The Role of The Long Non-coding RNA MALAT1 in Mediating Inflammation in The Human Osteoarthritis Joint

By

FAWZEYAH A Q ALNAJJAR

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Abstract

Osteoarthritis (OA) is a common degenerative and painful disorder affecting more than 22% of adults who are older than 40. According to Centre for Disease Control (CDC) 2020, more than 32.5 million US adults have OA. OA is a disease of the whole joint, including the cartilage, subchondral bone, synovium and skeletal muscle, and inflammatory processes contribute to the pathogenesis of the diseased joint tissues. Since lncRNAs have emerged as central regulators of the inflammatory response they may play a key role in mediating OA pathogenesis. For example, the Metastasis Associated Lung Adenocarcinoma Transcript 1 (MALAT1) lncRNA, has been implicated in contributing to the pathogenesis of many diseases by controlling the epigenetic transcriptional modulation of inflammatory genes in chronic inflammatory diseases. The aim of this thesis was therefore to investigate the role of lncRNAs, and in particular the lncRNA MALAT1, in OA pathogenesis by examining their association with the inflammatory response in OA and their expression in diseased OA joint tissues and OA joint tissue derived cells including synovial fibroblasts, articular chondrocytes and subchondral osteoblasts. In this thesis, I report for the first time the functional role of the lncRNA MALAT1 in OA mature osteoblasts, and the relationship between MALAT1 joint tissue expression and parameters of inflammation. A total of 16 tissues were utilized for the analysis of MALAT1 expression from different BMI cohorts of OA joints including 3 synovial membrane, 8 articular cartilage and 5 subchondral bone and

a total of 17 OA patient's demographic data were used, for articular cartilage and subchondral bone tissues, blood was collected from a total of 17 patients with end-stage OA and 6 non-OA patients from neck of femur fracture (NOF). The results of this thesis project showed that MALAT1 was expressed in all the primary cells obtained from OA joint tissues, and all primary cells induced IL-6 production upon stimulation with human protein recombinant IL-1β (1ng/mL) and TNF-α (10ng/mL). Notably, obese and over-weight OA patient cohorts induced more IL-6, compared to joint cells isolated from normal-weight OA patients. In OA articular cartilage tissues, MALAT1 expression was significantly associated with OA severity parameters in hip and knee joints, and with the levels of circulatory chemokines Dickkopf WNT signaling pathway inhibitor 1 (DKK1), Eotaxin and the levels of MIP3a chemotactic locally. In OA bone tissues MALAT1 expression showed a significant correlation with circulatory DKKI, Galectin1 and TNF-α. MALAT1 depletion effects on the functional phenotype of OA osteoblasts, was shown by modulating both PTGS2 expression and PGE2 prostaglandin production. However, the chronic effect of MALAT1-KD showed no noticeable effects on osteogenic differentiation activity, measured either by OPG production, ALP activity or the innate ability of osteoblasts to mineralise. In conclusion, the results of this thesis provide evidence that the lncRNA MALAT1 is associated with the inflammatory response in multiple cells of the OA joint, is highly expressed in both cartilage and subchondral bone tissue and in OA osteoblasts regulates inflammation by modulation of COX-2 prostaglandin production.

In The Name of God The Most Gracious, The Most Merciful

Peace be Upon Prophet Mohammad and His Progeny

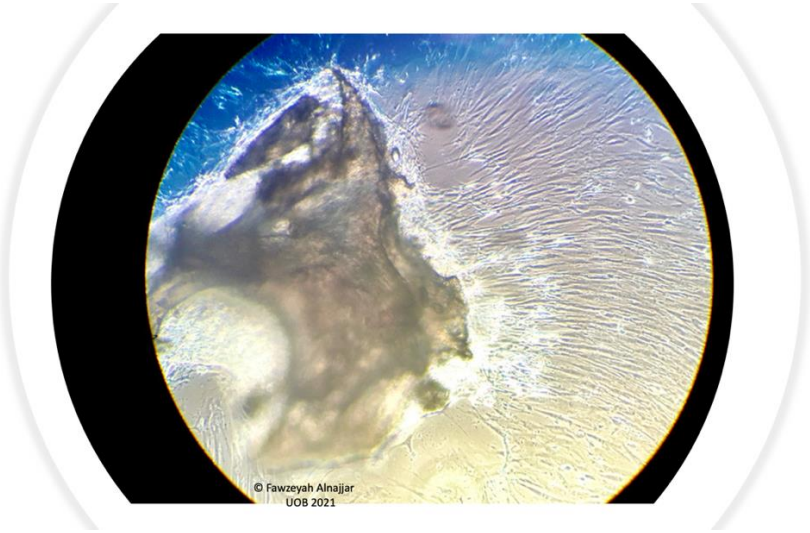
And All the Prophets and Messengers of God

Dedication

“As also in your own selves: Will ye not then, see?” [Holy Quran, Chapter 26, No. 51, verse 21]. This is an invitation from the Almighty God to see and think about the creation of ourselves. Bone was mentioned 12 times in the Holly Quran, ten times in occasions regarding recreation and two times in occasions of bone development. In the ten times the main topic was about starting life again from dead and porous bones, “What! – when we shall have become rotten (eroded) bones [Holy Quran, Chapter 30, No. 79, verse 21]. This is a clear indication of the effect of age on bone. Furthermore, bone development was mentioned twice, once at the early embryonic development and the other during recreation from dead bone. In the early development, human bones were created first and at nearly the same time it was covered with muscles “Then we made the sperm into a clot of congealed blood; then of that clot we made lump (foetus); then we made out of that lump bones and clothes the bones with flesh; then we developed out of it another creature. So blessed be Allah, the best to create!”

[Holy Quran, Chapter 18, No. 23, verse 14]. This confirms the current scientific knowledge about the embryology of bone and muscle which happens to be synchronous. However, when dead bone pieces are recreated, the bone is formed first and then after some time it will be coated with muscles, “Look further at the bones, how we bring them together and clothe them with flesh.” [Holy Quran, Chapter 2, No. 2, part of verse 259]. This discrepancy in the timing may indicate a cross talk between bone and muscle during development which is worth investigating.

This is an inspiration to the scientists who spend day and night thinking about solutions to lessen the suffer of others. May this little work motivate you to find remedy for bigger problems.



“Does man think that we cannot assemble his bones”

[Holly Quran, Chapter 29, No. 75, verse 3]

This image shows primary osteoblasts growing from a piece of bone in a culture flask.

To my Mother and Father

(I'm pretty sure they are happy for me now)

To my Dear Husband Abulkareem,

To Eiman / Bader, Fatemah Alzahraa, Mohammed,

Mariam / Jaffer, Ali, and Tiba / Jaffer

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Chapter 8. List of Abbreviations

For writing the units in this thesis I used the metric *System International d'unites* (SI) for quantities and units, which is well known in the world. Leaving a space following the digits before the units. Since the only SI unite for time is seconds, I will use (h) for hours.

I- General Abbreviations

Abbreviation	Full name
A	
ADAMTS	A disintegrin-like and metalloproteinase with thrombospondin motif
AITD	Autoimmune thyroid diseases
ANOVA	Analysis of Variance
AFPs	The articular fat pads
AS	Ankylosis Spondylitis
AZR	Alizarin Red Stain
B	
BMI	Basal metabolic index
BMPs	Bone morphogenetic factors
BMSCs	Bone marrow-derived mesenchymal cells
BSA	Bovine Serum Albumin
C	
CAT	Catalogue number
CDC	Centre for disease control

ceRNA	Competing endogenous RNA
circRNA	Circular RNA
COX-2	Cyclooxygenase-2
CT	Cycles Threshold
D	
DAMPs	Damage associated molecular patterns
DESeq2	Differentially expressed sequences Software
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
E	
eATP	Extracellular ATP
ECM	Extracellular Matrix
EDTA	Ethylenediamine Tetraacetic Acid
EMT	Epithelial-mesenchymal transformation
ER	Endoplasmic Reticulum
ESR	Erythrocyte Sedimentation Rate
eQTL	Expression quantitative trait loci
F	
FBS	Fetal bovine serum
FGFs	Fibroblast growth factors
FPKM	Fragments per kilobase of transcript per million mapped reads
G	
GA	Gouty arthritis
GAG	Glycosaminoglycan
GBD	Global Burden of Disease

GPCRs	G Protein Coupled Receptors
GWAS	Genome-wide associated scan
H	
hBMSCs	Human bone marrow-derived mesenchymal stem cells
hFOB	Human Fetal osteoblastic cell line
HRP	Horseradish-peroxidase
HSCs	Hematopoietic stem cells
HMGB1	High mobility group box 1
I	
IDD	Intervertebral disc degeneration
IGF-I	Insulin-like growth factor-I
IGV	Integrative Genomics Viewer
IL	Interleukin
IL-1β	Interleukin-1 β
IL-1RI	Interleukin receptor type I
IL-1RII	Interleukin receptor type II
IL-1Ra	Interleukin receptor type I agonist
IL-6	Interleukin-6
IPA	Ingenuity pathway analysis
IRF	Interferon regulatory transcription factor
J	
JSN	Joint space narrowing scores
K	
KD	Knockdown
KL	Kellgren Lawrence Scale
L	

LIF	Leukemia inhibitory factor
LOF	Loss of function
LNA	Locked nucleic acids
LncRNAs	Long noncoding RNAs
LPS	Lipopolysaccharide

M

MAL1-KD	MALAT1 Knockdown
M-CSF	Macrophage colony stimulating factor
MCP-1	monocytes chemoattractant protein 1
MEM	Minimum Essential Medium
miscRNA	Miscellaneous RNA
miRNA	Micro RNA
MMPs	Matrix metalloproteinases
mRNA	Messenger RNA
MT	Meniscal tear

N

ncRNAs	Noncoding RNAs
NGS	Next generation sequencing
NLRP3	NACT, LRR, and PYD domain containing protein 3
NO	Nitric Oxide
NOF	Neck-of-femur
NRES	National Research Ethics Committee
NSAIDS	Non-steroidal anti-inflammatory drug
NSCLC	Non-small lung cancer
NTC	Non-targeted negative control

NW	Normal weight
O	
OA	Osteoarthritis
OB	Obese
OPG	Osteoprotegerin
OPN	Osteopontin
OW	Overweight
P	
P	Passage
PBMCs	Peripheral blood mono-nuclear cells
PBS	Phosphate Buffered Saline
PGE-2	prostaglandin E2
piRNAs	Piwi-RNAs
PSA	Psoriatic arthritis
Q	
qRT-PCR	Quantitative reverse transcriptase polymerase chain reaction
R	
ROS	Reactive oxygen species
RE	Relative Expression
RIN	RNA integrity number
RNA	Ribonucleic acid
RNA-Seq	RNA sequencing
RPMI	Roswell Park Memorial Institute
RT	Room Temperature

S

SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEM	Standard Error of Mean
Shh	Sonic Hedgehog signaling
siRNA	Small interfering RNA
SIRT1	Sirtuin type 1
SLE	Systemic lupus erythematosus
snoRNAs	Small nucleolar RNAs
SOP	Standard Operating procedure
SS	Sjogren's syndrome

T

TBS	Tris-buffered saline
TBS-T	Tris-buffered saline- Tween
TIR	Toll-IL-1-receptor domain
TNAP	Tissue non-specific Alkaline phosphatase
T25	Tissue culture flask size 25cm
T75	Tissue culture flask size 75cm

U

UK	United Kingdom
3'UTR	3' untranslated region

W

W:H	Waist hip ratio

II- List of Genes

ADAMTS5	Disintegrin and Metalloproteinase with Thrombospondin Motifs S 5
AGO1	Argonaute RISC component 1
ALS2	Alsin Rho Guanine Nucleotide Exchange Factor
AKT3	AKT Serine/Threonine Kinase 3
ANRIL	Antisense Noncoding RNA in the INK4 Locus
APOA1	Apolipoprotein A1
BCL2L13	BCL2 like 13
CARHSP1	Calcium Regulated Heat Stable Protein
CARMN	Cardiac Mesoderm Enhancer-associated Non-Coding RNA
CCND1	Cyclin D1 protein
CDK6	Cyclin Dependent Kinase 6
C9orf64	Chromosome 9 Open Reading Frame 64
CHST3	Carbohydrate sulfotransferase 3
CILinc01	Chondrocyte Inflammatory Long intergenic ncRNA-01
CILinc01	Chondrocyte Inflammatory Long intergenic ncRNA-02
CKS1B	CDC28 Protein Kinase Regulatory Subunit 1B
CLCN7	Chloride Voltage-Gated Channel 7
COX-2	Cyclooxygenase-2
COL2A1	Collagen type II alpha 1 chain
CTBP1	C-terminal binding protein 1
CSF1R	Colony Stimulating Factor 1 Receptor
CTBP1-AS2	C-terminal binding protein 1 antisense RNA 2
CTD-2574D22.4	Cartilage Related Gene-2574D22.4

CST5	Cystatin D
DANCR	Differentiation Antagonizing Non-Protein Coding RNA
DKK1	Dickkopf WNT signaling pathway inhibitor 1
DKL1	Delta Like Non-Canonical Notch Ligand 1
DNM3OS	DNM3 Opposite Strand/Antisense RNA
DTX3	Deltex E3 Ubiquitin Ligase 3
E2F1	E2F Transcription Factor 1
E2F4	E2F Transcription Factor 4
ERBB3	Erb-B2 Receptor Tyrosine Kinase 3
ERK	Extracellular signal-regulated kinases
ESR1	Estrogen Receptor 1
EXOC5	Exocyst Complex Component 5
FGF5	Fibroblast Growth Factor 5
FBN2	Fibrillin 2
FnF14	Fibroblast growth factor inducible 14
FOXD2-AS1	FOXD2 Adjacent Opposite Strand RNA 1
FOXO	Forkhead box class O
FZD6	Frizzled Class Receptor 6
GAS5	Growth arrest-specific 5
GNB5	G protein Subunit Beta 5
GRIPAP1	GRIP Associated Protein 1
GWAS	Genome-wide association study
HECW1	E3 Ubiquitin-protein Ligase
H19	Human Gene 19
HMGB-1	High mobility group box-1
HNF4	Hepatocyte Nuclear Factor 4

HNF4A	Hepatocyte Nuclear Factor 4 Alpha
HOTAIR	HOX antisense intergenic RNA
INCENP	Inner Centromere Protein
JAK-STAT	Janus Kinase and Signal Transducer and activator of Transcription
JNK	C-jun N-terminal kinases
KLHL36	Kelch Like Family Member 36
KLF2	Kruppel Like Factor 2
KLF4	Kruppel Like Factor 4
LINC02289	Long Intergenic Non-coding 02289
LRCH3	Leucine Rich Repeats and Calponin Homology Domain Containing 3
LRFN3	Leucine Rich Repeat and Fibronectin Type III Domain
NAIF1	Nuclear Apoptosis Inducing Factor 1
NEAT1	Nuclear Paraspeckle Assembly Transcript 1
NEDD4	NEDD4 E3 Ubiquitin Protein Ligase
Nfat (family)	Nuclear Factor of Activated T-cells
NFATC1	Nuclear Factor of Activated T-cells Cytoplasmic 1
NFAT	Nuclear Factor of Activated T-cells
NFAT5	Nuclear Factor of Activated T-cells 5
NF-kB	Nuclear factor kB
NINJ2	Nerve Injury Induced J2
NKILA	NF-Kappa B Interacting LncRNA
NLRP3	NLR family pyrin domain containing 3
NPEPPS	Aminopeptidase Puromycin Sensitive
NR1D2	Nuclear Receptor Subfamily 1 Group D Member 2

NUPR1	Nuclear Protein 1
MALAT1	Metastases Associated Lung Adenocarcinoma Transcript
MAPK	Mitogen activated protein kinase
MCP-1	Monocytes chemoattractant protein 1
MED13	Mediator Complex Subunit 13
MEG3	Maternally Expressed 3
MET	MET proto-oncogene
miRNA	MicroRNA
miR-155-5P	Micro RNA-155-5P
miR-16-5p	Micro RNA-16-5P
miscRNA	Miscellaneous RNA
MMP3	Matrix Metallopeptidase 3
MTORC1	Mammalian Target of Rapamycin Complex 1
PACER	p50-associated COX-2 extragenic lncRNA
PART1	Prostate Androgen-Regulated Transcript 1
PCGEM1	Prostate-Specific Transcript1
P38	Protein 38
PGE2	Prostaglandin E2
PIK3K	Phosphatidylinositol 3-kinase
PI4KB	Phosphatidylinositol 4-kinase Beta
POLR2M	Pol II Synthesizes mRNA Precursors Subunit M
PMS2L2	PMS1 Homolog2 pseudogene 2
PRPF8	Pre-mRNA Processing Factor 8
PROC	Protein C Inactivator of Coagulation Factors Va and VIIIa
PTEN	Phosphate and Tensin
PTGS2	Prostaglandin-Endoperoxide Synthase 2
RANK	Receptor activator of nuclear factor-κB

RANKL	Receptor activator of nuclear factor-κB ligand
RCOR1	REST corepressor 1
RB1	Retinoblastoma 1
RP9	Retinitis Pigmentosa 9 pre-mRNA splicing factor
RP11-445H22.4	LncRNA RP11-445H22.4
Runx2	RUNX family Transcription Factor 2
PYROXD2	Pyridine Nucleotide-Disulphide Oxidoreductase Domine 2
SATB1	SATB Homeobox 1
SIRT1	Sirtuin type 1
SNHG	Small Nucleolar RNA Host Gene
snoRNA	Small nucleolar RNA
SMAD	SMAD transcription factors
SOX2	SRY-box transcription factor2
SOX4	SRY-box transcription factor4
SOX9	SRY-box transcription factor 9
SPON2	Spondin 2
SP1	SP1 transcription factor
SPP1	Secreted Phosphoprotein 1
SYVN1	Synovial Apoptosis Inhibitor 1
TACR1	Neurokinin 1/substance P receptor
TCEAI8	Transcriptional Elongated Factor
TGFBR2	TGF-beta receptor type-2 precursor
TLR4	Toll Like Receptor 4
TMSB4	Thymosin Beta 4 X-linked
TNF	Tumor necrotic super family
TNF-α	Tumor necrotic factor-α

TNFRI	Receptor tumor necrosis factor I
TNFRII	Receptor tumor necrosis factor II
TNFRSF1B	TNF Receptor Superfamily member 1B
TNFSF10	TNF Superfamily member 10
TNFSF11	TNF ligand superfamily member 11
TNFSF12	Tumor Necrosis Factor Superfamily member 12, also known as TNF-related weak inducer of Apoptosis (TWEAK)
Wnt/ β-catenin	Wingless-related integration site /β-catenin
WHAMM	WASP Homolog associated With Actin
XPRI	SPX domain-containing proteins
ZSCAN32	Zinc Finger and SCAN Domain Containing 32

List of Chemicals

Abbreviation	**Full name**
ALP	Alkaline Phosphatase
APS	Ammonium persulfate
BCA	Bicinchoninic acid
CO_2	Carbon dioxide
ddH_2O	d di-ionized water
ECL	Enhanced chemiluminescent detection reagent
HCL	Hydrochloric acid
Mg Cl_2	Magnesium chloride
MTS	Di-Methazole Tetrazolium
NADPH	Nicotinamide adenine dinucleotide phosphate
NAOH	Sodium Hydroxide

p-NPP	p-Nitrophenyl phosphate
RFH_2O	RNA free water
SDS	Sodium dodecyl sulfate
siRNA-LNA	Small interfering RNA
TBS	Tris Buffered Saline
TBS-T	Tris Buffered Saline and tween 20

List of Measuring Unites

Unites were abbreviated according to according to Oxford Advanced Learners Dictionary.

Abbreviation	**Full name**
cm	Centimetre
µm	Micrometre
L	Litre
mL	Millilitre
mM	Millimolar
U/mL	Units/mL
Kunitz units	Units used to measure DNase 1 enzyme
kg	Kilogram
gm	Gram
µg	Microgram
pg	Picogram
ng	Nanogram
min	Minute
hr	Hours

^{o}C	Degrees centigrade
%	Percentage
V	Volt
mA	Milli Amper

CHAPTER 1

Introduction and Background

1.1 Osteoarthritis as A Disease

Once regarded as a disease of the cartilage only, accumulating evidence now indicates that osteoarthritis (OA) is a multifactorial disease affecting many tissues forming the diarthrodial joints including the synovium, articular cartilage, subchondral bone, meniscus and the skeletal muscles surrounding joints [1]. This new way of looking at the pathogenicity of OA has advanced in recent years with increased understanding of the molecular mechanisms that drive cartilage degradation and bone remodelling [2], the establishment of OA animal models and improvement in genetic analysis including Next Generation RNA sequencing (NGS) and accompanied rapid advances in the bioinformatic analysis.

OA is a common form of degenerative arthritis causing, stiffness, pain, swelling, and motion disability in the affected joints. It is a major cause of disability in patients aged over 65 years and results in a decrease in both the physical quality of life [3] and the mental life of OA patients [4]. World-wide, it is a progressive disease that affects over 300 million people and exerts economic burden, according to the Global Burden of Disease (GBD) analysis study of 2017 [5]. According to the National Public Health Agenda for OA in 2020, OA was declared as a chronic disease affecting 1 in 7 adults in the USA, accounting for more than 32.5 million adults having OA [6]. Other statistics stated that 62% of people with OA are of the female gender [7] and world-wide 22% of adults aged over 40 have knee OA. [8] Furthermore, the prevalence of OA in England, according to Public Health UK / arthritis research UK, is such that approximately 1 in 5 adults aged over 45 and more have knee OA, and 1 in 9 have hip OA [9].

Looking at the natural history of OA and the risk factors contributing to the pathogenicity of the disease, it can be divided into two categories. The first category are those patients with OA who have modifiable risk factors such as a high body weight [Body Mass Index (BMI)] affecting specially weight-bearing joints, daily practice of overusing the joints, physical inactivity, and joint injury. The second category are OA patients with risk factors that can't be modified, such as age, gender, genetics, and race [6], (Figure 1.1).

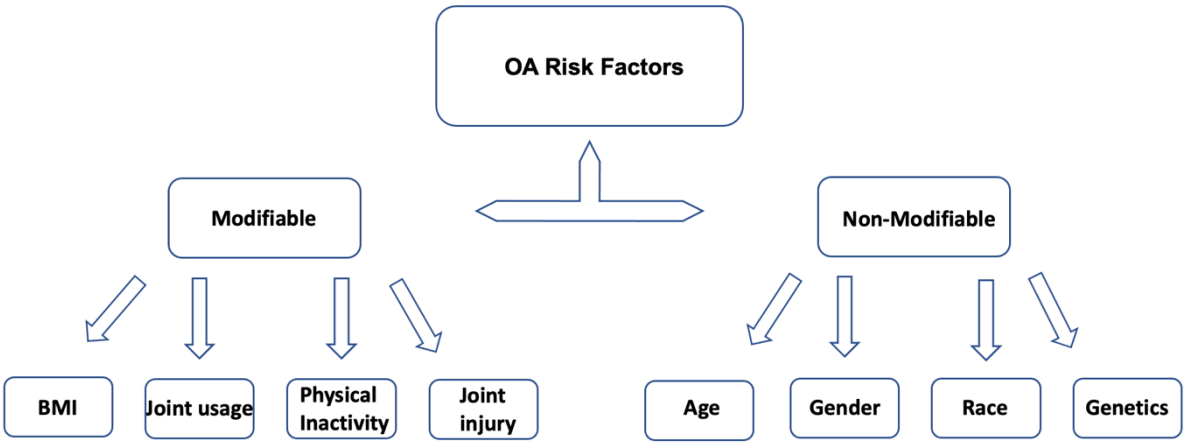


Figure 1.1 OA risk factors. BMI = Body Mass Index. Information obtained from [6]

Ageing is one of the major risk factors of OA. Age-related increase in inflammation (inflammageing) creates a chronic pro-inflammatory environment, with promotes degeneration of the joints [10]. Age related changes will be discussed in detail in the OA pathogenesis section. Gender is another risk factor that was recently related to OA. OA in particular joints, such as hand, foot and knee OA are more common in women than in men [11-12]. Low levels of sex hormones were associated with knee OA in women with OA [13]. Looking at the race risk factor, it was shown that African American have more hand and knee OA than other races [14]. In addition, one review indicated that the severity of pain and the incidence of disability due to OA is more common in African Americans, compared to Non-Hispanic White/Caucasian population [15].

The role of genetics in the predisposition of OA was studied by the genome-wide associated scan (GWAS), which identified 21 loci susceptible for OA [16]. Other studies reported that there is a 30%- 65% genetic risk for the development of OA [11,17]. Although there is no single gene responsible for OA, some studies indicated an association between mitochondrial DNA and knee OA progression and joint space width and hip O [16]. Other GWAS have shown that there are genetic differences among OA patients regarding the OA severity, patients' gender, and the joint anatomical site. To date, TGFA were recognised as a gene for hand, knee, and hip OA, SOX5 and EPHA5 for the weight bearing joints only including hip, knee, and spine [18]. Some data showed that a decreased risk of OA was associated with the SNP rs11688000 in the neurokinin 1 receptor gene (TACR1) [19]. In addition, other studies have shown the association of genes of the inflammatory pathway like IL-1β and its

receptors with hip and knee OA. Genetic variations were found in the genes affecting the inflammatory pathway and related to the severity of the disease [20-23].

A recent GWAS on 77,052 of knee, hip, knee and/or hip and any OA cases and 378,169 controls were analysed. The putative effector genes in this analysis were identified by integrating expression quantitative trait loci (eQTL) colocalization of data from human rare diseases, animal-model, and OA tissue expression. This analysis showed an enrichment of effector genes involved in the bone, collagen, and ECM development pathways including transforming growth factor 1 (TGF1), fibroblast growth factor 18 (FGF18), cathepsin K (CTSK), and interleukin 11 (IL-11). In addition, these eQTLs were therapeutically approved too [24].

From the study of Cindy G Boer, et al Cell,2021 [20], a GWAS was done across 826,690 individuals from which 177,517 had OA, using functional genomic data from primary tissues including articular cartilage, subchondral bone and osteophytic cartilage, they were able to clearly demonstrate the genetic effects in OA. Among most, the phenotypes related to pain in OA were identified, and identified key molecular effectors in the pathogenesis of OA and proposed therapeutic intervention for these targets. From this analysis 11 OA phenotypes were identified in the weight bearing knee, hip and spine, and non-weight bearing such as hand joints. Furthermore, from the 637 genes identified, 77 OA high-confidence effector genes were located related to skeletal development, neuronal function and development, joint degeneration, immune response and inflammation, muscle function and adipogenesis. These genes were based on three different lines of evidence obtained from combining different data obtained from fine-mapping, eQTL colocalization, animal model, human

musculoskeletal and neuronal phenotype, functional genomics, and causal inference data analysis.

Important effector genes obtained from this analysis include genes important in immunity and inflammation such as Toll like receptor 4 (TLR4) which encodes a protein that is important in immune cascades activation and is related to different musculoskeletal pathologies. TLR4 was related to RA and OA pathogenesis along with other genes expressed in different OA tissues derived cells including chondrocytes, synoviocytes and osteoblasts. TLR4, tumor necrosis factor ligand superfamily member 11 (TNFSF11) which encodes for the receptor activator of nuclear factor kappa-β ligand (RANKL). This cytokine was linked to inflammatory bone remodeling in RA and increased TNFSF11 was related to osteoclastogenesis [20]. Other genes important for bone growth included collagen type II alpha 1 chain (COL2A1), which encodes important proteins in cartilage and bone development. Some cartilage and bone diseases were found to be associated with COL2A1 such as spondyloepimetaphyseal dysplasia and early OA. Furthermore, Fibrillin 2 (FBN2) is one of the genes that encodes fibrillin glycoprotein important in ECM morphogenesis and bone remodeling.

Wnt family member gene 10B (WNT10B) is one of the genes important in Wnt1 pathway, was found to be one of the important effector genes related to OA pathogenesis and its mutation leads to limb defects [25]. In addition, other important effector genes were found to be form the endochondral pathway specifically for the TGF-β signaling access such as TGFB1 which controls downstream genes via the SMAD3-signaling in cartilage including carbohydrate sulfotransferase 3 (CHST3) [26]. The study also showed other genes related to cartilage and bone phenotypes in OA

including FGF-signaling cascade such as FGFR3, FGF18 and PIK3R1. The PIK3R1 encodes the p85a, p55a and p50a which are important for adipogenesis in OA. These GWAS provided a link between genetic involvement in the pathogenesis of OA and a possible therapeutic target in controlling this complex disease.

Arguably, the effects of the modifiable risk factors on the development of OA such as BMI, was shown in some studies indicating a higher susceptibility to hand OA in obese patients [27]. However, other studies showed an independency of the development of hip OA on obesity [28-29] and hand OA [30]. Recent research indicated that knee OA is directly associated with obesity, and they recommended that knee OA treatment should differ according to the severity of the patient's obesity [31,32]. Indeed, losing weight was associated with a lower cartilage degeneration in knee OA [33-34]. As it is clear there are different contradictory arguable findings on the obesity risk factor effect on OA and needs to be confirmed with further investigations. Daily joint usage in certain occupations, such as construction workers, fire fighters, military individuals and athletics who use there joints repetitively on a daily basis, were found to have more risk in developing OA [11,17, 34]. This also led us to the risk factor of joint injury which may be predicted to be more with the repetitive usage of joints especially in the previously mentioned occupations. The most common injury, especially in athletes, is meniscal tear (MT), which is associated with subsequent damage to the cartilage and development of knee OA [11,17], in some cases within a year [35]. Furthermore, it was reported that articular joint injury increases the risk of OA development by 20-folds [36]. Studies on the cross-sectional measurements of the high thigh muscle and OA incidence provides evidence that high muscle mass is protective effect against OA development [37]. Thus, individuals with physical inactivity resulting with reduced

strength of muscles surrounding the joint, may be predisposed to developing OA. Indeed, weakness in other muscles due to physical inactivity such as higher total extensor and vastus medialis were associated with an increase in the loss of the patellofemoral cartilage [38]. The aforementioned modifiable risk factors are under the individuals control by changing their daily lifestyle in order to minimize the risk of OA. Before discussing the pathogenesis of OA in different joints of the body, it's important to know how the joints were formed in the body in the early embryonic stages and the general anatomical and physiological characteristics.

1.2 Embryology, Anatomy and Physiology of Joints

Briefly, most of the joint tissues including cartilage, bone and connective tissues are formed from the mesenchyme. Joints of a limb are formed together with the bone of that limb. At the end of the fourth week of development a small limb bud containing mesenchyme will appear on the sides of the embryo. During the sixth week as theses buds grow, the mesenchyme within the buds will differentiate into hyaline cartilage and through the process of endochondral ossification, the hyaline cartilage with time will be transformed into future bones. Synovial joints forms within adjacent two cartilages from the interzone area forming the future joints [39]. The cells in the centre of this area will die forming the joint cavity, and the joints capsule and ligaments will be formed from the transformation of the surrounding mesenchymal cells. The joint capsule contains proprioceptors or mechanoreceptors, surrounded by sensory nerve endings

that sense the body position and balance positions (which are found also in the joint ligaments, muscles and tendons) [40].

1.2.1 Synovial Joints

The synovial joint is surrounded by a fibrous capsule which is lined at the intimal site by the synovium tissue. The synovium is formed from two layers, the lining and the sublining layers. The cellular content of the synovium layers is composed of synoviocytes of two types, the types of synoviocytes recognized include one which is located on the lining layer of the synovium and represents a tissue macrophage originating from blood-borne mononuclear cells and possess antigen presenting abilities in addition to fibroblasts. The other type represents a fibroblasts-like cell located at the sublining layer of the synovium together with other cells like macrophage, blood cells, adipose cells and some lymphocytes. The synovium secrets the synovial fluid providing lubrication and nourishment to the joint through vasculature and nerves within the synovium. The secreted synovial fluid is made up of specialized constituents such as hyaluronan, collagens, fibronectin [41] and lubricin. Hyaluronan and lubricin provide essential lubrication that protects against inflammatory cytokines and protects from cartilage degradation during OA [42-43]. The rest of the structures within the joint space include the articular cartilage, the subchondral bone, the menisci and the ligaments.

1.2.2 Articular Cartilage

The articular cartilage covers the ends of the subchondral bone, and it is formed from specialized connective tissue [44,45], the hyaline or articular cartilage [46]. This layer is composed of four zones the superficial, transitional and deep zone and the tidemark zone, which contains the calcified cartilage [47], Table 1.1.

The cellular component of the cartilage is composed of chondrocytes embedded in a specialized media, the Extracellular Matrix (ECM). The different layers of the cartilage are composed of differently oriented collagen fibres and varying proportions of cellular component in relation to the percentage of the collagen fibres in the matrix. This arrangement provides the maximum strength and resistance in the joint to assure a smooth and frictionless movement. The ECM is formed from different elements including collagen which lies at different orientations in a large amount of water accounting for 80% of the tissue, proteoglycans, structural proteins such as fibrillin and elastin and glycosaminoglycan (GAG) made up of chondroitin sulfate and keratin sulfate [44,47-48]. Most of the collagen in ECM is of type II and the remaining is of type I, IV, V, VI, IX, and XI collagen [39].

Table 1.1 Zones of Articular cartilage

Zone	Content	Orientation	Chondrocytes	Proteoglycans
Superficial	Type II collagen (highest conc*)	Parallel to joint	Flat	Lowest conc*
Intermediate	Type II collagen	Oblique to joint	Round	Abundant
Deep	Type II collagen	Perpendicular to joint	Round, arranged in columns	Highest conc*
Tidemark	Remnant of endochondral ossification	Made up of 2 layers	Superficial uncalcified, deep calcified	None

conc*= concentration

The articular cartilage is avascular, and it is not innervated, it gets its nourishments from the synovial fluid surrounding it. Being avascular means the articular cartilage has a reduced regenerative capability if damaged [49]. The process of chondrogenesis, which starts from the mesenchymal stem cells (MSCs) to a mature chondrocyte, is controlled by different signaling pathways. To mention some, fibroblast growth factors (FGFs), transforming growth factor β (TGF- β), wingless-related integration site / β-catenin (Wnt/ β-catenin), notch signaling and Sonic Hedgehog signaling (Shh) [50]. In addition to transcriptional and epigenetic control, where the most important transcriptional factor controlling the expression of chondrocyte phenotypes in chondrogenesis is SRY-box9 transcription factor9 (SOX9) [51]. The epigenetic control of chondrogenesis is performed by Histone deacetylase superfamily, which inhibits the

transcription of prochondrogenic genes [52], and noncoding microRNAs (miRNAs) which are important during pre-and post-natal chondrogenesis such as miRs-26b, -28, -130b [53]. The function of the articular cartilage is to act as a cushion protecting the subchondral bone by absorbing heavy loads, providing minimal friction with other parts of the joint [44] and ensuring smooth movement of the joint [48]. Physiologically, the chondrocyte functions are controlled by different factors such as growth factors, stress signals and cytokines [44]. The crosstalk between mature chondrocytes and the ECM occurs through integrins which are expressed on the adult chondrocytes, such as α1β1, α3β1, α5β1, αVβ1 and others. These receptors enable the chondrocytes to bind to collagen types II and VI in the ECM. By this interaction chondrocytes control the rate of cellular proliferation, differentiation and ECM remodelling [54].

1.2.3 Subchondral Bone

Immediately underneath the articular cartilage is the subchondral bone tissue. This tissue is composed of a superficial cortical bone and a deep cancellous bone. The cellular element of this tissue is formed from osteoblasts, osteocytes and osteoclasts. The osteoblasts are derived from the MSCs, which produce bone matrix and the osteoclasts are produced from hematopoietic stem cells (HSCs) and function in bone resorption. Osteocytes are derived from the osteoblasts and lie in lacunae surrounded by bone matrix. A balance between these three cells regulates bone remodelling under different physio-pathological conditions. The subchondral bone is controlled by different stimuli leading to bone remodelling, pain perception and angiogenesis. Bone remodelling is under control of different anabolic factors including insulin, insulin-like

growth factor-I (IGF-I) [55-56], bone morphogenetic factors (BMPs) [57], proteins in the Wnt signaling pathway [58] and serine-threonine kinase AKT [59]. Osteoblasts differentiate into osteocytes, which stays in lacunae in the matrix [60]. During this process osteoblasts express tissue non-specific Alkaline phosphatase (TNAP), which has a role in osteoblast mineralization in providing phosphatases to the ECM [48] and also expresses receptor activator of NF-kB ligand (RANKL), which controls osteoclast differentiation. The process of osteoclastogenesis starts with the interaction of RANKL expressing osteoblasts with pre-osteoclasts expressing RANK receptors leading to osteoclast differentiation [61]. This process is further controlled by osteoprotegerin (OPG), which competes for RANKL on osteoblasts [62-65]. The crosstalk between articular cartilage and the subchondral bone occurs by channels running through the two layers of the subchondral bone and connecting them to the articular cartilage. Nerves and blood vessels run through these channels carrying nourishments and stimulatory and/or inhibitory signals. Physiologically, the subchondral bone tissue gives the support to the joint by absorbing mechanical shock during movement and providing nourishments to the articular cartilage.

1.2.4 Articular Fat Pads

The role of the articular fat pads (AFPs) is generally understudied in the pathogenesis of OA. AFPs are composed of adipocytes that secretes adipokines and growth factors [66]. AFPs function as a source of progenitor cells, maintain stem cell niche, regulates immune responses [67], and regulates cell and ECM growth and repair. It has been

suggested that AFPs have a mechanical role as a cushion for the joints [66]. The role of AFPs in maintaining joint homeostasis is still under investigation.

1.2.5 Menisci

The other structure that also provides shock absorbance includes the menisci. This fibroelastic cartilaginous structure is crescent-shaped and in the knee, joint is located on the tibial plateau. It develops from the interzone. It is composed of fibrochondrocytes [68] and it is partially innervated and vascularized on only the outer one third of it, and the non-innervated parts absorb the mechanical shocks and decrease the load on the bones. It is composed of endostatin/collagen XVIII in the inner two-third part in adults [69]. The menisci are an important structure in the joint for maintaining joint integrity and loading, and if subjected to tear can initiate degeneration of the articular cartilage. Similarly, to the articular cartilage they have a limited regeneration capability [68]. The menisci are also controlled by different signaling stimuli and receive nourishments from the surrounding synovial fluid. The menisci functions in transferring weight across the joint together with the articular cartilage and its unique concave shape contributes to joint stability through accommodating the femoral condyles. [70].

1.2.6 Ligaments

The intra-articular and the extra-articular ligaments are structure that provide stability and prevent abnormal movement of the joints. These structures are made of connective tissues consisting of strong and long collagen fibres (fibrocartilage). These ligaments are covered with a superficial layer of epiligament containing nerves and blood vessels ending at the entheses. The ligaments provide the vasomotor control and the proprioception for a safe and a coordinated joint movement [44]. Altogether, knowing the normal anatomy of the different tissues forming joints, the crosstalk between different tissues and the physiological control under normal conditions will allow for a better understanding of the factors that contribute to the disturbances that may occur in these tissues leading to the development of OA. Also, it helps in predicting therapeutic interventions that may correct malfunctions close to the normal situation

1.3 OA Pathogenesis

OA was related for a long time only to articular cartilage degeneration caused by advancement in age. Today, it is regarded as a multifactorial disease of the whole joint in which a number of pathological processes contribute to its development including inflammation of the synovial membrane (Synovitis), osteophyte formation and subchondral bone sclerosis, in addition to cartilage degeneration [66]. Some researchers, [71] indicated that OA development may be regarded as a mechanobiological process, in which an injury to the cartilage may disturb the

mechanical loading on the joints, eliciting molecular factors that ignite certain mechanosensitive signaling pathways. This cascade of events leads either to functional alterations through inflammatory pathways (mechano-inflammation), or through pro-degenerative pathways driving repair and regeneration leading to chondroprotection. The balance between these two processes derives the fate of the functional status of the joint [71], (Figure 1.2). Importantly, accumulating evidence indicate a role for inflammation in the development of OA. Learning how the inflammatory processes elicit changes that may end in cartilage degeneration, synovitis or/and bone remodelling will increase our understanding of the pathogenesis of OA and contribute to reaching to effective therapeutic intervention targets within this complex process. Adding to this, the role of ncRNAs, the new players recently introduced in the pathogenesis of many diseases including OA, particularly lncRNAs and the epigenetic control exerted in the development of OA will be discussed in detail. Finally, the role of senescence and ageing will be introduced too.

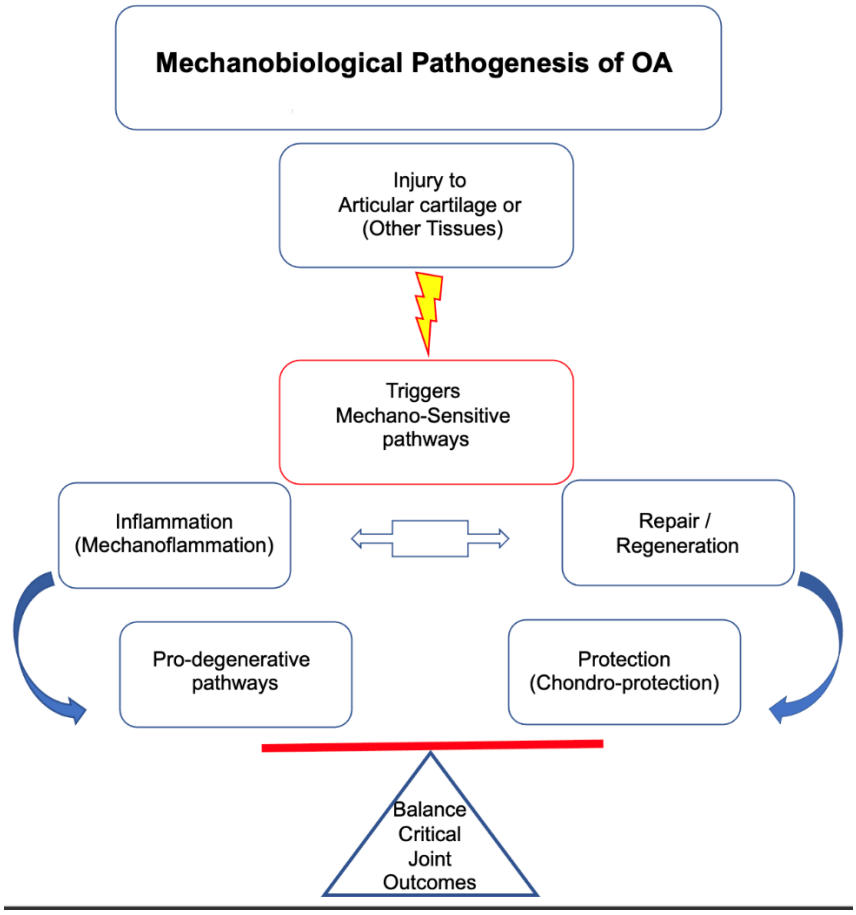


Figure 1.2 A possible scenario for OA taking the articular cartilage as an example. Information [71].

1.3.1 Role of Inflammation in The Pathogenesis of OA

One of the obvious symptoms of OA is inflammation and fluid collection in the joints in addition to stiffening, pain, bone deformities and movement restrictions [72-75]. Many factors may contribute to the etiology of OA including the risk factors mentioned earlier in this introduction, with higher age, female sex and obesity being more reported as the higher risk factors leading to OA [76]. Factors contributing to the pathogenesis of

OA such as altered catabolic and anabolic processes occur together after an injury to any tissue in the joint as shown in Fig 1.2, with the balance between these processes driving the outcome of joint tissue responses to injury. It is not known what initiates the release of certain modulators between these processes, but due to the presence of inflammation after tissue injury, some major pro-inflammatory cytokines such as Interleukin-1 β (IL-1β), Tumor necrotic factor-α (TNF-α), and Interleukin-6 (IL-6) cytokines were reported by many researchers to contribute to the pathogenesis of OA. The level of these pro-inflammatory mediators was increased in different joint tissues including the subchondral bone, the cartilage, the synovial membrane and the synovial fluid. [74, 77-79]. In addition, other pro-inflammatory cytokines were detected in the synovial fluid and chondrocytes of OA patients such as IL-15, IL-17, IL-18, IL-21 and IL-22, leukemia inhibitory factor (LIF), chemokines (IL-8, CCL2, CCL3, CCL4, CCL5), leukotrienes, [74,80], and adipokines (leptin, visfatin, adiponectin, resistin) [74,81]. It is worth mentioning here, the anti-inflammatory cytokines present including IL-4, IL-10, which affects the anabolic processes after tissue injury [79]. The proinflammatory cytokines mediate the release of other inflammatory cytokines including matrix metalloproteinases (MMPs), a disintegrin-like and metalloproteinase with thrombospondin motif (ADAMTS), cyclooxygenase-2 (COX-2), prostaglandin E2 (PGE-2) and nitric oxide (NO) [79]. These inflammatory cytokines, chemokines and adipokines trigger inflammatory signaling pathways which finally contribute to cartilage degeneration, synovitis and bone remodelling. In this introduction the major pro-inflammatory mediators of OA including IL-1 β, TNF- α and IL-6 will be introduced in detail.

1.3.1.1 IL-1β

IL-1 β is a pro-inflammatory cytokine from the IL-1 superfamily, produced by different cells forming the joint tissues like chondrocytes, osteoblasts, synovial fibroblasts, in addition to macrophages [81]. Other research indicated that IL-1β is produced by the NACT, LRR, and PYD domain containing protein 3 (NLRP3) intracellular sensing inflammasome protein and blood cells including monocytes, macrophages, and neutrophils. Both IL-1 β and IL-1α were found in the precursor form and the active precursor for IL-1α induces IL-1β [82]. An inflammatory response includes attracted blood cells that produce IL-1β, which is one in a family containing 11 cytokines including IL-18 and IL-1α. Some members of this family such as recombinant IL-1Ra have an anti-inflammatory function and were used for the treatment of inflammatory diseases [83-84]. It was found that the extracellular domains of the receptors for these cytokines supress inflammation. To mention some, IL1-R2 which is a receptor for IL-1β was shown to inhibit IL-1β [85].

The mechanism of IL-1β production with the NLRP3 inflammasome starts with a tissue injury that produces damage associated molecular patterns (DAMPs), such as reactive oxygen species (ROS) and IL-1α precursor. These precursors ignite the first signal to activate IL1 receptor type 1 (IL-1R1) and extracellular ATP (eATP), which causes the translocation of NF-kB into the nucleus and ends in the transcription and translation of the precursors for proinflammatory cytokines including IL-1β and IL-18. The second signal is initiated by eATP or DAMPs which cause K^+ efflux and NLRP3 activation. This activation leads to the cleavage of procaspase-1 into caspase 1, which in turn, cleaves pro IL-1β and pro-IL-18 into the mature IL-1β and IL-18 and will be released in the

extracellular space. Inflammatory processes start with the binding of IL-1β to its receptors (IL-1R1 and IL-1R3) forming a complex. IL-1β receptors lL-1R1 and IL-1R3 have an intracellular domain which contains Toll-IL-1-receptor domain (TIR), when these domains get in touch the inflammatory functions of IL-1β acts. The inflammatory functions of IL-1β are balanced and controlled by other anti-inflammatory members of IL-1β family [86].

Furthermore, IL-1β was reported to be elevated in many diseases including OA. The elevated levels in the tissues of OA patient's joints indicate an important role for this cytokine in the initiation and progression of the disease. IL-1β exerts its effect through binding to its cell surface receptor type I (IL-1RI), type II (IL-1RII) which was found to be increased in joint tissues of OA patients, and an antagonist IL-1 receptor antagonist (IL-1Ra). The antagonist has an ant-inflammatory effect, it is found at the same time in OA patients too, and it competes for binding to the same receptor for IL-1β in order to stop the inflammatory effects of IL-1β [87]. IL-1β binds to the receptor molecule forming a complex that initiates a signal to activate different molecules in Necrotic Factor- Kappa-light chain enhancer of activated B cells (NF-kB), and mitogen-activated protein kinase (MAPK) signaling pathways including three MAPK members p38, extracellular signal-regulated kinases (ERKs) and c-Jun N-terminal kinases (JNKs) [78]. In addition, IL-1β was found to regulate the expression of p50-associated COX-2 extragenic lncRNA (PACER) via the arachidonic acid pathway upregulating the synthesis of the prostaglandin-endoperoxide synthase 2 (PTGS-2) which has an anti-inflammatory effect on the joints [88]. Through the three members of MAPK family IL-1β induce the secretion of IL-6 and LIF as pro-inflammatory mediators [89].

1.3.1.2 TNF- α

TNF- α is another pro-inflammatory cytokine which was known due to its necrotic actions on certain cancers [79]. It is a member of the tumor necrotic super family (TNF). TNF-α exerts its biological effect by binding to the two-cell membrane-bound receptors tumor necrosis factor I, and II (TNFRI, TNFRII) that are found in all cells except red blood cells and unstimulated T-cells [90]. Binding to these receptors initiates signaling pathways related to cell proliferation, expression of pro-inflammatory genes and apoptosis such as NF-kB, MAPK (p38, ERK and JNK) [90]. These receptors were found to be upregulated in OA chondrocytes and synovial fibroblasts. TNF- α is shown in many studies to be elevated in OA and produced by synovial fibroblasts, chondrocytes, osteoblasts, and adipocytes May studies indicated that TNF- α plays a major role in OA together with IL-1β [74,87].

1.3.1.3 IL-6

The pro-inflammatory cytokine IL-6, known previously as B-cell differentiation factor [79-80]. It is a member of the IL-6 family, and it is produced by many cells including hematopoietic cells, immune cells, muscle cells and many other cells. In OA joints it is produced by chondrocytes, fibroblasts and osteoblasts [94-95]. It has been reported by many researchers to be detected in the synovial fluid of OA patients, which was correlated with OA joint pain [96-97], and elevated in the serum of OA patients, compared to healthy controls. It has a pro-inflammatory and an anti-inflammatory

action exerted through different pathways like: MIL-6R, sIL-6R, Janus-kinase/signal transduction and activator of transcription (JAK/STAT), MAPK, ERK and phosphoinositide 3-kinases (PI3K) [98-99]. Like IL β, IL-6 binds to a receptor IL-6R and this complex binds to a common protein (gp130), which then stimulates aforementioned signaling pathways [100-101]. IL-6 was reported to have a predictive value together with TNF- α of increased cartilage loss [102], and its reduced levels protects against OA development [103]. Furthermore, its expression in synovial fibroblasts was found to be associated with obese OA patients [104].

1.3.1.4 Other Cytokines

Other proinflammatory cytokines related to OA such as IL-15, IL-17, IL-18, IL-21 and IL-22 are summarized in (Table 1.2) and anti-inflammatory cytokines such as IL-4 and IL-10 (Table 1.3).

Table 1.2 Pro-inflammatory cytokines under investigation in OA

Cyto-kine	Family	Receptors	Cellular Source	In OA	Related To
IL-15	Pro-Inflamm-atory	Y-chain	Synovial fibroblasts, inflammatory cells, skeletal muscle cells	Concentration in synovial fluid of knee Oa correlates with MMPs	Early OA,
IL-17	IL-17	IL17Ra, IL17RC Found on chondrocytes, synovial fibroblasts	Th17[1], NK[2], γ δ-T cells	Not Known in human	Under investigation, pathogenic, protective
IL-18	IL-1, IFN- γ[3]	IL-18R α, IL-18R β, Expressed on chondrocytes	Chondrocytes, osteoblasts, synoviocytes, others	Found in plasma, synovial fluid, cartilage, upregulates COX-2, PGE-2, NO, MMPs	Radiographic OA severity, post-traumatic OA severity, cartilage degradation, IL-6 and TNF- α synthesis
IL-21	Pleiotropic cytokine[4]	IL-21R, Y-chain	NK, Th17, follicular T-cells	Found in synovial fluid	OA severity
IL-22	**IL-10**	IL-22R Increased in Chondrocytes	Th17, NK, Mac[6], Neut[7], fibroblasts	Found in Synovial fluid, synovial fibroblasts, Promote MMP-1	Synovial Inflammation

1.Th17= subtype of T helper cells 2. NK = Natural Killer cells 3. IFN- γ= Interferon – γ 4. Pleiotropic cytokine= of multiple cell types 5. Mac= Macrophages 6. Neut= Neutrophils [79].

Table 1.3 Anti-inflammatory cytokines under investigation in OA

Cyto-kine	Family	Receptors	Cellular Source	In OA	Related To
IL-4	Immune regulatory cytokine	Complex1(IL-4R α and IL-2Rγc), Complex 2(IL-4R α and IL13R α1)	Synovial fibroblasts, inflammatory cells, skeletal muscle cells	Decreases IL-1β, TNF-α, IL-6, COX-2, PGE-2, iNOS,	Chondro-protection
IL-10	Interferons	IL10Ra: IL10R1, IL10R2	Immune cells, chondrocytes	Stimulates IL-1β antagonist synthesis	Chondro-protection, antiapoptotic, anti-inflammatory

Data from [79].

1.3.1.5 Adipokines

Adipose tissues secreting cytokines (termed adipokines) are now regarded as key metabolic inflammatory factors that contribute to the pathogenesis of metabolic syndrome and chronic inflammatory disorders including OA [105] by acting on distal tissues, including the tissues of the joint. For example, the adipokines leptin, visfatin, adiponectin and resistin, chemerin, vaspin and omentin-1 have been implicated in type II diabetes, chronic heart disease and osteoarthritis. Some adipokines were found to be expressed in OA joints secreted by AFP [106] or/and from different tissues in the synovial joint including cartilage, synovium, osteophytes and meniscus. Leptin was found to be high in the synovial fluid of obese OA patients [107] and it was associated with the severity of OA in females [108]. In addition, leptin was reported to act as a pro-inflammatory mediator cooperating with other pro-inflammatory cytokines such as IL-

1 β and TNF- α, this was shown in a study by increase in the expression of these cytokines after stimulation with leptin [109-110]. Leptin was reported to induce inflammatory mediators IL-8 and IL-6 in synovial fibroblasts via JAK/STAT and PI3K signaling pathways respectively [111-112]. Furthermore, leptin contribute to cartilage degradation through positive association with MMP-1, MMP-3 [113], iNOS, COX-2 and PGE-2 via NF-kB and JNK pathway [109,114,115].

Visfatin, is another adipokine found in many diseases including OA [116,100]. It is released by adipocytes, inflammatory cells, synovium, chondrocytes, osteophytes and AFP [117]. In OA it was shown to be associated with K & L grade of the joint i.e the severity of OA joint damage), cartilage degradation biomarkers CTXII [118] and catabolic effects of inflammatory mediators on cartilage degeneration such as IL-1 β [119]. On the other hand, it also protects the cartilage from apoptosis and differentiation by activating sirtuin type 1 (SIRT1) histone deacetylase [120]. Its effect on the OA osteoblast was shown to increase the expression of IL-6 and monocytes chemoattractant protein 1(MCP-1) contributing to the pathogenesis of OA [121].

Adiponectin is an adipokine with an anti-diabetic and anti-atherogenic effects by binding to adiponectin receptor subtypes AdipoR1 and AdipoR2 receptors [109]. In OA, the serum levels of adiponectin were correlated with pain in joints of female patients [123] and the synovial fluid levels of adiponectin was positively correlated with the markers of cartilage degradation [124-125]. Adiponectin, like visfatin have a dual effect on OA cartilage a protective effect by downregulating IL-1β-induced MMP-13 [126] and a destructive effect by stimulating the catabolic action of IL-6, MMP-3 and MMP-13 [127].

Resistin adipokine plays a significant role affecting inflammation, obesity and OA. Plasma levels of resistin showed an association with radiographic knee OA in male overweight (OW)/obese (OB) patients [128-130], a pro-inflammatory role by correlating to IL-6 [131] and the synovial levels of resistin were associated with cartilage destruction [132].

Chemerin is an adipokine secreted by adipocytes, OA chondrocytes and synovial fibroblasts [133-134] and it may have a role in the initiation and progression of OA by controlling the pro-inflammatory processes in OA [135]. The Omentin-1 is an adipokine found in the extracellular fluid [136] recently identified and it is reported to be associated with sever radiographic OA cases [137]. Vaspin, adipokine was also recently identified which is expressed by OA cartilage, synovium, osteophyte, AFP and meniscus. In contrast to the previously mentioned adipokines, vaspin serum levels were found to be low in OA patients. This was indicated an anti-inflammatory, and anti-catabolic effects which was due to lowering IL-1β-mediated production of MMPs, COX-2, PGE2 and iNOS in chondrocytes [138]. In summation, many adipokines have been implicated in the pathogenesis of OA. However, the contradictory effects of these different adipokines across different tissues with the joint and the heterogeneity observed across different OA patient cohorts in expression and secretion of adipokines casts doubt on the true role of these factors in initiating and progressing OA disease.

1.3.1.6 Chemokines

Chemokines/chemotactic cytokines are a group of small proteins grouped according to the position of the cysteine residues of the N-terminal into C, CC, CXC and CX3C. Members of the CC group, where the two cysteine groups are next to each other include CCL2, CCL3, CCL4, CCL5. These cytokines were found to be upregulated in inflammation related to OA [74,79]. These chemokines guide immune cells to the site of inflammation acting in different cellular processes such as proliferation and differentiation (T-helper). CCL2 was reported to increase MMP-3 expression leading to degrading proteoglycans and cartilage degeneration by combining to Toll-like receptor-4 (TLR-4) [139]. Increased plasma levels of CCL3 were associated with the radiographic severity of OA [140], CCL3, CCL4 and CCL5 were upregulated in IL-1β treated chondrocytes [141]. In addition, CCL5 was upregulated in the synovial fluid of OA patients [97].

1.3.2 Non-coding RNAs, Role in Inflammation and OA Development

Non-coding RNAs (ncRNAs) are coded in the DNA, transcribed to RNA, but as their name suggests they do not code for any protein. ncRNAs are one of the major epigenetic regulatory mechanisms in mediating cellular processes, together with DNA methylation and histone modification [142]. ncRNAs are classified according to the length of the nucleotides into three main groups including short ncRNAs, long ncRNAs (lncRNAs) circular ncRNAs (circRNAs). Short ncRNAs nucleotide length is less than

30 nucleotides, and it includes different types such as small nucleolar RNAs (snoRNAs), small nuclear RNAs, Piwi-RNAs (piRNAS), small interfering RNAs (siRNAs) and miRNAs (miRNAs) having a length of 18-24 nucleotides. LncRNAs have nucleotide length of more than 200 and circRNAs are made up of 1-5 introns or exons in a loop like structure [143]. The mode of action of these ncRNAs is to regulate targeting gene expression at the post-transcriptional levels in the cytoplasm through binding to a specific messenger RNA (mRNA) ending in repressing or blocking it [144]. The other mode of action that was reported in many studies for lncRNAs and circRNAs is by sponging miRNAs in a competing endogenous way in order to reduce miRNAs that target mRNAs {competing endogenous RNA (ceRNA) hypothesis} [145-147]. Dysregulation in the epigenetic control on the signaling pathways and the cellular processes ends in different diseases including OA [148]. Many studies reported a role for ncRNAs in the pathogenesis of OA and the possibility of the use of these regulators in finding possible treatment for OA.

1.3.2.1 miRNAs in The Pathogenesis of OA

miRNAs were reported to regulate different cellular processes leading to the development of different diseases including OA by modulating inflammation [149-151], cellular differentiation [152], apoptosis [153-155] and ECM dysregulation [156-157]. miRNA's role in chondrocyte's differentiation and inflammation, will be introduced in this section. The regulatory roles of miRNAs in chondrogenesis, osteogenesis and osteoclastogenesis in OA, were shown to be related to the early stages of these

cellular processes from bone marrow-derived mesenchymal cells (BMSCs) [158-159]. miR-145 depresses the expression of a transcription factor related to chondrogenesis SOX9 leading to an increase in the levels of cartilage catabolic enzymes MMP13, and the loss of chondrocytes differentiation phenotype [160]. miR-125a-5p was reported to regulate osteoclast differentiation by targeting and supressing the TNF receptor superfamily member 1B gene (TNFRSF1B) [161]. In addition, miRNAs mediate the inflammatory processes in OA through signaling pathways such as NF-kB and through regulating pro-inflammatory cytokines such as IL-1β, TNF- α and IL-6. It was reported that miR-382-3p decreased NF-kB phosphorylation in IL-1β-treated OA chondrocytes [162], and miR-146a was shown to reduce NF-kB phosphorylation and inhibit the production of the pro-inflammatory cytokines [163]. Furthermore, miRNAs were reported to exert their epigenetic regulations through other signaling pathways including Wnt- β-catenin affecting chondrocytes as in the case with miR-145-5p [164-165] and synovial fibroblasts [166]. In addition, other miRNAs regulate cellular death by stimulating apoptosis in OA chondrocytes, through the Phosphoinositide 3-kinase (PI3K-AKT) signaling pathway, such as miR-486-5p, miR-363-3p and miR-455-3p [167-169].

1.3.2.2 LncRNAs in The Pathogenesis of OA

LncRNAs are a major group of ncRNAs which exerts epigenetic control on cellular processes in normal situations by maintaining homeostasis and in pathological conditions by producing tissue damage. Many studies reported a role for different

lncRNAs in the pathogenesis of many diseases [Appendix Table 9.3] and several of these lncRNAs have been identified as candidates for mediating inflammation in OA pathogenesis [170] including lncRNAs that are differentially expressed in human OA cartilage and lncRNAs associated with inflammatory obese OA synovial tissue [171].

The mode of action of lncRNAs in regulating the epigenetic control on gene expression in OA pathogenesis is mediated through a multitude of signaling pathways, dependent on the specific lncRNA. The three main inflammatory pathways in which lncRNAs exert its effect include the MAPK, JAK-STAT, and the NF-kB pathways. [172] LncRNA can regulate inflammatory pathways by initiating signaling cascades that modulates the release of pro-inflammatory cytokines [173-174]. Disturbances in the path of these signals contribute to the development of many mutations in some cytokines (interferons) involved in the activation of the JAK-STAT pathways [175]. Another mechanism in which lncRNA exert its genetic control, is by acting as a decoy to inhibit gene transcription [176], or it may act as a guide leading some genes to directly bind to its target [177]. Furthermore, some lncRNAs regulate the activities of genes by recruiting proteins in a complex manner by scaffolding in a “temporospatial” way inhibiting or activating functional genes [178-179]. The previously mentioned method for mode of action of ncRNAs in the genetic regulation: ceRNAs, was also reported as mechanism used by lncRNAs in regulating the activities of proteins of different inflammatory pathways by binding to the enhancer portion of the gene leading to its activation [180].

Some LncRNAs were reported to different roles in the pathogenicity of OA such as regulating cellular functions of chondrocytes, osteoblasts and synovial fibroblasts, including proliferation and apoptosis and ECM dysregulation [181]. LncRNA C-terminal

binding protein1 Antisense RNA2 (CTBP1-AS2) was shown to control cellular proliferation of OA chondrocytes. It was upregulated in synovial fluid of OA patients together with miR-130a [182]. LncRNA Growth arrest-special transcript 5a (GAS5a), which has a role in apoptosis, was found to be upregulated in OA chondrocytes, and its modulation of inflammatory responses was shown by decreasing pro-inflammatory cytokines TNF-α and IL-6 when the lncRNA was silenced [183]. In addition, some studies reported that upregulated lncRNA Human 19 (H19) promoted the apoptosis of chondrocyte treated with IL-1β and lipopolysaccharide (LPS) [181]. On the other hand, NF-KappaB Interacting LncRNA (NKILA) was shown to inhibit apoptosis of OA chondrocytes via miR-145/SP1/NF-kB axis [181]. Other lncRNA such as Prostate-Specific Transcript1 (PCGEM1), were found to regulate the survival of the synoviocytes, and GAS5a controlled its apoptosis [184]. Synovial fibroblasts proliferation was also controlled by the upregulation of the lncRNA: Antisense non-coding RNA in the INK4 locus (ANRIL) [185]. A number of lncRNAs were reported to control osteoblast proliferation through controlling osteogenesis, such as H19, Differentiation Antagonizing Non-Protein Coding RNA (DANCR), Metastasis Associated Lung Adenocarcinoma Transcript 1 (MALAT1), Maternally Expressed 3 (MEG3) and HOX Transcript Antisense RNA (HOTAIR) through regulating different signaling pathways related to osteogenic differentiation [186].

LncRNAs regulating the cartilage degradation and the ECM in OA tissues includes TM1P3 which promotes ECM of OA cartilage through miR-22/TGF- β/MMP13 axis [187]. HOTAIR which was shown to regulate MMPs responsible for cartilage degradation through miR-17-5p/Wnt/ β-catenin axis [188]. However, MEG3 lncRNA was shown to protect ECM degradation through miR-93/TGF-receptor B2

(TGFBR2)/TGF-β axis [189] and miR-361-5p/FOXO1 axis [190]. The epigenetic regulation of the lncRNA MALAT1 on OA joint will be introduced further in the next section.

1.3.3 MALAT1 in OA

MALAT1, also known as non-coding nuclear enriched abundant transcript 2 (NEAT2) was first identified in non-small lung cancer (NSCLC) and is the first lncRNA found to be associated with human diseases. It was found to be highly conserved in many mammalian species, playing different physiological and pathological rolls in cellular processes. The molecular functions of MALAT1 include alternative splicing, transcriptional regulation and post transcriptional regulation of genes including epigenetic regulation of gene expression by DNA methylation, histone modifications, chromatin modulation and ceRNA mechanisms [191].

MALAT1 is one of the most dysregulated lncRNAs in many diseases, but the mechanism of its action is not fully understood [192, Appendix 9.6]. Recently much evidence was published in research indicating its role in inflammatory processes. Like the rest of the lncRNAs, MALAT1 exerts its regulation on the three inflammatory pathways mentioned earlier. MALAT1 lncRNA was found to be one of the regulators of the inflammatory pathways MAPK by regulating the kinases like p38, JNK, and ERK. One of the possible modes of action of MALAT1 in its epigenetic regulation on different cellular processes was shown to be by sponging miRNAs and act as ceRNA (as

mentioned earlier in the mode of action of non-coding RNAs) [192]. It was shown that MALAT1 acts as a ceRNA for miR-150-5p competing with AKT3, which is important for proliferation and regulates apoptosis [193-196]. This led to suggesting MALAT1 as regulator of cellular proliferation, apoptosis and ECM dysregulation in OA chondrocytes by miR-150-5p/AKT3 axis [192]. MALAT1 has also been reported to regulate cellular proliferation through another signaling pathway ERK/p38/MAPK axis in skin fibroblasts. [197]. In addition, other studies showed that MALAT1 regulates senescence of photosensitive aged skin fibroblasts by controlling the ERK/MAPK pathway [198]. Many studies showed a roll for MALAT1 in the migration, and metastasis of malignant cells such as bladder cancer through MALAT1/SUZ/TGF-β-12 axis [199], lung [200], and colon cancer [201-202].

MALAT1 modulates inflammatory processes in many diseases including OA. Researchers indicated a possible role for MALAT1 in SLE, in which it inhibited the stimuli to NF-*K*B pathway reducing inflammatory cytokines leading to immunological diseases [203]. MALAT1 was shown to regulate inflammation also by regulating the production of hyperglycaemia induced pro-inflammatory mediators Il-6 and TNF in endothelial cells by activating p38 in MAPK pathway [198,204] and regulating the lipopolysaccharide- induced inflammatory responses through controlling the NF-kB pathway [205].

Due to its localization in nuclear speckles, where many pre-mRNA splicing factors are found, MALAT1 functions in physiological roles including direct and indirect transcription and pre-mRNAs splicing of genes by binding to and recruiting splicing factors such as SRSF1-SRSF3 [206-208]. Although MALAT1 has a role in many physiological cellular functions including differentiation, migration and tumor growth

and pathological functions including inflammation, diabetes, atherosclerosis, hypoxia, and angiogenesis [209], when knocked out (KO MALAT1) in mice models it didn't affect the normal physiological or developmental functions of the mouse. However, it was found that some other genes surrounding MALAT1 gene was affected, indicating a *cis*-like action for MALAT1 [210-212]. In cancer, MALAT1 was upregulated in many cancers and related to progression and metastasis such as, in lung, breast and liver tumors affecting many genes in a pathway [213]. In KO MALAT1 mouse models, cancer growth and metastasis were reduced in many types of cancer including esophageal and gallbladder cancers [214-215]. In addition, MALAT1 showed a differential expression under stress responses such as serum starvation and hypoxia which are important for angiogenesis and metastasis of tumors in a KO MALAT1 mice models [216-218]. It was reported that MALAT1 during hypoxic conditions was regulated by HIF1α transcription factor [219-220].

Furthermore, MALAT1 was shown in different studies to be dysregulated in pathological conditions related to diabetes including retinopathy, artherosclerosis, and renal disorders. This was found to be due to elevated levels of proinflammatory cytokines such as TNF- α, and IL-6 in cells with upregulated MALAT1 in the previously mentioned conditions [221]. The atherosclerotic lesions were reduced by MALAT1 reduction in KO MALAT1 mice models, and they related these atherosclerotic lesions to inflammatory responses [222-223]. Another example for MALAT1 modulation of inflammation and apoptosis was evident in preventing ischemic stroke in a mouse brain microvasculature. MALAT1 was shown to function as an anti-inflammatory and anti-apoptotic role in the by controlling the pro-inflammatory cytokines IL-6 and E-Selectin and the proapoptotic factor Bim in a NO MALAT1 mice model. This was found to be

due to direct binding of MALAT1 to Bim or E-Selectin preventing cell death and inflammation respectively. By this protecting the endothelial cells in the brain microvasculature from damage and preventing ischemia [224-225].

In addition to its role in cancer and inflammation, some studies showed the negative role of MALAT1 in immunity of mammalian cells against infection by using a NO MALAT1 mice model. It was found that in a NO MALAT1 mouse model, the levels of the immunosuppressive anti-inflammatory cytokine IL-10 were reduced in CD4+ T cells which affected the levels of malaria and visceral leishmania pathogen clearance. This was attributed to MALAT1 correlation with the proto-oncogene c-Maf transcription factor which regulates IL-10 in Th1 and TH2 cells [226].

Intriguingly**,** the role of MALAT1 in modulating inflammation in OA was shown by our group recently in the association of MALAT1 with inflammatory synovial fibroblast phenotypes in obese OA patients [171]. Other roles for MALAT1 in OA, were reported in regulating cellular processes in human OA joint cells such as chondrocytes and synovial fibroblasts. The role of MALAT1 in ECM dysregulation will be introduced at the end of this section. A study showed that MALAT1 downregulation reduced OA chondrocytes proliferation controlled by the P13K/Akt pathway, which is an important cell cycle regulating pathway [227]. Another study showed that MALAT1 was upregulated in OA chondrocytes, and it was associated with OA severity [191] This was further evident in the work of Liang et all on OA chondrocytes, who revealed that MALAT1 silencing of miR-127-5p promotes OA chondrocyte proliferation through controlling the levels of Osteopontin [228]. Furthermore, our group previously have shown that MALAT1 regulated the proliferation of synovial fibroblasts of the obese OA

cohort and that synovial fibroblast depleted MALAT1 showed a reduced rate of proliferation [171].

Furthermore, MALAT1 regulates cellular death by apoptosis in OA, through activating a number of pro-apoptotic mediators (caspase3, Bim, and Bax) through activation of p38 MAPK and nuclear factor k B (NFkB) pathways as in other diseases regulated by MALAT1 [229]. A study showed that MALAT1 knockdown (MALAT1-KD) inhibited OA chondrocytes proliferation and induced apoptosis in IL1-β-treated OA chondrocytes [191].

ECM is regulated by catabolic processes exerted by MMP gene family members and the anabolic effects of cartilage forming type II collagen and aggrecans. A study on the role of MALAT1 in regulating ECM degradation in Intervertebral disc degeneration (IDD) showed that MALAT1 reduced the effect of the proinflammatory cytokines IL-1β and TNF- α on inducing ECM degradation [230] In OA, other studies indicated a role for MALAT1 in regulating ECM pathogenesis by controlling members of the MMP and ADAMTS genes and demonstrated that MALAT1 depletion increased the expression of ECM degradation genes such as MMP-13 and ADAMTS-5 and decreased genes responsible for cartilage tissue formation such as type II collagen and aggrecan [192].

In summation, MALAT1 regulates cellular proliferation, apoptosis and ECM degradation by modulating inflammatory processes with different epigenetic regulation through different signaling pathways in OA joint tissue cells including chondrocytes and synovial fibroblasts. However, there is a gap in research on the role of MALAT1 in modulating the inflammatory processes and controlling bone remodelling in mature OA osteoblasts (Fig 1.3).

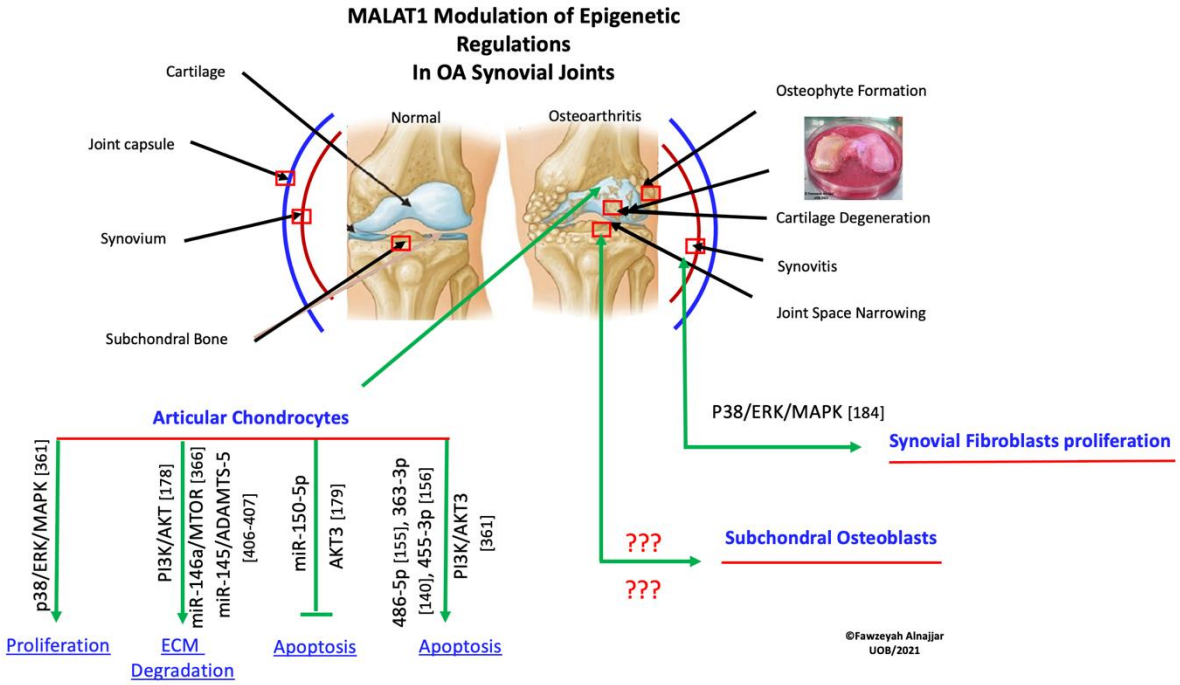


Figure 1.3 Reported MALAT1 modulation of epigenetic regulations in OA processes. MALAT1 was shown to modulate different cellular processes in human OA joint tissues extracted cells including synovial fibroblasts, articular chondrocytes and subchondral bone. Cellular processes are controlled with signaling pathways for example, Synovial fibroblasts and articular chondrocytes proliferation was shown to be regulated through p38/ERK/MAPK axis, articular chondrocytes ECM degradation was shown to be regulated vis PI3K/AKT, miR-146a/MTOR and miR-145/ADAMTS-5 axes and articular chondrocytes apoptosis was shown to be regulated through different miRNAs/PI3K/AKT3. What's missing is MALAT1 modulation of mature subchondral osteoblasts, which I'm trying to answer in this thesis project. The photo of the knees is by unknown author and licensed under CC BY-SA-NC.

1.3.4 Cartilage Degeneration in OA

This process was mentioned under different previous sections in this introduction, further information will be introduced here. One of the major changes in OA is cartilage degradation, which results from disturbances in the balance between anabolic and catabolic processes that disrupt homeostasis of the ECM in the cartilage. Under inflammatory conditions in OA these processes are controlled by signaling pathways, transcription growth factors, MMPs enzymes and inflammatory mediators including IL-1β, TNF- α and IL-6. IL-1β plays an important role in the pathophysiology of OA, by controlling the activities of chondrocytes in building the extracellular matrix by inducing ECM catabolic processes via MAPK signaling pathway. IL-1β reduces the expression of type II collagen and aggrecan proteoglycan genes via ERK/MAPK axis [231] and inhibits collagen synthesis via JNK/SOX9 axis [232]. IL-1β also induces ECM degradation enzymes including MMP-1, -3 and -13 and the aggrecanases ADAMTS-4 and ADAMTS 5 via ERK/p38/JNK axis [233]. At the same time IL-1β induces IL-6 and LIF secretion through the previously mentioned signaling pathways, promoting inflammation and thus further inducing the catabolic processes in OA cartilage ECM.

In addition, TNF- α and IL-6 were reported to be induced under the effect of IL-1β via NF-kB signaling pathway, which activates the secretion of ECM catabolic enzymes as mentioned previously in addition to other inflammatory mediators such as PGE-2, COX-2 and iNOS [234] and chemokines of the CCL family and IL8 [235] leading finally to the degradation of ECM.

The role of lncRNAs in regulating the articular chondrocytes in OA was shown in a pervious study where, the expression of two lncRNAs in particular PACER and HOTAIR, were reported to be downregulated and upregulated, respectively in OA. The

change in the expression of these lncRNAs induced chondrocytes apoptosis leading to cartilage destruction [236].

Collectively, IL-1 β TNF- α and IL-6, as pro-inflammatory mediators play a major role in cartilage degradation by reducing the expression of cartilage anabolic factors such as type II collagen and increasing cartilage catabolic factors such as promoting the release of aggrecanases such as ADAMTS-4 and ADAMTS-5, and the collagenases MMPs-1, MMP3 and MMP13, as well as the chemokines IL8 and CCL2 and 5, inflammatory mediators PGE-2, COX-2 and iNOS which act together to degrade cartilage thus promoting OA severity.

1.3.5 Bone Remodelling

Bone remodelling is also a balance between two cellular processes, bone formation and bone resorption, which are coupled together [237]. The balance of bone formation and bone resorption is important in adult life for maintaining homeostasis of the skeletal system. A key component of bone formation is osteoblastogenesis (the increase in osteoblast bone forming cells), whilst bone resorption is mediated by osteoclast cells, and thus the process of osteoclastogenesis. Osteoblastogenesis and osteoclastogenesis are controlled by multiple physiological factors such as hormones, Insulin and multiple growth factors, as well as a multitude of cytokines, chemokines, transcription factors such as TGF-β and signaling pathways such as TNF, PI3K/AKT and MAPK. Critically, this balance is disturbed in OA, with evidence of the thinking of

subchondral bone skeletal architecture and the formation of osteophytes (bony spurs) in the OA joint being characteristic features of OA [238].

In the recent years, increased understanding of the coupling between osteoblast and osteoclast activity has helped to better understand the process of bone homeostasis and the pathological remodelling of bone in disorders such as OA. Bone remodelling starts in areas of bone loss, and bone formation starts only after osteoprogenitor cells expand. This expansion is initiated by osteoclasts which trigger the osteoprogenitor reservoir to the proximity of the bone formation sites. These processes are regulated by many molecular events which are beyond the aim of this thesis. In brief, after osteoclasts resorb existing bone, a monolayer of progenitor reversal cells covers the area, the cells close to osteoclasts will have a phenotype that produce MMPs and RANKL (pro-resorptive cells). When the osteoclasts develop into a pro-osteogenic phenotype, it losses this phenotype and converts into a phenotype close to osteoblasts carrying the osteoblast lineage RUNX family Transcription Factor 2 (Runx2), alkaline phosphatase and type 3 collagen [239]. This shift between the two phenotypes, the pro-resorptive to a pro-osteogenic phenotype prepare for bone formation and expansion of the pro-osteogenic phenotype. This process continues until a certain density is reached, after that osteoblast differentiation starts and bone formation continues [240].

Under inflammatory condition such as OA, the subchondral bone will be regulated by damaged cartilage and the released catabolic enzymes. As a result, the different layers of the subchondral bone including the plate, the trabecular and the subarticular bone are affected, and bone remodelling starts leading to osteophyte formation and sclerosis [241]. Bone remodelling will be shifted towards bone resorption under the influence of

inflammatory mediators IL-1β, TNF- α, IL-6, glucocorticoids, histamine, and PGE-2 IL-1β, TNF- α, which stimulate osteoblasts to secret macrophage colony stimulating factor(M-CSF) and MCP-1 that attracts the recruitment of pre-osteoclasts to the remodelling area. OPG protein will control this process by binding RANKL competing with RANK on the pre-osteoclasts, and by this inhibiting maturation of the osteoclasts. This process is coupled by osteoblast recruitment to the area for differentiation and bone formation under the control TGF-β1 secreted by osteoclasts [242]. Furthermore, another study indicated a role for AKT1/FOXO3a/Bim axis in controlling bone remodelling by promoting osteoblast bone formation. This study showed that decreased AKT1 regulates increased osteoblasts mitochondrial apoptosis mediated by caspase 9, Bad and forkhead box class O (FOXOs) and decreased osteoblasts differentiation, by decreasing RANKL expression on osteoblasts leading to a decrease in osteoblast-mediated osteoclast maturation. [243-244].

1.3.6 Synovitis and Synovial Chondrocyte Cross Talk

Inflammation of the synovium (Synovitis) is one of the major causes of OA of the synovial joints. Synovitis was related to the severity of OA [245], and the concentration of the lubrican and the hyaluronic acid in the synovial fluid was affected by inflammation. Synovitis in early OA was evident histologically by the cellular proliferation and lymphocyte aggregation, it leads to the progression of OA [246] and modulates the release of pro-inflammatory mediators such as IL-1 β and TNF- α which affect other tissues nearby including articular cartilage and the subchondral bone.

Recently a study indicated the existence of a cross talk between the OA cartilage and the synovium. This study indicated that the pro-inflammatory cytokines present in the synovial fluid such as IL-1 β, TNF- α, IL-15 and IL-18 control the production of MMPs modulating cartilage degeneration. Using single cell sequencing a group of 12 upstream regulators of cytokines and growth factors that were expressed by synoviocytes and found in the synovial fluid were found to regulate OA cartilage phenotype changes. The study suggests that synovial cells regulate cartilage degradation in OA [247]. Furthermore, the extent of synovitis was shown to be reflected on the degree of pain in the joints of different OA patients [248]. This was suggested to be related to the high number of macrophages in the OA synovium [249]. Macrophages in the OA synovium were of two types M1 and M2, M1 exerts a catabolic effect on the cartilage by down regulating collagen type II and aggrecan synthesis and upregulating MMPs-1,3 and 9 [250], while M2 was found to have a protective effect modulating the release of anti-inflammatory cytokines IL-4 and IL-10. [251]. These differences at the single cell level were previously mentioned under the crosstalk between OA synovium and articular chondrocytes [252]. Anti-inflammatory drugs evaluated in clinical trials such as anti-TNF mAbs and IL-1 β antagonists have been found to be largely ineffective in OA compared to RA [248]. This may be related to the heterogenicity of the inflammatory cellular players in initiating synovitis in OA patients. Better understanding of the cellular and molecular changes leading to synovitis will lead to improvement of pharmacological therapies for the treatment of OA.

1.3.7 Signaling Pathways and Effective Genes

Accumulating evidence indicate the role for the following signaling pathways in the pathogenesis of OA Wnt/β-catenin, Hedgehog, TNF family of signaling pathways, MAPK-ERK and JNK [172], PI3K/AKT, JAK/STAT [175] and HIF-1 signalling [253]. In addition to growth factors such as TGF- β, Wnt3 α, Indian Hedgehog, and signaling mol Smad3, β-catenin, HIF-2 α and Runx2 [254]. VEGFA, MYC and CXCL12 were amongst the upregulated genes in relation to knee OA [253]. The non-coding RNAs including miRNAs and lncRNAs related to OA include those related to knee OA such as miR-101, miR-181a, miR-29, miR-9 and miR-221 [253]. Other miRNAs were reported in OA joints include, miR-150-5p [191], miR-486-5p [168], miR-363-3p [153], miR-455-3p [169], miR-145, miR-146a [93] and many others. The following lncRNAs were found to be related to OA including H19, DANCR, MEG3, MALAT1 and HOTAIR [186]. Better understanding of the molecular events underlying the pathogenesis of OA including the signalling pathways and genes related to these pathways may contribute to an in-depth recognition of the pathological processes leading to OA. In addition, this knowledge will lead to the identification of different OA phenotypes enabling the development of specialised therapeutics.

1.3.8 Thesis Hypothesis

To further investigate the role of lncRNAs and in particular MALAT1 lncRNA in OA, the hypothesis for this thesis project was that lncRNAs are key regulators in mediating joint

inflammation by impacting on the inflammatory phenotype of the cells in the OA joint, namely chondrocytes from the articular cartilage, osteoblasts from the subchondral bone and synovial fibroblasts from the synovial membrane. For this purpose, the lncRNA MALAT1, will be further studied in areas including the tissues of the OA joint in different patient cohorts including bone, synovium, adipose and cartilage expression in patients with knee, hip, and hand OA of varying BMI and gender, correlation of the expression of MALAT1 to inflammatory cytokines in synovial fluid and serum. In addition, determining the functional role of MALAT1 in the cells of the OA joint including chondrocytes, fibroblasts and osteoblasts and the expression and secretion of inflammatory genes in the three OA cellular elements.

CHAPTER 2

Material and Methods

2.1 Ethical Approval and Patient's Recruitment

UK National Research Ethics Committee (NRES 16/SS/0172) at the University of Birmingham approved ethical guidelines to be followed in collecting samples recruited for this study. Participants for this project were patients with OA undergoing elective joint replacement surgery for OA in different joints including both weightbearing joints such as hip, knee and toe joints and the non-weight bearing joints including hand, wrist, and shoulder joints. Normal (non-OA diseased) controls were patients undergoing total joint replacement for a fracture to the neck-of-femur (NOF) undergoing arthroplasty. Patients were recruited from two hospitals in the UK: The Royal Orthopaedic Hospital at Birmingham, and The Russell's Hall Hospital at Dudley. Participating volunteers were informed about the study's regulation and signed a written consent upon approval before sample collection. Prior to surgery and sample collection, patient's history was thoroughly documented. OA confirmation, osteophytes formation, hand OA, and the exitance of any exclusion criteria that may be a secondary cause of OA was confirmed with X-ray. Presence of certain diseases, joint injuries, and or any recent (2 weeks prior to surgery) anti-inflammatory medications such as NSAIDS or corticosteroid injections were exclusion criteria. Excluded diseases included diseases of the congenital stage and childhood like congenital hip dislocation, irregularities in the blood supply causing avascular necrosis in Perthes disease of childhood. Conditions of the growing stage of the bone at teens or before like, Slipped capital femoral epiphysis, old bone fractures like NOF and acetabular fractures. Ligament injuries such as Malalignment syndrome affecting both femoral and tibial ligaments, or Patella malalignment syndrome affecting the patellofemoral articulation. Cancer cases or patients with inflammatory diseases using immunosuppressive or steroidal drugs were

excluded from this study too. Demographic patient's characteristics (age and gender), blood pressure, presence of pain, other diseases, and medication used, were collected. Anthropometric parameters included weight in kg, height in cm, BMI kg/m^2 [weight/height]^2, waist to hip ratio [W:H], and the percentage of body fat, were measured. OA severity was assessed radiographically using the Kellgren Lawrence scale (KL), and joint space width measurement by joint space narrowing scores (JSN).

2.2 Sample Collection

All samples were collected following a standard procedure to insure consistency.
OA patients between 26 to 84 years old, of different cohorts and from different joints in the body were recruited for this study. Samples were collected from normal weight (NW) with BMI 18-24.9 kg, obese (OB) with BMI 25-29.9 kg, and overweight (OW) patients having BMI of 30+ kg.

2.2.1 Blood and Synovial Fluid

Prior to joint sample collection, blood samples (10 mL) were collected via venepuncture, and pre-operatively synovial fluid (0.5 mL – 2 mL) was aspirated from the joint. The blood samples were centrifuged at 3000 rpm for 10 min, serum was

separated and was aliquoted into 1mL cryovials. Serum and synovial fluid were snap-frozen in liquid nitrogen and stored at -80 ^{o}C for further use.

2.2.2 Tissue Biopsies

86 tissue biopsies were collected from different parts of joints (Table 2.1, 9.1) including whole femoral head in case of hip OA patients and synovium. The synovium was divided into two parts, one part was snap frozen to generate primary synovial fibroblast, and the other part was used to seed a culture flask and cultivate synovial fibroblasts. The articular cartilage on the femoral head was divided into two parts, one part was snap frozen and the other part was used to prepare a homogenised solution in a process for generating primary chondrocytes. From knee OA patients the synovium, the tibial-plateau, the femoral- condyle and the fossa were collected for generating fibroblasts and osteoblasts. From the hand, foot, toe and spine tiny pieces of bone, and synovium were collected.

A. **B.** **C.**

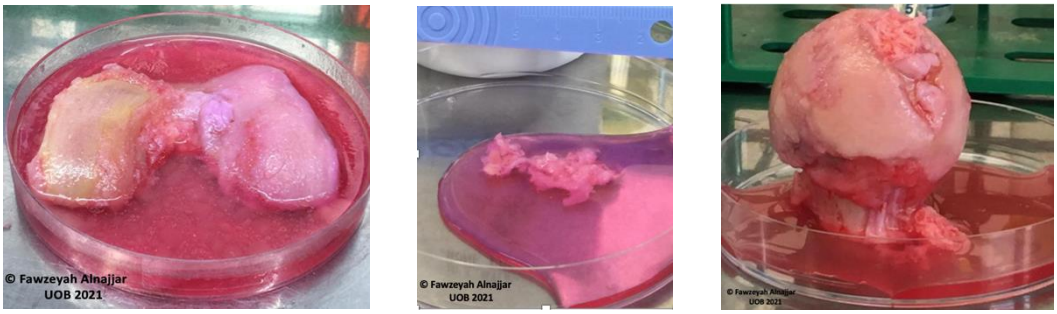


Figure. 2.1 Cartilage and bone biopsies. Three different biopsies from an OA Knee with the femoral condylum showing a clear cartilage degradation on the left side **(A)**. Bone biopsy from a hand OA joint **(B),** head of femur Joint showing the degraded cartilage **(C).**

Table 2.1 Total number of samples obtained of OA joints from different anatomical sites, used in this thesis project

Joint	Synovium	Cartilage	Bone	TOTAL
Hip	12	7	22	**41**
Knee	4	14	10	**28**
Hand	3	-	3	**6**
Wrist	1	-	2	**3**
Toe	-	-	1	**1**
Foot	-	-	6	**6**
Spine	-	-	1	**1**
TOTAL	**20**	**21**	**45**	**86**

2.3 Human Primary Cell Culture

Human primary cell culture was prepared from fresh joint tissue samples from archival cryopreserved primary cells from Dr. Simon W Jones cell bank, University of Birmingham, UK. Growth media specific to the particular cell type (fibroblasts, chondrocytes, or osteoblasts) were prepared and optimised for culturing of human primary cells depending on the cell type of the culture, and the purpose for the media whether growth, differentiation, or stimulation. All cell culture techniques were done in a class II safety cabinet under sterile conditions.

2.3.1 Media for Human Primary Cell Culture

2.3.1.1 Synovial Fibroblast Growth Media

Synovial fibroblast growth media contains optimal supplements for the growth of the fibroblasts. Roswell Park Memorial Institute- 1640 media (RPMI 1640, Sigma Life Sciences UK) basal media was supplemented with 10 % Fetal Bovine Serum (FBS) (LOT 42F4487K Gibco, UK) 100 U/mL Penicillin, 100 ug/mL Streptomycin, 1 % MEM Non-essential amino acids, 1 % Sodium Pyruvate (Sigma, UK), L-glutamine (2 mM) (Gibco Life Technologies).

2.3.1.2 Chondrocyte Growth Media

Chondrocyte growth media was prepared by supplementing Dulbecco's Modified Eagle Medium – High Glucose (DMEM) (Sigma Life Sciences UK) basal media with 10 % FBS, 100U/mL Penicillin, 100 ug/mL Streptomycin, 1 % MEM Non-essential amino acids, 1 % Sodium Pyruvate (Sigma, UK), L-glutamine (2 mM Gibco Life Technologies).

2.3.1.3 Osteoblast Differentiation Media

Osteoblast differentiation media contains, Dulbecco's Modified Eagle Medium – High Glucose (DMEM) (Sigma, UK) supplemented with 10 % FBS, 100 U/mL Penicillin, 100 ug/mL Streptomycin, 1 % MEM Non-essential amino acids, 1 % Sodium Pyruvate (Sigma, UK), L-glutamine (2 mM Gibco Life Technologies), 2 mM β-glycerophosphate disodium salt hydrate (G5422 Sigma, USA), 50 ug/mL L-Ascorbic acid (A8960 Sigma, Japan) and 10 nM Dexamethasone (D-2915 Sigma, USA).

2.3.1.4 Primary Cells Stimulation Media

When stimulating cells with different cytokines all the primary cells, (i.e., synovial fibroblasts, chondrocytes, and osteoblasts) the same growth media specific for the

specific type of the cell was used except for the 10 % FBS which was replaced with 0.1 % FBS.

2.3.2 Primary Cell culture techniques

2.3.2.1 Isolation and Culture of Primary Cells from Fresh Tissues

Fresh tissues of synovium, bone and cartilage biopsies were collected from patients undergoing surgery.

2.3.2.1.1 Synovial Fibroblasts

The synovial tissue biopsy was divided into portions, one portion weighing approximately 10 gm was wrapped in sliver foil sheet and snap-frozen in liquid nitrogen and stored either at either -80 ^{o}C or under liquid nitrogen if for long-term storage. Another portion of the synovial tissue biopsy was minced (using a sterile scalpel) into small pieces measuring approximately 1 mm^3. Approximately 8 small pieces were placed into a T25 tissue culture flask containing 6 mL fresh synovial fibroblast growth media (section 2.3.1.1). Flasks were incubated at 37 ^{o}C in a humidified atmosphere containing 5 % CO_2.

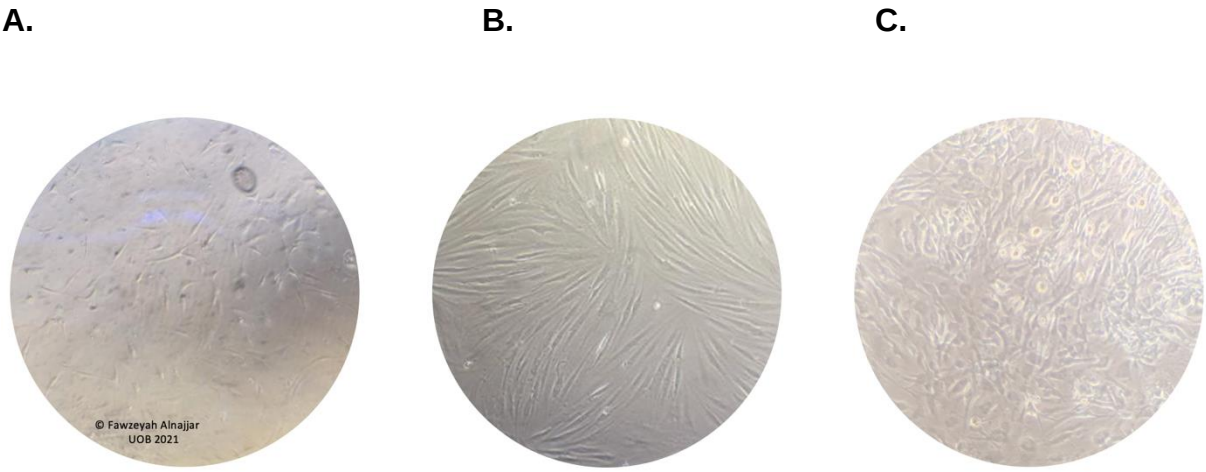


Figure 2.2 Primary Synovial fibroblast growth in a T25 culture flask containing fibroblast growth media. 1 week after seeding **(A)**, 3 weeks after seeding **(B)** and more than 85% confluency **(C).** Images taken with 10X objective.

2.3.2.1.2 Chondrocytes

Primary human chondrocytes are isolated from the fresh full-thickness articular cartilage, which was excised from the subchondral bone tissue using a scalpel. The excised cartilage was then cut into small pieces measuring $1mm^3$. The cartilage pieces were then digested in 2 mg/mL collagenase type 1A enzyme from Clostridium Histolyticum bacteria (Sigma Life Sciences UK) for 4 hr at 37 ^oC on a rotator until it was fully homogenised. The homogenate was strained with a 70 µm cell strainer and centrifuged for 5 min at 400 xg to pellet the isolated chondrocytes. The chondrocyte

pellet was washed in chondrocytes growth media (section 2.3.1.2), placed in a T25 tissue culture flask containing 6mL fresh chondrocytes growth media, and incubated at 37 ^{o}C in a humidified atmosphere containing 5 % CO_2. Primary human chondrocytes were prepared also from retrieved cryopreserved cells that have been thawed (section 2.3.4).

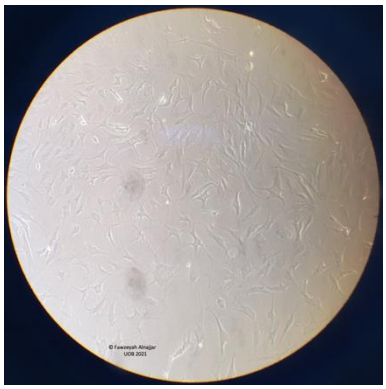


Figure 2.3 Primary chondrocytes growth from articular cartilage in a T25 culture flask containing chondrocytes growth media, 2 weeks after seeding, 10x objective.

2.3.2.1.3 Osteoblasts

Primary cell osteoblasts were obtained from subchondral bone tissue. The bone tissue was cut with a bone Friedman Rongeur tool (Integra Life Sciences, USA) into small chips sized approximately 3 mm^3. The chips were placed in a universal falcon tube containing osteoblast growth media (section 2.3.1.3) and washed for three times in

order to remove attached debris like blood and fat. Between 3-5 chips were placed in a T75 culture flask, containing 12 mL osteoblast differentiation media, and were incubated at 37 ^{o}C in a humidified atmosphere containing 5 % CO_2.

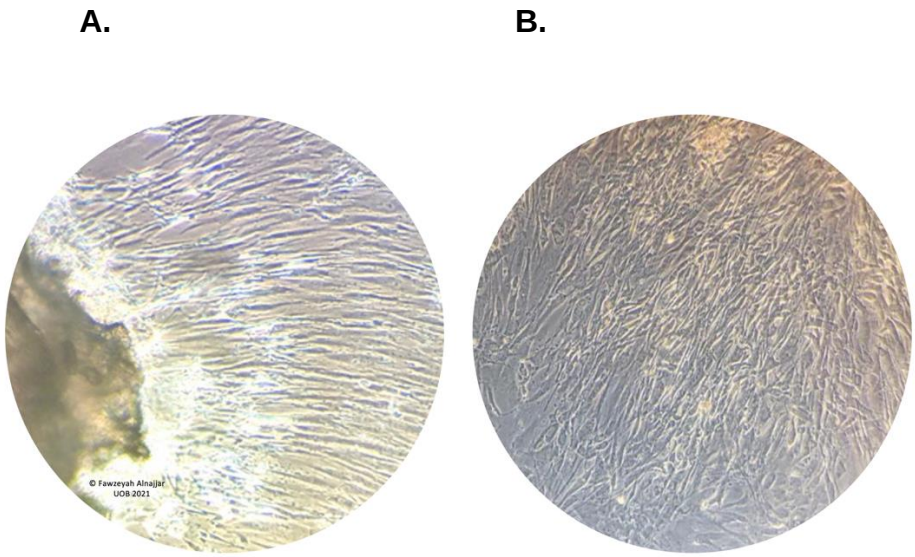


Figure 2.4 Primary Osteoblasts growth from subchondral bone in a T75 culture flask containing osteoblast differentiation media, after 2 weeks, showing early osteoblasts emerging from a bone chip and **(A)** and at more than 85% confluency **(B**), 10x objective.

2.3.3 Feeding, Splitting and Preserving cells

According to the Standard Operating Procedure Guidelines for synovial fibroblast Cell Culture (SOP) (Rheumatology Group Version 4 Nov 2006), cultured primary cells were feed every week or when the color of the media changed. Primary chondrocytes and osteoblasts were left for 5 days after seeding without changing the media, then the media was changed every 4 days. Generally, for all the cell lines flasks reaching cell confluency of 70-80 % were trypsinised with [2X] Trypsin-EDTA solution diluted 1:4 in Phosphate Buffered Saline (PBS) (Sigma, UK). At (P0) cells were cryo-preserved in freezing medium containing complete growth medium with 10 % Dimethyl Sulfoxide (DMSO) (Sigma UK) and split for expansion at (P2) at a rat of 1:3 depending on the growth rate of the cell line. At (P2-3) cells were used for experiments.

2.3.4 Retrieving Cryopreserved Primary Cells

All cell lines were recovered from liquid nitrogen by a fast-defrosting technique. Human primary fibroblasts and osteoblasts were cryopreserved at (P0). De-differentiated chondrocytes were cryopreserved at (P2) taking in consideration that de-differentiated chondrocytes lose the expression of collagen type II during prolonged cultivation.

2.4 Cytokine and Adipokine Simulation of Primary Cells

In order to mimic the *in-vivo* inflammatory processes, recombinant proteins including proinflammatory cytokines/adipokines IL-1β, & TNF-α, visfatin, and leptin were used to induce an inflammatory condition *in-vitro* (Table 2.2). Cultured primary cells (fibroblasts, chondrocytes, and osteoblasts) reaching 70-80 % confluent, were used for cytokine/adipokine stimulation.

Table 2.2 Recombinant Proteins

Rec. Protein	Source	Concentration	Sequence	Catalogue No./Company
L1β	E. Coli	1ng/mL	Unknown (153 aa residues)	19401-5UG/Sigma
TNF-α	E. Coli	10ng/mL	115 (aa N-terminal methionine)	GFH111/Cambridge Bioscience
Leptin	E. Coli	100ng/mL	Full length (aa 1-146)	GFH37-1000/ Cambridge Bioscience
Visfatin	E. Coli	500ng/mL	Full length (aa 1-491) and N-terminal His-Tag	4907-50/ Cambridge Bioscience

Rec. Protein = Recombinant protein.

2.4.1 Cytokine and Adipokine Optimization

60 x 10^3 trypsinised cells were seeded into each well of a 48 well plate (CLS3370 Corning® Merck, UK) in the relevant growth media (depending on the type of the cell) and incubated at 37^o C in an atmosphere of 5 % CO_2 for 24 hr prior to stimulation with cytokine or adipokine to allow for cell adherence. After 24 hr the media was removed and the cells were washed with PBS, and the media was replaced with stimulation media. Recombinant proteins of different concentration were diluted with the relevant primary cells' stimulation media and added to the cells (Table 2.3.) Plates were incubated at 37^o C in 5 % CO_2 atmosphere for different time slots of 2,4,6, and 24 hr. After each time slot, cell supernatant was collected and stored at -80 ^{o}C for further analysis, and the cells lysed for RNA extraction. According to the outcome of the different time slots examined, stimulation at 6 and 24 hr was selected for all the stimulation studies needed for this study.

2.5 Gene Expression Analysis

2.5.1 RNA Extraction

Cell lysis and RNA extraction was performed under standardized cell culture Class II cabinet and continued under laboratory fume cupboard. In order to ensure a pure RNA extraction without any contamination and to reduce the risk of RNA degradation specific procedures were followed each time. All surfaces on which RNA extraction to be carried on must be cleaned with a nuclease free reagent (RNaseZap®, Sigma,

USA), a set of calibrated pipettes was used only for RNA extraction procedures, and Eppendorf DNase or RNase microcentrifuge tubes (Eppendorf LoBind® tubes, UK) were used. RNA was extracted from frozen joint tissues and cells according to different experiments. The different techniques for tissue or cell's preparation for RNA extraction is detailed in the following sections.

2.5.1.1 Tissue RNA extraction

Tissues were prepared for total RNA extraction from snap-frozen OA subchondral bone by pulverising the frozen tissues into a fine powder and then using the standard TRizol® (Invitrogen-ThermoFisher, UK) RNA extraction method (Table 2.3).

Table 2.3. RNA Extraction using TRizol®

Steps	Reagents	Company
Homogenization	1mLTRizo® / 100mg tissue	Ambion Life Technologies
Phase- Separation	Phase- Separation	Sigma Life Science, USA
Precipitation	1.500µL Isopropanol /1mL TRizol®	1.Sigma Life Science, USA
	2. 2 µL Blue glycogen	2. Invitrogen ThermoFisher
Washing	1mL 75% ethanol/1mL TRizol®	VWR, BDH Chemicals, France
Resuspension	30-50 µL RNA'se-free water	Qiagen, UK

In this project 17 frozen subchondral bone tissues were collected from different joints including 8 knee, and 9 hip OA patients undergoing joint replacement surgery and were snap-frozen in liquid nitrogen and stored at -80 ^{o}C. For pulverisation, a Freezer Mill (Spex Sample Prep 6770 freezer mill, Stanmore, UK) was used. Briefly, the tank of the mill was filled to the label with liquid nitrogen and the frozen OA bone tissue pieces were placed in the cryotubes (Cryo Tubes® Merck, UK) and loaded into the mill. The tissue was pulverised into a fine powder after a 1 min pre-cooling and a 50sec cycle of 15 counts per second. Following the manufacturer's TRizol® RNA extraction protocol, for each 100 mg of powder, 1 mL of TRizol® was added into a 1.5 mL Eppendorf tube. Homogenization was facilitated by pipetting up and down for several times. The

general steps and reagents used for RNA extraction with TRizol® is detailed in Table 4. For the homogenization stage 100 mg of OA tissue powder was incubated with 1 mL TRizol® for 5 min at room temperature (RT), Chloroform was then added to the homogenate and centrifuged at10, 000 xg for 30 min at 4 ^{o}C. The aqueous phase was placed in a new tube and total RNA precipitated by adding 100 % isopropanol in addition to 2 µl of glycogen (15 mg/mL) in order to aid visualize of the RNA pellet upon subsequent steps. After overnight incubation at -20 ^{o}C in order to maximize RNA precipitation, the tube with the isopropanol suspended RNA was centrifuged at 10,000 xg for 10 min. at 4 ^{0} C. The supernatant was carefully discarded, and the RNA pellet was washed with 1 mL of 75 % Ethanol. The RNA samples were centrifuged again at 8000 xg for 5 min. at 4 ^{0}C, and the supernatant was discarded. RNA pellets were dried in a vacuum desiccator jar (DN150 DURAN, DWK Life Sciences) for approximately 20 min, and then re-suspended in 30-50 µl nuclease-free water.

2.5.1.2 RNA Extraction from Primary Cells

RNA was extracted from primary cells (fibroblasts, chondrocytes, and osteoblasts) using Rneasy mini columns kit (Qiagen, UK). To lyse the primary cultured cells a 70-80 % confluent primary cell culture flask was prepared for RNA extraction by removing the culture media and freezing it at -20 ^{o}C or at -80 ^{o}C for long term storage and subsequent protein analysis of secreted proteins. 350 µL/ well RLT buffer (Qiagen, UK) was added to each well in a 48 well plate, left for 5-10 min for the cells to lyse. The wells were scraped with a pipette tip to encourage cell lysis, and the cell lysates

were harvested in RNase free microcentrifuge tubes and RNA was extracted according to the manufacturer's protocol. Extracted RNA was suspended in 30-50 µL nuclease-free water.

2.5.1.3 RNA Quantification

Total RNA extracted was quantified by using Nano-Drop 2000 Spectrophotometer (ThermoFisher Scientific, USA). RNA concentration was measured at two different absorbance: 260 nm and 280 nm. 260/280 Ratios of 1.7 - 2 were regarded suitable for further gene analysis.

2.5.2 qRT-PCR

The relative expression of selected novel lncRNAs and specific mRNA were measured by Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) technique. Total RNA extracted from tissues or cells was further purified from any traces of DNA contamination using RNase-Free DNase kit (CAT No. 79254 Qiagen), In brief, and following the manufacturer's protocol any remining DNA was digested during the RNA extraction with reagents optimised for on-column DNA digestion including DNase 1 enzyme (1500 Kunits units) and RDD buffer, which were used for 15 min at 20-30 ^{0}C. A negative control, template free RNA was run for each primer

(Table 2.4) and primers were normalised to a housekeeping gene 18S (human, ThermoFisher Live Technologies). All the reactions were run separately in triplicates to ensure a statistically reliable result. The qRT-PCR reactions were performed using two detection chemistry SYBR Green qRT-PCR reagents (Bio-Rad), and TaqMan (ThermoFisher, UK). For the SYBR green chemistry, iTaq universal SYBR green one-step kit was used (Bio-Rad, UK).

Table 2.4 qRT-PCR Primer Sequence Details

Gene Symbol	Primer Sequences: 5`-3`	Amplicon Length (bp)	TM (°C)	GC%
LRCH3	F. GCAGCTCTAACTGACGGTGT	164	F.60	F.55
	R. AGAAATGTCTGTCCACTGTGCT		R.59	R.45
LINC02289	F. GAAGCATTTCCCCTTCCCCT	164	F.59	F.55
	R. GCTGTACTCAGTCGAAGCGT		R.60	R.55
PI4KB	F. GGCCGGAGCAAGTTTTGAAG	225	F.60	F.55
	R. CCCTCTTGTCCCATGCCAAT		R.60	R.55
PRPF8	F. AAAGCGGCCTCTTGTGTGAG	157	F.60	F.55
	R. TGCTGCCATTTTCGAGCTTTC		R.60	R.47
PTGS2	F. GGCCATGGGGTGGACTTAAA	181	F.59	F.55
	R. ACCGTAGATGCTCAGGGACT		R.60	R.55
NAIF1	F. ACCCTGACACAGATCCCCACA	175	F.62	F.57
	R. TGAGAGCAATGCGGCTCTTG		R.61	R.55
RP9	F. GGGAGAAAGCAACTTGATGGA	140	F.58	F.47
	R. GCACCTACTTTGGGAAAACACT		R.59	R.45
TNFSF12	F. AGCAGTGGTGGTATGGATGAAA	202	F.60	F.55
	R. ATGCCTGCTTCTCCCAAAGAAT		R.62	R.52

(F = Forward, R = Reverse)

Briefly, using a thermal cycler (C1000 Touch Thermal Cycler, Bio-Rad) and a 384 well plate (Bio-Rad, UK) 5 µL per well of a mixture of reagents was used, containing 1 µL template RNA (5 ng/µL), 2.5 µL master mix (containing DNA polymerase, buffers and

normalization dyes), 0.1 µL each of forward and reverse primers (customized primers Merck Life Science UK Limited) 0.06 µL reverse transcriptase enzyme, and 1.24 µL RNA free water. For the TaqMan detection reagents, the 5 µL mixture per well contains 1 µL template RNA (5 ng/µL), 2.5 µL master mix, 0.1 µL each of forward and reverse primers, 0.05 µL probe (validated probes ThermoFisher, UK), 0.06 µL reverse transcriptase enzyme, and 1.24 µL RNA free water. The run protocol included the following steps: in the first step Reverse Transcription run for one cycle of 10 min. at 50 ^{0}C, the second step Polymerase Activation run for one cycle for 1 min. at 95 ^{0}C, and the third step included 40 cycles of denaturation and annealing, denaturation for 10 sec. at 95 ^{0}C, and annealing for 30 sec. at 60 ^{0}C. All the relative expressions of genes were measured using the threshold cycles (CT), ΔCT formula:

$$\Delta CT = CT\ (template\ gene) - CT\ (housekeeping\ gene)$$

, and the relative fold gene expression was measured using the Livak-Scmittgen equation of $2^{-\Delta\Delta CT}$.

$$\Delta\Delta CT = highest\ \Delta CT\ (template) - \Delta CT\ (template)$$

2.5.3 LncRNA Primer Design

Integrative Genomics Viewer (IGV) software (©2013-2018 Broad Institute University of California, USA) was used to assess the mapping quality of transcripts of the following

long non-coding RNA (lncRNA): RP11-367F23.2, RP11-863P13.3, LINC01616, RP11-79H23.3, RP11-147L13.15, RP11-631N16.2, LINC01123, AF131217.1, MIR155HG, CARMN, LINC01021, MALAT1, RP11-362F19.1, AC068282.3, LINC01503, LINC01705, RP11-392O17.1, LINC00511, RP5-1086K13.1, MEG3. The sequencing mapping data from RNA sequences were analysed in reference to the human genome HG38, according to RNASeq: IGV Analysis Protocol v1, 2018, Mark J Pearson. (Figure 2.5).

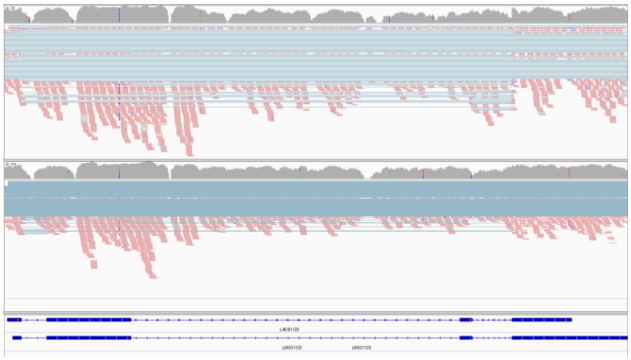


Figure 2.5 IGV software mapping of Transcriptomic Sequences relative to Human Genome HG38.

The sequence of the whole transcript was first defined, including individual exons. Then by looking at the ncRNA, the nearest coding gene was defined. Finally, specific regions for the primer design were defined; the prices chromosomal location for the selected exon was taken. Using the UCSC Table Browser

(http://genome.ucsc.edu/cgi-bin/hgTables), the specific sequence for the selected location was downloaded and primers for specific lncRNA were designed using Primer Express 3.0 software, (Applied Biosystems, ThermoFisher Scientific, UK). Designed primers were ordered from ThermoFisher, UK. For the customized primers, the Ref Seq for each primer was searched in the National Centre for Biotechnology Information (http://ncbi.nlm.nih.gov), a blast search was performed using the (blast.ncbi.nlm.nih.gov) site and primers were picked according to the location of the desired primer ensuring that it crossed an exon boundary. Selected primers were checked in the UCSC browser. Forward and reverse sequences were sent to (Merck Life Science UK Limited) to be synthesised.

2.6 Enzyme-Linked Immunosorbent Assay

Solid Phase Sandwich Enzyme-Linked Immunosorbent Assays (ELISA) of pre-validated kits (DuoSet ELISA Development system R&D systems, USA) were used to measure the concentration of human target proteins in different primary cell supernatants (fibroblasts, chondrocytes, osteoblasts) stimulated with or without cytokines. All antibodies used are tabulated in Table 2.5.

Table 2.5 Antibodies used to Detect Human Target Proteins

Target	Full name	Antibodies	Company
IL-6	Interleukin -6	Mouse anti-human IL-6 capture antibody, Biotinylated Goat Anti-Human IL-6 detection antibody	R&D systems USA
OPG	Osteoprotegerin	Mouse anti-human OPG capture antibody, Biotinylated Goat Anti-Human OPG detection antibody	R&D systems USA
TRANCE/RANKL	Tumor Necrosis Factor -related activation-induced cytokines/ Receptor activator of nuclear factor kappa-B Ligand	Mouse anti-human TRANCE capture antibody, Biotinylated Goat Anti-Human TRANCE detection antibody	R&D systems USA

In brief, following the manufacturer's protocol briefly, a 48 well microplate was coated with a capture (target specific) antibody overnight. Background antigens were blocked by adding 300 µL of Reagent Diluent (1 % Bovine Serum Albumin (BSA) in Phosphate Buffer Saline (PBS) to each well and incubating for 1 hr at RT. Diluted standards or samples were added to the wells, and this was sandwiched with incubation with 100 µL of diluted secondary (detection) antibody horseradish-peroxidase (Streptavidin HRP) labelled for 2 hrs at RT. 100 µL of the substrate solution was added and

incubated away from direct light for 20 min. 50 µL of stop solution was added, and the microplate was read immediately, using a microplate reader (Synergy-2 Bio-Tek, Bedfordshire, UK) set to OD450 nm and readings were corrected at OD550 nm . Unknown sample concentrations were extrapolated from a standard curve using GraphPad Prism Software v9 (GraphPad software, La Jolla, USA).

2.7 Western Immunoblotting

Proteins in cell lysates were analysed by western blotting technique. In General, total proteins were isolated from cell lysates, and the concentration of the proteins in the cell lysates were determined. The samples and gels were prepared, and proteins were blotted in a Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Next, proteins on the gel were transferred onto a supporting membrane, the membrane was incubated with primary and secondary antibodies. The bands on the membrane were detected on the site of antigen antibody complex formation. Finally, the bands were visualized imaged, and analysed compared to an internal control.

2.7.1 Protein Extraction from primary cells

Protein extraction should be as quick as possible and performed on ice and using buffer containing a protease inhibitor cocktail to reduce the risk of protein degradation

during extraction. Total proteins were isolated from cultured primary cells using 1 mL for (0.5-5 x 10^7) cells of RIPA (Merch Life Scientific UK, Limited) cell lysing buffer containing (50mM Tris-HCl, 150mM Sodium chloride, 1.0 % Igepal CA-630 (NP-40), 0.5 % sodium deoxycholate, and 0.1 % sodium dodecyl sulfate), after 10 min cells were scraped and harvested in a microcentrifuge tube which was frozen at -80 ^{o}C for future use.

2.7.2 Quantification of Total Protein Concentration in Cell Lysates

Total proteins concentration in cell lysates prepared in RIPA (section 2.6.1) were calculated using a BCA protein assay (CAT No.23225 Pierce^{TM} BCA Protein Assay Kit ThermoFisher, UK) containing a detergent of bicinchoninic acid (BCA) for a colorimetric total protein detection. Following the manufacturers protocol, total proteins were quantified against a serial dilution of standards of known protein concentration.

2.7.3 Electrophoresis and Immunoblotting

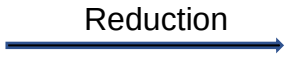
SDS-PAGE gel plates were prepared according to the amount of proteins available and were cast in a loading cassettes (Novex Mini Cell Electrophoresis Champers Invitrogen, UK) with 10 % separating gel containing: 10.0 mL Protogel, 12.5 mL ddH_2O, 7.5 mL (1.5M Tris HCL pH 8.8), 150 µL 10 % APS, 150 µL 20 % SDS, and 30 µL

Temed. 5 % of stacking gel was added on top of the set separating gel, containing: 2.6 mL Protogel, 12.2 mL ddH_2O, 5.0 mL (0.5M Tris HCL pH 6.5), 100 µL 10 % APS, 100 µL 20 % SDS, and 20 µL Temed. 15 µg total proteins of known concentrations were equally prepared in a ratio of 3:1 by mixing with 4x Laemmli sample buffer (Bio-Rad, USA) and was boiled at 100 ^{0}C for 10 min. The protein samples were loaded against a molecular weight marker (PageRuler^{TM} Ladder, Thermo Scientific, Lithuania). Current of (150 V 50 mA) was applied to the electrophoresis cell for 1 hr. Proteins were then transferred to a methanol pre-activated polyvinylidene difluoride (PVDF Bio-Rad, UK) membrane for 1.5 hr. Total proteins were validated PVDF membranes were incubated in 25 mL blocking solution of 3-5% BSA in Tris-buffered saline (TBS-T) and 0.1 % tween 20 (Sigma, USA) on a shaker for 1 hr at RT. The membrane next was incubated with optimized concentration of Rabbit primary antibody with a dilution of 1:1000 overnight at 4 ^{o}C on a shaker. After washing in TBS-T, the membrane was incubated in the anti-rabbit secondary antibody linked to horseradish peroxidase of 1: 5000 dilutions for 1 hr at RT on a shaker. Total proteins in the bands were detected using a sensitive enhanced chemiluminescent detection reagent ECL (Amersham^{TM} ECL Prime Western Blotting Detection Reagent GE Healthcare UK, Limited), at a ratio of 1:1 for 3 min (section 2.6.2). Luminescence produced by the reaction was visualised and imaged by the ChemiDoc^{TM} MP imaging System (Bio-Rad, UK). Images were analysed and bands were quantified using the Image Lab software (Life Science Research Bio-Rad, UK). For accurate western plotting, the results obtained were normalized to β-Actin as an internal control (Invitrogen, UK).

2.8 Proliferation Assay

To measure fibroblast cell proliferation rate, a colorimetric Di-Methazole Tetrazolium (3-(4,5dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (MTS) reagent kit was used (CellTiter 96® Aqueous One Solution kit Promega, USA). In this method metabolically active viable cells reduces the tetrazolium reagent by the NAD(P)H- dependent dehydrogenase enzymes located in the mitochondria of viable cells, generating a fluorescent product (formazan) in the supernatant. Following the manufacturer's protocol, cells were incubated with 20 µL of MTS reagent kit for 2 and 4 hr at 37 ^{o}C in 5 % CO_2 atmosphere. Next, the optical absorbance was measured at 490 nm using a plate reader (Synergy HT bio-teck, NorthStar Scientific LTD).

3-(4,5dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium



Coloured Formazan

Unknown concentrations were extrapolated from the MTS standard curve using Graph-pad Prism v9 (GraphPad software, La Jolla, USA).

2.9 MALAT1 Knockdown

To study the function of MALAT1 in the regulation of the genes responsible for protein expressions during inflammatory processes in OA, loss of function (LOF) approach was used. Small interfering RNA (siRNA) was used to transfect cells using a lipid delivering system to knockdown MALAT1. DNA-lipid complexes were prepared according to the manufacturer's instructions using lipids Lipofectamine 2000, 3000, and TransIT-X2® in a serum-free medium Opti-MEM (Gibco ThermoFisher, UK), Small interfering RNA (siRNA) were diluted in Opti-MEM. siRNA used for this project included non-targeted negative control (NTC) antisense LNA GapmeR standard negative control (LG00195873-DDA GeneGlobe Qiagen, UK), Human MALAT1 antisense Locked Nucleic Acid (LNA) GapmeR *invitro* premium (catalogue Design ID: 313359-9 GeneGlobe Qiagen, UK). DNA lipid complexes were added to the cells according to the lipid used, and the plate was incubated at 37 ^{0}C in a humidified atmosphere containing 5 % CO_2 for 24 hr. After the 24-hr transfection some cells were stimulated with IL-1β (1ng/ml) and incubated for an additional 4 hr at 37 ^{0}C in a humidified atmosphere containing 5 % CO_2, and some cells were left unstimulated. The percentage of MALAT1-KD gene expression was calculated from the following two formulas:

1- % MALAT1-KD relative to NTC = (100/ RE NTC) x RE MALAT1

Relative Expression (RE) $2^{-\Delta CT}$

2- % MALAT1-KD = 100 – (% MALAT1-KD relative to NTC)

2.10 RNA Sequencing

The transcriptomes quality was checked using Lexogen QuantSeq (Lexogen GmbH, Vienna Austria). RNA integrity was determined using Agilent Bioanalyser (California, USA) and RIN of ≥ 7 were selected to run the QuantSeq FWD kit protocol. 3` mRNA-Seq Illumina compatible libraries of sequences at the 3`polyadenylated reflecting the mRNA sequence were prepared. Using the next-generation sequencing (NGS) high-throughput, single reads of 76bp long were prepared. DeSeq2 bioinformatic software (Bioconductor, Open-source Software for Bioinformatics 2003-2021) was used to analyse the differential expression of different genes.

2.11 IPA Analysis

RNASeq were interpreted using Ingenuity Pathway Analysis software (IPA Qiagen, UK). Different pathways that were impacted by MALAT1-KD were analysed and upstream regulators and downstream effecters were predicted. Important control targets that may lead to therapeutic intervention were identified. The most common cellular processes and functions were highlighted and the pathological diseases in which these processes may contribute to were listed. Networks connecting important proteins in this study were determined. The connection between these networks and the pathological networks were established looking for possible causes related to OA initiation and progression. Differential expressed genes (fold change of ± >1.5, $p < 0.05$) were amended for further analysis.

2.12 Osteoblast Functional Studies

OA osteoblasts formation and differentiation were studied by analysing the metabolomics of the cultured osteoblasts. Mineralization i.e., calcium deposits in osteoblasts was detected by staining with Alizarin Red stain (AZR) ($C_{14H}7NAO_7S$) (CAT 130-22-3 Sigma, UK) and matrix maturation analysed by Alkaline Phosphatase Activity Assay (ALP). ALP is a liquid substrate containing p-Nitrophenyl phosphate (p-NPP) obtained from (Phosphatase, Alkaline, human placenta - CAT 524604 EMD Millipore Corp, USA) catalysis the following reaction:

p-NPP ($C_6H_4NNa_2O_6P$) + H_2O ⟶ p-nitrophenol ($C_6H_5NO_3$) + Pi

producing a colourful product which can be measured. The expression of proteins secreted during matrix proliferation including OPG, RANK/RANKL, were detected using ELISA of the supernatant of the MALAT1-KD osteoblasts. Primary osteoblasts used for these experiments were obtained from three OA osteoblasts including an OB hand, OB wrist and OB foot. All experiments were run independently and repeated three times.

2.12.1 Osteoblasts Mineralization Alizarin Red Staining

6 $x10^4$ per well OA osteoblasts were seeded in a 24 well plate in an osteoblast differentiation media (section 2.3.1.3) and cultured at 37 ^oC in 5 % CO_2 atmosphere for 21 days. The media was changed twice a week, and cell supernatants were collected

and frozen at -80 ^{o}C for different protein analysis in future. Osteoblasts were transfected with MALAT1 LNAs (section 2.9.2) twice a week, RNA extracted and MALAT1-KD was validated with qRT-PCR (section 2.8.1, 2.8.2). At day 21 osteoblast's differentiation was studied by the degree of mineralization produced at different nodes formed. The mineralized nodes were stained using alizarin red solution (0.5% AZR in 1% ammonia hydroxide at pH 4.5). Plates were incubated for 10 min at RT, and cells were washed with PBS. The reaction was stopped by incubating the osteoblasts in 10% cetyl pyridinium chloride ($C_{21}H_{38}ClN *H_2O$) (CAT 204-593-9 Merck Life sciences UK Limited) for 10 min. In order to quantify the osteoblast mineralization, the supernatant was collected, diluted 1:10 with 10% cetyl pyridinium chloride and absorbance was read at OD550 nm on a microplate reader. (Synergy-2 Bio-Tek, Bedfordshire, UK). Unknown sample concentrations were extrapolated from a standard curve using GraphPad Prism Software v9.0 (GraphPad software, La Jolla, USA).

2.12.2 Alkaline Phosphatase Activity Assay

ALP activity in MALAT1-KD primary osteoblasts were assayed at day 21 by lysing the osteoblasts in RIPA buffer diluted (1:5 with 1mM $MgCl_2$ and H_2O). Osteoblast lysates were diluted (1:5 in 1mM $MgCl_2$) and combined with diluted ALP to (100Units/mL in 1mM $MgCl_2$) and incubated at 37 ^{o}C in 5 % CO_2 atmosphere for 15 min. 20µL of 0.1N NAOH was added to each well in the plate to stop the reaction. Standards with known ALP concentration of 0.3Units/mL in 1mM $MgCl_2$ were serially diluted 1:2 or serial

dilution 1:2 of RIPA (+ 1mM MgCl2) were used to detect unknown ALP sample's concentration. The absorbance was read at OD405 nm on a microplate reader (Synergy-2 Bio-Tek, Bedfordshire, UK). The unknown sample concentrations were extrapolated from a standard curve using GraphPad Prism Software v9.0 (GraphPad software, La Jolla, USA).

2.12.3 OPG, RANK/RANKL Detection

6 x10^4 per well OA osteoblasts were seeded in a 24 well plate in an osteoblast differentiation media (section 2.3.1.3) and cultured at 37 ^oC in 5 % CO_2 atmosphere for 21 days. The media was changed twice a week, and cell supernatants that were secreted during primary osteoblast differentiation, were collected to detect OPG (Human OPG/TNFRSF11B CAT No. DY805 R&D Systems, USA), RANK/RANKL (Human TRANCE/RANKL/TNFSF11 CAT No. DY626 R&D Systems, USA) proteins using ELISA approach (section 2.5). The activity of MALAT1KD in osteoblasts expressing OPG was analysed by collecting cell supernatant after a short period of 24 hr of osteoblast MALAT1-KD and at longer periods of 4,7,10,14,17, and 22 days. Anti-human OPG was incubated with osteoblast's cell culture supernatant in a sandwich ELISA according to the manufacturers protocol. A seven-point standard curve was generated from recombinant human OPG standard starting from 4000 pg/mL. Unknown sample OPG concentrations were extrapolated from the standard curve using GraphPad Prism v9.0 software (GraphPad software, La Jolla, USA).

2.13 Data Handling and Statistical Analysis

All data in this study were analysed by GraphPad Prism Software v9.0 (GraphPad software, La Jolla, USA). The protocol through which I followed in analysing the data was as follows: Data sets were first checked for normal Gaussian distribution using one of the following normality tests D'Agostino-Pearson omnibus, Shapiro-Wilk, and Kolmogorov-Smirnov normality test with Dallal-Wilkinson-Lilliefor P value. Descriptive data analysis was done by calculating the mean, standard deviation, and standard error of mean (SEM). All data in this study were tabulated as ± SEM. For comparing two parametric data unpaired t test was used, and Mann-Witney U test for comparing the non-parametric data. For data sets containing more than two groups an ordinary one-way analysis of variance (ANOVA) was used to analyse the difference between the groups and for comparing the means of preselected pairs, a post hock Bonferroni test was used. A two-way ANOVA was used for analysing data sets having more than one independent categorical variables, and Bonferroni post hock test was used to rule out type one error when comparing groups. To study the correlation of two variables simple linear regression Person correlation was used. The confidence level for all the statistics used in this study was >95 %, with statistically significance test having p value of <0.05.

CHAPTER 3

LncRNA Profile in OA Synovial Fibroblasts

3. Expression Analysis of LncRNAs in OA Patient Synovial Fibroblasts

3.1 Background

The fast-growing knowledge in the area of ncRNA has led to the implication of their role in the initiation and/ or progression of OA. Many researchers have shown a role for lncRNA in the epigenetic regulation of different genes that contribute to the pathogenesis of many diseases including OA. Given the role of inflammation in OA, understanding the functional role of lncRNAs and epigenetic regulation of the cellular inflammatory responses have become an important area of research. Currently, it is established that both factors: inflammation and ncRNA epigenetic regulation of gene expression contribute to the development of OA [255,256]. In general, both types of the regulatory ncRNAs*:* small and long ncRNAs were found to regulate inflammatory responses in cartilage [257,258]. Molecular mechanisms such as epigenetic regulations of gene expression by lncRNA of chondrocytes and inflammatory mediators, has been intensely studied over the last 15 years [255,256].

Inflammatory mediators released as a consequence to cartilage degradation may be regulated by lncRNA. Examples of some lncRNAs that drive the production of cytokines during the inflammatory reactions in OA cartilage include PACER, CILinc01, CILinc02 [257], HOTAIR, GAS5, PMS2L2, RP11-445H22.4, H19, AND CTD-2574D22.4 [256]. However, the mechanism of action of these lncRNAs in the initiation or progression of OA is not clear yet. To investigate the potential role of lncRNAs in mediating OA joint inflammation in this study we examined the expression of a panel of 20 lncRNAs that our group had previously identified by RNASeq in synovial fibroblasts in a cohort of patients with hip OA [198].

The expression of the lncRNAs were evaluated by examining their genomic location and FPKM (Fragments Per Kilobase of transcript per Million mapped reads) using Integrated Genome Viewer, and by confirming their expression by qRT-PCR in synovial fibroblasts from hip OA patients who were either OB (inflammatory) or of NW (less inflammatory). The 20 lncRNAs selected were: AC068282.3, MALAT1, RP11-362F19.1, LINC01123, AF131217.1, CARMN, MIR155HG, LINC01503, LINC01705, RP11-392O17.1, LINC00511, RP5-1086K13.1, MEG3, LINC01021, RP11-631N16.2, RP11-367F23.2, RP11-863P13.3, LINC01616, RP11-79H23.3, RP11-147L13.15.

3.2 Methods

The mapping quality of the novel lncRNA was analysed using the FPKM for each lncRNA and the genomic location was used to determine the nearest upstream and downstream protein coding genes in order to predict a possible regulatory mechanism of the lncRNA under investigation. In an attempt to study the regulation exerted by lncRNAs on OA primary cells from different parts of the joints in the body, the expression of selected lncRNAs in OA synovial fibroblasts was quantified by qRT-PCR using RNA extracted from 6 OA hip samples consisting of 3 NW and 3 OB OA synovial fibroblasts. The mean age in males was 73.50 ± 4.5 and 76.67 ± 6.84 in females. The mean W:H ratio in males was 1.004 ± 0.023 and 0.79 ± 0.009 in females. Table 3.1 shows the demographic (gender and age) and anthropometrics (WHR) data for the samples used. IGV software was used for analysing the transcriptomic sequence obtained from OA fibroblasts, locating the nearest coding gene to the lncRNA

sequence under study, and identifying exon sequences within the target lncRNA suitable for qRT-PCR primer design.

Table 3.1 Demographic data for patient samples used in LncRNA Analysis.

Sample	BMI	Joint	Gender	Age	WHR[1]
MFX194	NW[2]	Hip	M[4]	78	1.026
MFX157	NW	Hip	F[5]	84	0.813
MFX182	NW	Hip	F	63	0.783
MFX176	OB[3]	Hip	M	69	0.981
MFX137	OB	Hip	F	83	0.766
MFX149	OW	Hip	F	63	0.796

[1]WHR=Ratio of waist to hip circumference,[2]NW=Normal weight,[3]OB= obese, [4]M= male,[5]F= female.

Data are represented as ±SEM.

3.3 Results

3.3.1 IGV Data of Mapping Genomic Location of different LncRNA in OB and NW Cohorts.

In total, 20 lncRNAs were significantly expressed (>1.5-fold change, $P<0.05$) in RNASeq obtained from 3 OB OA compared to 3 NW OA synovial fibroblasts. The selected lncRNAs were mapped by IGV with fold change ranging from 3.01 to -3.06, and primers were designed for further validation. For chromosomal location see Appendix (Table 9.2). Amongst the upregulated lncRNAs, two showed the highest

FPKM (Table 3.2) including: Maternally expressed 3 (MEG3) a nuclear gene with FPKM 57.289, fold change 1.514, $p<0.024$ (Figure 3.1.A). The closest upstream gene is Delta Like Non-Canonical Notch Ligand 1 (DKL1) and many miRNAs downstream. The second highest FPKM lncRNA is MALAT1 with FPKM 41.399, fold change 2.111, $p<0.0035$ (Figure 3.1.B).

The top three upregulated lncRNAs with highest fold change include: RP11-79H23.3, Rp11-362F19.1 and CARMN. RP11-79H23.3 with a FPKM 1.229, fold change 3.01 and $p< 0.0002$ (Figure 3.1.C). Two lncRNA lies close to RP-1179H23.3, PKIA lies downstream and IL7 upstream [259]. Rp11-362F19.1 (LINC02432) shows FPKM 1.18, fold change 2.24 and $p<0.01$ (Figure 3.1D), CARMN shows FPKM 1.57, fold change 1.97 and $p<0.00005$ (Figure 3.1.E). It *trans*-regulates IL7b which lies upstream and on the opposite strand of CARMN and downstream lies the gene networks regulating cardiac mesodermal functions [260].

The three most downregulated lncRNAs include: AC068282.3, lincRNA 01705 (LINC01705) and AF131217.1. AC068282.3, a novel transcript *trans*-regulates MAPK3K2 as an antisense, FPKM -0.568, fold change log2 -3.46 and $p<0.007$ (Figure 3.1.F). It *Cis*-regulates closest upstream gene MAP3K2 and 18kb downstream Protein C Inactivator of Coagulation Factors Va and VIIIa (PROC). LINC01705 (RP11-400N13.3) FPKM -1.43, fold change -2.59 and $p<0.01$(Figure 3.1.G). It *Cis*-regulates the closest upstream gene LINC02474 and downstream AL356108.1. AF131217.1… FPKM -9.364, fold change log2 -2.261 and $p<0.00005$ (Figure 3.1.H).

Table 3.2 FPKM and Fold change of selected LncRNAs differentially expressed in OB compared to NW OA synovial fibroblasts.

Gene Name	Fold Change Log2	*P*-value	FPKM
RP11-79H23.3	3.01	***0.0002***	1.229
RP11-362F19.1	2.24	***0.01***	1.18
CARMN	1.97	***0.00005***	1.57
RP5-1086K13.1	1.877	***0.013***	0.28
RP11-863P13.3	1.76	***0.01***	1.926
LINC01123	1.64	***0.02***	0.48
MIR155HG	1.58	***0.0005***	5.86
LINC01616	1.41	***0.04***	0.569
MALAT1	1.08	***0.0035***	***41.3993***
RP11-631N16.2	0.86	***0.004***	1.965
LINC01503	0.76	***0.03***	0.03
MEG3	0.59	***0.024***	***57.298***
LINC00511	-0.77	***0.036***	-2.11
LINC01021	-1.43	***0.01***	-1.845
RP11-147L13.15	-1.49	***0.025***	-0.514
RP11-392O17.1	-1.67	***0.04***	-1.24
RP11-367F2.32	-1.76	***0.01***	-1.207
AF131217.1	-2.26	***0.00005***	-9.364
LINC01705	-2.59	***0.01***	-1.43
AC068282.3	-3.46	***0.007***	-0.56

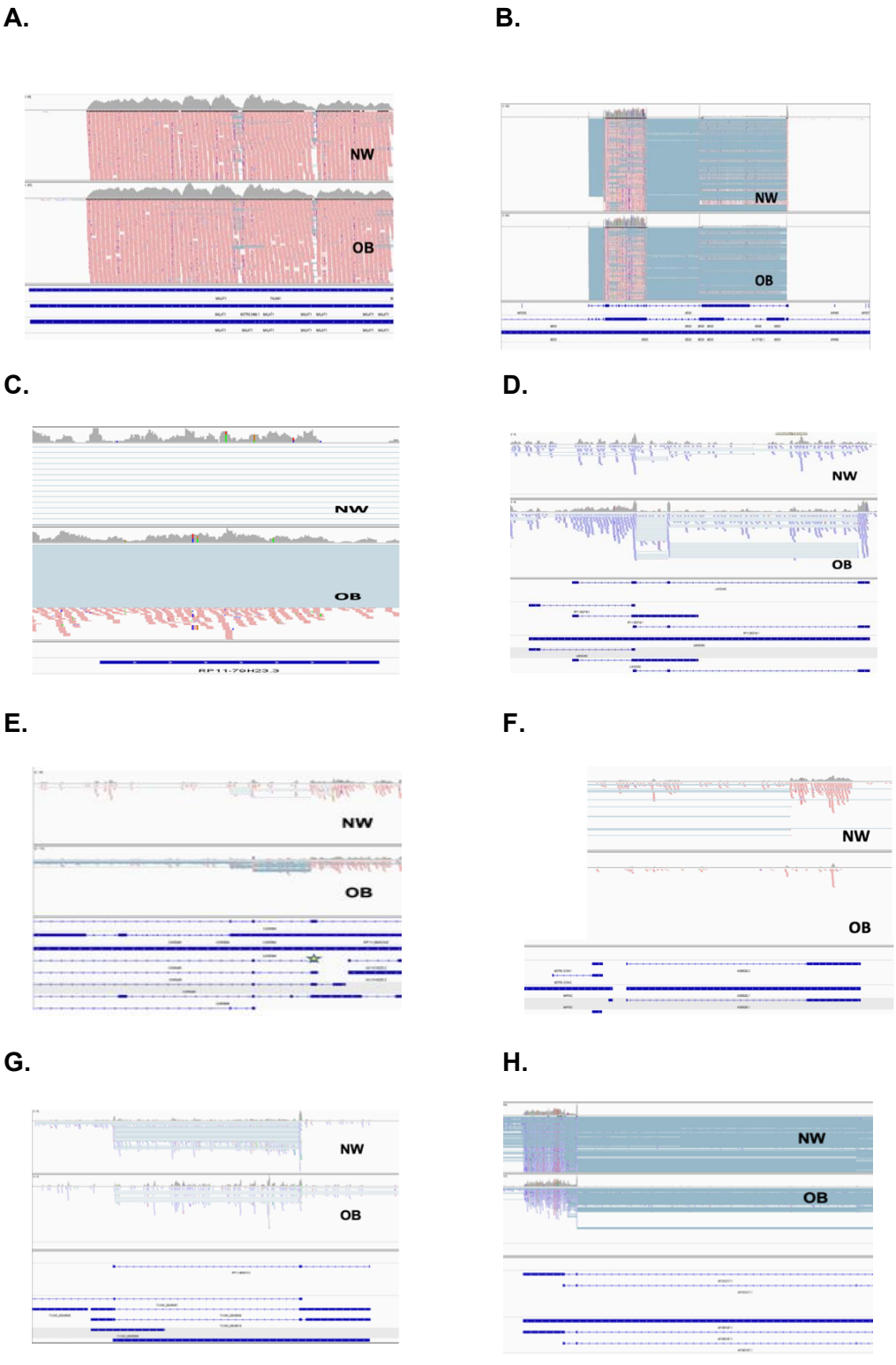


Figure 3.1 IGV analysis of lncRNAs in patient synovial fibroblasts. Highly expressed lncRNA in OB OA compared to NW OA: **1-** Upregulated lncRNA: MALAT1 **(A),** MEG3 **(B),** RP11-79H23.3 **(C),** RP11-362F19 **(D),** CARMN Exon4 **(E)**. **2-** Down-regulated lncRNA: AC068282.3 **(F),** LINC01705 and **(G),** AF131217.1 Exon 4 **(H)**.

3.3.2 qPCR Validation of Differentially Expressed LncRNA in OB and NW Fibroblasts.

The next step was to validate the expression of selected lncRNAs with qRT-PCR mRNA including: LINC01123, CARMN, RP11-79H23.3, RP11-631N16, AC068282.3, RP11-86P13P.13, MALAT1, AF131217.1 and LINC01705 (Table 3.3).

Table 3.3 mRNA Expression of selected lncRNAs in OB compared to and NW OA synovial fibroblasts, obtained by qRT-PCR.

Gene Name	Fold Change Log2	*P*-value
LINC01123	2.563	***0.04***
CARMN	2.318	***0.03***
RP11-79H23.3	2.235	0.16
RP11-631N16	1.498	0.6
AC068282.3	1.269	0.8
RP11-86P13P.13	1.21	0.4
MALAT1	1.118	0.99
AF131217.1	0.287	0.19
LINC01705	0.215	0.3

A significant relative expression was seen in two lncRNAs in OB compared to NW OA synovial fibroblasts in addition to showing the highest fold change of mRNA including LINC01123, $P < 0.04$, fold change 2.563 (Figure 3.2.I.A) and CARMN P <0.03, fold change 2.318 (Figure 3.2.I.B). LncRNA RP11-79H23.3 (Figure 3.2.I.C) showed fold change > 2, but the relative expression was not statistically significant in OB compared to NW OA synovial fibroblasts. The upregulated lncRNAs RP11-631N16 (Figure 3.2.I.D), RP11-86P13.3 (Figure 3.2.I.E), and MALAT1 (Figure 3.2.I.F) showed fold changes >1. On the other hand, the downregulated lncRNAs AC068282.3 (Figure 3.2.II.A), AF131217.1 (Figure 3.2.II.B) and LINC01705 (Figure 3.2.II.C) showed low fold change expression.

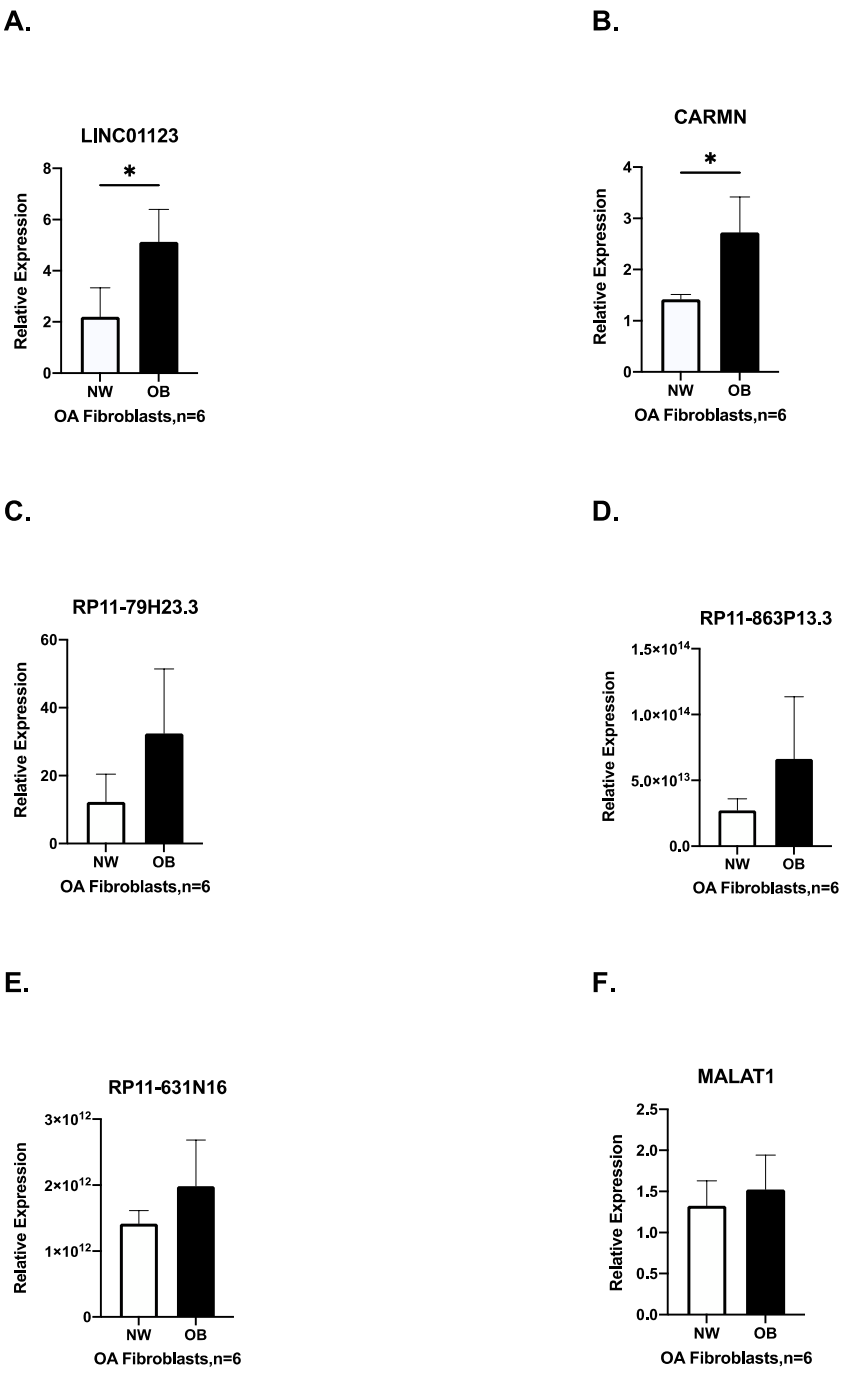
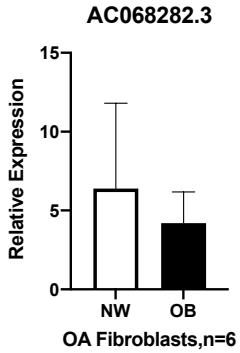


Figure 3.2.I Selected upregulated lncRNAs showing relative expression in OB compared to NW OA synovial fibroblasts measured by qRT-PCR. Two of the lncRNAs only showed significant difference in expression between NW and OB synovial fibroblasts including: LINC01123 EXON 2 *P*<0.04 **(A),** CARMN Exon 4 *P*<0.03 **(B),** while the rest of the upregulated lncRNAs expression were not significantly different in NW compared to OB synovial fibroblasts, RP11-79H23.3 **(C),** RP11-86P13P.13 **(E),** MALAT1 **(F)**.

A.



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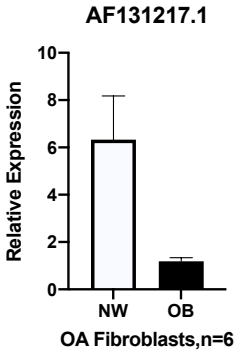

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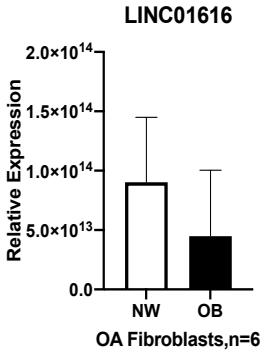


Figure 3.2.II Selected downregulated lncRNAs showing expression in OB compared to NW OA synovial fibroblasts, measured by qRT-PCR. AC068282.3 **(A),** AF131217.1 Exon 1 **(B),** LINC01705 **(C)**. None of the down-regulated lncRNAs showed any significant difference in expression between NW and OB synovial fibroblasts from OA patients.

In order to confirm the compatibility of the data extracted from the two approaches qRT-PCR and RA-Seq, next the correlation between the fold changes in lncRNAs relative expression obtained was analysed. A significant correlation was seen between the two approaches with $P < 0.02$. Although the correlation coefficient was not perfect $R^2 = 0.55$, but it was a positive correlation (Figure 3.3).

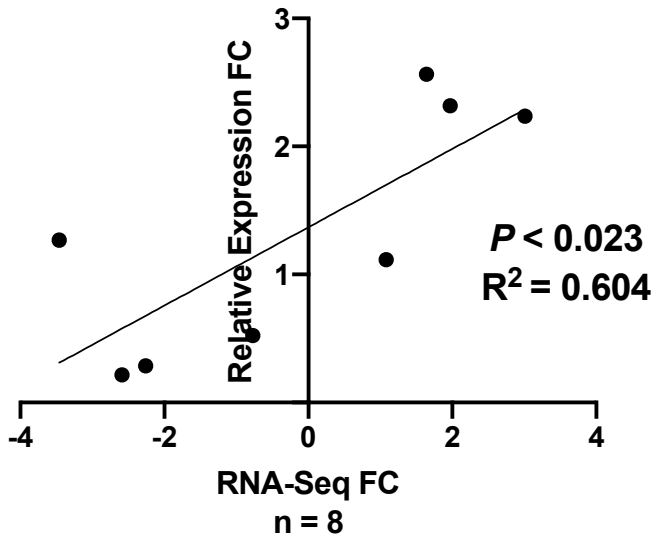


Fig. 3.3 Significant correlation between fold changes obtained from two methods: qRT-PCR (Relative expression FC) and relative expression measured by FPKM using RNA-Seq. The y-axis represents the relative expression FC of lncRNAs, and the X-axis represents RNA-Seq FC P <0.02 and $R^2 = 0.47$, of the lncRNAs between the NW and the OB synovial fibroblasts.

3.4 Discussion

Several lncRNAs have been identified as candidates for mediating OA inflammatory pathology, including lncRNAs that were published by our group and found to be differentially expressed in human OA cartilage including lncRNA PACER, CILinc01 and CILinc02. [257] Exploring the expression of lncRNAs in OA synovial fibroblasts across different patient cohorts will increase our understanding of its role in OA pathology. In this study, 20 novel lncRNAs were differentially expressed in NW and OB OA synovial fibroblasts and were selected based on FPKM mapping quality including: RP11-79H23.3, RP11-362F19.1, RP5-1086K13.1, RP11-863P13.3, CARMN, LINC01123, MIR155HG, LINC01616, MALAT1, RP11-631N16.2, LINC01503, MEG3, LINC00511, LINC01021, RP11-147L13.15, RP11-392O17.1, RP11-367F23.2, AF131217.1, LINC01705, AC068282.3.

12 lncRNAs were upregulated and 8 were downregulated in OB compared to NW OA synovial fibroblasts. Amongst the upregulated lncRNAs, MEG3 and MALAT1 were massively expressed in both NW and OB OA fibroblasts, indicated by showing the highest FPKM amongst the 20 lncRNAs. MEG3 is a nuclear gene which regulates p53 target gene and it is a lncRNA tumor suppressor. Its close proximity to DKL1 protein coding gene may suggest a role for MEG3 in regulating calcium ion binding and a role in Notch signaling pathway. DKL1 is a protein coding gene which was found to control calcium ion binding and negatively regulates Notch signaling pathway [261].

MALAT1 is a nuclear gene which is identified in many physiological processes and was found to be upregulated in many pathological diseases. MALAT1 is associated

with the miscRNA small noncoding RNA of 61 nucleotides which is produced during MALAT1 biogenesis by tRNA and transferred to the cytoplasm [262].

The top three upregulated lncRNAs include: RP11-79H23.3, Rp11-362F19.1 and CARMN. RP11-79H23.3, which is also known as LINC02605 or IL7 antisense, promotes inflammatory gene transcription via regulating IL-6 expression [263] and regulates PTEN expression in bladder cancer development [263]. There is not much information about lncRNA Rp11-362F19.1 in the literature, however many researchers indicated scientific evidence on the role of the Cardiac Mesoderm Enhancer-Associated Non-Coding RNA (CARMN) previously known as (miR-143HG), in regulating different biological and pathological processes including: regulating cardiac cell differentiation and homeostasis [259], being upregulated in Hirschsprung disease of the colon [264], and its expression was found to be related to heart hypertrophy [265].

The three most downregulated lncRNAs include: AC068282.3, LINC01705 and AF131217.1. AC068282.3, a novel transcript with close proximity to MAP3K2 which is regulated by Wnt signaling pathway [266]. Previous reports indicate that, LINC01705 which is also known as RP11-400N13.3 was related to collogen fibril organization and found to have a role in gastric cancer [267]. AF131217.1 lncRNA is a novel lncRNA that plays a role in developing atherosclerosis together with miR-128 axis [268]. The expression of selected lncRNAs were further validated by qRT-PCR in a total of 6 NW and OB OA synovial fibroblasts. Notably, two of the lncRNAs which were significantly expressed in RNASeq data including LINC01123 and CARMN also showed a significant differential expression of mRNA in OB compared to NW OA synovial

fibroblasts and were showing high fold change in the two BMI's. LINC01123 was found to facilitate cell proliferation and promote glycolysis in lung cancer [269]. The upregulated lncRNAs in the RNA-Seq analysis data including RP11-79H23.3, RP11-631N16, RP11-86P13P.13 and MALAT1 showed higher mRNA expression in OB compared to NW OA synovial fibroblasts. However, the downregulated lncRNAs including: AC068282.3, AF131217.1 and LINC01705 from RNA-Seq showed lower mRNA expression in OB compared to NW OA synovial fibroblasts. A significant positive correlation was seen between the expression fold change of lncRNAs in data obtained from RNA-Seq analysis and the mRNA obtained from qRT-PCR approach, confirming the reliability of the data obtained at this stage of the project.

Although, it was confirmed that these lncRNAs were expressed in synovial fibroblasts the expression of many of these transcripts was very low. An exception to this was lncRNA MALAT1, which was found to be abundantly expressed in both NW and OB OA synovial fibroblasts, in accordance with previous research that showed a similar result in chondrocytes. They found that MALAT1 induces chondrocyte proliferation through regulating metabolites in the PI3K/Akt pathway, which may also be true for synovial fibroblasts [270].

One of the drawbacks from lncRNAs lie in its low expression and poor conservation in different mammalian species. MALAT1 is widely investigated recently in many diseases including cancer due to its conserved nature in many species and the high rate of its expression in many pathological conditions [271,272]. Of interest in this project, the high differential expression of MALAT1 in obese OA synovial fibroblasts and (the relatively low expression of the other lncRNAs), prioritized its selection as a

guide in the next approach. Focusing on determining the expression of MALAT1 in OA synovial fibroblasts from different joints and in different OA patient cohorts may work as a road map in reaching to the understanding of the etiological factors that may contribute to the pathogenesis of OA.

CHAPTER 4.

MALAT1 and The Inflammatory Response

4.1 Background

The cross talk between inflammatory responses and the regulatory epigenetic control of gene expression by lncRNAs were highlighted in a recent review by Pearson and Jones [170]. They highlighted four inflammatory pathways that were epigenetically regulated by lncRNAs including NF-κB, Arachidonic acid pathway, MAPK signaling pathway, and TLR signaling pathway. For the purpose of this chapter, no further details will be given here, a detailed review was given in the introduction. The focus of this chapter will be on the regulatory role played by MALAT1 on the pro-inflammatory mediators of OA in different cell types from the joints.

Previously, it was reported that MALAT1 was involved in mediating activity of the p38 MAPK pathway, controlling the production of a number of pro-inflammatory cytokines that have been implicated in OA pathogenesis [273]. Furthermore, Jones group [171] showed an association between MALAT1 and the inflammatory joint tissues from OB OA patient cohort. This association between inflammatory responses and the lncRNAs epigenetic regulation of the genes are involved in the pathogenesis of OA is evident. However, the mechanism by which this is achieved, and the functions and mode of actions of the many lncRNA found in OA joint tissues (including MALAT1) are still not clear [273-275]. Although, some of the lncRNAs were found to have different pathological contribution to the development of the OA disease, there are not enough information on the role of one of the thousands of lncRNAs in relation to OA [273-276-277). In the previous chapter, it was indicated that the lncRNA MALAT1 was highly expressed in NW and OB OA. Therefore, the next step and the aim of this part of the study, was to determine the expression of MALAT1 in OA and non-OA synovial fibroblasts isolated from different joints from OA patients of varying adiposity (obese,

normal-weight, overweight) when subjected to inflammatory challenge including IL-1β and TNF-α.

4.2 MALAT1 and The Inflammatory Response in OA Synovial Fibroblasts

4.2.1 Methods

Primary cells obtained from the synovial tissue of 11 OA hip and knee joints with different BMI's were used for this study (Table 2.1). An *in-vitro* model of an inflammatory cell was established, and the primary cells were stimulated with two pro-inflammatory cytokines IL-1β and TNF-α. Briefly, the recombinant proteins were used at a final concentration of 1ng/mL, and 10ng/mL respectively, it was diluted with complete culture media and added to the cells for 6 and 24 h. Total RNA was extracted, qRT-PCR was used to examine the expression of IL-6 and MALAT1 mRNA and IL-6 protein secretion was quantified by ELISA. For this purpose, synovial fibroblasts from n=11 OA patients, passages 0-3 were utilised, including 3NW OA patients (1 hand and 2 hip) and 5 OB OA patients (1 hand and 4 hip), 3OW OA patients (1 hip and 2 knee), primary cells were used. One way *ANOVA* was performed to compare between the mean of all the groups, and Sidak's multiple comparison test was used to compare between pairs of groups.

4.2.2 Results

The effect of cytokine stimulation of OA primary human cells on the production of IL-6

4.2.2.1 IL-6 induction in IL-1β Stimulated synovial fibroblasts

All but one of the 11 patient synovial fibroblast cells were found to be responsive to stimulation with IL-1β, with significant induction in the expression of IL-6 mRNA observed at either 6 h or 24 h (Figure 4.1A, 4.1B). The exception was the obese hand OA synovial fibroblasts, which did not show any induction at IL-6 mRNA at either 6h or 24 h of IL-1β stimulation. Comparing IL-1β stimulated NW fibroblasts from different joints, IL-6 induction was seen more in hand joints than hip joints at 6 h and 24 h compared to OB fibroblasts which showed significant difference in IL-6 induction between hip and hand joints at 24 h ($P < 0.006$), showing a robust induction in OB hip joints than hand joints, with the obese hand OA fibroblasts being low-responsive at 6 and 24 h. Interestingly, IL-1β stimulated OW fibroblasts from different joints showed a significant difference in IL-6 induction between hip and knee joints at 6 h ($P < 0.0001$), (Figure 4.1C, 4.3.1.D). Looking at the fold change difference between IL-1β stimulated and non-stimulated fibroblasts, IL-6 induction was significantly different in the two groups in different BMI cohorts and joint locations. At 6 h, IL-6 induction was significantly different in IL-1β stimulated and non-stimulated fibroblasts from NW hip ($P < 0.01$), and hand joints ($P < 0.0001$), and fibroblasts from OW knee joints ($P < 0.0001$), (Figure 4.1.E). At 24 h, only fibroblasts from OW knee joints showed a significant difference in the fold change of IL-6 induction between IL-1β stimulated and non-stimulated fibroblasts ($P < 0.0001$), (Figure 4.1.F).

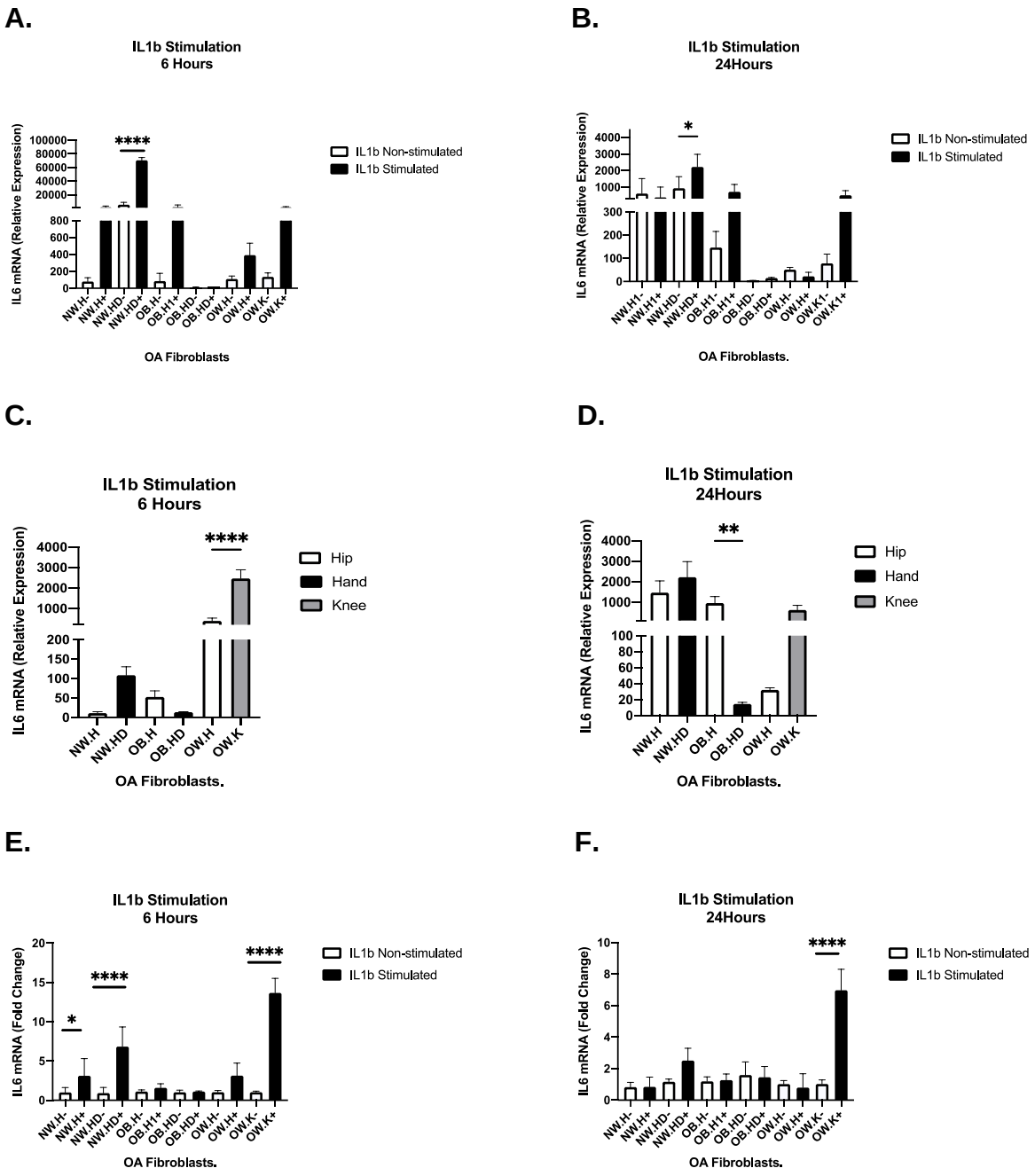


Figure 4.1 IL-6 induction in IL-1β stimulated synovial fibroblasts. Expression of IL-6 mRNA in stimulated and non-stimulated fibroblasts from different BMI and different joints at 6 h **(A)** at 24 h **(B)**. Comparison between the expression of IL-6 induction in stimulated NW compared to OB and OW synovial fibroblasts at 6 h **(C),** at 24 h **(D)**. Comparison between fold change expression of IL-6 mRNA of IL-1β stimulated and non-stimulated synovial fibroblasts at 6 h **(E),** at 24 h **(F)**. Bars represent mean expression ± SEM n=3 biological replicates. In total n=11 OA patients, (n=2) NW.H = NW Hip, (n=1) NW. HD = NW Hand, (n=4) OB.H = OB Hip, (n=1) OB. HD = OB Hand, (n=1) OW.H = OW Hip, (n=2) OW. K = OW Knee.

4.2.2.2 IL-6 induction in TNF-α Stimulated synovial fibroblasts

IL-6 mRNA was significantly expressed in TNF-α stimulated synovial fibroblasts compared to non-stimulated synovial fibroblasts from hip joints of NW patients ($P < 0.0001$) and OB patients ($P < 0.0001$), while OB hand and OW hip and knee were not responsive to TNF-α stimulation at 6 h. At 24 h the opposite was seen, in which a significant induction in IL-6 was seen in TNF-α stimulated synovial fibroblasts compared to non-stimulated synovial fibroblasts in OB hand ($P < 0.0004$) and OW hip and knee joints ($P < 0.0001$) and ($P < 0.0001$), respectively, (Figure 4.2.A & B).

Comparing TNF-α stimulated NW synovial fibroblasts in different joints, a significant difference in IL-6 induction was seen between hip and hand joints at 6 and 24 h ($P < 0.0001$) both, and in OW synovial fibroblasts from hip and knee joints at 6 and 24 h ($P < 0.0001$) both. There was no significant difference in IL-6 mRNA expression in TNF-α stimulated OB synovial fibroblasts between hip and hand joints. Noticeably, OB hand synovial fibroblasts showed a similar response to stimulation with TNF-α as to IL1 β stimulation at 6 and 24 h (Figure 4.2.C & D).

Looking at the fold change in IL-6 induction between TNF-α stimulated compared to non-stimulated synovial fibroblasts at 6 h, all hip with different BMI cohorts NW, OB and OW showed a significant difference in IL-6 mRNA fold change expression between the two groups ($P < 0.001$), ($P < 0.0005$), ($P < 0.001$) respectively, and OW knee ($P < 0.0001$), with the OB hand showing a non- significant difference in IL-6 mRNA expression fold change between TNF-α stimulated compared to non-stimulated synovial fibroblasts. At 24 h, a significant difference in the IL-6 mRNA expression fold

change was seen between TNF-α stimulated compared to non-stimulated synovial fibroblasts in OB hip joint (P <0.0001), and OW hip joint (P <0.01), (Figure 4.2.E & F).

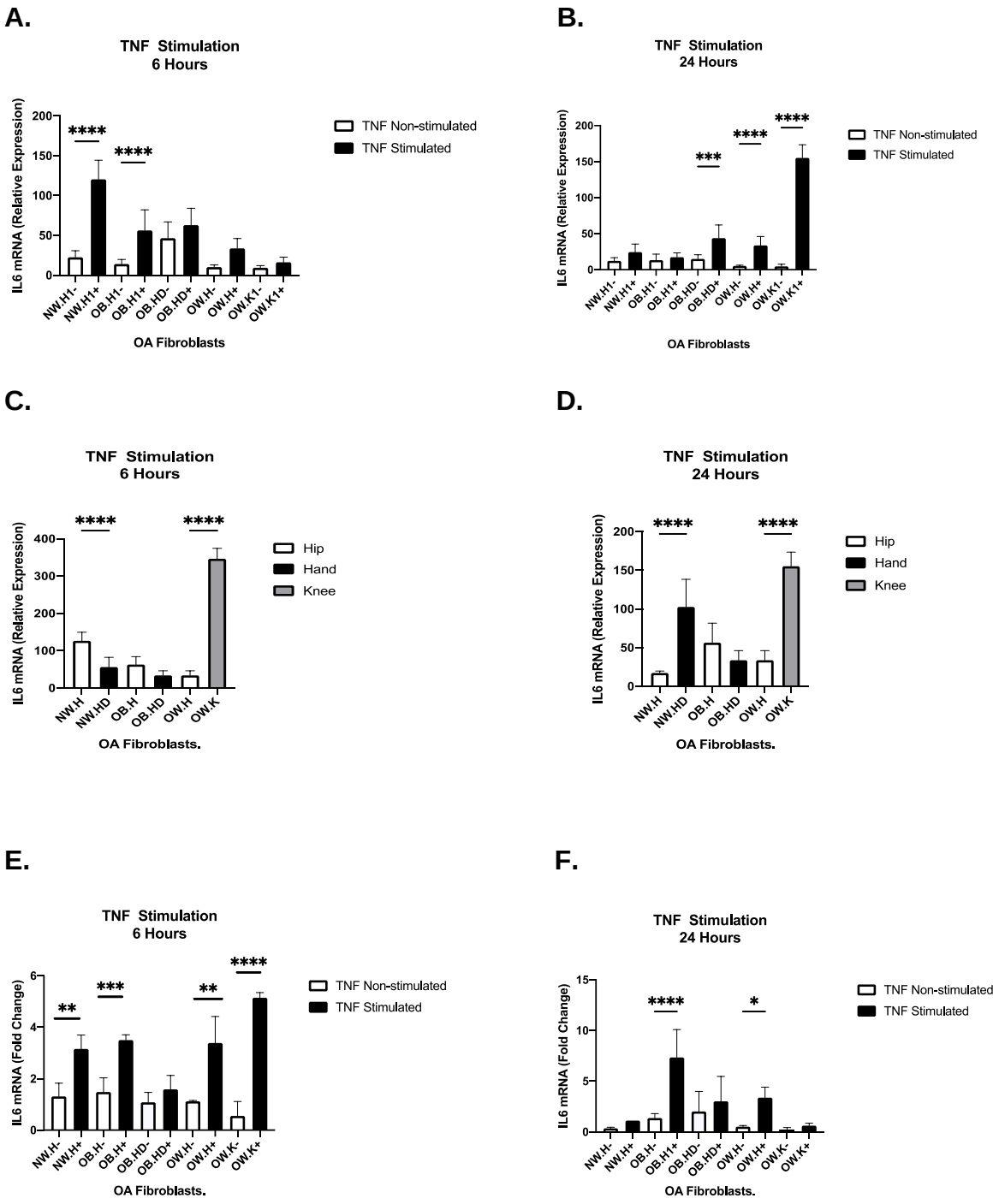


Figure 4.2 IL-6 induction in TNF- α stimulated synovial fibroblasts. Expression of IL-6 in stimulated and non-stimulated fibroblasts from different BMI and different joints at 6 h **(A),** at 24 h **(B)**. Comparison between the expression of IL-6 induction in stimulated NW compared to OB and OW synovial fibroblasts at 6 h **(C)**, at 24 h **(D)**. Comparison between fold change expression of IL-6 mRNA of TNF- α stimulated and non-stimulated synovial fibroblasts at 6 H **(E)** and 24 H **(F)**. Bars represent mean expression ± SEM n=3 biological replicates. In total n=7 OA patients, (n=1) NW.H = NW Hip, (n=2) OB.H = OB Hip, (n=1) OB. HD = OB Hand, (n=1) OW.H = OW Hip, (n=2) OW. K = OW Knee.

4.2.2.3 MALAT1 Expression in IL-1β stimulated synovial fibroblasts

The next step was to look into the expression of MALAT1 in IL-1β stimulated synovial fibroblasts obtained from 2 BMI cohorts NW and OB in different OA joints including hand, hip and knee. For this analysis two NW hand and hip, and one OB hip, were used. At 6 h all the groups expressed MALAT1 except OB hip and MALAT1 was expressed significantly in IL-1β stimulated synovial fibroblasts from NW hand compared to non-stimulated (P <0.0001). At 24 h of IL-1β stimulation only NW hip synovial fibroblasts showed MALAT1 expression, but it was not significant. (Figure 4.3.A & B).

Comparing IL-1β stimulated synovial fibroblasts between different BMI cohorts, there was a significant difference in MALAT1 expression in NW compared to OB groups (P <0.01) (Figure 4.3.C & B). Analysing the fold change difference in MALAT1 expression between IL-1β stimulated and non-stimulated synovial fibroblasts of different groups, at 6 h a significant difference in the fold change expression of MALAT1 was seen in NW hand (P <0.0001) and NW hip (P <0.001) (Figure 4.3.E & F).

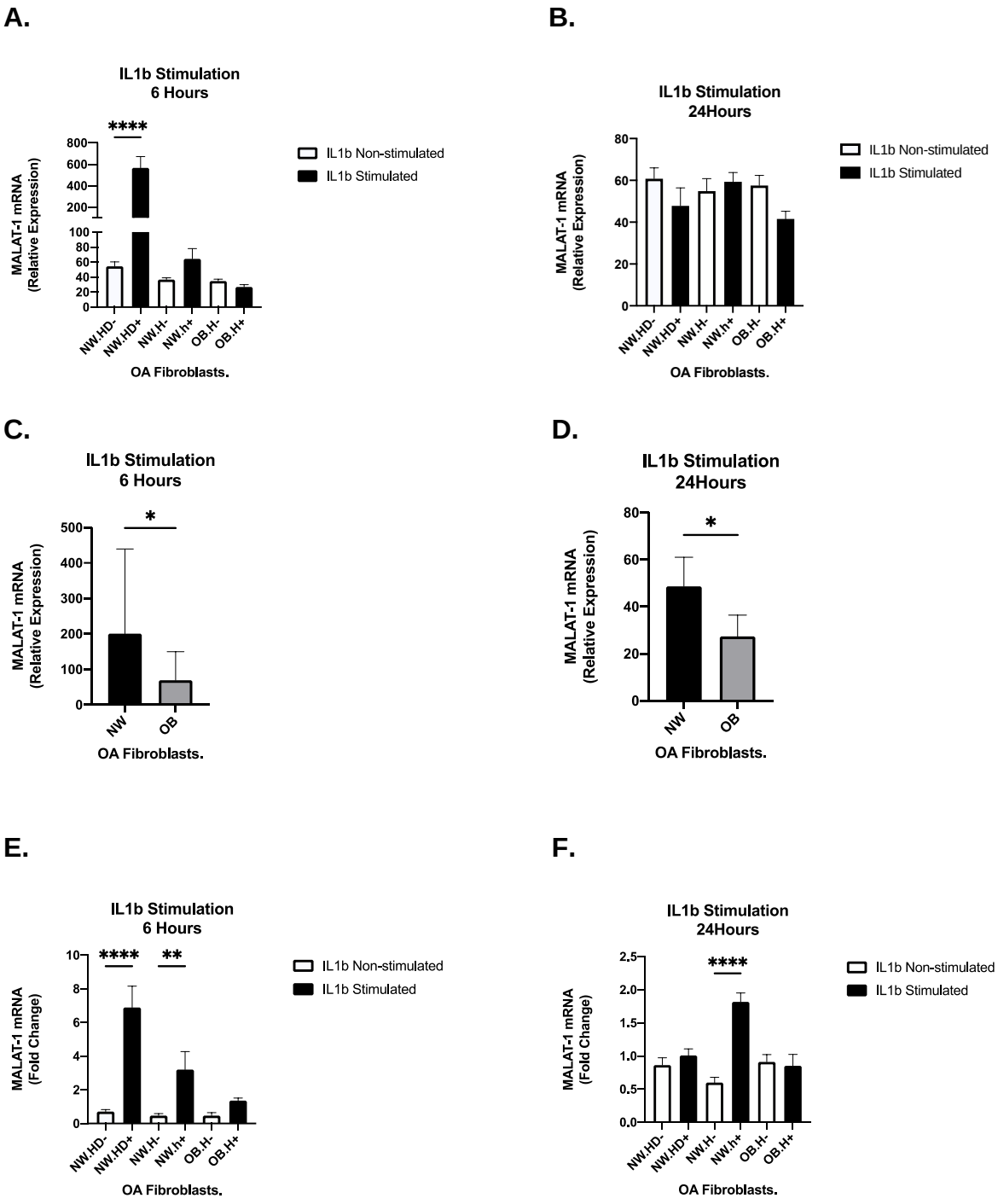


Figure 4.3 MALAT1 expression in IL-1β stimulated synovial fibroblasts. Expression of MALAT1 in stimulated and non-stimulated fibroblasts from different BMI and different joints at 6 h **(A),** at 24 h **(B)**. Comparison between the expression of IL-6 induction in stimulated NW compared to OB and OW synovial fibroblasts at 6 h **(C)**, at 24 h **(D)**. Comparison between fold change expression of IL-6 mRNA of TNF- α stimulated and non-stimulated synovial fibroblasts at 6 H **(E)** and 24 H **(F)**. Bars represent mean expression ± SEM n=3 biological replicates. In total n=7 OA patients, (n=1) NW.H = NW Hip, (n=2) OB.H = OB Hip, (n=1) OB. HD = OB Hand, (n=1) OW.H = OW Hip, (n=2) OW. K = OW Knee.

4.3 MALAT1 and The Inflammatory Response in OA Chondrocytes

4.3.1 Methods

4.3.1.1 IL-1β Stimulation of Articular Chondrocytes

Articular cartilage from 8 OA biopsies obtained from hip and knee joints with different BMI (Table 2.1), including 5 NW hip, 1 OB hip and 2 OB knee were used. The primary chondrocytes were stimulated with two pro-inflammatory cytokines IL-1β and TNF-α. Briefly, the recombinant proteins were used at a final concentration of 1ng/mL, and 10ng/mL respectively, it was diluted with complete culture media and added to the cells for 6 and 24 h. Total RNA was extracted, qRT-PCR was used to examine the expression of IL-6 and MALAT1 mRNA and IL-6 protein secretion was quantified by ELISA. One way *ANOVA* was performed to compare between the mean of all the groups, and Sidak's multiple comparison test was used to compare between pairs of groups. Primary chondrocytes of passages 0-2 were used in this experiment.

4.3.2 Results

4.3.2.1 IL-6 induction in IL-1β Stimulated Chondrocytes

Chondrocytes from OB hip showed a significant IL-6 induction at 6 h in response to IL-1β stimulation compared to non-stimulated chondrocytes (P <0.0001), and only one out of the 5 NW hip joints showed response to IL-1β stimulation (P <0.002). At 24 h, chondrocytes from 2 of the 5 NW hip joints were responsive to IL-1β stimulation, but

this did not reach a significant level. In the OB cohort, hip and one of the knee joints were significantly responsive to IL-1β stimulation compared to non-stimulated chondrocytes (P <0.0001), (Figure 4.4.A & B).

Comparing IL-1β stimulated chondrocytes from different BMI cohorts and joints, at 6 h chondrocytes from OB hip showed a significant high level of IL-6 induction compared to chondrocytes from both NW hip and OB knee joints (P <0.0001). The same was seen at 24 h in addition to a significant difference in IL-6 induction in IL-1β stimulated chondrocytes from NW hip joints compared to OB knee joints (P <0.002), (Figure 4.4.C & D).

The fold change difference in IL-6 mRNA expression between IL-1β stimulated and non-stimulated chondrocytes at 6 h was significant in one of the 5 NW hip joints (P <0.0002), OB hip (P <0.0001) and OB knee (P <0.04). At 24 h, a significant difference in IL-6 mRNA expression was seen between IL-1β stimulated and non-stimulated chondrocytes from one NW hip joint (P <0.0001) and in 2 OB knee joints (P <0.0001), (Figure 4.4.E & F).

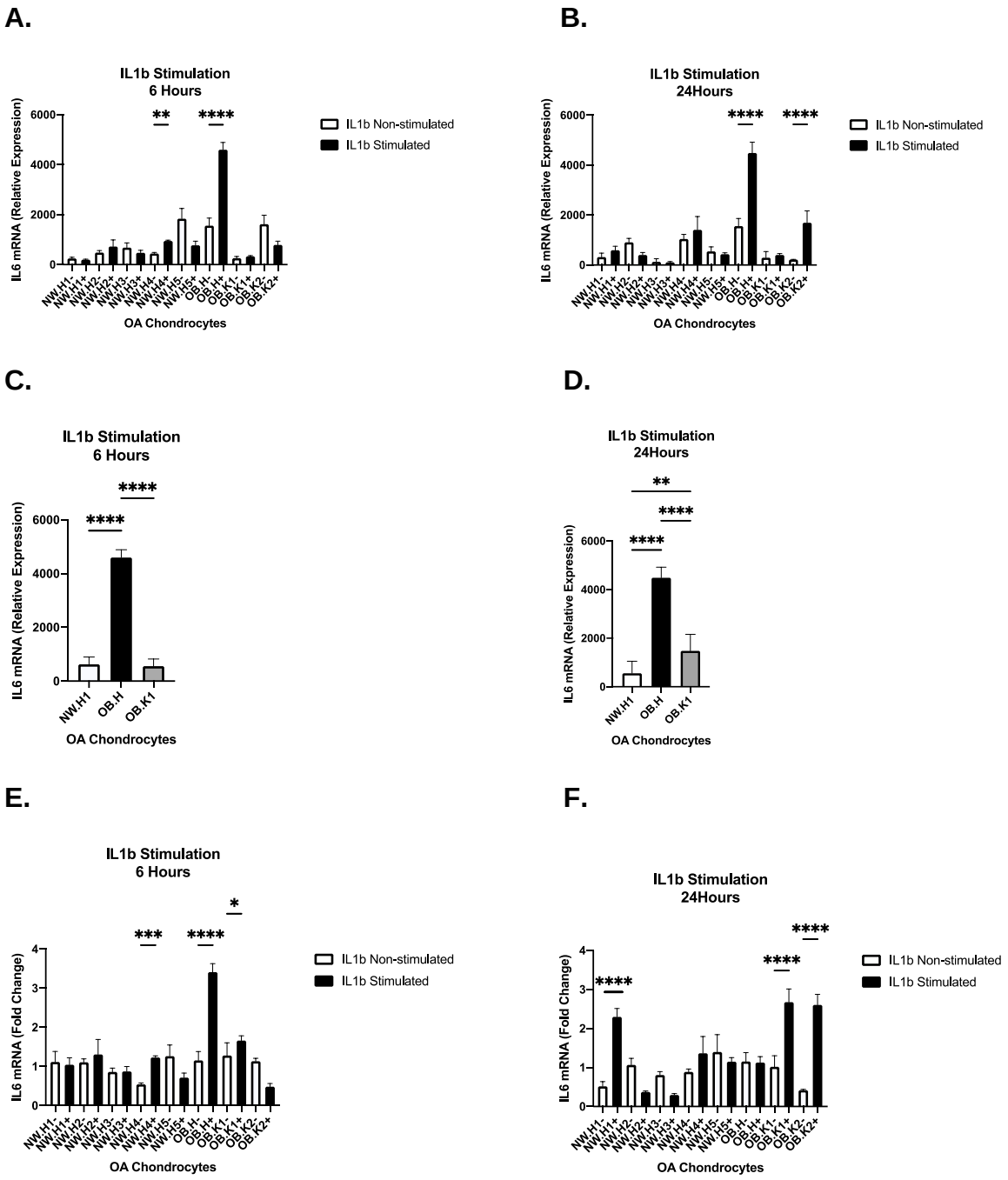


Figure 4.4 IL-6 induction in IL-1β stimulated chondrocytes. Expression of IL-6 mRNA in stimulated and non-stimulated chondrocytes from different BMI and different joints **(A)** at 6 h **(B)** at 24 h. Comparison between the IL-6 induction in stimulated NW compared to OB chondrocytes **(C)** at 6 h, **(D)** at 24 h. Comparison between fold change expression of IL-6 mRNA of IL-1β stimulated and non-stimulated chondrocytes in all the groups, at 6 H **(E)** and 24 H **(F)**. Bars represent mean expression ± SEM n=3 biological replicates. In total n=8 OA patients, (n=5) NW.H = NW Hip, (n=1) OB.H = OB Hip, (n=2) OB.K = OB knee.

4.3.2.2 IL-6 Quantification

In order to confirm that IL-1β stimulation of the osteoblasts has provoked an inflammatory response, we examined whether the induction of IL-6 gene expression observed after recombinant cytokine stimulation was also accompanied by an increase in the secretion of IL-6 protein. To this end, IL-6 protein was quantified in cell supernatants of IL-1β stimulated and unstimulated cells using ELISA. A total of 7 chondrocytes were utilized for this purpose of the NW (n=4) and OB (n=3) cohort from OA hip joint, after stimulation with IL-1β for 6 and 24 h and compared to non-stimulated controls. IL-6 protein was detected in all of the samples and its expression in response to chondrocytes stimulation validated IL-6 gene expression in stimulated chondrocytes detected by qRT-PCR (Figure 4.5 A & B).

IL-6 protein in the NW mean ± SEM (223 ± 68.57) cohort was more responsive to stimulation than the OB mean ± SEM (92.57 ± 83.56) cohort at 6 h, while at 24 h the opposite was seen, IL-6 protein in the NW mean ± SEM (260 ± 46.39) and in OB mean ± SEM (301.9 ± 61) (Figure 4.5 C).

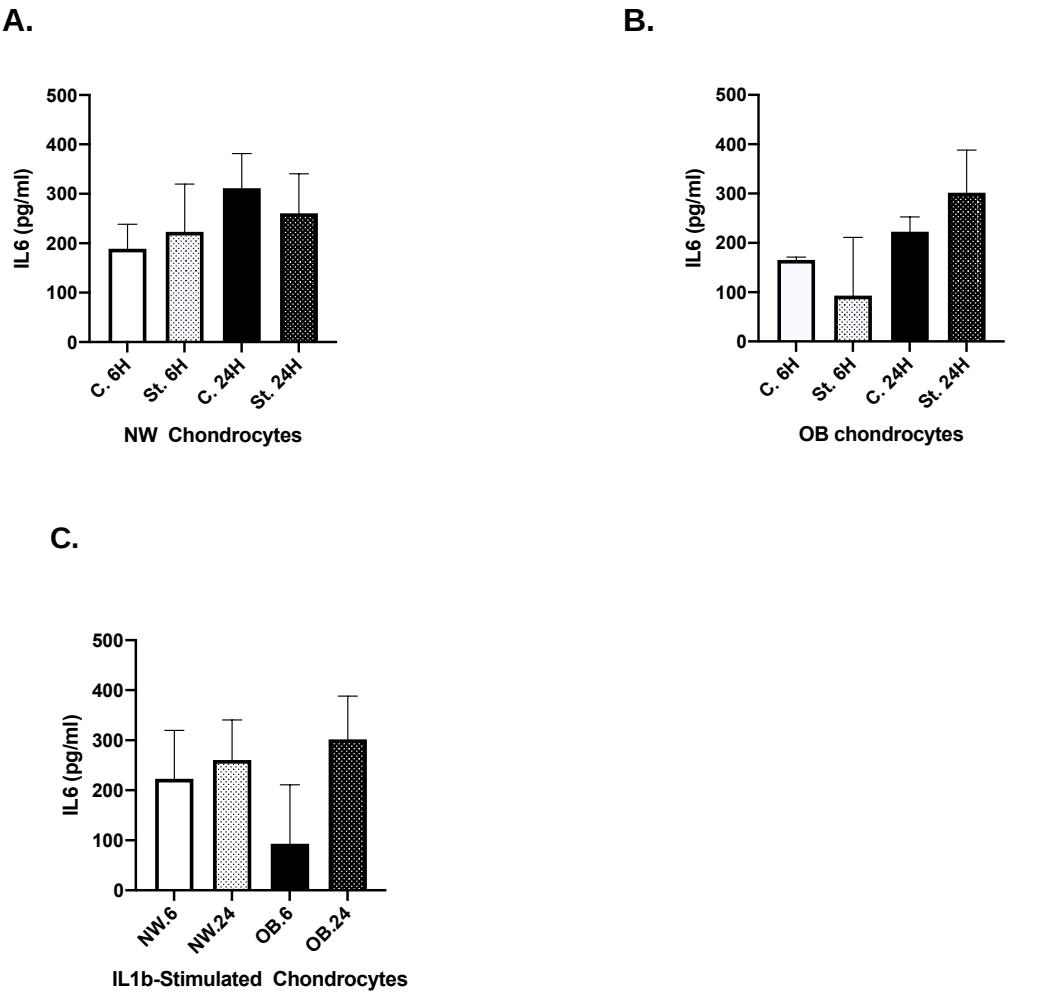


Figure 4.5 IL-6 validation in IL-1β stimulated chondrocytes using ELISA. Comparison between IL-6 concentration in IL-1β stimulated to non-stimulated chondrocytes at 6 h **(A)** and at 24 h **(B)**. IL-6 induction in NW and OB stimulated at 6 and 24 h **(C).** Bars represent ±SEM (n=3) biological replicates in (n=7) OA patients, C = control, St. = stimulated.

4.3.2.3 MALAT1 Expression in IL-1β Stimulated Chondrocytes

Next, the expression of MALAT1 was analysed in IL-1β stimulated chondrocytes obtained from 2 BMI cohorts NW and OB in different OA joints including hip and knee.

For this analysis 4 NW hip and 1 OB hip and 3 OB knee, were used. At 6 h MALAT1 was expressed more in response to IL-1β stimulation in NW. hip1, OB hip and all OB knee, but this expression was not statistically significant except in OB knee1 (P <0.0001) (Figure 4.6.A & B). At 24 h, all BMI cohorts were responsive to IL-1β stimulation and significant MALAT1 expression was seen in NW hip3 (P <0.0001) and OB knee3 (P <0.02), (Figure 4.6.A & B).

Comparing MALAT1 expression within groups of stimulated chondrocytes at 6 h, a significant difference was seen between NW hip (P <0.0001) and between hip and knee joints of the OB cohort (P <0.0002). At 24 h, a significant difference of MALAT1 expression was seen between the same groups as in at 6 h IL-1β stimulation (P <0.04) and (P <0.01) respectively (Figure 4.6.C & D).

Analysing the fold change difference in MALAT1 expression in response to IL-1β stimulation and non-stimulated chondrocytes, at 6 h NW hip1 and OB hip joints showed a significant difference in MALAT1 fold change between the two groups (P <0.003) and (P <0.001) respectively. While at 24 h, none of the different cohorts showed a significant difference in MALAT1 expression fold change in response to IL-1β stimulation (Figure 4.6.E & F).

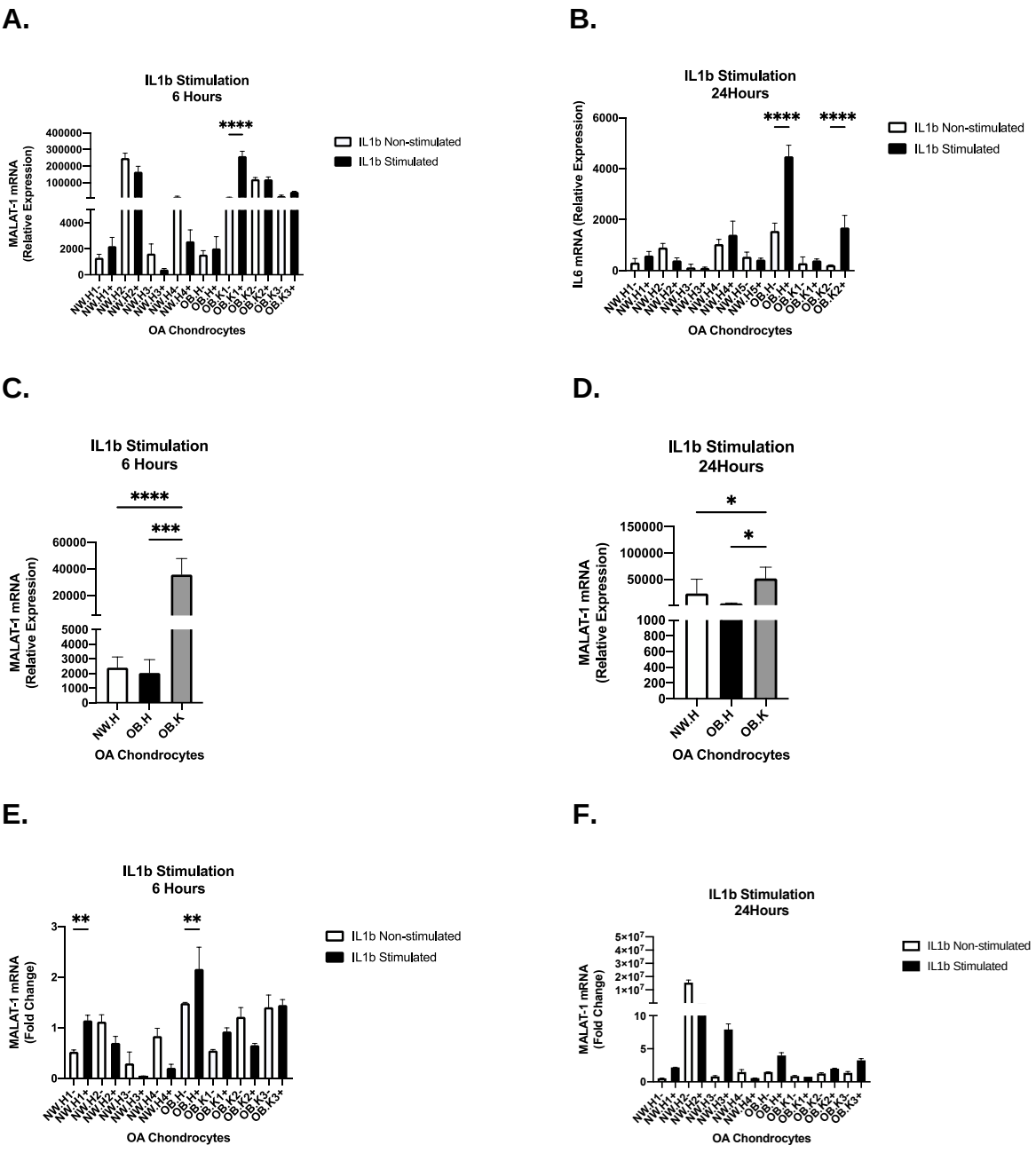


Figure 4.6 MALAT1 expression in IL-1β stimulated chondrocytes. Expression of MALAT1 in stimulated and non-stimulated chondrocytes from different BMI and different joints at 6 h **(A)**, at 24 h **(B**. Comparison between the expression of MALAT1 in stimulated NW compared to OB chondrocytes at 6 h **(C)**, at 24 h **(D)**. Comparison between fold change expression of MALAT1mRNA of IL-1β stimulated and non-stimulated chondrocytes in different groups at 6 H **(E)** and 24 H **(F)**. Bars represent mean expression ± SEM n=3 biological replicates. In total n=8 OA patients, (n=5) NW.H = NW Hip, (n=1) OB.H = OB Hip, (n=2) OB. K= OB knee.

4.4 IL-1β Stimulation of OA Osteoblasts

4.4.1 Results

4.4.1.1 IL-6 induction in IL-1β Stimulated OA Osteoblasts

Primary osteoblasts from subchondral bone of 8 OA patients (from different BMI cohorts and different OA anatomical joints including knee, toe, hip, wrist, and hand) were stimulated with IL-1β for 6 and 24 h or left unstimulated. At 6 h 7/8 osteoblasts were responsive to IL-1β stimulation except for one of the NW knee osteoblast samples. OW foot osteoblasts showed a significant response to IL-1β stimulation at 6 and 24h ($P < 0.0001$). A second NW patient (NW hip2) osteoblasts showed a slight response to stimulation at 6 h and showed a significant IL-6 induction in stimulated osteoblasts at 24 h ($P < 0.006$) (Figure 4.7.I. A & B).

Comparing the fold change of IL-6 mRNA in IL-1β stimulated compared to non-stimulated, at 6 h the following groups showed significant difference NW hip1 ($P < 0.004$), OB hand ($P < 0.0001$), OB wrist ($P < 0.01$), OW toe and foot ($P < 0.0001$). At 24 h NW hip1 and hip2 showed significant difference in IL-6 mRNA expression ($P < 0.0001$) and OW foot ($P < 0.0002$) (Figure 4.7.I. C & D).

Next, the differences of IL-6 induction in stimulated osteoblasts within different groups, was examined. At 6 h, osteoblasts from OW foot joints showed a profound IL-6 induction and a significant difference in IL-6 mRNA was seen between osteoblasts from OW toe and foot joints ($P < 0.0001$). At 24 h, the degree of IL-1β mediated IL-6 induction across the different osteoblast patient samples was more variable, with significant differences in IL-6 mRNA in osteoblasts obtained from NW hip compared to

NW knee ($P < 0.0001$), OB knee compared to OB hand ($P < 0.0007$), OB hand compared to OB wrist ($P < 0.004$), and OW toe compared to OW foot ($P < 0.0001$) (Figure 4.7.II.A & B).

IL-6 mRNA expression in stimulated osteoblasts also showed significant difference between different BMI including NW to OB BMI cohorts and between NW to OW at 6 h ($P < 0.0001$). At 24 h, IL-6 mRNA of OB stimulated osteoblasts was significantly different to OW osteoblasts only ($P < 0.01$) (Figure 4.7.II.C&D).

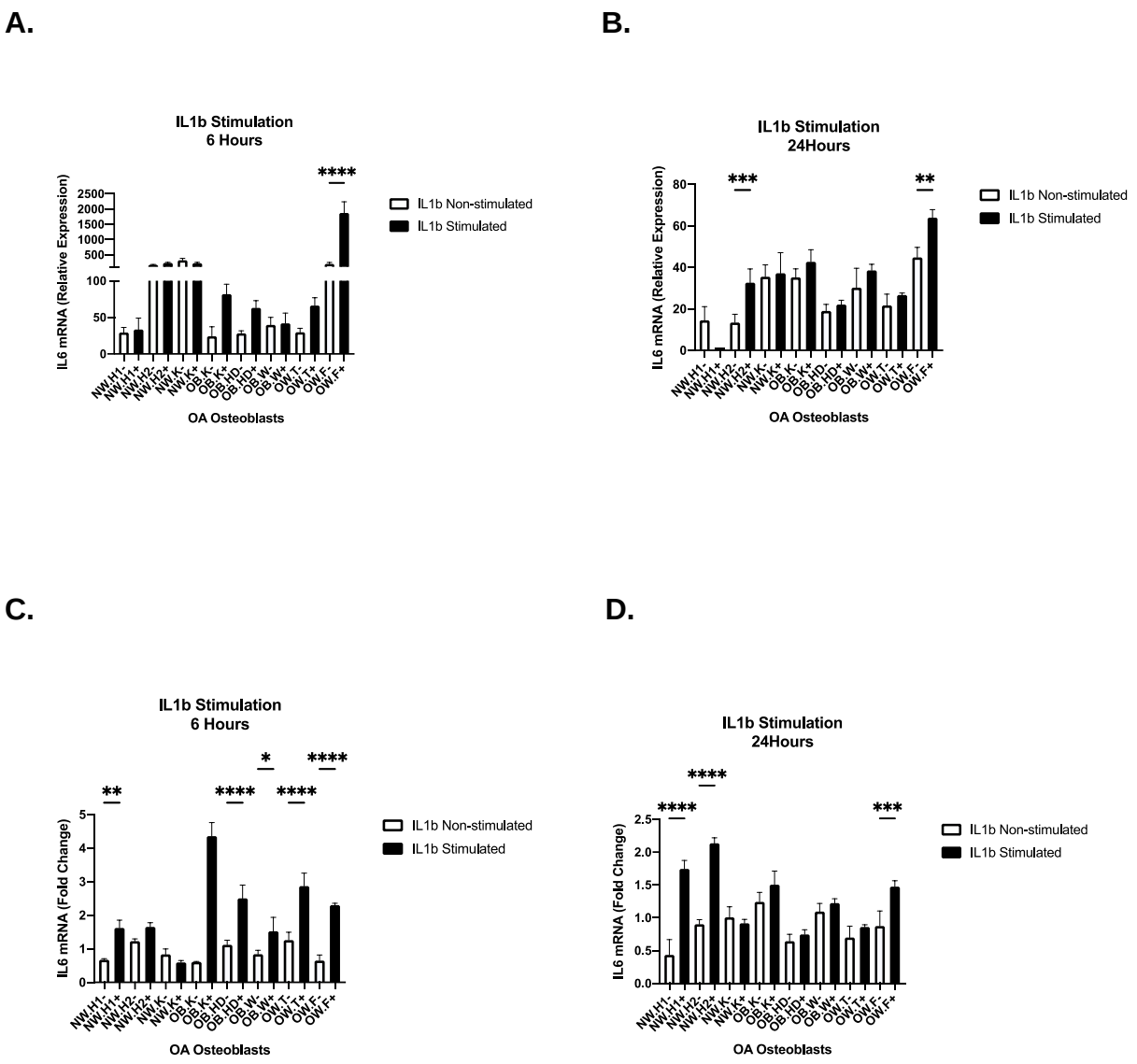
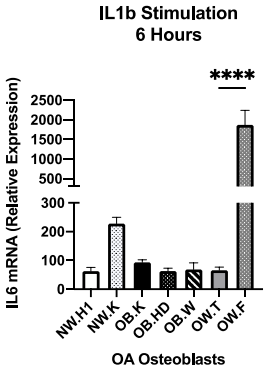


Figure 4.7.I IL-6 induction in IL-1β stimulated osteoblasts. Expression of IL-6 mRNA in stimulated and non-stimulated osteoblasts from different BMI and different joints at 6 h **(A)**, at 24 h **(B)**. Comparison between the fold change of IL-6 mRNA expression in stimulated and non-stimulated osteoblasts at 6 h **(C)**, at 24 h **(D)**. Bars represent mean expression ± SEM n=3 biological replicates. In total n=8 OA patients, (n=1) NW. K = NW knee, (n=2) NW. H= NW hip. (n=1) OB. K = OB Knee, (n=1) OB. HD = OB hand, (n=1) OB. W = OB wrist, (n=1) OW. T = OW toe and (n=1) OW. F = OB foot.

A.



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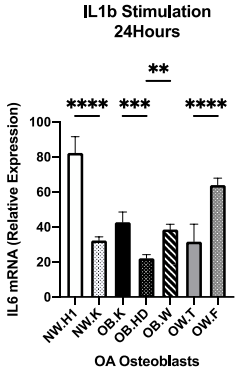

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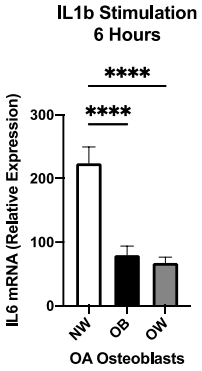

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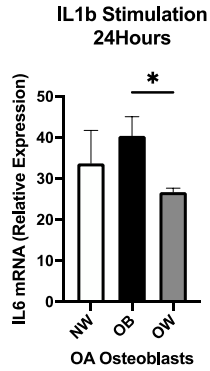


Figure 4.7.II IL-6 induction in IL-1β Osteoblasts Comparison between IL-6 induction in response to IL-1β stimulation within groups at 6 h **(A)**, and at 24 h **(B).** Comparison between IL-6 induction in response to IL-1β stimulation in different BMI's at 6 h **(C)**, and at 24 h **(D).** Bars represent mean expression ± SEM n=3 biological replicates. In total n=8 OA patients, (n=1) NW. K = NW knee, (n=2) NW. H= NW hip. (n=1) OB. K = OB Knee, (n=1) OB. HD = OB hand, (n=1) OB. W = OB wrist, (n=1) OW. T = OW toe and (n=1) OW. F = OB foot.

4.4.1.2 MALAT1 Expression in IL-1β Stimulated OA Osteoblasts

The next step after IL-6 induction was to analyse MALAT1 expression in IL-1β stimulated and non-stimulated osteoblasts. At 6 h, MALAT1 expression was increased in response to IL-1β stimulation in osteoblasts from patients with different BMI and in osteoblasts from different joints. An exception to this was the non-weight bearing joints OB hand and wrist, where MALAT1 expression was not statistically increased compared to non-stimulated. In contrast, a significant MALAT1 expression was seen in osteoblasts from weight-bearing hip and foot joints at 6 h (P <0.0001). At 24 h MALAT1 was slightly expressed in osteoblasts from the non-weight bearing joints of hands and wrist, but in general was more highly expressed in osteoblasts from the weight- bearing NW hip (P <0.0002) and OW foot (P <0.0001) joints. (Figure 4.8.I.A & B).

Comparing the fold change of MALAT1 expression between the stimulated and non-stimulated osteoblasts, at 6 h all the weight-bearing joints of different BMI including NW hip, OB knee, and OW foot showed an increase in the fold change of MALAT1 expression in stimulated osteoblasts compared to non-stimulated, with a significant difference shown in OW foot (P <0.0001) and none of the OB hand or wrist. At 24 h MALAT1 expression fold change was more in stimulated compared to non-stimulated osteoblasts in all the groups except for OB knee. A significant difference in the fold change expression of MALAT1 between stimulated and non-stimulated osteoblasts was seen in NW hip (P <0.0001), OB knee = (P <0.0001), OB wrist and OW foot (P <0.0001) (Figure 4.8.I.C & D).

Comparing within the groups of different BMI and stimulated osteoblasts from different OA joints, at 6 h MALAT1 expression in osteoblasts from NW hip were significantly different to osteoblasts from OB knee, OB hand and OB w (P <0.0001), and to OW foot (P <0.0004). MALAT1 expression in osteoblasts from OW foot, were significantly different to osteoblasts from OB knee, hand, and wrist (P <0.0001). At 24 h MALAT1 expression in osteoblasts from NW hip were significantly different to osteoblasts from OB knee, hand, wrist and OW foot (P <0.0001) and osteoblasts from OW foot to OB knee, hand, wrist (P <0.0001) (Figure 4.8.II.A & B).

Comparing MALAT1 expression in stimulated osteoblasts from different BMI of NW, OB and OW, at 6h a significant difference was seen in osteoblasts from NW BMI compared to both OB BMI (P <0.0001) and OW BMI (P <0.0008). MALAT1 expression in stimulated osteoblasts were significantly different in osteoblasts from OB BMI compared to OW BMI (P <0.0001). At 24 h the same differences in MALAT1 expression were seen in stimulated osteoblasts from NW compared to both OB (P <0.0002) and OW BMI (P <0.0001), and osteoblasts from OB compared to OW BMI (P <0.0001) (Figure 4.8.II.C & D).

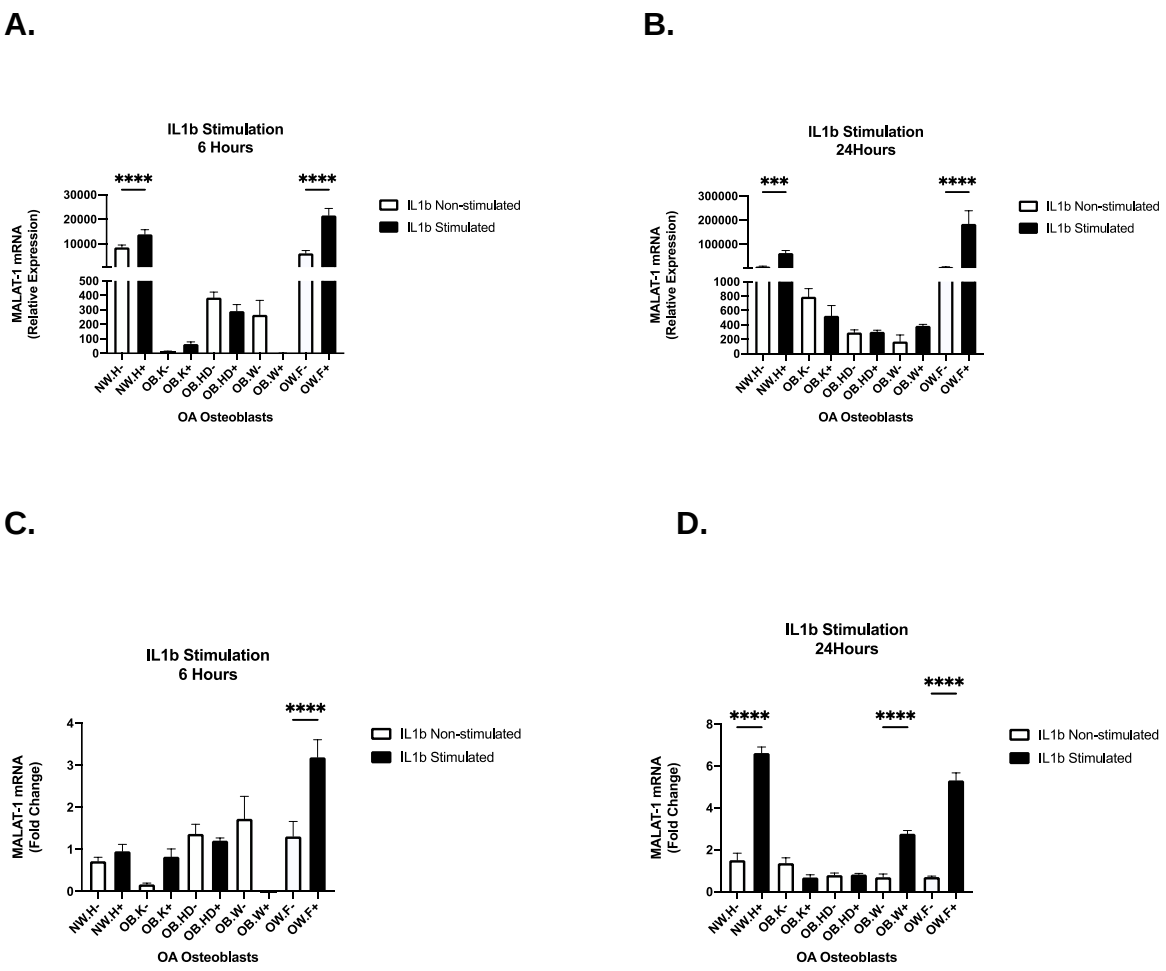
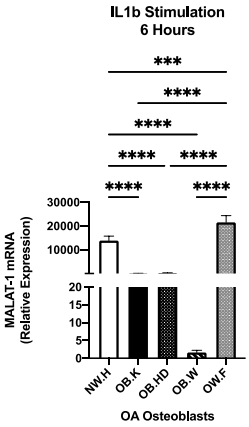


Figure 4.8.I MALAT1 expression in IL-1β stimulated osteoblasts. Expression of MALAT1 in stimulated and non-stimulated osteoblasts from different BMI and different joints at 6 h **(A)**, at 24 h **(B)**. Comparison between fold change expression of MALAT1 in stimulated NW compared to OB osteoblasts at 6 h **(C)**, at 24 h **(D)**. Bars represent mean expression ± SEM n=3 biological replicates. In total n=5 OA patients, (n=1) NW.H = NW hip, (n=1) OB. K = OB knee, (n=1) OB. HD = OB hand, (n=1) OB. W = OB wrist, (n=1) and (n=1) OW. F = OB foot.

A.



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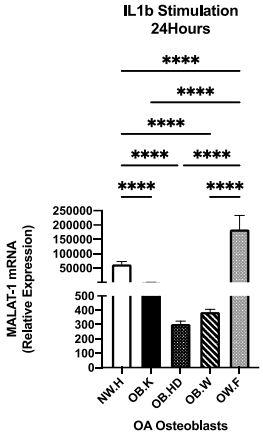

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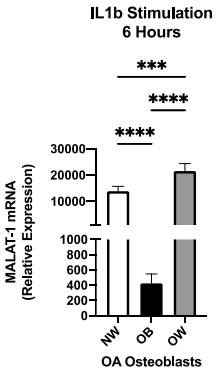

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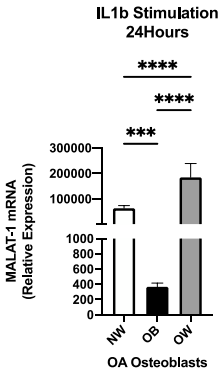


Figure 4.8.II MALAT1 expression in IL-1β stimulated osteoblasts. Comparison between MALAT1mRNA expression in IL-1β stimulated osteoblasts of different OA cohort from different joints, at 6 h **(8)**, at 24 h **(B)**. Comparison between MALAT1mRNA expression in stimulated osteoblasts different BMI's, at 6 h **(C)**, at 24 h **(D)**. Bars represent mean expression ± SEM n=3 biological replicates. In total n=5 OA patients, (n=1) NW.H = NW hip, (n=1) OB. K = OB knee, (n=1) OB. HD = OB hand, (n=1) OB. W = OB wrist, (n=1) and (n=1) OW. F = OB foot.

4.5 Discussion

The aim of this chapter was to investigate MALAT1 long non-coding RNA in the *in vitro* inflammatory models of primary cells from different cohorts of OA joints. Human protein recombinant IL-1β and TNF- α were used to stimulate synovial fibroblasts, articular chondrocytes and osteoblasts from cartilage and subchondral bone of OA joints for 6 and 24 h. To date, this is the first project to study the expression of MALAT1 upon inflammatory challenge in different human joint cells from patients with OA, including osteoblasts, chondrocytes, and fibroblasts, as well as the first to compare cells from different anatomical joints. Primary cells were cultured from OA non-weight bearing joints including hand and wrist and the weight-bearing joints including hip, knee, foot, and toe.

The pathological process behind the initiation and progression of OA is still not fully understood. Depending on the fact that OA pathology involves the whole joint indicated by the general features of OA including synovitis, articular cartilage degeneration and remodelling of the subchondral bone, this project was designed to utilize the main primary cells forming joints in the experimental analysis including synovial fibroblasts, chondrocytes, and osteoblasts.

Inflammation is reported to contribute to the pathogenesis of many diseases including rheumatoid arthritis (RA), and cancer [279]. Increasing evidence indicate a role for inflammation in the pathogenesis of OA [280]. It was reported in literature that the pro-inflammatory cytokines including IL-1β and TNF-α and IL-6 were elevated in synovial fibroblasts, articular cartilage and subchondral bone [275]. Moreover, researchers indicated that the main two inflammatory mediators IL-1β and TNF-α exert a crucial

role in the pathological disturbances of the synovial membrane causing (synovitis) and synovitis was proven to play a role in the initiation of OA [279]. Interestingly, our group demonstrated previously that the proinflammatory cytokines Adipokine and leptin in obese OA patients resulted in an increased level of IL-6 mediated by crosstalk between synovial fibroblasts and chondrocytes [78].

Furthermore, these pro-inflammatory cytokines play a role in cartilage and extracellular matrix degradation by influencing the production of other proinflammatory cytokines with a pivotal role in the development of these pathological process including matrix metalloproteinases (MMPS) MMP-1,3 and 13 and prostaglandins [275], [281]. IL-1β were elevated in the subchondral bone layer of OA patients [248]. In another study, IL-1β and TNF-α were shown to modulate osteoblast resorption by inhibiting alkaline-phosphatase expression and the production of osteocalcin [283-284].

Taking these findings in account, an *in vitro* inflammatory model was designed to apply to primary cells cultured from different tissues of OA joints in order to mimic the *in vivo* inflammatory environment. For this purpose, primary synovial fibroblasts, articular chondrocytes, and osteoblasts were stimulated with IL-1β and TNF-α and IL-6 induction was analysed. In accordance with the previously published research [275, 281-284], all the primary cells were responsive to IL-1β stimulation in the production of IL-6 cytokine with varying degrees. In this project the synovial fibroblasts obtained from OW knee showed greater response after one day stimulation compared to synovial fibroblasts obtained from NW or OB joints. A similar behaviour was noticed in stimulation with TNF-α. IL-1β stimulated articular chondrocytes, rather showed more IL-6 induction in the OB compared to the NW OA joints. IL-1β stimulated osteoblasts

also showed an increase in IL-6 mRNA in the OB cohorts compared to the OW and NW joints after one day stimulation. This was validated with the detection of higher levels of IL-6 secretion in a one day stimulated chondrocytes from OB joints compared to non-stimulated. This coincides with our group's previous findings in OA OB synovial fibroblasts secreting more IL-6 compared to NW synovial fibroblasts from OA joints [171].

lncRNA have been recently taking the attention of many researchers, and its role in many diseases were investigated and confirmed. LncRNA contribute to the pathogenesis of many diseases including OA but still the mechanism of their action is not fully understood. Several lncRNAs have been identified as candidates for mediating OA inflammatory pathology [170], including lncRNAs that are differentially expressed in human OA cartilage, lncRNAs associated with the IL-1β inflammatory response [170] and lncRNAs associated with inflammatory obese OA synovial tissue. HOTAIR regulating MMP levels [285] and with Small nucleolar RNA host gene 5 (SNHG5) [286], HOTTIP, RP11-445H22.4, lncRNA-CIR [287], and HoxA13 [288] cause cartilage degradation. Other lncRNA related to OA pathology mentioned in research include Maternally expressed gene 3 (MEG3) [289], Prostate specific gene (PCGEMI), Growth arrest specific 5 (GAS5) [290], and Small nucleolar host gene 1 (SNHG1) [291]. MALAT1 epigenetic regulation was reported in many diseases including cancer, immunological, and inflammatory diseases. However, the role of MALAT1 in cells originating from different tissues of joints affected with OA has not been fully investigated. Since inflammatory changes were suggested to have a role in the pathogenesis of OA [290]. Since MALAT1 like other lncRNAs was expressed in immune cells [170,292], this indicates a role in the immune cell functions. This raises

the question whether MALAT1 might have a role in regulating inflammatory pathways and may have a possible role in the pathogenesis of OA.

Recently, evidence has emerged from our group [170] and others on the role of lncRNAs epigenetically mediating cellular inflammation. In particular, several lncRNAs have been identified that exhibit differential expression in OA diseased cartilage or are associated with inflammatory OA tissues. Using bioinformatics software to analyse RNAseq data of the synovial fibroblast transcriptome and have validated by qRT-PCR the differential expression of a number of lncRNAs, including MALAT1, in obese OA synovial fibroblasts.

MALAT1 is one of the most dysregulated lncRNA in many diseases, and recently much evidence was published in research indicating its role in inflammatory processes. Like the rest of the lncRNA, MALAT1 exerts its regulation on the three inflammatory pathways mentioned earlier. MALAT1 lncRNA was found to be one of the regulators of the inflammatory pathway MAPK by regulating the kinases like p38, JNK, and ERK. This kind of regulation was seen in many conditions like in glioma metastasis [293], and diabetes related dysfunction in rodents [294,295]. Researchers also, indicated a possible role for MALAT1 in SLE. This lncRNA worked by inhibiting the stimuli to NF-*K*B pathway reducing inflammatory cytokines leading to immunological diseases [296]. Determining the expression of these lncRNAs (in particular MALAT1) in OA joint tissues across different patient cohorts, and determining their functional role in isolated OA, synovial fibroblasts, chondrocytes and osteoblasts will increase our understanding of the cellular molecular mechanisms that drive OA disease. Furthermore, it may identify candidate targets for the development of novel therapeutics that can modify disease progression.

My hypothesis for this part of the project is that MALAT1 lncRNA has a regulatory role in mediating joint inflammation by impacting on the inflammatory phenotype of the cells in the OA joint, namely chondrocytes from the articular cartilage, osteoblasts from the subchondral bone and synovial fibroblasts from the synovial membrane. For this purpose, a total of 16 tissues were utilized for the analysis of MALAT1 expression from different BMI cohorts of OA joints including 3 synovial membrane, 8 articular cartilage and 5 subchondral bone.

In this project it was shown for the first time that MALAT1 expression was increased in primary human OA chondrocytes and primary human OA osteoblasts during a cytokine-induced inflammatory response. This data provides further support for the role of MALAT1 in mediating inflammatory responses in the OA joint. All cohorts expressed MALAT1 under *in vitro* inflammatory challenges with varying degrees. The synovial fibroblasts showed more MALAT1 expression in NW compared to OB joints. This finding is in contrast to our group's previous finding [171], which may be due to the limited number of samples included in this project. The articular chondrocytes expressed more MALAT1 in OB compared to NW OA joints. This association between MALAT1 expression and IL-6 secretion being higher in OB compared to NW articular chondrocytes, may indicate a regulatory role for MALAT1 in articular chondrocytes from OA joints. In addition, in this project MALAT1 expression in osteoblasts was more in OW compared to OB or NW OA joints. This may suggest a role for MALAT1 expression in OB osteoblasts cultured from OA subchondral bone. More samples are needed in order to prove this finding statistically.

The limitation of this study was in the shortage of patient replicates of a similar BMI from same OA joints, which limited the comparative analysis between tissues from certain joints like hand, wrist, and toe. However, results from this project showed a general overview on the expression of MALAT1 under inflammatory conditions in tissues from different BMI cohorts of weight-bearing and non-weight bearing OA joints.

In conclusion all primary cells including synovial fibroblasts, articular chondrocytes and osteoblasts cultured from OA joints of different BMI cohorts showed IL-6 induction upon stimulation with pro-inflammatory cytokines and this was associated with the expression of MALAT1 long non-coding RNA. This finding leads to the next stage of the project which is to explore MALAT1 profiling in OA cohorts of bone and cartilage tissues and look into its possible correlation with BMI, age, gender, and OA severity.

CHAPTER 5

MALAT1 Profile in Bone and Cartilage

5.1 Background

Despite initially considered to be a disease solely of the articular cartilage, it is now widely accepted that OA is a disease that encompasses the whole joint, including underlying subchondral bone tissue [297,298]. Pathological changes to OA subchondral bone tissue, characterized by trabecular thickening [299], abnormal type I Collagen production [300-301] and the formation of osteophytes, occurs early in the disease course of OA [302] and involves changes to the osteoblast phenotype [303]. Furthermore, in animals that are prone to developing OA trabecular thickening occurs prior to cartilage degeneration [304] and thus it has been suggested that this alteration to the bone architecture may pathologically alter the loading biomechanics, thus promoting cartilage degeneration [297,305].

Recently, long non-coding RNAs (lncRNAs) have emerged as novel epigenetic regulators of gene transcription [306-307] and of mediating several cellular processes including cellular proliferation, cell cycle control, apoptosis, and the innate inflammatory response [308-309]. As such, lncRNAs have now been implicated in the pathology of a number of chronic inflammatory diseases [310-311], including OA [312]. Indeed, lncRNAs have been identified that are differentially expressed in OA diseased cartilage and which mediate the IL-1β inflammatory response in OA chondrocytes [170,257]. In addition, lncRNA's were found to regulate chondrogenesis, [313], and increase chondrogenic differentiation leading to cartilage formation [314]. The process of cartilage degeneration leading to diseases like OA was proven to be regulated with a number of LncRNAs including MALAT1 [315].

The role of MALAT1 in regulating chondrogenesis and cartilage development was proven to act through different pathways. It was shown that MALAT1 regulates chondrocytes through PI3K/Akt pathway [270,316].

In bone tissues and osteoblasts, the expression of MALAT1 was reported to be greater in the bone tissue of patients who exhibit aseptic loosening following a hip replacement [317] and its upregulation has been implicated in lumber intervertebral disc degeneration [318]. In vitro, knockdown of MALAT1 has been demonstrated to inhibit the proliferation of the human osteoblast cell line hFOB 1.19 [316], and MALAT1 sponging of the microRNA miR-30 has been shown to promote the osteoblast differentiation of mesenchymal stem cells by inducing RUNX2 expression [319]. Furthermore, MALAT1 is associated with abnormal osteogenic and adipogenic differentiation of BMSCs in the patients with osteonecrosis of the femoral head [320]. Furthermore, we recently reported that MALAT1 lncRNA modulates the inflammatory phenotype of synovial fibroblasts in the OA synovial joint lining by mediating the production of CXCL8 [171]. However, importantly, MALAT1 lncRNA has now emerged as a central mediator of osteoblast function and bone homeostasis.

The aim of this chapter is to profile MALAT1 expression in cartilage and bone tissues of normal weight and obese OA patients, and correlate the findings with Anthropometrics (age, BMI, waist/ hip ratio), joint health parameters (osteophyte formation, joint severity KL scale and joint space narrowing scores), and serum / synovial cytokine concentration (Chemokines, chemotactic, proinflammatory cytokines).

5.2 Methods

Ethical approvals (UK NRES 16/SS/0172 & NRES 14/ES/1044) were obtained for this study. An informed consent was obtained from all subjects involved in the study. A total of 17 OA patient's demographic data were used for the articular cartilage tissues, including 10 males and 7 females. In addition, subchondral bone tissue and blood was collected from a total of 17 patients (9 males, 8 females) with end-stage OA and from neck of femur fracture (NOF) (n = 6) from non-OA patients, who were undergoing surgery at Russell's Hall Hospital (Dudley, UK) or the Royal Orthopaedic Hospital (Birmingham, UK). Exclusion criteria includes patients with secondary causes with OA, such as avascular necrosis, Perthes disease, developmental dysplasia, previous acetabular or femoral neck fractures and slipped upper femoral epiphysis. Presence of OA in different body joints including end-stage knee and /or hip OA, was confirmed by radiographic assessment and the severity of OA was determined by Kellgren Lawrence scale (KL), and joint space narrowing scores. A detailed patient history was documented for each patient including age, gender, weight, height, BMI, central adiposity measured by waist/hip ratio (W:H) and body fat %, presence of hand OA, surgery, and medications used. The serum concentration of 24 cytokines were obtained using Luminex multiplex platform (Luminex R&D systems Minnesota, USA), diluted to 1:2 in assay buffer according to the manufacturer's protocol.

5.3 Results

5.3.1 MALAT1 Expression in Articular Cartilage Tissues

5.3.1.1 Patients Demographics

The OA patient characteristics used in the chapter are detailed in Table 5.1, and in total involved 17 patients including articular cartilage tissues from 6 hip (4 NW, 2 OB) and 11 knee joints (5 NW, 6 OB).

Table 5.1 OA patient characteristics in articular cartilage

	All OA	Knee OA	Hip OA
Age	68.59 ± 2.07	64.91 ± 2.39	75.33 ± 1.98
Gender (M/F)	10:7	4:7	6:0
Height (cm)	166.2 ± 1.9	168.9 ± 1.9	169.3 ± 3.4
Weight (Kg)	82.4 ± 5.8	94.5 ± 6.2	60.1 ± 3.5
BMI (kg/m^2)	28.9 ± 1.7	30.2 ± 2.4	26.5 ± 2
% Fat	35.4 ± 3.1	39.6 ± 2.4	25.4 ± 4
WHR[1]	0.89 ± 0.02	0.9 ± 0.03	0.89 ± 0.001
KL Grade	4 (3.5-4)	4 (3.25-4)	4 (3.5-4)

[1] WHR= Ratio of waist to hip circumference. All values represent mean ± SEM, except KL grade which is shown as median (25th-75th percentile).

5.3.1.2 Correlation between patient anthropometrics in articular cartilage

Looking at the patient's characteristics, a significant correlation was seen between age and BMI in knee joints (R^2 = 0.65, *P* = 0.002) (Figure 5.1.A), but not in hip joints (R^2 = 0.01, *P* = 0.5) (Figure 5.1.B).

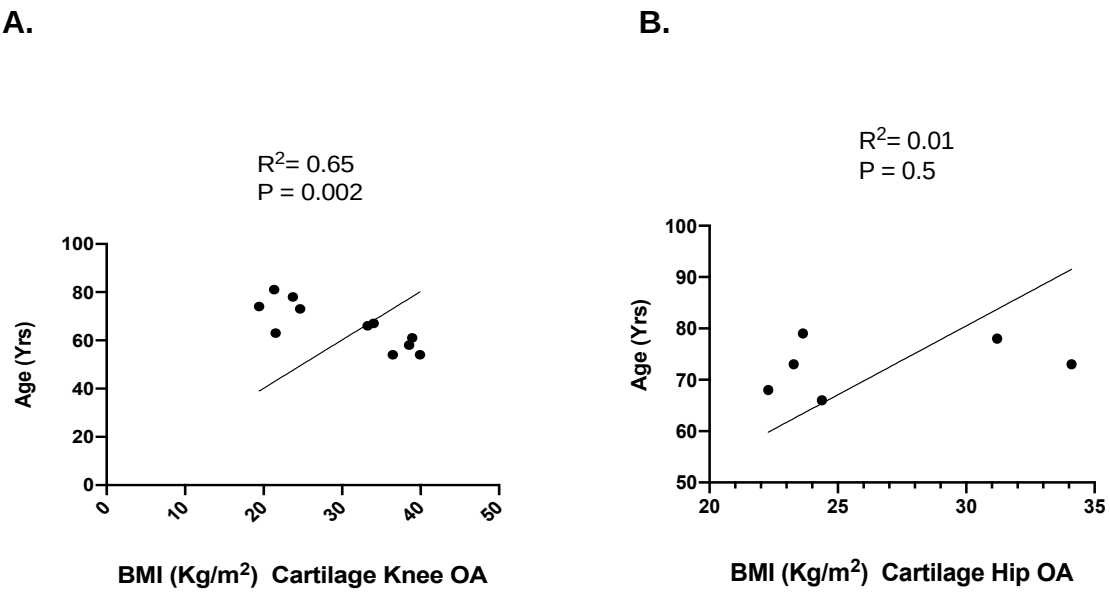
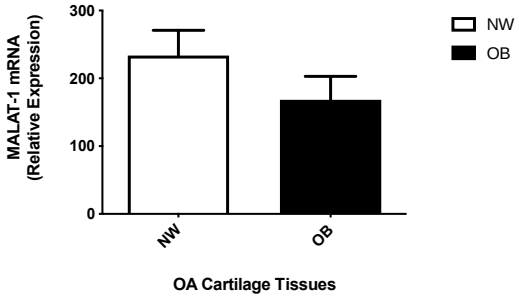


Figure 5.1 Association between age and BMI in articular cartilage in OA knee joints, n=11 patients **(A)** in OA hip, n=6 patients **(B)**. There was no significant correlation between age and BMI in the hip or knee OA joints.

5.3.1.3 Correlation of MALAT1 expression in articular cartilage with anthropometrics

MALAT1 expression was studied in 10 male and 7 female cartilage tissues from knee and hip joints of NW and OB OA patients. There was no significant difference in MALAT1 expression between cartilage obtained from NW and OB OA joints (Figure 5.2.A). However, a significant difference was observed in MALAT1 expression between cartilage from OA OB hip compared to OB knee joints and in cartilage from NW knee compared to OB knee ($P < 0.04$), (Figure 5.2.B). On average, MALAT1 expression in articular cartilage was higher in male compared to female OA patients but this difference was not statistically significant (Figure 5.2.C).

A.



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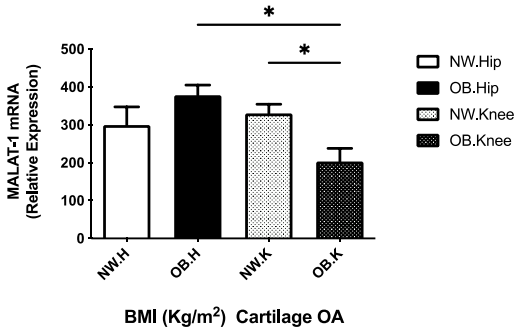


C.

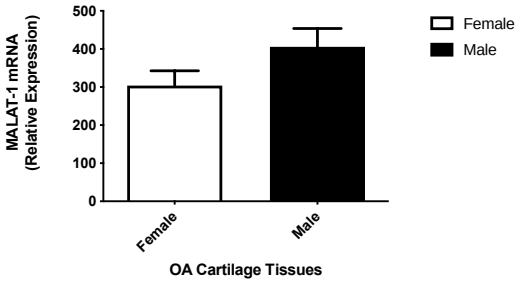


Figure 5.2 MALAT1 expression in articular cartilage of different OA and cohorts. NW vs OB joints, **(A)** Hip and knee joints **(B)**. NW.H = normal weight hip, OB.H = Obese hip, NW. K = NW knee, OB. K = OB knee. *n* = 17 patients, MALAT1 expression in cartilage of different gender **(C).** Female n=7patients and male n=10 patients.

MALAT1 expression in articular cartilage was not correlated with any other anthropometric measures including age, weight, hight, waist:hip ratio (W:H) in male and female and/or NW and OB OA joints and fat % (Figure 5.3.I A-F) and (Figure 5.3.I A-C).

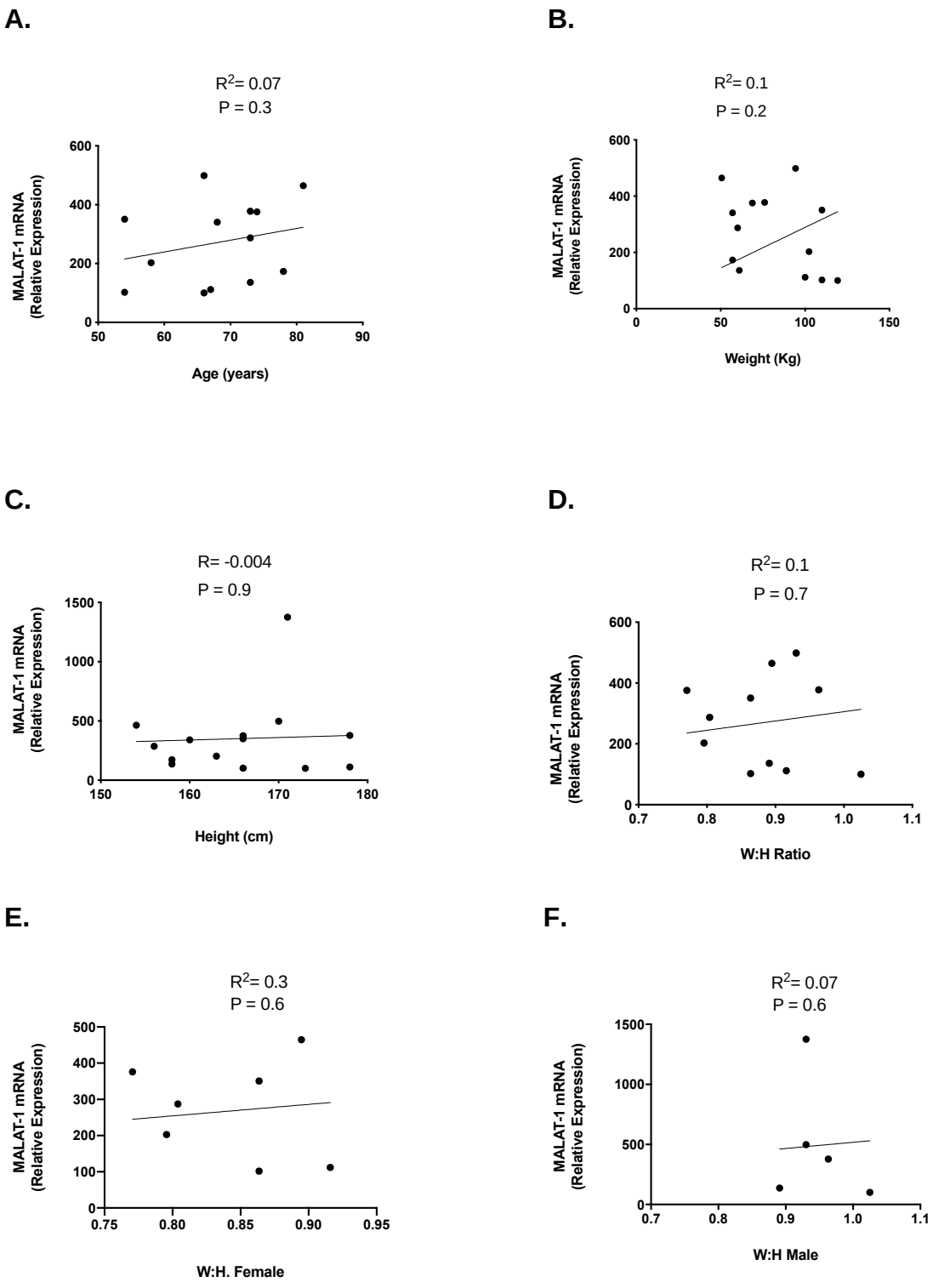


Figure 5.3.I MALAT1 expression in articular cartilage, correlation with different anthropometric measures including age n=17 patients **(A)**, weight n=17 patients **(B)**, height n=17 patients **(C)**, W:H ratio n=14 **(D)**, W:H ratio in female n=7 patients **(F)**.

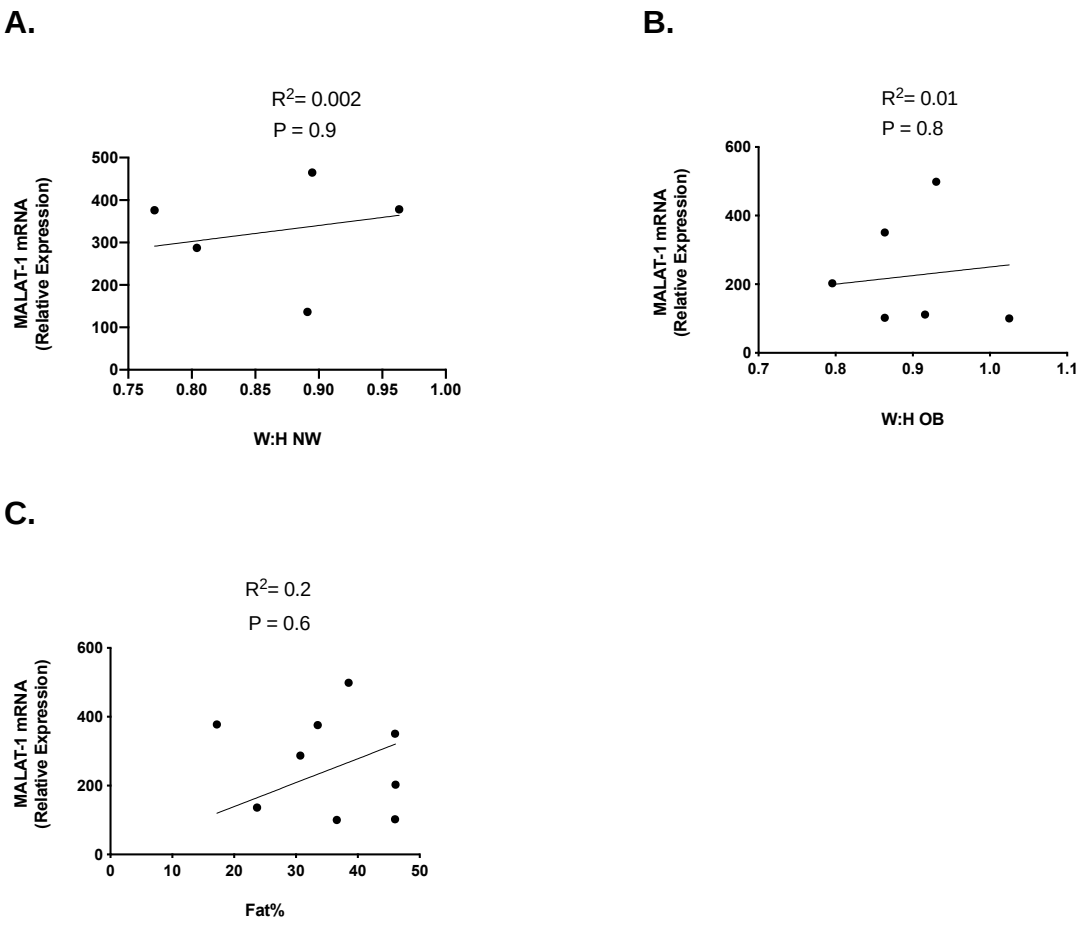


Figure 5.3.II MALAT1 expression in articular cartilage, correlation with different anthropometric measures including age n=17 patients **(A)**, weight n=17 patients **(B)**, height n=17 patients **(C)**, W:H ratio n=14 **(D)**, W:H ratio in female n=7 patients **(F)**.

5.3.1.4 Correlation of MALAT1 expression in articular cartilage with joint health parameters

MALAT1 expression in articular cartilage association with joint health parameters was analysed next. None of the joint health parameters including osteophyte formation, presence of hand OA, KL scale and JSN scores in (mm), showed any association with MALAT1 expression in cartilage (Figure 4.5. A-G).

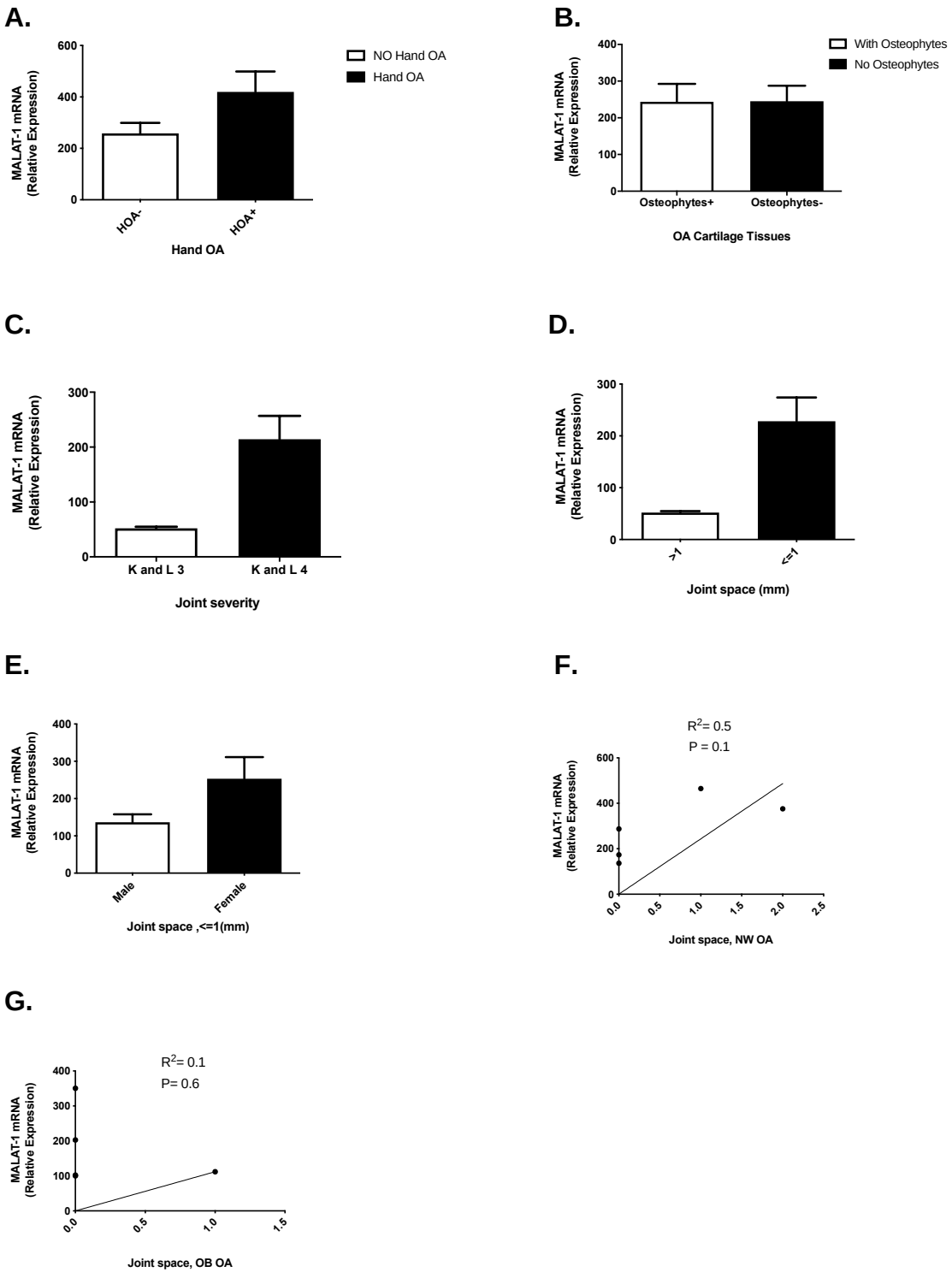


Figure 5.4 MALAT1 expression in articular cartilage joint health parameters including presence of hand OA [HDOA (+)] n = 3 patients, [HDOA (-)] n = 13 patients **(A)**, presence of osteophytes, [osteophytes (+)] n = 10 patients, [osteophytes (-)] n = 4 patients **(B)**, [KL=3] n = 3 patients, [KL=4] n = 11 patients **(C)**, joint spaces [>1 n] = 3, patients [<=1] n = 10 patients **(D)**, joint spaces <=1 with gender [male] n = 5 patients, [female] n = 6, patients **(E)**, joint spaces in NW OA n = 6 patients **(F)**, joint spaces in OB OA n = 8 patients **(G)**.

5.3.2 MALAT1 expression in Subchondral Bone Tissues

5.3.2.1 Patients Demographics

17 OA patient characteristics detailed in Table 5.2, including subchondral bone tissues from 9 hip (5 NW, 4 OB) and 8 knee joints (3 NW, 5 OB).

Table 5.2 OA patient characteristics in subchondral bone

	All OA	Knee OA	Hip OA
Age (years)	65.00 ± 2.22	66.56 ± 2.75	62.00 ± 4.5
Gender (M/F)	9:8	4:4	5:4
Height (cm)	167.2 ± 3.2	164.8 ± 5.4	169.4 ± 3.8
Weight (Kg)	82.4 ± 4.2	84.2 ± 7.9	80.8 ± 4.3
BMI (kg/m^2)	29.7 ± 1.6	31.1 ± 2.7	28.4 ± 1.9
% Fat	34.8 ± 2.4	35.0 ± 5.1	34.8 ± 2.3
WHR[1]	0.92 ± 0.02	0.93 ± 0.03	0.91 ± 0.03
KL Grade	4 (3.5-4)	4 (3.25-4)	4 (3.5-4)

[1] WHR= Ratio of waist to hip circumference. All values represent mean ± SEM, except KL grade which is shown as median (25th-75th percentile).

5.3.2.2 Correlation between patient anthropometrics in OA bone

Looking at the patient's characteristics, no significant correlation was seen between age and BMI in OA bone from knee or hip joints. (Figure 5.5.A & B).

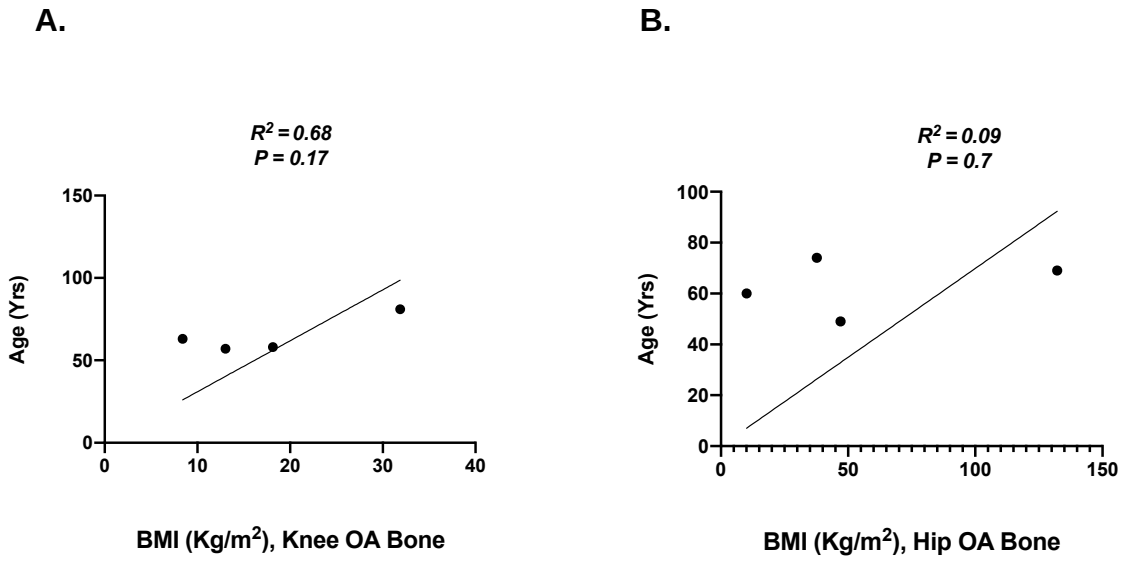
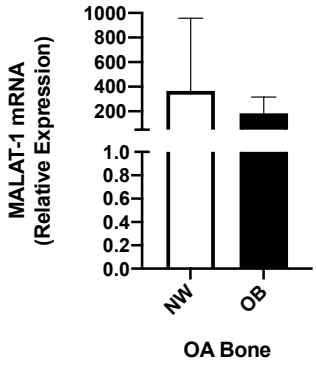


Figure 5.5 Association between age and BMI in OA bone tissues from knee joints, n=8 patients **(A)**, hip joints, n=9 patients **(B)**. There was no significant correlation between age and BMI in bone tissues from OA knee or hip joint.

5.3.2.3 Correlation of MALAT1 expression in OA bone tissues with anthro-pometrics

MALAT1 expression was studied in 9 male and 8 female subchondral bone tissues from knee and hip joints of NW and OB OA patients. There was no significant difference in MALAT1 expression between bone obtained from NW and OB OA joints (Figure 5.6.A), or between different joints (Figure 5.6.B). However, a significant difference was seen in MALAT1 expression and gender ($P < 0.04$), were OA bone tissues from male showed significantly more MALAT1 expression compared to female gender (Figure 5.7.A). 2-way ANOVA analysis of MALAT1 expression in different gender comparing knee verses hip joints showed a significant difference between the groups ($P = 0.0002$), significant difference in MALAT1 expression between gender ($P < 0.0001$) and between different joints in males and female ($P <0.0001$) (Figure 5.7.B). MALAT1 expression was significantly correlated with waist:hip ratio (W:H) in females ($R^2 = 0.99$, $P = 0.004$) (Figure 5.7.E) and was not correlated with any other anthropometric measures including age, weight, hight, waist:hip ratio (W:H) in male and/or NW and OB OA joints and fat % (Figure 5.8.I A-F) and (Figure 5.8.II A-C).

A.



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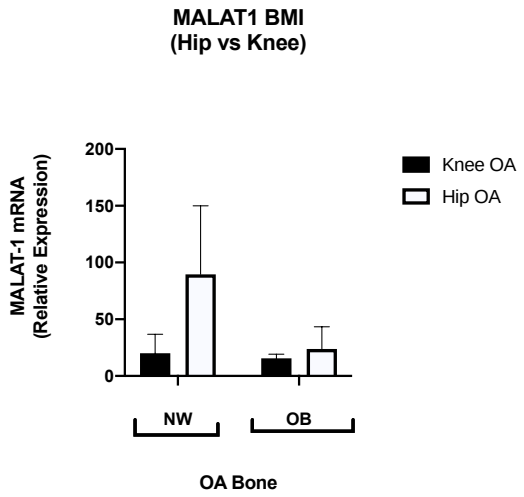
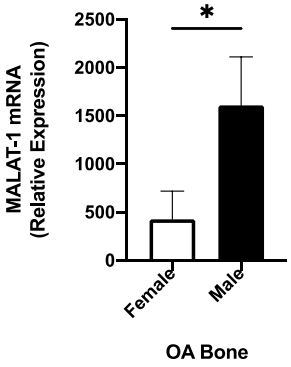


Figure 5.6 MALAT1 expression in in subchondral bone of different OA BMI cohorts including NW vs OB joints, n=8 and n= 9 patients respectively **(A)**. 2-way ANOVA: MALAT1 BMI (Hip vs knee) joints, NW.H = normal weight hip n=5 patients, OB.H = Obese hip n=4 patients, NW. K = NW knee n=3 patients, OB. K = OB knee n=5 patients **(B)**.

A.



B.

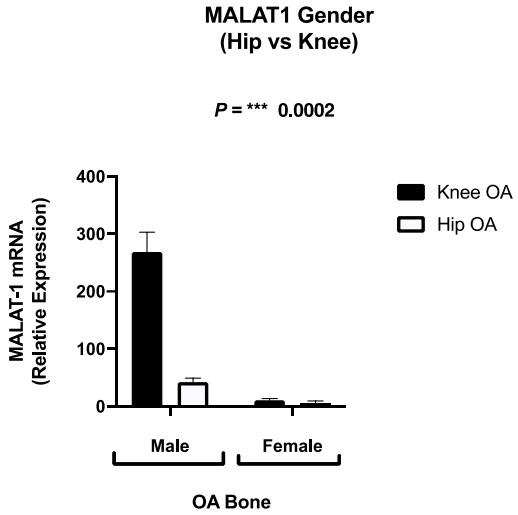


Figure 5.7 MALAT1 expression in OA bone of different gender. Female n=8 patients, male n=9 patients. A significant difference was seen in MALAT1experession, and gender of the OA bone tissues *P=0.04* **(A)**. 2-way ANOVA: MALAT1 (Hip vs knee) joints in male verses female, NW.H = normal weight hip n=5 patients, OB.H = Obese hip n=4 patients, NW. K = NW knee n=3 patients, OB. K = OB knee n=5 patients **(B)**.

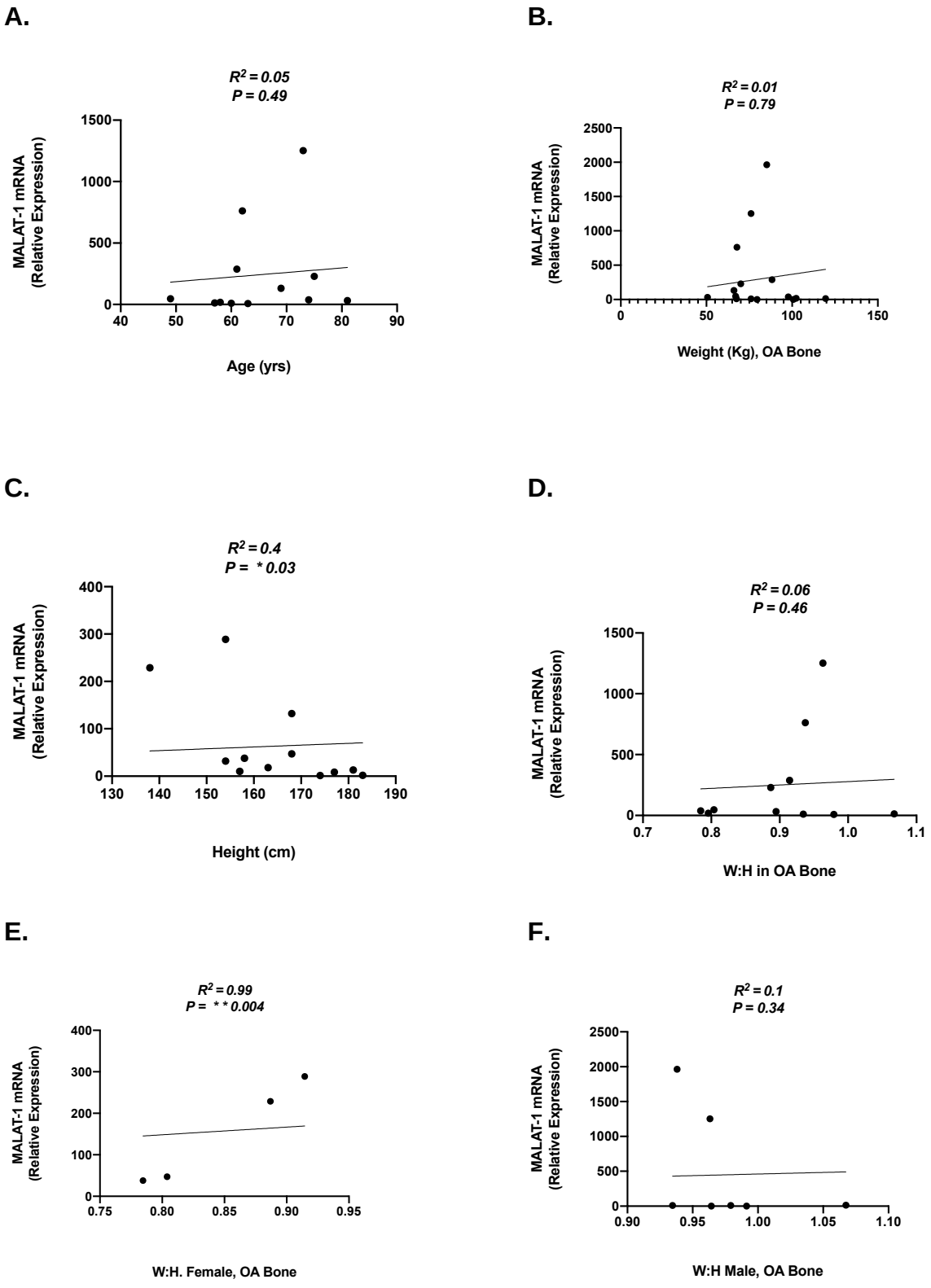


Figure 5.8.I MALAT1 expression in subchondral bone correlation with different anthropometric measures including age n=17 patients **(A)**, weight n=17 patients **(B)**, height n=17 patients **(C)**, W:H ratio n=16 patients **(D)**, W:H ratio in female n=7 patients **(E)** and W:H ratio in male n=9 patients **(F)**.

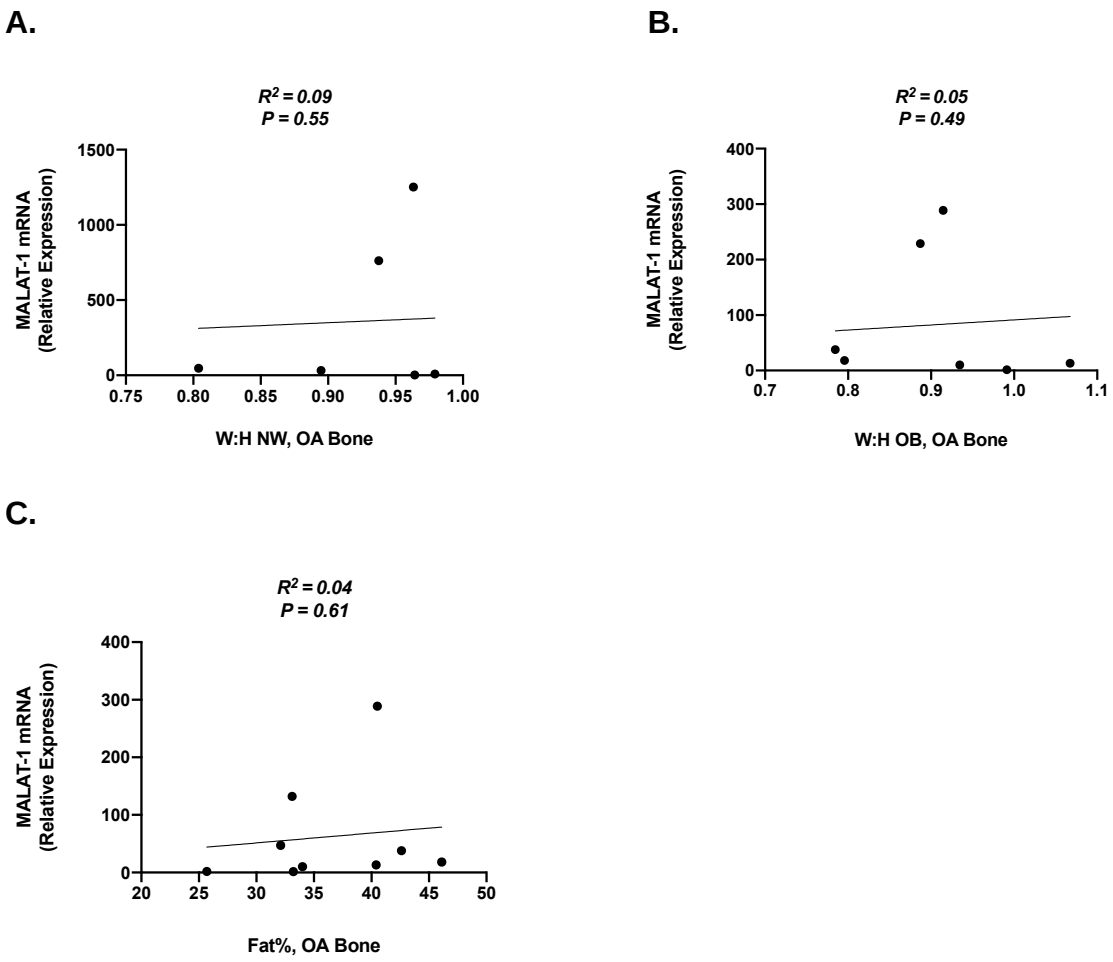


Figure 5.8.II MALAT1 expression in subchondral bone correlation with different anthropometric measures including W:H ratio in NW**,** n=7 patients **(A)**, W:H ratio in OB n=9 patients, (**B)** and fat % n=12 patients **(C)**.

5.3.2.4 Correlation of MALAT1 expression in subchondral bone with joint health parameters

Next, MALAT1 expression in subchondral bone and its association with joint health parameters was analysed. None of the joint health parameters including osteophytes formation, presence of hand OA, KL scale and JSN scores, showed any association with MALAT1 expression in subchondral bone (Figure 5.9. A-D).

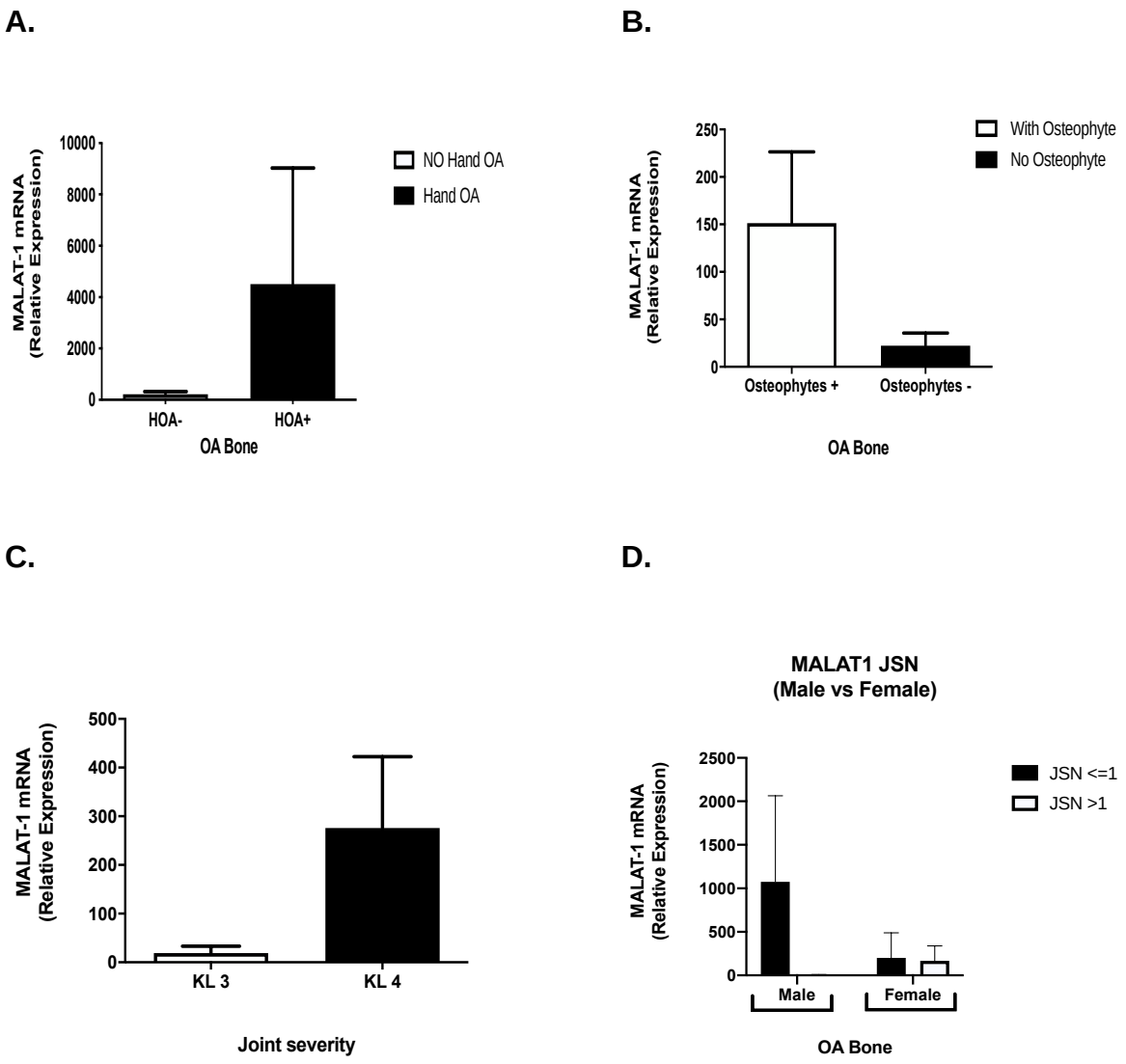


Figure 5.9 MALAT1 expression in subchondral bone and joint health parameters including presence of hand OA, HDOA (+) n = 2 patients, no HDOA (-) n = 15 patients **(A)**, presence of osteophytes, osteophytes (+) n = 12 patients, without osteophytes (-) n = 5 patients **(B)**, KL=3 n = 4 patients, KL=4 n = 13 patients **(C)**, joint spaces >1 male, female n=2 patients, <=1 male, female n=6 patients **(D)**.

5.3.3 The relationship between MALAT1 expression in articular cartilage and subchondral bone tissues with the concentration of inflammatory cytokines in serum and synovial in OA hip and knee joints

5.3.3.1 The relationship between MALAT1 expression in articular cartilage with serum and synovial inflammatory cytokines

The next step was to measure a panel of serum and synovial pro-inflammatory cytokines and chemokines by Luminex multiplex assay in 17 OA patients, in order to determine the relationship between local and circulatory levels of pre-inflammatory factors with the expression of MALAT1 in the articular cartilage. A total of 5 adipokines (visfatin, resistin, leptin, adiponectin and chemerin), 4 chemotactics (MIP1a, MIP1b, MIP3a, and MCP1), 8 chemokines (Dkk1, galectin1, Eotaxin, amphiregulin, aggrecan, FABP4, serpin E1, and Ip10), and 6 pro-inflammatory cytokines (ILIβ. IL-6, IL7, IL10, IL15, and TNFα), (Tables 5.3.I, 5.3.II and 5.4) were measured.

None of the five tested adipokines in the serum or the synovial fluid showed any correlation with MALAT1 expression in the OA cartilage (Figure 5.10 & 11). The same was true for the four chemotactics in serum (Figure 5.12) and the synovial fluid except for one chemotactic MIP3a (R^2 = 0.75, *P* = 0.006), which was significantly correlated with MALAT1 expression in the synovial fluid (Figure 5.13). Two only of the 8 chemokines tested in serum were significantly correlated with MALAT1 expression in OA cartilage, including DKK1 (R^2 = 0.528, *P* = 0.027) (Figure 5.14.I.A) and Eotaxin (R^2 = 0.646, *P* = 0.009) (Figure 5.14.I.C), but none in the synovial fluid of the OA cartilage (Figures 5.15.I and 5.15.II).

In addition, none of the 6 pro-inflammatory cytokines in the serum or the synovial fluid showed any correlation with MALAT1 expression in cartilage from OA joints (Figure 5.16.I – 5.17.II).

Table 5.3.I Correlation of MALAT1 expression in articular cartilage with serum inflammatory cytokines, in 17 OA patients including 6 hip and 11 knee OA patients

(pg/mL)	P(two-tailed)	R^2
Visfatin	0.066	0.299
Resistin	0.278	0.145
Leptin	0.338	0.092
Adiponectin	0.913	0.001
Chemerin	0.284	0.114
Dkk1	***0.027***	0.528
Galectin1	0.244	0.103
Eotaxin	***0.009***	0.646
Amphiregulin	0.775	0.006
Aggrecan	0.304	0.081
FABP4	0.987	1.956e-005
Serpin E1	0.271	0.099
IP10	0.959	0.0002

Cytokines measured by Luminex multiplex and expressed as pg/mL

Table 5.3.II Correlation of MALAT1 expression in articular cartilage with serum inflammatory cytokines, in 17 OA patients including 6 hip and 11 knee OA patients

(pg/mL)	P(two-tailed)	R^2
MIP1a	0.289	0.111
MIP1	0.685	0.017
MCP1	0.209	0.189
MIP3a	0.219	0.146
IL1β	0.542	0.065
IL-6	0.498	0.121
IL7	0.441	0.076
IL10	0.112	0.285
IL15	0.857	0.005
TNF-α	0.263	0.123
Gp130	0.263	0.202

Cytokines measured by Luminex multiplex and expressed as pg/mL

Table 5.4 Correlation of MALAT1 expression in articular cartilage with synovial inflammatory cytokines in 17 OA patients including 6 hip and 11 knee OA patients

(pg/mL)	P(two-tailed)	R^2
Resistin	0.469	0.137
Leptin	0.118	0.496
Adiponectin	0.388	0.189
Chemerin	0.723	0.019
Dkk1	0.768	0.528
Galectin1	0.677	0.038
Eotaxin	0.283	0.577
Amphiregulin	0.382	0.155
Aggrecan	0.603	0.058
FABP4	0.382	0.155
Serpin E1	0.623	0.052
IP10	0.255	0.248
MIP1a	0.492	0.125
MIP1b	0.145	0.562
MCP1	0.186	0.271
MIP3a	***0.006***	0.746
IL1b	0.206	0.362
IL-6	0.818	0.011
IL7	0.277	0.229
IL10	0.060	0.539
IL15	0.803	NA*
TNF-α	0.585	0.111
Gp130	0.603	0.073

*NA = Non-Available, Cytokines measured by Luminex multiplex and expressed as pg/mL.

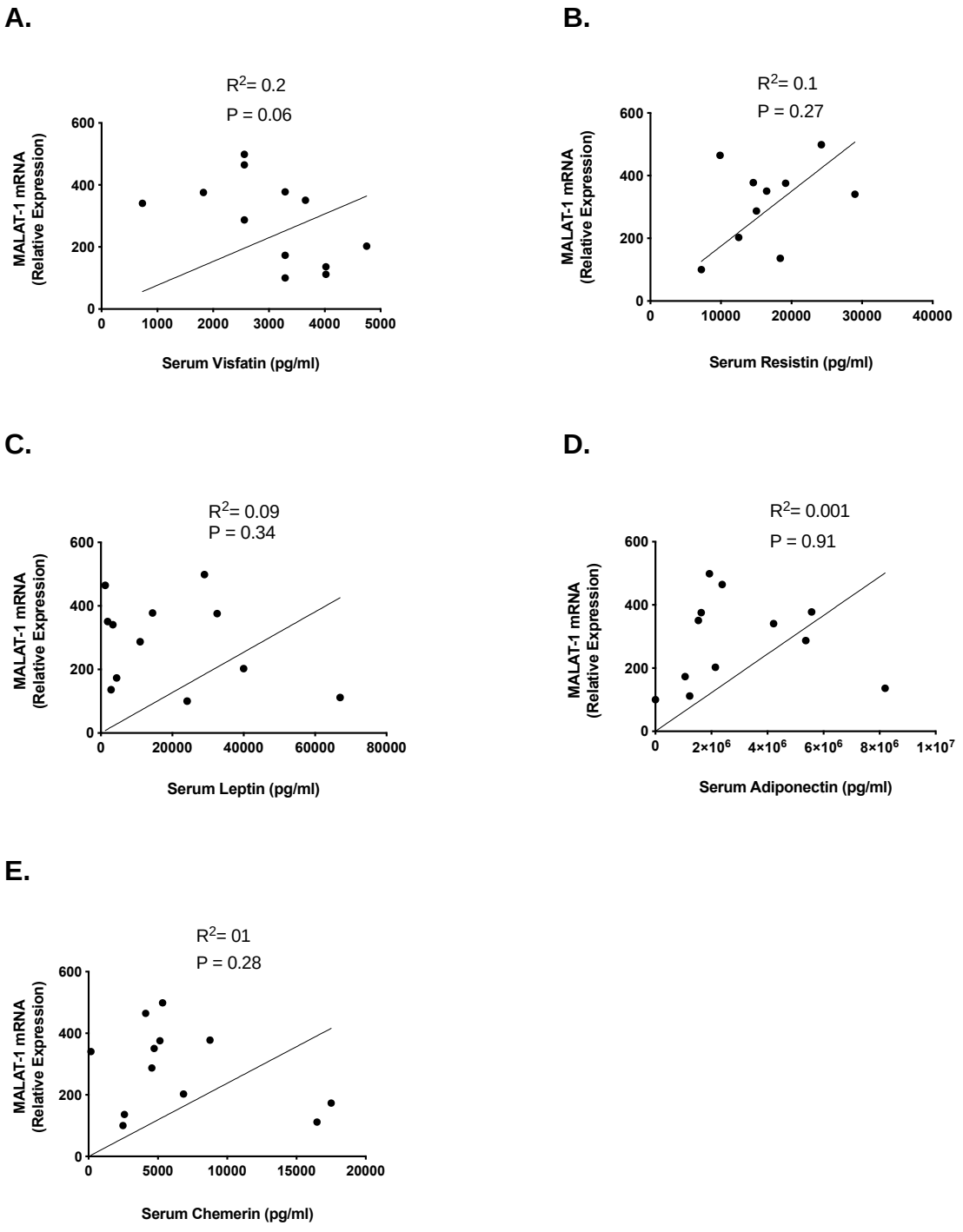


Figure 5.10 MALAT1 expression in articular cartilage correlation with serum adipokines in hip and knee OA joints including visfatin n = 16 patients **(A)**, resistin n = 14 patients **(B)**, leptin n = 16 patients **(C)**, adiponectin n = 16 patients **(D)**, chemerin n = 16 patients **(E)**.

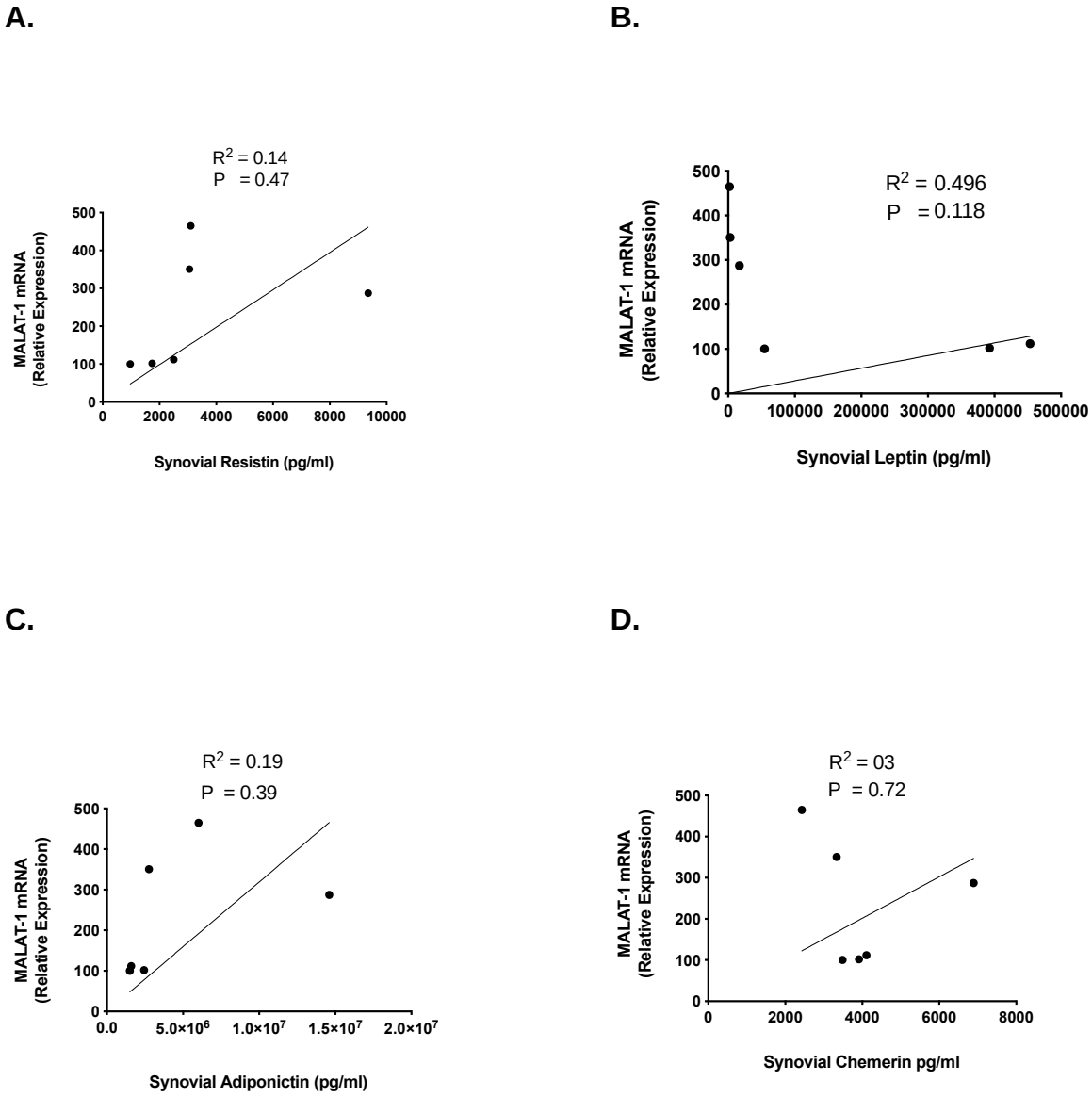


Figure 5.11 MALAT1 expression in articular cartilage correlation with synovial adipokines in hip and knee OA joints including resistin **(A)**, leptin **(B)**, adiponectin **(C)**, chemerin n = 8 patients **(D)**.

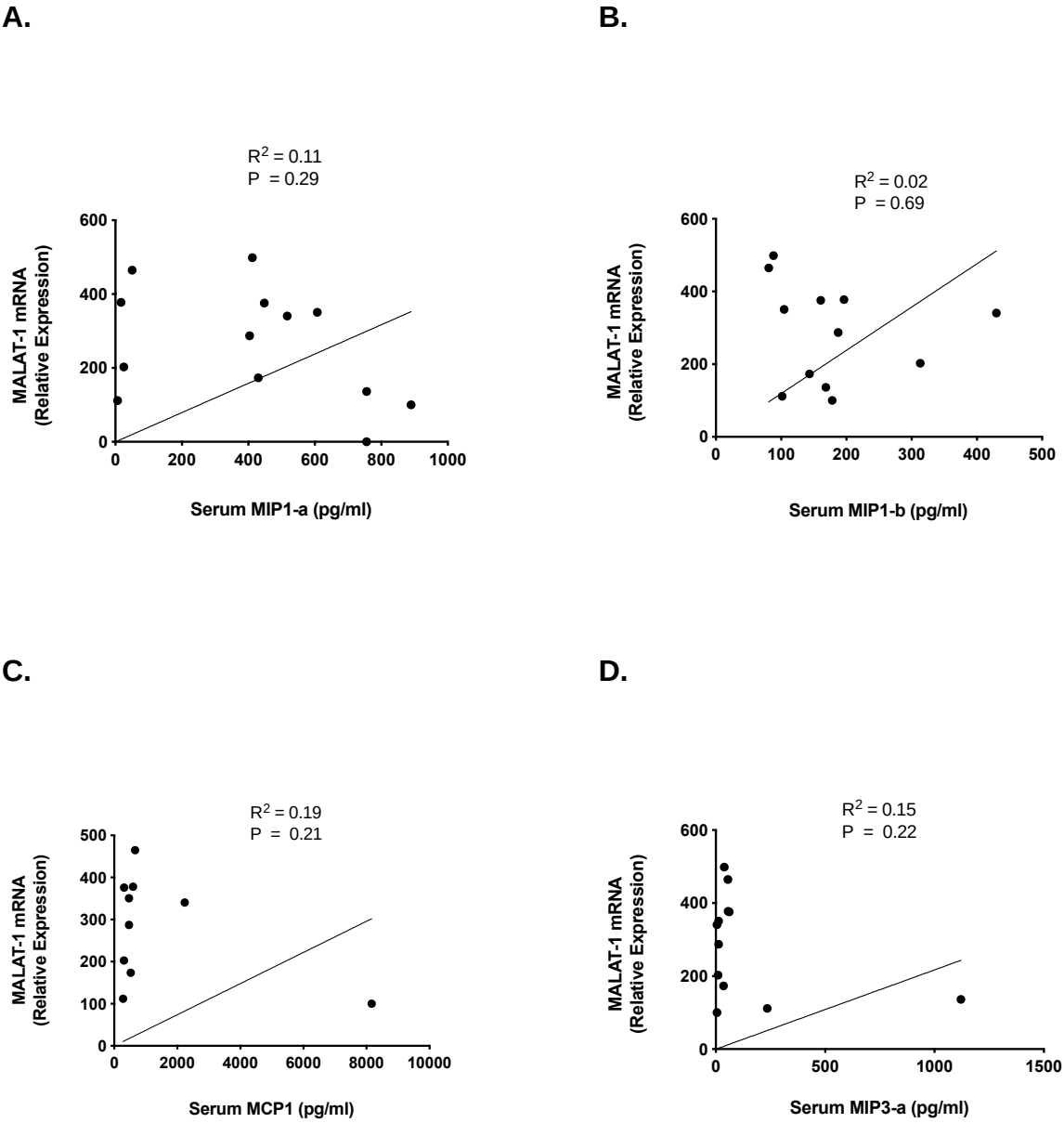


Figure 5.12 MALAT1 expression in articular cartilage correlation with serum chemotactics in OA patients including MIP1-a n = 16 patients **(A)**, MIP1-b n = 16 patients **(B),** MCP1 n = 15 patients **(C)** MIP3-a n = 16 patients **(D)**.

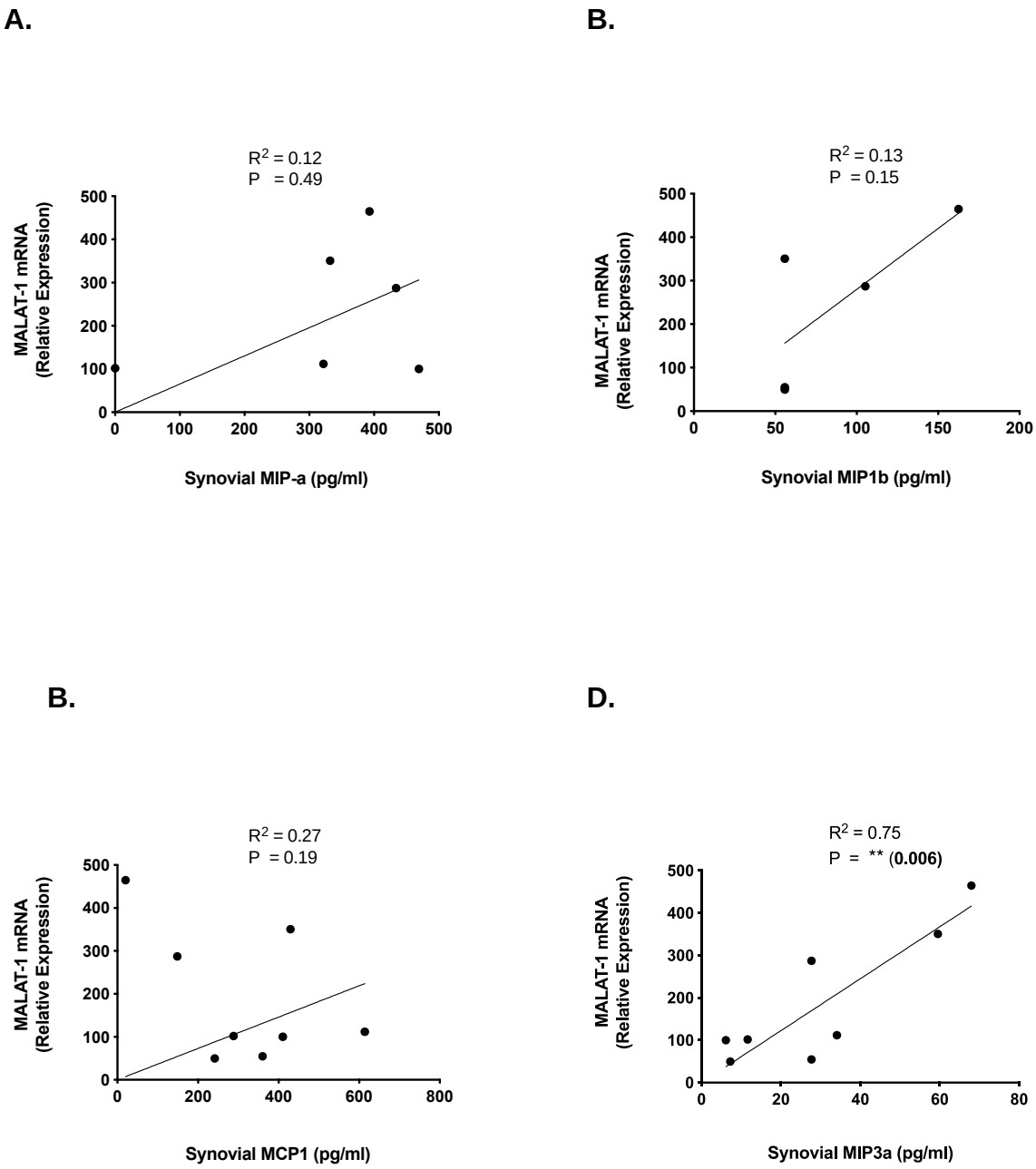


Figure 5.13 MALAT1 expression in articular cartilage correlation with synovial chemotactics in hip and knee OA joints including MIP1-a n = 7 patients **(A)**, MIP1-b n = 5 patients **(B)**, MCP1 n = 8 patients **(C)**, MIP3-a n = 8 patients **(D)**.

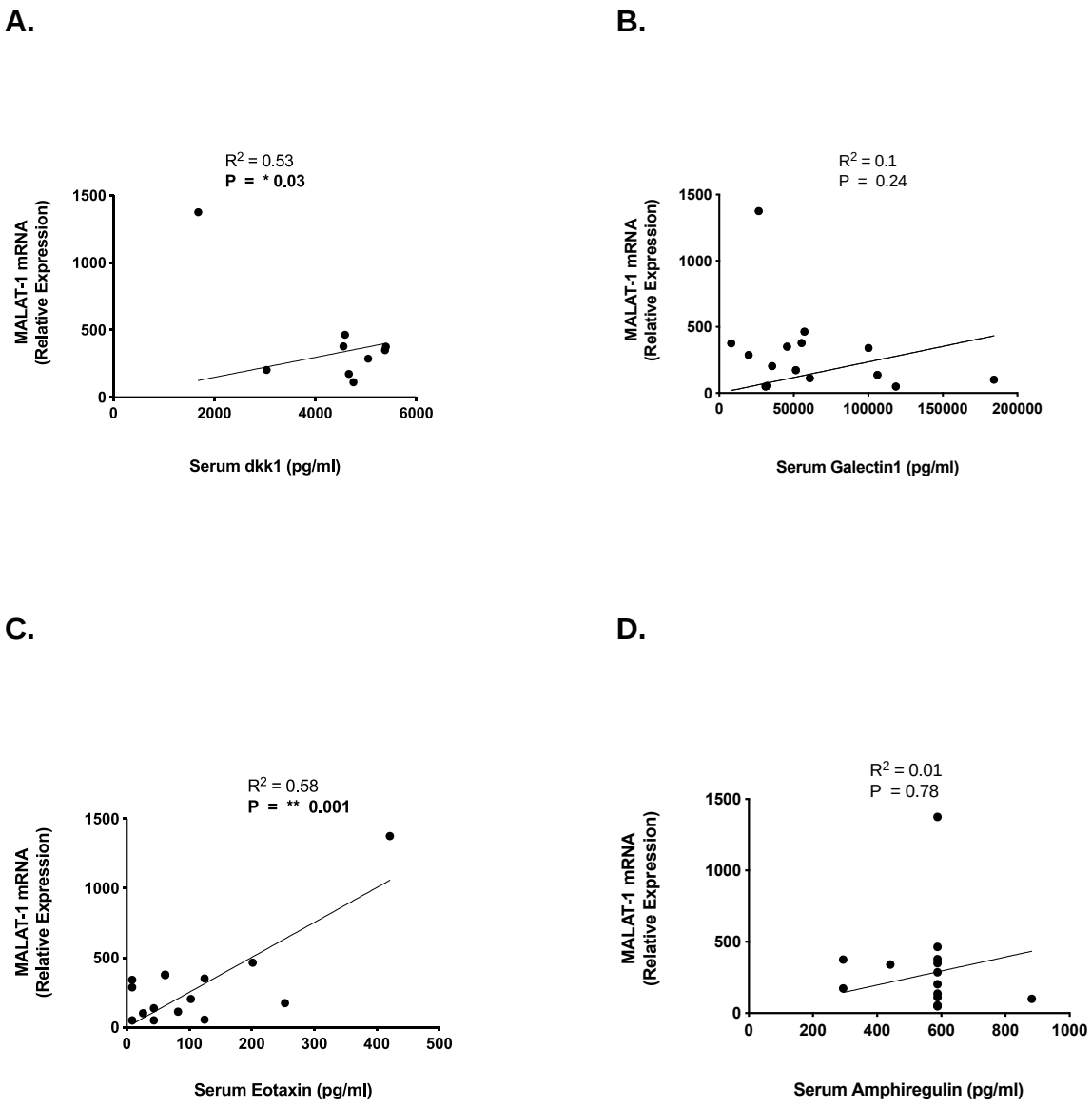


Figure 5.14.I MALAT1 expression in articular cartilage correlation with serum chemokines in hip and knee OA joints including DKK1 **(A)**, Galectin1 **(B)**, Eotaxin **(C)**, amphiregulin **(D)**.

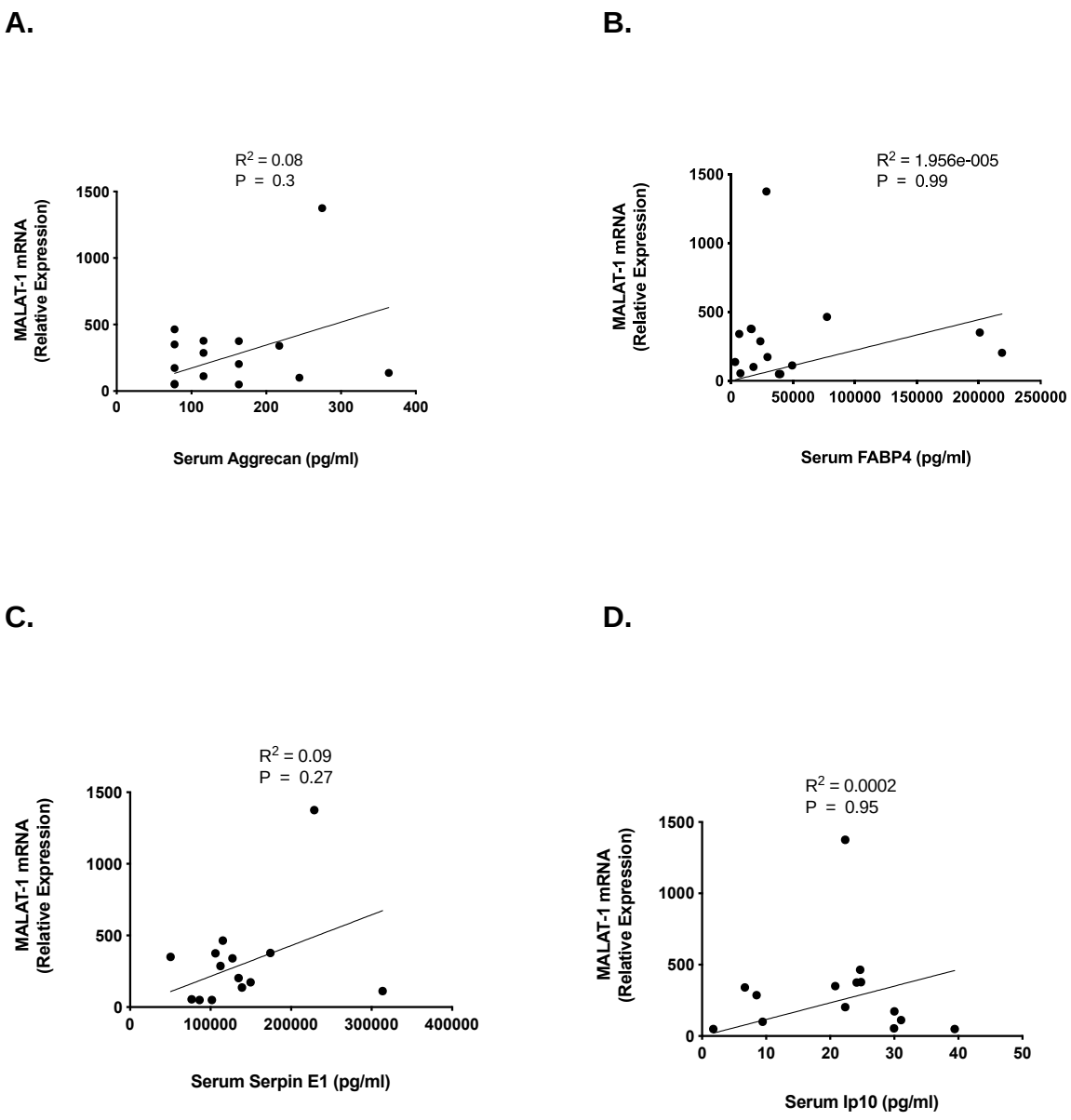


Figure 5.14.II MALAT1 expression in articular cartilage correlation with serum chemokines in hip and knee OA joints including Aggrecan **(A)**, FABP4 **(B)**, Serpin E1 **(C)**, IP10 n = 15 patients **(D)**.

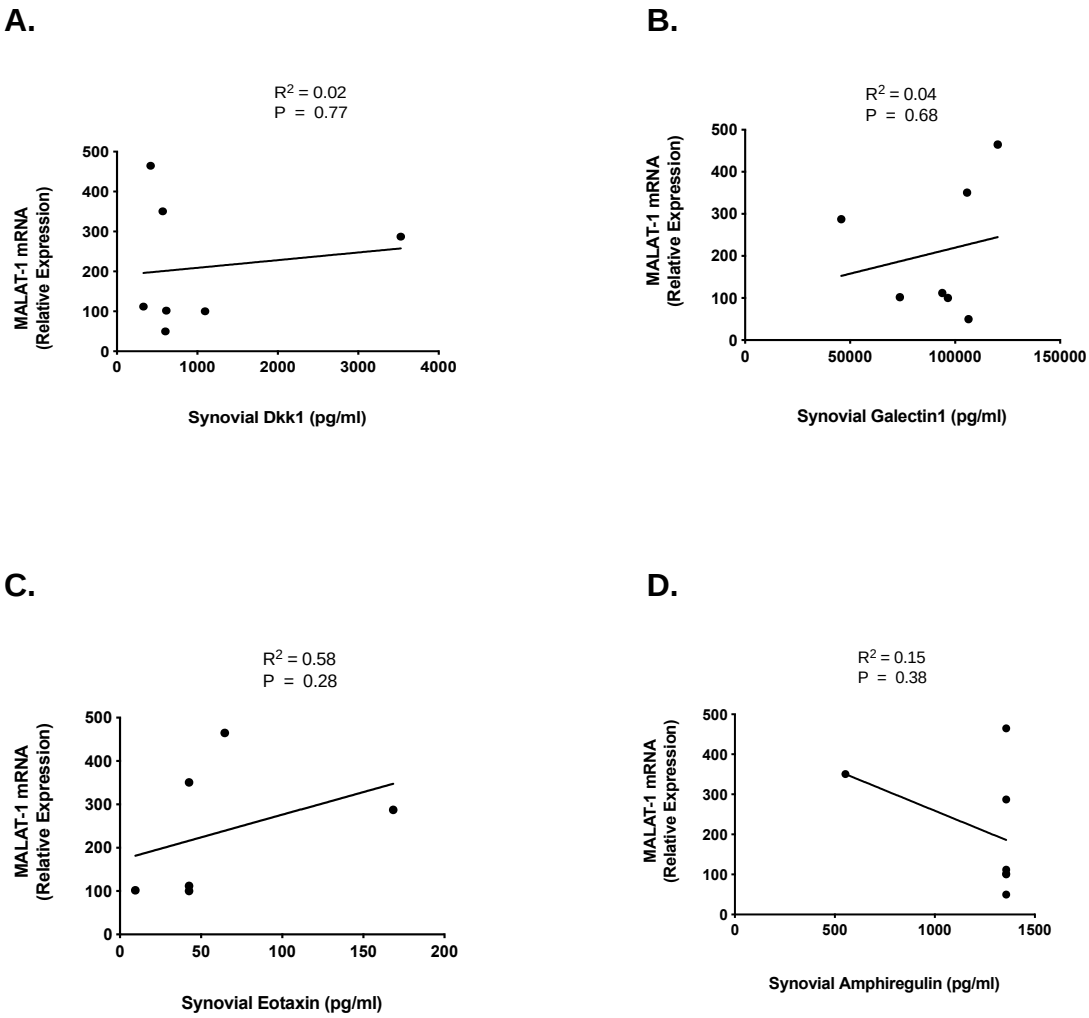


Figure 5.15.I MALAT1 expression in articular cartilage correlation with synovial chemokines in hip and knee OA joints including DKK1 n = 8 patients **(A)**, Galectin1 n = 8 patients **(B)**, Eotaxin n = 6 patients **(C),** Amphiregulin n = 8 patients **(D)**,

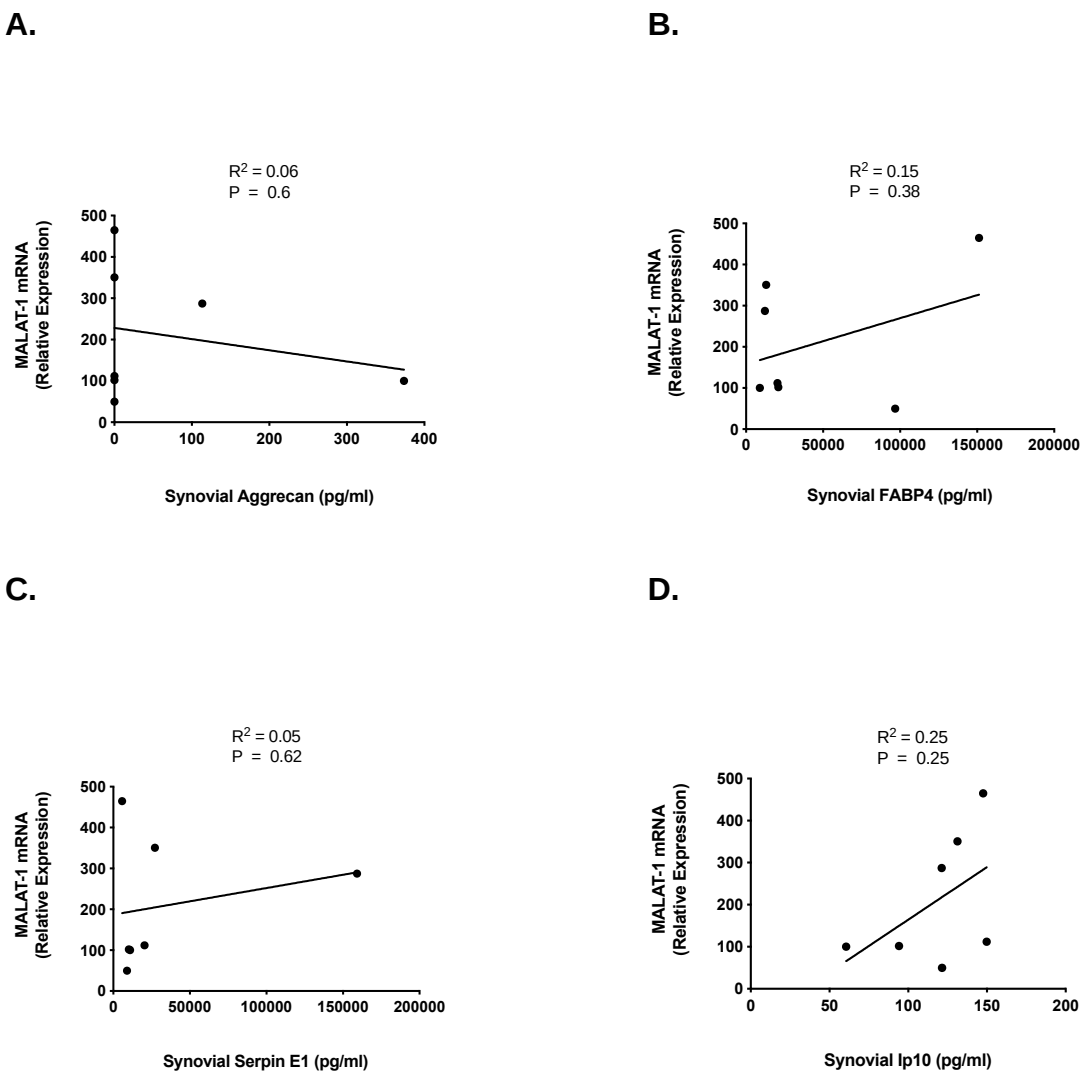


Figure 5.15.II MALAT1 expression in articular cartilage correlation with synovial chemokines in hip and knee OA joints including Aggrecan n = 8 patients **(A)**, FABP4 n = 8 patients **(B)**, Serpin E1 n = 8 patients **(C)**, IP10, n = 8 patients **(D)**.

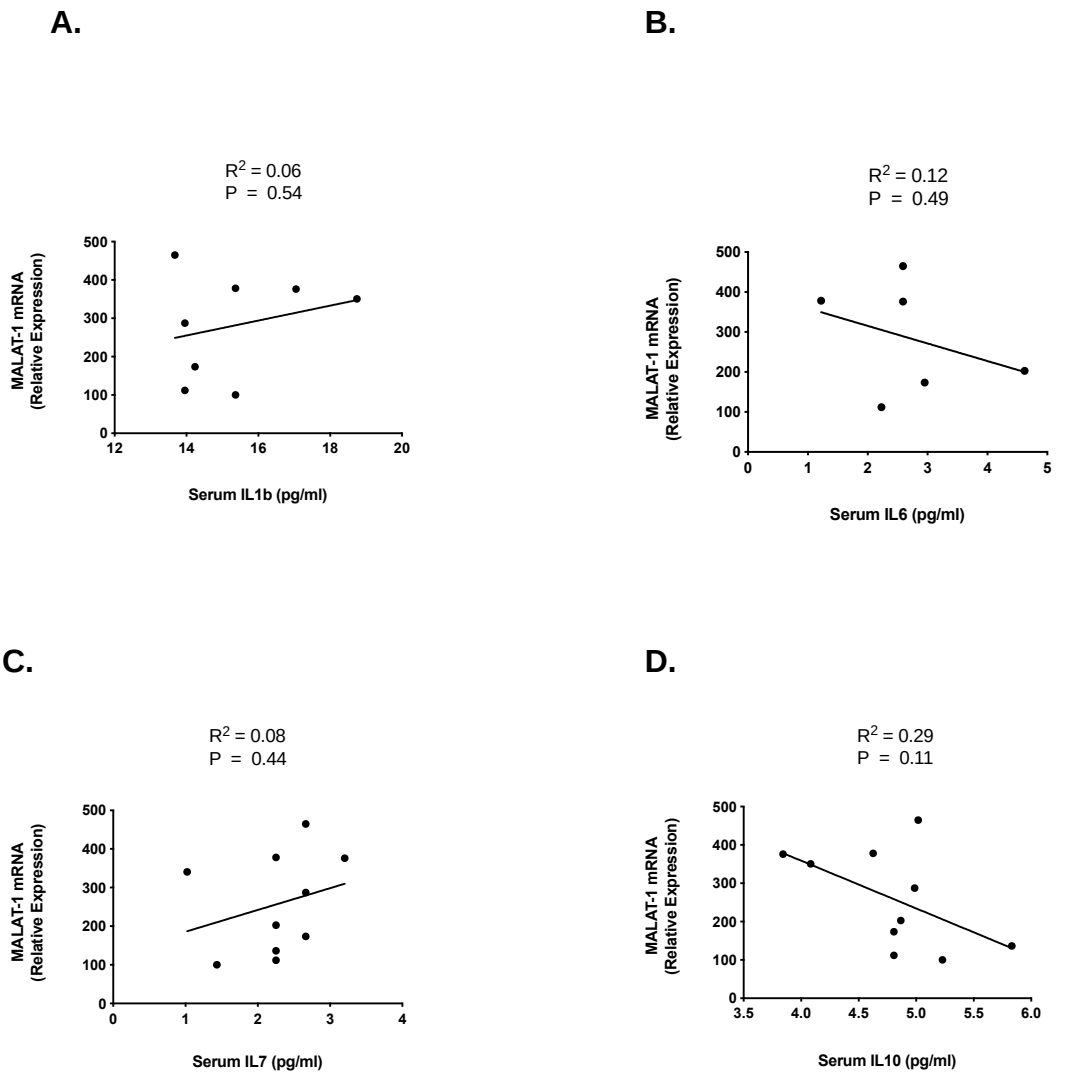
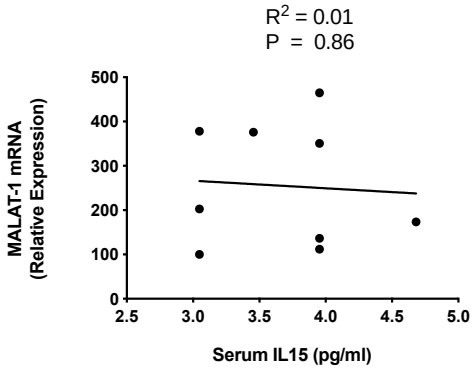
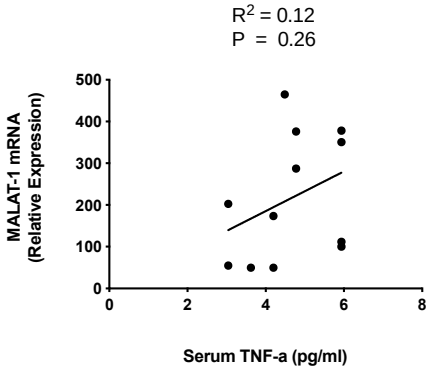


Figure 5.16.I MALAT1 expression in articular cartilage correlation with serum cytokines in OA patients including IL1β n = 15 patients **(A)**, IL-6 n = 12 patients **(B)**, IL7 n = 15 patients **(C)**, IL10 n = 15 patients **(D)**.

A.



B.



C.

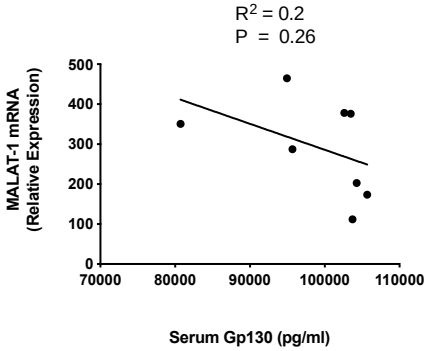


Figure 5.16.II MALAT1 expression in articular cartilage correlation with serum cytokines in OA patients including IL15 n = 15 patients **(A)**, TNFα n = 15 patients **(B)**, gp130 n = 15 patients **(C)**.

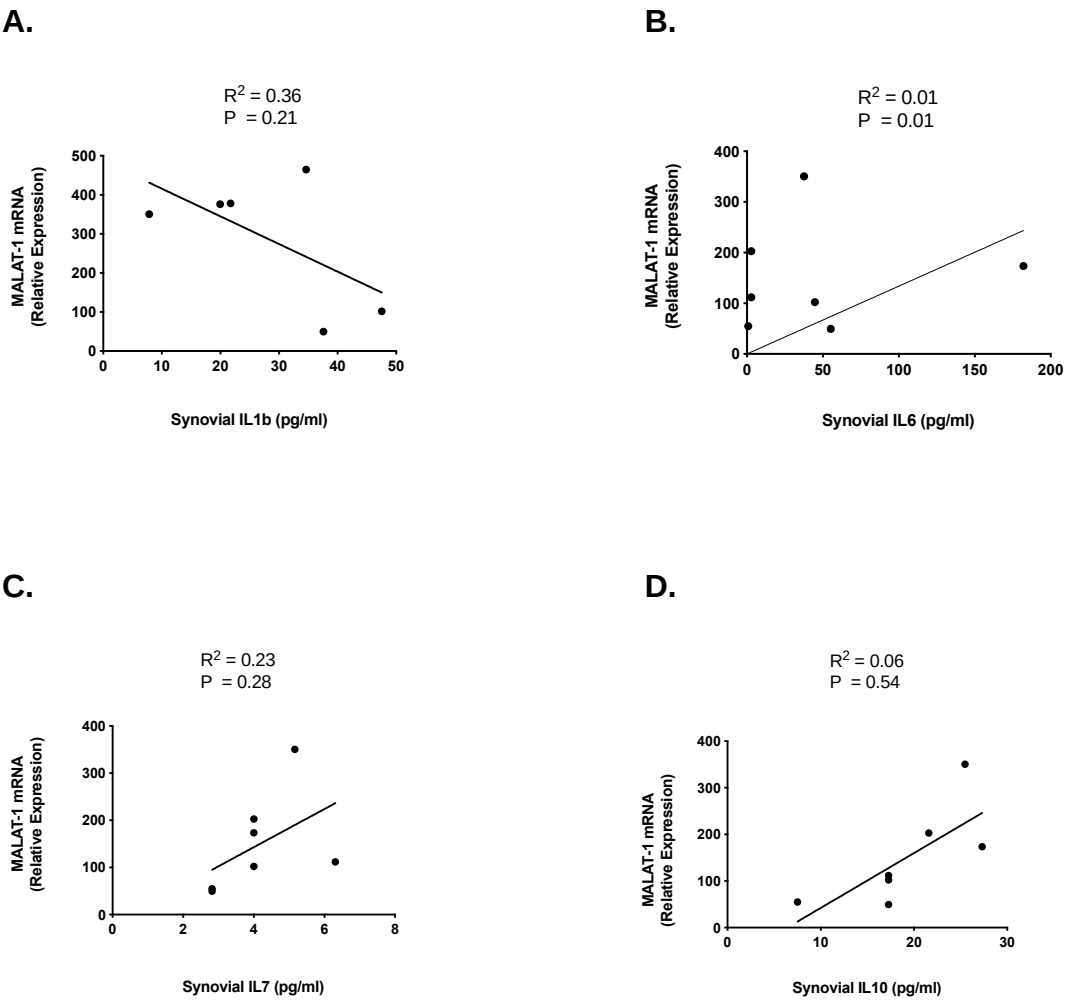
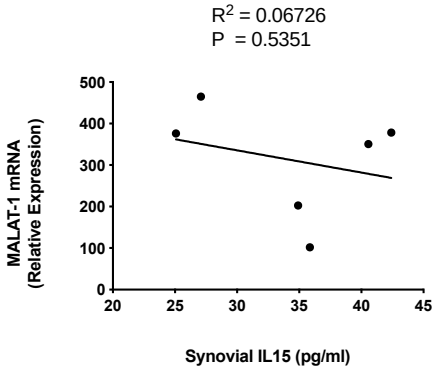
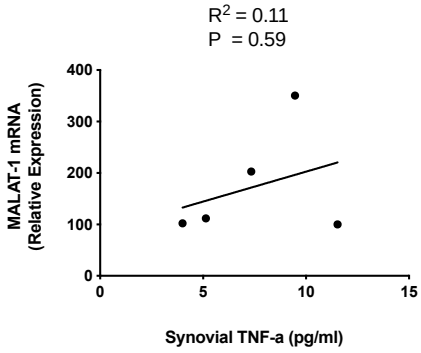


Figure 5.17.I MALAT1 expression in articular cartilage correlation with synovial cytokines in hip and knee OA joints including**)** IL1β n = 15 patients **(A)**, IL-6 n = 12 patients **(B)**, IL7 n = 15 patients **(C)**, IL10 n = 15 patients **(D)**.

A.



B.



C.

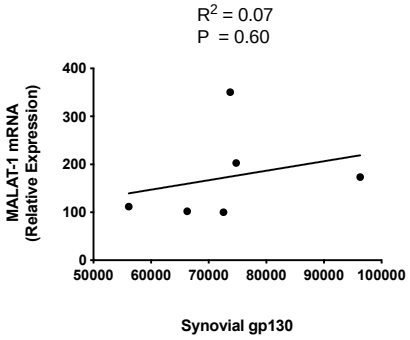


Figure 5.17.II MALAT1 expression in articular cartilage correlation with synovial cytokines in hip and knee OA joints including IL15 n = 15 patients **(A)**, TNFα n = 15 patients **(B)**, gp130 n = 15 patients **(C)**.

5.3.3.2 Correlation of MALAT1 expression in subchondral bone with serum and synovial inflammatory cytokines

The analysis was taken further on to measure all the serum and synovial inflammatory cytokines by Luminex multiplex assay. A total of 5 adipokines (visfatin, resistin, leptin, adiponectin and chemerin), 4 chemotactics (MIP1a, MIP1b, MIP3a, and MCP1), 8 chemokines (DKK1, galectin1, Eotaxin, amphiregulin, aggrecan, FABP4, serpin E1, and Ip10), and 6 pro-inflammatory cytokines (ILIβ. IL-6, IL7, IL10, IL15, and TNFα), (Tables 5.5 and 5.6) were measured. One of the five tested serum adipokines chemerin, showed a significant correlation with MALAT1 expression in the OA bone (*P* <0.0001) (Figure 5.18.E). The same was true for the four chemotactics in serum and synovial fluid (Figure 5.19 & 20). Two only of the 8 chemokines tested in serum were significantly correlated with MALAT1 expression in OA subchondral bone, including DKK1 (R^2 = 0.415, *P* = 0.033) (Figure 5.22.I.A) and galectin1 (R^2 = 0.413, *P* = 0.045) (Figure 5.22.I.B), but none in the synovial fluid of the OA subchondral bone (Figure 5.23.I – 5.23.II).

In addition, one of the 6 pro-inflammatory cytokines in the serum, TNF-α showed a significant correlation with MALAT1 expression (R^2 = 0.559, *P* = 0.013) (Figure 5.24.II.B), and none of the synovial fluid showed any correlation with MALAT1 expression in the OA subchondral bone tissues (Figure 5.25.I – 5.25.II).

Table 5.5 Correlation of MALAT1 expression with serum inflammatory cytokines

(pg/mL)	P(two-tailed)	R^2
Visfatin	0.973	0.000
Resistin	0.339	0.131
Leptin	0.569	0.037
Adiponectin	0.721	0.015
Chemerin	***0.723***	0.823
Dkk1	***0.033***	0.415
Galectin1	***0.045***	0.413
Eotaxin	0.205	0.172
Amphiregulin	0.376	0.088
Aggrecan	0.743	0.013
FABP4	0.608	0.030
Serpin E1	0.861	0.004
IP10	0.995	0.000
MIP1a	0.532	0.040
MIP1b	0.636	0.026
MCP1	0.531	0.040
MIP3a	0.882	0.002
IL1b	0.655	0.019
IL-6	0.595	0.060
IL7	0.427	0.092
IL10	0.481	0.064
IL15	0.441	0.076
TNF-α	***0.013***	0.559
Gp130	0.769	0.011

Cytokines measured by Luminex multiplex and expressed as pg/mL

Table 5.6 Correlation of MALAT1 expression with synovial inflammatory cytokines

(pg/mL)	P(two-tailed)	R^2
Resistin	0.591	0.062
Leptin	0.905	0.004
Adiponectin	0.382	0.155
Chemerin	0.250	0.253
Dkk1	0.527	0.085
Galectin1	0.603	0.058
Eotaxin	0.616	0.069
Amphiregulin	0.389	0.151
Aggrecan	0.686	0.035
FABP4	0.545	0.078
Serpin E1	0.553	0.075
IP10	0.226	0.276
MIP1a	0.817	0.015
MIP1b	0.995	0.00
MCP1	0.325	0.161
MIP3a	0.224	0.438
IL1b	0.219	0.346
IL-6	0.467	0.139
IL7	0.520	0.110
IL10	0.502	0.119
IL15	0.132	0.471
TNF-α	0.305	0.257
Gp130	0.865	0.008

Cytokines measured by Luminex multiplex and expressed as pg/mL.

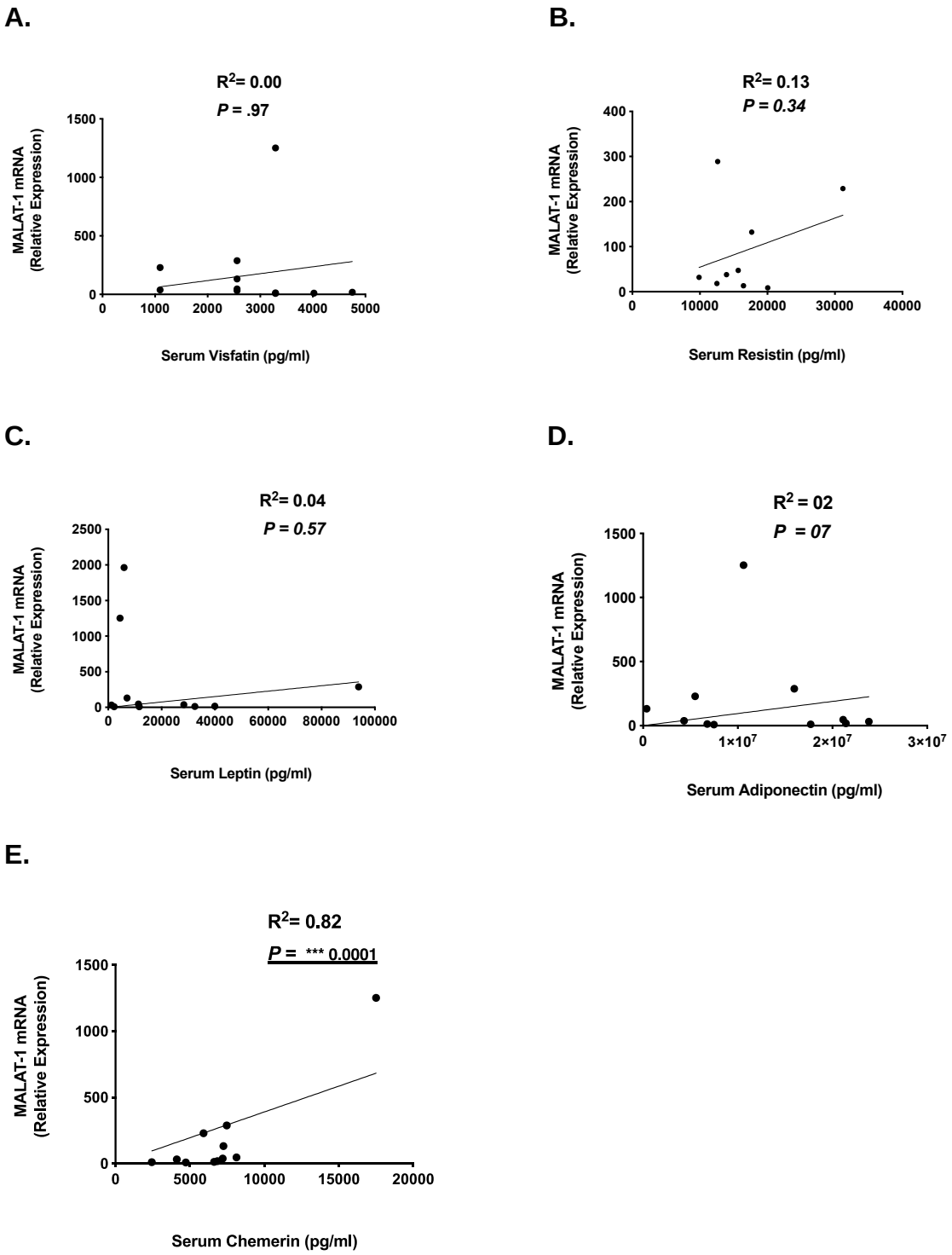


Figure 5.18 MALAT1 expression in subchondral bone correlation with serum adipokines including visfatin n = 16 patients **(A)**, resistin n = 14 patients **(B)**, leptin n = 15 patients **(C)**, adiponectin n = 16 patients **(D)**, chemerin n = 16 patients. MALAT1 expression showed a significant association with serum chemerin $P < 0.0001$ **(E)**.

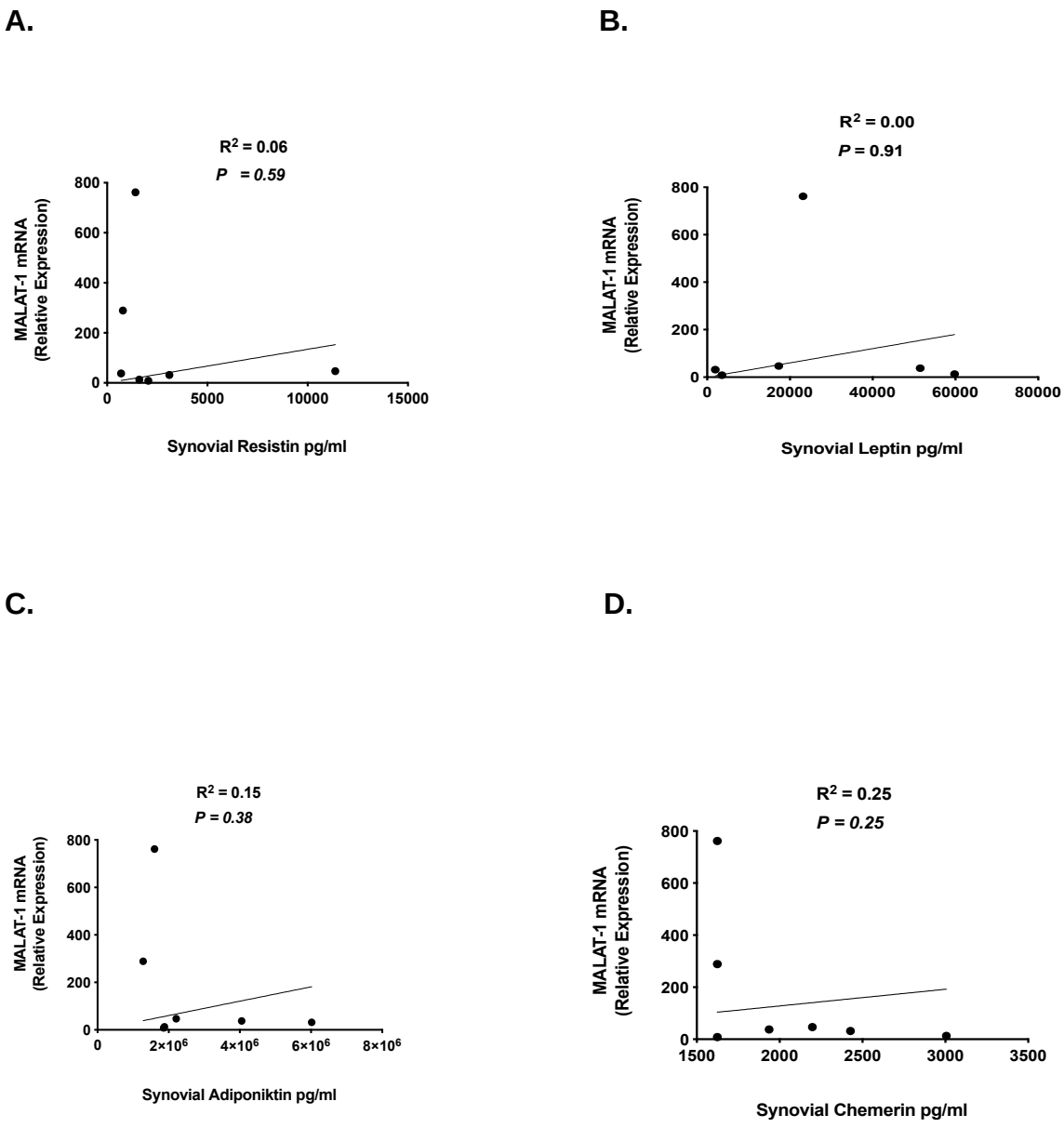


Figure 5.19 MALAT1 expression in subchondral bone correlation with synovial adipokines including**)** resistin **(A)**, leptin **(B)**, adiponectin **(C),** chemerin n = 8 patients **(D)**.

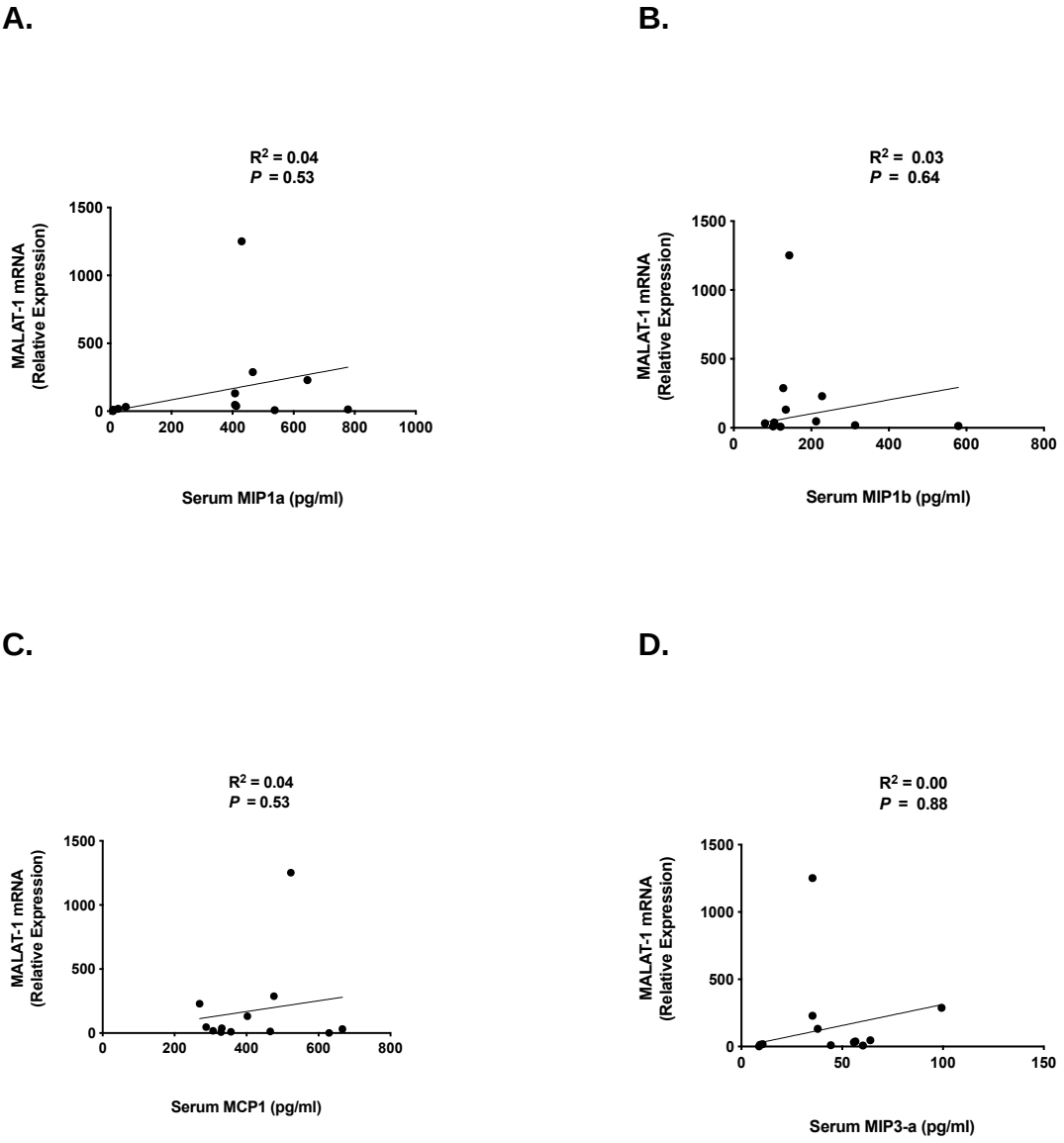


Figure 5.20 MALAT1 expression in subchondral bone correlation with serum chemotactics including MIP1-a n = 16 patients **(A)**, MIP1-b n = 16 patients **(B)**, MCP1 n = 15 patients **(C)** MIP3-a n = 16 patients **(D)**.

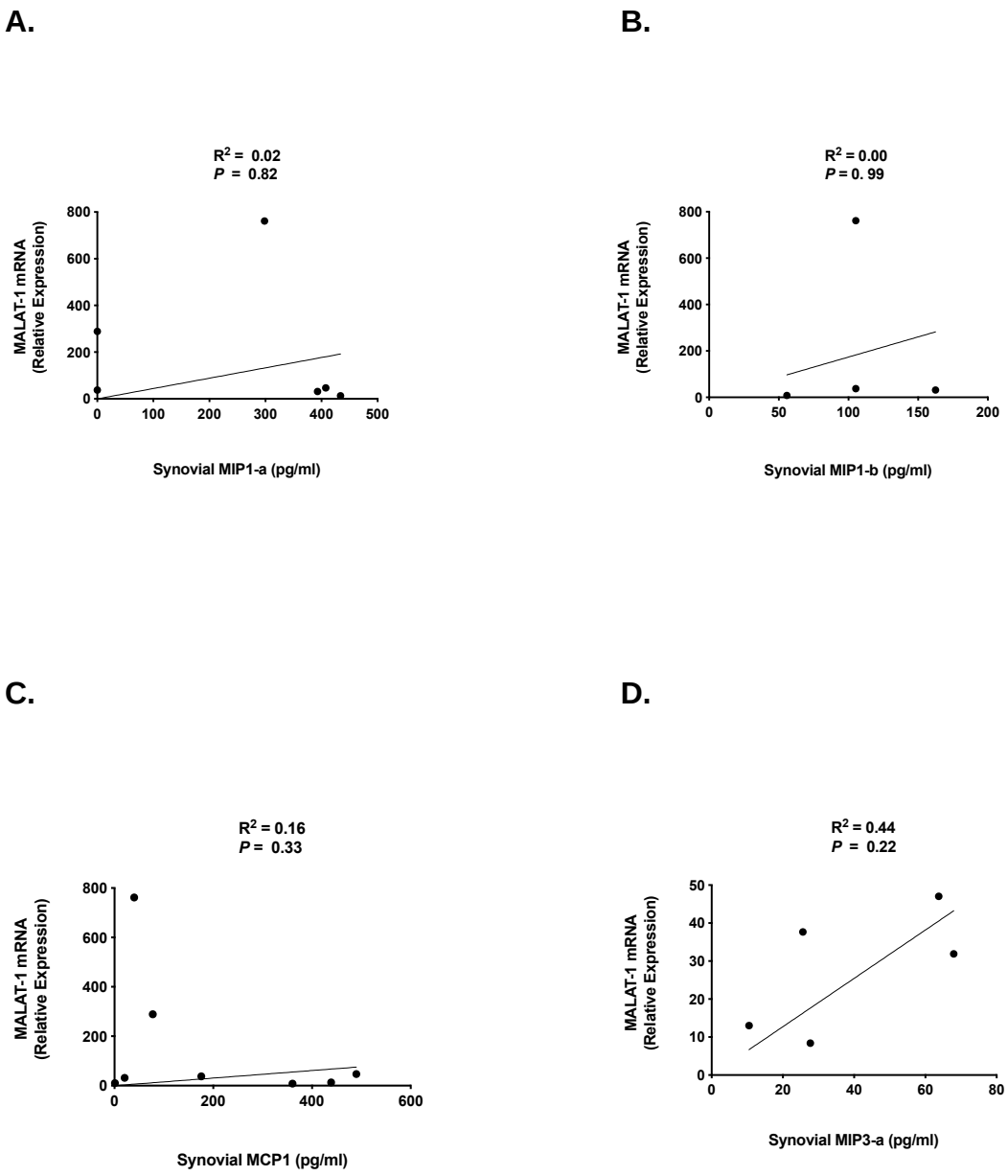


Figure 5.21 MALAT1 expression in subchondral bone correlation with synovial chemotactics including MIP1-a n = 7 patients **(A)**, MIP1-b n = 5 patients **(B)**, MCP1 n = 9 patients **(C)**, MIP3-a n = 8 patients **(D)**.

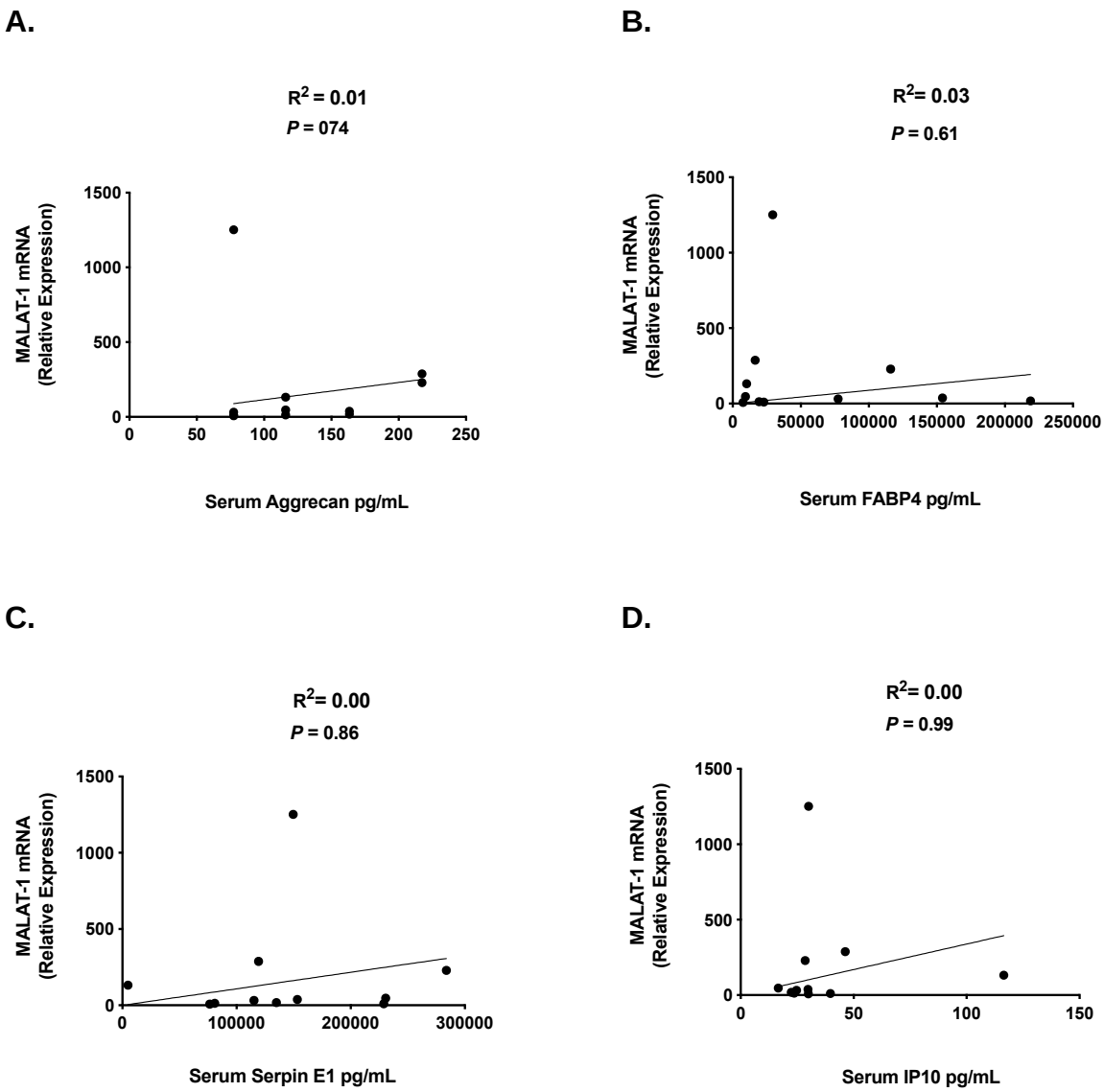
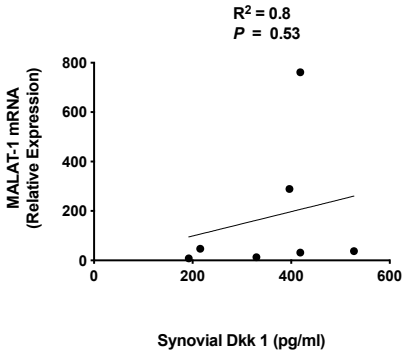
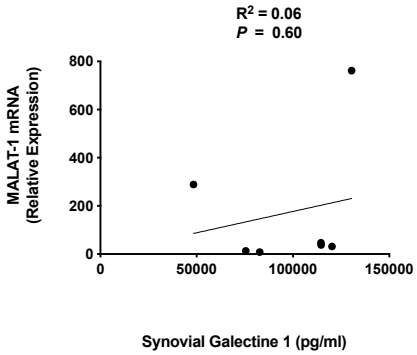


Figure 5.22.I MALAT1 expression in subchondral bone correlation with serum chemokines including Aggrecan **(A)**, FABP4 **(B)**, Serpin E1 **(C)**, IP10, n = 16 patients **(D)**.

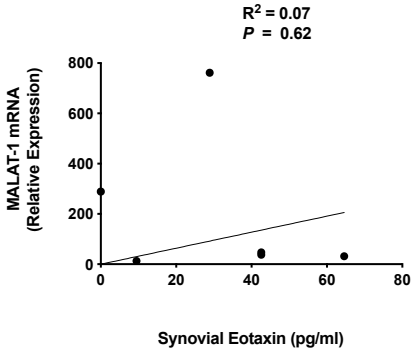
A.



B.



C.



D.

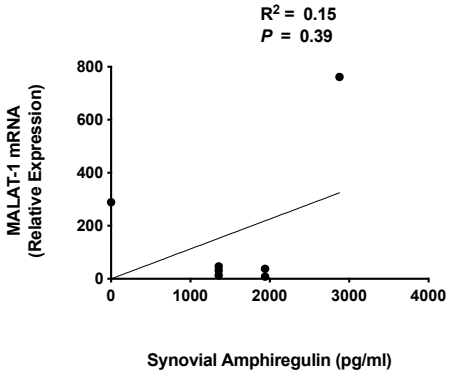


Figure 5.22.II MALAT1 expression in subchondral bone correlation with synovial chemokines including DKK1 n = 8 patients **(A)**, Galectin1 n = 8 patients **(B)**, Eotaxin n = 7 patients **(C)**, Amphiregulin n = 8 patients **(D)**.

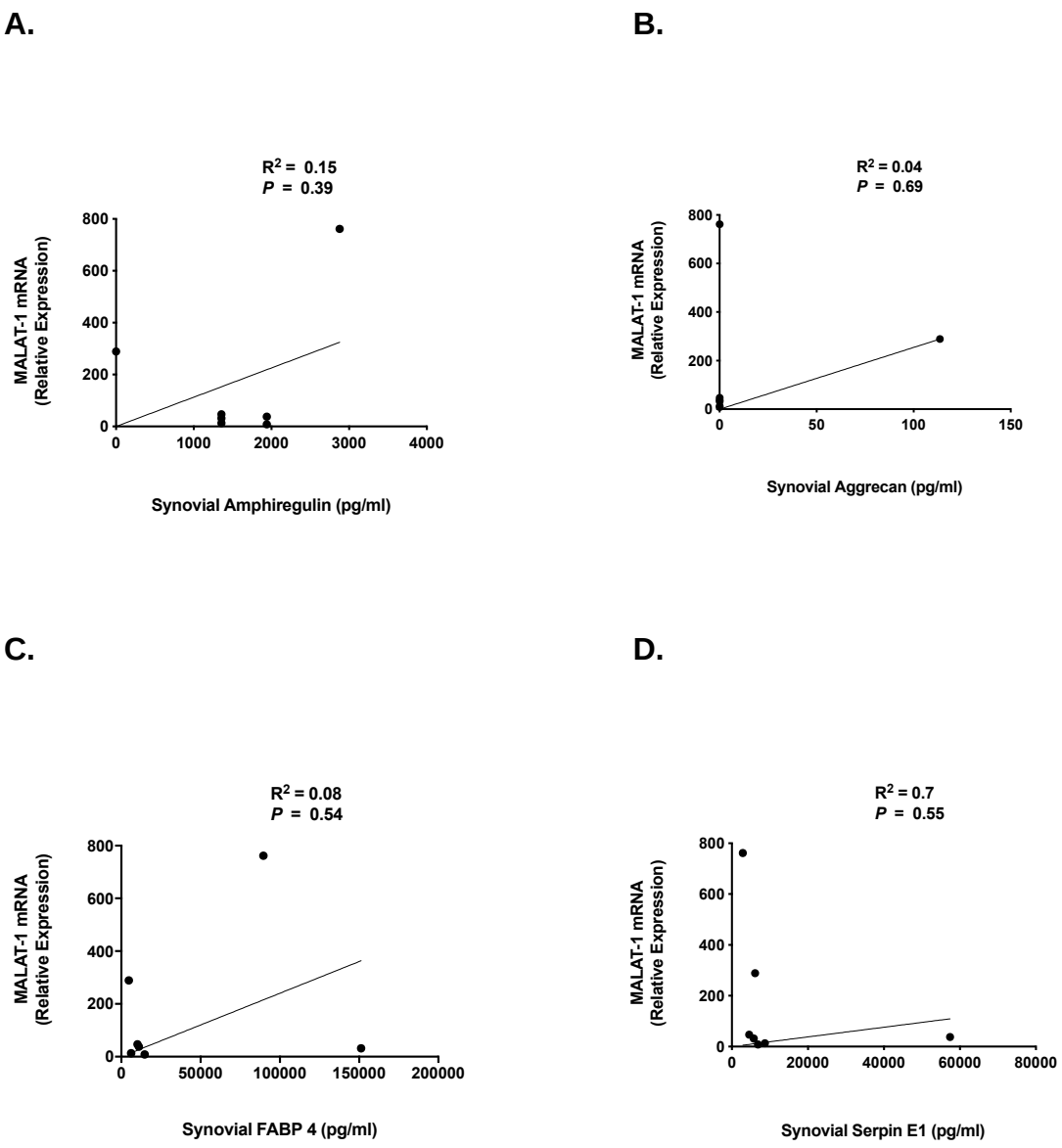


Figure 5.23 MALAT1 expression in subchondral bone correlation with synovial chemokines including Aggrecan n = 8 patients **(A)**, FABP4 n = 8 patients **(B)**, Serpin E1 n = 8 patients **(C)**, IP10 n = 8 patients **(D)**.

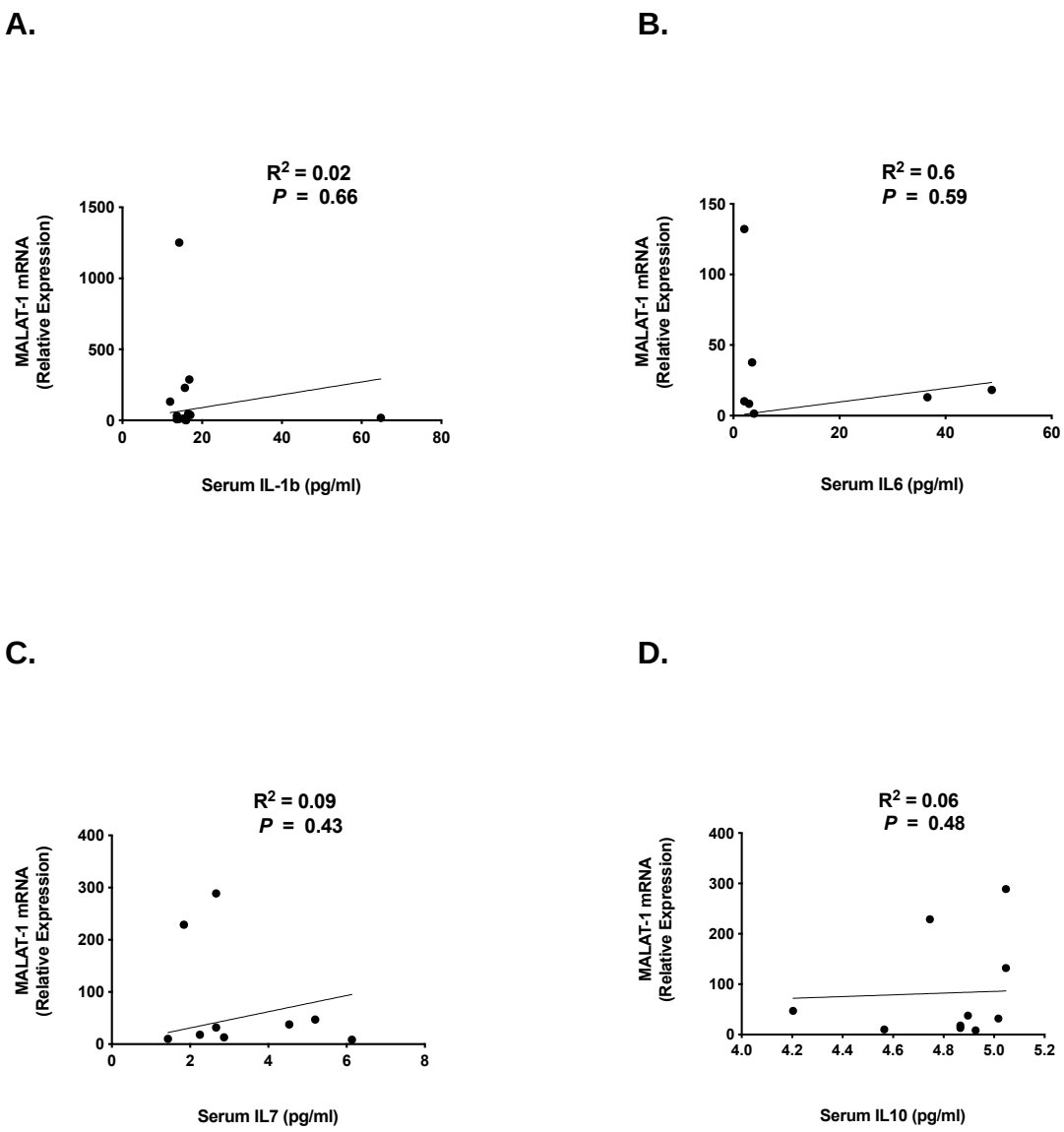


Figure 5.24.I MALAT1 expression in subchondral bone correlation with serum cytokines including IL1β n=16 patients **(A)**, IL-6 n=10 patients **(B)**, IL7 n=16 patients **(C)**, IL10 n=16 patients **(D)**.

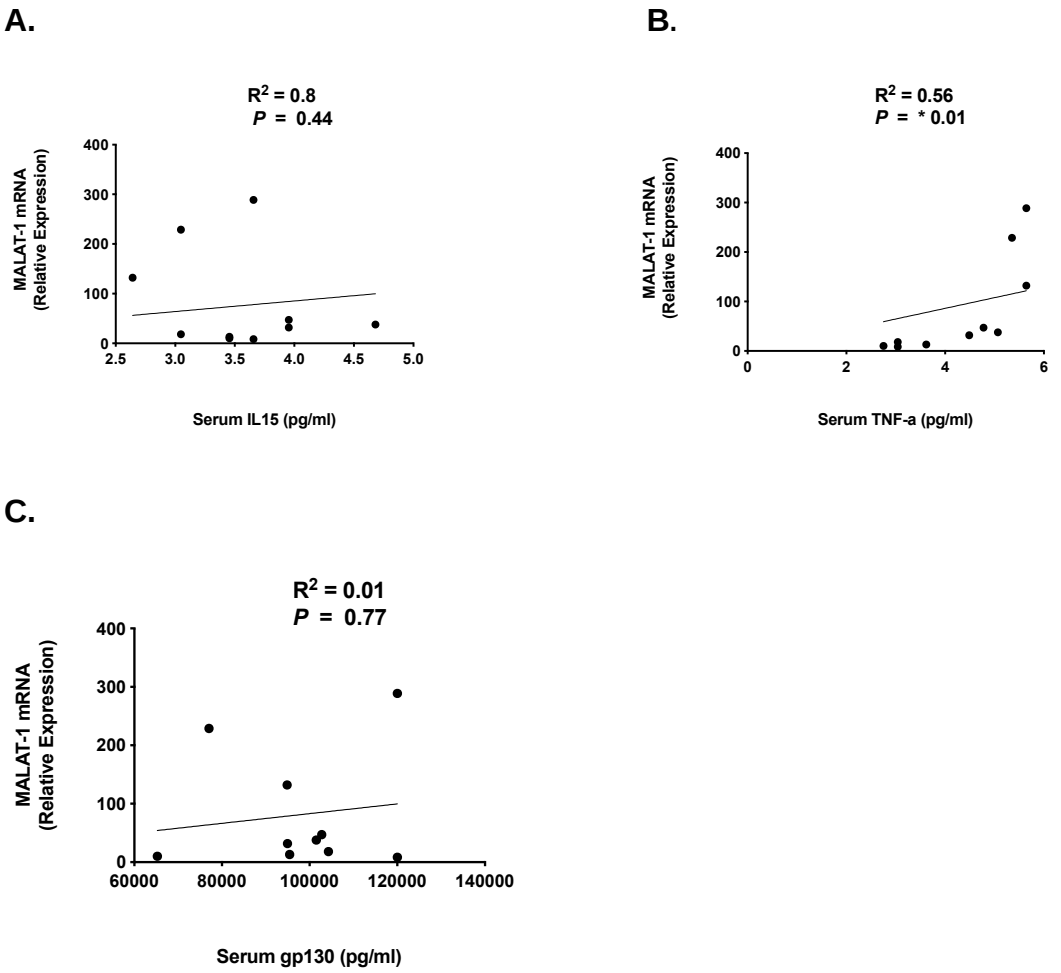


Figure 5.24.II MALAT1 expression in subchondral bone correlation with serum cytokines including IL15 n=16 patients **(A)**, TNFα, $P < 0.01$ n=16 patients **(B)**, gp130 n=16 patients **(C)**.

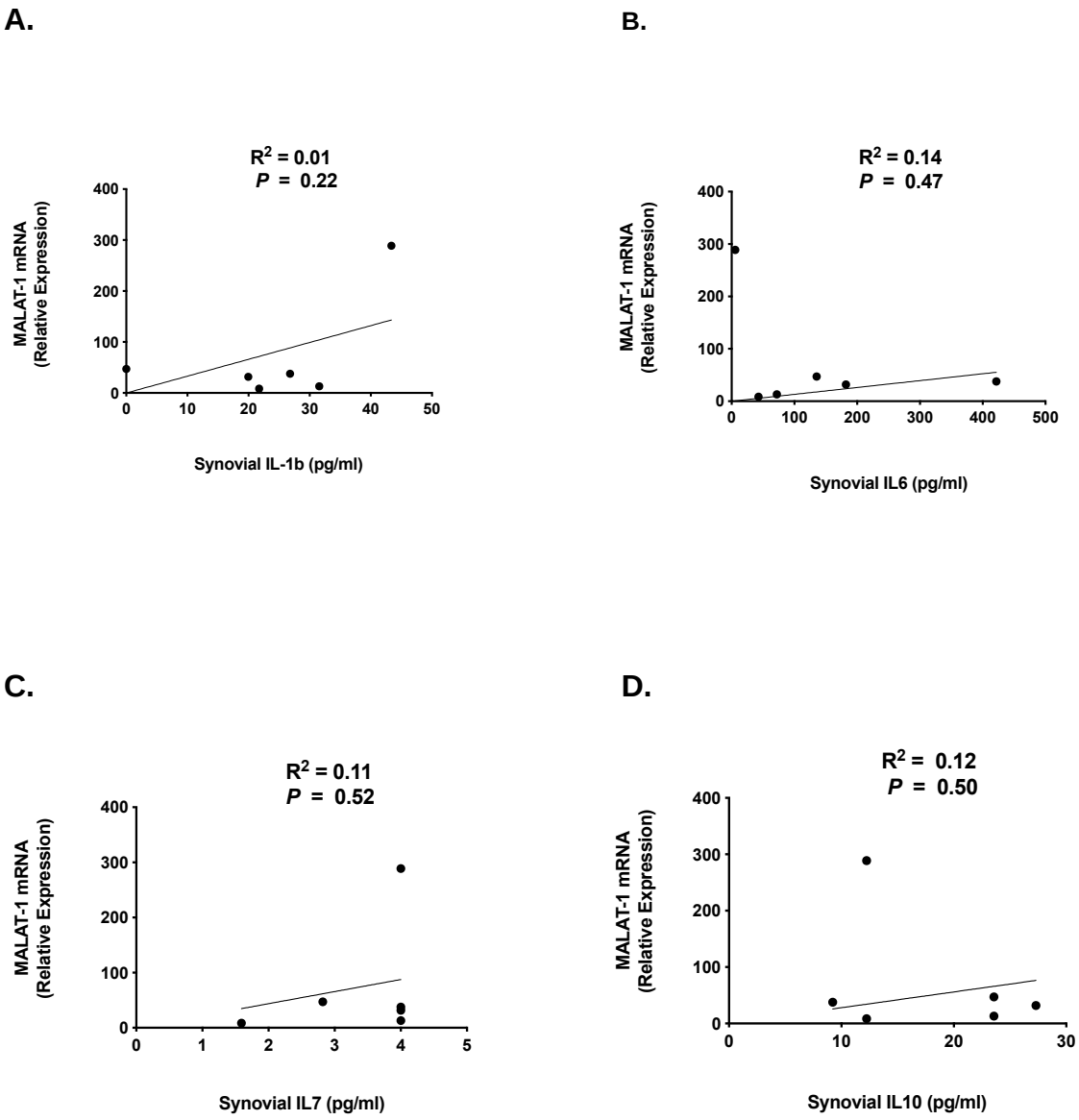


Figure 5.25.I MALAT1 expression in subchondral bone correlation with synovial cytokines including**)** IL1β **(A)**, IL-6 **(B)**, IL7 **(C)**, IL10 **(D)**.

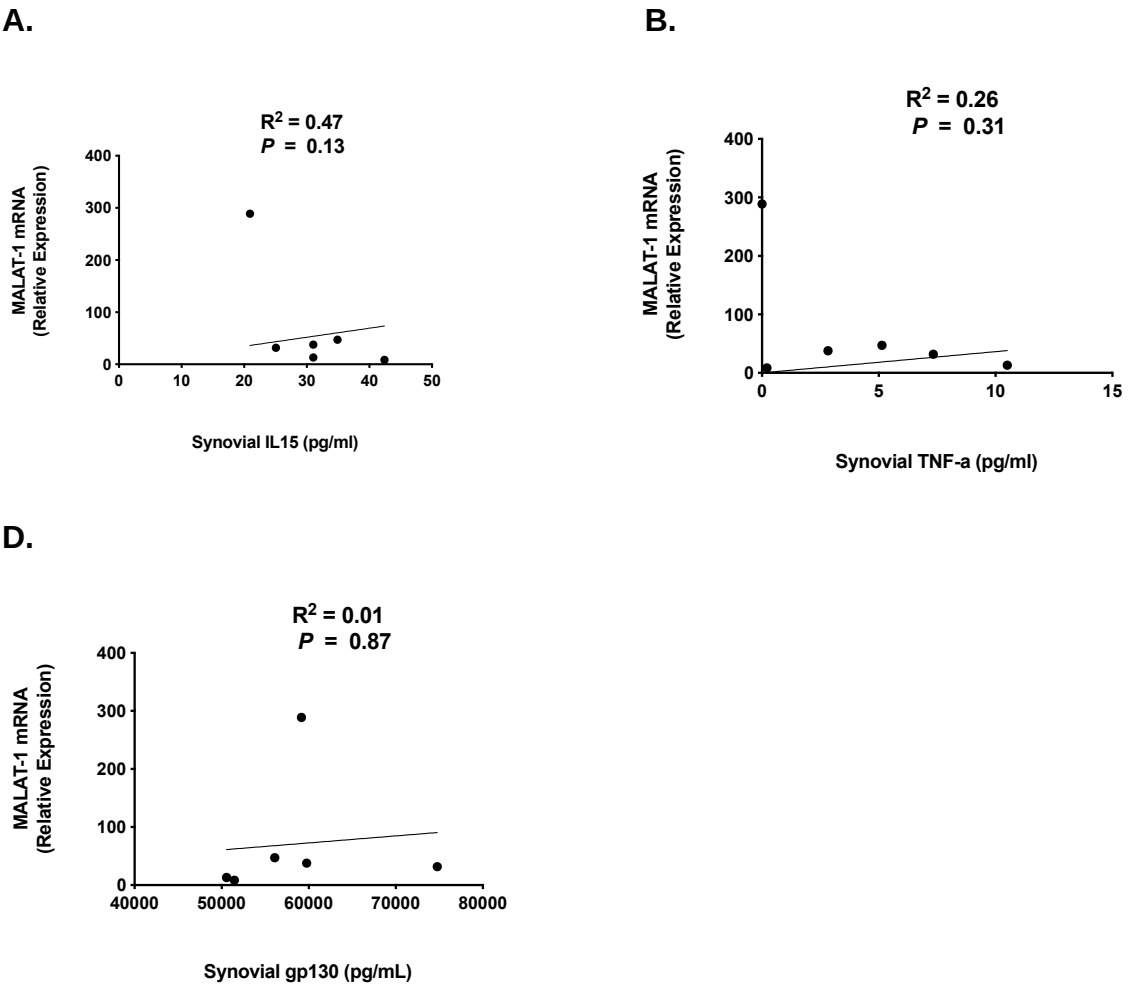


Figure 5.25.II MALAT1 expression in subchondral bone correlation with synovial cytokines including IL15 **(A)**, TNFα **(B)**, gp130 n = 8 patients **(C)**.

5.4 Discussion

The expression of lncRNA MALAT1 in OA patients was profiled in cartilage and subchondral bone of NW or OB patient's hip and knee joints. For this purpose, the association of MALAT1 expression to different joint damage severity parameters and inflammation was analysed in a number of ways. Examining the correlation between MALAT1 expression in the joint with the concentration of cytokines in both the systemic circulation (serum) and locally in the synovial fluid, joint health parameters including osteophyte formation, KL scale, hand OA, joint space narrowing scores and demographic parameters including gender, age, weight, height, BMI, W:H, body fat.

Looking at the articular cartilage tissues of OA joints in this study, a significant correlation was seen between age and BMI in knee OA, indicating a role of obesity in and OA prevalence in the ageing population. This is further supported by the reported fact that body adiposity increases with age reflected on BMI in knee OA [321]. Furthermore, MALAT1 expression was correlated with the severity of OA in hip and knee joints indicated by high KL scale and a lower JSN scores (<=1mm), indicating a possible role for MALAT1 in end -stage joint OA.

The next interesting finding was in the measured serum and synovial inflammatory parameters, were chemokines Dkk1 and Eotaxin showed a significant correlation with MALAT1 expression in the serum of OA patient's cartilage. Whereas MIP3a chemotactic was significantly correlated with MALAT1 expression in the synovial fluid of the joints of OA patient's cartilage. This pattern of elevated inflammatory mediators and its significant association with MALAT1 expression in end-stage OA cartilage again re-enforces the suggestion of the possible involvement of MALAT1 as an

inflammatory mediator in the pathogenesis of OA or the possibility of MALAT1 association with cartilage breakdown which is one of the features of OA. A similar finding of increased levels of proinflammatory mediators including proinflammatory chemokines were reported previously in human OA cartilage [322].

Serum Dkk1 is an inhibitor of canonical Wnt pathway, and it was reported in another study to be associated with hip OA [323] Eotaxin is a chemokine indicating matrix protein breakdown due to cartilage degradation which is one of the main OA features. [324]. This chemokine was reported to induce MMP-3 gene expression in human chondrocytes. [325] This regulation was reported to be induced by IL1β which is produced by the chondrocytes of the OA joints [326-327]. Based on the previously mentioned published reports, it is possible that MALAT1 lncRNA may have a regulatory role in controlling chondrocytes during the inflammatory process in OA. MIP3a stands for Macrophage inflammatory protein 3 alpha active protein or CCL20. It is a strong chemoattractant to lymphocytes and is induced by inflammatory cytokines like TNF. The presence of this protein indicates the state of inflammation in the joint and its significant correlation with MALAT1 expression may confirm the suggestion of the role that this lncRNA may play as a mediator of the inflammatory changes affecting the cartilage tissues of the OA joints [328].

17 OA patient's subchondral bone from different joints were used, in which the population's demographics, anthropometrics, joint health and inflammatory cytokines were examined. The findings showed a significant correlation in BMI with gender in which more MALAT1 was expressed in the subchondral bone from joints of male OA

patients compared to female and there was a significant difference in MALAT1 expression between the different joints in males and females.

Although there were some limitations in the analysis because of missing data for W:H ratio for 2 patients, and body fat % was not recorded for 5 patients, a significant correlation was seen between MALAT1 expression and the degree of adiposity of patients in the female group. W:H ratio is a measure of adiposity in patients, and previous studies indicated that obese individuals are more prone of having OA in their joints [329]. The most interesting finding was in the significant association between MALAT1 expression and height. An increase in height indicates more bone mass, which increased the risk of developing radiographic knee OA [330]. It was also proved that some patients with knee OA show a specific phenotype for increased bone mass [331]. These reports are in accordance with the findings of the current study. The correlation of MALAT1 and height indicates a possibility for MALAT1 regulation on bone in OA joints.

In this study, the proinflammatory cytokines showed a similar finding like in cartilage, in which Dkk1 chemokine in the serum was significantly correlated with MALAT1 expression. However, galectin1 instead of Eotaxin showed significant correlation with MALAT1 expression in serum. This finding was supported with a study done on knee OA in which Dkk1 expression was increased [332]. Dkk1 which is a chemokine, and one of the inflammatory cytokines investigated in this study, functions in inhibiting the Wnt/β-catenin signaling acting on skeletal tissues [333] and regulates bone regeneration via parathyroid hormone [334]. MALAT1 significant correlation with increased Dkk1 expression in bone from OA joints, may indicate an epigenetic

regulation of MALAT1 on inflammatory cytokines associated with OA pathogenesis. Importantly, MALAT1 correlation with height and increased Dkk1 expression in bone may support the previous assumption perfectly as it was shown that high levels of Dkk-2 were seen in subchondral bone cell culture [335]. It is worth to mention her that, Dkk-1 and Dkk-2 are from the same DICKKOPF related proteins known to regulate bone functions.

Galectin1 is a chemokine from lectin family, more specifically galectins which was found to regulate adhesion and growth of mammalian tissues in many cancers. It was increased in expression in bone from OA joints in this study, which is in accordance with a previous study that showed an increase in galectin expression in subchondral bone of OA joints [336]. MALAT1 expression significant correlation with galectin in bone may indicate a common initiator that triggers the activation of both regulators. This may be true, as galectin-1 exerts its effects on different cells in OA joints through inflammatory mediators like NFκB [337]. It is possible that inflammatory chemokine galectin-1 which binds to cell surface may activates certain signaling pathways together with MALAT1 contributing to OA pathogenesis. Future work on a larger population is required to prove this. Indeed, in this study a third serum chemokine which may be related to the NFκB pathway, TNF-α, showed a significant correlation with MALAT1 expression in the subchondral bone from OA joints. TNF-α was reported to in one study that it stimulates Nucleosomal high mobility group box-1 (HMGB-1) which was suggested to have a role in the thickening of the subchondral bone in OA patients. [338] Furthermore, TNF-α was reported to be one of the main pro-inflammatory cytokines related to the pathogenesis of OA by stimulating MMPs and angiogenic substances [339]. It’s worth mentioning her that in this project, Eotaxin

significant correlation to MALAT1 expression in the articular cartilage together with TNF-α significant correlation with MALAT1 in the subchondral bone, may strongly suggest a possible role for MALAT1 in modulating MMPs which were proven to contribute to the cartilage degradation in OA. This suggestion is built on the reported findings that Eotaxin expresses MMP-3 gene in human chondrocytes [325] and the reported finding that TNF-α stimulates MMPs-1,3 and 13 in OA patients [339]. Further analysis with more samples is needed to validate these suggestions.

In the next chapter, MALAT1 knockdown and the effect of this knockdown on a number of functional aspects of OA osteoblasts will be studied in detail.

CHAPTER 6

The Expression and Functional Role of MALAT1 in OA Primary Osteoblasts

6.1 Background

LncRNA regulation of gene's expression has been implicated in the initiation and progression of several chronic diseases including OA, but the mechanism on how this is achieved is not clear and many lncRNAs remain understudied with regard to their functional role and mechanisms of action [340]. The lncRNA MALAT1 has been implicated in a number of physiological and pathological processes including the epigenetic regulation of inflammatory pathways. Furthermore, it is one of the first lncRNAs that was reported in different studies to be associated with diseases in human including cancer development and progression [341]. Similar to other lncRNAs,

MALAT1 exerts its regulation on three main inflammatory pathways including the Mitogen activated protein kinase (MAPK), Janus Kinase and Signal Transducer and activator of Transcription (JAK-STAT), and the Nuclear factor kB (NF- ΚB) pathways [342]. This regulation is accomplished by specific mechanisms including initiating signalling cascades that activates protein receptors downstream in the NF- ΚB pathway [343-344]. MALAT1 lncRNA regulates the inflammatory pathway MAPK by regulating the kinases like protein 38 (p38), C-jun N-terminal kinases (JNK), and extracellular signal-regulated kinases (ERK). Activation of these kinases by pro-inflammatory stimuli produces a signal in the MAPK pathway leading to the development of inflammatory changes in the cells. This mode of regulation was seen in many conditions such as in glioma metastasis [345], and diabetes related dysfunction in rodents [276,346-348]. Researchers indicated a possible role for MALAT1 in Systemic lupus erythematosus (SLE) by inhibiting the stimulation of the

NF-KB pathway, thus reducing inflammatory cytokines leading to immunological diseases [348].

With regards to the potential role of MALAT1 in OA pathology, MALAT1 has been implicated in modulating both bone and cartilage remodelling [192]. For example. MALAT1 regulation of osteoblasts were shown in some studies indicating that this lncRNA plays a vital role in the initiation of osteolysis [349], promotion of osteogenesis differentiation of osteoblasts [350], and mediation of osteogenesis in adipose-derived mesenchymal stem cells [351]. In bone disease, MALAT1 together with other lncRNAs regulated abnormal osteogenesis of human bone marrow stromal cells [352]. On the other hand, osteoblast proliferation was inhibited in vitro by binding of receptor activator of nuclear factor-κB (RANK) receptor to its legend receptor activator of nuclear factor-κB ligand (RANKL) regulated by increased MALAT1 expression [353].

In addition, since MALAT1 like other lncRNAs is highly expressed in immune cells [354-355], this could indicate that, it plays an important role in immune cell functions, and therefore in the development of different immune related diseases such as RA. This raises the question that MALAT1 might have a role in regulating inflammatory pathways and may have a possible role in the pathogenesis of OA. Furthermore, advancements in RNA high-throughput sequencing technologies, and the new approaches in detecting the functional role of lncRNAs like loss and gain of function approaches may help in the detection of the functional role of MALAT1 in the initiation or progression of OA.

In the previous chapter, MALAT1 expression was profiled in articular cartilage and subchondral bone of OA joints and its association with the parameters of joint damage.

It was expressed more in joints with sever OA having high KL scale of 4 and lower JSN scores <= 1. Given these findings, the next step was to determine the expression of this lncRNA under inflammatory conditions and the functional role of MALAT1 expression in both primary osteoblasts and chondrocytes. However, restrictions applied to UK universities, the reduction in the laboratory working hours due to COVID-19 pandemic and the delay in the availability of laboratory reagents resulted in shortening my project and therefore this chapter will focus on the expression and function of MALAT1 in OA osteoblasts only.

6.2 Methods

First MALAT1 expression was studied in primary cells incubated with the pro-inflammatory cytokine IL-1β (1ng/mL) for 6 and 24 h. In order to study the function of MALAT1 in the regulation of the genes responsible for protein expressions during inflammatory processes in OA, the next step was to use loss of function (LOF) approach. Locked nucleic acids (LNA1 or LNA2) was used to target the knockdown of MALAT1 expression via using a lipid delivering system to transfect the primary cells. A non-targeted control LNA (NTC) was used for comparison. In a 96 well plate, 10,000 primary osteoblasts or chondrocytes per well, were cultured and the next day DNA-lipid complexes Lipofectamine 2000, 3000, or TransIT-X2® in a serum-free medium Opti-MEM were added to the cells according to the lipid used and left for 24 hours. After one day transfection some cells were stimulated with IL-1β for 4 h and some cells were left unstimulated. After optimizing MALAT1-KD in OA chondrocytes and

osteoblasts, the effects of MALAT1-KD were studied on the transcriptomics of primary osteoblasts and next, the effect of this knockdown was further investigated to evaluate any modulation on the innate metabolic functions of osteoblasts as bone forming cells.

6.3 Results

6.3.1 Inflammatory Response in IL-1β-stimulated Osteoblasts

Primary osteoblasts (n=3 OA patients) were stimulated with IL-1β (1ng/mL) for 6 or 24 h. A significant IL6 expression was noticed after 24 h treatment with IL-1β compared to non-treated osteoblasts (P = 0.03). Comparing IL-6 induction in IL-1β treated cells for 6 h to cells treated for 24 h, there was a 10-fold more IL-6 induced in cells treated for 24 h compared to cells treated for 6 h. Comparing treated cells with those non-treated for 24 h, IL-6 induction was 23-fold more induced in treated cells compared to nontreated. (Figure 6.1.A).

6.3.2 MALAT1 Expression in OA Osteoblasts Under *in vitro* Inflammatory Conditions

MALAT1 expression in IL-1β stimulated osteoblasts was determined by qRT-PCR. A significant MALAT1 expression was noticed after 24 h treatment with IL-1β compared

to non-treated osteoblasts (*P* = 0.02) (Figure 6.1.B). Comparing MALAT1 expression between the treated osteoblasts at 6 h and 24 h, MALAT1 was expressed 52-fold more in treated osteoblasts for 24 h compared to osteoblasts treated for 6 h. Comparing MALAT1 expression between IL-1β treated and non-treated for 24 h, MALAT1 was expressed 40-fold more in treated osteoblasts compared to non-treated osteoblasts.

It's noteworthy, to mention that OA chondrocytes were also responsive to IL-1β stimulation and IL6 was induced in treated cells. Also, LNA's induced MALAT1-KD in OA chondrocytes. (Appendix Fig 9.5) In this chapter only the results of primary osteoblasts will be shown.

A. **B.**

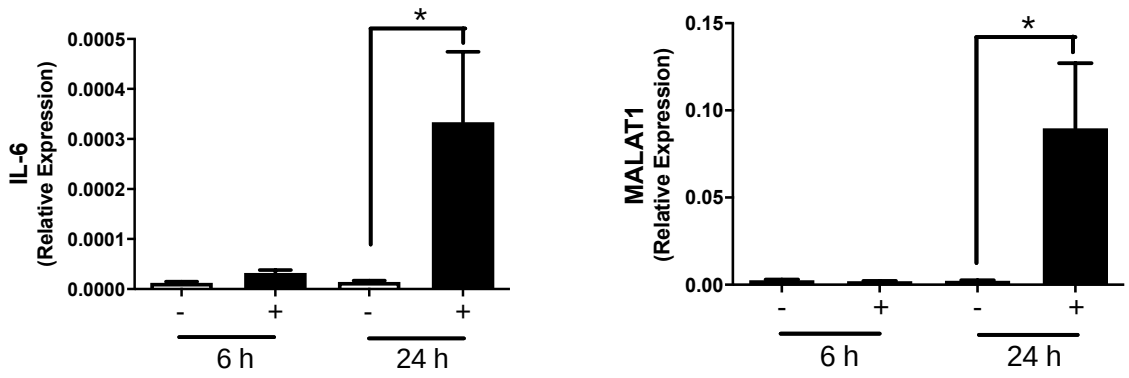


Figure 6.1 IL-6 pro-inflammatory cytokine and MALAT1 expression in IL-1β stimulated and non-stimulated primary osteoblasts IL-6 expression **(A)**, MALAT1expression for 6 and 24 h in (-) non-stimulated and (+) IL-1β stimulated osteoblasts (**B)**.

6.3.3 MALAT1 knockdown in OA osteoblasts

Next, MALAT1-KD was performed in OA primary osteoblasts obtained from the subchondral bone of different BMI cohort and different joints including hip, hand, wrist and foot. 24 h MALAT1-KD in primary osteoblasts from OW foot showed 97% knockdown in LNA-2 and 46.45% knockdown in LNA-1 (Figure 6.2.A) and 24 h MALAT1-KD in primary osteoblasts from NW hip showed 90% knockdown in LNA-2 and 80% knockdown in LNA-1 (Figure 6.2.B).

We then examined if the LNAs could also deplete the expression of MALAT1 in osteoblasts stimulated with IL-1β. To this end, osteoblasts from OB hand and obese wrist OA patients were transfected with MALAT1 LNAs or NTC control for 24h and then stimulated +/- IL-1β. Despite stimulation with IL-1β, expression of MALAT1 was significantly depleted in both OB hand OA osteoblasts (Figure 6.2.C & D) and OB wrist OA joints (Figure 6.2.E & F) by >90% with either MALAT1 LNA1 or LNA2 compared to the control LNA.

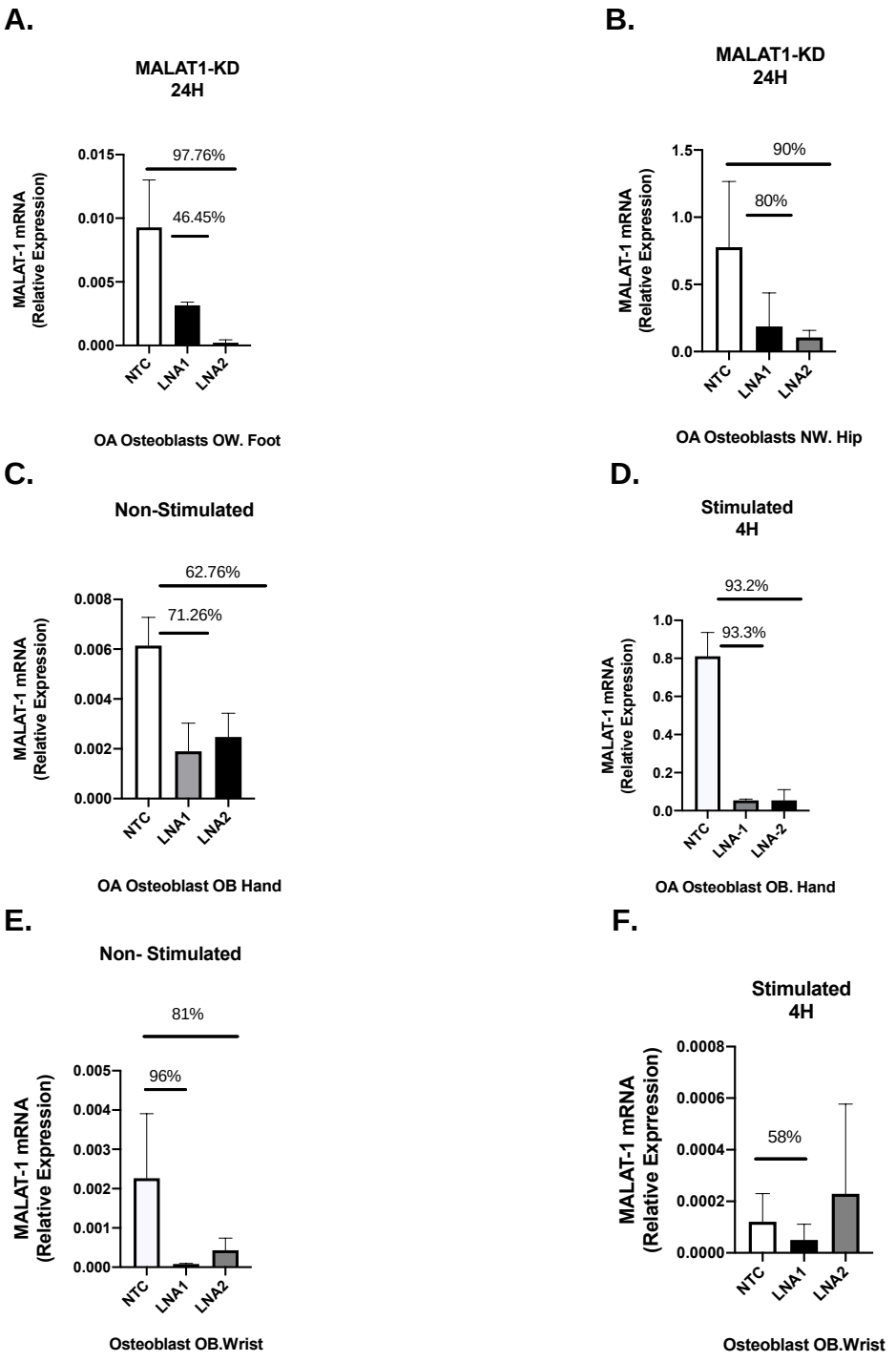


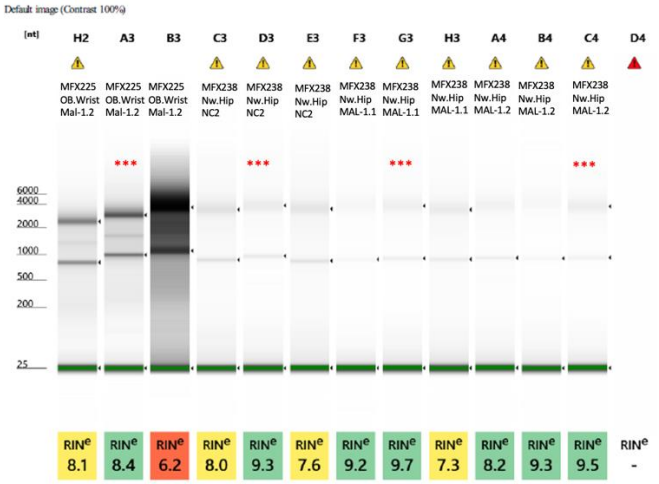
Figure 6.2 MALAT1-KD in OA primary osteoblasts. 24 h MALAT1-KD in OA primary osteoblasts from OW foot using Lipofectamine 3000 **(A)**, 24 h MALAT1-KD in OA primary osteoblasts from NW hip using Lipofectamine 3000 **(B)**, MALAT1-KD in non-stimulated **(C)** and IL-1β stimulated primary osteoblasts from OA OB hand **(D)**, MALAT1-KD in non-stimulated **(E)** and IL-1β stimulated primary osteoblasts from OA OB Wrist **(F)** using Lipofectamine 3000. NTC = Non target control, LNA1= Locked nucleic acid for MALAT1-1 and LNA2 = Locked nucleic acid for MALAT1-2.

6.3.4 MALAT1-KD modulation of OA osteoblast transcriptome

6.3.4.1 RNA Sequencing

Successful MALAT1-KD in primary osteoblasts from different BMI cohort and different OA joint location led to the next step in this project which was to investigate the possible effects of the lncRNA MALAT1-KD on the transcriptomic sequences of primary OA osteoblasts. MALAT1 loss-of-function using the aforementioned LNAs, was performed in n=3 OA patient osteoblasts (including primary OA osteoblasts form 2 OB joints (hand and wrist) and 1 NW hip joint). Following transfection, total RNA was extracted by Qiagen mini columns and RNA quality evaluated using the Agilent Bioanalyser. All RNA samples were of high quality, with RIN values from 8.2-10 (Figure 6.3A, 6.4B) and were therefore deemed of sufficient quality (RIN > 7) to be selected for RNA sequencing analysis using the Lexogen, QuantSeq 3` kit. Star Aligner version 2.5.2b was used to prepare sequenced reads libraries which were mapped to the hg38 reference human genome. DESeq2 version software was used to determine the differential gene expression analysis and to compute the log2 fold changes.

A.



B.

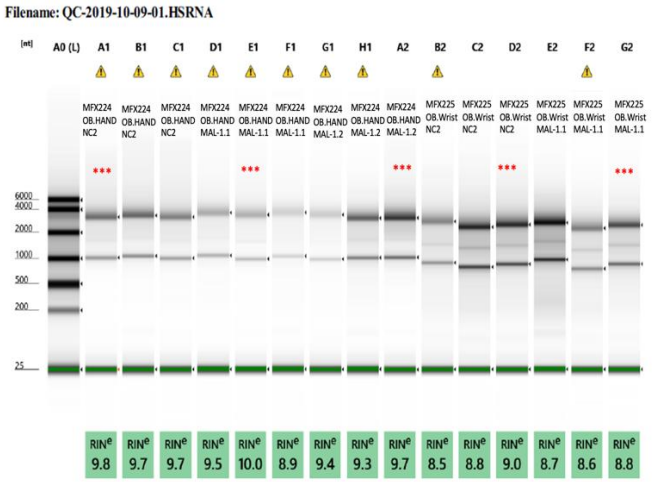


Figure 6.3 Quality Analysis of RNA obtained from MALAT1-KD OA primary osteoblasts. MALAT1-KD primary osteoblasts were obtained from three OA joints including OB hand, OB wrist and NW hip, with RIN ranged from 8.2 – 10. RIN = RNA integrity number **(A-B)**.

From DESeq2 analysis, a total of more than 60,000 transcripts were detected in the RNA extracted from OA primary osteoblasts transfected with LNA1 or LNA2. The total number of significantly differentially expressed transcripts were 489 in MALAT1 LNA1 compared to NTC (>1.5-fold change, $P \leq 0.05$) and 343 transcripts in MALAT1 LNA2 compared to NTC. A total of 155 transcripts were significantly differential expressed transcripts (>1.5-fold change, $P \leq 0.05$) common between MALAT1 LNA's 1 & 2 (Table 6.1). The transcripts were of different types including protein coding mRNA, miscellaneous RNA (miscRNA), small nucleolar RNA (snoRNA), processed transcripts, pseudogenes, lincRNA, antisense lncRNA, sense lncRNA, sense novel lncRNA, and bidirectional RNA. Table 6.1 shows the number of each group in MALAT1-LNA1 compared to NTC and in MALAT1-LNA2 compared to NTC. Table 6.2 shows a list of significant novel lincRNA, sense and antisense transcripts and table 6.3 shows a list of significant lincRNA expressed in MALAT1-LNA1 and LNA2 compared to NTC.

Table 6.1 The number of significant differentially expressed transcript groups in MALAT1-LNA1, MALAT1-LNA2 Compared to NTC and common between both LNA's

Transcript Group	MALAT1-LNA1/NTC	MALAT1-LNA2/NTC	Common
Protein coding mRNA	453	303	146
Pseudogenes	3	0	1
miscRNA	2	1	0
snoRNA	0	0	0
miRNA	0	0	0
Processed transcripts	1	2	1
LincRNA	17	18	2
Antisense	10	14	4
Sense	1	1	1
Sense novel lncRNA	2	4	0
Bidirectional lncRNA	0	0	0
Total	**489**	**343**	**155**

Table 6.2 List of significant novel lincRNA, sense and antisense genes expressed in OA MALAT1 KD osteoblasts (LNA1 & 2 Compared to NTC)

Gene Name	F Change	P-value	Gene Type	NTC/LNA
AC026356.2	8.737	0.002	Sense-intronic	2
AL022311.1	-8.343	0.018	Sense overlapping	1&2
AC087721.1	-22.300	1.756E-09	Sense-intronic	1&2
AC037198.1	-10.752	0.001	Sense-intronic	1&2
AC099778.1	-9.273	0.004	Antisense to PTPN23	1&2
AL138921.1	-7.636	0.040	Antisense	1&2
AC079298.3	9.674	0.003	Antisense to DCHS2	2
AC008443.6	-6.732	0.044	Antisense	2
AC008764.6	-9.824	0.002	LincRNA	1&2
AC092747.4	-9.734	0.003	LincRNA	1&2
AL158152.1	9.481	0.004	LincRNA	1
AL1391056.1	10.505	0.000	LincRNA	2
AC245060.5	-10.969	0.000	LincRNA	2
AC010618.3	9.900	0.002	LincRNA	2
AP002784.1	8.971	0.005	LincRNA	2
AC116667.1	8.250	0.006	LincRNA	2
AC107068.1	-7.994	0.018	LincRNA	2
AL160396.2	-7.283	0.048	LincRNA	2

Table 6.3 List of significant lincRNA expressed in OA MALAT1- KD osteoblasts (LNA1 & 2 compared to NTC)

Gene Name	F Change	P-value	Gene Description	NTC/LNA
LINC00662	9.388	0.000	Non-protein coding RNA 662	1
MIR1938HG	10.668	0.001	Host gene	1
PPP4RI-ASI	9.730	0.001	Antisense RNA1	1
LINC00467	8.230	0.003	Non-protein coding RNA 467	1
FAM225A	9.459	0.004	Family with sequence similarity 225 member A	1&2
LINC02289	-8.080	0.004	Non-protein coding RNA 2289	1&2
ZNF582-AS1	9.244	0.005	Antisense RNA1	1
ILF3-DT	-7.820	0.005	Divergent transcript	1
MIR29B2CHG	8.989	0.006	Host gene	1
LINC02511	8.275	0.007	Non-protein coding RNA 2511	1
MIR155HG	8.275	0.017	Host gene	1&2
LINC01963	-7.035	0.032	Non-protein coding RNA1963	1
CARMN	-6407	0.033	Cardiac mesoderm enhancer-associated non-coding RNA	1
LINC02405	-7.210	0.033	Non-protein coding RNA 2405	1
LINC00886	-9.110	0.000	Non-protein coding RNA 886	2
LINC0294	10.182	0.002	Non-protein coding RNA 294	2
MIRLET7BHG	9.772	0.002	Host gene	2
LINC00607	8.699	0.005	Non-protein coding RNA 607	2
LINC00968	8.811	0.007	Non-protein coding RNA 968	2

6.3.4.2 Analysis of Differentially Expressed Genes in Common to Both LNA1 and LNA2

Given that any RNA approach is as to infer off-target effects (i.e., modulation of genes that are not specific to the function of MALAT1) we focused on the analysis of genes that were differentially expressed with both the LNA1 and LNA2 MALAT1 LNAs. In total, 155 transcripts were significant differentially expressed in RNA extracted from

MALAT1 depleted primary OA osteoblasts transfected with MALAT1-LNA1 or MALAT1-LNA2.

Of these transcripts, 82 transcripts were upregulated and 73 were downregulated. These transcripts were significantly differentially expressed (>1.5-fold change, $P \leq 0.05$) in MALAT1-KD OA primary osteoblasts transfected with either LNA1 or LNA2 compared to NTC control LNA (Table 6.4). In addition, a comparison between the upregulated and downregulated genes in MALAT1-KD LNA1, LNA2 and NTC is presented in the heat map showing the (Figure 6.4.A) and the Venn diagram (Figure 6.4.B).

A. **B.**

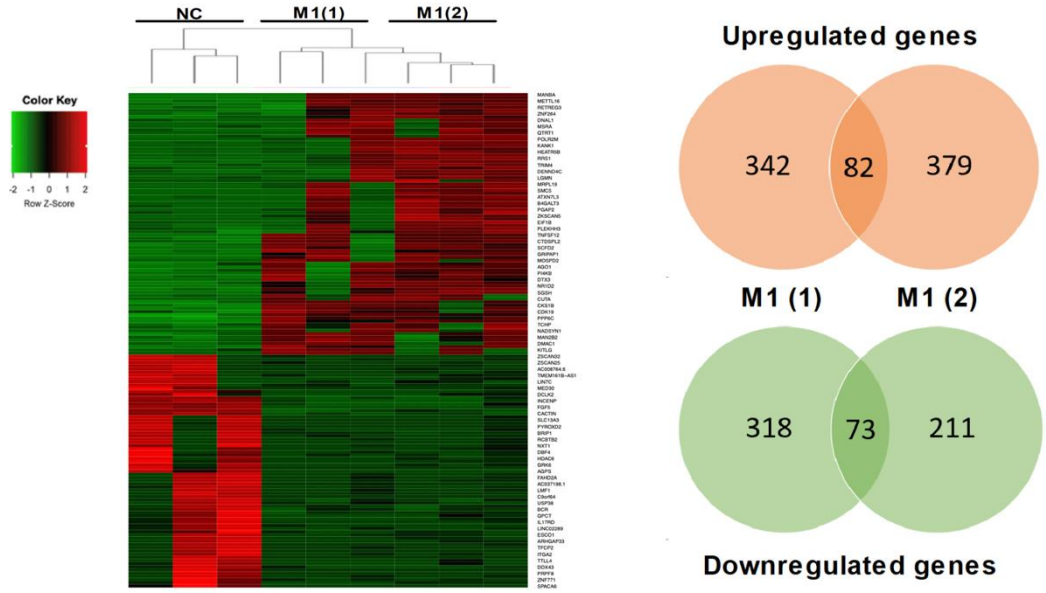


Figure 6.4 Significant differentially expressed upregulated and downregulated genes obtained from RNASeq analysis of MALAT1-KD primary osteoblasts. **(A)** Heatmap showing upregulated positive Z scores (red) and downregulated negative Z scores (Green) in M1(1) = LNA1, M1(2) = LNA2 compared to NC = NTC. **(B)** Venn diagram showing common upregulated (orange) and downregulated (green) genes between the LNAs 1 & 2.

Table 6.4 Significant differentially expressed most upregulated and downregulated genes in RNA sequence from primary OA MALAT1-KD osteoblasts common between MALAT1-LNA1 and LNA2 compared to NTC

Gene	Gene Name	Log2 Fold Change	p-value
Most Upregulated			
TNFSF12	TNF Superfamily member 12	11.4	0.00
DTX3	Deltex E3 ubiquitin ligase 3	11.1	0.00
POLR2M	RNA polymerase II subunit M	10.9	0.00
PIK4B	Phosphatidylinositol 4-kinase beta	10.8	0.02
CKS1B	CDC28 protein kinase regulatory subunit 1B	10.7	0.00
PTGS2	Prostaglandin-endoperoxide synthase 2	10.6	0.00
NR1D2	Nuclear receptor subfamily 1 group D member 2	10.6	0.00
KLHL36	Kelch like family member	10.5	0.00
RCOR1	REST corepressor 1	10.5	0.00
GRIPAP1	GRIP associated protein 1	10.5	0.00
Most Downregulated			
NAIF1	Nuclear apoptosis inducing factor 1	-10.8	0.00
INCENP	Inner centromere protein	-10.7	0.00
C9orf64	Chromosome 9 open reading frame 64	-10.6	0.01
HECW1	HECT, C2 and WW domain containing E3 ubiquitin protein ligase 1	-10.6	0.10
FGF5	Fibroblast growth factor 5	-10.5	0.00
WHAMM	WASP homolog associated with actin, Golgi membranes and microtubules	-10.4	0.01
LRFN3	Leucine rich repeat and fibronectin type III domain containing 3	-10.4	0.00
ZSCAN32	Zinc finger and SCAN domain-containing protein 32	-10.3	0.00
PYROXD2	Pyridine nucleotide-disulphide oxidoreductase domain 2	-10.3	0.01
NINJ2	Ninjurin 2	-10.2	0.02

6.3.4.3 Pathway Analysis

To further understand the implications of these MALAT1 depleted osteoblasts transcriptomes, we next conducted pathway analysis using Ingenuity Pathway Analysis software (IPA). IPA was used to further examine the MALAT1 depleted differentially expressed genes by aligning them to canonical pathways and cellular processes. Transcriptomes were generally evaluated by using the pathway analysis tool to predict the most significant canonical pathways that are changing based on gene expression, top upstream regulators, downstream regulators, disease and functions altered, regulator effects and networks affected due to gene expression in RNA from primary osteoblasts due to MALAT1-LNA1 and MALAT1-LNA2 transfection compared to NTC. The first step was to evaluate the general transcriptomes in the RNA extracted from osteoblasts transfected with either LNAs and the second step was then dedicated to the analysis of the common genes in transfection with LNA1 and LNA2.

The pathway analysis tool was used to analyse a list of canonical pathways that were predicted to be stimulated by significantly associated with the differentially expressed genes following MALAT1-KD. Predicted pathways, the -log*P* values of the association between pathway and gene dataset and the molecules related to these pathways is shown in Table 6.5.I – 6.5.III. The most significant canonical pathways affected in the RNASeq transcripts included phosphatidylcholine biosynthesis, fMLP signaling in neutrophils, NAD biosynthesis, eicosanoid biosynthesis and prostanoid biosynthesis (Figure 6.5.A). Cellular processes significantly affected included cell to cell signaling,

DNA replication, cellular growth and proliferation and cellular development (Figure 6.5.B).

Table 6.5.I List of canonical pathways related to OA pathogenesis

Pathway	-log (p-value)	Molecules
PI3K/AKT Signaling	1.0	IL17RD, ITGA2, PTGS2
IL-8 Signaling	0.87	GNB5, NOX4, PTGS2
Osteoarthritis Pathway	0.41	ITGA2, PTGS2
AMPK Signaling	0.41	ADRA1B, HMGCR
Adipogenesis pathway	0.68	HDAC6, NR1D2
eNOS Signaling	0.57	HSPA14, ITPR2
Actin Cytoskeleton Signaling	0.39	FGF5, ITGA2
NF-κB Activation by Viruses	0.39	ITGA2
IL-1 Signaling	0.35	GNB5
IL-17 Signaling	0.39	PTGS2
Cyclins and Cell Cycle Regulation	0.39	HDAC6
Protein Kinase A Signaling	0.60	ANAPC4, GNB5, ITPR2, PTGS2
Prostanoid Biosynthesis	2.75	PTGIS, PTGS2
Eicosanoid Signaling	2.06	PLA2R1, PTGIS, PTGS2
fMLP Signaling in Neutrophils	1.42	GNB5, ITPR2, NOX4

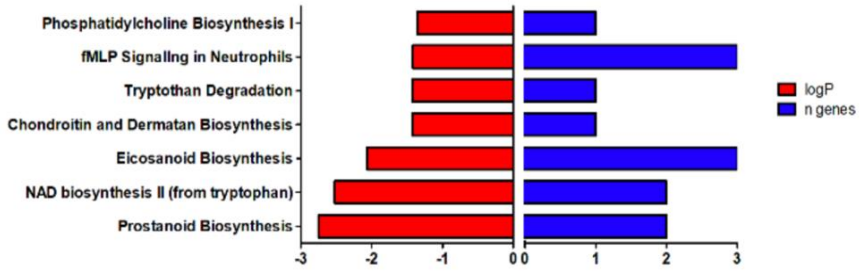
Table 6.5.II List of canonical pathways related to OA pathogenesis

Pathway	-log (p-value)	Molecules
Cell Cycle Control of Chromosomal Replication	1.3	CDK19, DBF4
Signaling by Rho Family GTPases	1.15	CDH8, GNB5, ITGA2, NOX4
Phospholipase C Signaling	1.08	GNB5, HDAC6, ITGA2, ITPR2
Tec Kinase Signaling	1.06	GNB5, ITGA2, TNFSF12
Protein Ubiquitination Pathway	1.01	ANAPC4, DNAJC13, HSPA14, USP38
Acyl-CoA Hydrolysis	1.1	PPT1
Death Receptor Signaling	0.35	TNFSF12
D-myo-inositol (1,4,5)-Trisphosphate Biosynthesis	0.83	PI4KB
G Protein Signaling Mediated by Tubby	0.74	GNB5
Antiproliferative Role of Somatostatin Receptor 2	0.41	GNB5
Notch Signaling	0.68	DTX3
Role of IL-17A in Arthritis	0.53	PTGS2
Regulation of Cellular Mechanics by Calpain Protease	0.47	ITGA2
Cell Cycle: G2/M DNA Damage	0.57	CKS1B

Table 6.5.III List of canonical pathways related to OA pathogenesis

Pathway	-log (p-value)	Molecules
Cell Cycle: G1/S Checkpoint Regulation	0.48	HDAC6
Calcium-induced T Lymphocyte Apoptosis	0.47	ITPR2
Role of CHK Proteins in Cell Cycle Checkpoint Control	0.51	TLK2
Role of NFAT in Regulation of the Immune Response	0.49	GNB5, ITPR2
HOTAIR Regulatory Pathway	0.57	AGO1, RCOR1
Inhibition of ARE-Mediated mRNA Degradation Pathway	0.74	AGO1, TNFSF12
CCR3 Signaling in Eosinophils	0.74	GNB5, ITPR2
Calcium Signaling		
Regulation of Actin-based Motility by Rho	0.42	HDAC6, ITPR2
CCR5 Signaling in Macrophages	0.34	ITGA2
PAK Signaling	0.34	GNB5
Senescence Pathway	0.33	ITGA2
CD28 Signaling in T Helper Cells	0.28	ANAPC4, ITPR2
Telomerase Signaling	0.27	ITPR2
Neuroinflammation Signaling Pathway	0.30	HDAC6
PI3K Signaling in B Lymphocytes	0.24	NOX4, PTGS2

A.



B.

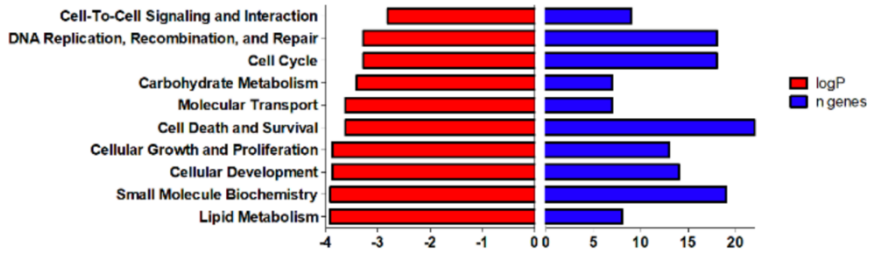


Figure 6.5 Top canonical pathways (A) and top cellular processes **(B)** determined by IPA analysis of differentially expressed genes (>1.5-fold change, $P \leq 0.05$) in OA osteoblasts devoid MALAT. (Red) representing logP = values of significant difference between pathway/process and the gene dataset and (blue) represents n = numbers of genes within the dataset aligned to the pathway/process.

6.3.4.3.1 Regulator Effects Analysis

Next, IPA was used to predicate the top upstream regulators including transcription regulators, peptidase, kinase, transporter, G-protein coupled complexes, cytokines receptors and others (Table 6.6).

Table 6.6 Top upstream regulators showing the genes, its type and the target molecules in the dataset

Gene Name	*P*-Value	Molecule Type	Target Molecules in Dataset
MED13	0.004	Transcription regulator	HMGCR, KITLG, PHKA1
HNF4A	0.004	Transcription regulator	AP3M1, BRIP1, C9orf64, CRYZL1, CTDSPL2, DHX8, DNAL1, FAHD2A, HDAC6, INCENP, IVNS1ABP, LIN7C, MRPL49, NOC3L, NOX4, PI4KB, PPP6C, RNF44, SGSH, TBC1D17, TEAD3, TRIM4, UPF3B, ZKSCAN5, ZNF317.
APOA1	0.004	Transporter	HMGCR, PTGIS, PTGS2
MTORC1	0.005	Complex	HMGCR, NOX4, PTGS2
F2RL1	0.008	G-protein coupled receptor	FGF5, KITLG, PTGS2
CST5	0.011	Other	AGO1, NOC3L, OTUD4, PRPF8, RRS1, WDR36
TCF7L2	0.023	Transcription regulator	CCNYL1, CDK19, MOSPD2, NINJ2, PI4KB, REEP3
NFAT5	0.023	Transcription regulator	NOX4, PTGIS, SEMA4C
miR-155-5p	0.024	Mature microRNA	MOSPD2, MSH2, PTGS2, RCOR1
SATB1	0.026	Transcription regulator	CDK19, KITLG, PTGS2, TNFSF12
Nfat (family)	0.032	Group	ITPR2, NOX4, PTGS2
NFATC1	0.033	Transcription regulator	HMGCR, NOX4, PTGS2
KLF2	0.034	Transcription regulator	FGF5, KITLG, PTGIS, PTGS2
MMP3	0.034	Peptidase	CACTIN, DHX8, PTGS2
MET	0.035	Kinase	NOX4, PTGS2, TCHP
SPP1	0.035	Cytokine	HMGCR, ITGA2, NDUFA9, PTGS2
TGFBR2	0.045	Kinase	FGF5, MAN2B2, PTGIS, PTGS2
miR-16-5p	0.048	Mature microRNA	ITGA2, KITLG, MSH2, PTGS2

6.3.4.3.2 Regulator Analysis

The next step was to analyse the top 20 significant upregulated genes (Table 6.7) and the top 20 significant downregulated genes (Table 6.8) obtained from datasets of RNASeq from MALAT1-KD OA osteoblasts transfected with LNA1 and LNA2, and then identifying candidate upstream regulators (either as upstream activators or inhibitors), which could explain the differentially expressed gene output in primary osteoblasts depleted of MALAT1.

Table 6.7 List of top 20 significant upregulated protein-coding genes analysed from RNASeq of MALAT1-KD OA osteoblasts transfected with LNA1 and LNA2

Gene Name	F Change	P-value	Gene Description
PI4KB	12.3	0.01	Phosphatidylinositol 4-kinase beta
CARD6	11.8	0.01	Caspase recruitment domain family member
FNDC3A	11.8	0.01	Fibronectin type III domain containing 3A
GNPDA2	11.7	0.01	Glucosamine-6-phosphate deaminase 2
			PR/SET domain 4
SLAIN2	11.6	0.01	SLAIN motif family member 2
TPMT	11.5	0.01	Thiopurine S-methyltransferase
TNFSF12	11.4	0.00	TNF superfamily member
EDEM1	11.4	0.02	ER degradation enhancing alpha-
			mannosidase like protein
DTX3	11.1	0.00	Deltex E3 ubiquitin ligase 3
KLHL36	11.0	0.02	Kelch like family member
FAM167A	10.9	0.01	Family with sequence similarity 167
			member A
PRDM4	10.9	0.01	PR/SET domain 4
MPST	10.9	0.02	Mercaptopyruvate sulfur transferase
POLR2M	10.9	0.00	RNA polymerase II subunit M
CKS1B	10.7	0.00	CDC28 protein kinase regulatory subunit 1B
KIAA2026	10.7	0.01	KIAA2026 protein coding gene
SCFD2	10.6	0.02	Sec.1 family domain containing
PTGS2	10.6	0.00	Prostaglandin-endoperoxide synthase 2
NR1D2	10.6	0.00	Nuclear receptor subfamily 1 group D
			member 2
KLHL36	10.5	0.00	Kelch like family member 36

Table 6.8 List of top 20 significant downregulated protein-coding genes analysed from RNASeq of MALAT1-KD OA osteoblasts transfected with LNA1 and LNA2

Gene Name	Fold Change	P-value	Gene Description
PYCARD	-11.4	0.02	PYD and CARD domain containing
CYB5R2	-11.2	0.02	Cytochrome b5 reductase
SLC37A3	-10.9	0.02	Solute carrier family 37 member
NAIF1	-10.8	0.00	Nuclear apoptosis inducing factor 1
INCENP	-10.7	0.00	Inner centromere protein
TWNK	-10.7	0.01	Twinkle mtDNA helicase
RBMX2	-10.7	0.01	RNA binding motif protein X-linked
ZNF445	-10.7	0.02	Zinc finger protein 445
C9ORF64	-10.6	0.01	Chromosome 9 open reading frame 64
HECW1	-10.6	0.01	HECT, C2 and WW domain containing E3 ubiquitin protein liqase 1
PSD3	-10.6	0.01	Pleckstrin and Sec7 domain containing
FGF5	-10.5	0.00	Fibroblast growth factor 5
SMYD4	-10.5	0.02	SET and MYND domain containing
CCDC47	-10.5	0.03	Coiled-coil domain containing
WHAMM	-10.4	0.01	WASP homolog-associated protein with actin membranes and microtubules
LRFN3	-10.4	0.00	Leucine rich repeat and fibronectin type III domain containing 3
MRGPRF	-10.4	0.02	MAS related GPR family member F
CACTIN	-10.3	0.02	Cactin, spliceosome C complex subunit
ZSCAN32	-10.3	0.00	Zinc finger and SCAN domain containing 32
PYROXD2	-10.3	0.01	Pyridine nucleotide-disulphide oxidoreductase domain 2

6.3.4.3.3 Upstream Regulator Analysis

The regulator effects analysis in IPA was applied on the datasets to predict the upstream and downstream regulators that caused changes in gene expression affecting pathway interactions to study the effect of these genes on target molecules

that may play an important role in the pathogenesis of OA. In total 30 genes were predicted as significant upstream regulators, from which 15 were the most significant upstream activated genes including HNF4A, Nfat and MMP3, (Table 6.9). Interestingly, one or the common target molecules for these regulators is PTGS2. 15 other genes were the most significant upstream inhibited genes including B4GALT3, KANK1 and MANBA (Table 6.10).

Table 6.9 List of the most significant activated upstream regulator genes

Gene Name	Gene name / Molecule type	*P* Value	Target Genes
Genistein	Isoflavone / Chemical drug	0.01	GRK6, HMGCR, LGMN, MANBA, PPT1, PTGS2, SGSH
CST5	Cystatin D / Other	0.01	AGO1, NOC3L, OTUD4, PRPF8, RRS1, WDR36
TCF7L2	Transcription factor 7 like 2 / Transcription regulator	0.02	CCNYL1, CDK19, MOSPD2, NINJ2, PI4KB, REEP3
miR-155-5p	Mature microRNA	0.02	MOSPD2, MSH2, PTGS2, RCOR1
KLF2	Kruppel like factor 2 / host transcription factor	0.02	CDK19, KITLG, PTGS2, TNFSF12
MET	MET proto-oncogene / receptor tyrosine kinase	0.03	FGF5, KITLG, PTGIS, PTGS2
SPP1	Secreted phosphoprotein 1 / chemical drug	0.03	ITGA2, MSH2, PTGS2, RCBTB2
NFATC1	Nuclear factor of activated T cells 1 / Transcription regulator	0.03	ITPR2, NOX4, PTGS2
Nfat	Nuclear factor of activated T cells / Group	0.03	ITPR2, NOX4, PTGS2
MMP3	Matrix metallopeptidase 3 / Peptidase	0.03	CACTIN, DHX8, PTGS2
miR-16-5p	Mature microRNA	0.04	ITGA2, KITLG, MSH2, PTGS2

Table 6.10 List of the most significant inhibited upstream regulator genes

Gene Name	Gene name / Molecule type	*P*- Value	Target Genes
B4GALT3	Beta-1,4-galactosyltransferase 3 / Enzyme	0.00	Activated HRAS
KANK1	KN motif and ankyrin repeat domains 1 / Transcription regulator	0.00	Activated SOX2
MANBA	Mannosidase beta / Enzyme	0.00	Activated Genistein
HSPA14	Heat shock protein family A (Hsp 70) member 14 / Peptidase	0.00	Activated MYC
NADSYN1	NAD synthetase 1 / Enzyme	0.00	Activated SMARCA4
CEP250	Centrosomal protein 250 / Other	0.00	Inhibited ESR
LGMN	Legumain / Peptidase	0.00	Inhibited MYC
AGO1	Argonaute RISC component 1 / Translation Regulator	0.00	Activated Estrogen receptor
IVNS1ABP	Influenza virus NS1A binding protein / Others	0.00	Activated EGFR
MSH2	MutS homolog 2 / Enzyme	0.00	Inhibited JUN
NOC3L	NOC3 like DNA replication regulator / Others	0.00	Inhibited TGFB1
AFMID	Arylformamidase / Enzyme	0.00	Activated MYC
FAM167A	Family with sequence similarity 167 member A / Other	0.00	Activated SMARCA4
DPY30	Dpy-30 histone methyltransferase complex regulatory sub Deltex E3 ubiquitin ligase 3 / Other	0.00	Activated SOX2
HMGCR	3-hydroxy-3-methylglutaryl-CoA reductase / Enzyme	0.00	Inhibited FBXW7

6.3.4.3.4 Diseases and Functions Analysis

In this analysis, the relationship of mRNA transcripts in certain diseases and the effects on the pathogenesis of diseases and functional aspects were predicted. mRNA for different groups were predicted to contribute to the pathogenesis of osteoarthritis including growth factors, cytokines, transcription factors, transmembrane receptors and enzymes. Upregulation, expression and down-regulation of mRNA in different tissues of a joint was related to OA in human (Figure 6.6) and (Table 6.11).

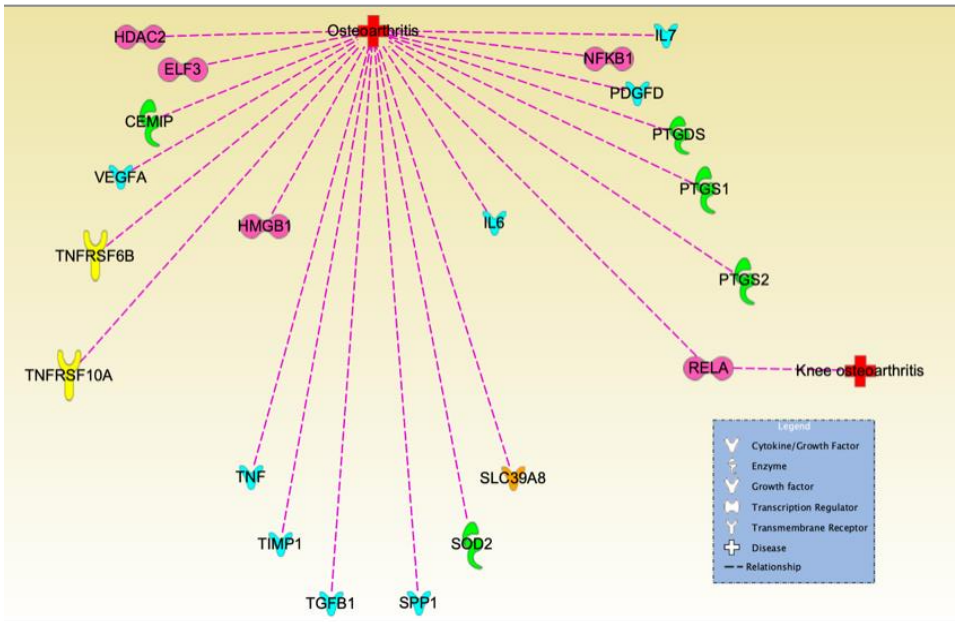


Figure 6.6 Using the disease and functions analysis, a relationship was built between OA disease and molecules with possible contribution to the pathogenesis of OA according to data from research gathered by the software. CK and growth factors (blue), enzymes (green), transmembrane receptors (yellow), transcription regulators (pink) and growth factors (orange).

Table 6.11 mRNA expression relationship with human OA in MALAT1-KD primary osteoblasts extracted from IPA data

mRNA-code	mRNA-name	Type	Tissue/Cells	Expression
PTGS2	Prostaglandin-Endoperoxide synthase 2	Enzyme	Articular cartilage	Upregulated
PTGDS	Prostaglandin D2 synthase	Enzyme	Articular cartilage	Upregulated
TIMP1	TIMP1 Metallopeptidase inhibitor 1	Cytokine	Articular cartilage	Upregulated
TNF	Tumor necrotic factor	Cytokine	Articular cartilage (Knee)	Upregulated
VEGFA	Vascular endothelial growth factor A	Growth factor	Articular cartilage	Upregulated
IL7	Interleukin 7	Cytokine	Chondrocytes	Upregulated
RELA	RELA Proto-Oncogene, NF-KB subunit	Transcription regulator	Synovial membrane	Upregulated
PDGFD	Platelet derived growth factor D	Growth factor	Synovial membrane	Upregulated
TNFRSF10A	TNF receptor superfamily member 10a	Transmembrane receptor	Synovial membrane	Upregulated
SOD2	Superoxide Dismutase 2	Enzyme	Synovial fibroblasts	Upregulated
HMGB1	High mobility group box 1	Transcription regulator	Synovial fibroblasts	Upregulated
TGFB1	Tumor growth factor beta 1	Growth factor	Subchondral bone	Upregulated
PTGS 1	Prostaglandin-Endoperoxide synthase 1	Enzyme	Synovium tissue	Expression
NF-KB1	Nuclear factor kappa B 1	Transcription regulator	Synovium tissue	Expression
TNFRSF6B	TNF receptor superfamily member 6b	Transmembrane receptor	Synovial fibroblasts	Expression
SPP1	Secreted phosphoprotein 1	Cytokine	Synovial fibroblasts	Expression
IL6	Interleukin 6	Cytokine	Synovial membrane	Downregulated

6.3.4.3.5 Networks Analysis

Next, an analysis was performed to examine important key networks that could be related to the inflammatory processes and the identified differentially expressed genes related to MALAT1-KD in primary OA osteoblasts datasets from this thesis project including genes associated with NF-kB network (Figure 6.6) and PTGS2 (Figure 6.7) as an example.

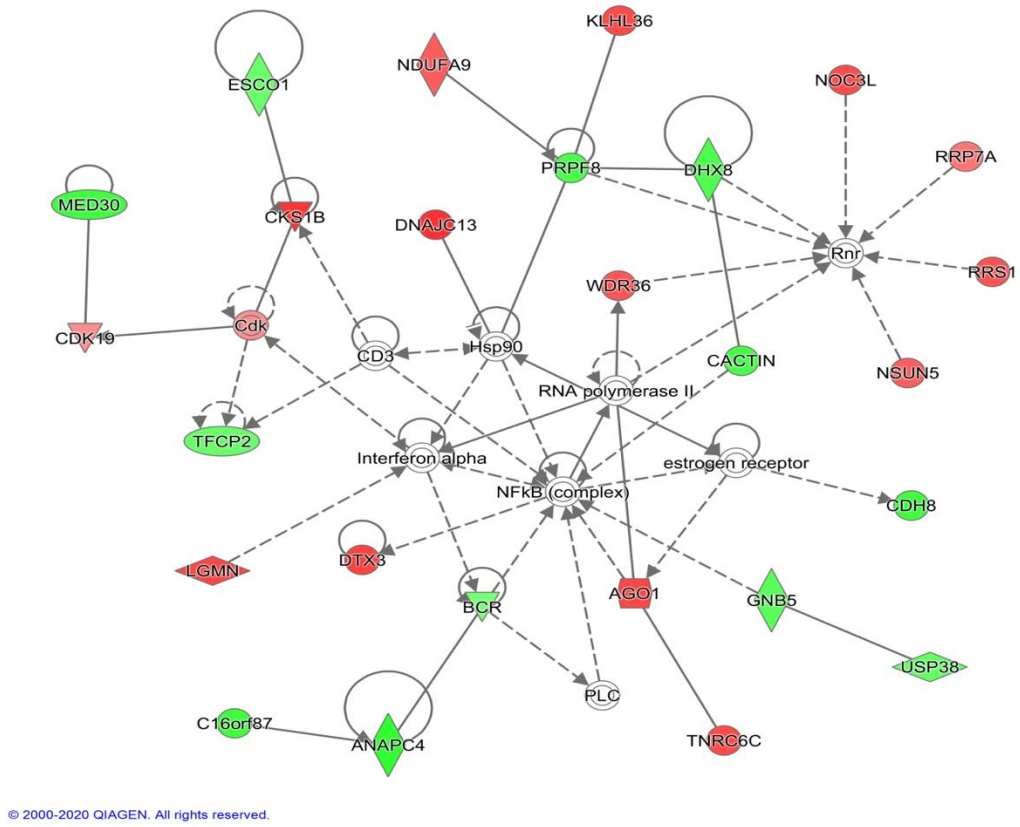


Figure 6.7 NF-kB network (grey), indicating that it was not upregulated, nor downregulated. It also shows its relationship with other nodes through a direct relation (un-dashed line) to RNA polymerase II, and indirectly (dashed line) related to Interferon alpha, estrogen receptor, and upregulated (red) DTX3. Different predicted upregulated (red) molecules including AGO1 (which is directly related to TNRC6C (un-dashed line), indirectly regulates NF-kB complex. The predicted down regulated (green) molecules like BCR, (which is directly affected by the downregulation of ANAPC4) for example. © 2000-2020 QIAGEN.

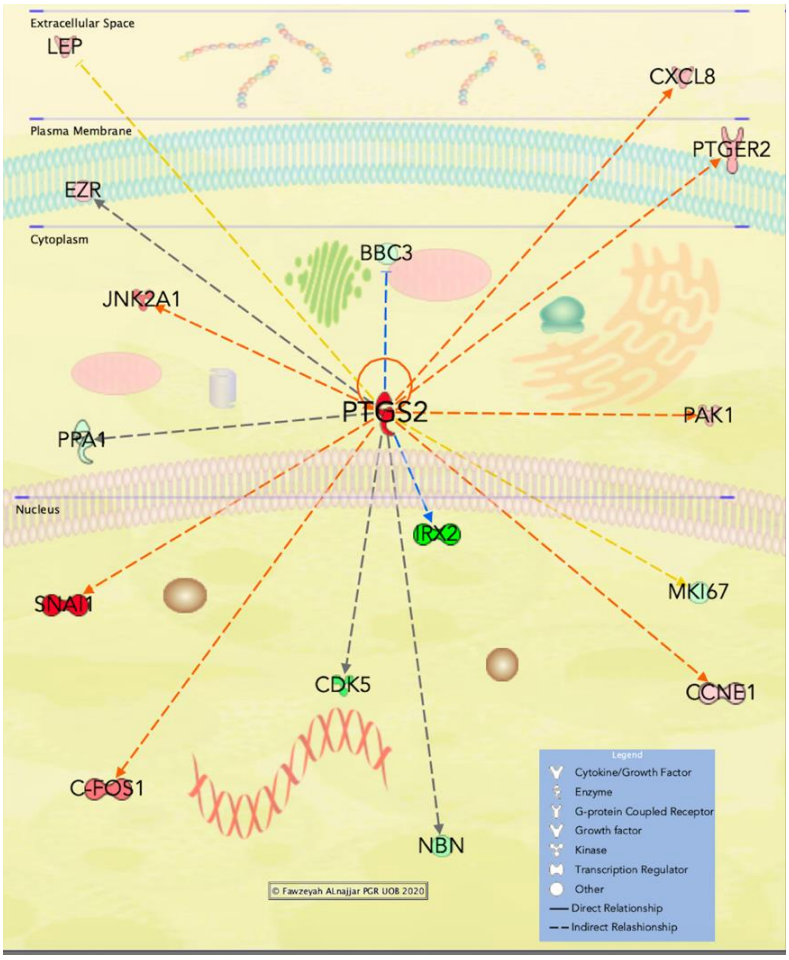


Figure 6.8 PTGS2 network designed by Fawzeyah A. Alnajjar using ingenuity IPA 2000-2020 Qiagen tools. Showing the relationship between the upregulated mRNA for PTGS2 with other genes in different cellular compartments. The following genes are related positively to PTGS2 (relationship shown in dashed lines ended with an arrow) including C-X-C Motif chemokines ligand 8 (CXCL8) in the extracellular space, Prostaglandin E receptor 2 (PTGER2) transmembrane receptors and Ezrin (EZR) protein, JNK2A1 or MAPK9 variant 1 (JNK2A1), P21 activated kinase 1 (PAK1), Inorganic pyrophosphatase 1 (PPA1) in the cytoplasm and Iroquois homeobox 2 (IRX2), Marker of proliferation Ki-67 (MKI67), Cyclin E1 (CCNE1), Nibrin coding gene (NBN), Cyclin dependent kinase 5 (CDK5), c-FOS proto-oncogene 1 (C-FOS1) and Snail family transcriptional repressor 1 (SNAI1) in the nuclease. Only 2 genes show a negative relationship (shown in dashed lines ended with a line) with PTGS2 including leptin (LEP) in the extracellular space and BCL2 binding component 3 (BBC3) in the cytoplasm. With the red color indicating an upregulated gene, while the green indicates a downregulated gene. The pink and the light green indicating a lower degree of upregulation and downregulation respectively.

6.3.5 qPCR Validation of Genes from RNASeq Data

In order to validate the presence of some of the upregulated or downregulated genes from the RNASeq analysis a number of genes were selected in order to predict a possible association with OA pathogenesis based on the following criteria: IPA predicted roles in important pathways related to OA, high reads in the eRNAs, fold change and *P* values and according to previously published reports. 19 genes were selected and designed according to gene design protocol put together by our group. These primers were tested in MALAT1-KD OA osteoblasts including upregulated genes Prostaglandin-Endoperoxide Synthase-2 (PTGS2), Argonaute RISC component1 (AGO), Phosphatidylinositol 4-kinase Beta (PI4KB), and Leucine Rich Repeats and Calponin Homology Domain Containing-3 (LRCH3), and downregulated genes including Nuclear Apoptosis Inducing Factor1 (NAIF1), Aminopeptidase Puromycin Sensitive (NPEPPS), G protein Subunit Beta5 (GNB5), Calcium Regulated Heat Stable Protein-1 (CARHSP1), Chloride Voltage-Gated Channel-7 (CLCN7), Long Intergenic Non-coding-02289 (LINC02289), Spondin-2 (SPON2), Alsin Rho Guanine Nucleotide Exchange Factor-2 (ALS2), NEDD4 E3 Ubiquitin Protein Ligase (NEDD4) , SPX domain-containing proteins (XPRI), Exocyst Complex Component -5 (EXOC5), (Transcriptional Elongated Factor AI-8 (TCEAI8) and Hepatocyte Nuclear Factor-4 (HNF4) (Table 6.13).

Table 6.12 Designed primers for gene qRT-PCR validation in OA MALAT1-KD OA osteoblasts.

No.	Primer	Log2 Fold Change	*P* adj.
	Upregulated		
1	PTGS2	10.6	0.03
2	AGO1	10.2	0.04
3	RP9	9.5	0.03
4	PI4KB	9.4	0.05
5	LRCH3	9.2	0.02
	Downregulated		
6	NAIF1	-10.8	0.04
7	PRPF8	-9.6	0.05
8	NPEPPS	-9.3	0.02
9	GNB5	-8.6	0.04
10	CARHSP1	-8.2	0.02
11	CLCN7	-8.2	0.03
12	LINC02289	-8.1	0.05
13	SPON2	-7.7	0.03
14	ALS2	-7.6	0.03
15	NEDD4	-7.6	0.35
16	XPRI	-7.6	0.04
17	EXOC5	-7.5	0.03
18	TCEAI8	-7.4	0.03
19	HNF4	-1.6	0.04

Among the upregulated genes LRCH3 showed a significant expression in MALAT1-KD OA osteoblasts in LNA2 compared to NTC control LNA ($P < 0.02$) (Figure 6.10.I.A). From the downregulated genes NPEPPS was significantly expressed in LNA2 compared to NTC control LNA ($P < 0.0001$) (Figure 6.10.I.B), ALS2 was significantly expressed in LNA1 compared to NTC ($P < 0.0002$) and LNA2 compared to NTC ($P < 0.01$) (Figure 6.10.I.C) and EXOC5 showed significant expression in LNA2 compared to NTC control LNA ($P < 0.001$) (Figure 6.10.II.B). Other genes were expressed in LNA1 or LNA2, but this expression didn't reach a statistically significant level including PI4KB, PRPF8 and NEDD4. Some of the top upregulated like PTGS2 or downregulated like NAIF1 genes were not expressed in qRT-PCR but it was possible to detect COX-2 (encoded by PTGS2 gene) protein by Western blotting in MALAT1-KD osteoblasts (Figure 6.11) which was in consistence with the upregulated levels of PTGS2 in MALAT1 depleted osteoblasts by qRT-PCR.

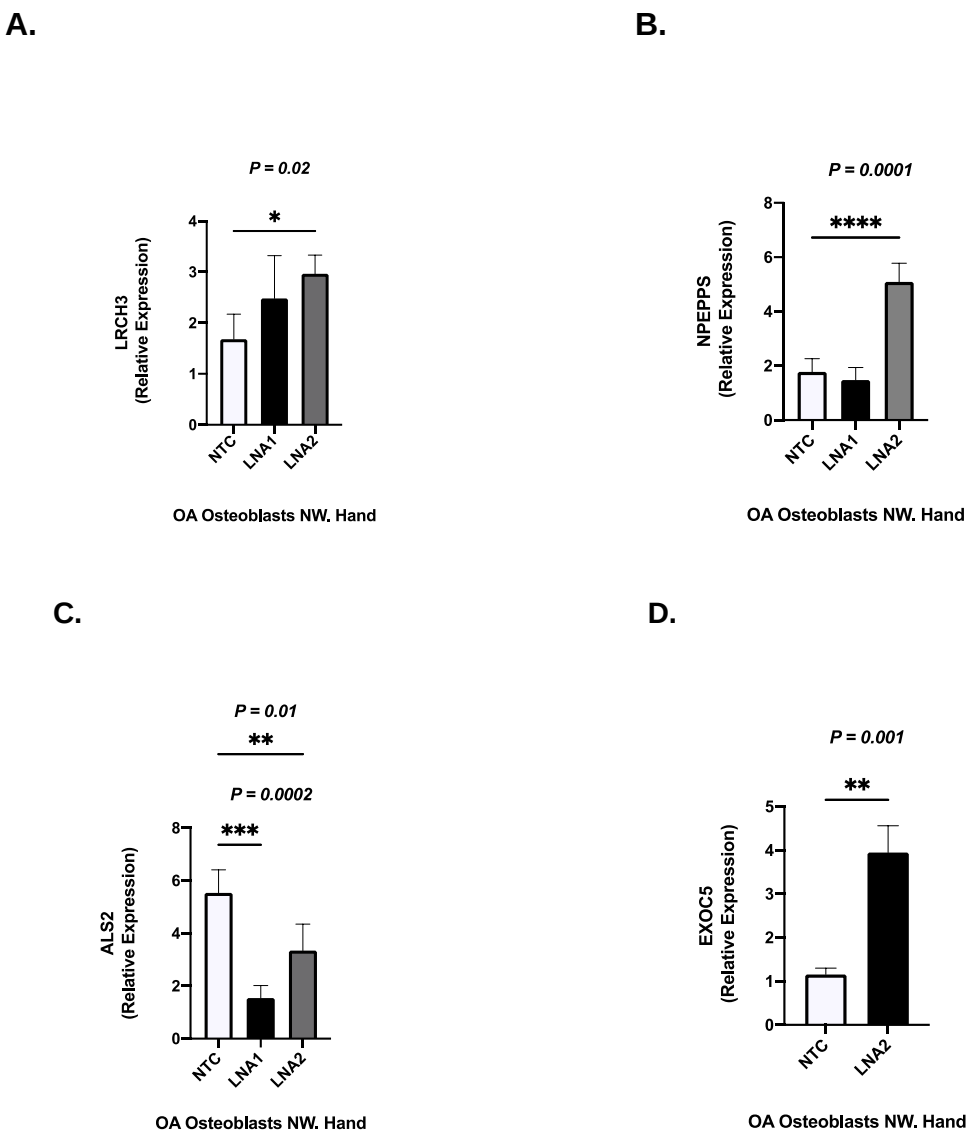
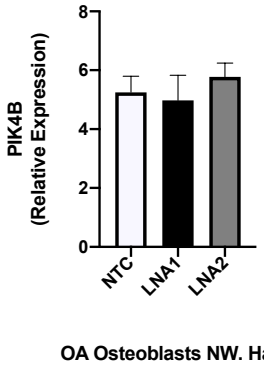
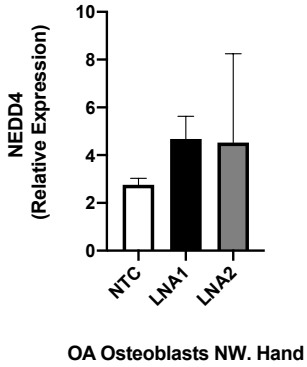


Figure 6.10.I qRT-PCR Gene validation of a panel of selected upregulated and downregulated genes. Significantly expressed upregulated genes include LRCH3 gene ($P < 0.02$) in LNA2 compared to NTC **(A)** and the downregulated genes including NPEPPS gene ($P < 0.02$) **(B)**, ALS2 ($P < 0.0002$) in LNA1 compared to NTC and ($P < 0.01$) in LNA2 compared to NTC **(C)** and EXOC5 ($P < 0.001$) in LNA2 compared to NTC **(D)**.

A.



B.



C.

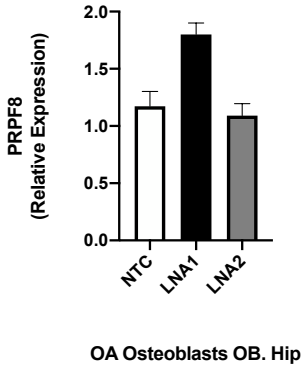


Figure 6.10.II qRT-PCR Gene validation of a panel of selected upregulated and downregulated genes. Upregulated genes include PI4KB gene **(A)** and the downregulated genes including NEDD4 **(B)** and PRPF8 **(C).** None of these genes showed a significant expression in LNA or LNA2 compared to NTC.

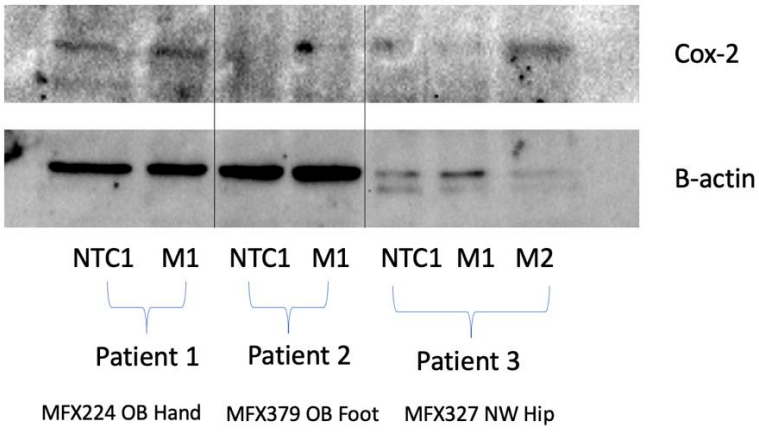


Figure 6.11 Western blotting measuring the protein levels of COX-2. COX-2 protein levels were detected by western blotting in MALAt1-KD NW osteoblast from OA hip joint in LNA2 compared to B-actin control. This protein is encoded by PTGS2 gene, which was upregulated by MALAT1-KD.

6.3.6 MALAT 1 Expression Modulation of Metabolic Functions of Primary Osteoblasts

The last step in this project was to study whether MALAT1-KD modulates the metabolic functions of OA primary osteoblasts with regards to their innate role in bone formation. To this end, the metabolic functions of primary osteoblasts during bone remodelling was tested *in vitro* by measuring the levels of OPG production in the supernatants of prolonged MALAT1-KD, performed twice a week on cultured osteoblasts for a period of 3 weeks with either LNA1 or 2 or NTC LNA. RNASeq analysis reviled that MALAT1-KD induced the expression of PTGS2 gene, for this fact the next step was to measure PGE2 production by ELISA, from the collected supernatants of IL-1β stimulated and

non-stimulated primary osteoblasts. ALP activity was measured next by using the cell lysates and at the end of the 3 weeks mineralisation was measured and quantified by staining the cells with Alizarin red stain.

6.3.6.1 Osteoblast OPG, and PGE2 production

MALAT1-KD was maintained for 3 weeks by transfecting the osteoblasts twice a week. A sustained MALAT1 expression knockdown was achieved ranging between 65-85% (Figure 6.12.I.A). OPG production determined by ELISA, was increased throughout the 3 weeks in both the control (NTC) and MALAT1-LNA transfected OA osteoblasts, with similar rate of production and the total amount of OPG production (Figure 6.12.I.B). Next, the effect of MALAT1-KD on the basal and the 24 h IL-1β mediated production of PGE2 in OA osteoblasts was determined using ELISA. A significant difference in the levels of PGE2 was seen in IL-1β stimulated compared to non-stimulated osteoblasts (2-fold, $P < 0.05$) (Figure 6.12. I.C & D).

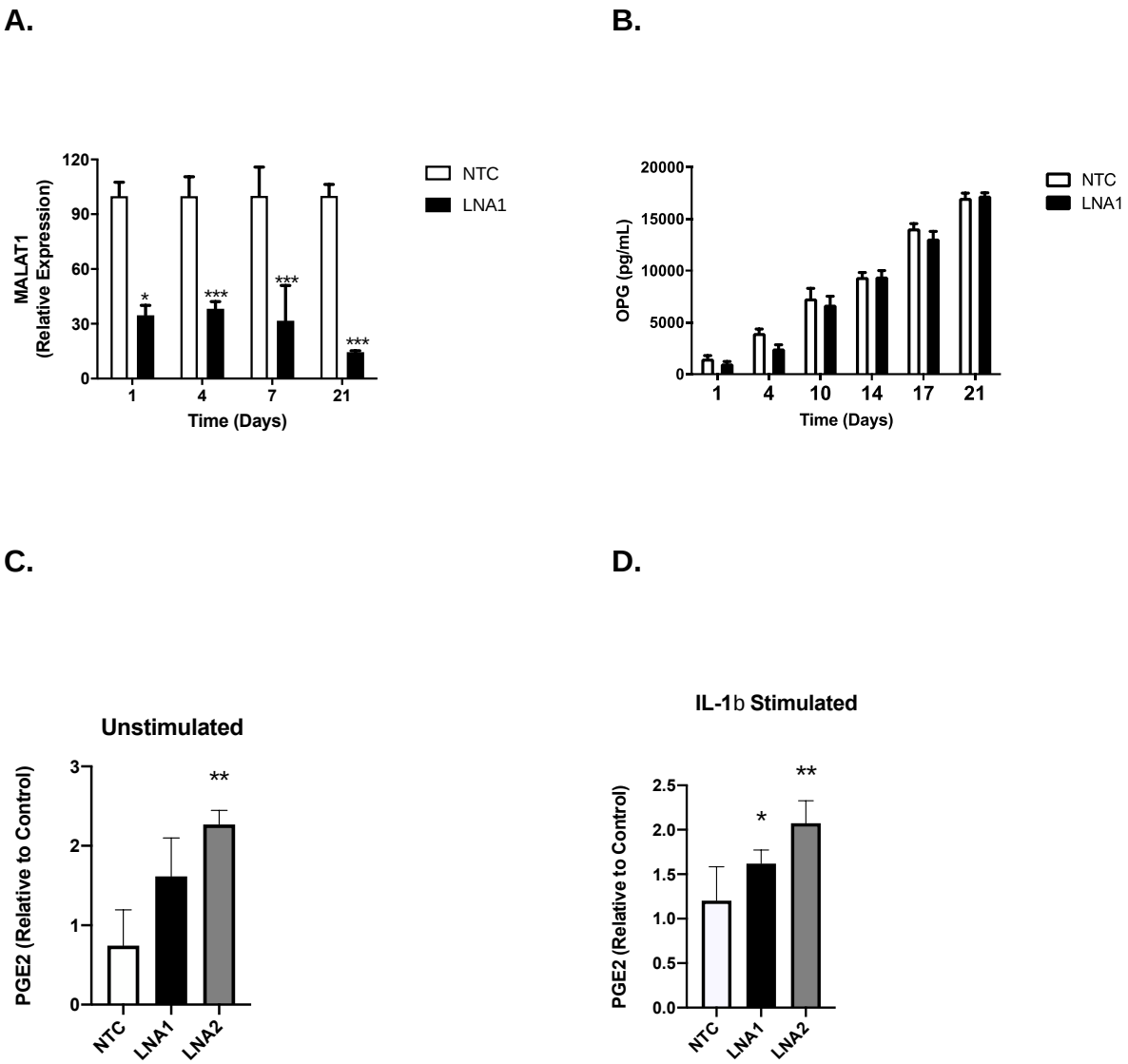


Figure 6.12.I MALAT1-KD modulation of metabolic functions of OA osteoblasts. Sustained MALAT1-KD expression for 21 days in OA osteoblasts (*n* = 6), following LNA1 (Black) targeting MALAT1 compared to NTC (white) LNA control **(A)** OPG production (pg/mL) determined by ELISA for over 21 days in LNA1 (black) and NTC (White) control LNA in OA osteoblasts (*n* = 6) **(B)**, MALAT1-KD modulation of PGE2 production, significantly difference seen in non-stimulated osteoblasts **(C)** compared to**,** IL-1β stimulated OA osteoblasts ($P < 0.01$) (*n* = 6) **(D)**.

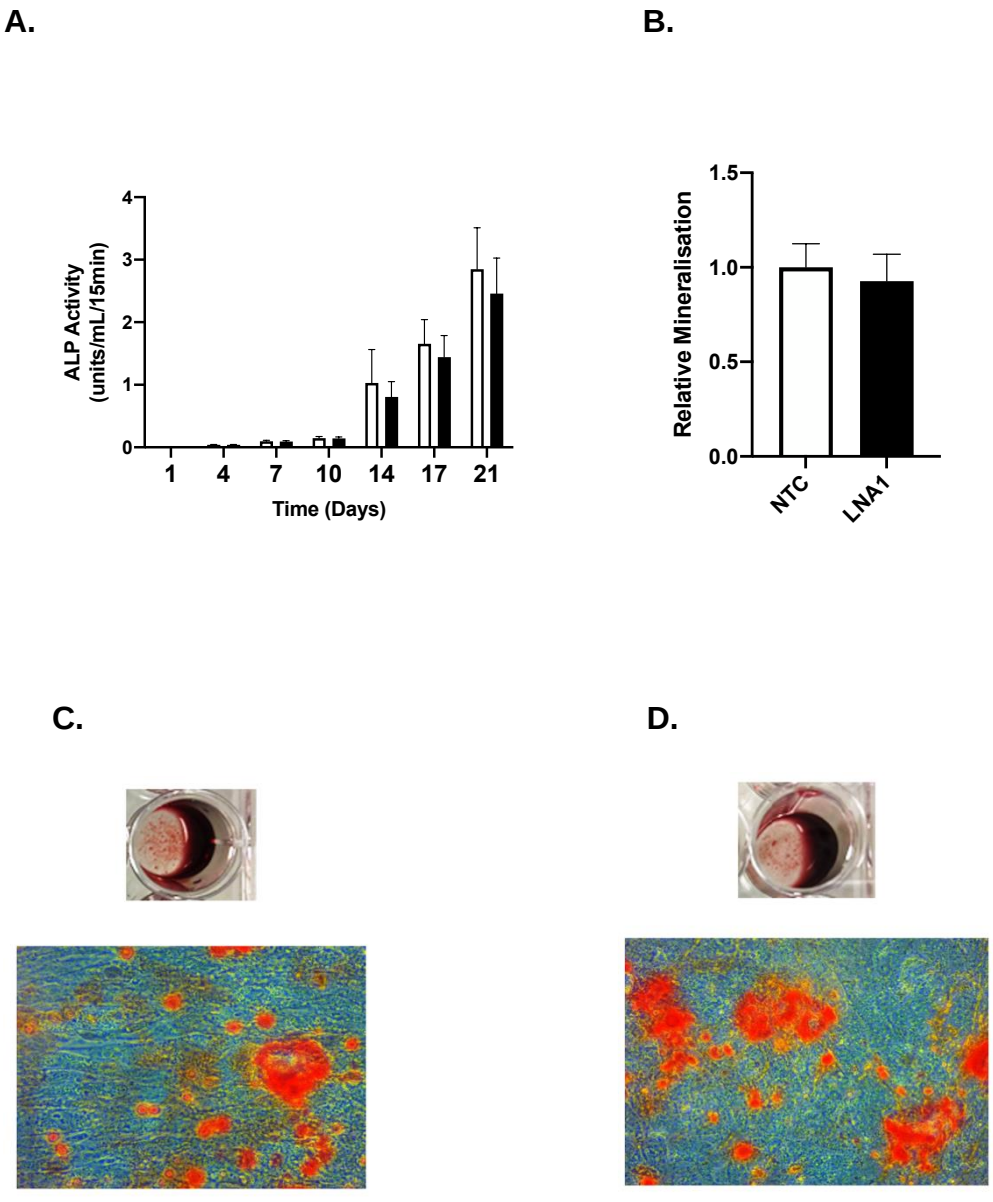


Figure 6.12.II MALAT1-KD modulation of metabolic functions of OA osteoblasts. ALP activity in NTC (white) compared to MALAT1 LNA transfected OA osteoblasts (*n* = 6) for more than 21 days **(A)**, Relative amount of OA osteoblast mineralization quantification of absorbance after 21 days of transfection with NTC (white) or MALAT1 LNA (Black), after staining with Alizarin red **(B)**, light microscopic images (x 40 magnification) showing Alizarin red stained mineralised bone nodules in both NTC **(C)** and MALAT1 LNA targeted OA osteoblasts **(D)**.

6.3.6.2 Osteoblast ALP activity and mineralisation

ALP activity was measured in NTC and MALAT1 targeted LNA of 6 transfected OA osteoblast's lysates, after more than 21 days. No significant difference in the ALP activity was seen during this time between the OA osteoblast's transfected MALAT1 LNA and the NTC LNA control (Figure 6.12.II.A). Next, the ability of MALAT1-KD OA osteoblasts in nodule formation was studied. There was no noticeable difference in nodule formation between osteoblast's transfected MALAT1 LNA and the NTC LNA control for more than 21 days (Figure 6.12.II.B). After staining with Alizarin red mineralization was quantified. No significant difference was seen in the mineralisation between osteoblast's transfected MALAT1 LNA and the NTC LNA control (Figure 6.12.II.C & D).

6.4 Discussion

The aim of this stage of the project was to knockdown MALAT1 in primary OA osteoblasts from subchondral bone and to detect its expression under *in vitro* inflammatory challenges. MALAT1 loss of function in primary osteoblasts paved the way for investigating the possible role of this lncRNA in modulating the functional phenotype of these cells aiming at identifying novel OA therapeutic intervention points that target the bone. In this thesis project, MALAT1 was knocked down in OA osteoblasts and its expression was induced in IL-1β stimulated osteoblasts.

Upon depletion of MALAT1 in OA osteoblasts, the most significantly activated pathways were related to the production of Inflammatory prostacyclins and eicosanoids indicating a modulatory role for the lncRNA MALAT1 in the transcriptomic inflammatory phenotype of OA osteoblasts. This finding was associated to the differential expression of the top upregulated gene, TNFSF12 (>11-fold upregulated), in MALAT1 depleted osteoblasts. This is in agreement with the previously published reports which indicates that, TNFSF12 is a TNF superfamily member that encodes TNF-related weak inducer of apoptosis (TWEAK) cytokine acting as a mediator of inflammatory bone remodelling [356]. Indeed, a therapeutic protocol was proposed by Du Y Y et all. in which they reduced bone resorption in rheumatoid arthritis by inhibiting the signalling pathway for TWEAK/fibroblast growth factor inducible 14 (FnF14) [357]. It was also reported that, TWEAK/FnF14 together stimulated the inflammatory cascades in NF-kB classical and alternative pathways [358].

The other important finding of this project was the significant upregulated expression of PTGS2 gene in MALAT1-KD osteoblasts (>10-fold upregulation). This was further

validated with the detection of COX-2 protein by western blotting in MALAT1 depleted OA osteoblasts. The whole picture was completed with the significant secretion of PGE2 in the basal and the *in vitro* inflammatory induced cells, which is detected more in induced MALAT1-KD OA osteoblasts compared to the control osteoblasts. This is in-accordance with the reported finding which indicates that PTGS2 gene encodes for COX-2 enzyme that mediates the production of inflammatory prostaglandins including the putative OA pain mediator PGE2 [359-360]. The TWEAK/FnF14 axis was used in inhibiting downstream cellular proteins that may contribute to the initiation of many diseases including cardiovascular diseases and cancer. From the findings of this project this axis may contribute to pain initiation in OA of the joints. It is well known that pain in OA patients radiate from the subchondral bone because of the innervation of the bone with sensory neurons [361]. In addition, bone marrow lesions (identified as hyper-intense regions on T1-weighted MRI scans) and bone shape in OA patients were associated with bone pain [362]. Li X, et al, have reported a scenario in reducing the pain and inflammation in OA patients, through the possible action of PGE2 on the E prostanoid receptors EP2 and EP4 that sensitizes nociceptors, acting synergistically with IL-1β to induce IL-6 and iNOS expression [363] in addition to selective COX-2 inhibitors such as celecoxib [364] This takes us to the results of this project, that MALAT1 expression in OA osteoblasts regulates COX-2 expression as well as both basal and IL-1β induced PGE2 production, indicating a possible role for MALAT1 in regulating inflammatory pain in the bone.

Looking at the effect of chronic MALAT1-KD on the metabolic functions of primary OA osteoblasts, a sustained MALAT1 knockdown during a time period of 3 weeks, didn't induced any significant difference in the secretion of OPG or in the activity of ALP and

ability of the osteoblast to form mineralised bone nodules. This disagrees with previous publications which have implicated that MALAT1 mediates both OPG production in osteoblasts [365] as well as ALP activity and mineralization during osteoblast differentiation [366]. This may be explained by the complexity of the underlining molecular mechanisms by which lncRNAs mediate their functions. It has been proposed that one of the pro-osteogenic functions exerted by a number of lncRNAs, including MALAT1 is by acting as miRNA sponges [367]. This was proved by different reports which indicate a role for MALAT1 in mediating osteoblast differentiation by sponging of a number of miRNAs including miR-204 [365], miR-30 [366] and miR-143 [368]. It's worth mentioning here that, in this thesis project no miRNAs were identified from the analysis of the OA osteoblast transcriptome following MALAT1 depletion. This may be explained by the fact that in this project, total RNA was isolated using mini columns which would likely have excluded many miRNAs from the RNA collected.

To conclude, MALTA1 was expressed in OA subchondral bone, and it was induced in osteoblasts under inflammatory conditions. MALAT1-KD in OA osteoblasts modulated the production of PGE2 prostaglandin which may suggest an important possible role for MALAT1 in the development of OA bone pain and inflammation.

CHAPTER 7

Discussion

7.1 General Discussion

98% of the transcribed RNAs in the human genome belong to the non-coding genes [369] It is now nearly more than 60 years since the discovery of the first ncRNA, transfer RNA and ribosomal RNA in the 1950s, since then many ncRNAs were identified due to advances in genomic technologies including microarrays, RNA sequencing and bioinformatics analysis. Three main groups of noncoding RNAs including miRNA, lncRNA and circRNAs which were regarded as junk for a period of time, were reported lately to be involved in many important biological and pathophysiological functions [370-371] in many diseases including cancer, cardiovascular diseases, inflammatory and autoimmune diseases [372-376].

Accumulating evidence shows that ncRNAs play important roles in the epigenetic regulation of gene expression leading to changes to expression without causing any changes in the DNA sequence [377]. ncRNAs were reported to contribute to different epigenetic gene expression mechanisms including histone modification, DNA methylation, heterochromatin formation and gene silencing [369,372,378-379]. LncRNAs represent an important group of ncRNAs, which were recently reported to have important roles at the level of transcriptional and post-transcriptional [380-381] gene regulation in many diseases including roles in the transcription, chromatin remodelling, activation and transport of transcription factors, oligomerization of activator proteins, alternative splicing, inactivation of transcriptional promoter and repression of gene clusters epigenetically [382-384]. In addition, lncRNAs were reported to contribute to both physiological and pathological processes including cellular proliferation, migration, apoptosis, gene imprinting [385]. The role of lncRNAs

in inflammatory joint diseases including RA and OA has also been reported [349], including by our group in 2016 and 2020 [170, 311]. Based on the recent report of our group [171] and other research groups world-wide inflammatory processes contribute to the pathogenesis of OA [386-388] and lncRNAs were reported to contribute to OA pathogenesis [389-390]. LncRNA MALAT1 was extensively studied as a lncRNA that contributes to the pathogenesis of many diseases by controlling the epigenetic transcriptional modulation of inflammatory genes in chronic inflammatory diseases.

To-date, OA is regarded as a disease of the whole joint, affecting all the tissues of the joint including the main tissues forming any diarthrodial joints articular cartilage, synovium and synovial fluid, and subchondral bones. In addition to other tissues in the joint including the skeletal muscle, meniscus, and ligaments [392-394]. In order to investigate the effect of particular lncRNAs in the pathogenesis of OA, it is therefore important that its expression and functional role are considered across multiple tissue types. The most recent reports from our group indicated a role of MALAT1 in regulating the inflammatory phenotype of the synovial fibroblasts of OB OA patients [171], and its role in regulating P38 in MAPK inflammatory signalling pathway [170]. In addition, it was reported that in articular chondrocytes MALAT1 regulates chondrocytes proliferation through modulating metabolites of the PI3K/Akt signalling pathway [395] and promotes cell proliferation, apoptosis and ECM degradation of OA chondrocytes by controlling miR-150-5p/AKT pathway [192]. However, the role of MALAT1 in mature osteoblasts of OA patients has not yet been studied, although some studies have been reported on the role of MALAT1 in human fetal osteoblastic cell line (hFOB) cell lines [396-397].

The aim of this thesis was therefore to investigate the expression of lncRNAs that were associated with the inflammatory response in OA, and in particular to examine both the expression and functional role of the lncRNA MALAT1 in OA joint tissues and derived primary cells. [Diagram 7.1].

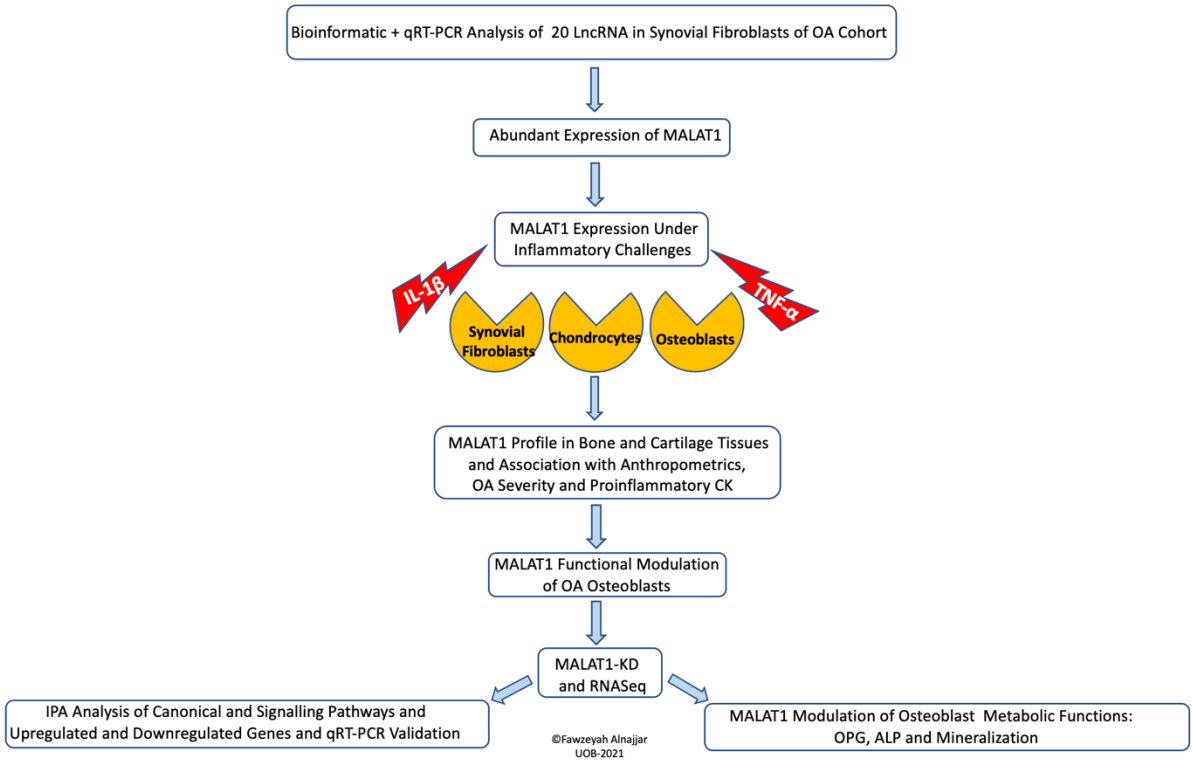


Diagram 7.1 Sketch for the steps applied in this thesis

For this purpose, Bioinformatic analysis and qRT-PCR was used to explore the expression of a panel of 20 lncRNA in synovial fibroblasts of OB and NW OA joints. IGV analysis and qRT-PCR reviled an abundant expression of MALAT1 in NW and OB OA fibroblasts. Further, to examine the association between the expression of MALAT1 during the inflammatory response across different primary cells isolated from of OA joint tissues, namely synovial fibroblasts (from synovial tissues), chondrocytes (from articular cartilage) and osteoblasts (from subchondral bone). To my knowledge this is the first study to look into MALAT1 expression upon inflammatory challenges in all three of these major primary cells from different OA joints.

All the primary cells were responsive to IL-1β and TNF-α stimulation, in terms of the induction of the cytokine IL-6 and this was consistent in cells from different OA patient cohort and anatomical joints. This finding is in agreement with previous reports indicating a key role for IL-1β and TNF-α pro-inflammatory cytokines in the pathogenesis of OA from early stages producing pathological changes affecting all the primary cells in all tissues forming the joint including chondrocytes and osteoblasts [394]. However, of interest, overall, when challenged with pro-inflammatory cytokines joint cells from obese and over-weight OA patient cohorts induced more IL-6, compared to joint cells isolated from normal-weight OA patients.

Since X. Yang et al, indicated a role for MALAT1 in the onset of osteolysis [397], based on this finding, the next step of this project was to explore the expression of MALAT1 and its association with IL-6 induction under *in vitro* inflammatory conditions, in primary OA cells. Importantly, MALAT1 was expressed in association with IL-6 induction in chondrocytes and osteoblasts from OW/OB OA cohorts compared to NW OA,

suggesting it plays an important role in the inflammatory response. This finding agrees with the previous report from our group on the expression of MALAT1 being upregulated in more inflammatory synovial fibroblasts [171]. Furthermore, it was reported that MALAT1 expression was related to inflammatory chondrocytes which were induced by IL-1β pro-inflammatory cytokine [398]. Another study indicated that MALAT1, TNF- α1, IL-6, MMP13 and caspse-3 were increased in *in vitro and in vivo* OA models [399].

Before looking at the functional role of MALAT1 in OA osteoblasts, the expression of MALAT1 was profiled in OA bone and cartilage tissues and this expression was further analysed in correlation with different anthropometric measures, joint damage severity parameters and the systemic and local concentration of a panel of cytokines and chemokines. Notably, MALAT1 expression in cartilage tissues from different cohorts was associated with the severity of the OA disease (based on x-ray radiographic analysis of cartilage damage and joint space narrowing). Indeed, a previous study indicated the role of MALAT1 on articular cartilage in the progression of OA disease by controlling miR-146a-PI3K/Akt/mTOR axis in an OA model with lipopolysaccharide (LPS) induced articular cartilage [400]. However, MALAT1 expression in subchondral bone tissues showed a significant correlation with gender being expressed more in male OA than female. This may be explained by the finding of a previous study that MALAT1 has a role in the pathological changes in organs that are under the effect of sex hormones and cancers of the reproductive system including prostate cancer [401], cervical cancer [402]. Interestingly, MALAT1 was also more highly expressed more in taller OA patients and in females with higher central adiposity. This finding may be explained by the report from previous studies that MALAT1 regulates pathways

controlling energy homeostasis and this is further supported by the work of Ebrahimi et al. who pointed out in their study that MALAT1 has a role in obesity pathogenesis by controlling lipogenic and adipogenic genes [403].

Comparing the joint tissue expression of MALAT1 with biomarkers of inflammation in the circulation provided further evidence for the association between MALAT1 and inflammation. The circulatory levels of the pro-inflammatory chemokines DKK1 and Eotaxin were significantly associated with MALAT1 expression OA cartilage tissues, indicating a potential role of MALAT1 in the modulation in the Wnt/ß catenin signaling pathway by inhibiting it due to increased DKK1 levels, leading to OA cartilage destruction which is one of the main features of OA. This finding was supported by the work of Jian Tian et al, and other researchers, in which they showed that Wnt/ ß catenin signaling pathway is important in maintaining the homeostats of human OA cartilage [404-406]. In addition, locally, circulatory levels of MIP3a pro-inflammatory chemotactic were significantly correlated with MALAT1 expression in OA cartilage. It is known that chemokines have a catabolic effect on OA articular cartilage [407] through a pathway which is triggered by the combination of chemokines and its receptors on chondrocytes leading to the release of matrix-degrading proteinases ending in a catabolic effect on the cartilage [408]. Consequently, from the significant correlation of MALAT1 with MIP3a chemokine and its modulation of OA cartilage, this chemokine could be regarded as a therapeutic target by inhibiting its interaction with its receptor on chondrocytes leading to a reduction in the catabolic effect of this chemokine on OA cartilage.

Comparing the association with MALAT1 expression in OA subchondral bone tissue, the circulatory levels of the pro-inflammatory chemokines DKK1, galectin1,TNF-α cytokine, and Chemerin adipokine were found to be significantly correlated with MALAT1 expression. In accordance with the findings of this project, previous research has indicated that DKK1 is a kee endogenous inhibitor of the Wnt signaling pathway, acting to regulate osteoblastogenesis by modulating the balance between bone resorption and bone formation [409-410]. However, the previous research [409] also indicated an elevation of the OPG in the serum from OA patients which contrasts with the finding of this project. OPG in this project was not correlated with MALAT1 expression, which maybe is due to the heterogenic nature of OA, given the relatively small OA patient cohort examined in this thesis.

The relatively high concentration of galectin1 detected in the circulation and its significant association with MALAT1 expression in OA bone is notable. Previous research indicates a role for galectin 1 in inflammation [411], this may support the findings of the current project. Other research pointed out another role for galectin 1, which is in regulating the immune processes in RA [412-413]. It is conceivable therefore that the findings in this thesis are indicative of MALAT1 mediating immune processes in OA bone via galectin1. However, clearly further research is required to discover whether MALAT1/galectin1 act together to modulate subchondral bone during the progression of OA.

The thesis provided clear evidence to show that MALAT1 was induced under inflammatory challenges in OA primary osteoblasts and its knockdown modulated the functional phenotype of OA osteoblasts by modulating both PTGS2 expression and

PGE2 prostaglandin production. However, the chronic effect of MALAT1-KD had no noticeable effects on osteogenic differentiation activity measured either by OPG production, ALP activity or the innate ability of osteoblasts to mineralise. Importantly, PGE2 is known to mediate inflammation and pain and is a putative mediator of OA pain, being produced in cyclooxygenase 2 (COX-2) pathway by converting Arachidonic acid to Prostaglandin H2 using PTGS2 and converting Prostaglandin H2 to PGE2 using PTGE2 [414]. It can be inferred from the data of this thesis that, MALAT1 may have a possible role in triggering inflammatory processes contributing to the pathogenesis of OA and in pain production by controlling PTGS2 expression and PGE2 production. This conclusion was also supported in previous research that indicated a role for PGE2 in regulating bone remodelling in addition to its effects on inflammation and its production is dependent on COX-1 and COX-2 [414-415].

The absence of evidence on the effect of chronic MALAT1-KD on osteogenic differentiation in this project could be indicative of the limited functional effect of MALAT1-KD on mature osteoblast cells. Previous research has indicated a role for MALAT1 in regulating the molecular events in human osteoblast differentiation from MSCSs [416], through regulating certain miRNAs [417] including miR-30 and miR-214 [418-420]. Furthermore, MALAT1 was previously reported to inhibit osteogenic differentiation of MSCSs in rats [421]. The afro-mentioned evidence indicating the role of lncRNA MALAT1 in controlling the osteogenic copiabilities of MSCSs may explain the missed effect of chronic MALAT1-KD on metabolic osteogenic effects in this thesis.

7.2 MALAT1 in Other Arthritic Joint Diseases

In this part of the discussion, I will discuss my results in the context of the role of MALAT1 lncRNA in other joint diseases. One of the findings regarding lncRNAs is their utility as diagnostic and prognostic biomarkers, particularly because many lncRNAs have been reported to play a role in regulating the immunity and inflammatory based process in certain diseases. LncRNA were found to modulate immune cells including T, B and NK immune cells and regulate inflammatory signaling pathways contributing to the pathogenesis of auto-immune diseases including RA, SLE, Autoimmune thyroid diseases (AITD) and Sjogren's syndrome (SS) [422] and inflammatory diseases like Psoriasis of the skin [423]. Researchers attributed the modulation of inflammatory and immune-based processes to the crosstalk between lncRNA, immune cells and inflammatory signaling pathways like TNF-α. MALAT1 lncRNA was shown to control inflammation in systemic lupus erythematosus (SLE) by modulating the levels of IL-21 and SIRT1 in monocytes of SLE patients [424]. In addition, a number of lncRNA including MALAT1, MEG3 and NEAT1 were found to be dysregulated in RA autoimmune disease. MALAT1 amongst other lncRNAs were found to be a good predictor marker for RA disease were found to be increased in the peripheral blood mono-nuclear cells (PBMCs) and the plasma of RA patients [425]. Furthermore, previous reports indicated that in RA, MALAT1 contributes to the pathogenesis of RA by controlling the fibroblast-like synoviocytes growth and apoptosis by increasing the expression of caspase-3 and 9 [426]. The aforementioned evidence agrees with the current project, in which it was shown that MALAT1 is associated with the inflammatory response in cells from the OA joint.

The role of MALAT1 in the development of immune cells was shown in some autoimmune diseases like SLA [424] and recently, some evidence was published showing a high expression of lncRNA MALAT1 in the skin and serum of patients with skin Psoriasis, modulating the immune cell function and contributing to the inflammatory process during disease via the TNF-α signaling pathway [427]. Psoriatic arthritis (PSA) is a disease of the skin and joints with an autoimmune basis and recently published evidence for inflammatory processes contributing to its pathogenesis. Inflammatory mediators such as cytokines, type-I interferon, and TNF-α were found to be contributing to enthesitis and synovitis, features of PSA, in addition to other factors like autoimmune disorders (T cell infiltration), environmental and genetic factors contributing to disease initiation and progression [428]. All of these findings are in agreement with the findings of this thesis project, regarding association of MALAT1 expression with the inflammatory response, and the correlation between the joint tissue expression of MALAT1 in cartilage and subchondral bone with circulatory level of pro-inflammatory cytokines including DKK1, galectin1 and TNF-α [429].

MALAT1 has also been implicated in the pathogenesis of Gouty arthritis (GA), which is a form of inflammatory arthritis with a metabolic pathway disorder and genetic factors. This disease is caused by purine disturbances leading to hyperuricemia and increase in uric acid excretion by the kidneys [430], together with monosodium urate (MSU) crystals deposition on joints provoking inflammatory reactions [431]. Recently noncoding RNAs including lncRNAs were found to contribute to the pathogenesis of this disease. Before going into the lncRNAs contributing to gouty arthritis disease, it is worth mentioning that research indicated a role for pro-inflammatory mediators IL-1, IL-6 and TNF-α in the pathogenesis of GA [432]. Many different ncRNAs were found

to contribute to the pathogenesis of GA, which will be discussed in detail in the next part of this discussion. A recent publication indicated a role for MALAT1 lncRNA in regulating GA via controlling the inflammation produced by macrophages due to the disposition of MSU in the joints. They showed that MALAT1 sponges miR-876-5p and by this upregulates the expression of another lncRNA, termed NLR family pyrin domain containing 3 (NLRP3). Inflammation in GA was modulated through MALAT1/miR-876-5p/NLRP3 axis which was through downstream TNF-kB signaling pathway [431]. Indeed, this role for MALAT1 in controlling macrophages during inflammatory processes were proved with another research which indicated this role for MALAT1 too [433].

Taking all together, the key finding of this thesis and the role of MALAT1 in different other arthritic diseases including RA, PSA, AS, and GA, indicates that MALAT1 may be a kee regulator of inflammatory processes in multiple arthritic joint diseases through TNF signaling pathway, and it may also contribute to the regulation of immune-based processes in the pathogenesis of these diseases. This seems to be achieved according to the competing endogenous RNA (ceRNA) hypothesis [434], in which MALAT1 competes for binding with a miRNA with another coding RNA to exert its effect on a downstream gene through multiple mechanisms including silencing and/or posttranscriptional modifications. Thus, MALAT1 could be as a therapeutic target in the treatment of such diseases.

7.3 MALAT1 Modulation of Other Non-coding in OA

Accumulating evidence indicate a role for MALAT1 lncRNA in the pathogenesis of OA by modulating other members of noncoding RNAs including miRNAs. MALTA1 was proven to work as a sponge for several miRNAs including miR-150-5p, miR-127-5p, miR-145, and miR-146a, to control some pathophysiological processes in the progression of OA through gene-coding proteins, by direct targeting of miRNAs which leads to inhibition of the expression of the miRNAs. MiR-150-5p was negatively controlled by overexpressed MALAT1 in IL-1ß stimulated chondrocytes, leading to an increase in AKT Serine/Threonine Kinase 3 gene- coding protein (AKT3), an important protein for cellular growth and proliferation. MALAT1 regulated these functions by sponging miR-150-5p leading to promoting proliferation, inhibiting apoptosis and increasing degradation of the extracellular matrix, through the axis of miR-150-5p/AKT3/MALAT1 and predicting OA progression [192,435]. Another miRNA, miR-127-5p was modulated by MALAT1 control and found to inhibit human chondrocytes proliferation through Osteopontin (OPN). OPN contributes to the pathogenesis of OA by promoting the proliferation of chondrocytes in addition to other cytokines through PI3K/Akt signaling pathway [192-436]. MALAT1 directly targets miR-127-5p to inhibit its expression which will rescue OPN proliferation of OA chondrocytes through PI3K /Akt pathway downstream [395]. Furthermore, MALAT1/miR-145 was reported to modulate IL-1ß induced chondrocyte viability and cartilage matrix degradation by regulating MMPs such as ADAMTS5 in human OA [437]. It was reported to be upregulated in IL-1ß induced chondrocytes and positively related to articular cartilage degradation [438]. In addition, miR-145 was reported to modulate cartilage

homeostasis [439] and promoted OA cartilage degradation through TNF-α pathway [440]. It was found that MALAT1 modulates the expression of miR-145 by directly targeting miR-145 in OA articular cartilage and at the same time modulating the miR-145 target ADAMTS5. Together, these data suggest that MALAT1 controls OA articular cartilage ECM degradation which is induced by IL-1 ß through a miR-145/ADAMTS5 axis [255,265,441]. Other reports also indicated MALAT1 regulation of the proliferation of LPS treated articular chondrocytes via the control of miR-146a through PI3K/Akt/ mTOR signaling pathway axis [442] MALAT1 regulates OA progression through targeting the PI3K/Akt/mTOR axis which controls the apoptosis of LPS-treated chondrocytes OA cell model, ECM catabolism and inflammation. As mentioned earlier in this chapter, in articular cartilage MALAT1 regulates chondrocyte proliferation through modulating metabolites of the PI3K/Akt signalling pathway [395] and as with the previously mentioned miRNAs, MALAT1 was reported to reduce the expression of miR-146a leading to the modulation of LPS-treated chondrocytes proliferation. At the same time miR-146a targets PI3K gene and modulates downstream Akt and mTOR genes. These findings indicate a role for MALAT1 in regulating LPS-treated OA chondrocyte's ECM by sponging miR-146a through downstream signaling pathway PI3K/Akt/mTOR axis [359].

7.4 PGE2, Bone Pain and MALAT1 in Osteoporosis

It has previously been reported that PGE2 regulates bone homeostasis and regeneration as well as pain sensation through its action on sensory nerves. Bone tissue is highly innervated by sensory and sympathetic nerves [441]. Catabolic activities in bone, such as regulating the levels of calcium, minerals, glucose [442] and fatty acids are induced by sympathetic innervation through signaling in the hypothalamus [443-445]. Furthermore, it has been shown that osteoblasts secrete greater PGE2 in pathological bone remodelling conditions such as osteoporosis. PGE2 activates the PGE2 receptor 4 (EP4) in sensory nerves and PGE2 was found to regulate bone formation by inhibiting sympathetic activity through central nervous system (CNS) [446]. PGE2 results in pain sensation through its signaling axis PGE2/EP4 and by activating different pain channels via inducing different ions like calcium and sodium through transient receptor ion channels [447]. This was shown by the presence of high levels of PGE2 in vertebral endplates that activated sensory nerves ending in spinal pain through PGE2/EP4 signaling axis, which when blocked, spinal pain was ameliorated [448]. Furthermore, it was reported that cyclooxygenase (COX) and prostaglandin enzymes such as COX-2, controls the production of PGE2 [449-450] and inhibitors of COX-2 (NSAIDs) are widely used in medications to treat musculoskeletal pain [451].

In osteoporosis, bone density decreases and the mechanical load on the surface of the bone changes leading to inducing osteoblasts on the bone surface to produce more PGE2, to modulate bone remodelling and maintain bone mass via sympathetic nerves [452].

Looking at the regulation of osteogenesis in osteoporosis, recent research indicated a role for MALAT1 in the osteogenic capabilities of hBMSCs. MALAT1 modulated the hBMSCs differentiation in osteoporosis by directly targeting miR-96 [453] and miR143 via downstream osteoblast-specific transcription factor gene, Osterix (Osx). However, another group indicated that MALAT1 inhibits BMSCs osteogenesis in osteoporosis via MAPK signaling pathway in rats, in which they found that p38 MAPK and ERK1/2 were higher in the MALAT1 devoid BMSCs [454-455]. This differences in reporting MALAT1 modulation of osteogenesis may be attributed to the fact that MALAT1 is widely expressed in different cell types and controls multiple downstream genes in many different signalling pathways.

Recently, a group of researchers investigated whether there is specific miRNA/ceRNA networks different from miRNA/ceRNA networks that regulated OA. They reported two miRNA/ceRNA networks related to lncRNAs MALAT1 and NEAT1 competing for miRNA hsa-miR-32-3p with down-stream genes SP1 transcription factor (SP1) / Frizzled Class Receptor 6 (FZD6) and targeting miRNA has-miR-22-3p to control PTEN, ESR1, Erb-B2 Receptor Tyrosine Kinase 3 (ERBB3), Colony Stimulating Factor 1 Receptor (CSF1R) and Cyclin Dependent Kinase 6 (CDK6) modulating osteoporosis and not OA [456-457]. Looking at more evidence for the role of MALAT1 in osteoporosis, Xucheng Yang, et al. looked at the exosomes containing MALAT1 from BMSCs in osteoporosis in mice, they proved that MALAT1 in exosomes from BMSCs targeted miR-34c and by this it promoted the expression of the protein- coding Special AT-rich sequence-binding protein 2 Homeobox 2 (SATB2), that encodes a DNA-binding protein involved in regulating gene transcription [458]. Altogether, PGE2 and MALAT1 modulate bone homeostasis by different mechanisms. PGE2 modulates

bone homeostasis by controlling bone metabolic activities and produces pain via sensory and sympathetic innervation, which may be a protective mechanism reflecting bone health. On the other hand, LncRNA MALAT1 via ceRNA's modulate osteogenesis as in the osteoporosis model. In the current project, we were able to show that MALAT1 modulates PTGS2 expression and PGE2 production in OA osteoblasts. One of the limitations of this project was the extraction and purification of RNA, which excluded several miRNAs from analysis in the studies where the effect of MALA1-KD was investigated in osteoblasts. Importantly, it may be useful to use MALAT1 lncRNA as a therapeutic intervention plan to control bone homeostasis and to reduce pain perception in OA and osteoporosis. This will be discussed further at the end of this chapter.

7.5 MALAT1 Regulation of Inflammation in other Fibrotic-based Diseases

Fibrosis, a pathological process which is caused by the disposition of ECM elements occurs in the multiple tissue types and driven by factors including inflammation and infection [459]. Although it is also a tissue repair mechanism in response to injury, uncontrolled it can lead to tissue damage and functional disturbances. It is a very serious problem causing a heavy burden on health system providers world-wide. Accumulating evidence indicates a role for the lncRNA MALAT1 in the pathogenesis of fibrosis in different organs including the heart, kidney, lung and liver by regulating different genes that contribute to the process of fibrosis. MALAT1 causes fibrosis

through different mechanisms including regulating the expression of ECM elements and inflammatory mediators via miRNAs that in-turn activate or inhibit genes in key fibrotic signaling pathways including transforming growth factor-ß1 (TGF- ß1) / SMAD transcription factors (SMAD) and the Wnt/ß-catenin signaling pathways [460-461]. MALAT1 is expressed widely in the body which makes its detection in body fluids and secreted exosomes a potential diagnostic biomarker of fibrosis in different organs.

The general pathological processes by which MALAT1 contributes to the formation of fibrosis in different organs of the body include the maintenance of ECM homeostasis by stimulating the proliferation of fibroblasts via regulation of the cell cycle [462], and the transformation of cells into myofibroblasts, which are capable of secreting ECM elements including Fibrin and collagen. Furthermore, MALAT1 regulates ECM degradation by inhibiting MMP enzymes [463] and mediates epithelial-mesenchymal transformation (EMT) to facilitate the transfer of mediators of the development of fibrosis by controlling several pathways via targeting miRNAs such as, miR126-5p [464], miR-145 [465] and miR-22 [466] which in-turn affect downstream genes in signaling pathways related to EMT.

Given the data in this thesis, and that of other authors, it appears likely that one mechanism by which MALAT1 contributes to fibrosis is through mediating inflammation, through promoting the secretion of proinflammatory factors such as cytokines including TNF-α, IFN-y and TGF-ß, chemokines and trigger immune-inflammatory responses, resulting in the modulation of the phenotype of fibroblasts/myofibroblasts [467].

Myocardial infarction (MI) is one of the major pathologies of the heart leading to death. One of the consequences of MI is activation of cardiac fibroblasts and proliferation of

ECM elements, i.e fibrosis [468]. MALAT1 expression was reported to be upregulated in MI [469]. As mentioned earlier MALAT1 sponges miRNAS and control protein coding genes, here in MI MALAT1 was found to sponge miR-145, activating TGF- ß1 and a pr-protein converting enzyme Furin that is important for myocardial fibrosis process [470]. In the liver, fibrosis is developed from different liver diseases including chronic hepatitis and cancer [471]. Hepatic stellate cells (HSC) get activated due to inflammatory injury and initiates the ECM disposition resulting in fibrosis [472]. MALAT1 was found to activate HSC through (SIRT1) which is a deacetylase that inhibits TGF- ß signaling pathway resulting in promoting fibrosis [473-474]. In the lungs, the alveolar epithelial cells play an important role in fibrosis resulting from different pulmonary diseases such as silicosis [475] caused long exposure to silica or bleomycin [476]. Silica stimulates inflammatory reactions in the pulmonary epithelial cells and macrophages secret huge amount of proinflammatory factors activating fibroblasts which initiate EMT process [477]. MALAT1 induces fibrosis in Silicosis by targeting miR-503 which activates the PI3K/AKT/mTOR/Snail signaling pathway inducing EMT leading to fibrosis [478]. In the kidney, renal fibrosis is the end stage of chronic renal diseases. Inflammatory challenges results in the release of inflammatory mediators affecting the renal interstitial fibroblasts through the TGF- ß1 and ends in accumulating ECM elements initiating fibrosis [479]. MALAT1 induces renal fibrosis through targeting miR-145 which lead to the activation of focal adhesion kinase (FAK) pathway which induces TGF- ß1 related renal fibrosis [480]. Taken together, these data support a central role for MALAT1 in the pathogenesis of fibrosis by different mechanisms including inflammation and ceRNA hypothesis, which may be dependent on the organ and its cellular composition.

7.6 Potential Therapeutic Targeting of lncRNAs

Nucleic acid-based therapeutics are a rapidly growing new class of therapeutic, which are quick to develop due to their relatively benign safety profiles. Furthermore, the ability to target a non-coding RNA has the potential advantage of preventing a multitude of pathogenic processes, rather than trying to neutralize a single functional pathogenic protein. Recently, RNA-based therapeutics have been approved in both cancer and infectious diseases [481-482]. Furthermore, the first miRNA-targeting therapeutic is now in advance stages for the treatment of hepatitis C virus infection providing first clinical proof-of-concept studies for miRNA targeting in therapy, and several oligonucleotide-based therapeutics have evaluated in preclinical models of arthritis [483].

Data obtained from this thesis project and emerging data on lncRNAs provides the possibility of therapeutically targeting lncRNAs, including MALAT1, to regulate the pathological inflammatory response in the joint for the treatment of OA. These axes originate from the proven modulation of the lncRNA MALAT1 of certain cellular functions through biological molecules including MALAT1/DKK1, MALAT1/MIP3a, MALAT1/Galectin1/TNF, PGE2/EP4 and TWEAK/FnF14. MALAT1/DKK1 and the inhibition of the Wnt/ ß-catenin signaling pathway, may influence cellular processes controlled by this axis. In addition, to MALAT1/MIP3a axis that regulates the catabolic processes in OA cartilage. The other axis may be MALAT1/Galectin1/TNF in regulating inflammation in OA. These recommendations need to be further investigated in the hope for looking for a new treatment that cures OA at the molecular levels.

7.7 Final Conclusion

The results of this thesis revealed an abundant expression of MALAT1 in NW and OB OA fibroblasts. Its expression was associated with *in vitro* inflammatory responses to IL-1β and TNF-α across the different primary cells isolated from OA joint tissues from different patient cohort and anatomical joints. Notably, MALAT1 was shown to play an important role in the inflammatory responses in chondrocytes and osteoblasts from OA joints from OA OB/OW cohort compared to NW OA patients. MALAT1 expression in cartilage tissues from different cohorts, was associated with the severity of the OA disease and was significantly associated with the circulatory DKK1, Eotaxin and MIP3a indicating a possible role for MALAT1 in modulating the Wnt/ ß-catenin signaling pathway, and the degradation of OA cartilage.

Interestingly, in bone tissues from different OA cohorts, MALAT1 expression showed a significant correlation with gender being expressed more in male OA than female, and it was highly expressed in taller OA patients and in females with higher central adiposity. Importantly, MALAT1 expression was significantly correlated with the circulatory DKKI, galectin1 and TNF-α indicating a role for MALAT1 mediating immune processes in OA bone via galectin1.

The effects of MALAT1 depletion on the functional phenotype of OA osteoblasts, was shown in this thesis by modulating both PTGS2 expression and PGE2 prostaglandin production. However, the chronic effect of MALAT1-KD showed no noticeable effects on osteogenic differentiation activity, measured either by OPG production, ALP activity or the innate ability of osteoblasts to mineralise. The results of this thesis provide

evidence that the lncRNA MALAT1 is associated with the inflammatory response in multiple cells of the OA joint, is highly expressed in both cartilage and subchondral bone tissue and in OA osteoblasts regulates inflammation and pain by modulation of COX-2 prostaglandin production and regulating PGE2 secretion.

CHAPTER 8

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CHAPTER 9

Appendix

9.1 Appendix to Chapter 2

Table 9.1 Detailed types of samples obtained from different joints.

Joint		Synovium	Cartilage	Bone	Blood	Synovial Fluid
Hip						
No.	Sample					
1	MFX007	✓			✓	✓
2	MFX044	✓			✓	✓
3	MFX087	✓			✓	✓
4	MFX098	✓			✓	✓
5	MFX116	✓			✓	✓
6	MFX148	✓			✓	✓
7	MFX153	✓			✓	✓
8	MFX184	✓			✓	✓
9	MFX185	✓			✓	✓
10	MFX191	✓			✓	✓
11	MFX195	✓			✓	✓
12	MFX205	✓			✓	✓
13	MFX212	✓			✓	✓
14	MFX213	✓			✓	✓
15	MFX216	✓			✓	✓
16	MFX235	✓			✓	✓
17	MFX237	✓			✓	✓
18	ADI131	✓			✓	✓

19	ADI133	✓			✓	✓
20	MFX062	✓	✓		✓	✓
21	RHH021		✓		✓	✓
22	RHH063		✓		✓	✓
23	RHH065		✓		✓	✓
24	RHH094		✓		✓	✓
25	RHH143		✓		✓	✓
26	ADI004		✓		✓	✓
27	ADI029		✓		✓	✓
28	ADI069		✓		✓	✓
29	ADI105		✓		✓	✓
30	ADI141		✓	✓	✓	✓
31	RHH009			✓	✓	✓
32	RHH194			✓	✓	✓
33	JP23			✓	✓	✓
34	MFX230			✓	✓	✓
35	MFX232			✓	✓	✓
36	MFX238			✓	✓	✓
37	MFX299			✓	✓	✓
38	MFX329			✓	✓	✓
	Knee					
No.	**Sample**					
1	RHH003	✓			✓	✓
2	RHH051	✓			✓	✓
3	MFX167	✓			✓	✓
4	MFX227	✓			✓	✓

5	MFX219			✓	✓	✓
6	MFX220			✓	✓	✓
7	MFX236			✓	✓	✓
8	MFX237			✓	✓	✓
9	MFX303			✓	✓	✓
10	RHH008		✓		✓	✓
11	RHH057		✓		✓	✓
12	RHH060		✓		✓	✓
13	RHH062		✓		✓	✓
14	RHH066		✓		✓	✓
15	RHH070		✓		✓	✓
16	RHH077		✓		✓	✓
17	RHH098		✓		✓	✓
18	RHH212		✓		✓	✓
19	RHH197		✓	✓	✓	✓
20	RHH194		✓	✓	✓	✓
21	MFX229		✓	✓	✓	✓
22	MFX306		✓	✓	✓	✓
23	MFX329		✓	✓	✓	✓
	Hand					
No.	**Sample**					
1	RHH001	✓			✓	✓
2	RHH002	✓			✓	✓
3	MFX224	✓		✓	✓	✓
4	MFX327			✓	✓	✓
5	MFX230			✓	✓	✓

Wrist					
No.	**Sample**				
1	MFX225	✔	✔	✔	✔
2	MFX291		✔	✔	✔
Foot					
No.	**Sample**				
1	MFX218		✔	✔	✔
2	MFX223		✔	✔	✔
3	MFX228		✔	✔	✔
4	MFX267		✔	✔	✔
5	MFX291		✔	✔	✔
6	MFX379		✔	✔	✔
Toe					
No.	**Sample**				
1	MFX221		✔	✔	✔
Spine					
No.	**Sample**				
1	RHH081		✔	✔	✔

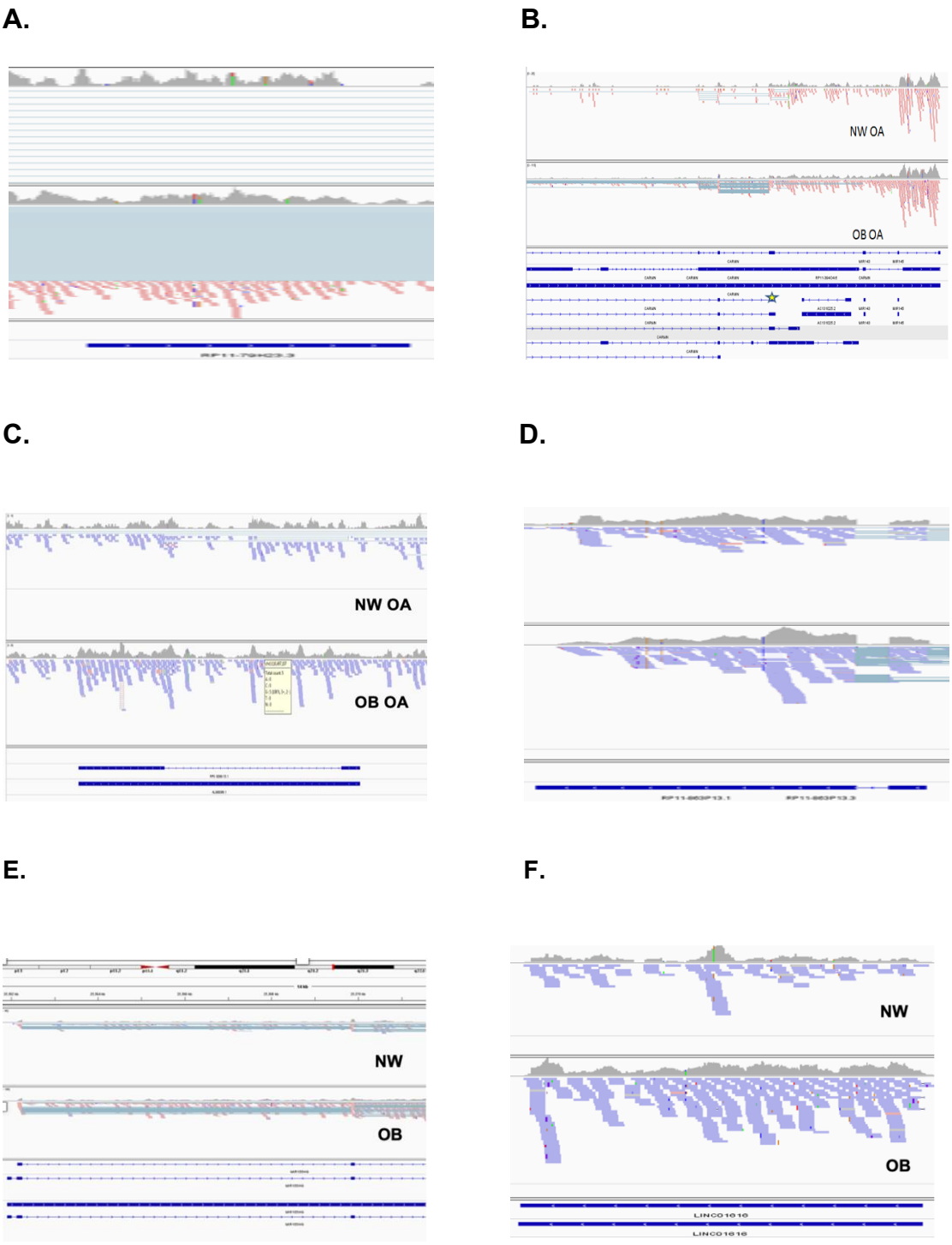
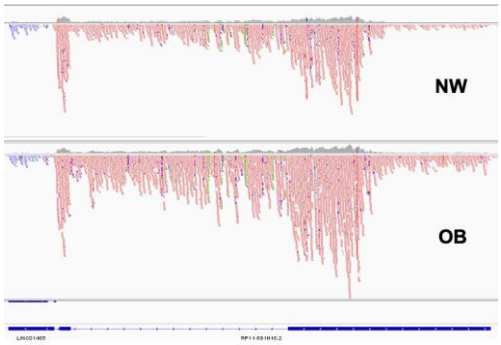
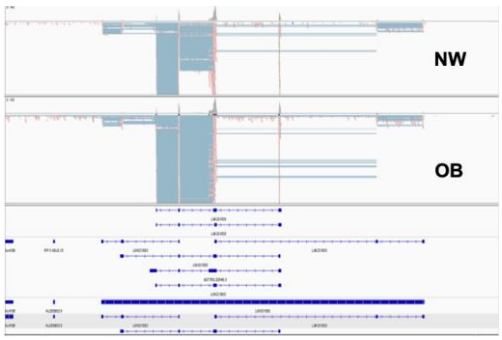


Fig 9.1.I IGV LncRNAs in OA synovial fibroblasts(A) RP11-79H23.3 highly expressed lncRNA fold change 3.01, *P*< 0.0002. **(B)** RP11-362F19.1 highly expressed lncRNA fold change 2.24, *P*< 0.01. **(C)**RP5-1086K13.1 highly expressed lncRNA fold change 1.87, *p*<0.013. **(D)** RP11-863P13.3 highly expressed lncRNA fold change 1.76, *p*< 0.013. **(E)** MIR155HG highly expressed lncRNA fold change 1.64, *p*<0.02. **(F)** LINC01616 highly expressed lncRNA fold change 1.64, *p*<0.02.

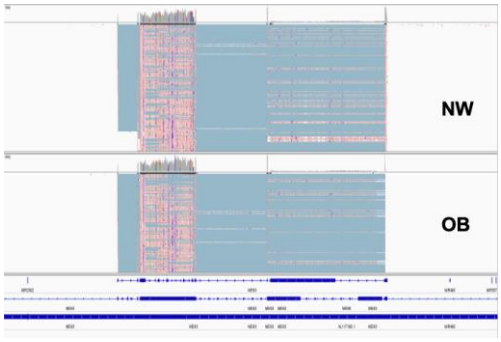
A.



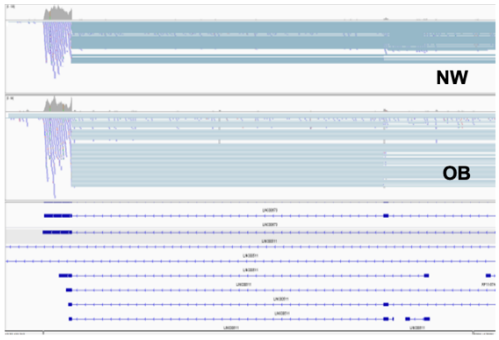
B.



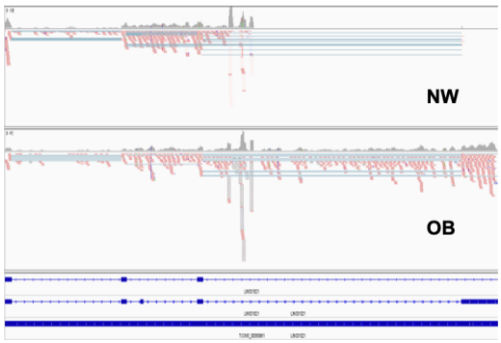
C.



D.



E.



F.

G.

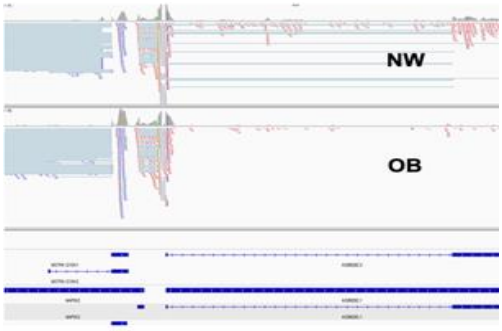


Fig 9.1.II IGV LncRNAs in OA synovial fibroblasts (A) RP1-631N16.2 highly expressed lncRNA fold change 0.86, *p*< 0.004. **(B)** LINC01503 highly expressed lncRNA fold change 0.76, *p*<0.03. **(C)** MEG3 highly expressed lncRNA fold change 0.59, *p*<0.024. **(D)** LINC00511 highly expressed lncRNA fold change - 0.77, *p*<0.036. **(E)** LINC01021 highly expressed lncRNA fold change - 1.43, *p*<0.01. **(F)** RP11-392O17.1 highly expressed lncRNA fold change - 1.67, *p*<0.04. **(G)** AC068282. 3 highly expressed lncRNA fold change – 3.46, *p*<0.007.

Table 9.2 Significant LncRNA Details.

Gene Name	Chromosome Locus
RP11-79H23.3	chr8:7974976479752757
RP11-362F19.1	chr4:141319449-141332618
CARMN	chr5:149406688-149432835
RP5-1086K13.1	chr1:116493015-116499212
RP11-863P13.3	chr16:8817729888186929
LINC01123	chr2:109,986,938-109,996,140
MIR155HG	chr21:25561908-25575168
LINC01616	chr11:2998011329982392
MALAT1	chr11:65497761-65506516
RP11-631N16.2	chr12:6260275262622213
LINC01503	chr9:129332299-129359538
MEG3	chr14:100779409-101027415
LINC00511	chr17:72323122-72640472
LINC01021	chr5:27472291-27496401
RP11-147L13.15	chr17:6820548968207493
RP11-392O17.1	chr1:219409522-219459369
RP11-367F2.32	chr9:9099694991002198
AF131217.1	chr21:28439345-28674848
LINC01705	chr1:222041704-222064763
AC068282.3	chr2:127389129-127400580

Table 9.3 LncRNAs Role in Different Diseases (Article Review) (A-C)

A.

LncRNA	Organism	Expression	Role in Vivo	Role in Vitro/Function	Refernce
2-TTTY14	1- Human	Gastric tissues	Role not known	Identified as a lncRNA signature in Gastric cancer(GC) with another three lncRNAs (LINC01018, LOC553137, and MIR44352HG), as a new candidate indicator with the potential to predict the survival in GC patients.	Miao Y, Sui J, Xu SY, Liang GY, Pu YP, Yin LH. Comprehensive analysis of a novel four-lncRNA signature as a prognostic biomarker for human gastric cancer. Oncotarget. 2017 Aug 24;8(43):75007-75024. doi: 10.18632/oncotarget.20496. eCollection 2017 Sep 26.
	2-Human	Oral squamous cell carcinoma		Influnce the survival rate of Oral Squamous Cell Carcinoma/ Expressed more in Prostate and stomach.	Li S, Chen X, Liu X, Yu Y, Pan H, Haak R, Schmidt J, Ziebolz D, Schmalz G. Complex integrated analysis of lncRNAs-miRNAs-mRNAs in oral squamous cell carcinoma. Oral Oncol. 2017 Oct;73:1-9.
3- LINC01123	1- Human	Prostate	Role not known	Part of the four LncRNA signiture for Prosate cancer/Prostate cancer signiture	Alessia Cimadamore, Silvia Gasparrini, Roberta Mazzucchelli, Andrea Doria,Liang Cheng, Antonio Lopez-Beltran,Matteo Santoni, Marina Scarpelli, and Rodolfo Montironi. Long Non-coding RNAs in Prostate Cancer with Emphasis on Second Chromosome Locus Associated with Prostate-1 Expression. Front Oncol. 2017; 7: 305.
	2- Human	Bile duct	Role not known	Upregulated in intrahepatic cholangiocarcinoma (iCCA)	Wenhui Yang, Yuan Li, Xia Song, Jun Xu,and Jun Xie. Genome-wide analysis of long noncoding RNA and mRNA co-expression profile in intrahepatic cholangiocarcinoma tissue by RNA sequencing. Oncotarget. 2017 Apr 18; 8(16): 26591–26599.

B.

LncRNA	Organism	Expression	Role in Vivo/ Function	Role in Vitro / Function	Refernce
6- LINC00511	1- Mouse	1- Lung cell lines (NCSLC) 2- Lung normal epithelial cell lines (16HBE). 3- BALB/cnude mouse xenograft model.	By repressing cell proliferation causing growth delay, and mediating apoptosis / Knockdown of INC00511 reduces tumorigensis and increase survival.	1- It acts as a ceRNA for miR-377-3p, leading to de-suppression of E2F3, miR-377-3p, a central oncogene in facilitating NSCLC progression. 2,3- By epigenetical suppression of p57. / function: 1- Oncogenic in NSCLC . 2- Facilitate cell growth, migration and invasion. 3-Repress cell apoptosis	Cheng-Cao Sun, Shu-Jun Li, Guang Li, Rui-Xi Hua, Xu-Hong Zhou, and De-Jia Li. Long Intergenic Noncoding RNA 00511 Acts as an Oncogene in Non-small-cell Lung Cancer by Binding to EZH2 and Suppressing p57. Mol Ther Nucleic Acids. 2016 Nov; 5(11): e385.
	2- Human	Tca-8113 cell line (tongue squamous cell carcinoma TSCC)		LINC00511 songe miR-765 and causing the depression of laminin subunit gamma 2 (LAMC2), inhibitting TSCC cells proliferation, invasion and cell cycle distribution. It regulates TSCC progression	Ding J, Yang C, Yang S. LINC00511 interacts with miR-765 and modulates tongue squamous cell carcinoma progression by targeting LAMC2. J Oral Pathol Med. 2018 Jan 9. doi: 10.1111/jop.12677.
	3- Human	Breast tissues and cell lines (MDA-MB-453, MCF-7).		LINC00511 associated with triple three type of breast cancer and the mechanism of oncogensess is by gene amplification. Oncogene role in breast cancer	Xu S, Kong D, Chen Q, Ping Y, Pang D Oncogenic long noncoding RNA landscape in breast cancer. Molecular Cancer. 2017 Jul 24;16(1):129. doi: 10.1186/s12943-017-0696-6.

C.

LncRNA	Organism	Expression	Role in Vivo/ Function	Role in Vitro / Function	Refernce
7- CARMN	Human	Heart Tissue		Super inhancer associated LncRNA controls some of cardiac functions.	CARMEN, a human super enhancer-associated long noncoding RNA. Journal of Molecular and Cellular Cardiology Volume 89, Part A, December 2015, Pages 98-112
8-LINC01021	Human	Colorectal tissue	P53 upregulated regulator of p53 levels, called PURPL/ p53 target gene		Hunten S, Kaller M, Drepper F, et al.p53-Regualted networks of protein, mRNA, miRNA, and lncRNA expression revealed by integrated pulsed stable isotope labelling with amino acids in cell culture (pSILAC) and next generation sequencing (NGS) analyses. Mol Cell Proteomics. 2015 Oct;14(10):2609-29.
9- LINC00599	Human	Oral squamous cell carcinoma		Found ultered in mucosal cells of the patients with oral squamous cell carcinoma	Morandi L, Gissi D, Tarsitano A, et al. CpG location and methylation level are curcial factors for the early detection of oral squamous cell carcinoma in brushing samples using bisulfite sequencing of a 13-gene panel. Clin Epigenetics. 2017 Aug 15;9:85.
10- MIR3142HG	Human	lymph nods		Induced to microbial infection and proinflammatory cytokines.	Taganov KD, Boldin MP, Chang KJ, et al. NF-kappaB-dependent induction of microRNA miR-146, an inhibitor targeted to signalling proteins of innate immune responses. Proc Natl Acad Sci USA. 2006 Aug 15;103(33):12481-6.
11- SMILR				Smooth muscle induced, inhancer of proliferation	Ballantyne MD, Pinel K, Dakin R, et al. Smooth muscle enriched long noncoding RNA(SMILR) regulated cell proliferation. Circulation. 2016 May 24;133(21):2050-65.

Table 9.4 MALAT1 (Article Review) (A-D)

A.

Organism	Expression (tissue)	In Vivo Data		In Vitro Data		Reference
		Mechanism of action	Function	Mechanism of action	Function	
1- Rats	Cardiac	Activate P38 MAPK/NFkB	Induce dysfunction & inflammation			1-
				Upregulation of pro-apoptotic (caspase3, Bim,Bax) and Proinflammatory cytokines (NF-κB ,E-selectin, MCP-1 and IL-6)		2-
2- Mouse	Endothelial cells				Proapoptosis & Proinflammatory	
3- Human	Liver			Chemokines CXCL5	Steatohepatitis & Fibrosis	3-
4- Human	Bronchial cell lines			Down-regulation of E-cadherin, up-regulation of N-cadherin Interact with EZH2 in CMVECs	Associated with Epithelial-Mesenchymal transition	4-
					Induces permeability and apoptosis of (LPS) in (CMVECs)	5-
5- Rats	Cardiac			By serum Amyloid antigen 3(SAA3)	Increases cytokines (TNFα, IL-6) in response to LPS treatment	6-
6- Mouse	cardiac			Induce expression of the proapoptotic(Bim), proinflammatory cytokines (MCP-1, IL-6, and E-selectin)	Reduce ischemic cerebral vascular and parenchymal damages.	7-
7- Mouse	Brain (BMECs)					
				Inhibits NF-jB DNA biniding decreasing the production of inflamatory cytokines (IL-6, TNFα)	Autonegative feedback regulator of NF-jB in innate immune responses	8-
8- Human cell lines	THP1 macrophages					
	Umblical vein endothelial cells				Regulates glucose - induced upregulation of inflammatory mediators (IL-6, TNFα)	9-
9- Human 10- STZ induced diabetic Rats & db/db mice		Regulates endothelial cell functions by crosstalk between MALAT1 and MAPK pathway.	Regulates diabetic induced microvascular functions	Through activation of SAA3		
	Retinal tissues					10-

B.

11- Human & mice	Bladder cancer cells	By MALAT1/SUZ 12 pathway	Promotes bladder cancer metastasis	MALAT1 is associated with suppressor zeste 12 (suz12), which decreases E-cadherin expression and increase N-cadherin and fibronectin expression., inducing EMT by TGF- β.	Mediator of TGF- β induced Epithelial -Mesenchymal Transition (EMT).	11-
12- Human	Bladder cancer cells	By miR-200 targeting MALAT1 in a reciprocal way controlling the proliferation, invasion, metastases, polarization, actin filaments formation, and EMT in EEC cells.	MALAT1/miR-200c sponge regulates invasion and migration of Endometrioid Endometrial carcinoma. (EEC)	By activating Wnt signaling pathway through increasing EMT-associated ZEB1, ZEB2 and Slug levels, and decrease E-cadherin levels.	Tumor metastasis by promoting EMT process	12-
13- Human cell lines ??	Xenograft-tumor model			BY CXCL5 chemokine (downstream gene for MALAT1)		13-
14- Human	Lung cells			By MALAT1 mediated FGF2 protein secretion from Tumor associated macrophages (TAMs).	Regulates the migration & invasion of NSCLC.	14-
15- Human	Thyroid cancer tissues and cells			Through transcriptional and post-transcriptional regulation of motility related gene expression (CTHRC1, CCT4, HMMR, ROD1).	Inhibits inflammatory cytokines release, promotes proliferation, migration, and invasion of FTC133 cells and induces vasculature formation.	15-
16- Human	Lung cells			By upstream regulator of Snail gene expression & increases the effects of tumor associated dendertic-cell mediated colon cancer(TADC-CM) and CCL5 on cell migration, invasion, and EMT	Promotes cell motility of lung adenocarcinoma cells.	16-
17- Human	Colon cancer cell line SW480 and SW620				Plays important role in colon cancer growth and metastasis	17-

C.

18- Human	Breast cancer cell lines (MCF-7 and MDA-MB-231)			By binding to CD133 (PROM1, a marker of cancer stem cells, which facilitates EMT in various cancer promoter region to control its expression).	Regulates CD133 (marker for cancer stem cells).
19- Human-	Circulating endothelial progenitor cells (EPCs).			Through regulation of TGFBR2 and SMAD3 pathways by miR-145 (tumor suppressor gene).	MALAT1 modulates TGF-β1-induced EndMT of EPCs
20- STZ-induced diabetic Rates & Human	Rat's kidney cortex / high glucose-treated HK-2 human proximal tubular epithelial cells.	By regulating miR-23c which controls *ELAVL1 gene expression of the protein NLRP3*.	MALAT1 regulates miR-23c role in diabetic nephropathy.	MALAT1 acts as an endogenous sponge of miR-23c (small regulatory RNA) by downregulating it which removes the inhibition on its target gene *ELAVL1*, increasing its expression which leads to pyroptosis by inducing the downstream protein NLRP3	Pro-pyroptotic role for MALAT1 in the pathogensis of diabetic nephropathy.
21- Human	OA Chondrocytes			By regulating Osteopontin mediated chondrocyte proliferation through controlling the levels of Osteopontin and PI3kK/Akt pathways downstream.	MALAT1 acts as a sponge to miR-127-5p during OA progression.
22- Human	RA Fibroblast like synoviocytes (RAFLS)			By inhibiting the activation of PI3K/AKT pathway By regulating the ERK/mitogen-activated protein kinase signaling pathway.	MALAT1 induces apoptosis
23- Human	Normal and silenced fibroblasts.	Skin			MALAT1 may participate in UVB-induced photo-aging of fibroblasts.

D.

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9.2 Appendix to Chapter 4

9.2.1 Optimisation of Synovial Fibroblast Proliferation

It was decided to determine optimal seeding density and time course using MTS reagents to assess cellular metabolic activity as a marker of cell number. We first established a standard curve for synovial fibroblasts using MTS reagent. Synovial fibroblasts were seeded as 500, 1000, 2000, 4000, 8000 cells/well in a 96-well plate. After 24 hr absorbance was read at 490 nm on a plate-reader. The standard curve was generated and found to be cell number = (Absorbance x 24998) + 3000. Following the standard curve, fibroblasts were seeded at 1000, 5000, 10000, 150000, and 20000 cells/well and cultured for 1, 2 or 3 days in serum free fibroblast media. At each time-point absorbance was read at 490 nm on a plate-reader as before. For each seeding density and time-point, n=3 replicate wells were seeded, as well as a negative control containing media and the MTS reagent only. Analysis of the cell viability test was done by calculating the actual absorbance by subtracting the absorbance of the sample from the absorbance of the control.

9.2.2 Comparison of Cellular Proliferation in Different BMI of OA Synovial Fibroblast in MTS Analysis

We then investigated whether synovial fibroblasts isolated from OA patients of varying BMI exhibited different proliferative rates. To this end, five patient synovial fibroblast primary cell cultures were utilised, including one NW, two OB, and 2 OW were selected

for the MTS analysis. Cultured cells reaching 70-80 % confluency was trypsinised and seeded into a 96 well plate with two different concentrations of cells: 5000, and 10000 cells per well, in fresh fibroblast growth media and incubated at 37 ^{0}C, and 5 % CO_2 overnight. The next day cells attachment to the surface of the plate, 20 µl of the pre-warmed MTS reagent was diluted with serum free fibroblast media, added to each well and the plate was returned to the incubator. After 2, and 4 hr the reading was taken at 490 nm absorbance from the plates by a plate reader. Demographic data for these cell lines is presented in (Table 9.5).

Table 9.5 Demographic data for OA patient's synovial fibroblast samples.

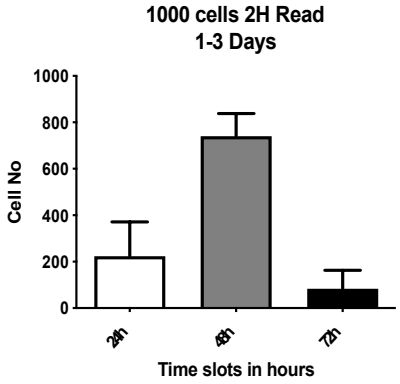
Sample	BMI	Joint	Gender	Age (years)	WHR
NW-1 RHH002	NW	Hand	M	53	0.915
OW-1 MFX098	OW	Knee	F	79	0.893
OW-2 MFX185	OW	Hip	M	78	0.97
OB-1 MFX062	OB	Hip	M	82	0.992
OB-2 MFX212	OB	Hip	F	72	0.902

NW = Normal weight, OB = Obese, M = Male, F = Female, WHR = Waist/ hip ratio.

9.2.3 Optimisation of MTS Experimental Conditions

Cellular proliferation at density of 1000 cells/well increased by approximately three folds in day 2 in comparison to day one and decreased by approximately four folds on the third day (Figure 9.3A). On the other hand, cellular proliferation continued to increase at different incubation times from 1-3 days at the rest of the concentrations used in this study (5000, 10000, 15000, and 20000) cells/well (Figure 9.3). Comparing between the different time of incubation, cell concentrations of 5000 and 10000 showed a distinctive increasing rate in cell proliferation from 1-3 days (Figure 9.3.B,C). These 2 cell concentrations were used as standards to run an MTS cell viability test in comparing NW, OB, and OW OA synovial fibroblast cell lines.

A.



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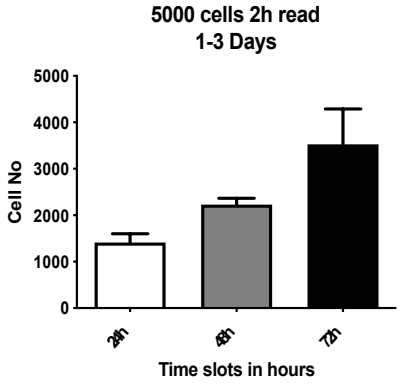

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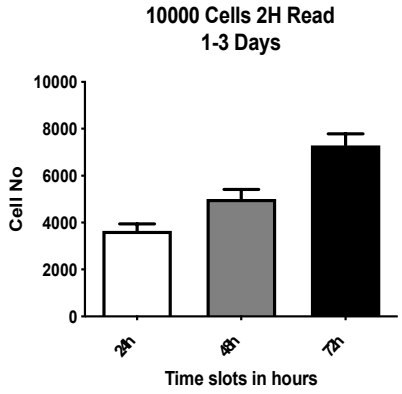
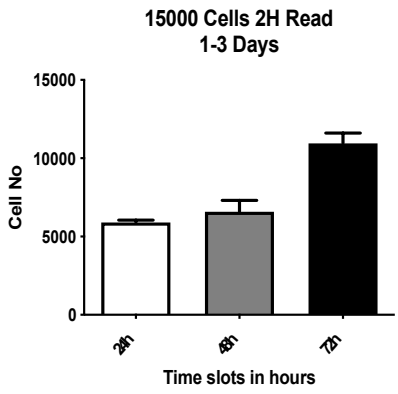


D.



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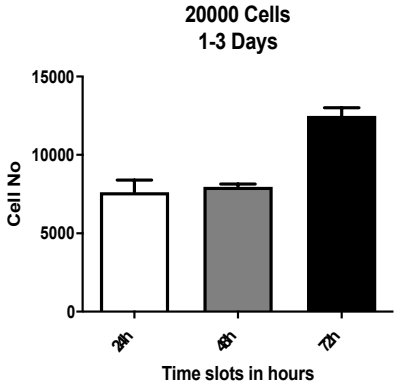


Fig. 9.3 (A-E) Optimisation of experimental of MTS conditions. The effect of obese OA synovial fibroblast, seeding densities on the time-course of proliferation. Bars represent mean ± SEM.

9.3.4 Comparison of cellular proliferation in Different BMI of OA Synovial Fibroblast

A significant difference between the mean of OA synovial fibroblast proliferation for 10 days was seen in different BMI, $p<0.0001$ seeded with different cell numbers 5000 and 10000 cells/well. All synovial fibroblasts cohort showed significant difference in the mean of cellular proliferation at the third and sixth days, $p<0.0001$ in all conditions except 10000 cell/well density of NW hand and OB hip synovial fibroblasts, $p<0.01$, and $p<0.05$ respectively. Table 9.6 shows the details of synovial fibroblasts proliferation for 10 days for the different cohorts.

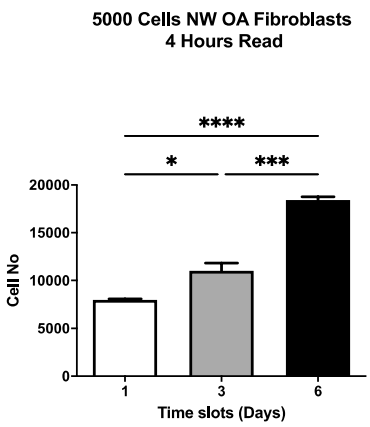
Table App.9.6 Significant difference of mean OA synovial fibroblasts cohort's proliferation after 4 hours for 10 days

Cell Viability	5000 Cells/well					10000 Cells/Well				
	NW	OW		OB		NW	OW		OB	
	1	1	2	1	2	1	1	2	1	2
All groups	***0.0001***	***0.0003***	***0.0004***	***0.0001***	***0.004***	***0.002***	***0.002***	***0.0001***	***0.0001***	***0.008***
Days:1&3	***0.01***	NS	NS	NS	NS	NS	NS	***0.01***	NS	NS
Days:1&6	***0.0001***	***0.0007***	***0.001***	***0.00001***	NS	***0.002***	***0.002***	***0.0001***	***0.0001***	***0.009***
Days:3&6	***0.0002***	***0.0006***	***0.0006***	***0.00001***	***0.001***	***0.03***	***0.01***	***0.0001***	***0.0002***	***0.04***

NW = Normal weight, OW = Overweight, OB = Obese.

OA Synovial fibroblasts seeded at 10000 cell/well showed a significant rate of proliferation for 6 days in different cohorts. Figure App.4.2 shows details of the proliferation of synovial fibroblasts seeded at 5000 and 10000 cells/well in NW (Figure 9.4.A,B), OW (Figure 9.4.C-F) and OB (Figure .9.4.G-J).

A.



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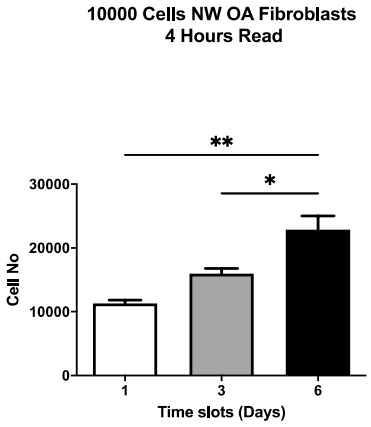

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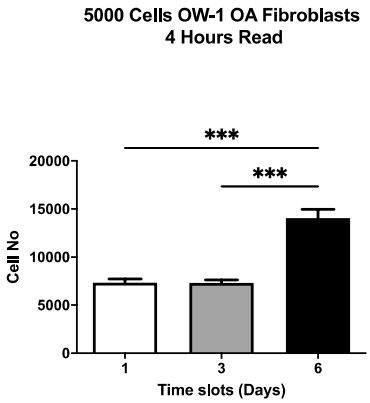
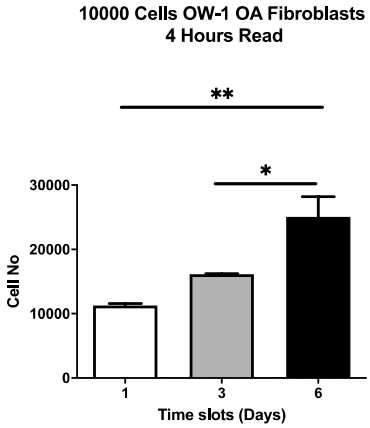
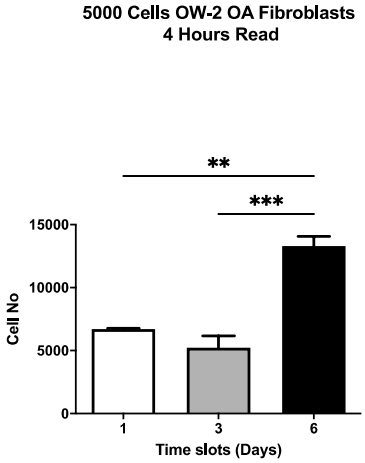

5000 Cells OW-1 OA Fibroblasts
4 Hours Read
***
***
20000
15000
10000
5000
0
Cell No
1
3
6
Time slots (Days)

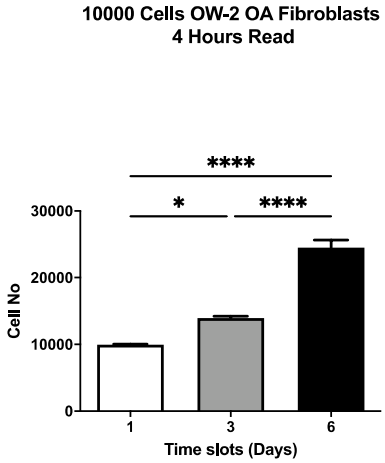
D.


10000 Cells OW-1 OA Fibroblasts
4 Hours Read
**
*
30000
20000
10000
0
Cell No
1
3
6
Time slots (Days)

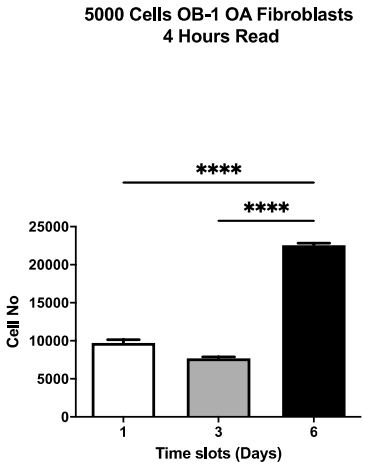
E.


5000 Cells OW-2 OA Fibroblasts
4 Hours Read
**
***
15000
10000
5000
0
Cell No
1
3
6
Time slots (Days)

F.


10000 Cells OW-2 OA Fibroblasts
4 Hours Read
****
*
****
30000
20000
10000
0
Cell No
1
3
6
Time slots (Days)

G.



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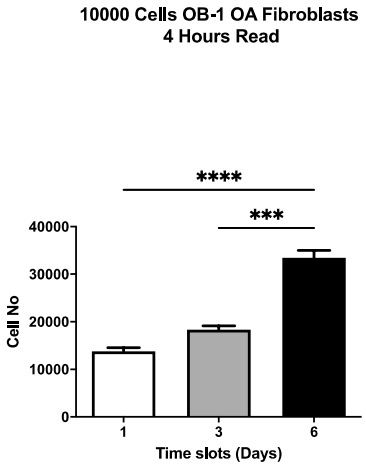
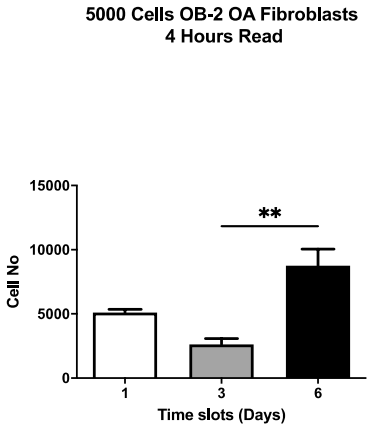


I.



J.

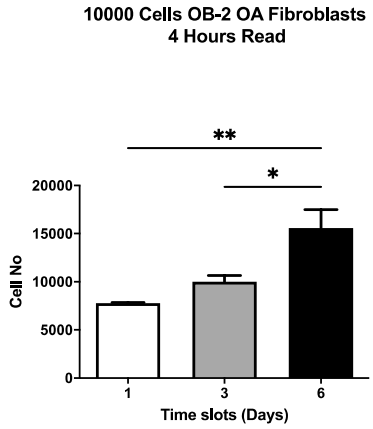


Fig. App.9.4 Cellular proliferation of five OA synovial fibroblast with different BMI cohorts: NW, OW and OB at 5000 and 10000 cells per well at days 1,3 and 6. Bars represent mean ± SEM.

9.3 Appendix to Chapter 6

9.3.1 MALAT1 knockdown in OA chondrocytes

MALAT1-KD was optimized first using different lipid delivering systems, LNA concentration and incubation timing. A concentration of 30uM of LNA was best used with Lipofectamine 3000 and incubated for 24 h for the maximum knockdown. Primary chondrocytes from OA OB knee joints showed more knock down than in primary chondrocytes from OA NW hip joints (Figure 9.5.A&B).

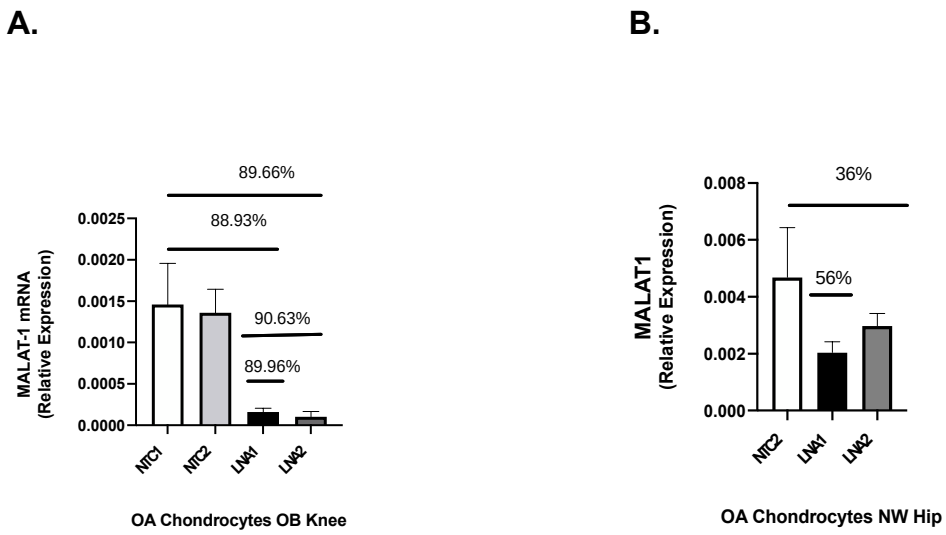


Figure App.9.5 MALAT1-KD in OA primary chondrocytes. (A) MALAT1-KD in chondrocytes from OA OB knee using Lipofectamine 3000, **(B)** MALAT1-KD in chondrocytes from OA NW hip using Lipofectamine 3000. NTC1 & NTC2 = Non target control 1 & 2, LNA1= siRNA-LNA1 and LNA2 = siRNA-LNA2.

9.4 Future planes

For future planes, I would like to complete a review article which I have started already on OA therapeutics and would like to write an article on MALAT1 Modulation of DKK1 in OA. In addition, if I get the opportunity, I would like to investigate the rest of the top upregulated and down regulated genes in MALAT1-KD OA osteoblasts, which we could not finish due to limitation of time for this thesis project and the delays due to COVID-19 in reagents and PCR plates.

9.5 Publication Extracted from This Project

I. Publications

1. **Fawzeyah A Alnajjar**, Edward T Davis, Simon W Jones. *"*The expression and function of Metastases Associated Lung Adenocarcinoma Transcript-1 long non-coding RNA in subchondral bone and osteoblasts in osteoarthritis patients*." Abstract submitted (November 2020) to Osteoarthritis research society international world congress (OARSI)* **2021***.*

2. **Fawzeyah A Alnajjar**, Archana Sharma-Oates, Susanne N Wijesinghe, et al. The expression and function of Metastasis associated Lung Adenocarcinoma transcript-1 long non-coding RNA in Subchondral bone and osteoblasts from patients with osteoarthritis. Cells 2021 Apr 1; 10;4:786. doi.org/ 10.3390 /ceels10040786.

II. Poster

1. Fawzeyah A Q Alnajjar, Simon W Jones. The role of MALAT1 in the pathogenesis of osteoarthritis. *"The role of MALAT1 Long non-coding RNA in the pathogenesis of osteoarthritis".* Poster day, College of Medicine and Dental Sciences, University of Birmingham, UK, **April 2019.**

2. Fawzeyah A Q Alnajjar, Edward T Davis, Simon W Jones. *"The expression and function of Metastases Associated Lung Adenocarcinoma Transcript-1 long non-coding RNA in subchondral bone and osteoblasts in osteoarthritis patients."* Poster to be submitted to *OARSI*, **2021**.

III. Oral Presentations

1. *The role of obesity and novel non-coding RNAs in mediating the inflammatory synovial fibroblast phenotype in OA patients*. Presented to The Ageing and Trauma Research Group, Institute of Inflammation and ageing, College of Medicine and Dental Sciences, University of Birmingham, UK, October **2018**.

2. *The Role of MALAT-1 LncRNA in Osteoarthritis*. Presented to The Ageing and Trauma Research Group, Institute of Inflammation and ageing, College of Medicine and Dental Sciences, University of Birmingham, UK, May **2019**.

3. *The role of LncRNA MALAT-1 in regulating the inflammatory pathways in osteoarthritis.* Presented to The Ageing and Trauma Research Group, Institute of Inflammation and ageing, College of Medicine and Dental Sciences, University of Birmingham, UK, October **2019.**

4. *The expression of selected LncRNA in IL1β stimulated synovial fibroblasts (IGV analysis).* Presented to The Musculoskeletal inflammation Research Group, Institute of Inflammation and ageing, College of Medicine and Dental Sciences, University of Birmingham, UK, May **2018**.

5. *IL6 induction in IL1β stimulated OA hip, knee, hand, wrist, foot, and toe fibroblasts, chondrocytes, and osteoblasts.* Presented to The Musculoskeletal inflammation Research Group, Institute of Inflammation and ageing, College of Medicine and Dental Sciences, University of Birmingham, UK, July **2019**.