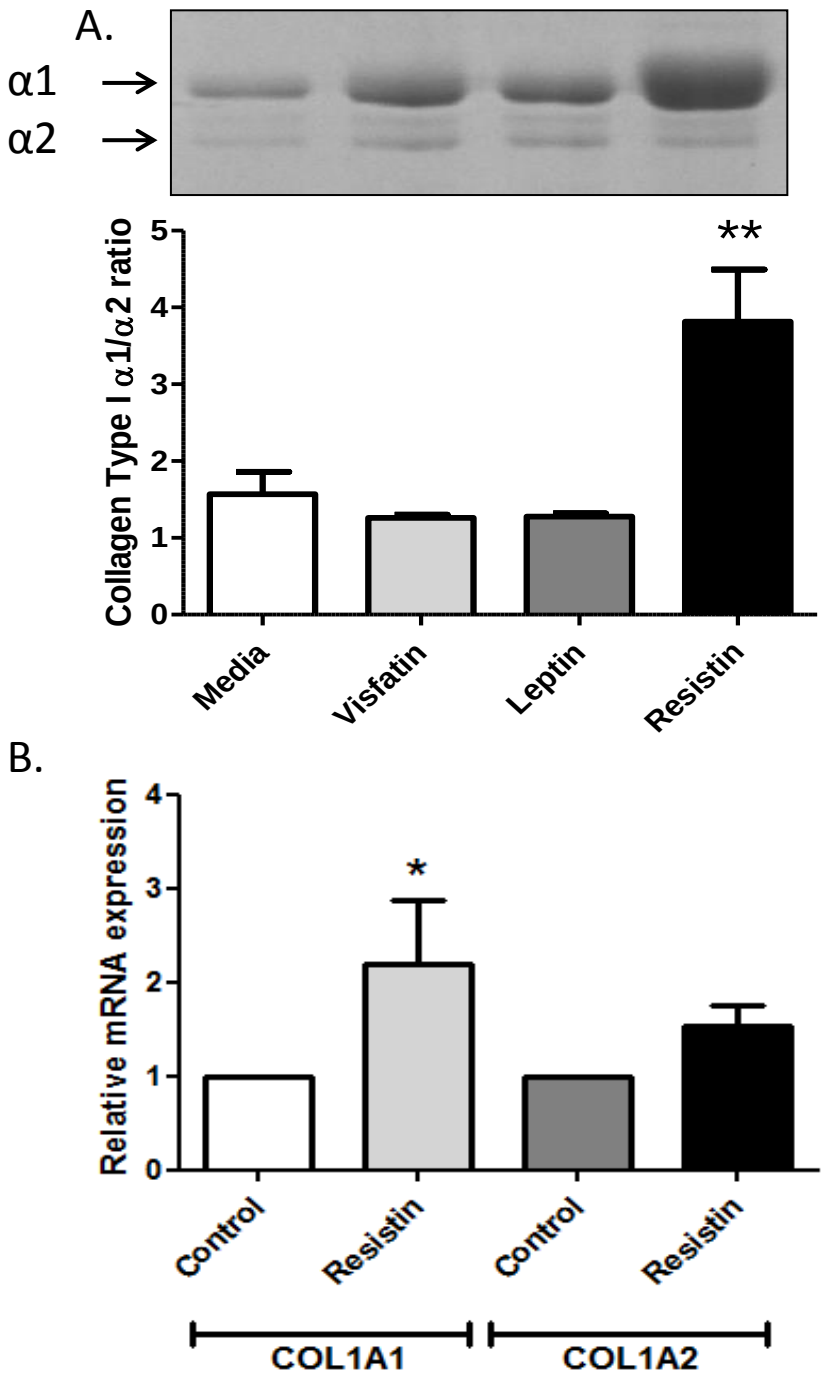


Figure 4.3. Resistin pathological alters NW bone collagen composition.

A. A typical coomassie gel of subchondral bone collagen following 4wk adipokine stimulation. The ratios of α1/α2 quantities demonstrate an increase in α1/α2 ratio and therefore the increase in homotrimer formation (n=4) (** = $p<0.01$). **B.** COL1A1 and COL1A2 relative gene expression following acute (24hr) stimulation with resistin (n=4) (*=$p<0.05$).

4.2.3 Resistin stimulated osteoblasts exhibited increase alkaline phosphatase activity and bone nodule formation.

Primary osteoblasts were cultured from the subchondral bone tissue of three OA patients, and stimulated with recombinant resistin or IL1β for 2 weeks. Following 2 weeks of stimulation, there was a significant ($p<0.05$) increase in alkaline phosphatase activity in resistin stimulated osteoblasts (Figure 4.4A) compared to non-stimulated cells (0.001 units/mg vs 0.0003 units/mg respectively). A small increase in alkaline phosphatase activity was also observed in those osteoblasts stimulated with IL1β (0.0006 units/mg), compared to control (0.0003 units/mg), although this did not reach statistical significance (data not shown). Stimulation of osteoblasts with resistin also increased their basal oxygen consumption rate (OCR) 2.2±2.1 fold ($p=0.002$, $n=10$ Figure 4B). Bone nodule formation was significantly increased following 4 weeks of resistin stimulation, as depicted by alizarin red staining of the calcium containing nodules. Alizarin stain was quantified with cetyl peridium chloride, which showed an increase in bone nodule formation following resistin stimulation compared to unstimulated control osteoblasts (0.16 vs 0.26 nm) (Figure 4.4C). Despite increase alkaline phosphatase activity and bone nodule formation, we depicted no change in osteoblasts proliferation upon stimulation with resistin (Figure 4.4D).

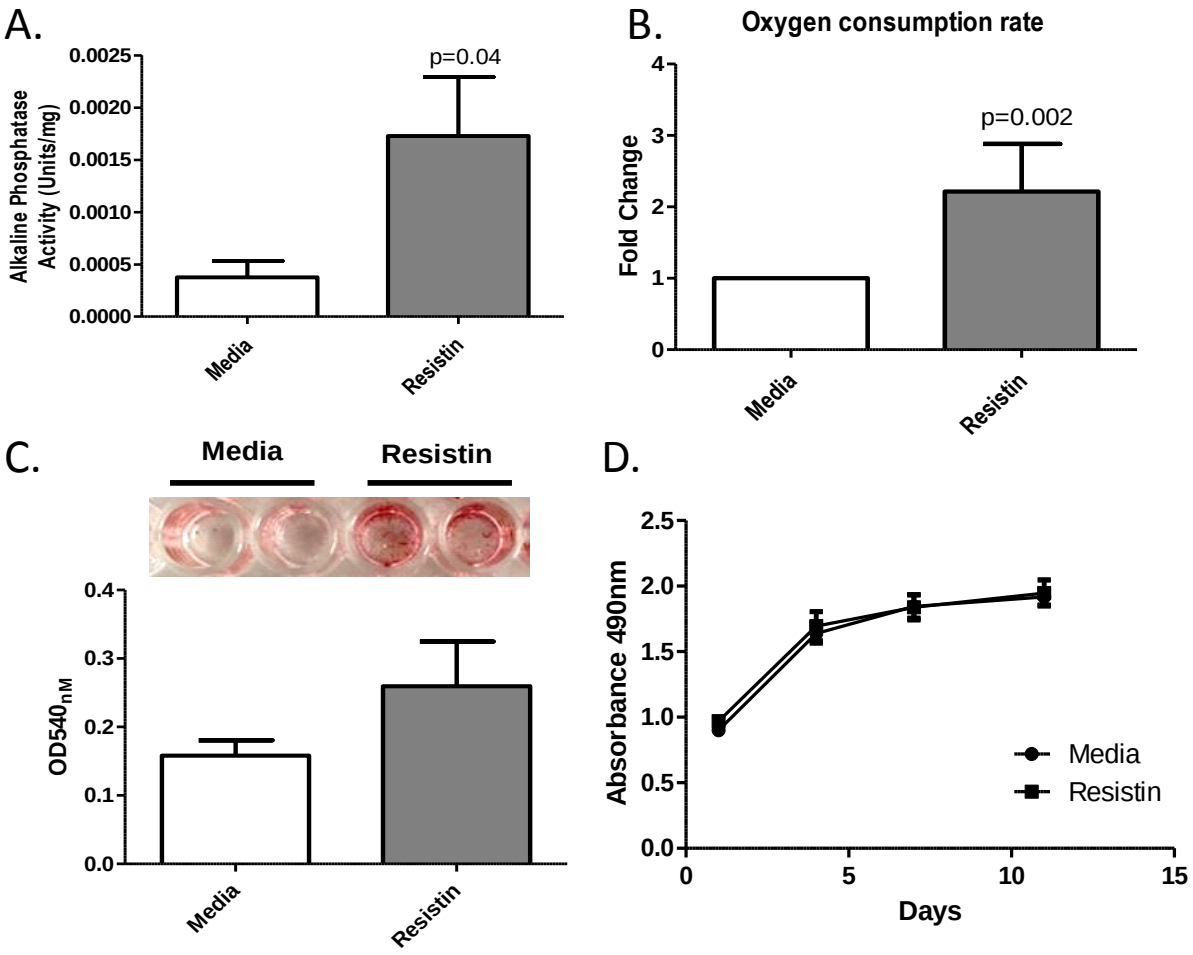


Figure 4.4. Functional impact of resistin on the metabolic activity and bone nodule formation of human primary osteoblasts.

A. Alkaline phosphatase activity of human primary osteoblasts from the femoral subchondral bone following 2wk resistin stimulation (n=4) (*=p<0.05) and normalised to total protein following cell lysis. B. Oxygen consumption rate fold change following 24hr stimulation of primary human osteoblast cells with 500ng/mL resistin. OCR values (pmol/min) were normalized to total protein following cell lysis prior to fold change calculation. C. Alizarin red staining following 4wk resistin stimulation (n=4). A representative image of alizarin red staining is located above the graph with the intensity of alizarin red staining increasing with increased bone nodule formation. Alizarin Red staining was quantified using 10% cetyl pyridinium chloride and read at

OD_{540nm} on a microplate Reader (Biotek, Elx808). D. Time-course of human primary osteoblasts cell proliferation following stimulation with and without resistin (n=4) as determined using an MTS assay.

4.2.4 Wnt signalling pathway gene expression is upregulated following 24 hours of resistin stimulation.

In an effort to understand the signalling mechanisms behind these resistin-mediated changes in bone pathology, we stimulated primary OA osteoblasts for 24 h with recombinant resistin and measured 84 canonical Wnt pathway genes by qRT-PCR. Of the 84 genes analysed, 14 genes were significantly upregulated >1.4 fold in the resistin stimulated cells, compared to unstimulated cells. Pathway analysis using IPA predicted that this pattern of gene expression would lead to an increase in β-catenin expression and activity (Table 4.2, Figure 4.5A). This was then confirmed in primary osteoblasts where we demonstrated nuclear translocation of β-catenin within 30 min of recombinant resistin stimulation (Figure 4.5B), quantified by an increase in the percentage of β-catenin staining within the nucleus (10.2 ± 3.9% vs 17.6 ± 8.9% in media and resistin stimulated osteoblasts respectively (Figure 4.5C)).

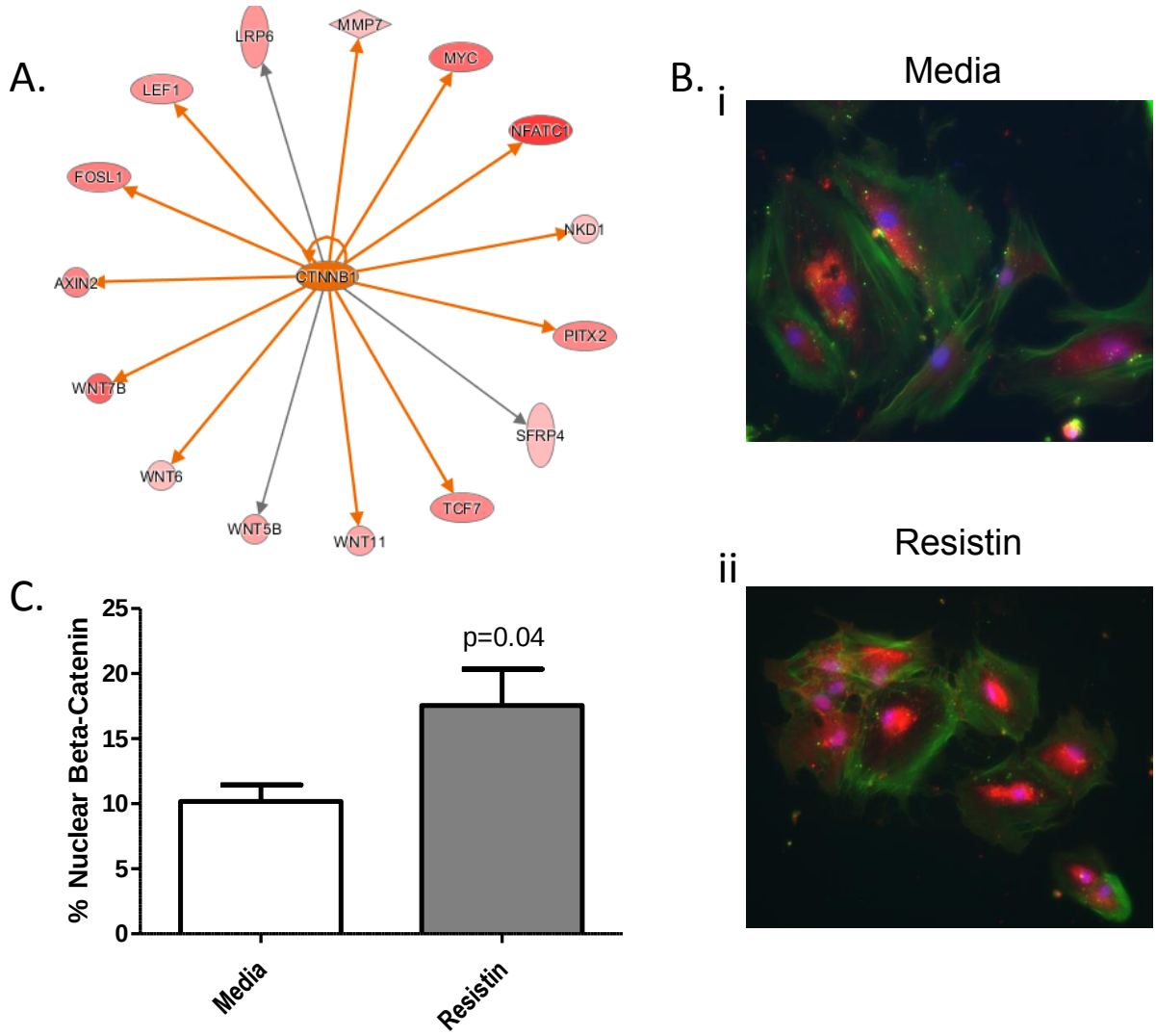


Figure 4.5. Analysis of the Wnt signalling pathway in human primary osteoblasts following resistin stimulation.

A. IPA analysis of Wnt genes upregulated with resistin stimulation and the predicted increase in β-catenin expression. Orange arrows = predicted activation, grey arrows = effect not predicted. **B.** Representative images of nuclear translocation of β-catenin in human osteoblasts following i) media only ii) 30min of resistin stimulation (500ng/mL). Green = α-Actin, Blue = Hoechst, and Red = β-catenin (n=3). **C.** Quantification of nuclear translocation of β-catenin using Image J software. Percentage nuclear β-catenin refers to the subset within the area of hoechst staining

compared to the whole cell and is an average of 6 images per biological replicate (n=3 biological replicates) (*=p<0.05).

Table 4.2. Wnt Signalling pathway genes upregulated following the stimulation of primary osteoblasts with recombinant resistin for 24 hours.

GENE	ALIAS	FOLD CHANGE	p value
WNT5A	Wnt Family Member 5A	1.56	0.004
WNT2B	Wnt Family Member 2B	1.62	0.004
NFATC1	Nuclear Factor of Activated T-Cells	1.42	0.005
WNT7B	Wnt Family Member 7B	1.94	0.015
MYC	c-Myc	1.71	0.018
FOSL1	FOS Like Antigen 1	1.50	0.031
AXIN2	Axis Inhibition protein 2	3.07	0.035
BOD1	Biorientation of chromosomes in cell division 1	1.64	0.036
GSK3β	Glycogen Synthase Kinase 3 Beta	1.40	0.038
PITX2	Paired like Homeodomain 2	3.94	0.038
TCF7	Transcription Factor 7	1.71	0.039
FRZB	Frizzled-Related Protein	1.76	0.039
FZD4	Frizzled Class receptor 4	1.75	0.044
CHSY1	Chondroitin Sulfate Synthase 1	1.62	0.044

Data expressed is mean fold change, and p values were calculated on log10 transformed data.

4.3 Discussion

To our knowledge, this is the first study to examine the relationship between adiposity and bone collagen type I structure in patients with hip OA, and to identify a potential role of resistin in altering the biosynthetic activity of osteoblasts via activation of the canonical WNT signalling pathway.

Collagen structure and alignment is pivotal to the structural integrity of bone [281, 318], therefore increased proportion of collagen type I homotrimer in OA bone has negative mechanical consequences [281]. In 2002 Bailey *et al.,* demonstrated an increased in collagen homotrimer content in OA subchondral bone when compared to fracture neck of femur patients, resulting in poorly organised collagen matrix, reduced mineralisation of the bone, and increased lysine hydroxylation [281]. The type I collagen α2 chain is thought to be integral to maintaining the triple helix structure of collagen [134], increasing the interaction of the hydrophobic side chains and thus limiting water content, and allowing for greater cross-linking of the collagen molecules [135]. Furthermore, a study using computational models of murine type I collagen fibrils concluded that the homotrimer formation results in a 'kinking' of the fibril, which provides greater flexibility, smaller bending stiffness, and a smaller persistence length [319].

In this study, we have demonstrated a modest increase in collagen homotrimer content in weight-matched OA bone when compared to the non-OA NOF# bone samples. However, the increase in collagen homotrimer content observed in the OA patient samples was accentuated in OW/OB patients compared to NW OA patients, suggesting that adiposity may be a primary driver of collagen malformation, leading to accentuated sclerotic bone development and OA progression [281]. Structural

abnormalities of bone in relation to adiposity were further confirmed via micro-CT. In particular, we have demonstrated an increase in BS/BV ratio in the OW/OB patient bone samples, compared to NW samples; an indication of higher rates of bone turnover occurring as a result of greater bone surface area [320].

In terms of OA, modifications in trabecular bone thickness remain controversial. Some studies have found OA subchondral bone to have thicker trabeculae [316, 321], compared to non-OA bone. Conversely, in this study we have shown a decrease in trabecular thickness in the subchondral bone, suggesting more of an osteoporotic phenotype than an OA phenotype. However Buckland-Wright (2004) suggested that concurrent with the flattening and deformation of the articular cartilage in the knee, comes improved load transmission and reduced mechanical stress [125]. The increase in sclerotic bone present within the subchondral plate reduces load within the deeper subarticular region and subsequent development of a localized osteoporosis. Therefore, the change in trabecular thickness we have observed within our study may in part be due alterations in hip alignment and force transduction, and in reference to Buckland-Wright (2004), may be due to excessive flattening of the articular cartilage due to the excessive load-bearing evident in individuals of greater adiposity. Further studies to determine trabecular thickness across a larger area of the femoral head, together with analysis of cartilage integrity and assessments of patient gait prior to elective joint surgery could therefore prove informative.

Critically, since bone is a highly vascularized tissue bone health can be highly affected by circulating adipokines as a result of increased adiposity. As previously described in Chapter 3, in the present chapter we analysed the serum concentration of a panel of 24 adipokines in 150 patients with OA who were either NW or OW/OB.

Notably, in addition to leptin, we found that there was a significant increase in the concentration of the adipokine resistin in OW/OB patient serum compared to NW serum ($p<0.05$). The association of resistin to obesity is in support of previous research [322, 323] and can therefore provide a systemic driver that may alter bone pathologies in OB individuals.

Resistin has raised significant interest in terms of bone pathology in OA. Termed due to its relationship to insulin resistance in mice [28, 324], resistin is a dimeric protein secreted from adipocytes, macrophages and abundantly expressed in bone marrow cells [259]. In RA, resistin has been shown to accumulate within inflamed joints, and correlates with the degree of inflammation and the expression of inflammatory cytokines including TNFα, IL1β and IL6. Despite these data, very little is understood with regards to the role of resistin in driving pathological changes in the bone.

Resistin has been reported to promote MC3T3-E1 preosteoblast proliferation, however little is known with regards to human primary osteoblasts and the overall impact on bone remodelling or collagen formation. In the present study, we have shown that resistin promotes a shift in NW bone towards an OW/OB phenotype, with regards to collagen homotrimer formation. This suggests that resistin may accentuate the formation of homotrimer-rich, sclerotic bone in OA patients who are overweight or obese. We also showed an increase in bone nodule formation following stimulation of primary human osteoblasts with recombinant resistin for 4 weeks. This is counterintuitive to data expressed in recent studies that have shown a negative correlation with serum resistin levels and bone mineral density [325, 326]. It is important to note however that resistin has been shown to increase cytosolic calcium in HSC (human hepatic stellate cells) [327], and that an increase in cytosolic

calcium in osteoblasts has been shown to contribute to increased extracellular hydroxyapatite formation [328]. We propose therefore that resistin could be increasing cytosolic calcium within our primary human osteoblast cells, and thus drives the formation of extracellular hydroxyapatite crystals. However this theory requires further study. Furthermore, previous data suggesting serum resistin is negatively associated with BMD is based upon correlation data, and correlation does not imply causation.

In accordance with previous research, we observed an increase in alkaline phosphatase activity following a 14 day stimulation of osteoblasts with resistin (Figure 4.4A) [314]. Alkaline phosphatase was one of the first components recognised to be essential in osteogenesis and its role in normal and pathological calcification is undeniable [329, 330]. Expressed by mature osteoblasts, alkaline phosphatase activity has been shown to be higher in osteoblasts from OA bone, with speculation that this may be due to an increased differentiation of pre-osteoblasts into a mature osteoblast phenotype [331, 332], though this remains to be elucidated. This data further supports the role of resistin in driving bone nodule formation. With no change in proliferation between resistin-stimulated and media-stimulated human primary osteoblasts, we have shown that resistin may play a significant role in the maturation and osteoid development phase of bone formation, and not in the recruitment of osteoblasts to the site of bone formation.

The WNT pathway is a principal regulatory pathway associated with both alkaline phosphatase expression and activity, and osteoblastic differentiation [333, 334]. The canonical WNT pathway is initiated through the binding of WNT to the Frizzled receptor and low density lipoprotein receptor related protein 5 and 6 (LRP5 and LRP6). AXIN then dissociates from GSK3β, allowing for the translocation of β-catenin

into the nucleus to bind with TCF/LEF transcription factors, and subsequently activate gene transcription from the RunX2 promoter [335, 336]. Confidence in the role of the WNT signalling pathway in bone formation emerged through the discovery of a high bone mass phenotype in LRP5 gain-of-function mutations, in comparison to loss-of-function mutations that lead to an osteoporosis-pseudoglioma syndrome [337, 338]. Our data demonstrates that resistin causes the translocation of β-catenin to the nucleus, via the activation of a number of WNT signalling genes including WNT5A, WNT2B, AXIN2 and FZD4, thus supporting our role for resistin in driving bone formation.

The present study has some limitations. Firstly, all samples were obtained from patients undergoing total joint replacement and therefore, suffering from advanced OA. This means that the data attained using patient samples excludes any patients with less severe OA. Furthermore, due to the cross-sectional design of this study, it is only possible to suggest causal relationships, which can only be confirmed with longitudinal research. It is important to note, that despite gene analysis of the WNT signalling pathway, there was no attempt to determine if the many SNPs identified within the WNT signalling pathway in previous research were present in our primary osteoblast samples. SNPs within any of the measured genes within the signalling pathway could directly regulate gene expression and subsequent functional outputs including bone nodule formation and osteoblast biosynthetic activity. For example, it has been previously shown that gain of function SNP mutations in the LRP5 Wnt co-receptor results in an increase in bone mass [339, 340]. In addition, Arg324Gly substitution in the FRZB, a gene increased by resistin stimulation, resulted in a loss of Wnt antagonism in females with hip OA [341]. Therefore if the primary osteoblast cells used within this study had the Arg324Gly substitution in the FRZB, it is possible

that the effect we identified with resistin stimulation is exaggerated and not a true reflection of the effects of resistin on bone formation,

Further, as mentioned in chapter 3, for all patients we received a medical history and current medication list at the time of surgery. Due to the vast array of medications the patients were receiving, it was not possible to assess each therapeutic for its effect on bone metabolism. Furthermore, we received information of medications at the time of surgery, not those that have been received in the years prior to surgery which may impact bone turnover including glucocorticoids and thiazide diuretics. All patients that went for surgery were fasted prior to surgery so the fed state of patients remained constant throughout, however in our NOF# patients, the time between fracture and surgery varied and therefore may provide a source of variability within our data. Though most surgeries were completed within 24 hours of the fracture, some resulted in a delay to 72 hours in order to obtain medical clearance.

To conclude, these data have demonstrated that resistin promotes an increase in the biosynthetic activity of human primary osteoblasts resulting in collagen type I homotrimer formation. The data suggests that resistin may affect bone turnover through activation of the canonical WNT-signalling pathway and subsequent β-catenin translocation and gene transcription. However, further studies are required to fully understand the mechanistic role of resistin in affecting the dynamic balance of bone maintenance. In a broader context, given the mounting evidence that bone changes precede cartilage alterations [123], establishing adipokines as a link between these bone changes and obesity, as well as identifying their role in OA pathogenesis, may lead to the development of adipokine-targeted drugs as novel DMOADs.

Chapter 5: Visfatin (NAMPT) drives cartilage catabolism leading to proteoglycan loss and inflammation.

5.1 Introduction

As previously mentioned in Chapter 1 (section 1.7.4), one adipokine that has received much attention in recent years with regards to OA is visfatin, a highly conserved ubiquitously expressed protein, which was originally defined as a pre-B cell colony enhancing factor [266]. Numerous potential roles for visfatin have now been proposed including; catalysing the conversion of nicotinamide and phosphoribosyl-pyrophosphates to nicotinamide mononucleotide [267], and acting as an insulin mimetic [268] a growth factor [269], or as an inflammatory cytokine able to induce TNFα, IL6 and IL1β [270].

Visfatin is termed an 'adipokine-enzyme' due to its enzymatic nature when in a homodimeric conformation [271], and is secreted independently from the Golgi apparatus and the endoplasmic reticulum from visceral adipose tissue [272]. Visfatin exists in an intracellular (iVisfatin) and extracellular (eVisfatin) form. eVisfatin has received a lot of attention in current literature due to its elevated expression and potential contribution to a number of pathological conditions associated with ageing including diabetes [273] and obesity [274-277]. Although the biosynthesis of nicotinamide adenine dinucleotide is well established as one of the functional roles of iVisfatin, the pathophysiological relevance and the functional consequence of elevated levels of eVisfatin in disease states, including in osteoarthritis is not fully established.

Previous studies have shown that visfatin expression is increased in human chondrocytes upon stimulation with IL1β and that the stimulation of chondrocytes with recombinant visfatin can modulate the expression of matrix proteases, aggrecan proteoglycan [278] and the production of IL6 [271]. These studies suggest that

visfatin may drive both pro-inflammatory and pro-degradative effects within joint cartilage. However, critically these studies were conducted in isolated chondrocytes and not on articular cartilage tissue, where the chondrocytes are embedded within a collagen and proteoglycan matrix and thus are of a different phenotype [342-344]. Currently, there are no studies which have reported the functional effects of visfatin on human *ex-vivo* OA articular cartilage tissue, where effects on the cartilage proteoglycan integrity can be observed. Furthermore, no studies have as yet reported on the expression of visfatin in the joint tissues of patients with hip OA, or how the joint tissue expression of visfatin in this patient cohort varies with BMI.

The aim of this study was therefore to evaluate the serum and joint tissue expression of visfatin in patients with hip OA of varying BMI and to determine the functional role of visfatin in articular cartilage from patients with hip OA using a human *ex vivo* cartilage explant model.

5.2. Participant recruitment and sample collection

As detailed in section 2.1, patients with hip OA (age 45-80 years) undergoing elective total hip joint replacement surgery (K and L grade 3-4) were recruited (Ethical approval NRES 14-ES-1440). For this particular study, serum was collected from n=24 hip OA patients who were classified as of normal-weight, over-weight or obese. For comparison to non-OA, serum was also collected from normal-weight (n=23), over-weight (n=24) and obese (n=22) individuals without OA from the Birmingham 1000 Elders cohort (NRES 10/H1202/45). From n=9 patients with hip OA, at the time of surgery, the femoral heads were collected and a portion of subcutaneous adipose tissue, gluteus maximus skeletal muscle and synovium from around the joint collected (Table 5.1). Furthermore, synovial fluid was aspirated from n=3 OW/OB patients and from n=3 NW patients.

Table 5.1: Patient characteristics

	Normal weight	Overweight	Obese	p=
No. of Non OA patient # (Female %)	23 (52)	24 (50)	22 (50)	-
OA patient # (Female %)	24 (50)	24 (50)	24 (50)	-
Non OA Male Age (yr.)	67±4.9	66±3.8	67±5.5	0.78
OA Male Age (yr.)	67±4.4	67±7.4	60±7.3	***0.05***
Non OA Female Age (yr.)	67±4.3	65±6	66±4.1	0.25
OA Female Age (yr.)	63±4.2	61±10.2	65±8.1	0.8
Non OA Male BMI (kg/m^2)	22±0.7	27±0.3	33±1.4	***<0.001***
OA Male BMI (kg/m^2)	23±1.3	27±1.5	33±2.7	***<0.001***
Non OA Female BMI (kg/m^2)	20±0.7	28±0.9	33±2.1	***<0.001***
OA Female BMI (kg/m^2)	21±0.9	28±1.1	34±2	***<0.001***
Non OA Male W:H	0.89±0.08	0.93±0.04	0.95±0.07	0.17
OA Male W:H	0.90±0.03	0.96±0.07	1.00±0.06	***0.001***
Non OA Female W:H	0.77±0.02	0.87±0.07	0.86±0.06	***0.001***
OA Female W:H	0.80±0.06	0.86±0.04	0.88±0.06	***0.01***

Abbreviation: # = number, W:H = waist to hip ratio, BMI = Body Mass Index.

5.3 Results.

5.3.1 The serum profile of visfatin in males and females of varying BMI is not related to hip OA disease status or to biomarkers of cartilage remodelling.

Both the non-OA and OA population were divided into three statistically-matched BMI groups (normal-weight, overweight and obese). Before analysis of all study participants' serum samples, we first verified the specificity of the visfatin ELISA kit by comparing western blot densitometry values and ng/µL concentrations as determined by the ELISA kit (Figure 5.1).

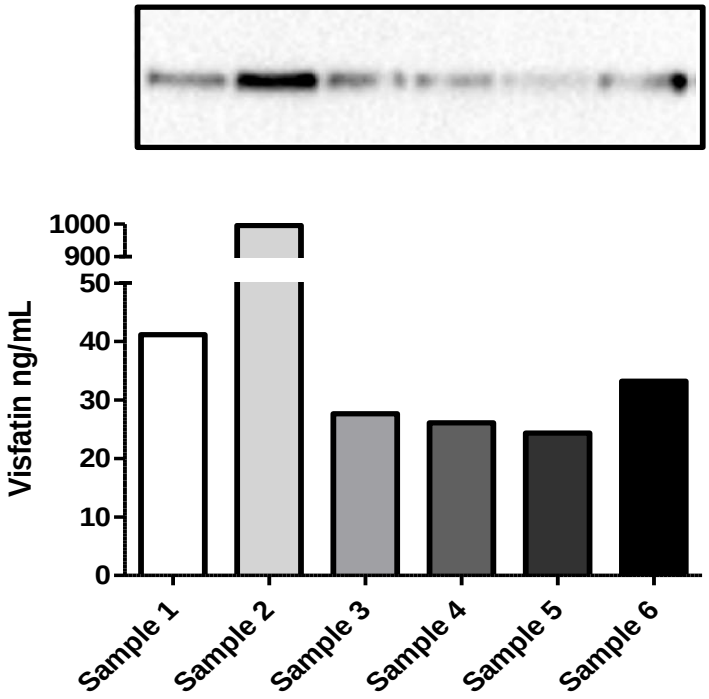


Figure 5.1. Verification of the visfatin ELISA kit via western blot of serum samples.

Bar chart represents values quantified by ELISA. Specificity of the visfatin ELISA kit was verified by comparing western blot densitometry values and ng/µL concentrations as determined by the visfatin ELISA kit.

Serum visfatin levels demonstrated no significant correlation with BMI in either males or females without OA (R^2=0.09 (95%CI -0.05 to 0.746) and R^2=0.00 (95% CI -0.42 to 0.36) respectively), or with BMI in males with hip OA (R^2 = 0.03 (95% CI -0.49 to 0.14)). However in females with hip OA, there was a significant correlation of serum visfatin expression levels and BMI (R^2 = 0.2 (95% CI -0.11 to 0.53) (Figure 5.2).

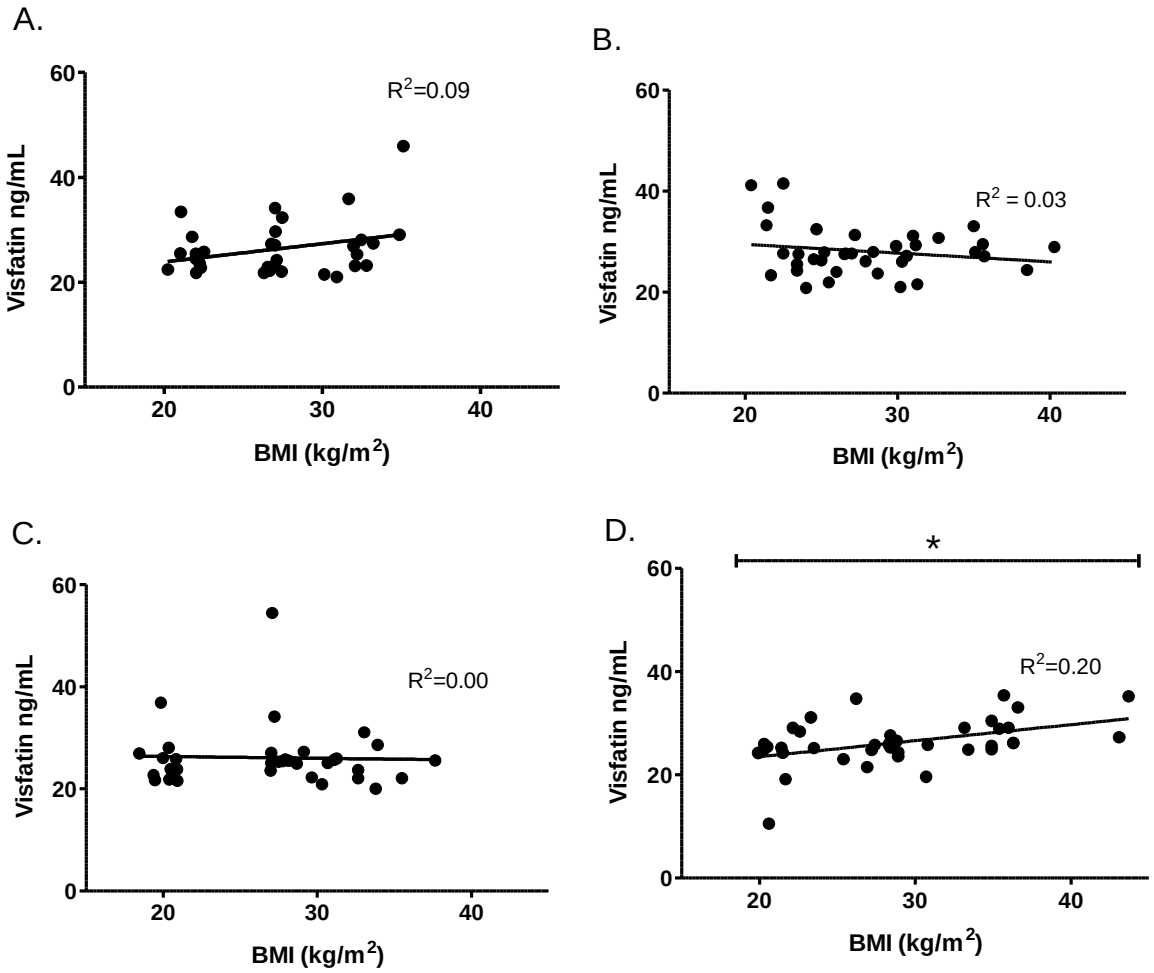


Figure 5.2. The correlation between serum visfatin concentration and BMI in patients with or without hip OA.

Serum visfatin expression levels were determined by ELISA. A. Male control (n=35) patients, B. male OA patients (n=36), C. female control patients (n=35), D. female OA patients (n=36). *=correlation is significant to $p<0.05$.

To examine if serum visfatin concentrations were associated with the degree of cartilage remodelling, serum biomarkers associated with cartilage degradation (COMP) and cartilage synthesis (Type IIA collagen neopeptide; PIIANP) were measured. We found no significant correlation associated between serum visfatin

concentrations with either serum COMP or serum PIIANP in any of the patient cohorts (Figure 5.3A and 5.3B). However, serum visfatin concentrations were negatively associated with CTX-II and BMI in OA females only ($p<0.05$) (Figure 5.4).

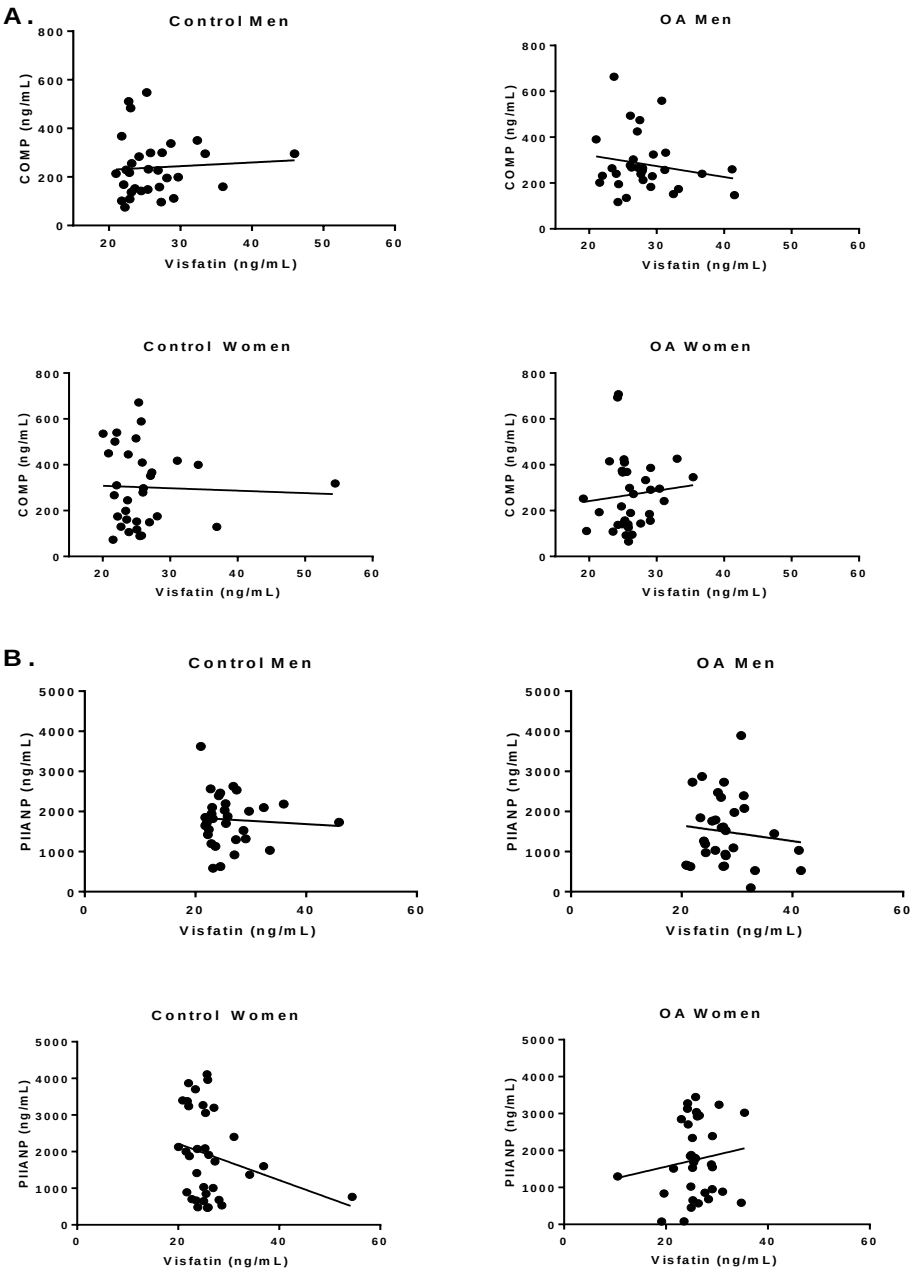


Figure 5.3. Serum concentrations of COMP and PIIANP in males and females with hip OA.

A. Cartilage oligomeric matrix propeptide (COMP) serum concentration and its correlation with serum visfatin concentrations in males and females with and without hip OA (n=63 control, n=66 OA). B. Type IIA Collagen N-propeptide serum

concentration and its correlation with serum visfatin concentrations in males and females with and without hip OA. (n=62 control, n=66 OA).

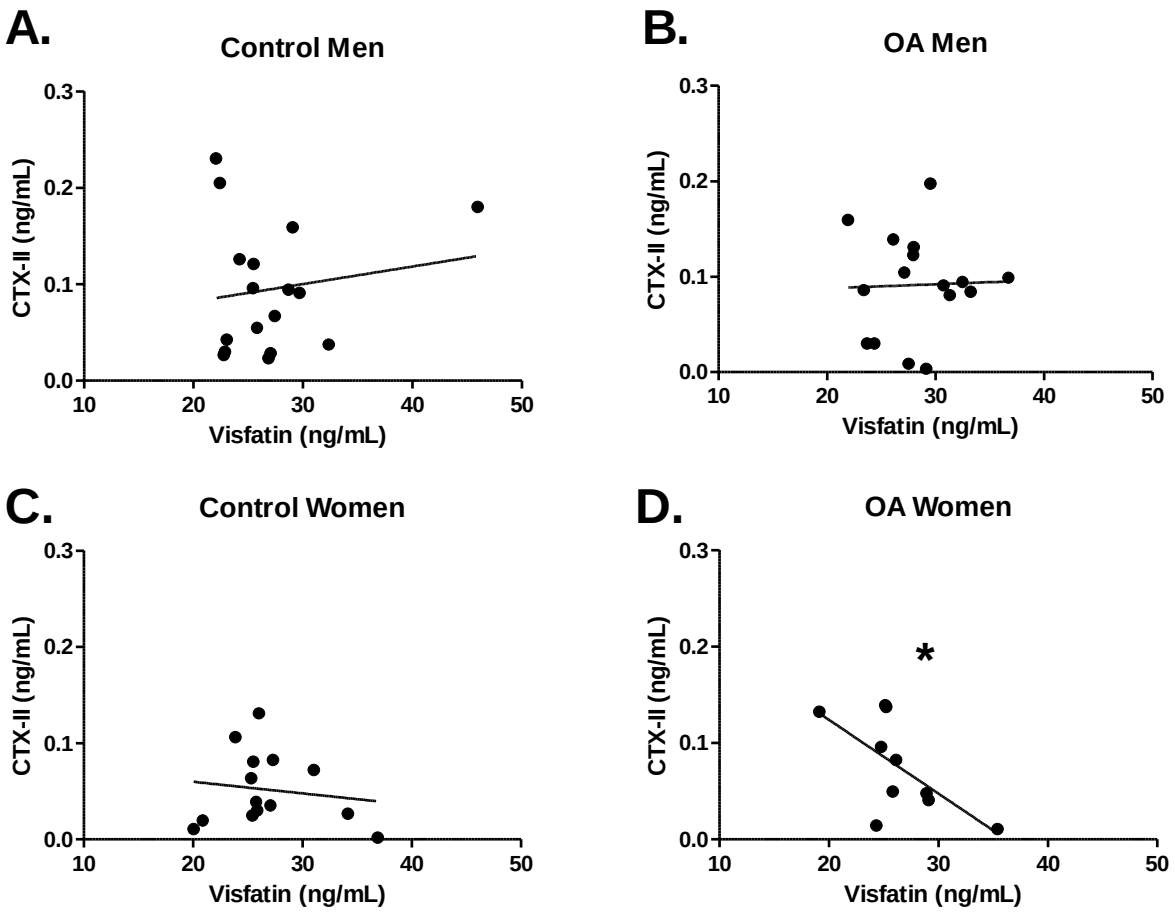


Figure 5.4. C-terminal cross-linked telopeptide type II collagen serum concentration and its correlation with serum visfatin concentrations.

A. Male Control patients (n=22); B. Male OA patients (n=16); C. Female Control patients (n=14); D. Female OA patients (n=10). * = $p<0.05$. Visfatin and CTX-II concentrations were determined by ELISA.

5.3.2 Visfatin is expressed locally by the tissues of the hip OA joint and is elevated in the tissues of obese hip OA patients.

The presence of visfatin, under denaturing conditions, was examined in the synovial fluid as well as the tissues that encompass the hip joint, namely cartilage, bone, skeletal muscle, synovium and adipose and compared to serum (Figure 5.5A). Electrophoretic mobility was confirmed with his-tagged recombinant visfatin. Notably, when compared w/w to cartilage, bone and muscle, visfatin was found to be highly expressed in adipose tissue from the hip joint. Expression of visfatin was significantly greater in synovial fibroblasts (0.19 ± 0.05 vs. 1.65 ± 0.22, p=0.02) from obese (OB) hip OA patients (n=3), compared to normal weight (NW) hip OA patients (n=3; Figure 5.5B and C). Visfatin expression in adipose tissue displayed significant patient variability, but on average also appeared to be greater in OB patients compared to NW patients (Figure 5.5B and C).

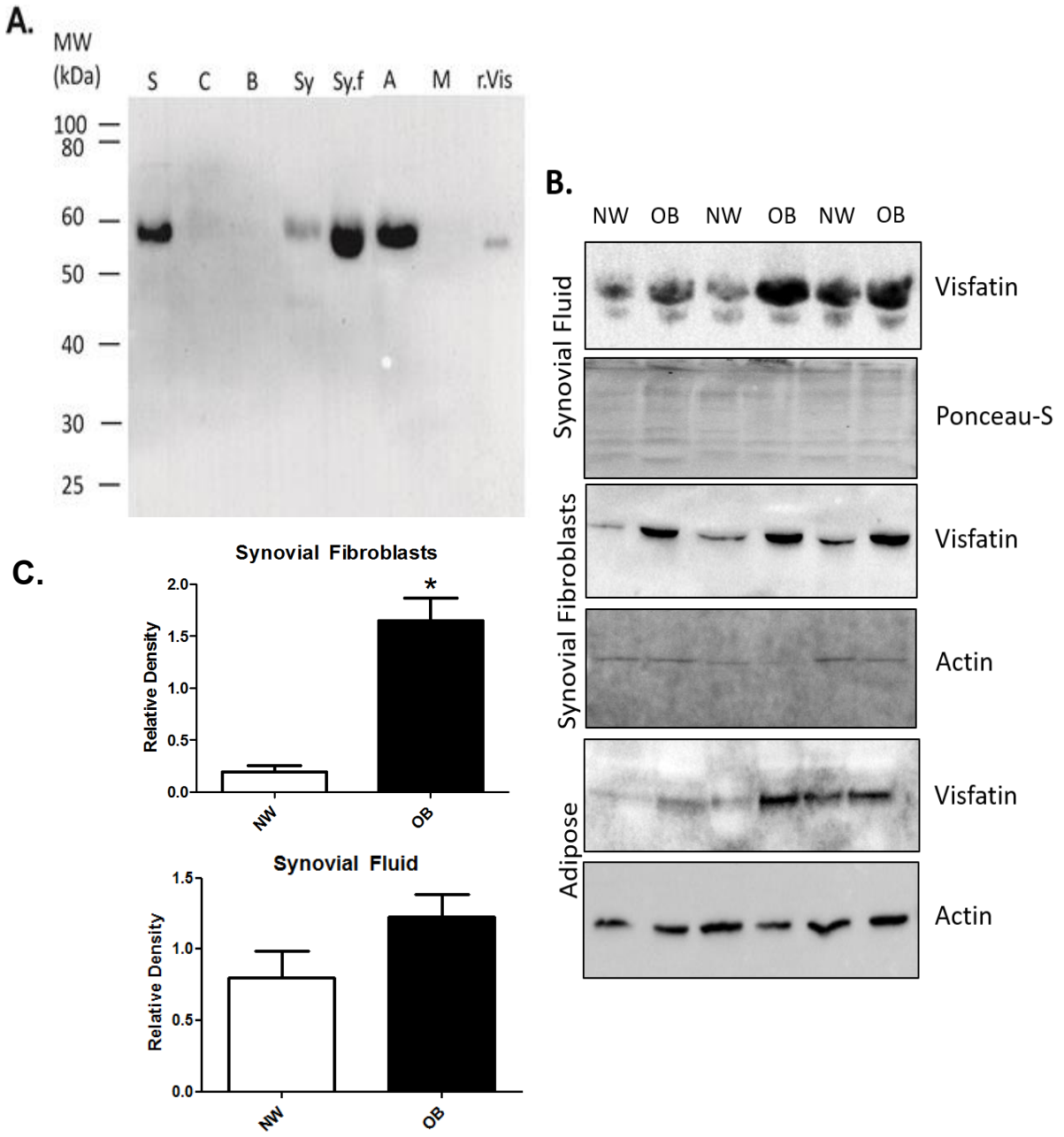


Figure 5.5. The expression of visfatin in hip OA joint tissues.

A. Expression of visfatin under reducing conditions in serum (S), cartilage (C), bone (B), synovium (Sy), synovial fluid (Sy.f), adipose(A), and muscle (M). Recombinant visfatin (r.Vis) indicates molecular weight region. B. Tissue panel of sample western blot from normal-weight (NW) and obese (OB) individuals with hip OA. All samples were normalised to µg of total protein loaded and equal loading was confirmed by

ponceau-S staining and actin expression. C. Western blots were analysed using Image J software and the densitometry was compared in NW and OB patients.

5.3.3 Visfatin induces the production of matrix metalloproteases in human hip OA cartilage.

Having observed that visfatin is expressed locally by the tissues of the OA joint we next examined the effect of stimulating OA cartilage explants with visfatin, compared to IL1β, on the production of a panel of MMPs by Luminex. To this end, for each experimental condition, 5 cartilage explants were prepared from each of 9 individual femoral head cartilage patient samples. Visfatin stimulation led to significant increases in a number of disease relevant catabolic proteases including MMP-1 (4-fold), MMP-2 (3-fold), MMP-3 (3-fold), MMP-7 (2.2-fold), MMP-8 (1.3-fold), MMP-9 (1.2-fold), MMP-10 (1.5-fold), and MMP-13 (5-fold) (Figure 5.6A-D). Visfatin induced the production of MMPs in both NW and OB cartilage, although no significant difference was observed between the degree of fold-change induction between NW and OB cartilage (Figure 5.6E).

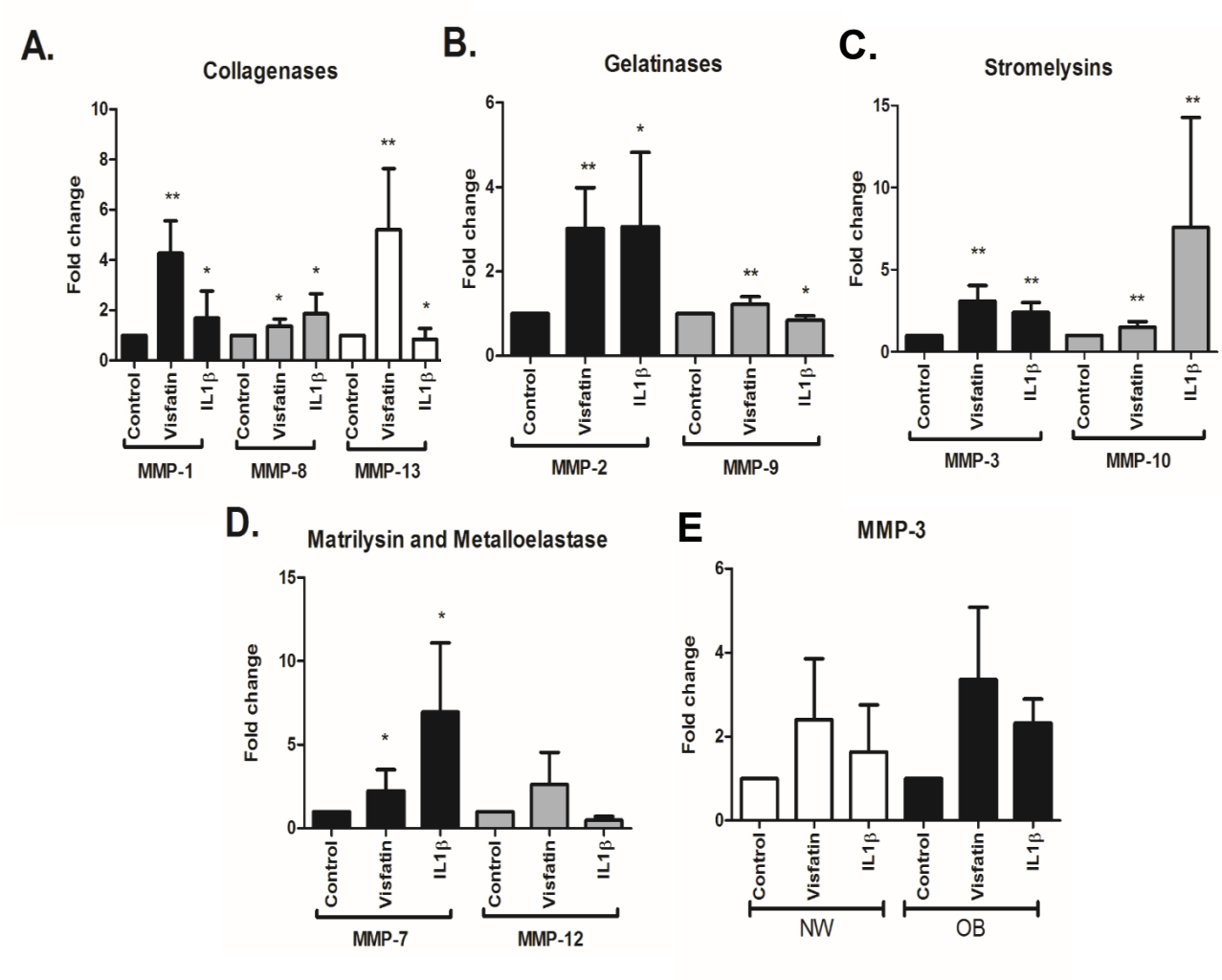


Figure 5.6. Visfatin induces the production of cartilage catabolic proteases in hip OA cartilage.

MMP secretion from cartilage explants following visfatin stimulation (500ng/mL) and IL1β (1ng/uL) using Luminex technology, and separated into the MMP classification (n=9 individual patients (5 explants per patient)). A. Collagenases classification. B. Gelatinases classification C. Stromelysins classification, D. Matrilysins and Metalloelastase classification. E. MMP3 secretion from NW versus OB patient explants following visfatin stimulation (n=4 and n=5 respectively). *=p<0.05, ** = p<0.01, ***=p<0.001, significantly different between treatment and control values.

5.3.4 Visfatin induces the secretion of pro-inflammatory cytokines and chemokines in human hip OA cartilage.

We then examined the functional role of visfatin on human OA cartilage cytokine production. This time, per condition, we prepared 5 cartilage explants from femoral head cartilage samples of n=4 individual patients with hip OA and stimulated them with or without human recombinant visfatin (500 ng/ml) for 24 h. The effect of visfatin on the production of a panel of 44 known pro-inflammatory cytokines and chemokines was determined using Proseek technology. Following stimulation with visfatin there was a notable increase in the production of 15 pro-inflammatory cytokines and chemokines, with significant increases in CCL4, MCP-1 and in CCL20 in the tissue culture supernatant following visfatin stimulation compared to the media only control (20-fold, 4-fold and 7-fold respectively)(Figure 5.7).

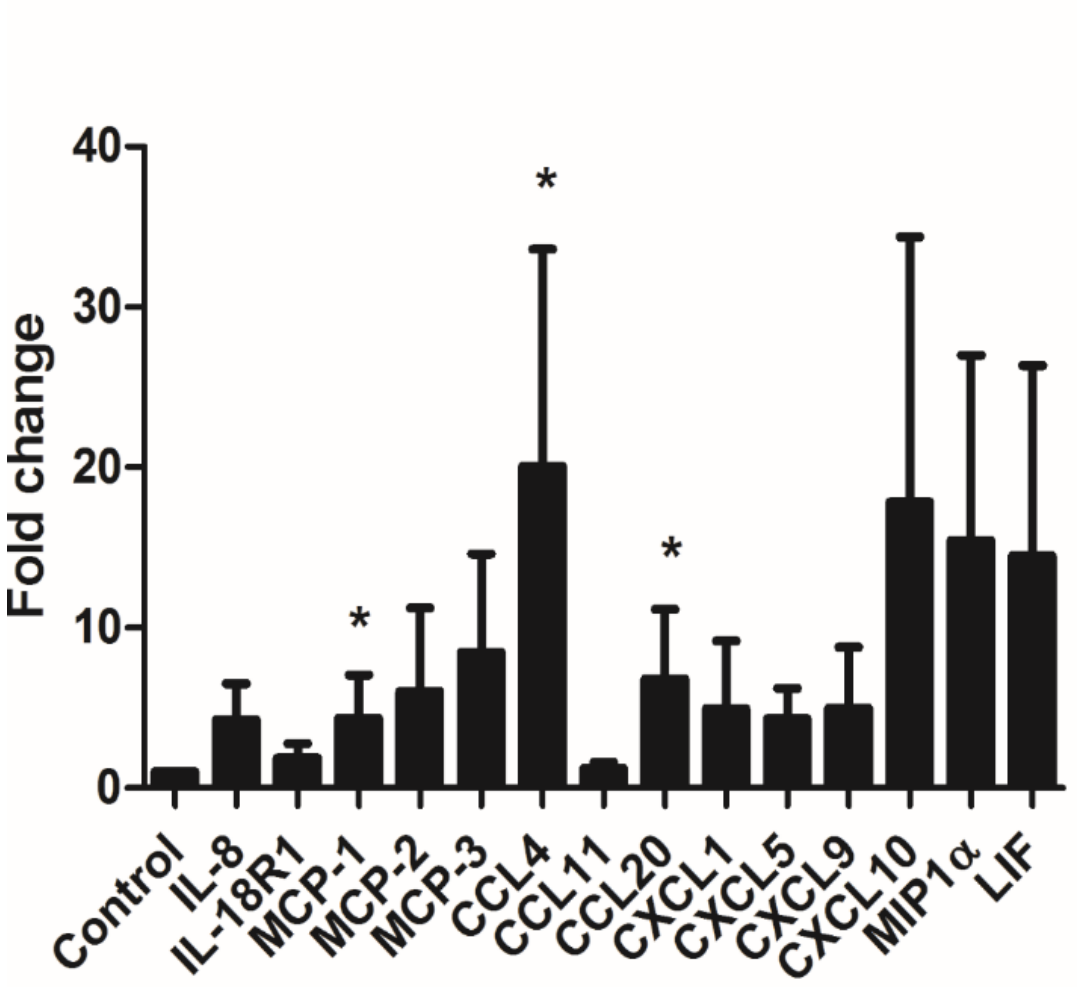


Figure 5.7. Visfatin induces the production of pro-inflammatory cytokines in hip OA cartilage.

24h cytokine production in tissue culture supernatants following visfatin stimulated cartilage explants. Cytokines and chemokines were measured using Proseek technology in tissue culture supernatants of media and visfatin (500ng/mL) stimulated explants (n=4 individual patients).

5.3.5 Visfatin induces greater production of IL6 in obese cartilage compared to normal-weight hip OA cartilage.

We then examined the effect of visfatin stimulation on the production of IL6 by ELISA in both NW (n=4) and OB (n=4) cartilage, compared to IL1β stimulation. Stimulation of both NW and OB cartilage for 24 h with visfatin led to a significant production in IL6. However, the fold-change induction in the production of IL6 was significantly much greater in OB cartilage explants compared to NW cartilage explants (7-fold vs >1500-fold respectively $p<0.05$) (Figure 5.8). This increase in IL6 was also shown to be independent of bacterial endotoxin contamination within the recombinant protein as demonstrated in Appendix 5.

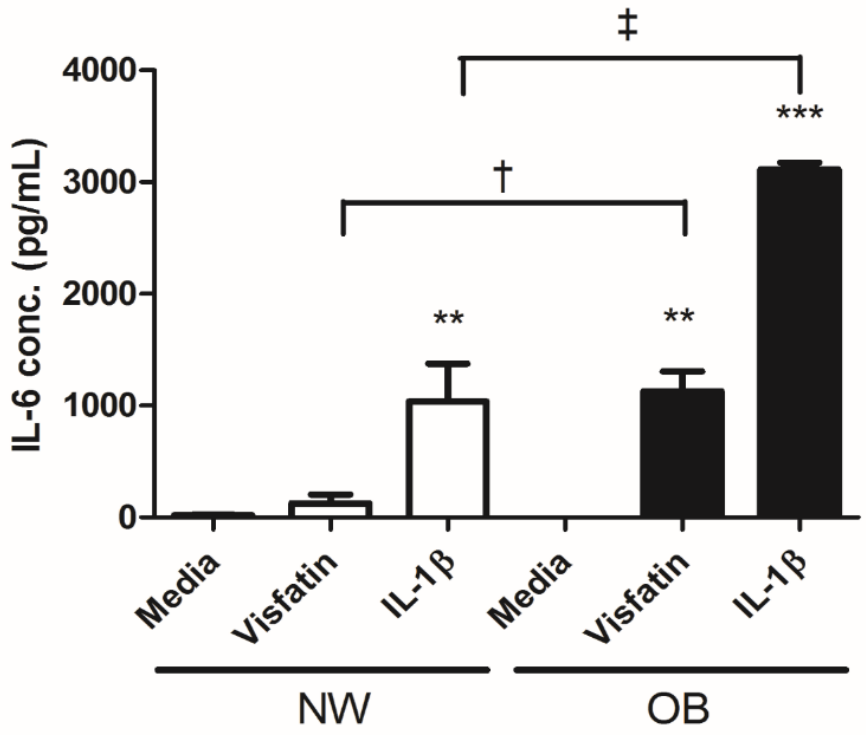


Figure 5.8. IL6 secretion following visfatin and IL1β stimulation.

Production of IL6 following visfatin and IL1β stimulation of NW (n=4) and OB (n=4) cartilage. N=5 explants from 4 individual patients per BMI group. *=$p<0.05$, ** =

p<0.01, ***=p<0.001, significantly different between treatment and control values. †=p<0.01, ‡=p<0.001, significantly different between NW and OB values.

5.3.6 Visfatin stimulates loss of proteoglycan matrix in human hip OA cartilage.

To determine whether the visfatin-mediated increase in the production of both pro-inflammatory cytokines and cartilage proteases was associated with cartilage proteoglycan degradation we then measured the release of sulfated GAG (sGAG) into human cartilage explant culture supernatants as a marker of proteoglycan loss. Stimulation of cartilage explants with visfatin for 24 h induced an increase in the release of sGAG in both NW and OB cartilage explants, demonstrating that visfatin promoted proteoglycan loss (Figure 5.9). Of note, both basal (non-stimulated) and visfatin-stimulated sGAG levels were higher in OB cartilage, compared to NW cartilage.

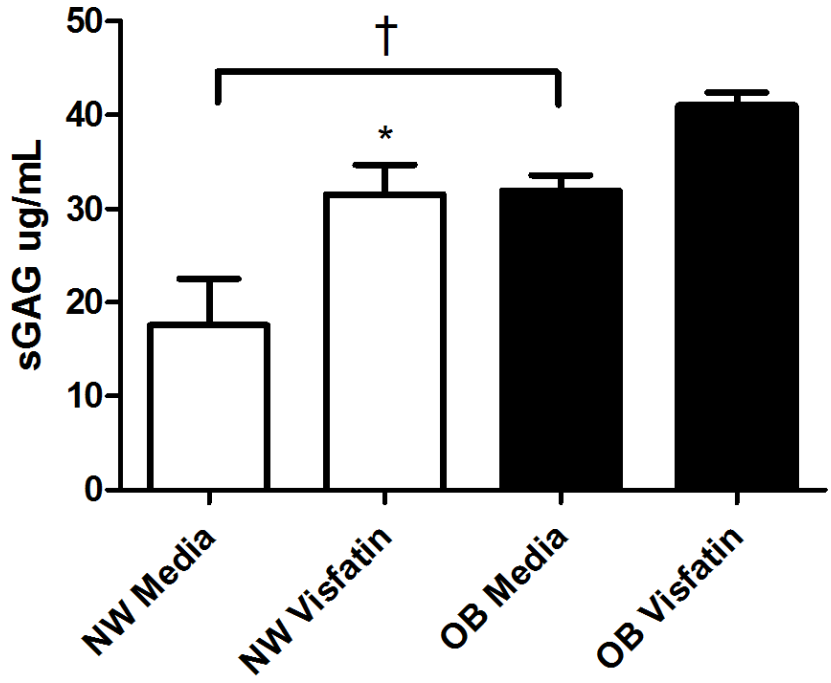


Figure 5.9. Visfatin induces proteoglycan loss.

sGAG secretion into tissue culture supernatants following visfatin stimulation of human cartilage explants from normal weight (NW) and obese patients (OB) (n= 4 individual patients (5 explants per patient) *=$p<0.05$, significant difference between treatment and control values. † = $p<0.05$, significantly different between NW and OB values.

5.3.7 Visfatin co-localises with MMP13 in areas of cartilage fibrillation.

Visfatin's role in cartilage degeneration was further investigated through IHC analysis of human hip OA femoral head sections. Staining for visfatin expression was more pronounced in areas of cartilage fibrillation and degeneration, when compared to areas of full thickness cartilage located on the same femoral head, as shown in Figure 5.10A (i-iv). Furthermore, there was increased expression and co-localization of visfatin with MMP-13 in the pericellular extracellular matrix zone surrounding

chondrocytes in areas of fibrillation, compared with chondrocytes in full thickness cartilage (Figure 5.10B, i-vi).

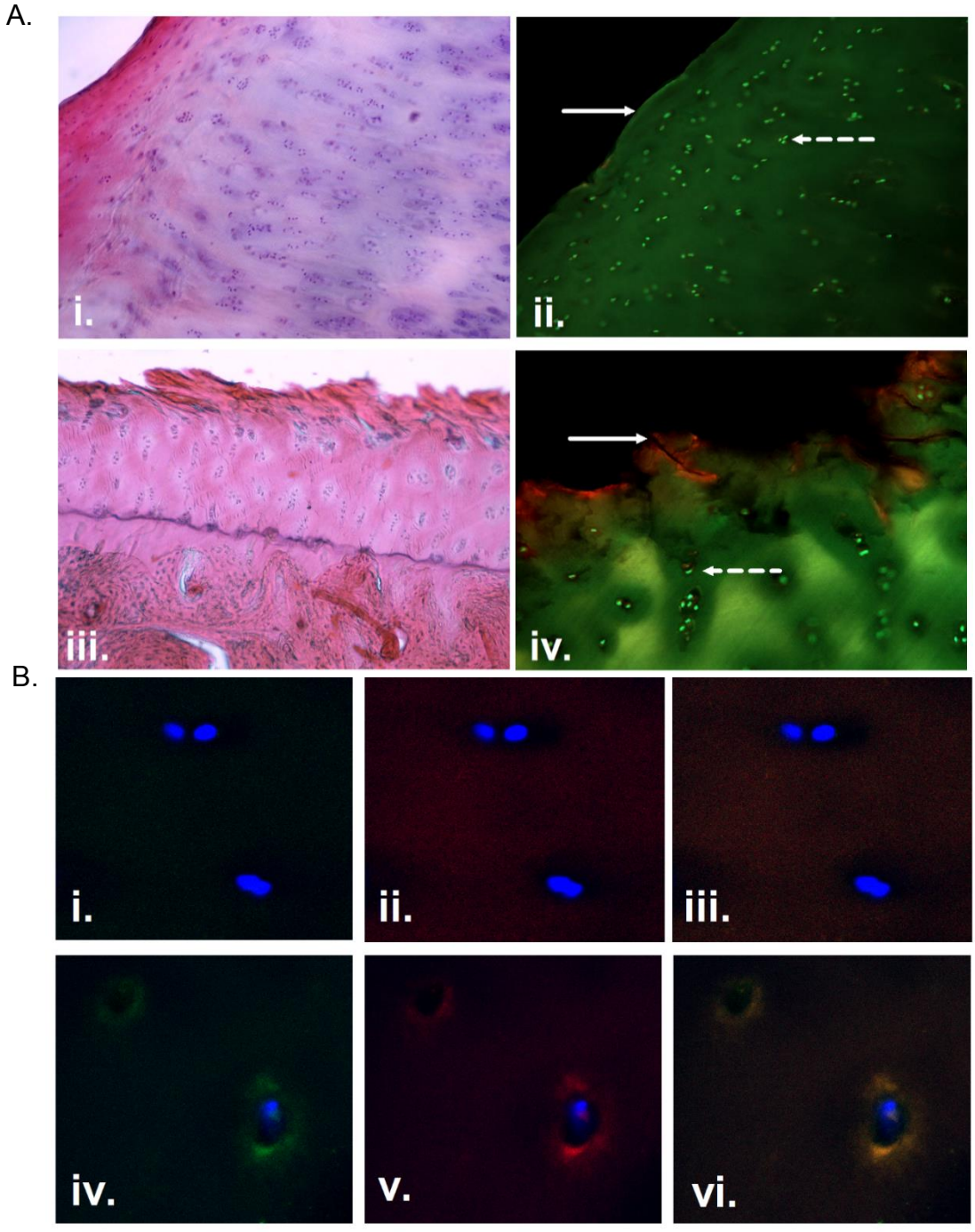


Figure 5.10. Visfatin expression is associated with areas of cartilage damage.

A. IHC of cartilage on a human femoral head showing full thickness and fibrillated cartilage. i. H and E staining of full thickness cartilage. ii fluorescent images of full thickness cartilage (α-visfatin – shown in red, α-NFkB shown in green; n=4 individual

patients). Solid arrow represents area of smooth cartilage; dotted arrow demonstrates low visfatin expression within the pericellular area of chondrocytes. iii H and E staining of degraded and fibrillated cartilage (25x magnification). iv fluorescent images of degraded and fibrillated cartilage (α-visfatin – shown in red, α-NFkB shown in green; n=4 individual patients) (25x magnification). Solid arrow represents area of smooth cartilage; dotted arrow demonstrates increased visfatin expression within the pericellular area of chondrocytes. B. Co-expression of MMP-13 and visfatin in degraded and fibrillated cartilage (63x magnification). i. MMP-13 expression in chondrocytes of full thickness cartilage. ii. Visfatin expression in chondrocytes of full thickness cartilage. iii. Co-staining of visfatin and MMP-13 in chondrocytes of full thickness cartilage. iv. MMP-13 expression in chondrocytes of degraded cartilage. ii Visfatin expression in chondrocytes of degraded cartilage. iii Co-staining of visfatin and MMP-13 in chondrocytes of degraded cartilage.

5.4 Discussion.

To our knowledge, this study is the first to report increased expression of the pro-inflammatory mediators CCL4, CCL20 and MCP-1, increased expression of MMP-1, -2, -3, -7, -8, -9, -10 and -13, and subsequent proteoglycan loss as a functional effect of visfatin on human OA *ex vivo* articular cartilage tissue. Furthermore it is the first to show that visfatin is co-expressed with MMP-13 within localised areas of human hip OA cartilage fibrillation and wear.

Visfatin plasma concentrations have previously been reported to correlate with BMI in individuals without OA undergoing abdominal surgery [345], albeit no relationship was found between visfatin and visceral fat mass, and a third of those recruited had a form of glucose intolerance. Importantly, our analyses found no significant correlation between serum visfatin levels and BMI in males regardless of OA disease status, or between visfatin and BMI in females without hip OA. However, in contrast to males with OA, our data did find a significant positive correlation between serum visfatin expression levels and BMI in females with hip OA, suggestive of sexual dimorphism in the circulatory levels of visfatin in patients with hip OA disease. Furthermore we noted no correlation between serum visfatin concentrations and visceral fat mass, as measured by waist to hip ratio, as previously reported [345].

In support for a systemic driver of OA in females, Maillefert *et al.,* (2003) conducted a longitudinal study of patients with painful hip OA noting a higher incidence of multi-joint OA in females with hip OA [231]. However, it is important to note that in our study the relative serum concentrations of visfatin were not vastly different between all study participants. Furthermore, we found no positive correlations between visfatin and biomarkers of cartilage remodelling. Therefore, it would appear that if visfatin signalling activity is elevated in patients with OA disease, it is more likely to

be due to either localised increases in visfatin expression, or perhaps elevated expression of its unidentified receptor within joint cartilage. This was demonstrated by an increased concentration of visfatin within the synovial fluid of obese individuals when compared to normal weight individuals with OA. In addition, visfatin has previously been reported to be increased within the synovial fluid of patients with knee OA, where its levels positively correlated with biomarkers of cartilage degradation [346].

Previously it has been reported that visfatin is increased within the synovial fluid [346], infrapatellar fat pad, serum and osteophytes [347] of patients with knee OA, compared to non-OA tissues. Importantly, we have now shown that visfatin is expressed locally by all the tissues of the hip OA joint (including cartilage, bone, synovium and adipose) and that visfatin expression is elevated in the synovial fluid and synovial fibroblasts of obese hip OA patients compared to normal-weight hip OA patients. Furthermore, our histochemical analysis of OA femoral head sections reveals for the first time that visfatin expression is highly localized to areas of cartilage fibrillation, where it is co-localized with MMP13 in the pericellular extracellular matrix zone surrounding chondrocytes.

Our studies to examine the functional role of visfatin in human OA patient-derived cartilage tissue provide perhaps the most significant findings of this study and demonstrate that visfatin induces both pro-inflammatory and pro-degradative effects on human hip OA articular cartilage tissue. Visfatin induced significant increases in the production of the collagenases MMP-1, MMP-8 and MMP-13, the gelatinases MMP-2 and MMP-9, stromelysins MMP-3 and MMP-10 and matrilysin MMP-7. Of all the collagenases, MMP-1 is most associated with newly formed collagen molecule breakdown, suggesting that visfatin may prevent cartilage repair as well as inducing

cartilage resorption. Visfatin also induced a significant increase in the production of the gelatinase MMP-9. Gelatinases have remained largely under-researched in osteoarthritis. However, it was recently suggested that MMP-9 was fundamental to the activation of pro-MMP-13 [348], indicating that the partnership between MMP-9 and MMP-13 accelerated collagenase digestion. Our finding therefore that visfatin induces the production of both MMP-9 and MMP-13 is notable.

Visfatin stimulation of hip OA cartilage explants also led to increased secretion of several pro-inflammatory mediators, suggesting that visfatin induces a marked inflammatory response in OA cartilage tissue. In particular, there was a significant increase in the production of IL6, MCP-1, CCL20 and also increased secretion of the chemokine ligand CCL4. Our finding that visfatin induces the production of CCL4 from articular cartilage is intriguing since it has previously been reported that CCL4 within OA synovial fluid is responsible for a large proportion of monocyte chemotactic activity [349]. This suggests that visfatin activity within the joint could play a key role in mediating the movement of monocytes into the synovial fluid contributing towards synovitis.

A key finding was the differential response of normal-weight and obese OA articular cartilage tissue to visfatin stimulation, with significantly greater IL6 production induced by visfatin in cartilage from obese OA patients. Pallu and colleagues (2010) previously noted a similar phenomenon in isolated cultured chondrocyte in response to leptin, where the leptin-mediated expression of TIMP2 and MMP-13 was dependent on the BMI of the patients from which the chondrocytes were isolated [350]. Furthermore, although in this study we did not measure the effect of visfatin on the activity of the aggrecanases (ADAMTS4/ADAMTS5), visfatin stimulation of cartilage tissue led to an increase in the release of sGAG, indicative of aggrecan

proteoglycan loss and increased activity of the aggrecanases [220]. However, of significance, both basal and visfatin-stimulated production of sGAG was greater in articular cartilage from obese hip OA patients than in articular cartilage from normal-weight hip OA patients. These findings suggest that visfatin may mediate greater inflammatory-mediated cartilage damage in obese hip OA patients, than in normal-weight hip OA patients.

This study has some limitations. Firstly, all OA tissues in this study were received from patients undergoing joint replacement surgery and were therefore at an advanced stage of disease. The absence of joint tissue from early OA patients means we can only speculate into the potential role of visfatin in early OA disease initiation. Conducting functional studies on human joint cartilage from patients with early stage OA is inherently difficult due to the inaccessibility of tissue from patients not requiring joint surgery. Secondly, our sample sizes were not large enough to draw any definitive conclusions. Within this study it was not possible to measure the gait of the patients. As gait can alter in OA and therefore change hip cartilage wear patterns, the cartilage used within this study may have received little mechanical loading and therefore the typical biological processes seen in OA may not be a true reflection of cartilage during degeneration and wear. Furthermore, we are unaware of the physical fitness and activity levels of these patients at the point of surgery which could alter cartilage health and pain. Anthropometric indices such as BMI also do not allow us to distinguish between patients with large muscle mass and large fat mass.

In conclusion, we have shown that the adipokine visfatin is expressed locally within the tissues of the hip OA joint and is co-expressed with MMP-13 in the pericellular zones of chondrocytes of fibrillated human OA cartilage tissue. Furthermore, we have

shown that visfatin markedly induces both pro-inflammatory and pro-degradative effects on human hip OA cartilage tissue, particular in cartilage from obese patients. These data suggest that visfatin is a central mediator of cartilage degeneration in patients with hip OA. Targeted inhibition of visfatin signalling within the hip OA joint could therefore be a rewarding strategy for developing a novel therapeutic.

Chapter 6: Identification of the receptor of extracellular visfatin (eVisfatin)

6.1 Background.

Visfatin has received a significant amount of attention in current literature due to its elevated expression and suggested contribution to a number of pathological conditions associated with ageing including diabetes [273], obesity [274-277], and osteoarthritis [271, 346, 351]. It exists in a compartmentalized fashion, with intracellular visfatin (iVisfatin) contributing to NAD^+ biosynthesis through the conversion of nicotinamide into nicotinamide mononucleotide (NMN), and allowing essential metabolic regulation [352]. Conversely extracellular visfatin (eVisfatin), originally isolated and named pre B-cell colony-enhancing factor 1 (PBEF) due to its role in B-cell maturation [353], has demonstrated significant cytokinic effects both in vitro and in vivo [271, 346, 351]. Specifically in OA, as detailed in the previous chapter, visfatin has been shown to increase prostaglandin E2 synthesis [354], IL6 [351], and IL1B [278] production from articular chondrocytes, and we have now shown that visfatin induces both a pro-inflammatory and pro-degradative effect on articular OA cartilage tissue.

The mechanisms by which visfatin exert these effects remain unclear. Currently there is no secretory signal sequence identified, suggesting that increased eVisfatin concentrations occur through cellular apoptosis, and a receptor has yet to be identified [355].

Some researchers believe the cytokinic effect of eVisfatin is achieved indirectly through increased NAD^+ production. Specifically, following a reduction in intracellular NAD^+ levels, Van Gool and colleagues measured a subsequent decrease in TNF-alpha from dendritic cells and macrophages [356]. Of note, cells exhibited no synthesis and secretory dysfunction due to the lower NAD^+ concentrations as evident

by the unaltered secretion of RANTES. Therefore, many researchers hypothesise a NAD^+ dependent mechanism for altering inflammatory cytokine production and secretion.

It is important to note however that APO866 (FK866), the inhibitor of intracellular visfatin used in these studies, is often reconstituted in dimethyl sulphoxide (DMSO). DMSO is considered a exceptional solvent for many polar and non-polar compounds [357, 358], but also has significant anti-inflammatory potential at concentrations as low as 0.5% due to the supressed expression of prostaglandins and pro-inflammatory genes including IL6, CXCL1 and CXCL2 [359]. Indeed, the anti-inflammatory properties of DMSO have led to a surge in research focused on the use of DMSO for the treatment of inflammatory conditions including rheumatoid arthritis and osteoarthritis [360, 361]. Therefore its use in assessing inflammatory responses in vitro may be a source of experimental error and bias.

The quest to determine the visfatin receptor has also been controversial. In 2005, Fukuhara and colleagues defined visfatin as an 'insulin mimetic' through binding and activating the insulin receptor and lowering plasma glucose levels in mice [268]. However, 3 years after publication, the Fukuhara lab retracted their publication after numerous questions were raised following an investigation of their research, despite continuing to stand by their findings.

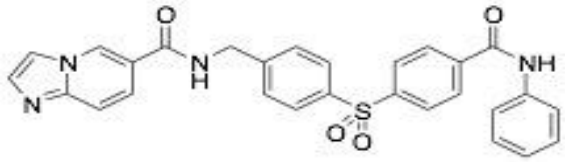
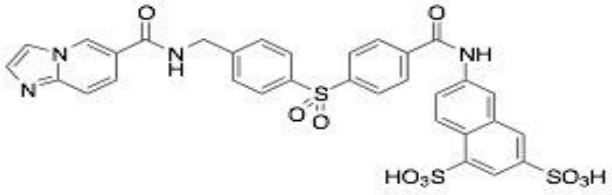
The aim of this chapter was firstly to ascertain if the inflammatory response noted in chapter 5 was independent of visfatin enzymatic activity. Secondly, if eVisfatin signalling was NAD^+ independent, to then identify potential receptor candidates for eVisfatin that could provide novel targets for disease modifying therapeutics, without affecting essential metabolic regulation by NAD^+.

6.2 Results

6.2.1 Does eVisfatin increase NAD^+ production and cause a subsequent increase in pro-inflammatory cytokines?

Visfatin exists in two forms, iVisfatin which is the rate limiting enzyme in NAD+ production, and eVisfatin which we propose has significant cytokinic effects, independent of the intracellular pool. In order to determine if eVisfatin's role in driving catabolic degradation of cartilage and inflammation is independent of NAD+ production, we used both cell-penetrating and non-cell penetrating visfatin inhibitors to inhibit iVisfatin and eVisfatin together, or eVisfatin alone. Inhibitors were developed and provided by Dr. Sam Butterworth, School of Pharmacy, University of Birmingham. Table 6.1 details the inhibitors developed by Dr. Sam Butterworth, through the adaptation of compounds published by Zheng and colleagues (2013) [362].

Table 6.1. Cell penetrating and non-cell penetrating visfatin inhibitors.

Inhibitor	MW	Function	Structure
SB57	510.56	Cell penetrating	
SB58	720.75	Non-cell penetrating	

Primary human chondrocytes were pre incubated for 1 hr with inhibitor compounds then co-stimulated with inhibitor and recombinant visfatin, and an NAD activity assay was performed as detailed in section 2.17 NAD activity assay. As demonstrated in Figure 6.1A, the cell-penetrating compound SB57 significantly decreased NAD production after 1800 seconds at 10nM and 1uM ($p<0.0001$) (Control vs SB57; 258900 vs. 96432 RLU and 258900 vs. 76725 RLU at 10nM and 1uM respectively). This effect was maintained for 3600 seconds (Control vs SB57; 516260 vs. 196084 RFU and 516260 vs. 156184 RLU at 10nM and 1uM respectively). There was no significant reduction in NAD^+ production with the lowest concentration of SB57 (0.1nM) at any time point. Conversely, there was no significant change in NAD production when using the SB58, non-cell penetrating compound at any concentration or time-point (Figure 6.1B).

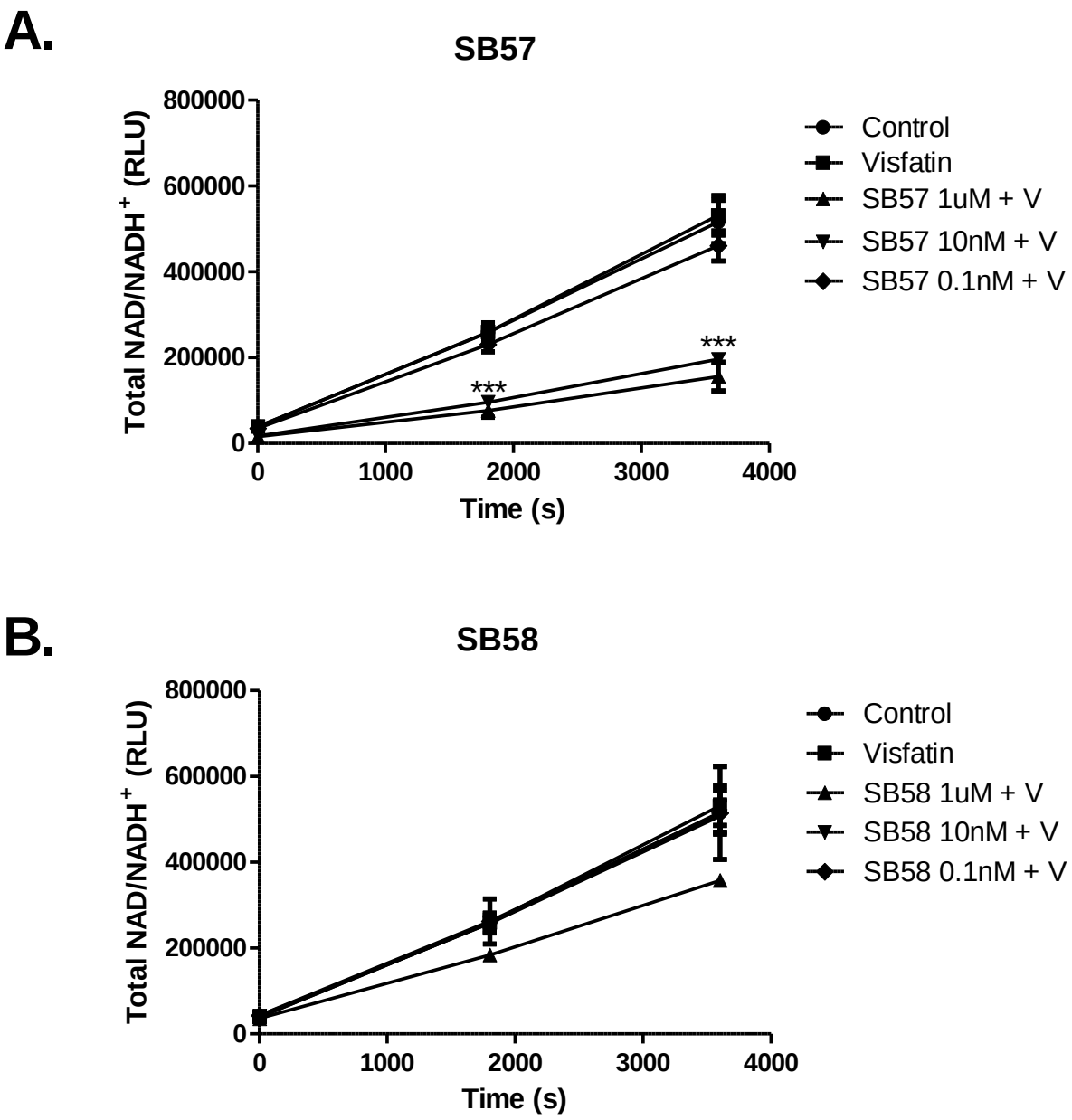


Figure 6.1. NAD$^+$ production following visfatin and visfatin plus SB inhibitor stimulation of chondrocytes.

Primary human chondrocytes were pre-incubated for 1 hr with inhibitor compounds then co-stimulated with inhibitor and recombinant visfatin, and an NAD activity assay was performed as detailed in section 2.17 NAD activity assay. ***=p<0.0001.

The addition of recombinant visfatin also had no effect on NAD^+ production as shown in Figure 6.2, suggestive of an enzymatic independent role of eVisfatin.

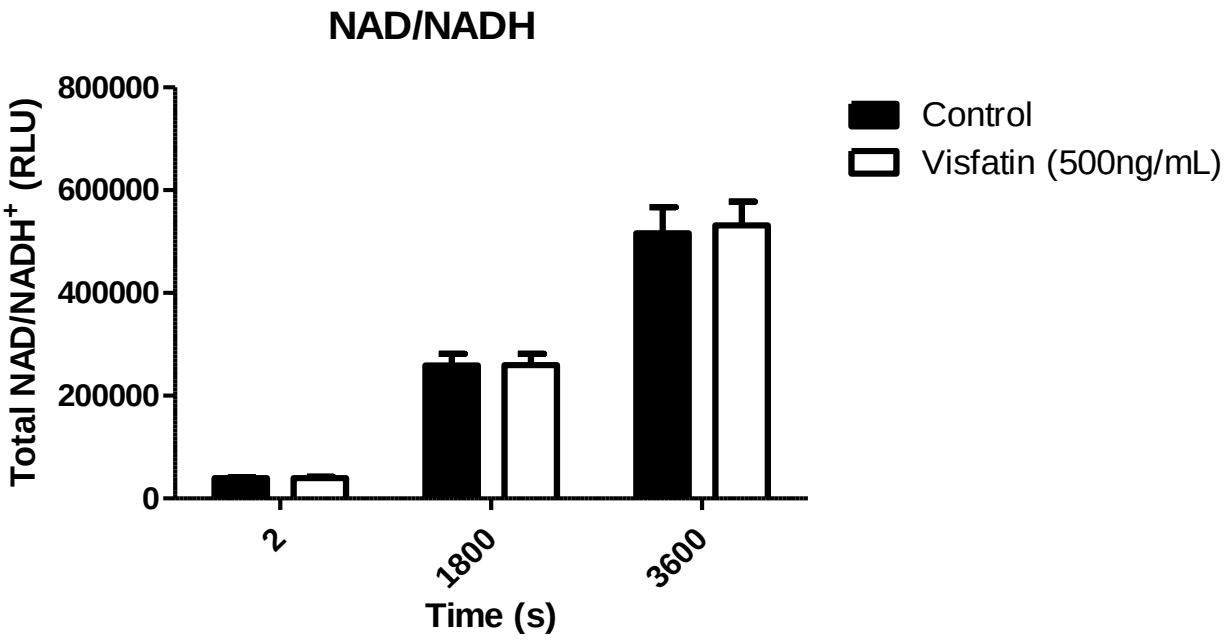


Figure 6.2. NAD^+ production over 2-3600seconds following cell lysis with the addition of recombinant visfatin.

Primary human chondrocytes were incubated with recombinant visfatin, and an NAD activity assay was performed as detailed in section 2.17 NAD activity assay.

In order to confirm an NAD^+ independent mechanism of cytokine production, IL6 protein concentration in the tissue culture supernatant was measured. Recombinant visfatin stimulation of human primary chondrocytes significantly increased IL6 production after 24 hours (96.7 ± 51.5pg/mL vs 245.8 ± 45.75pg/mL in control and visfatin stimulated respectively). With the addition of the SB57 inhibitor, there was a small reduction in the visfatin-stimulated IL6 secretion. However, this was not significant and was not in a dose dependent manner (1uM: 160.5 ± 86.3pg/mL.

10nM: 150.0 ± 32.2pg/ml. 0.1nM 155.7 ± 55.6pg/mL) (Figure 6.3A). A similar result was noted with the addition of the SB58 inhibitor, though the decrease in visfatin stimulated IL6 production was more modest than with the addition of the SB57 inhibitor (1uM: 187.5 ± 62.3pg/mL. 10nM: 183.9 ± 42.2pg/mL. 0.1nM 212.8 ± 165.1pg/mL) (Figure 6.3B).

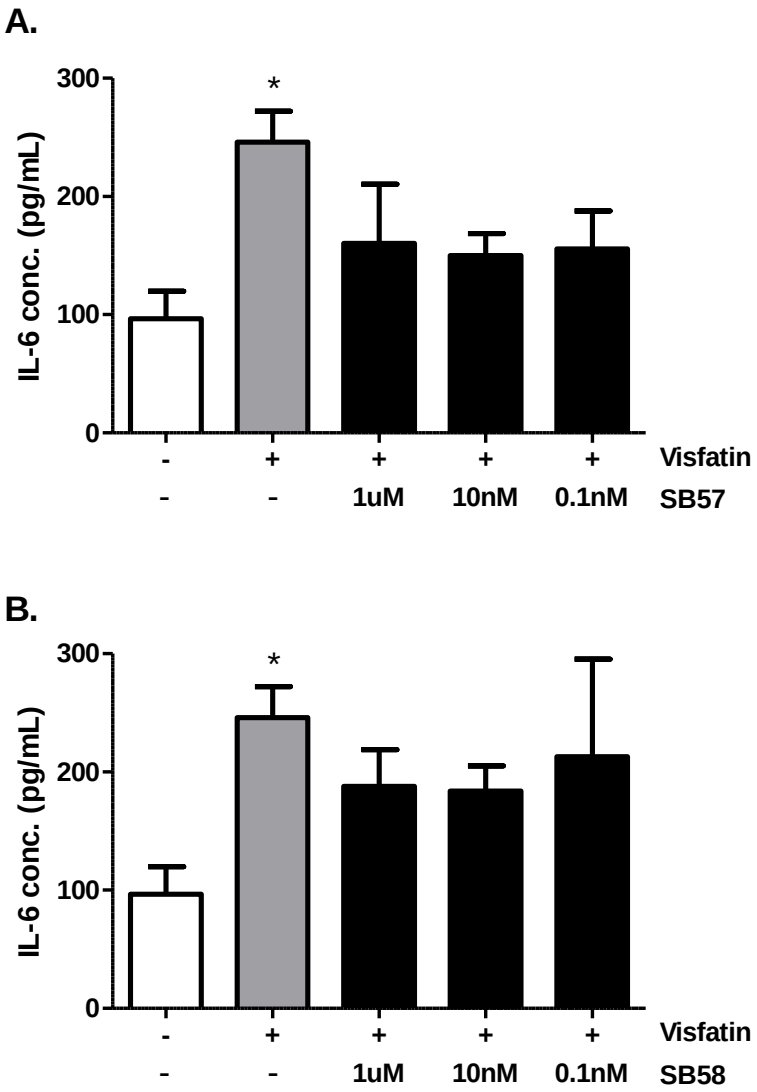


Figure 6.3. IL6 secretion following visfatin and visfatin plus SB inhibitor stimulation of chondrocytes.

A. IL6 secretion from chondrocytes stimulated for 24 hours with 500ng/mL visfatin in the presence of the intracellular inhibitor of visfatin, SB57 (n=5, *=p<0.05). B. IL6 secretion from chondrocytes stimulated for 24 hours with 500ng/mL visfatin in the presence of the extracellular inhibitor of visfatin, SB58 (n=5, *=p<0.05). Primary human chondrocytes were pre incubated for 1 hr with inhibitor compounds then co-

stimulated with inhibitor and recombinant visfatin for 24 hours prior to an IL6 ELISA of tissue culture supernatants.

6.2.2 Identifying potential receptor candidates for eVisfatin

Our previous data suggests that in human primary chondrocytes visfatin-mediated IL-6 secretion is independent of intracellular enzymatic NAMPT activity. It is therefore conceivable that visfatin exerts its actions by binding to a receptor on the surface of the chondrocytes, which initiates a downstream signalling cascade resulting in induction of cytokine and MMP production. In order to screen for a candidate visfatin receptor, a receptor binding screen was performed using Retrogenix target deconvolution platform (www.retrogenix.com). Retrogenix target deconvolution technology utilises expression vector arrays, encoding over 70% of known plasma membrane receptors, which is spotted on to slides for reverse transfection into human cells (HEK293). The test molecules are applied and specific binging confirmed via the appropriate detection system. Retrogenix technology demonstrates a broad coverage of plasma membrane proteins and was therefore considered most likely to identify the specific target of visfatin compared to a standard protein array, or immunoprecipitation followed by mass spectrometry.

In the primary screen, more than 2500 known plasma membrane proteins were screened for binding with His-tagged visfatin or His-tagged EGF which served as a negative control. Figure 6.4 illustrates the human membrane protein cDNA array in HEK293 cells. zsGreen, encoded within the cDNA library vectors, confirmed successful transfection. As detailed in figure 6.4, transfections were standardized across the test ligands and secondary only slides, although individual receptor

expression differed. Interactions were detected using a mouse anti-His antibody (Millipore) followed by an Alexa Fluor 647 anti-mouse antibody (Life Technologies). Figure 6.4 demonstrates some cross-reactivity between His-Visfatin, His-EGF and the no test ligand slides, which must be considered during the data analysis. In an attempt to remedy this cross-reactivity, bead technology was utilised to increase the specificity of the assay.

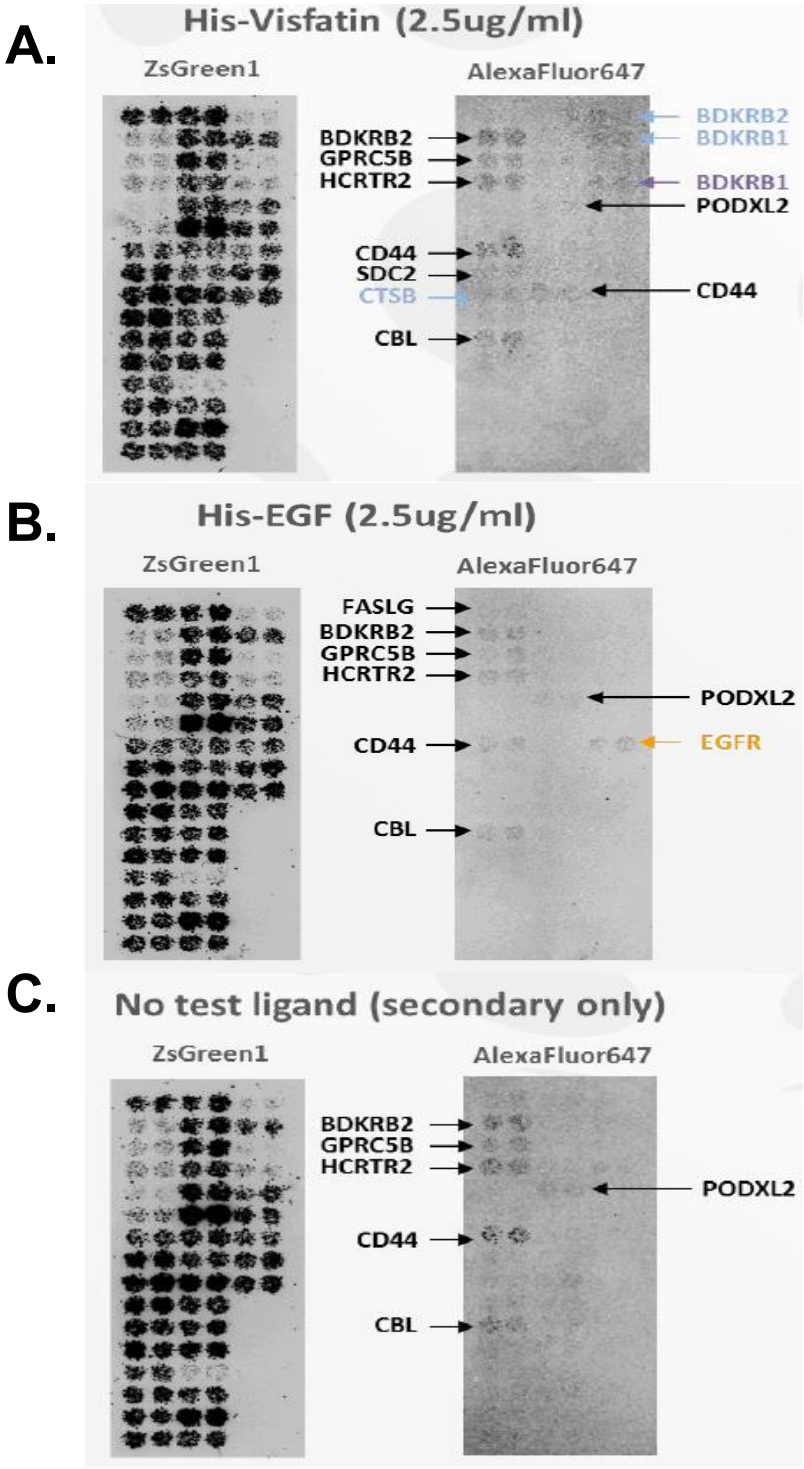


Figure 6.4. An example of Retrogenix Cell Microarray technology slides utilizing HEK293 cells over-expressing cell membrane receptor proteins. HEK293 cells were reverse transfected with cell membrane receptors and samples were detected using a mouse anti-His antibody (Millipore) followed by an Alexa Fluor 647 anti-mouse antibody (Life Technologies). ZsGreen1 confirmed successful transfection.

Using recombinant protein tagged bead technology, Retrogenix repeated the first screen. As shown in Figure 6.5, signal intensity was marginally increased in comparison to Figure 6.4, however there remained some unspecific binding in the His-EGF tagged slides. Table 6.2 details the findings from the preliminary and confirmatory screens using the recombinant and recombinant-bead tagged technology.

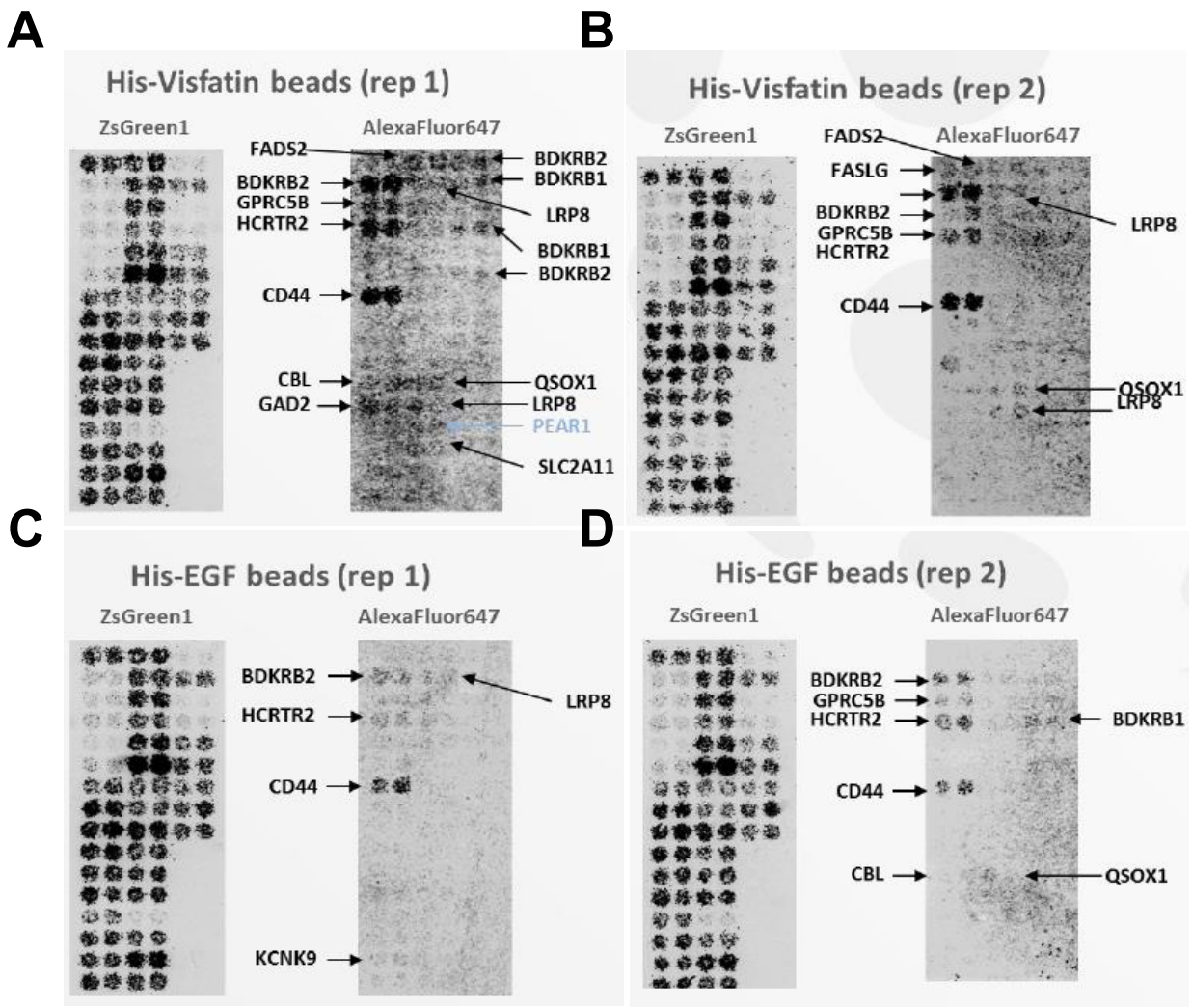


Figure 6.5. An example of Retrogenix Cell Microarray technology utilising recombinant protein attached to beads for increased specificity and sensitivity. HEK293 cells were reverse transfected with cell membrane receptors and samples were detected using a mouse anti-His antibody (Millipore) followed by an Alexa Fluor 647 anti-mouse antibody (Life Technologies). ZsGreen1 confirmed successful transfection.

Table 6.2. Positive hit results from the Retrogenix Cell Microarray technology assays.

Gene ID	Sample Id and dose													
	His-Visfatin 2.5ug/ml	His-Visfatin 2.5ug/ml	His-Visfatin 2.5ug/ml	His-Visfatin 2.5ug/ml	His-EGF 2.5ug/ml	His-EGF 2.5ug/ml	No test ligand	No test ligand	His-Visfatin labelled beads	His-Visfatin labelled beads	His-Visfatin labelled beads	His-Visfatin labelled beads	His-EGF labelled beads	His-EGF labelled beads
	Primary (P) or Confirmation (C) screen													
	P	P	C	C	C	C	C	C	P	P	C	C	C	C
FASLG	+	+	-/+	-/+	+	+	-/+	-/+	-/+	-/+		+		-/+
GPRC5B	+	+	+/++	+/++	+/++	+/++	+/++	+/++	N/A	N/A	++	+	-/+	++
HCRTR2	-/+	-/+	++	++	+/++	+/++	+/++	+/++	N/A	N/A	+++	+/++	++	+++
GABBR1	-/+	-/+							N/A	N/A				-/+
CD44	++/+++	++/+++	++/+++	++/+++	+/++	+/++	++	++	+++	+++	+++	+++	++/+++	+++
SDC2	-/+	-/+	+	+	-/+	-/+	-/+	-/+	N/A	N/A			-/+	++/++ +
CTSB	-/+	-/+	+	+					N/A	N/A				+
CTSL2	-/+	-/+	-/+	-/+	-/+	-/+	-/+	-/+	N/A	N/A	-/+		-/+	
CBL	+	+	++	++	+/++	+/++	+/++	+/++	+	+	+	-/+		++
GAD2	-/+	-/+	-/+	-/+					N/A	N/A	+/++	-/+	-/+	-/+
KCNJ11	-/+	-/+							N/A	N/A				
TSPAN9	-/+	-/+							N/A	N/A				
KCNK9	-/+	-/+							-/+	-/+	-/+		+	+/++
KCNJ12	-/+	-/+							-/+	-/+		-/+	-/+	+
FADS2	-/+	-/+	N/A	N/A					-/+	-/+	+/++	+		+
LRP8	-/+	-/+							N/A	N/A	+	+	+	+
F7	-/+	-/+							-/+	-/+	-/+	-/+	-/+	+
HNRNPU	-/+	-/+					-/+	-/+	N/A	N/A	-/+		-/+	-/+

Gene ID	Sample Id and dose													
	His-Visfatin 2.5ug/ml	His-Visfatin 2.5ug/ml	His-Visfatin 2.5ug/ml	His-Visfatin 2.5ug/ml	His-EGF 2.5ug/ml	His-EGF 2.5ug/ml	No test ligand	No test ligand	His-Visfatin labelled beads	His-Visfatin labelled beads	His-Visfatin labelled beads	His-Visfatin labelled beads	His-EGF labelled beads	His-EGF labelled beads
	Primary (P) or Confirmation (C) screen													
	P	P	C	C	C	C	C	C	P	P	C	C	C	C
PODXL2	-/+	-/+	+	+	+	+	+	+	N/A	N/A			-/+	
LFNG	-/+	-/+							-/+	-/+	-/+			
PLA2G3	-/+	-/+					-/+	-/+	-/+	-/+	-/+			
GPR61	N/A	N/A							-/+	-/+				
CD44	-/+	-/+	+	+	-/+	-/+	-/+	-/+	N/A	N/A		-/+	-/+	
SLC7A8	-/+	-/+							-/+	N/A				
QSOX1	-/+	-/+			-/+	-/+	-/+	-/+	N/A	N/A	+/++	+	-/+	++
LRP8	++	++			-/+	-/+	-/+	-/+	++	++	+/++	+	-/+	+
PEAR1	-/+	-/+							-/+	-/+	+			
SLC2A11	-/+	-/+							-/+	?	+		-/+	
ATP6V1G3	N/A	N/A							N/A	-/+				
BDKRB1	N/A	N/A							N/A	N/A	+			
BDKRB2	N/A	N/A	+	+					N/A	N/A	+/++	-/+		+
BDKRB1	N/A	N/A	+	+					N/A	N/A	+/++	-/+		+
BDKRB1	N/A	N/A							N/A	N/A	-/+	-/+		-/+
BDKRB1	N/A	N/A	+/++	+/++	-/+	-/+	-/+	-/+	N/A	N/A	++	-/+		++
BDKRB1	N/A	N/A	-/+	-/+					N/A	N/A			N/A	-/+
BDKRB2	N/A	N/A							N/A	N/A	+			
EGFR	N/A	N/A			+/++	+/++			N/A	N/A				
Negative	N/A	N/A							N/A	N/A				
Negative	N/A	N/A							N/A	N/A				

Table 6.3 details the positive hits identified during the primary or confirmatory screen for our test ligands binding to a known receptor, using both standard and bead technology. While no receptor provided a robust and specific signal for His-tagged visfatin binding, there were a number of potential candidate "hits" that could be a potential receptor for visfatin. Specifically, CD44, GAD2, KCNJ11, LFNG, SLC7A8, LRP8, PEAR1, BDKRB1 and BDKRB2 all exhibited greater affinity for the His-tagged visfatin ligand compared to the His-tagged EGF ligand. Of note, BDKRB1 and 2 were identified on numerous occasions as a positive hit for His-tagged visfatin ligand.

6.2.3 Confirmation of visfatin receptor identification.

It is unfeasible to attempt to confirm all identified potential receptors associated with the His-visfatin ligand, therefore after an extensive literature search and on the strength of the Retrogenix Cell Microarray technology assays, the Bradykinin receptors B1 (BDKRB1) and B2 (BDKRB2) were pursued. Firstly, we considered that if either BDRRB1 or BDKRB2 was the receptor for visfatin, that stimulation of primary chondrocytes with visfatin might be expected to affect its expression. Therefore, primary human OA articular chondrocytes 3 individual hip OA patients were either stimulated for 24h with recombinant visfatin (500ng/uL) or left unstimulated, and the expression of BDKRB1 and BDKRB2 was determined by qRT-PCR. In chondrocytes stimulated with visfatin there was a small down-regulation in BDKRB1 expression (0.8 ± 0.5 fold) and a moderate upregulation in BDKRB2 r expression (1.5 ± 0.3 fold) compared to un-stimulated chondrocytes. Although neither of these findings reached statistical significance (Figure 6.6) we conducted

further studies with BDKRB2 given the greater change in expression observed, compared to BDRRB1

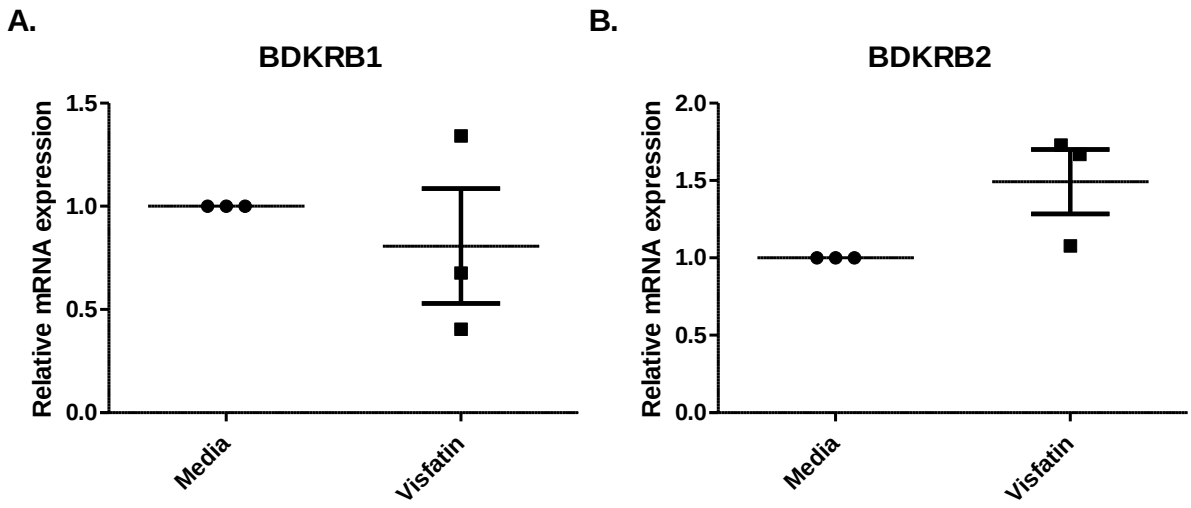


Figure 6.6 BDKRB1 and BDKRB2 gene expression following 24 hr recombinant visfatin stimulation.

Human primary chondrocytes were stimulated for 24hr with 500ng/mL visfatin (n=3) and data expressed as relative mRNA expression compared to the unstimulated, media control.

6.2.4 The effect of BDKRB2 knockdown on cytokine secretion.

In order to determine whether the functional effects of visfatin on chondrocytes were mediated via binding and signalling through BDKRB2 we performed loss of function (LOF) studies using siRNA. Firstly, it was necessary to optimise siRNA transfection. Three unique siRNA duplexes targeted toward human BDKRB2, or a non-targeting control (Origene, U.S.), were transfected into human primary chondrocytes using the Nucleofector 4D system (Lonza, U.S.). Cells were plated for 24hr prior to RNA

isolation and qRT-PCR. BDKRB1 and BDKRB2 gene expression were both analysed to ensure there was no compensatory increase in BDKRB1, or off-target knockdown effects following BDKRB2 knockdown.

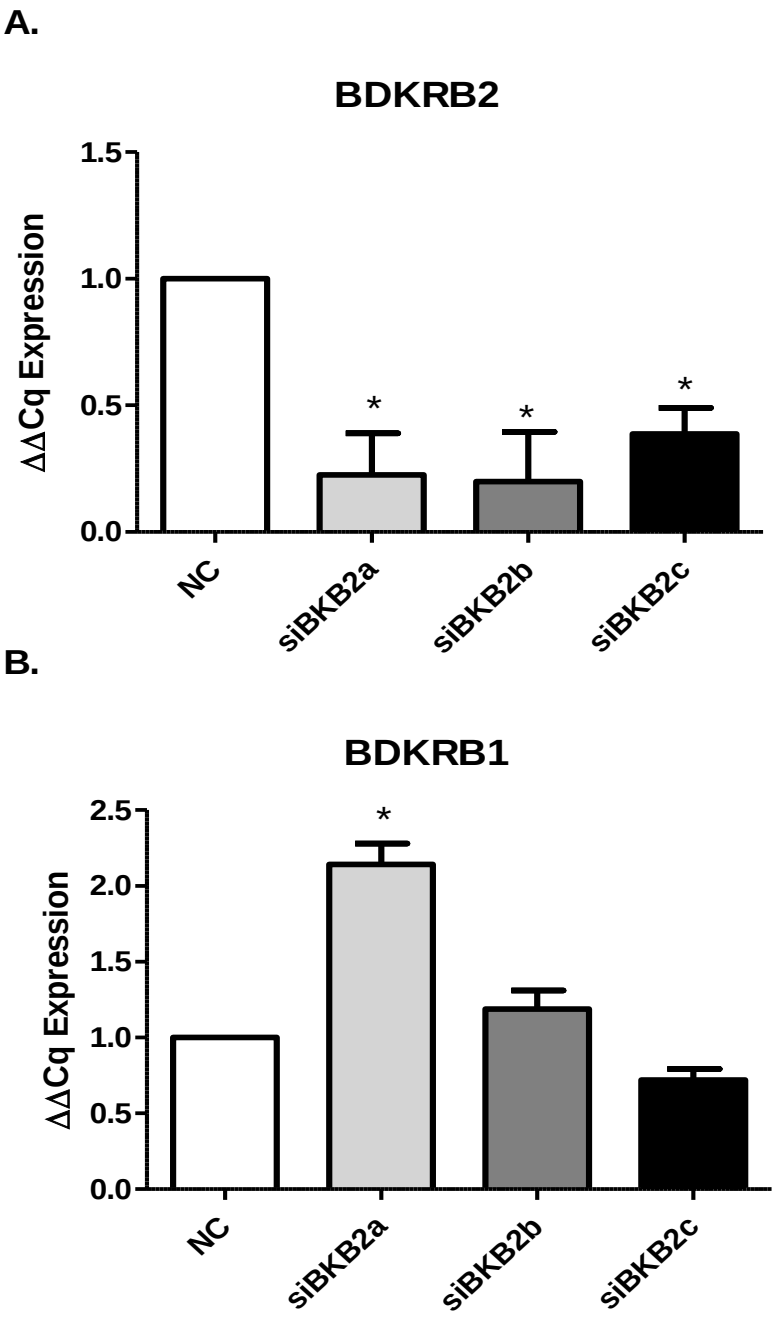


Figure 6.7. Bradykinin receptor expression following BDKRB2 knockdown.

Relative expression of BDKRB2 (**A**) and BDKRB1 (**B**) in primary chondrocytes 24h after transfection with 3 individual BDKRB1 siRNA duplexes (siBKB2a-c), compared to non-targeting control siRNA transfection (NC). Relative expression was determined by ΔΔCq, normalised to the house-keeping gene ACTB.

BDKRB2 gene expression was significantly decreased with all targeting siRNAs (Figure 6.7A). siBKB2a exhibited the greatest knockdown (85 ± 21% knockdown) followed by siBKB2b (80.1 ± 27.7% knockdown) and siBKB2c (61.3 ± 17.9% knockdown). However, Figure 6.7B shows a significant increase in BDKRB1 gene expression relative to NC gene expression following transfection with siBKB2a (114 ± 24% gene expression compared to NC, $p<0.0001$). BDKRB1 expression remained unchanged in chondrocytes transfected with either siBKB2b or siBKB2c.

Based upon these siRNA knockdown data siBKB2b or siBKB2c were used to determine whether BDKRB2 knockdown affected the cytokinic functional role of visfatin. Human primary chondrocyte cells transfected with siBKB2b or siBKB2c, or with NC siRNA were plated for 24 hr. Cells were then either stimulated with his-tagged visfatin (500ng/mL) or cultured in fresh growth media alone for a further 24 hr. Total RNA was then isolated from the cells and tissue culture supernatants were collected for IL-6 cytokine analysis.

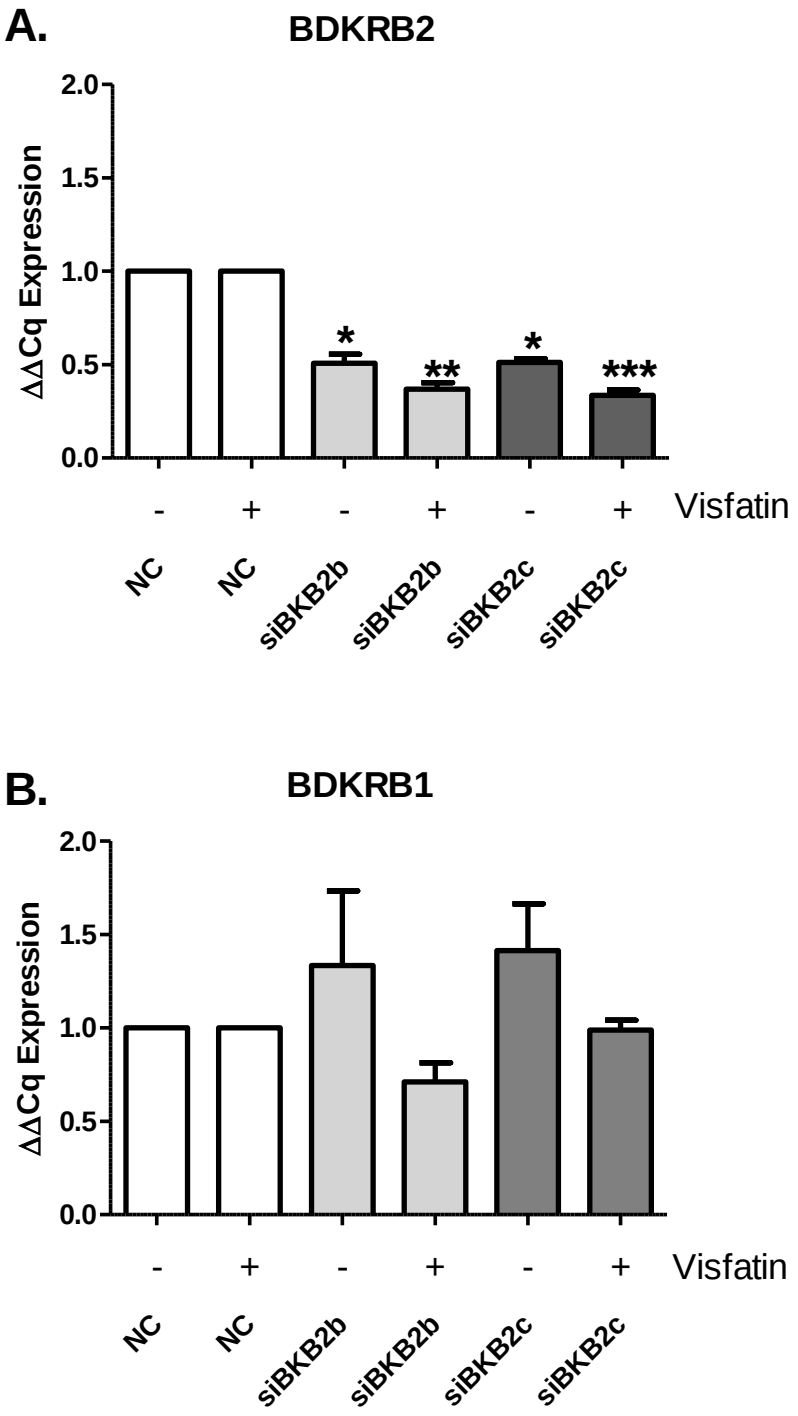


Figure 6.8. Bradykinin receptor gene expression following BDKRB2 knockdown and visfatin stimulation.

Relative expression of BDKRB2 **(A)** and BDKRB1 **(B)** in primary chondrocytes 48hr post-transfection, either with or without 24 hr visfatin stimulation, n=3. Transfection

was with 2 individual BDKRB1 siRNA duplexes (siBKB2b-c), compared to non-targeting control siRNA transfection (NC). Relative expression was determined by ΔΔCq, normalised to the house-keeping gene ACTB. +/- refer to with or without 500ng/mL visfatin stimulation.

As noted before, siBKB2b and siBKB2c induced a marked and significant knockdown in the expression of BDKRB2, Significantly, Figure 6.8 demonstrates that this knockdown was sustained for up to 48h following transfection. Relative to cells transfected with NC siRNA, siBKB2b and siBKB2c knocked down expression of BDKRB2 by 45.5 ± 13.1% and 42.9 ± 14.9% respectively in cells left unstimulated. There was no significant difference in the degree of BDKRB2 knockdown in cells that had been stimulated for 24h with visfatin, compared to those cells that had been cultured in growth media alone. Relative to cells transfected with NC siRNA and stimulated with visfatin, siBKB2b and siBKB2c knocked down BDKRB2 expression by 63.1 ± 8.6% and 66.4 ± 6.7% respectively. Importantly, there was no significant effect on the expression of BDKRB1 with either siBKB2b or siBKB2c, regardless of whether cells had been stimulated with visfatin (Figure 6.8B). Therefore, with sufficient KD of BDKRB2 in all siRNA cell transfections, without a subsequent BDKRB1 compensation or off-target knockdown, tissue culture supernatants were collected for IL6 analysis.

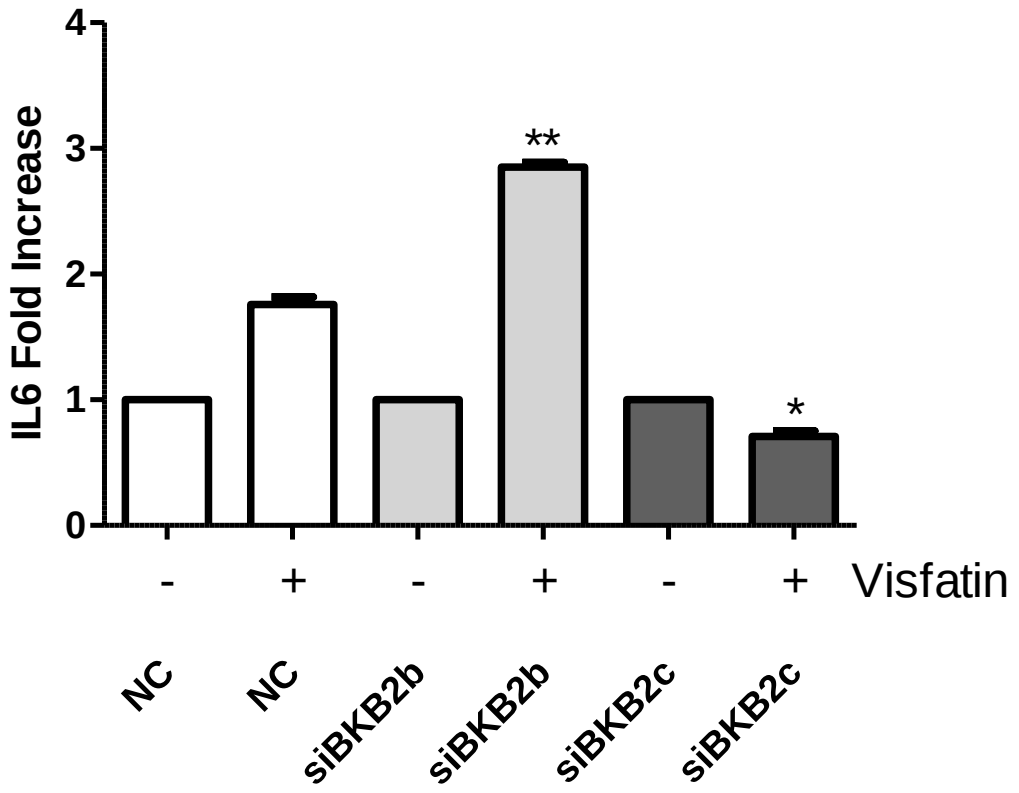


Figure 6.9. IL6 protein content following BDKRB2 knockdown and visfatin stimulation.

IL6 tissue culture media protein content in BDKRB2 KD cells compared to cells non-targeting control 48hr post-transfection, either with or without 24 hr visfatin stimulation, samples were assayed in triplicate (n=3 biological replicates).

As expected IL6 was induced following 24h stimulation of visfatin in NC-transfected cells (1.73 ± 0.19 fold). However, the effect of visfatin stimulation on cells depleted of BDKRB2 gave converse results, with an increase in IL-6 in cells transfected with siBKB2b, and no induction of IL-6 in cells transfected siBKB2b (2.85 ± 0.09 fold increase ($p<0.01$) and 0.73 ± 0.09 fold ($p<0.05$) decrease respectively).

6.3 Discussion

This is the first study to demonstrate that the enzymatic activity of visfatin is not necessary to elicit a cytokinic response in human primary articular chondrocytes. Through inhibiting iVisfatin and eVisfatin, we demonstrated a reduction in NAD^+ content without inhibiting the IL6 and MMP-13 response. While this has not been published in chondrocyte cells lines previously, there are a number of studies to support the enzymatic/NAD^+ independent role of eVisfatin in pro-inflammation.

Interestingly, one such study by Audrito *et al.,* (2015) demonstrated a role of eVisfatin in driving the differentiation of resting monocytes into tumor-supporting M2 macrophages. In cancer cell lines, M2 macrophages are responsible for the secretion of tumour-promoting cytokines including IL6 and IL8 [363]. The researchers noted an enzymatically independent role of eVisfatin activation of the signalling pathways (ERK1/2, STAT3 and NFκB) associated with tumour-promoting cytokine release through the use of an enzymatically inactive eVisfatin mutant (H247E). This was further supported by Li and colleagues (2008) who demonstrated a non-enzymatic, protective role of eVisfatin, preventing ER stress induced apoptosis in macrophages. Through inducing IL6 secretion, eVisfatin is able to activate the pro-survival signal transducer STAT3 [364]. Furthermore, this anti-apoptotic role of eVisfatin is achieved using the monomeric form, and thus the enzymatically inactive form, of eVisfatin. The authors therefore suggested that although enzymatic activity requires dimerization of the protein, the monomer form, which comes from apoptotic or necrotic cells during an inflammatory event, may retain cytokine-like activity [364].

Contrary to the above, visfatin has been shown to be secreted by synovium, cartilage and bone in its dimeric and enzymatically active form and visfatin

significantly increased IL6, KCP and MCP1 secretion in chondrocytes [271]. Furthermore, due to up to a 94% decrease in cytokine expression following FK866 inhibition of eVisfatin, the authors acknowledged that targeting visfatin activity via FK866 can prevent NAD^+ synthesis and therefore provide a new therapeutic perspective in OA. However, this and many other studies have been unable to prove that the binding of FK866 to the nicotinamide pocket of visfatin does not prevent alternative proteins from binding to, or being bound by visfatin. Furthermore, the addition of nicotinamide, a product of visfatin enzymatic activity, to the FK866 stimulated cells has yet to reverse the inhibition of the cytokinic response. Finally, in this study there was no difference in NAD^+ content following visfatin stimulation when compared to unstimulated cells, suggesting that NAD^+ cannot be associated with the pro-inflammatory response.

The above data supports the notion that eVisfatin could elicit a pro-inflammatory response in the absence of enzymatic activity. As systemic visfatin expression has been shown to be elevated in obesity and type II diabetes [365, 366], it was prudent to suggest that visfatin may interact with an unknown receptor to initiate a downstream signalling cascade and elicit a regulatory response. In this study, the Retrogenix target deconvolution platform identified the genes BDKRB1 and BDKRB2 as potential receptors for eVisfatin, with BDKRB2 further supported by PCR data following visfatin stimulation.

The kinin system is composed of kininogens which are activated by kallikreins to produce two peptides known as bradykinin and kallidin [367]. Bradykinin has previously been identified in the regulation of blood pressure and vascular permeability, however recent research has implicated bradykinin and in particular the bradykinin receptors in OA pathogenesis and pain [367]. Bradykinin has previously

been shown to be increased in the synovial fluid of arthritis patients, including those with OA [368] [369]. Furthermore, there is constitutive expression of BDKRB2 throughout the tissues of the joints, in particular in the synovial lining cells and fibroblasts [368, 370], and activates synoviocytes and chondrocytes [371], to initiate inflammatory pathways and alter tissue homeostasis.

In this study depletion of BDKRB2 expression in primary chondrocytes using two different siRNA duplexes produced converse results on the effect of visfatin-mediated IL-6 secretion. Therefore, it was not possible to conclusively determine if BDKRB2 plays a role in visfatin signalling. RNA interference is a widely used technique, however at times is plagued by ineffectiveness (leading to falsely negative data) and non-specificity (leading to falsely positive data). Evidence would suggest that siRNAs in particular are able to affect the translation of off target transcripts containing a partially complimentary sequence [372, 373]. Future research may therefore benefit from a stable knockdown approach utilising shRNAs, or utilising a wider panel of siRNA duplexes.

While there appears to be no evidence of direct NAMPT and bradykinin receptor interaction in published OA literature, there is some suggestion of visfatin mediating endothelial dysfunction. In 2011, Vallejo *et al.,* found that systemic visfatin was able to impair the vasorelaxant response to endothelium dependent vasodilators including bradykinin without affecting the endothelial independent relaxant properties of the vasculature muscle [374]. The authors conclude that visfatin enzymatic activity is essential for this inhibitory effect, as proven by the restoration of the relaxant properties in human mesenteric arteries with APO866. However, it is unknown whether APO866 causes a conformational change in visfatin structure and prevents

its binding to the receptor. Furthermore, there is no reference to the mechanism by which visfatin may prevent bradykinin signalling, for example, it is possible that through competitive binding of visfatin to the B2-bradykinin receptor, visfatin is able to prevent bradykinin signalling.

There are limitations to this research. Recombinant visfatin used within this study had dimeric confirmation therefore it is assumed to be enzymatically active, however this was not confirmed. Furthermore, B2-bradykinin receptor was selected based upon most promising target deconvolution data and based upon previous literature, however there were a number of potential hits from the target deconvolution data that warrant further investigation. Furthermore, it was not possible to conclude the functional effect of BDKRB2 KD using siRNAs alone as two RNAs directed to BDKRB2 exhibited contrary findings. Finally it is important to consider the potential for siRNA to induce an innate cytokine response, as demonstrated by a six fold induction of IL6 in U87-$CD4^+$ $CCR5^+$ cells transfected with multiple siRNAs [375].

Chapter 7: General Discussion

7. General Discussion.

OA is the leading cause of joint degeneration, pain and disability in the UK, with over 8.75 million individuals over the age of 45 years seeking treatment. Characterised as a disease of articular cartilage degeneration and subchondral bone remodelling, many patients experience limited movement and are unable to perform everyday tasks. Furthermore, with OA disease prevalence increasing with age, these figures are set to worsen given the ageing population. Of preventable risk factors, obesity has been defined as having the most significant impact on the progression of OA. A pivotal study published in 2001 was the first to truly illustrate the impact of obesity of the progression of OA in the knee. The researchers showed a significant correlation of the risk of knee OA with increasing BMI, from men and women over the age of 45 years. Surprisingly, of individuals with a BMI of greater than 36, there was a 14-fold increase in the risk of knee OA compared to healthy weight-matched individuals [377]. This finding supported previous data obtained by Oliveria *et al.,* (1999) who examined the effect of obesity on symptomatic OA of the hands, hips and knees in 134 case control pairs of women aged between 20-89 years [378]. The researchers identified body weight and body mass index as predictors of incident OA in all three joints, with odds ratios ranging from 3-10.5 in the lower and extreme tertiles.

The discovery that adipose tissue secretes cytokines (adipokines) has led to the current understanding of adipose tissue as an endocrine organ [379]. Adipokine release has been mechanistically linked to metabolic complications and the metabolic syndrome [27, 33] by contributing to the low-level pro-inflammatory state seen commonly in obese individuals [28]. Critically, adipokine signalling is now receiving much attention in relation to OA joint pathophysiology largely due to the association between obesity and OA [28, 380] in both weight-bearing and non-weight

bearing joints (e.g. hands) [381]. Furthermore, differential expression of particular adipokines have been reported in OA serum and synovial fluid, which are capable to modulating cartilage catabolic and anabolic pathways [380].

Current therapeutics of OA focus primarily on pain relief and restoration of joint function, however due to the heterogeneity of OA, pain sources can often remain elusive. Despite the high prevalence of OA within the UK alone, there remains an unmet clinical need for therapeutics to prevent OA development. Non-steroidal anti-inflammatory drugs (NSAIDs) and narcotics demonstrate promising disease-modifying capabilities, however the side effects are less desirable, for example heart failure, persistent headaches, stomach ulcers and anaemia [15]. Acetaminophen toxicity has also been demonstrated, despite being the primary therapeutic subscribed by clinicians, and is thus is to be prescribed with caution [14]. Most recently, OARSI guidelines [16] suggest a more holistic approach to OA treatment, including acupuncture, weight loss, and increased patient/doctor communication as treatment for patients with symptomatic OA [16].

When this project was conceived, there was growing evidence to suggest that adipokines could provide a systemic functional link between obesity and the increased prevalence of multi-joint OA. However, any functional studies using intact human hip cartilage and bone tissue to determine the role of adipokines in mediating OA pathology were lacking. One aim of this thesis was therefore to determine the adipokine expression profile in the serum and joint fluid of OA patients, in comparison to non-OA NOF# patients, and to determine their association with joint damage and BMI. Of the 24 proteins screened, 11 proteins were differentially expressed in OA patients compared to NOF# identifying candidate cytokines or adipokines that may be integral to the progression of joint destruction. Many of

these proteins are associated with, and secreted during, the inflammatory process and could potentially provide early diagnostic value to OA.

In reference to BMI, this study found serum resistin concentration was significantly increased with BMI and had a significant association with joint disease severity as determined by joint space. After further investigation, we noted an increase in the biosynthetic activity of human primary osteoblasts following resistin stimulation and collagen type I homotrimer formation through the activation of the canonical WNT-signalling pathway and subsequent β-catenin translocation.

As well as identifying its role in OA pathogenesis, through characterising the role and mechanism of resistin in bone turnover, it may be possible to modulate OA and other bone pathologies or diseases typified by increased bone formation or WNT/ β-catenin signalling. For example, Ankylosing spondylitis (AS), a rheumatic disease typified by chronic inflammation that can affect the spine and sacroiliac joints at an early age [382, 383]. One indices of AS is the development of bony growths, known as syndesmophytes, within spinal ligaments which impact the mobility of the spine leading ultimately to spinal fusion [384]. Spinal inflammation has been shown to be a key regulator of syndesmophytes development with 3 times the number of syndesmophytes on the vertebral edges developing in the presence of inflammation at baseline compared with the vertebral edges without inflammation, after 2 years [385]. Interestingly, patients with AS have been shown to have elevated levels of serum resistin compared to a control population (11.6ng/mL vs. 6.6ng/mL respectively), however there was no association between resistin concentration and disease severity [386]. As the main feature of AS is new bone formation, there is sufficient reason to suggest that increased circulating resistin expression may

provide a key link to syndesmophyte development and may provide a new therapeutic targets to modify the progression of AS.

So far, focus has been on the role of resistin in driving adverse disease pathologies, however it may be prudent to consider the use of resistin as a therapeutic modulator of bone pathologies which are characterised by increased bone resorption. Osteoporosis, for example, is characterized by an age associated imbalance of osteoclastic and osteoblastic activity and therefore dysregulated bone remodelling [387], whereby the degree of bone resorption is higher than bone formation. Therefore the aim of therapeutics for osteoporosis is to readjust the balance of bone formation and bone resorption. In this study it has been shown that resistin increases biosynthetic activity of osteoblasts, therefore providing a potential for recombinant resistin to provide a novel therapeutic for osteoporosis. Furthermore, it has been shown that circulating resistin levels are lower in patients with osteoporosis compared with healthy controls [326], therefore it may be possible to utilise recombinant resistin systemically without increasing risk of colorectal cancer, AS, or osteoarthritis.

Similarly to resistin, this study found that visfatin expression was increased with BMI, however this was shown in synovial fluid, not serum. It is generally accepted that proteins elevated in the synovial fluid are more likely to impact cartilage turnover, compared with bone, because nutrients are typically transported to chondrocyte cells via diffusion through the matrix from the synovial fluid [295]. Therefore focus was placed on understanding the role of visfatin in mediating cartilage pathology in OA patients. Upon further investigation, visfatin was demonstrated to mediate the secretion of pro-inflammatory cytokines and chemokines from human cartilage tissue including CCL4, CCL20 and MCP-1. Furthermore, visfatin was shown to be pro-

degradative with an increased expression of MMP-1, -2, -3, -7, -8, -9, -10 and -13, and subsequent proteoglycan. In support, visfatin was shown to be co-expressed with MMP-13 in the pericellular zones of chondrocytes of fibrillated human OA cartilage tissue. Therefore with this data it is possible to conclude that visfatin is a catabolic mediator of cartilage degeneration in OA. As well as identifying its role in OA cartilage pathogenesis, this study attempted to determine the mechanism by which eVisfatin is able to enter the cell and exert is pro-inflammatory effects. While it was not possible to confirm that BDKRB2 was a receptor of visfatin, it was noted that visfatin does not need to be enzymatically active to increase IL6 secretion from human chondrocytes.

The concentration of visfatin used within this study was based upon previous research and within the physiological limits found within human joint tissue [271]. Following 24 hr incubation of OA joint tissues within serum free medium, visfatin was secreted by all joint tissues (synovium, 628 ± 106 ng/g tissue; subchondral bone, 195 ± 26 ng/g tissue; cartilage, 152 ± 46 ng/g tissue) [271], and with an estimated half-life of 30 hr [388], we estimate that the standard concentration used within this study of 500ng/mL is well within that which is seen *in vivo*.

Visfatin is known to be expressed in its active form in cartilage, subchondral bone, and to a greater extent, synovial membrane suggesting a potential paracrine role of visfatin within the joint [271]. The mechanism by which visfatin is able to up-regulate MMPs and pro-inflammatory cytokines has not been fully investigated in this study. However, Kim *et al.,* (2013) have suggested an indirect relationship between visfatin stimulation and subsequent IL6 expression, which is achieved through the activation of JAK2/STAT3 [389]. Furthermore it was concluded that IL6 is able to mediate visfatin-induced angiogenesis from rat aortic rings. In terms of OA, this information

is notable, as it may be a key mechanism for vascular intrusion from the bone into the cartilage of OA patients.

7.1 Weight-loss as a therapeutic for OA disease

As well as being more prone to OA disease, there is evidence that obese individuals have poorer outcomes following joint replacement surgery when compared to their normal weight counterparts. Following a multicentre cohort of over 20,000 primary total hip replacements, increased BMI was found to be associated with decreased mobility over a 15 year period [390]. Interestingly there was no difference in pain experienced by obese individuals when compared to their normal weight counterparts. In the short term, obese individuals have also been shown to have increased post-surgical complications, with an increased hazard ratio for surgical site infections and dislocations from 3.5 to 4.1 in obese patients compared to the normal weight patient group [391]. Therefore therapeutic strategies aimed at improving metabolic health and reducing gross adiposity as well as adipokine profiles could be beneficial to patients with OA disease.

Weight loss has previously been shown to have an advantageous effect on individuals with OA. Through a meta-analysis of randomised controlled trials (RCTs), a significant improvement in OA-induced disability is achieved with an average weight reduction of 5.1% [392]. Interestingly, this degree of weight loss is sufficient for significant improvements to comorbidities often associated with OA, including high blood pressure and improved glucose tolerance [393]. In terms of primary prevention, the Framingham study demonstrated that a 5kg reduction in weight over a 10 year period was sufficient to decrease the likelihood of women

developing OA by 50% [394]. However this effect was only seen in overweight or obese women, with no significant reduction in OA risk in those with a normal weight baseline prior to weight loss [394]. However, this was not supported by the PRevention of knee Osteoarthritis in Overweight Females (PROOF) study, who found no significant effects on knee OA incidence after the prescription of a diet and exercise program over 2.5 years [395]. However this may be due to low compliance to the diet and physical activity program (28%) and at 2.5 years there was only a -0.5kg weight loss in both the control and intervention group.

One mechanism to explain how weight loss may improve symptomatic OA progression is due to mechanical factors such as a change in gait pattern or a decrease in loading on the joints. For example, for every 0.5kg of body weight lost, there is a 2kg reduction in the load exerted through the knee per step [396]. However Aaboe *et al.,* (2011) confirmed that with weight loss, participants increased their self-selected walking speed, thus increasing their joint loading by ~44N/0.1m/s speed increment, and counteracted any reduction in joint loads due to the weight loss [397]. Therefore, the notion that weight loss reduces forces experienced by the weight bearing joints needs further investigation, particularly allowing for natural speed selection.

In addition to the potential reduction in joint loading, weight loss may improve symptomatic OA progression indirectly though the modulation of inflammatory adipokines. Though under-researched, some studies have demonstrated an alteration in serum adipokine levels in accordance with weight loss and a reduction in adiposity. In 2015, Albadah *et al.,* (2015) investigated the effect of weight loss on serum adipokines, and their association with serum osteocalcin, a bone formation biomarker, in obese males. Following a 4-month dietary program, BMI significantly

decreased from 39.7 ± 7.6 to 37.8 ± 7.6, as did bone-specific alkaline phosphatase and the adipokine resistin [398]. Inversely to weight loss, there was an increase in serum adiponectin, yet no adipokines were associated with circulating osteocalcin levels. This data was supported by King *et al.,* (2015) who found that obese patients who lost an average of 13% body weight over 12 months had significantly greater circulating adiponectin protein content, and significantly lower circulating leptin protein content [399]. Furthermore, the increase in adiponectin and leptin was associated with a reduction in the loss of cartilage volume, which is reflected by the reduction in circulating COMP protein levels [399].

The question remains whether weight loss via either dietary or physical activity is a viable and effective option for the therapeutic modulation of OA. In fact, challenging OA patients to go beyond the short-term musculoskeletal benefits and focus on the long-term behavioural outcomes is one of the main challenges facing clinicians and researchers today. While recent studies have shown that females placed on an intermittent fasting diet (5:2 diet) achieve similar levels of weight loss and reductions in circulatory levels of the adipokine leptin as those placed on a continuous calorie-controlled diet [400, 401], it still remains to be seen if this diet is able to be adhered to more readily. As such, bariatric surgery prevalence has dramatically increased in the morbidly obese population over the last 10 years [402]. However how inflammatory cytokines may be modulated following surgical weight loss remains controversial. Six weeks following bariatric surgery, previous research has shown a significant reduction in circulating visfatin concentrations with a dramatic reduction in BMI (45 ± 4.7 vs. 39.2 ± 4.6). Furthermore, increases in the adiponectin serum content were reported, suggesting that rapid weight reduction following bariatric surgery may improve the metabolic health of patients in the short–term [403].

Interestingly, this data is the opposite to that published by Botella-Carretero *et al.,* (2008), who found visfatin levels increased following an average 30% baseline body weight loss after bariatric surgery [404].

Through understanding the interplay between OA and obesity it may be possibly to not only to identify and develop novel therapeutics, but also improve the quality of life in those OA patients unable to meet the criteria for joint replacement surgery. For example, OA patients who are Class II obese are currently not eligible for surgery. Current disparity within research studies may be due to heterogeneous populations recruited into each study, therefore it is important to identify the appropriate patient group for treatment to maximise success.

7.2 Current clinical therapeutics for adipokine modulation

There is strong evidence to support the relationship between obesity and OA, however this area still requires further research to understand the mechanisms of adipose tissue and joint tissue cross-talk. A number of adipokines have been implicated in connecting obesity, osteoarthritis and inflammation. Therefore, with further research it may be possible to develop novel therapeutics that target adipokine-mediated signalling pathways.

The important role of resistin in OA bone pathology has been clearly detailed within this thesis (Chapter 4) with an increase in the biosynthetic activity of human primary osteoblasts, activation of the canonical WNT-signalling pathway and subsequent β-catenin translocation and gene transcription. Currently, therapeutic development directed towards resistin is challenging due to the lack of a known receptor. However, several cholesterol lowering drugs have shown the potential to lower

resistin concentrations. For example, HMG-CoA reductase inhibitors, also known as 'statins', have shown the greatest potential with Grosso *et al.,* (2014) demonstrating a significant reduction in resistin in the plasma of coronary artery disease and metabolic syndrome patients treated with simvastatin [405]. Interestingly, there was also a significant increase in adiponectin and a significant decrease in leptin plasma concentrations following simvastatin treatment. This therefore demonstrates the novel ability of statins to reduce circulating adipokines, and provide a potential drug repurposing for the treatment of OA. Further research into the ability to repurpose statins into intra-articular injection may be prudent, particularly to offset adipokine-mediated cartilage degeneration and synovitis.

In this thesis, visfatin was identified as a key adipokine that is increased in the synovial fluid of obese OA patients, and associated with cartilage degeneration and inflammation. However, similarly to resistin, the development of effective therapeutics is hampered by the lack of a known receptor. FK866 is a well characterised inhibitor of visfatin, known to competitively bind to the NAD binding site with great affinity [406-408], and could potentially provide a novel therapeutic to visfatin. In a collagen induced arthritis mouse model, two doses of 10mg/kg FK866 daily resulted in a significant decrease in IL6, TNFα, and IL1β, and reduced disease severity.

Another important visfatin inhibitor is CHS 828, a cyanoguanidine compound currently in phase II clinical trials for its anti-tumour activity [409-411]. The mechanisms by which CHS 828 inhibits visfatin are still unknown. However, there is some suggestion of CHS 828 inhibiting NAD synthesis through binding to the NAD binding pocket of visfatin [407, 412, 413]. Therefore it shares its active binding site with FK866, and can be defined as a competitive inhibitor of visfatin.

One of the most widely studied adipokines is leptin, a product of the *ob* gene, which is known to regulate metabolism and appetite [248]. Leptin is increased in OA synovial fluid compared to non-OA controls [251]. Recombinant leptin, either alone or synergistically with IL-1β, induces MMP-1 and MMP-13 expression in primary human chondrocytes [254]. Furthermore, it increases the production of inflammatory mediators including IL1β, IL6, IL8 and PGE2 [255]. Due to the pro-inflammatory nature of leptin and its potential to drive cartilage catabolism, the recent development of peptide-based and antibody-based leptin antagonists could provide an effective therapeutic [414, 415].

7.3 Stratification of obese OA patients for novel therapeutics

Critically, for the development of novel therapeutics, there are many factors that can alter the adipokine concentration in the surrounding joint tissues and circulation. Co-morbidities associated with OA are also known to alter the metabolic profile, including type II diabetes, cardiovascular disease, hypertension and renal function impairment [416], and have also been shown to alter the circulating adipokine profile [417-419]. For example, resistin was first identified as a mouse adipocyte gene product representing a link between obesity and diabetes [258], and since then has been shown to cause a dysregulation of glucose production from the liver, subsequently leading to insulin resistance [420, 421]. In humans there is an indication of a resistin gene polymorphism that is associated with metabolic abnormalities. Smith and colleagues (2003) identified a G/G genotype of a SNP, -180C>G, in the promoter of resistin which increases resistin promoter activity within adipocytes, and was associated with increased oxidative stress and insulin

resistance [422]. Therefore, it would be appropriate to screen co-morbidities and potential gene polymorphisms that may alter the adipokine profile in order to have the most success in therapeutic intervention, whether through drugs or weight loss.

One cohort where adipokines may play a central role in recovery and the long-term development of OA is in fracture patients. Post traumatic OA is attributed to 12% of symptomatic OA of the hip, knee and ankle [423]. Therefore, identification of proteins modulated immediately post fracture could provide important insight into the key pathways and mechanisms that predispose these individuals to developing OA in later life. To this end, Wei *et al.,* (2008) demonstrated an increase in circulating leptin concentrations following fracture in rats, with peak serum leptin concentration achieved at 4-weeks post-fracture [424]. In addition, there were a peak number of leptin positive cells within the callus region at 8 weeks following the fracture incidence. While the circulating leptin levels returned to near baseline values within 12 weeks in this study, it is unknown whether local concentrations of leptin were modulated in the long-term, and may contribute to joint destruction. Resistin has also shown to be increased both systemically and locally with 1 week following traumatic joint injury, and displayed catabolic properties in cartilage turnover including the induction of pro-inflammatory cytokine release and proteoglycan breakdown as determined by cartilage cultured in resistin-treated monocyte media [305]. While this study is limited by its cross-sectional nature, and does not provide information on the BMI of patients, it is important to consider the potential impact of adipokines in post-traumatic OA patients, independent of BMI state.

Gender may also be an important factor to consider for trials of particular OA anti-inflammatory therapeutics. For example, free levels of leptin have been reported to

be higher in the joints of females with knee OA, compared to males [425]. This finding could explain why the association between obesity and OA risk is reported to be greater in females [234], and suggests that therapeutics targeting leptin signalling could be more efficacious in female OA patients. Furthermore, a longitudinal prospective follow-up study found that females exhibited a greater incidence of poly-articular OA, more rapid structural progression, and a more severe symptomatic disease [231]. The higher incidence of multi-joint in females with hip OA could indicate the presence of a systemic inflammatory OA driver which is not present to the same degree, in males [231]. In addition, the increase in incidence of OA in females rises dramatically post-menopause [426, 427].

This knowledge of the diverse OA patient population necessitates the requirement for clear patient stratification in clinical trials. The impact of not selecting the appropriate patient population was illustrated by clinical trials with the drug Iressa (AstraZeneca), an EGFR-targeted therapy for non-small cell lung cancer patients. Iressa originally failed to meet its clinical end point during a PhII trial. However, subsequent analysis showed those patients with EGFR mutation [428] had improved survival. Not selecting the appropriate patient population masked the beneficial effect of the drug in the original clinical study. Iressa was eventually approved 5 years after the original PhIII trial and several large and costly follow-up clinical studies [428]. Therefore identifying clinical patient-selection biomarkers such as adipokines for clinical trials of OA and obesity therapeutics is highly important to achieve success.

7.4 Future considerations and conclusion

The aim of this thesis was to determine the OA adipokine expression profile and how the functional roles of adipokines may differ across different OA patient cohorts, including patients of varying BMI.

Numerous studies have examined the effect of adipokines on cartilage tissue damage. However, very few have performed a longitudinal-based study to determine the role adipokines have in initiating joint damage. All patients tissues received in this study were from patients undergoing joint replacement surgery and were therefore at an advance stage of OA disease. The lack of patient samples with a less severe disease state makes us unable to determine if joint damage precedes a heightened adipokine expression or vice versa. Future research investigating the temporal role of adipokines in early OA samples would be advantageous for therapeutic development, as well as longitudinal studies to provide invaluable evidence to support the role of adipokines in OA disease progression. However, the cost of such studies makes them difficult to implement, and obtaining joint tissues from patients with early OA and during progression is clearly difficult. The Chingford 1000 Women study have gone to some lengths to address this gap in the research through retrospective musculoskeletal data collection that evolved into a prospective population based longitudinal cohort of women.

It is worth noting that during this study we observed patient samples that responded to adipokine stimulations (e.g. 50 fold increase in IL6 expression), and other patient samples that did not respond or were much less responsive (e.g. 5 fold increase in IL6 expression) despite being collected, stimulated and analysed on the same days. The study performed by Pallu and colleagues (2010) may provide a potential explanation for our responders and non-responders, in which they studied the BMI-

dependent effect of leptin stimulation in chondrocytes [350]. The authors demonstrated an up- regulation of tissue inhibitors of metalloproteinases (TIMPs) following leptin stimulation, however this was only present in normal and overweight individuals, and decreased as the BMI of the patients increased. Furthermore, leptin induced MMP-13 expression was more sensitive in individuals with greater BMIs, with MMP-13 expression detected in the lowest leptin concentrations. The sensitivity to leptin stimulations may be heightened in obese patients because of regular exposure to adipokines with the joint.

Ultimately, through determining both the expression and functional role of adipokines in cartilage and bone joint tissue from patients with OA potentially new targets for therapeutic modification have previously been identified. In particular, the findings presented in this thesis on the expression and functional role of resistin and visfatin suggest that future therapeutic entities designed to modulate their pathological signalling pathways in the joint could modify OA disease progression in patients who are over-weight or obese.

Chapter 8: References

8. References.

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Chapter 9: Appendix

Appendix Table 1. Serum cytokine and adipokine profiles in relation to K and L grade.

pg/mL	≤ Grade 3 (n=33)	Grade 4 (n=94)	P
TNFα	4.57±1.29	4.85±2.22	0.45
IL10	4.56±0.97	5.80±13.54	0.46
IL1β	20.50±20.24	17.93±16.75	0.55
Dkk1	3378±1487	3107±1754	0.44
MIP1α	292.8±232.4	333.7±288.3	0.37
Galectin 1	46163±29263	45658±33922	0.98
Chemerin	7262±4126	6111±4346	0.23
Eotaxin	145.9±135.0	176.1±184.1	0.38
gp130	88725±34836	81579±35223	0.38
IP10	27.13±13.11	38.88±70.84	0.17
MCP1	472.9±920.4	806.7±2088.8	0.43
IL7	3.87±1.99	3.19±1.61	0.15
MIP3α	36.92±28.4	109.7±487.5	0.11
Amphiregulin	530.3±151.5	616.4±226.7	0.07
IL15	3.98 ±1.09	5.06±5.46	0.14
Aggrecan	166.9±126.5	245.5±340.6	0.13
Resistin	13605±4742	16086±8683	0.09
SerpinE1	146579±72341	139417±63931	0.67
Adiponectin	$1.15 \times 10^7 \pm 6.73 \times 10^6$	$1.06 \times 10^7 \pm 5.86 \times 10^6$	0.55
IL6	2.32±1.43	5.45±11.77	0.57
LIF	-	-	-
Leptin	27768±41003	22023±29084	0.51
FABP4	20237±12649	29936±36710	0.06
MIP1β	143.8±52.5	147.2±78.7	0.85

Appendix Table 2. Serum cytokine and adipokine profiles in relation to joint space.

pg/mL	≥1.5 (mm) (n=30)	<1.5(mm) (n=85)	P
TNFα	4.59±1.33	4.86±2.22	0.44
IL10	4.71±1.48	5.78±13.7	0.50
IL1β	17.50±16.42	20.72±20.37	0.38
Dkk1	3379±1582	3106±1726	0.41
MIP1α	289.4±238.2	334.9±286.5	0.37
Galectin 1	42639±29901	46896±33660	0.49
Chemerin	6294±3628	6451±4534	0.84
Eotaxin	131.2±134.75	181.2±183.1	0.10
gp130	88342±35118	81557±35145	0.35
IP10	26.46±13.54	39.12±70.77	0.10
MCP1	792.8±2092	477.9±919.6	0.40
IL7	3.40±1.55	3.34±1.79	0.87
MIP3α	31.44±27.8	111.5±487.2	0.11
Amphiregulin	529.2±162.0	618.9±225.0	0.03
IL15	3.89±1.15	5.12±5.51	0.06
Aggrecan	159.9±126.0	250.0±343.4	0.06
Resistin	13159±75207	16239±8543	0.03
SerpinE1	146052±67484	139598±65714	0.38
Adiponectin	$1.17x10^7 \pm 6.43x10^6$	$1.06x10^7 \pm 5.97x10^6$	0.38
IL6	2.44±1.30	5.51±11.78	0.87
LIF	-	-	-
Leptin	28316±40926	21830±29080	0.41
FABP4	20237±12649	29726±36633	0.20
MIP1β	146.4±60.2	146.2±77.4	1.00

Appendix Table 3. Serum cytokine and adipokine profiles in relation to hand OA.

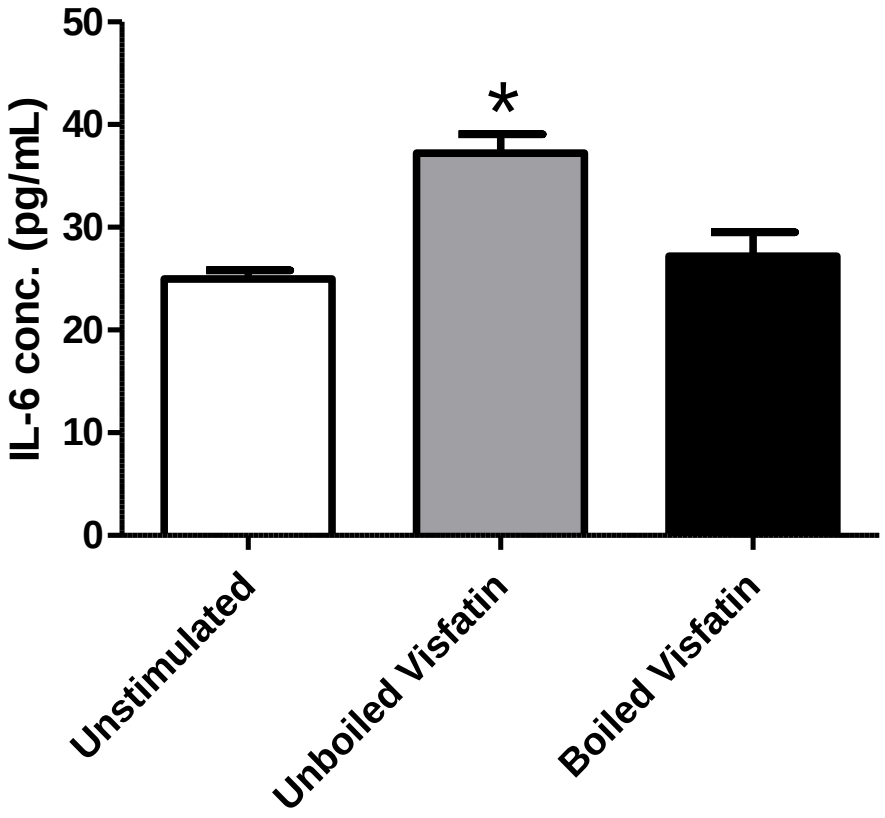
pg/mL	Non Hand OA (n=40)	Hand OA (n=106)	P
TNFα	4.99±2.9	4.5±1.5	0.95
IL10	4.29±2.04	4.24±1.6	0.76
IL1β	22.21±22.6	17.23±17.6	0.57
Dkk1	3260±1998	3068±1522	0.39
MIP1α	327.1±282.8	325.6±259.6	1.00
Galectin 1	41723±27788	46925±32731	0.71
Chemerin	6400±4261	7193±5134	0.54
Eotaxin	194.3±215.6	124.5±182.7	0.74
gp130	79898±41052	91191±31559	0.20
IP10	36.89±75.26	33.7±50.4	0.24
MCP1	333.7±192.8	602.1±1455	0.76
IL7	3.63±2.00	3.47±1.93	0.66
MIP3α	194.77±739.8	39.58±45.3	0.79
Amphiregulin	583.9±161.4	599.7±233.7	0.77
IL15	6.2±8.01	3.36±1.7	0.08
Aggrecan	314.7±475	183.4±157	0.74
Resistin	14310±6855	14426±8507	0.84
SerpinE1	142471±64768	142797±65225	0.81
Adiponectin	$1.09x10^7$±6.30 $x10^6$	$1.14 x10^7$±7.11 $x10^6$	0.84
IL6	6.35±13.2	6.31±11.10	0.19
LIF	-	-	-
Leptin	21849±21602	23116±34646	0.52
FABP4	27460±31704	24296±30345	0.11
MIP1β	133.9±56.9	138.4±81.93	0.87

Appendix Table 4. Wnt signalling pathway gene regulation following the stimulation of primary osteoblasts with resistin.

Gene	Fold Change	SD	p value
WNT5A	1.56	0.17	0.00
WNT2B	1.62	0.19	0.00
NFATC1	1.42	0.13	0.00
WNT7B	1.94	0.50	0.01
MYC	1.71	0.34	0.02
FOSL1	1.50	0.29	0.03
AXIN2	3.07	1.54	0.04
BOD1	1.64	0.40	0.04
GSK3B	1.40	0.25	0.04
PITX2	3.94	2.42	0.04
TCF7	1.71	0.48	0.04
FRZB	1.76	0.55	0.04
FZD4	1.75	0.52	0.04
CHSY1	1.62	0.44	0.04
FZD6	1.46	0.34	0.05
LEF1	1.52	0.36	0.06
RUVBL1	1.56	0.46	0.06
LRP6	1.96	0.86	0.06
BTRC	1.63	0.56	0.07
WNT5B	1.42	0.41	0.08
WNT11	2.44	0.34	0.09
EP300	2.81	1.68	0.10
CTNNB1	2.59	1.80	0.10
AXIN1	1.75	1.79	0.10
KREMEN1	3.51	0.68	0.10
MT1A	1.46	2.62	0.11
FGF4	2.51	0.47	0.11
CSNK1A1	1.59	1.49	0.12
FZD5	4.01	0.68	0.12
DVL1	2.30	2.95	0.13
CTNNBIP1	12.89	1.31	0.14
DVL2	0.86	17.93	0.15
WNT3	4.64	1.42	0.15
SFRP4	3.97	0.11	0.16
MMP7	11.09	4.03	0.17
FBXW11	1.31	4.25	0.17
WNT6	9.55	17.78	0.17
NKD1	5.01	0.31	0.17
WNT7A	2.71	14.95	0.19
WNT1	40.12	0.82	0.22
CYP4V2	1.50	5.76	0.23
PPARD	2.21	2.36	0.23

Gene	Fold Change	SD	p value
TCF7L1	1.81	0.40	0.23
FZD9	2.35	75.22	0.24
WNT8A	4.29	0.58	0.25
WNT10A	4.65	1.52	0.25
RHOA	1.57	1.14	0.27
PORCN	1.80	1.62	0.27
SOX17	3.69	4.77	0.28
WNT4	6.38	5.28	0.28
FZD1	1.18	3.74	0.28
MTSS1	1.36	0.68	0.29
WNT3A	1.79	1.13	0.30
MTFP1	1.55	3.72	0.32
CALM1	1.23	10.09	0.33
WNT2	3.35	0.27	0.33
SKP2	1.57	0.54	0.35
VANGL2	25.98	1.36	0.35
WIF1	1.46	0.88	0.36
DAAM1	1.86	0.36	0.38
NLK	1.48	4.38	0.39
LRP5	2.50	0.87	0.39
WNT9A	4.75	49.48	0.39
CXADR	2.30	1.60	0.44
SFRP1	2.02	1.09	0.45
HSPA12A	1.37	0.85	0.47
NAV2	1.65	0.23	0.47
DKK1	1.85	0.75	0.49
FZD2	1.35	2.03	0.51
MAPK8	1.59	7.34	0.51
CCND1	1.17	1.39	0.54
DKK3	4.18	2.28	0.57
PRICKLE1	1.27	2.08	0.60
PRMT6	0.95	0.60	0.60
CCND2	3.28	1.36	0.61
CTBP1	1.54	1.05	0.66
WISP1	0.98	0.58	0.66
FZD8	2.00	0.97	0.73
FZD3	1.04	0.42	0.85
FZD7	1.19	1.30	0.87
DAB2	1.02	5.97	0.87
APC	1.32	0.52	0.95
JUN	1.16	0.49	0.96
FRAT1	1.90	3.90	0.98

Appendix 5 – Confirmation of pro-inflammatory action of visfatin, independent of bacterial endotoxin contamination of the recombinant protein.



Appendix 5. Secreted IL6 concentration following 24 hr stimulation of chondrocytes with recombinant visfatin.

Chondrocytes were stimulated with recombinant visfatin that had either been maintained at 4°C or boiled at 100°C for 10 minutes.*=$p<0.05$.

Appendix 6 - Publications and review articles published during the completion of this PhD.

1. Newton, Ede MP*; **Philp, AM*;** Philp, A; Richardson, SM; Mohammad, S; Jones, SW. (2016). Povidone-Iodine Has a Profound Effect on In Vitro Osteoblast Proliferation and Metabolic Function and Inhibits Their Ability to Mineralize and Form Bone. Spine, 41 (9): 729-734.

   *Joint first authorship.

2. **Philp, AM**; Pearson, MJ; Lord, JM; Davis, ET; Jones, SW. (2016). eNAMPT is localised to areas of cartilage damage in patients with hip osteoarthritis and drives cartilage catabolism leading to proteoglycan loss and inflammation. Osteoarthritis and Cartilage, (24): S388

3. **Philp, AM**; Collier, RL; Bartlett, DB; Lord, JM; Davis, ET; Jones, SW. (2016). Evidence of abnormal type I collagen composition in obese patients with OA. Osteoarthritis and Cartilage, (24): S388-S389

4. **Philp, Ashleigh M**; Raja, Saroop; Philp, Andrew; Ede, MP; Jones, Simon W. (2016). The effect of Vancomycin and Gentamicin Antibiotics on Human Osteoblast Proliferation, Metabolic Function and Bone Mineralisation. Spine, Epub.

5. Pearson, MJ; **Philp, AM**; Heward, JA; Roux, BT; Walsh, DA; Davis, ET; Lindsay, MA; Jones, SW. (2016). Long Intergenic Noncoding RNAs Mediate the Human Chondrocyte Inflammatory Response and Are Differentially Expressed in Osteoarthritis Cartilage. Arthritis and Rheumatology, 68 (4): 845-856.

6. **Philp, AM**; Davis, ET; Jones, SW. (2016). Developing anti-inflammatory therapeutics for patients with osteoarthritis. Rheumatology, Epub.

7. **Philp, AM**; Collier RL, Grover LM; Davis, ET and Jones, SW. (2016). Resistin drives the abnormal type I collagen phenotype of obese subchondral bone in patients with hip osteoarthritis. Journal of Bone and Mineral Research. In review.