Investigation of Inflammatory Mechanisms in Models of Osteoarthritic Pain

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ABSTRACT

Background： Osteoarthritis (OA) is a highly prevalent joint degenerative disorder among the older population. The main symptoms of OA are chronic pain, swelling and stiffness of joint. OA histopathology is characterized by cartilage damage, synovial inflammation and remodelling of subchondral bone. Resolvins are endogenous lipid mediators produced from Ω-3 poly-unsaturated fatty acids (PUFAs) during resolution of inflammation. The main biological functions of resolvins include anti-inflammation and resolution of inflammation. Currently, the emerging anti-nociceptive roles of some resolvins have been reported in various models of pain. However, roles of resolvins and the resolvin receptor system on osteoarthritic pain are unknown.

Objectives: This thesis assesses the therapeutic potential of a resolvin precursor on OA pain and investigates the underlying mechanisms of action and resolvin receptor system in OA.

Methods： Monosodium iodoacetate (MIA) and medial meniscus transection (MNX) -induced joint damage was used as models of OA pain. 17(R)-HDoHE (300ng/300µl) or vehicle (1% ethanol in saline, 300µl) was acutely or chronically administered at day 14 post model induction and pain behaviour was measured to determine the analgesic effects of the drug in these models. Haematoxylin and eosin (H&E) staining was used to assess joint histopathology. Gene expression of resolvin receptors, inflammatory cytokines and metabolic enzymes were measured by reverse transcription quantitative polymerase chain reaction (RT-qPCR) in associated tissues from the models and human OA samples.

Results: Pain behaviour and joint histopathology were established in both the MIA and MNX models. Expression of chemokine-like receptor 1(ChemR23) was lower in the synovia and higher in the spinal cord in the MIA model. 15-lipoxygenase (15-LOX) was expressed at a lower level in both synovia and spinal cord in the MIA model. Negative correlations were revealed between synovial ChemR23 expression and pain behaviour at both day 14 and 35 in the MIA model. ChemR23 expression in the spinal cord was positively correlated with pain behaviour at day 35 in the MIA model.

Expression of formyl peptide receptor 2 (ALX), some inflammatory cytokines and metabolic enzymes was lower in the synovia in the MNX model but expression of 5-lipoxygenase-activating protein (FLAP) was higher. Expression of ALX in the synovia

was positively correlated with tumor necrosis factor alfa (TNFα), interleukin 1 beta (IL1β) and cyclooxygenase 2(COX2) but negatively correlated with 5-LOX expression in the MIA model. Expression of ALX in the spinal cord was positively correlated with pain behaviour at day 14 but then the converse was true at day 35. Expression of ALX in the spinal cord was negatively correlated with IL6 in the MIA model.

17(R)HDoHE attenuated pain behaviour in both the MIA and MNX models following acute, chronic and discontinuous administration. Effects of acute administration of 17(R)HDoHE on pain behaviour were associated with an up-regulation in the expression of IL6 and decreased 5-LOX expression in the synovia of MIA model. A trend towards down-regulation of pro-inflammatory cytokines and associated enzymes by 17(R)HDoHE was observed in the acute study in the MIA model. Repeated administration of 17(R)HDoHE produced robust and sustained inhibitory effects on pain behaviour, but no change in joint histopathology. Pain behaviour was attenuated when 17(R)HDoHE was administered but returned to levels seen in vehicle treated rats after 7 days after drug cessation.

In human OA samples, expression of ChemR23 was significantly higher than expression of ALX in both synovia and medial tibial plateau. ChemR23 expression was positively correlated with expression of 5-LOX in both synovia and medial tibial plateau and negatively correlated with 15-LOX2 expression in the medial tibial plateau from OA patients. There was a significantly positive correlation between ChemR23 expression and IL6 and 15-LOX1 expression in the medial tibial plateau. In addition, there was a significantly positive correlation between ALX and IL6 and 15-LOX1 expression in both synovia and medial tibial plateau. Expression of ALX, TNFα, IL6, COX2 and 5-LOX in the medial tibial plateau from OA patients was lower, compared to expression in bone from femoral heads obtained from trauma patients.

Conclusions: These findings support anti-nociceptive and anti-inflammatory roles of resolvins and provide evidence that resolvins may be potential novel drugs to treat OA pain.

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PUBLICATIONS AND PRESENTATIONS

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ABBREVIATIONS (Alphabetic)

18-HpEPE	18R-hydroperoxy-EPE
5-HT	Serotonin
18R/S-HEPE	18R/S-hydroxy-5Z, 8Z, 11Z, 14Z, 16E-eicosapentaenoic acid,
17(R) HDoHE	17R-hydroxy-4Z, 7Z, 10Z, 13Z, 15E, 19Z-docosahexaenoic acid
17S-HDHA	17S-hydroxy-4Z, 7Z, 10Z, 13Z, 15E, 19Z-docosahexaenoic acid
ACC	Anterior cingulate cortex
ACL	Anterior cruciate ligament
ADAMs	A disintegrin and metalloproteinases
ADAMTS	A disintegrin and metalloproteinase with thrombospondin motifs
AIA	Adjuvant-induced arthritis
ALX	Formyl peptide receptor 2
ASICs	Acid sensing ion channels
ATF3	Activating transcription factor-3
ATP	Adenosine triphosphate
AT-RvD	Aspirin triggered D-series resolvin
BDNF	Brain derived neurotrophic factor
BLT1	Leukotriene B4 receptor
BMI	Body mass index
BMPs	Bone morphogenetic proteins
CCL	Chemokine (C-C motif) ligand
CAMKII	Calcium/calmodulin-dependent protein kinase II
cAMP	Cyclic adenosine monophosphate
CCI	Chronic constriction injury
CD	Cluster of differentiation
CFA	Complete Freud’s adjuvant
CGRP	Calcium gene-related peptide
ChemR23	Chemokine-like receptor 1

COX	Cyclooxygenases
CREB	cAMP response element binding protein
CRP	C-reactive protein
CXCL	Chemokine (C-X-C motif) ligand
CYP2J2	Cytochrome P450, family 2, subfamily J, polypeptide 2
DAMPs	Damage-associated molecular patterns
DHA	Docosahexaenoic acid
DMM	Destabilisation of the medial meniscus
DRG	Dorsal root ganglia
EPA	Eicosapentaenoic acid
EPHX2	Epoxide hydrolase 2
ERKs	Extracellular-regulated kinases
FLAP	5-lipoxygenase-activating protein
GABA	Gamma-aminobutyric acid
GAP43	Growth associated protein-43
GFAP	Glial fibrillary acidic protein
GPCRs	G protein-coupled receptors
H&E	Haematoxylin and eosin
IL	Interleukin
iNOS	Inducible nitric oxide synthase
LOX	Lipoxygenase
LPS	Lipopolysaccharide
LTB4	Leukotriene B4
LTP	Long-term potential
MAPK	Mitogen-activated protein kinases
MIA	Monosodium iodoacetate
MCP-1	Monocyte chemo-attractant protein-1
MMPs	Matrix metalloproteinases

MNX	Medial meniscus transection OA
mPGES1	Microsomal prostaglandin E synthase-1
MT-MMPs	Membrane-type MMPs
NE	Norepinephrine
NF-kB	Nuclear factors-κB
NGF	Nerve growth factor
NMDA	N-methyl-D-aspartate
NSAIDs	Non-steroidal anti-inflammatory drugs
OPG	Osteoprotegerin
OA	Osteoarthritis
PAG	Periaqueduct gray
PD1/NPD1	ProtectinD1/neuropeptide 1
PGs	Prostaglandins
PI3K	Phosphoinositide 3-kinase
PK	Protein kinase
PMNs	Polymorphonuclear leukocytes
PPT	Pressure pain threshold
PRRs	Pattern-recognition receptors
PWT	Paw withdrawal threshold
PUFAs	Poly-unsaturated fatty acids
RA	Rheumatoid arthritis
RAGE	Receptor for advanced glycation end-products
RANKL	Receptor activator of nuclear factor κB ligand
RT-PCR	Reverse transcription polymerase chain reaction
RvD:	Resolvin D
RvE	Resolvin E
RVM	Rostroventral medulla
SNL	Spinal nerve ligation

SP	Substance P
SPMs	Specialized pro-resolving lipid mediators
TGFβ1	Transforming growth factor beta 1
TIMPs	Tissue inhibitors of metalloproteinases
TLR	Toll-like receptor
TNFα	Tumour necrosis factor alpha
TREM1	Triggering receptor expressed on myeloid cells 1
TRP	Transient receptor potential
Trk	Tyrosine kinase
VAS	Visual analog scale
VEGF	Vascular endothelial growth factor
WB	Weight bearing
WDR	Wide dynamic range

Contents

1 Introduction

1.1 Osteoarthritis

1.1.1 Definition and classification

Osteoarthritis (OA) is a highly prevalent degenerative joint disease that involves loss of cartilage, remodelling of subchondral bone and synovitis. OA can manifest in various joints, such as hand, knee and hip (Hunter 2011) . The main symptoms of OA are pain and stiffness of joints, which lead to disability (Goldring and Goldring 2007). There is no agreed criteria for OA as it could be defined by radiographic assessment, clinical symptoms or self-report.

The radiographic grading system established by Kellgren and Lawrence is widely used for scoring the severity of OA into grades 0-4 (Kellgren and Lawrence 1957). In 1995, Osteoarthritis Research Society International (OARSI) published a radiographic atlas of OA and revised it in 2007 to include detailed scores for different joints of OA (hand, hip and knee) (Altman and Gold 2007). The application of different imaging approaches, such as magnetic resonance imaging (MRI), ultrasound and computed tomography, for assessing the development of structural changes within the joint, in particular the early stages of the disease, are improving. These approaches should in the future provide three-dimensional and dynamic images of morphology of joint that provide a stereo view of structures and help better understanding the dynamic progression of OA (Guermazi et al. 2013).

Symptomatic OA was described as a heterogeneous group of conditions that lead to joint symptoms and signs which are associated with defective integrity of articular cartilage, in addition to related changes in the underlying bone and at the joint margins by a subcommittee of the American Rheumatism Association in 1986. They subclassified primary and secondary OA and developed criteria for diagnosis. (Altman et al. 1986). The details of criteria for diagnosis of OA will be discussed in 1.1.2.

Although people with OA experience pain, which can be chronic, this symptom is not well correlated with the radiographic grading of the joint. A population survey of 2865 people showed that only 4.7% of subjects showed both radiographic OA and current knee pain and no single clinical sign can predict radiographic OA well (Claessens et

al. 1990). More recently, a systematic literature review revealed that 15-76% OA patients with knee pain have radiographic OA and 15-81% of patients with radiographic OA have pain (Bedson and Croft 2008). The prevailing current opinion is that OA describes a range of disorders resulting in structural and symptomatic failure of synovial joints rather than a single disease or process to one component of the joint (Brandt et al. 2006).

1.1.2 Symptoms and Diagnosis

The major symptoms of OA are pain, joint stiffness, swelling of joint, joint crepitus and limitation of motion (Haq et al. 2003). Joint pain is often associated with physical activity, which then limits the amount of physical activity individuals undertake. Joint instability limits the range of motion in activity and crepitus (grating or crunching sound) is present at the later stages of OA (Felson 2006; Sinusas 2012).

The diagnosis of OA is based on clinical symptoms and radiography or MRI images. The American College of Rheumatology (ACR) developed the clinical and radiographic criteria of OA as joint pain, osteophytes and one of the following: age greater than 50 years, morning stiffness less than 30 minutes, or crepitus which showed 91% of sensitivity and 86% of specificity for clinical examination of idiopathic OA (**Table1.1**) (Altman et al. 1986).

Clinical and laboratory	Clinical and radiographic	Clinical
Knee pain+	Knee pain +	Knee pain+
at least 5 of 9:	at least 1 of 3:	at least 3 of 6:
Age >50 year	Age >50 years	Age >50 year
Stiffness <30 min	Stiffness <30 min	Stiffness <30 min
Crepitus	Crepitus+osteophytes	Crepitus
Bony tenderness		Bony tenderness
Bony enlargement		Bony enlargement
No palpable warmth		No palpable warmth
ESR* <40 mm/hour		
RF <1:40		
SF OA		
92% sensitive	91% sensitive	95% sensitive
75% specific	86% specific	69% specific

Table 1.1 Criteria of diagnosis of OA is based on combination of clinical signs, radiographic features and laboratory test. * ESR = erythrocyte sedimentation rate (Westergren); RF = rheumatoid factor; SF OA = synovial fluid signs of OA (clear, viscous, or white blood cell count <2,W/mm^3). If the alternative criteria reach 4 of 6 in the clinical category, the sensitivity and specificity of diagnosis will be 84% and 89% respective (Altman et al. 1986).

OARSI used radiographic scoring of osteophytes, joint space narrowing, malalignment, subchondral sclerosis and subchondral cyst as OA features (Altman and Gold 2007). The European League Against Rheumatism (EULAR) recommends combining clinical symptoms, risk factors, imaging and laboratory testing for the diagnosis of OA. Persistent pain, morning stiffness of joint, reduced function of joint, crepitus, limitation of movement and bony enlargement are the predominant six features used (Zhang et al. 2009).

1.1.3 Incidence and prevalence

The prevalence of OA is increased with ageing, and is higher in females. It is estimated that 9.6% of men and 18.0% of women aged at 60 or over have symptomatic OA world-wide. Levels are higher when considering the US and Europe (14.1% of men and 22.8% of women aged 45 years and over) (Woolf and Pfleger 2003). The Framingham Study for 1041 people aged at 71-100 years reported 26.2% woman and 13.4% man had symptomatic hand OA (Zhang 2002). A further 9-year follow-up study of Framingham OA study showed age-standardised prevalence of symptomatic OA was 15.9% for woman and 8.2% for man respectively (Haugen et al. 2011). The age-standardised prevalence of hand OA was 44.2% for women and 37.7% for men respectively (Haugen et al. 2011). Recently, OA in the hip and knee was ranked as the 11th highest contributor to global disability (Cross et al. 2014). The appearance of symptoms versus radiographic OA has been evaluated in the Johnston County Osteoarthritis Project of 2637 cases on hip symptoms, radiographic hip OA and symptomatic hip OA for African-Americans and Caucasians aged $\geqslant$45 years; 36% of the subjects had hip symptoms, 27.6% had radiographic hip OA and 9.7% had symptomatic OA. The prevalence of those categories was increased with age (45–54 years, 55–64 years, 65–74 years, and 75 years and older) (Jordan et al. 2009). In the UK, it is estimated that over 8.75 million people have OA, 49% of woman and 42% of man at 75 years old or over, and a third of people aged 45 or older have sought treatment for OA) (Aruk 2013) . As people live longer and levels of obesity increase, it is predicted that the prevalence of OA will increase further. Obesity is a main risk factor for the prevalence of OA as overload to the joint may result in cartilage damage. In addition, adipose tissue can release a number of adipocytokines such as TNFα, IL1β, leptin and resistin which accelerate progression of OA (Pottie et al. 2006; Sowers and Karvonen-Gutierrez 2010). A 10-year follow-up study has shown obesity was associated with hand OA and higher body mass index was associated with higher risk in knee OA (Grotle et al. 2008). OA is becoming a major public health problem worldwide that undermines the quality of life and represents a major economic burden on countries with large population with OA.

1.1.4 Aetiology and risk factors

The aetiology of OA is heterogeneous and multiple factors are involved in the pathogenesis and progression of OA. Nevertheless, the susceptibility of OA has been ascribed to systemic and local biomechanical factors which exert mechanical stress on joints and thus change microenvironment around the joints (Jordan et al. 2000; Johnson and Hunter 2014) **(Fig 1.1).**

Systemically, genetics (Cicuttini and Spector 1996), age and gender (Felson et al. 1987), bone density (Foss and Byers 1972) and nutritional factors such as diet and obesity and vitamins (C,D and E) (Mcalindon and Felson 1997) are risk factors associated with OA. Epidemiological studies of family history and family clustering showed more than 50% of OA was heritable. Genetic factors affected 39-65% of radiographic hand and knee OA in women, 60% of hip OA and 70% of spine OA (Spector and Macgregor 2004). OA is more prevalent in old people as aging changes musculoskeletal system (muscles and ligaments) and joint tissues such as cartilage and meniscus (Shane Anderson and Loeser 2010). The Baltimore longitudinal cohort study reported an association of high bone mineral density with increased incidence of knee OA (Hochberg et al. 2004), The Multi-centre Osteoarthritis Study (MOST) of 1754 subjects also reported higher bone mineral density of femoral neck and whole body increased the risk of OA (Nevitt et al. 2010).

A number of factors including, obesity (Cicuttini et al. 1997), joint injury (Gelber et al. 2000) , muscle strength (Kathy D. Hall et al. 1993) and occupation (Rossignol et al. 2005) have been implicated in the initiation and progression of OA.

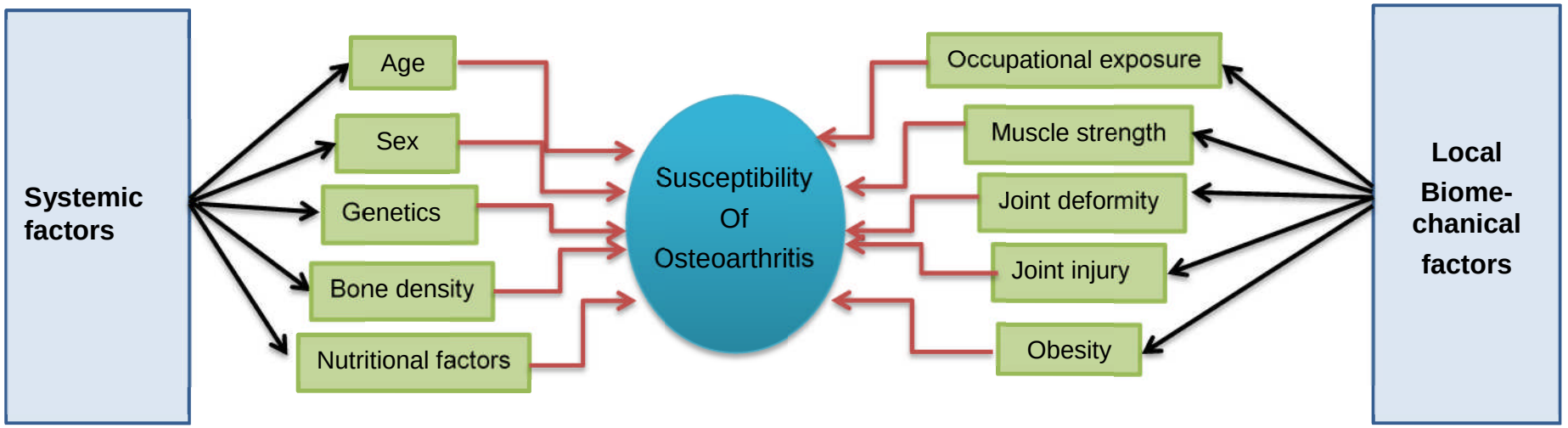


Fig 1.1 Susceptibility of OA with systemic factors and local biomechanical risk factors. Age, sex, obesity, genetic and instability of joints are involved in initiation and progression of OA. Figure was adapted from Jordan et al (2000).

1.1.5 Pathophysiology

OA joints are characterized by articular cartilage degradation, meniscus damage, subchondral bone remodelling and sclerosis, synovial hypertrophy, osteophytes formation and mal-alignment of muscle and ligament (Hunter 2011). **(Fig 1.2)**

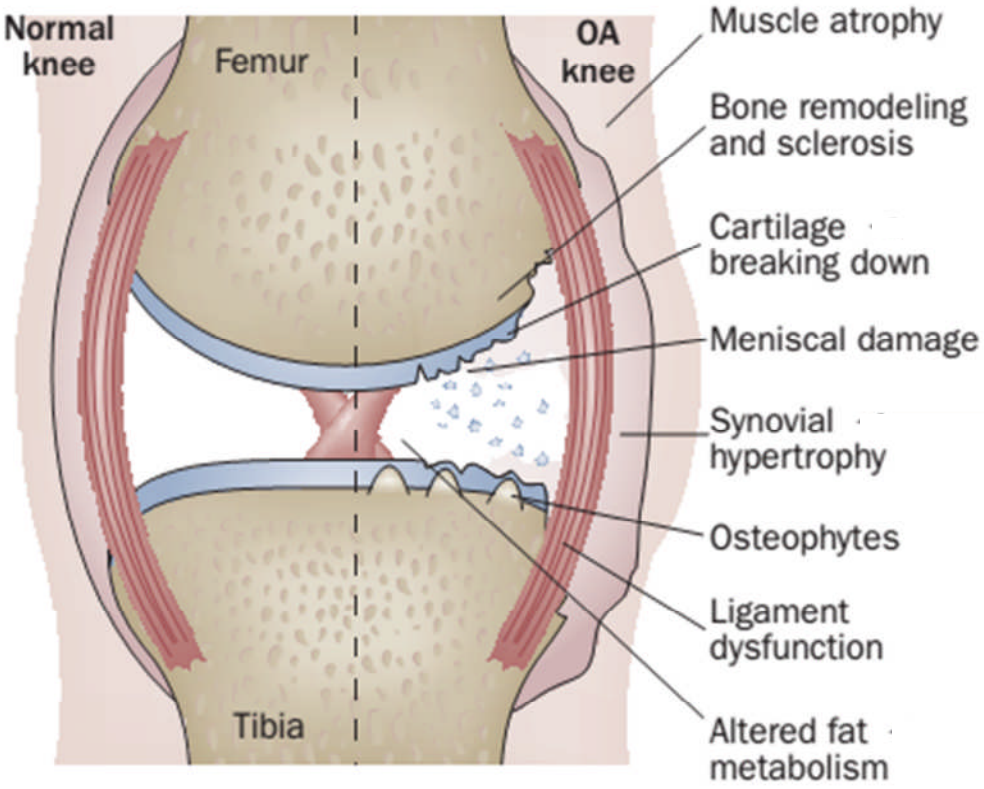


Fig 1.2 Comparison of an OA joint and normal joint. The OA joint shows degradation of cartilage, meniscus damage, remodelling of subchondral bone, thickness of synovium, osteophytes and mal-alignment of muscle and ligament compared to normal joint.

Figure was adapted from Hunter et al (2011).

Under normal conditions, the turnover of cartilage in adult is very slow and the synthesis and degradation of extra-cellular matrix component is regulated. Under osteoarthritic condition, however, this homeostasis is disrupted which leads to apoptosis of chondrocytes, loss of articular cartilage and degeneration of joints (Sandell and Aigner 2001; Goldring and Marcu 2009). In the early stage of OA, chondrocytes, the only cell type producing proteoglycan, collagen and proteinases in cartilage, proliferate to compensate the loss of cartilage. However, as OA progresses, the balance is disrupted. Chondrocytes cannot compensate the degradation of extra-cellular matrix components and the anabolism shifts to catabolism which exacerbates the deterioration of joints (Stockwell 1991; Middleton and Tyler 1992; Lories and Luyten 2011).

During this process, a number of proteases, mediators and factors including matrix metalloproteinases (MMPs), a disintegrin and metalloproteinases (ADAMs), bone morphogenetic proteins (BMPs), inflammatory cytokines and growth factors promote OA progression.

MMPs are a family of highly homologus endopeptidases that degrade most components of the extracellular matrix (Birkedal-Hansen et al. 1993). Currently, 24 different MMP genes have been identified and 23 members are present in man. These MMPs belong to 6 different groups: collagenases (MMP1, 8, 13, and MMP18), gelatinases (MMP2 and MMP9), stromelysins (MMP3, MMP10 and MMP11), matrilysins (MMP7 and MMP26), membrane-type MMPs (MMP14-17 and MMP24-25) and others (MMP12,19-23,27-28) (Nagase et al. 2006). MMPs play multiple biological roles in bone formation and remodelling, tissue morphogenesis, angiogenesis, inflammation, wound healing, cell migration and proteolysis (Brewa; et al. 1999; Vu and Werb 2000; Parks et al. 2004; Page-Mccaw et al. 2007). Physiologically, the activity of MMPs is regulated by endogenous inhibitors and tissue inhibitors of metalloproteinases (TIMPs) which have 4 homologous isomers (TIMP1-4). The balance of MMP-TIMP has been shown to shift to MMPs under pathological conditions and loss regulation of MMPs may contribute to a number of diseases such as arthritis, cancer and cardiovascular diseases (Nagase and Woessner 1999; Visse and Nagase 2003).

Clinical studies have shown that concentrations of metalloproteinases, TIMPs and proteoglycan fragments are significantly increased in synovial fluid of OA patients compared to controls (Lohmander et al. 1993; Tchetverikov et al. 2005). MMPs, membrane-type MMPs (MT-MMPs), ADAMs and a disintegrin and metalloproteinase

with thrombospondin motifs (ADAMTS) including MMP1-3, MMP8-10, MMP12-14, ADAMTS1, 4, 5,9,15, and ADAMTS 16 are produced by chondrocytes, osteoclasts and synovial fibroblasts in OA (Murphy and Nagase 2008). These proteinases cleave different types of collagen (I, II and III.), proteoglycan and aggrecan which are the main extracellular components that lead to degradation of bone. (Martel-Pelletier et al. 2001; Murphy et al. 2002; Murphy and Nagase 2008). Significant degradation of type II collagen by MMP1 and MMP13 has been demonstrated in cartilage from OA patients with total knee replacement. Furthermore, the synthesis of MMP13 was modulated by TNFα and IL1β in a dose-dependent manner (Mitchell et al. 1996; Reboul et al. 1996). Imai showed expression of MT1-MMP (MMP14) in OA cartilage and activated pro-MMP2 which can cause destruction of cartilage (Imai et al. 1997). Increased MMP1 and MMP2 have been shown in co-cultured OA chondrocytes and normal osteoblast taken from trauma patients (Prasadam et al. 2012).

Although cartilage has been the most heavily investigated of the structures exhibiting pathological damage associated with OA, remodelling of subchondral bone, formation of osteophytes and synovial inflammation are all associated with pathogenesis of OA. More recent studies have revealed that changes in subchondral bone precede cartilage damage (Carlson et al. 1994) and stiffness of subchondral bone can cause deformity of cartilage and accelerate the fibrillation of cartilage (Radin and Rose 1986; Carlson et al. 1994; Burr and Gallant 2012). Radiographic imaging of OA has shown bone sclerosis, osteophytes and joint space narrowing in joints of hand, knee and foot (Jacobson et al. 2008). Animal models of OA showed resorption of subchondral bone at early stage but increased formation at late-stages of OA (Hayami et al. 2006). Osteophytes are present in the femoral condyle and medial tibial plateau of animal models of OA (Hayami et al. 2006). Inhibition of bone resorption with alendrolate decreased resorption of subchondral bone and attenuated formation of osteophytes in a dose-dependent manner in animal models of OA (Hayami et al. 2004). Previous work from our group demonstrated that pre-emptive treatment of osteoprotegerin-Fc (OPG-Fc) prevented ostephytes formation, decreased osteoclast number and attenuated osteoarthritic pain in an animal model of OA (Sagar et al. 2014).

Although OA was traditionally considered a degenerative joint disorder induced by ‘wear and tear’ mechanical stress, more recently the role of inflammation in the

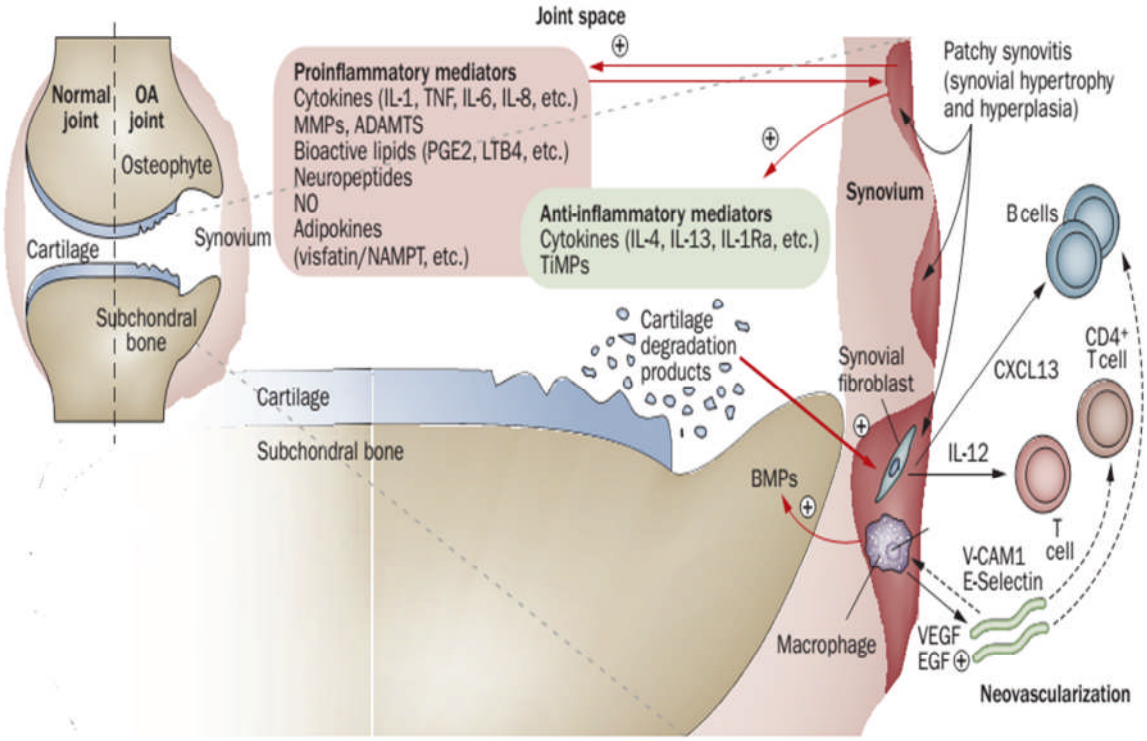
pathogenesis of OA has been recognized (Berenbaum 2013). 31% of OA patients have severe synovial inflammation and 62.5% of patients with mild or moderate synovial inflammation (Haywood et al. 2003). A longitudinal arthroscopic study has shown 50% of patients have abnormal synovia with 21% having synovial inflammation (Ayral et al. 2005). OA patients with inflamed synovia (synovitis) have significantly higher chondropathy compared to OA patients with normal synovia (Ayral et al. 2005) and synovitis is significantly associated with OA pain (Baker et al. 2010).

Numerous studies have investigated the inflammatory factors associated with OA.

Synovial tissue from early OA patients (arthroscopic manifestations of OA) showed significantly increased infiltration of cluster of differentiation 4+ (CD4+) T-cell, CD68+ macrophage and formation of blood vessels compared to late OA patients (knee joint arthroplasty) (Benito et al. 2005). Moreover, the immune cell numbers which secreted TNFα and IL1β are also increased in early OA patients compared to late OA patients. The inflammation in early OA was associated with the increased nuclear factors-κB (NF-kB), a nuclear transcription factor that controls transcription of DNA, and COX2 (Benito et al. 2005). These findings may indicate a major role of inflammation in the initiation of OA. Nevertheless, whether inflammation, or degradation of cartilage, occurs first in OA remains to be determined. It has been proposed that iinflammation may be initiated by fragments of cartilage degradation which activate the innate immune system via pattern-recognition receptors (PRRs) and damage-associated molecular patterns (DAMPs) on chondrocytes in cartilage and synoviocytes (macrophage and fibroblast-like synoviocyte) in synovium (Berenbaum 2013; Liu-Bryan 2013). Conversely, inflammatory mediators released by those cells activate more chondrocytes and synoviocytes, amplifying the activation of immune system and accelerate loss of cartilage (Goldring and Otero 2011; Sokolove and Lepus 2013). Microarray gene expression profiling from OA patients has shown cytokines (IL6, IL8 etc.), chemokines (chemokine (C-X-C motif) ligand (CXCL5), CXCL6, etc.), FLAP, 5-LOX anabolic protein (triggering receptor expressed on myeloid cells 1(TREM1), alarmin S100A9, etc.) and catabolic proteinases (MMP3, MMP9, cathepsin H etc.) were significantly up-regulated in inflamed synovia compared to normal area of synovia from the same patient (Lambert et al. 2014). They further confirmed activation of Wnt signalling pathway in inflammatory synovia (Lambert et al. 2014). Cultured synovial cells from OA patients produced TNF-α, IL-1β and large amounts

of IL-6, IL-8, monocyte chemo-attractant protein-1(MCP-1), MMPs (1, 3, 9, and 13) and TIMP1 (Bondeson et al. 2006; Bondeson et al. 2010). Wang et al. found significantly higher expression of complement (C3 and C5), MMPs (MMP1, 3, 13), ADAMTS4-5 and CCL2, 5 in synovial tissue in OA (Wang et al. 2011). Depletion of C5 and C6 significantly reduced cartilage degeneration and C5 dramatically decreased levels of MMPs, ADAMTS and CCLs, which suggests an important contribution of inflammatory complements to the pathogenesis of osteoarthritis (Wang et al. 2011).

The complex mixture of inflammatory mediators and enzymes in joints indicate both innate (PRRs, DAMPs and complement system) and adaptive immune system contribute to pathophysiology of OA. The roles of inflammatory mediators are regulated by various signalling pathways such toll-like receptors, receptor for advanced glycation end-products (RAGE), NF-κB and Wnt (Haseeb and Haqqi 2013; Liu-Bryan 2013; Liu-Bryan and Terkeltaub 2014). A summary of the major features of OA pathophysiology and involvement of multiple inflammatory mediators and enzymes in joint damage of OA is described in **Fig1.3**.



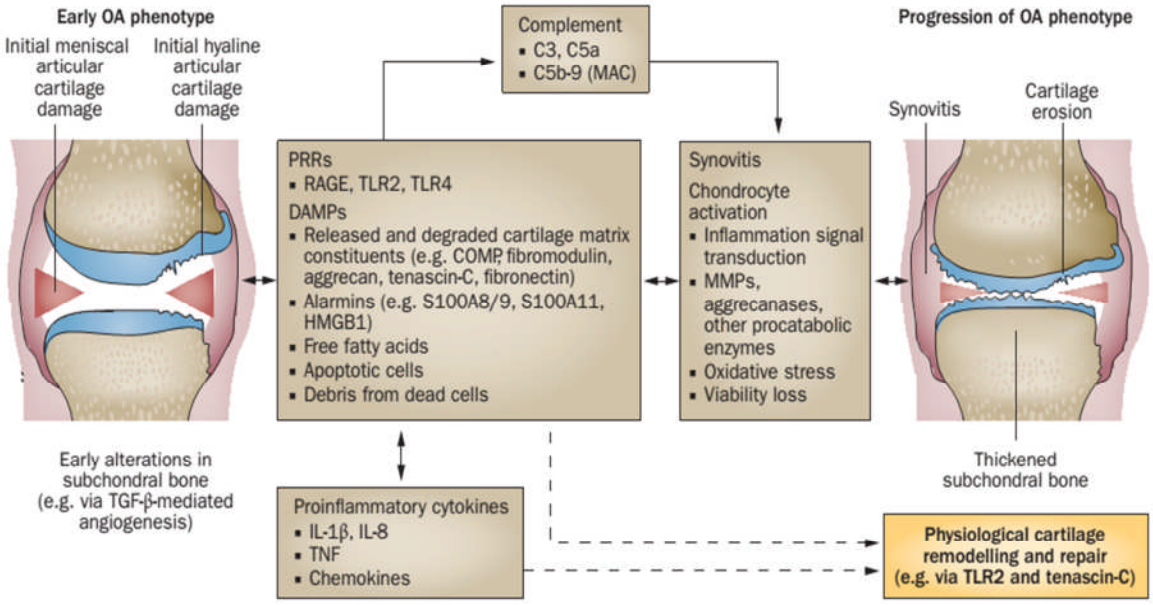


Fig 1.3 Pathophysiology of normal joint and OA joint and mediators networks in the progression of OA. Degradation products of cartilage activates macrophage and synovial fibroblast via the innate immune system and these cells release a number of inflammatory mediators into the joint space which degrade cartilage and exacerbate deterioration of the OA joint. The released mediators also activate immune cells and induce adaptive immune system during progression of OA. Figures were from Sallam and Berenbaum (2010) and Liu-Bryan and Terkeltaub (2014).

As described above, mechanical stress of the joint contributes to OA and is associated with risk factors such as obesity, trauma or joint instability (Guilak 2011; Heijink et al. 2012; Buckwalter et al. 2013). Biomechanical loading of the joint may interact with multiple biochemical factors, including Ca^{2+} signalling, cytokines, MMPs and growth (Sanchez-Adams et al. 2014). However, the mechanism(s) by which mechanical force could be transduced to biochemical signals and lead to pathogenesis of OA is undefined. The roles of ion channels or integrins to mechanical stress responses of chondrocytes have been studied (Grandolfo et al. 1998; Millward-Sadler et al. 1999; Mobasheri et al. 2012). Recently, Piezo1 and Piezo2 were identified as essential components of mechanical activated channels for mechanotransduction (Coste et al. 2010). Lee et al further confirmed mechanosensitivity of Piezo1 and Piezo2 in mechanotransduction of chondrocytes. Blocking the Piezo channels reduced chondrocyte death after mechanical injury in an explant cartilage injury model

(Lee et al. 2014). Transient receptor potential V4 (TRPV4) is also involved in mechanical signal transduction in chondrocytes (O'conor et al. 2014).

Clearly, the pathophysiology and pathogenesis resulting in OA is complicated, involving in a series of factors, mediators and signalling pathways at the cellular and molecular levels. It is therefore not surprising that the specific mechanisms underlying initiation and progression of OA remain elusive.

1.1.6 Treatment

As a result of the heterogeneous aetiology and pathogenesis of OA, management of OA is not achieved by a single therapy. The ACR recommends using non-pharmacological and pharmacological therapy or surgery to treat OA according to severity. The non-pharmacological management focuses on disease education, weight loss and increasing activity. Analgesic treatments used are non-steroidal anti-inflammatory drugs (NSAIDs), intra-articular steroid injection and opioids. The final choice to treat OA is osteotomy or total joint replacement (Hochberg et al. 1995; Hunter and Felson 2006; Hochberg et al. 2012). The OARSI recommendation for treatment, based on systematic literature reviews and consensus of 16 experts, is 12 non-pharmacological modalities (self-management, loss weight, exercises, etc.), 8 pharmacological modalities including acetaminophen, COX2 non-selective and selective NSAIDs, capsaicin, intra-articular injection of corticosteroids and hyaluronates etc. for symptom relief and structure modification and 5 surgical modalities (including total joint replacement, osteotomy, arthroscopic debridement, etc.) to treat OA (Zhang et al. 2007; Zhang et al. 2008). The EULAR used more than 20 experts from various European countries and based on research evidence to make recommendation with Delphi consensus methods for management of knee OA, hip OA and hand OA in 2003, 2005 and 2007 respectively. They also recommended the combination of non-pharmacological and pharmacological therapy and surgery for treatment of OA (Jordan et al. 2003; Zhang et al. 2005; Zhang et al. 2007). All these recommendations are based on various treatment propositions for specific subgroups of OA patients. Although conventional drugs are effective in relieving pain and other symptoms of OA, adverse effects, such as gastrointestinal bleeding, cardiovascular, hepatic and renal injury limit their use (Vaile and Paul 1998; Sarzi-Puttini et al. 2005; Shi and Klotz 2007).

Currently, some of preclinical and clinical analgesic drugs such as anti-nerve growth factor (NGF) drugs, bisphosphonate, duloxetine, poly-unsaturated fatty acids (PUFAs) and stem cells are emerging and under investigation to find novel treatment for OA and OA pain. The human soluble NGF receptor, tyrosine kinase Ad 5 (TrkAd5), was shown to inhibit pain behaviour in destabilisation of the medial meniscus (DMM) surgery model of OA via neutralizing NGF (Mcnamee et al. 2010). Tanezumab, an anti- NGF monoclonal antibody, reduced joint pain and improved joint function in knee OA in a dose-dependent manner, compared to placebo. However, it also showed some side effects such as headache, respiratory tract infection or paresthesia in OA patients (Lane et al. 2010). Subcutaneous injection of tiludronate, a bisphosphonate, attenuated pain scoring, improved the joint histology and decreased synovitis in the anterior cruciate ligament (ACL) transection model of dog, compared to placebo (Moreau et al. 2011). Bisphosphonate reduced scale pain scores (numeric rating scale) in OA patients compared to OA patients without using bisphosphonates at the first three years (Laslett et al. 2014). Duloxetine, a serotonin and norepinephrine reuptake inhibitor, reduced pain score in knee OA compared to placebo-control but also had some side effects (Chappell et al. 2009). In another 16-week randomized placebo-control trial, duloxetine was shown to reduce pain scores and improved physical function in OA patients, compared to placebo (Abou-Raya et al. 2012). There is evidence to show PUFAs could down-regulate IL1α-induced ADAMTS-4, ADAMTS-5, COX2, MMP3, MMP13, TNFα and IL1β in bovine chondrocytes, which are essential enzymes and inflammatory cytokines in the initiation and progression of OA (Zainal et al. 2009). Stem cells isolated from caprine (goat) bone marrow have been shown to regenerate the medial meniscus and reduce degeneration of cartilage, osteophytes and subchondral sclerosis after 6 weeks of intra-articular injection in a combined (ACL) transection and medial meniscectomy goat model of OA (Murphy et al. 2003). In a more recent 1 year follow-up study, mesenchymal stem cells from human bone marrow have been transplanted into four OA patients and revealed that pain on visual analog scale (VAS) was improved (Davatchi et al. 2011). Intra-articular injection of adipose-derived stem cells (ASCs) attenuated synovial thickening and degradation of cartilage at early stage of OA (Ter Huurne et al. 2012). Intra-articular injection of adipose tissue derived mesenchymal stem cells (MSCs) demonstrated recovery of cartilage and reduction of cartilage de-

fect in medial femoral and medial tibial condyle by histological MRI and arthroscopic assessment (Jo et al. 2014).

Some disease modifying osteoarthritis drugs (DMOADs), for example, MMP inhibitors, inhibitors to IL1, TNFα, inducible nitric oxide synthase (iNOS), bone morphogenetic protein 7 (BMP7), vitamin D, fibroblast grow factors, receptor activator of nuclear factor κB ligand (RANKL), calcitonin, cathepsin K and lubricin, are under investigation to modify progression of OA (Hunter 2011; Martel-Pelletier et al. 2012).

Traditional drugs such as steroids, NSAIDs and opioids are the mainstay of analgesic treatments until joint replacement, thus investigation of potential novel therapeutic treatment for OA pain continues to be of clinical importance.

1.2 Pain

1.2.1 Introduction to pain

Pain provides an early warning of tissue damage such as skin burning, finger pricking and etc. This endogenous self-guarding system helps us to avoid external and internal painful stimuli and congenital deficits in painful sensitivity can be life-threatening (Scholz and Woolf 2002) . However, pain associated with disease can also be maladaptive, serving little protection purpose and is difficult to treat (Scholz and Woolf 2002). The sensation of pain not only refers to the detection of a painful stimulus (nociception) but also the emotional component which gives rise to the unpleasant experience of pain, and the cognitive responses such as attention (Price 2000; Linton and Shaw 2011). Thus, pain is defined as 'an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage' by International Association for the Study of Pain (IASP) (IASP, 2012).

The importance of pain has been recognized for centuries from the initial thoughts of ancient Aristotle (384–322 BC) to the advanced understanding in the past century (Perl 2007). In 1906, Sherrington published the integrative action of the nervous system which proposed that pain was normally produced from tissue injury and signalling of noxious events (nociception) (Burke 2007; Perl 2007). Understanding of the mechanisms of pain was pioneered by Patrick Wall and Ronald Melzack who initiated the gate control theory of pain (Melzack and Wall 1965) and Edward Perl who defined the existence of Aδ and C-fibre "nociceptors" to specific noxious stimulation (Burgess and Perl 1967; Bessou and Perl 1969) in the 1960s. The phrase "central sensitization" was first proposed by Woolf in 1980s and the mechanisms underpinning these events continue to be studied and investigated (Woolf 2007). Later on, David Julius and colleagues identified TRP channels as key receptors involved in the nociceptive pathways (Caterina et al. 1997; Caterina et al. 1999; Caterina et al. 2000). Many other ion channels have been actively studied, not least sodium (Na_v) channels which have essential roles in driving pain signalling. Ablation of $Na_v1.7$ and $Na_v1.8$ in nociceptors blocks pain behaviour in the models of inflammatory pain (Nassar et al. 2004; Abrahamsen et al. 2008) and loss of $Na_v1.7$ results in a congenital inability to detect painful stimuli (Fertleman et al. 2006). Pain is a broad term and is primarily divided into four types: nociceptive pain , inflammatory pain, neuropathic

pain and functional pain (Woolf 2004) . Allodynia and hyperalgesia are clinical terms to describe painful responses to normally non-noxious stimulation and facilitated responses to an overt painful stimulusus (Sandkuhler 2009; Jensen and Finnerup 2014).

1.2.2 Nociceptors

Nociceptors are a specialized class of primary afferents that respond to intense, noxious (mechanical, thermal and chemical) stimuli (McMahon et al. 2013). Nociceptors are the free nerve endings that innervate organs or tissues and the axon conducts electrical signals to the central ending which terminates in the dorsal horn of the spinal cord. (Woolf and Ma 2007; Dubin and Patapoutian 2010). Nociceptors are broadly classified into Aδ and C fibres depending on their conductance, which is associated with diameter and myelination (Coutaux et al. 2005; Fein 2012). A fibers are myelinated and have faster conduction velocity (Aβ fibre: 35-75 m/s and Aδ : 5-30 m/s) whereas the un-myelinated C-fibres have a slower conductance velocity at 0.5-2 m/s (Purves et al. 2012). Aβ fibres predominantly detect non painful stimuli although there is evidence that some of these fibres can also detect painful stimuli.

As a fast conducting efferent fibre, Aδ fibres respond to initial sensations of pain (sharp pain) such as cutting and prickling, while C-fibres contribute to the 'second pain' such as burning and gnawing (Giordano 2005). Primary afferent fibre nociceptors have heterogeneous properties and respond to multiple stimulus modalities, mechanical, thermal and chemical (polymodal) stimuli (McMahon et al. 2013). The nociceptive input is modulated by multiple mechanisms including, peripheral sensitization, central sensitization and disinhibition (Woolf 2004).

1.2.3 Nociception and peripheral sensitization of pain

Noxious stimuli are detected by polymodal nociceptors C-fibre and Aδ fibre nociceptors which express TRP channels, acid sensing ion channels (ASICs), sodium channel (Na_v1.6-1.9), calcium channels, potassium channels, G protein-coupled receptors (GPCRs), cytokine receptors and P2X receptors (Mccleskey and Gold 1999; Gold and Gebhart 2010). The stimulus is transduced by these channels and receptors and then encoded into electrical signals that initiate action generation in the sensory neurons (Gold and Gebhart 2010) (**Fig1.4**).

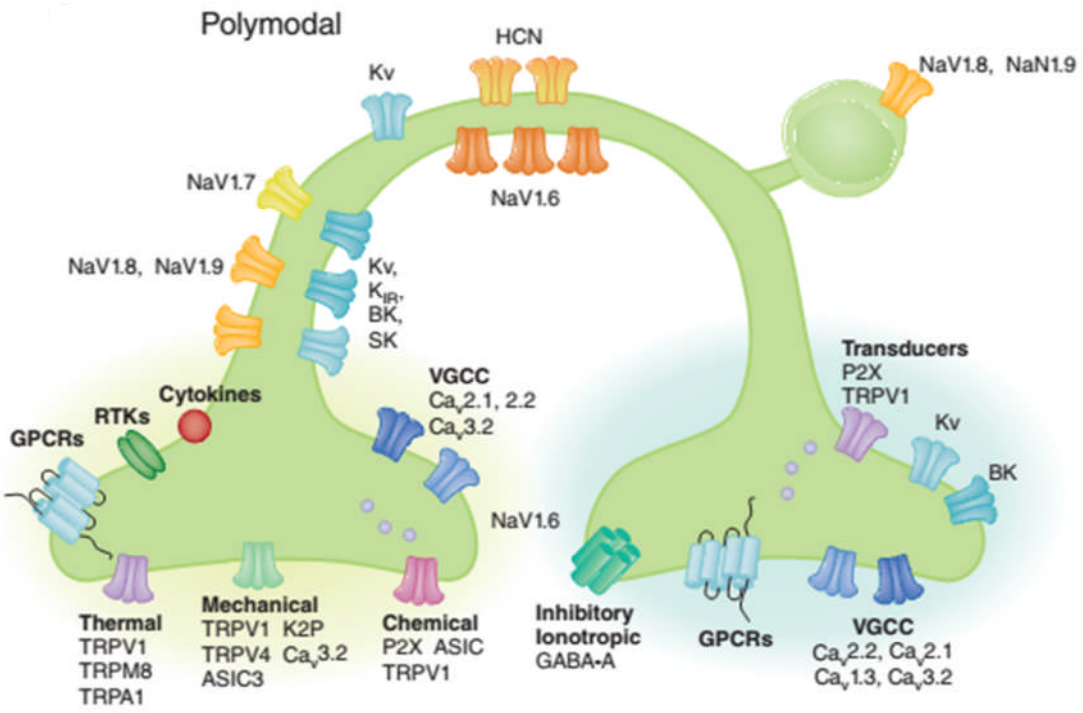


Fig 1.4 Transducers including ion channels and receptors are expressed in the terminals of heterogeneous nociceptors. (Figure was modified from Gold and Gebhart 2010)

Peripheral sensitization is a stimulus-evoked increase in response and decreased threshold of the peripheral ending of the nociceptive neurons (Woolf and Ma 2007). Peripheral sensitization is mediated by tissue damage and inflammation, which results in the elevation of a multitude of mediators such as prostaglandins, NGF, cytokines, chemokines, H^+ and adenosine triphosphate (ATP) in the receptive field of the primary sensory neurone and initiates a series of signalling cascades in the nociceptors (Hucho and Levine 2007; Von Hehn et al. 2012). These mediators can act directly on ion channels or interact with their cognate receptors, which activate the down-stream signalling pathways such as protein kinase A (PKA), PKC, phosphoinositide 3-kinase (PI3K) and extracellular-regulated kinases (ERKs) via the secondary messengers (cyclic adenosine monophosphate (cAMP) and Ca^{2+}) (Julius and Basbaum 2001). In turn, these activated kinases can depolarize ion channels (TRP channels and sodium channels) in the nociceptors (Wang and Woolf 2005) and elevates levels of intracellular Ca^{2+}, which enhances the sensitivity of sensory neurons

to the stimulation by lower intensity stimuli (**Fig 1.5**) (Levine and Alessandri-Haber 2007).

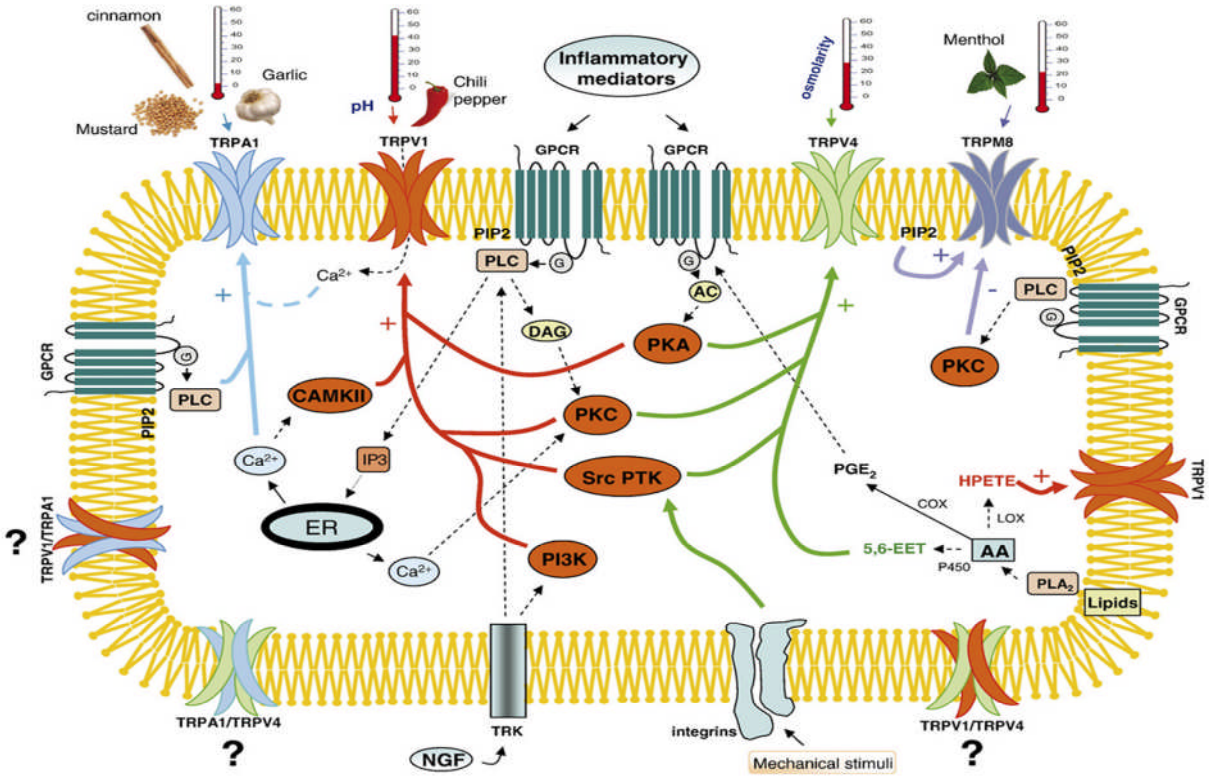


Fig 1.5 Signal cascades of peripheral sensitization in terminals of primary sensory fibres (nociceptors). Noxious stimuli and inflammatory mediators act directly on ion channels or interact with receptors, which activate the down-stream signal ling cascades via cAMP, and Ca^{2+} to sensitize the peripheral terminals of the nociceptors.Figure was from Levine and Alessandri-Haber (2007).

1.2.4 Central sensitization of pain

Peripheral nociceptive information is conducted to secondary order neurons in the spinal cord via the primary afferent neurone axon. The primary afferent fibres terminate in specific laminae in the dorsal horn of the spinal cord (Braz et al. 2014). Aβ fibres relay non-noxious input and project to the deep laminae (inner lamina II and lamina V) of the dorsal horn. Aδ fibres project to outer lamina II and lamina V of the dorsal horn of the spinal cord. The peptidergic C fibres, defined by release of neuropeptides such as calcium gene-related peptide (CGRP) or substance P(SP), project to the superficial dorsal horn (lamina I and outer lamina II) of spinal cord and non-peptidergic C fibres project to the inner lamina II of the dorsal horn of the spinal cord (**Fig1.6**) (Basbaum et al. 2009).

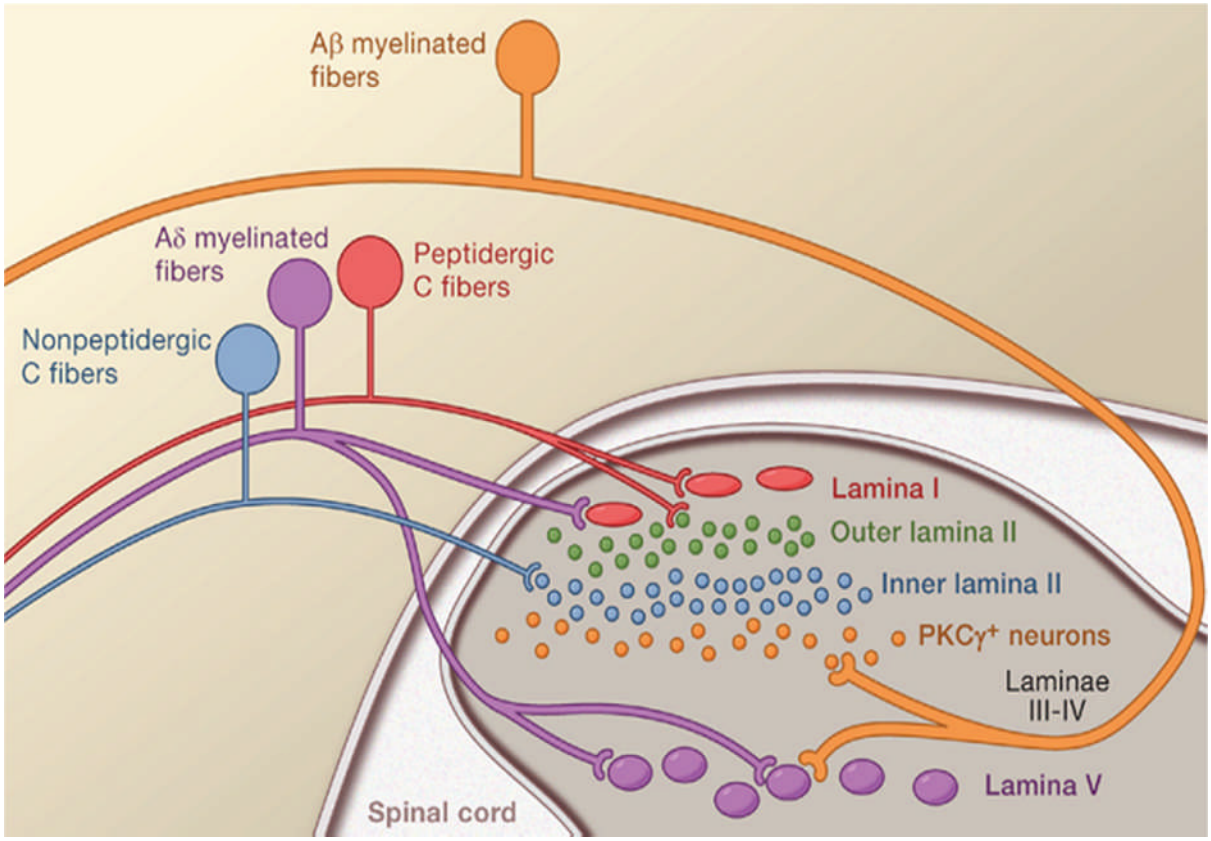


Fig 1.6 The projection of primary afferent fibres to the dorsal horn of spinal cord. Figure was from Basbaum et al (2009).

As the nociceptive signals are processed by the second order neurons in the spinal cord, responses are subject to modulation by central sensitization and descending controls from the brainstem. Central sensitization was first described by Woolf (Woolf 2007). Central sensitization is characterized by the decreased threshold or the increased excitability of neurons in the dorsal horn and the enlargement of the peripheral receptive fields, which results in pain hypersensitivity (Woolf 2011). Central sensitization has an induction and maintenance phase. The induction of central sensitization may start from the wind-up of neurons elicited by repeated low frequency stimulation of C-fibres (Li et al. 1999; Herrero et al. 2000). Maintenance of central sensitization is associated with changes in neurotransmitter signalling such as glutamate/ N-methyl-D-aspartate (NMDA) receptor mediated sensitization (Woolf and Thompson 1990), loss of gamma-aminobutyric acid (GABAergic) and glycinergic control (Sivilotti and Woolf 1994; Malan et al. 2002) and glial-neuronal interaction (Calvo et al. 2012). In the superficial dorsal horn of the spinal cord, Aδ and C nociceptors release a great number of neurotransmitters such as glutamate, brain derived neurotrophic factor (BDNF), CGRP and SP (Ji et al. 2003). These neurotrans-

mitters activate post synaptic neurons via their cognate receptors and the subsequent sustained depolarization leads to the activation of the NMDA receptors by glutamate and sustained influxes of Ca^{2+} (Woolf 2000; Latremoliere and Woolf 2009). These signals are critical steps to generation of central sensitization as they activate the downstream signal cascades including calcium/calmodulin-dependent protein kinase II (CAMKII), cAMP –dependent PKA and Ca^{2+}-dependent PKC, which phosphorylate the receptors expressed by the neurons to increase sensitivity and also activate ERK1/2 and cAMP response element binding protein (CREB) to drive transcription of c-fos, COX2, NK1 and TrkB (**Fig 1.7**) (Latremoliere and Woolf 2009). In addition, inflammation induces phenotype shifts in responses to Aβ fibre input leading to increases in the excitability of neurons and enhanced synaptic transmission in the dorsal horn of spinal cord to maintain the central sensitization (Neumann et al. 1996).

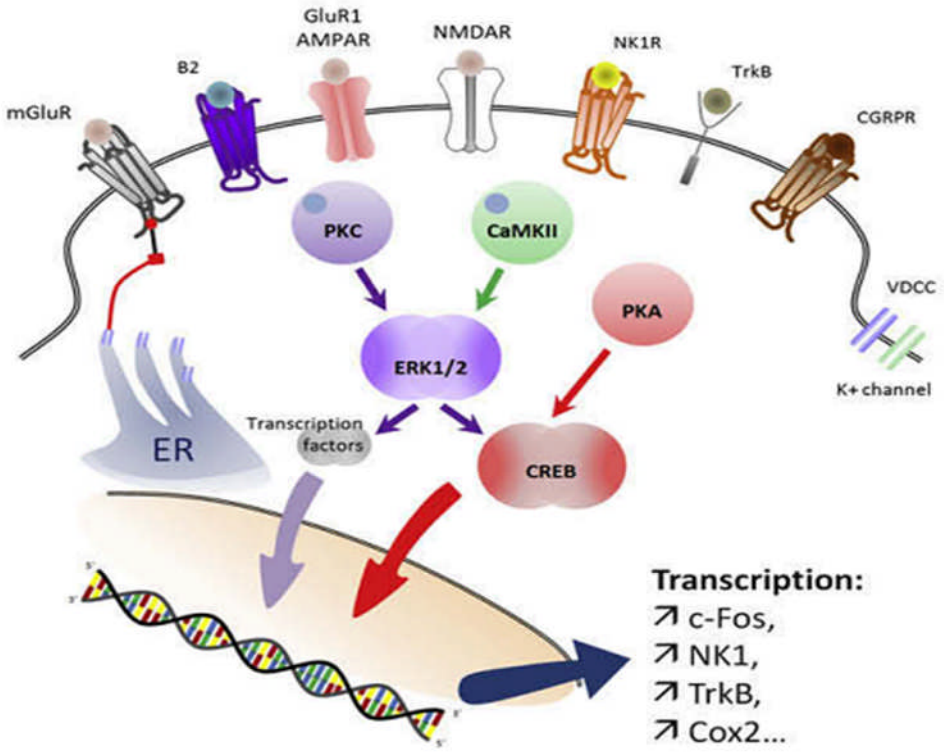


Fig 1.7 Signalling cascades involved in the generation of central sensitization. Receptors expressed by the post-synaptic neurons are activated by their corresponding neurotransmitters; kinases are activated to drive the down-stream signalling cascades leading to the central sensitization. Figure was from Latremoliere and Woolf (2009).

Under normal conditions, nociceptive signals are modulated by inhibitory interneurons and descending fibres. It is proposed that disruption of these inhibitory systems may contribute to the chronic pain. This is what Melzack and Wall described as the gate control theory of pain (Melzack and Wall 1965). However, disinhibition mechanisms are debated as some suggested the disinhibition results from death of GABAergic interneurons (Ibuki et al. 1997; Moore et al. 2002) while others showed there was not obvious evidence that the neurons or GABA receptors were lost in neuropathic pain models (Polgar et al. 2004; Polgar et al. 2005; Polgar and Todd 2008). Some other factors such BDNF and TRPV1 were reported to be involved in the disinhibition of inhibitory neurotransmitters or long-term depression of GABAergic interneurons (Coull et al. 2005; Kim et al. 2012; Chen et al. 2014). Importantly, disinhibition can also contribute to alterations in Aβ fibre input as blocking GABAergic and glycinergic receptors increased Aβ fibre input to dorsal horn neurons (Torsney and Macdermott 2006; Schoffnegger et al. 2008).

The interactions of glial cells (microglia and astrocytes) and neurons have emerged as an important component of the maintenance of central sensitization and chronic pain. Under the normal conditions, microglia are quiescent in the gray matter of spinal cord; however, they become activated following damage to peripheral tissues or the central nervous system. Activated microglia have altered morphology and release inflammatory mediators such as cytokines (TNFα, IL1β and IL6), BDNF and cathepsin S (Mifflin and Kerr 2013). These mediators bind to cognate receptors expressed by neurons and activate the down-stream signal cascades which increase the excitability of neurons. In return, neurotransmitters released from neurons bind to receptors such as P2X receptors, toll-like receptor 4 (TLR4), C-C chemokine receptor type 2 (CCR2) and CX3CR1 expressed by microglia and promote the activation of microglia (Mcmahon and Malcangio 2009). This interaction between microglia and neurons contributes to the central sensitization in the dorsal horn of spinal cord. Studies showed that spinal administration of ATP activated microglia and produced pronounced tactile allodynia in naïve rats which supports the role of microglia in chronic pain states (Tsuda et al. 2003; Coull et al. 2005). TLR is another emerging family of receptors expressed by microglia contribute to central sensitization. TLR4 expressed by microglia was shown to maintain the behavioural hypersensitivity as genetic deletion of TLR4 and administration of TLR4 antisense oligodeoxynucleotide

(ODN) attenuated pain behaviour and microglia activation in a neuropathic model (Tanga et al. 2005). A recent study revealed TLR2 mediated up-regulation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase 2 (Nox2) which was critical for microglia activation after nerved injury and subsequent pain hypersensitivity (Lim et al. 2013).

Astrocytes are also implicated in pain modulation but their contribution to central sensitization is less clear. Astrocytes activation (reactive gliosis) is evident at later stages of models of chronic pain, they can release numerous neurotransmitters such as glutamate, D-serine or ATP which activate synaptic NMDA receptors on neurons leading to cross-talk between astrocyte and microglia (Ren and Dubner 2010). In addition, astrocytes can release inflammatory mediators such as IL1β and CCL2 and express TLRs, which sensitize NMDA receptor and contribute to central sensitization (Liu et al. 2012). The neuroimmune interaction for central sensitization is described in **Fig 1.8**.

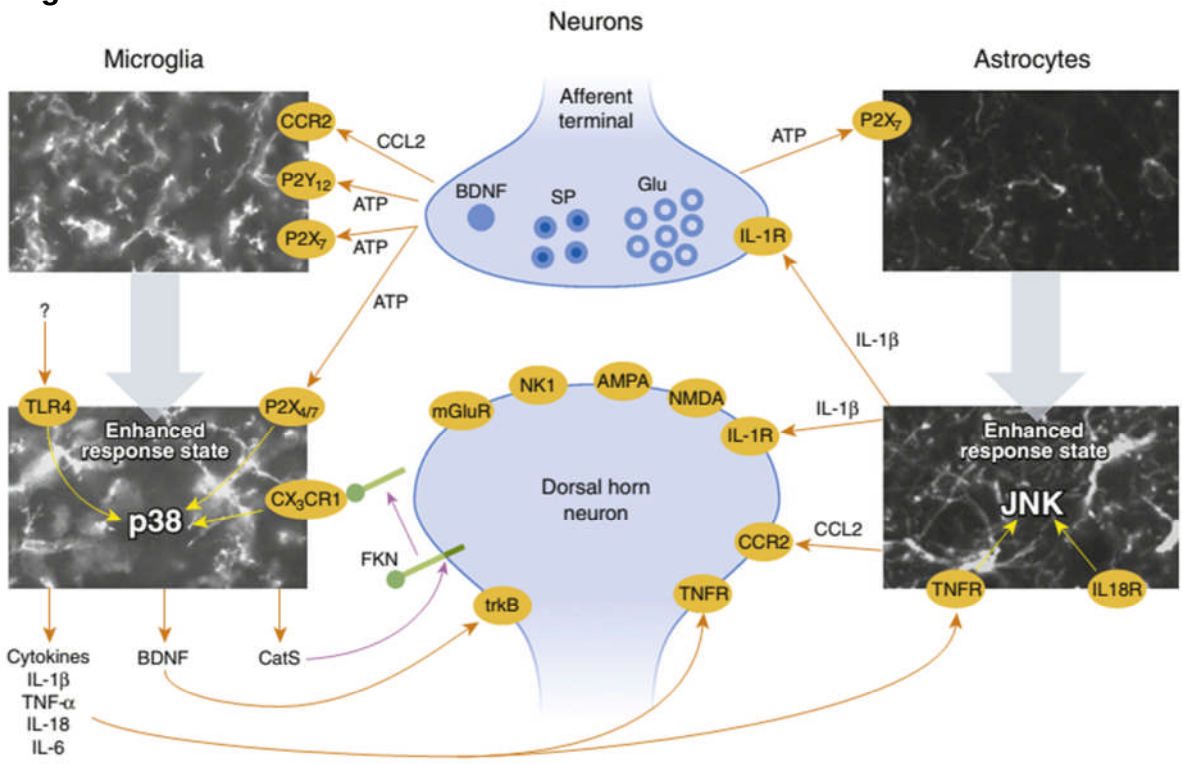


Fig 1.8 The interaction between glial cells and neurons induce and maintain central sensitization in the dorsal horn of spinal cord. The primary afferent terminal fibres release neurotransmitters which activated receptors expressed by neurons in the dorsal horn. The released ATP and chemokines activate glial cells which change morphology and release inflammatory mediators. In turn, these mediators sensitize neurons in the dorsal horn and contribute to central sensitization. Figure was from Mcmahon and Malcangio (2009).

1.2.5 The ascending and descending pain pathways

The nociceptive output from the second-order neurons crosses over the anterior white commissure of the spinal cord, ascends to the thalamus along the spinothalamic tract and projects to the cingulate and sensory cortex in brain. Nociceptive information is also sent to the medulla via spinoreticular and spinomesencephalic tracts. (Almeida et al. 2004; Tracey and Mantyh 2007).

A number of studies have characterized the responses of thalamic neurons to noxious stimulation. Neuronal firing was observed in the ventroposterolateral (VPL) nucleus of the thalamus in naïve rats and chronic constriction injury (CCI) -induced rats after mechanical stimulation and activity of VPL neurons was blocked by an NMDA-glycine antagonist, morphine and NMDA channel blocker (Bordi and Quartaroli 2000).

The anterior cingulate cortex (ACC) has been shown to play a major role in pain processing. Positron emission tomography revealed that the anterior cingulate cortex has significantly higher activity following pain-related signals (unpleasant) (Rainville et al. 1997). Two forms of long-term potential (LTP) have been demonstrated in the ACC and blocking pre-LTP in ACC prevented allodynia in nerve injury mice compared to saline treated mice (Koga et al. 2015).

In addition to the ascending pathways, pain is also modulated by the descending circuit which consists of various areas including rostral ACC, amygdala, periaqueduct gray (PAG) and rostroventral medulla (RVM) (Rainville 2002; Ossipov et al. 2010). Neurons are excited in amygdala by noxious stimuli to the somatic tissues such as joint, skin and muscle (Neugebauer and Li 2002). A recent study revealed that the mGlu1 receptor was associated with the pain-related increase of synaptic transmission and decrease of inhibitory transmission of neurons in amygdala (Ren and Neugebauer 2010). Stimulation of the PAG inhibits nociceptive responses of neurons in the dorsal horn of the spinal cord and this disinhibition of neurons was attributed to the release of some neurotransmitters such as serotonin (5-HT), norepinephrine (NE), Gly, and GABA in the spinal cord (Cui et al. 1999). Further study has shown different subtypes of 5-HT receptors produce differential effects on neuronal excitability in the PAG (Jeong et al. 2013). The RVM receives input from PAG and two main subpopulations of neurons have been described in the RVM. The ‘on cells’ increase firing and ‘off cells’ decrease firing prior to withdrawal reflex, these cells send information to the neurons in the dorsal horn of the spinal cord (Fields 2004). There

is good evidence that this system is involved in chronic pain mechanisms in that 'on cells' and 'off cells' in RVM are sensitized by innocuous and noxious stimuli in spinal nerve ligation (SNL) -induced neuropathic pain (Carlson et al. 2007) and SNL- induced allodynia was reversed by intrathecal injection of lidocaine in RVM (De Felice et al. 2011). NMDA receptors, serotonin and neurokinin-1 receptors in RVM are implicated in facilitating pain transmission via the descending pain pathway (Zhang and Hammond 2009; Da Silva et al. 2010; Wei et al. 2010).

Overall, pain is modulated by both ascending and descending pathways between spinal cord and cerebral cortex via various neurotransmitters and signalling pathways.

1.3 Contribution of inflammation to pain

Inflammation is a physiological or pathological process initiated by noxious stimuli such as infection or tissue injury (Medzhitov 2008). It modulates pain by the release of multiple inflammatory mediators from immune cells (Moalem and Tracey 2006). The contribution of pro-inflammatory mediators such as TNFα, IL1β and prostaglandins to pain are well documented in previous studies (Dray 1995; Moalem and Tracey 2006). Herein, the section has a brief induction to the contribution of key inflammatory mediators relevant to pain.

Intra-plantar repeated injection of TNFα, IL1β and IL8 produced persistent mechanical nociceptor hypersensitivity (Cunha et al. 1992; Sachs et al. 2002). Expression of TNFα, IL1β, IL6 and iNOS was up-regulated in spinal cord glial cells via activation of NFκB and mitogen-activated protein kinases (MAPKs) after stimulation with supernatant from damaged sensory neurons (Kim et al. 2007). TNFα and IL6 can increase neuronal hyperexcitability and inhibit the inhibitory interneurons respectively and IL1β shows both of the effects (Kawasaki et al. 2008). Moreover, spinal administration of all these cytokines can produce heat hyperalgesia via phosphorylation of CREB (Kawasaki et al. 2008).

The contribution of IL1β to thermal hyperalgesia has been demonstrated in a drug interventions study in the Complete Freud's adjuvant (CFA)-induced model (Safieh-Garabedian et al. 1995) and IL1β produces the inflammatory pain hypersensitivity by up-regulation of spinal COX2 and prostaglandin E2 (PGE2) (Samad et al. 2001). Spinal administration of IL1β and TNFα increases spinal expression of COX2 and

produces thermal hyperalgesia (Narita et al. 2008). Moreover, expression of NF-κB is up-regulated in the spinal cord of CFA model (Narita et al. 2008). Subcutaneous injection of lL1β increases formalin-induced behavioural responses and pERK and p-p38 in neurons and microglia, indicating lL-1β-induced central sensitization of nociception via MAPK pathway (Yang et al. 2013). Interestingly, lL1β also induces rapid LTP of glycinergic synapse, which was shown for the first time in superficial dorsal horn of the spinal cord (Chirila et al. 2014).

Local sciatic injection of TNFα produces transient thermal hyperalgesia and mechanical allodynia and nerve edema (Wagner and Myers 1996). Intramuscular injection of TNFα produces muscular hyperalgesia and increased protein level of CGRP, NGF and PGE2 (Schäfers et al. 2003). Knock down of TNFα receptor (p55) abolishes thermal and mechanical hyperalgesia in a neuropathic injury model (brachial plexus avulsion) and the mechanical allodynia was reversed by TNFα antibody and TNFα synthesis inhibitor (Quintao et al. 2006). TNFα increases expression of BDNF in trigeminal ganglion sensory neurons via influx of sodium channel and p38-MAPK (Balkowiec-Iskra et al. 2011). Treatment of TNFα also has been demonstrated to increase BDNF, TrkB, CGRP and SP in dorsal root ganglia (DRG) neurons, which are essential neurotransmitters for pain transmission (Lin et al. 2011).

Intradermal injection of PGE2 reduces mechanical threshold of C-fibres (Chen et al. 1998) and direct application of PGE2 to PAG and RVM decreases paw withdrawal latency, activates ‘on cells’ and inhibits ‘off cells’, which indicates PGE2-induced hyperalgesia (Heinricher et al. 2004). PGE2 induces thermal hyperalgesia and inflammatory nociception via EP1 receptor and sensitizes TRPV1 (Moriyama et al. 2005). PGE2 and its analogue were also revealed to increase the expression of IL6 in DRG neuron via the receptor EP4 and activation of PKA, PKC, ERK, CREB and NFκB in a neuropathic pain model. (St-Jacques and Ma 2011). EP1 receptor antagonist and microsomal prostaglandin E synthase-1(mPGES1) knock out reduce the activation of microglia and PGE2 blocked microglial transmission, indicating a role for PGE2 in maintenance of pain (Kunori et al. 2011). A recent study reported that PGE2 stimulated externalization of it receptor EP_4 in DRG neurons, which further supports PGE2 in nociceptive sensitization and results in inflammatory pain (St-Jacques and Ma 2013).

NGF is heavily implicated in the sensitization of nociceptors in inflamed tissue (Koltzenburg et al. 1999). The biological effects that endogenous NGF is associated with chronic pain were firstly demonstrated by using TrkA-IgG to sequester NGF in sensory neurons. Infusion of TrkA-IgG to the skin of the lower leg reduces capsaicin and carrageenan-induced thermal hyperalgesia and decreased CGRP in sensory neurons (Mcmahon et al. 1995). Intra-plantar injection of NGF produces thermal hyperalgesia and recruited neutrophil accumulation and this effects was blocked by 5-LOX inhibitor (Bennett et al. 1998). Expression of NGF is increased during inflammation (Woolf et al. 1998). Anti-NGF neutralizing antibody attenuates thermal and mechanical hypersensitivity and down regulates levels for SP , CGRP and c-fos in dorsal horn neurons, indicating role for NGF in inflammatory pain (Woolf et al. 1998). Both an anti-NGF antibody and an anti-TrkA antibody reverse inflammatory (formalin-induced and CFA) and neuropathic (CCI and SNL) pain, which indicates the critical role of NGF in established pain (Ugolini et al. 2007; Wild et al. 2007).

A mountain of studies has investigated the role of pro-inflammatory mediators in pain induction. On the other hand, there are also some cytokines such as IL10 and IL4, which have anti-inflammatory and anti-nociceptive roles. IL10 reduces endotoxin and carrageenan-induced inflammatory pain and CCI-induced neuropathic pain and down-regulates the expression of IL1β and NGF (Kanaan et al. 1998; Wagner et al. 1998; Willemen et al. 2014). Naïve IL4 knock out mice show tactile allodynia and CCI-induced increase of TNFα, IL1β, IL10 and IL13 (Uceyler et al. 2011). Expression of IL4 is up-regulated in injured sciatic nerve (Kiguchi et al. 2015). Treatment of IL4 could inhibit partial sciatic nerve ligation-induced expression IL1β and CCL3 in macrophages and attenuate tactile allodynia and thermal hyperalgesia (Kiguchi et al. 2015). IL4-transfected DRG neurons reduces SNL-induced mechanical allodynia and thermal hyperalgesia and inhibits the expression of c-fos, IL1β, PGE2 and p-p38 MAPK in the model (Hao et al. 2006).

1.4 Osteoarthritic pain

1.4.1 Peripheral and central mechanisms contribute to OA pain

Pain is the cardinal symptom that drives patients to seek treatment for OA. Osteoarthritic pain is studied world-wide in preclinical and clinical aspects which provide a mountain of evidences that sensitization of pain appears in OA. This sensitization of pain results from changes in nociceptors in joints and neuropathic components which transduce pain signals in spinal cord, brain stem, thalamus and cerebral cortex (Schaible 2012). However, when does pain appear and what causes pain in OA is still elusive. As cartilage is not -innervated and avascular, the sources of pain must be due to joint-associated structures such as synovia, bone marrow, meniscus, muscles and ligaments (Konttinen et al. 2012). Activity and aberrant loading of joints may be the major driving force contributing to initial pain in OA (Felson 2009).

Inflammatory mediators released from chondrocytes, macrophages and fibroblasts in joints are likely to contribute to OA pain. The mechanical stimulation of joints and inflammatory mediators can directly sensitize nerve fibres in the joints by acting on ion channels or receptors in the nociceptors, which are essential for initiation of OA pain at peripheral sites (Schaible et al. 2009; Schaible et al. 2011). Pro-inflammatory cytokines such as TNFα, ILβ, IL6 and IL17 can directly activate the nociceptive system by binding to their receptors, which induce peripheral sensitization of pain (Schaible 2014).

Intra-articular injection of TNFα increases the sensitization of C-fibre to joint stimulation in a dose-dependent manner and enhances neuronal excitability in isolated DRG neurons in naïve rats (Richter et al. 2010). Both peripheral and spinal administration of TNFα increases neuronal hyperexcitability in the spinal cord after joint stimulation (Konig et al. 2014). Intra-articular injection of IL1β to temporomandibular joints induces joint pathology and joint pain (Lai et al. 2006). Pathology and pain behaviour are dependent on spinal expression of IL1β, indicating mechanisms from primary injury site to central nervous system (Fiorentino et al. 2008). Increased expression of IL1β indicates higher pain scores in symptomatic knee OA (Attur et al. 2011). NGF was firstly detected and was shown to be up-regulated in human osteoarthritic chondrocytes in 2002, which implicates a role of NGF in OA pathogenesis (Iannone et al. 2002). Later on, the increase in NGF was revealed in the vascular channels of osteochondral junction, which also contained CGRP immunoreactive sensory nerve fi-

bres, indicating an association of NGF with OA pain (Walsh et al. 2010). Intra-articular injection of NGF produces weight bearing asymmetry and mechanical allodynia in naïve rats. Moreover, weight bearing asymmetry in OA rats was exacerbated by intra-articular injection of NGF (Ashraf et al. 2014). In addition, intra-articular injection of NGF increases joint extension induced neuronal firing and expands the receptive field of spinal neurons in MIA-induced OA model (Sagar et al. 2015).

TRP channels are a family of ion channels involved in pain transduction and transmission (Julius 2013). Some members of TRP channels have been found in chondrocytes and synovial fibroblasts of OA patients (Engler et al. 2007; Gavenis et al. 2009). Expression of TRPV1 was increased in human OA synovia and intra-articular injection of TRV1 antagonist reverses mechanically evoked firing of joint afferents and weigh bearing asymmetry in the MIA-induced OA model (Kelly et al. 2013). Oral dosing of a TRPV1 antagonist (A-889425) attenuated pain behaviour and spontaneous firing of spinal wide dynamic range (WDR) and nociceptive specific (NS) neurons in the MIA model (Chu et al. 2011). Systemic administration of a TRPA1 antagonist (A-967079) decreased WDR neuronal response in the MIA model after noxious mechanical stimulation (Mcgaraughty et al. 2010). Sodium channels such as $Na_v1.8$ expressed by small nerve fibres are also implicated, block of $Na_v1.8$ reduced the neuronal response in joint afferents induced by noxious rotation and attenuates pain behaviour in the MIA model (Schuelert and Mcdougall 2012) . ASIC3 was up-regulated in joint afferents and strongly associated with pain behaviour in the MIA model of OA pain and ASIC3 selective peptide blocker (APETx2) reverses the pain behaviour via down-regulation of ASIC3 (Lzumi et al. 2012).

There is mounting evidence that peripheral and central sensitization contribute to OA pain (Arendt-Nielsen et al. 2010). CGRP-immunoreactive and SP-immunoreactive fibres were shown to increase in soft tissues and synovium in the hip joints of OA patients, indicating a role for these neuropeptides in OA pain (Saxler et al. 2007). Neuronal responses (Aδ and C fibre) evoked by electrical or natural stimulation wereincreased in the dorsal horn of MIA rats compared to saline rats, indicating central mechanism of OA pain (Harvey and Dickenson 2009). Activation of microglia and astrocyte were observed in MIA-induced OA and microglia activation was correlated with distal allodynia, which further supports a role for central sensitization in OA pain (Sagar et al. 2011). The increase of CGRP, activating transcription factor-3 (ATF3)

and growth associated protein-43 (GAP43) immunoreactive neurons in DRG cell bodies and microglia in the dorsal horn suggests there may be neuropathic components in the MIA model (Orita et al. 2011). CGRP was up-regulated in the dorsal horn of spinal cord and blocking CGRP attenuates pain hypersensitivity in the model (Ogbonna et al. 2013). Descending serotonergic facilitation mediates neuronal responses in the deep dorsal horn in MIA-induced pain (Rahman et al. 2009), and milnacipran, a serotonin/noradrenaline reuptake inhibitor, inhibited neuronal responses in the deep dorsal horn and reversed OA pain via descending serotonergic and noradrenergic pathways (Burnham and Dickenson 2013). Phosphorylation of ERK1/2 and p38 MAPK, critical intracellular transduction signals for central sensitization, is up-regulated in MIA-induced OA (Lee et al. 2011).

Overall, multiple peripheral and central components contribute to OA pain (Malfait and Schnitzer 2013) (**Fig1.9**).

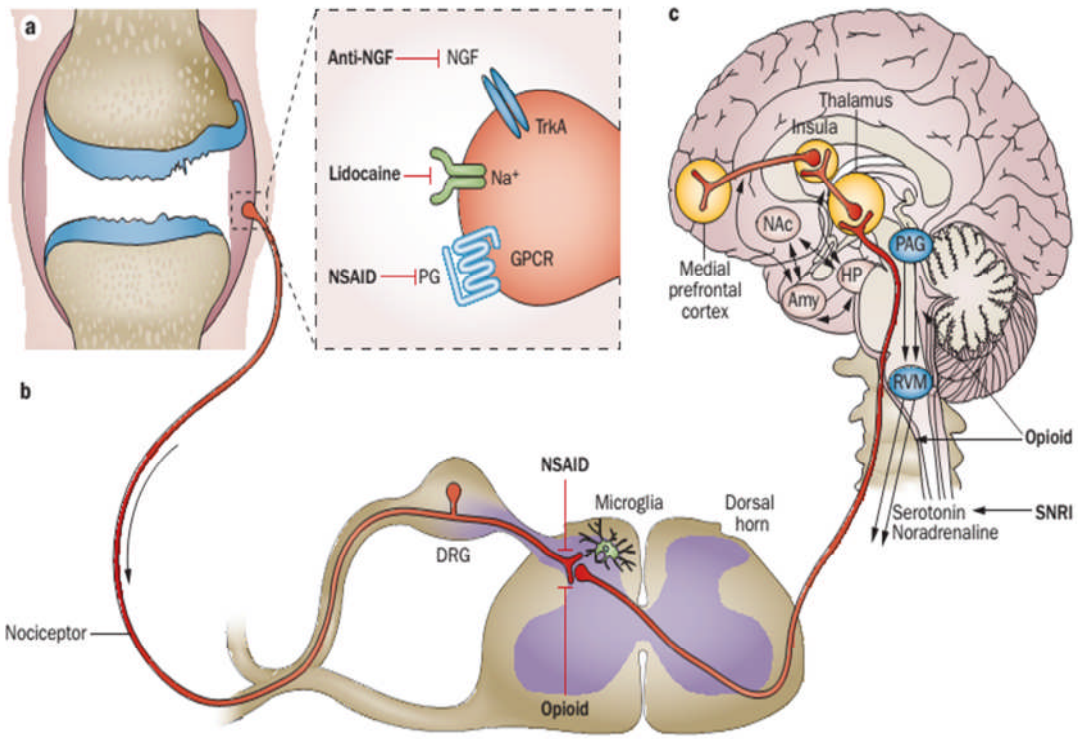


Fig 1.9 The transmission pathway of OA pain from peripheral site (knee joint) to central nervous system (brain) and its potential analgesic targets. Figure was from Malfait and Schnitzer (2013). Abbreviations: Amy, amygdala; HP, hippocampus; NAc, nucleus accumbens; PAG, peri-aqueductal grey; PG, prostaglandin; RVM, rostral ventromedial medulla; SNRI, serotonin noradrenaline reuptake inhibitor.

1.4.2 Inflammatory mediators and pain in osteoarthritis

As described above, inflammation is closely associated with both pathogenesis of OA (see 1.1.5) and pain (see 1.3) and alteration of inflammatory mediators and established pain behaviour have been shown in OA.

Inflammatory cytokines, such as TNFα, TNFβ, IL1β, IL2, IL12, IL17 and interferon-γ (IFNγ) were significantly higher and expression of IL10 was significantly lower in the synovia of OA patients (Vernal et al. 2008). Expression of TNFα, MMP3 and MMP9 was significantly higher and IL10 was significantly lower in serum of OA patients. In the synovia and cartilage, expression of MMP-2, -3, -9 and IL10 was significantly higher in OA patients (Wassilew et al. 2010). In addition, all the tested cytokines (TNFα, IL1α, IL8, and IL10), MMP-3, -9, and TIMP-1 were higher in the subchondral bone in OA patients (Hulejova et al. 2007). Moreover, TNFα was significantly correlated with MMP1 and MMP3 in synovial tissue (Wassilew et al. 2010). Expression of COX2, mPGES1, iNOS was increased in OA chondrocytes after treatment with advanced glycation end products (AGEs)- bovine serum albumin, which has been shown to involve in tissue stiffness in articular cartilage and signalling pathways for pro-inflammatory responses via the receptor for AGEs (Loeser et al. 2005; Nah et al. 2008). Expression of TNFα, IL6, RANKL was expressed higher in human OA compared to control (Toncheva et al. 2009). IL6, IL8 and MMP13 expression was increased in osteophytes from OA patients and mechanical stimulation increased expression of IL6 and IL8 (Sakao et al. 2009). IL18 increased COX2 and PGE2 in synoviocytes, and up-regulated expression of COX2, PGE2 and TNFα in chondrocytes in OA patients (Fu et al. 2012). Expression of IL6, IL8, MCP1, vascular endothelial growth factor (VEGF) was up-regulated in human chondrocytes when stimulated with OA synovial fluid (Hoff et al. 2013). Expression of IL6 and TNFα were increased in the synovial fluid from injury joint of canine OA compared to contralateral joint (Venn et al. 1993). Expression of TNFα, COX2, iNOS and NF-κB was significantly higher in the cartilage of MIA-induced OA joint (Kim et al. 2012).

The anti-inflammatory cytokines (IL4, IL10 and IL13) reduced TNFα-induced COX2 and PGE2 in synovial fibroblast from human OA (Alaaeddine et al. 1999). IL13 reduced expression of TNFα, IL1β and increased IL1Rα in synovial membrane of OA (Jovanovic et al. 1998).

All these studies show that pro-inflammatory mediators greatly contribute to pathogenesis of OA such as acceleration of cartilage degradation, inhibition of proteoglycan synthesis and induction of chondrocytes apoptosis and anti-inflammatory cytokines played the converse role (Sutton et al. 2009; Bondeson et al. 2010).

In OA patients, the pressure pain threshold (PPT) was significantly lower than in healthy volunteers, indicating high pain sensitivity, hyperalgesia and higher disability scores in the patients (Imamura et al. 2008). OA patients had lower PPTs and higher heat pain rating. In addition, expression of C-reactive protein (CRP) and IL6 was higher in OA patients and enhanced pain sensitivity was associated with increased CRP and IL6, suggesting contribution of inflammation to human OA pain (Lee et al. 2011). Central sensitization has been investigated in OA patients via PPTs and spreading sensitization. Patients with severe pain had lower PPTs and negative correlation was found between PPTs and visual analogue scale (VAS), indicating central sensitization of pain in OA (Arendt-Nielsen et al. 2010).

Various models of OA have been used to investigate OA pain, such as the MIA-induced model (Guingamp et al. 1997; Combe et al. 2004), MNX model (Bove et al. 2006; Mapp et al. 2010; Mapp et al. 2013) and DMM model (Malfait et al. 2010).

MIA is a glycolysis inhibitor that results in cartilage degeneration and joint damage (Janusz et al. 2001). Intra-articular injection of MIA induces apoptosis of chondrocytes and causes loss of cartilage and lesions of subchondral bone, similar to those observed in human OA (Dunham et al. 1993; Guzman et al. 2003). Thus, this model is widely used to investigate the peripheral and central mechanisms of osteoarthritic pain. Herein, I mainly focus on the contribution of inflammatory mediators to OA pain in this model.

Pain behaviour and synovial inflammation were observed in the MIA model from day 3 until day 14, indicating a quickly developed pain behaviour and joint pathology in this model (Beyreuther et al. 2007). Linear regression has shown activity of NFκB and expression of IL1β and IL6 were correlated with pain-related sensitivity in MIA model, indicating an important role of inflammatory signals in OA pain (Bowles et al. 2014). Expression of COX1 and COX2 in the spinal cord and pain behaviour were enhanced in the MIA model, suggesting essential roles of COXs in OA pain (Prochazkova et al. 2009). Mechanical allodynia developed in MIA-induced OA from day 4 until day 28 post-MIA injection, pro-inflammatory cytokines (TNFα,IL6 and

NGF) were increased in synovia and capsule from joints in the model and numbers of activated microglia in the spinal cord and CGRP-immunoreacitve neurons in DRG were increased in this model, indicating sensory innervated joint of inflammatory pain to neuropathic pain in the model (Orita et al. 2011). Expression of TNFα, IL1β, neuropeptide Y and galanin increased but CGRP and SP decreased in DRG in the MIA model, which was similar to SNL neuropathic model. In addition, expression of TNFα, IL1α, IL1β, IL13,IL17 ,VEGF and thymus chemokine was increased and expression of IL4 and IL10 was decreased in the spinal cord of MIA model (Im et al. 2010). Mechanical allodynia developed in MIA model and expression of IL1β, IL6, IL15, iNOS, MMP13, and receptor for advanced glycation end product (RAGE) was enhanced in the model, which was attenuated by coenzyme Q10 (Lee et al. 2013). Pain behaviour established and chemokines such as CCL2, CCL 7 and CCL9 were also increased in joints of MIA model (Dawes et al. 2013). In a recent study, expression of IL1β, IL6, nitrotyrosine and iNOS was increased and pain behaviour was established in the MIA model and IL1β induced high expression of MMP3, MMP13 and ADAMTS5 in human chondrocytes, which were all attenuated by ursodeoxycholic acid (Moon et al. 2014). Pain behaviour in the MIA model is associated with activation of pERK in neurons and p-p38-MAPK in microglia of the spinal cord (Lee et al. 2011) and was maintained by activated microglia and astrocytes (Sagar et al. 2011). Pain behaviour, joint pathology and expression of ATF3, neuropeptide Y and GAP43 were increased in the MIA model, indicating peripheral fibre regeneration and neuronal injury in this model (Ferreira-Gomes et al. 2012; Thakur et al. 2012). These alterations of inflammatory cytokines and neuropeptides in the sensory and spinal neurons further support the critical roles of these mediators in peripheral and central sensitization of OA pain in the MIA model (Im et al. 2010).

Some other models were also used to investigate OA pain as mentioned above. Pain behaviour, synovial inflammation and angiogenesis have been demonstrated in the MNX-induced OA model (Ashraf et al. 2011; Mapp et al. 2013). Moreover, dexamethasone, indomethacin and PPI-2458 (angiogenesis inhibitor) reduced synovial inflammation and angiogenesis as well as pain behaviour, which suggests a role for-inflammation in OA pain in the MNX model (Ashraf et al. 2011). Decreased paw withdrawal threshold has been shown in DMM-induced OA rats compared to sham control and ADAMTS-5 knock out mice didn't show the behaviour (Malfait et al.

2010). RT-PCR assay revealed that expression of MMP3, CCL21, and insulin-like growth factor (IGF-1) was significantly higher in the DMM mice, compared to sham mice, and expression of CCR7 and IL33 was higher in young DMM mice (12 weeks old) (Loeser et al. 2012).

1.4.3 Treatments for OA pain

As treatment for OA pain has been detailed in the previous section (see 1.1.6). Here I just make a brief summary for the treatment of OA pain. Some traditional drugs such as non-selective NSAIDs (Orita et al. 2011; Balmaceda 2014), COX2 selective-inhibitors (Stengaard-Pedersen et al. 2004; Laine et al. 2008), cannabinoids agonists (Richardson et al. 2008; Burston et al. 2013), opioids (Avouac et al. 2007; Vonsy et al. 2009), corticosteroids (Jones and Doherty 1996; Huebner et al. 2014) and hyaluronic acid (Petrella et al. 2008; Ikeuchi et al. 2014) are used to treat OA pain. However, some side effects have been demonstrated in these drugs such as gastrointestinal bleeding and renal toxicity with NSAIDs (Murray and Brater 1993; Makris et al. 2010; Sostres et al. 2010) and tolerance for opioids (Dumas and Pollack 2008). Some potential drugs are under preclinical investigation but finding novel drugs with high potency and safety are still of great interest to improve life quality of OA patients.

1.5 Resolvins

1.5.1 Biosynthesis of resolvins from Ω-3 poly-unsaturated fatty acids

Resolvins are endogenous lipid mediators generated during resolution of inflammation. They were identified for the first time in the exudates from TNFα-induced inflammation in a mouse air-pouch model after treatment with docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA) and aspirin (Serhan et al. 2000; Serhan et al. 2002). These findings have revealed that resolving inflammation with Ω-3 PUFAs is an active process producing a number of specialized pro-resolving lipid mediators (SPMs) such as resolvins, protectins and maresins, and contributes to restoration of tissue homeostasis and functional recovery (Buckley et al. 2014; Serhan 2014). Resolvins are the main products of DHA and EPA and are classified into D-series and E-series respectively (Serhan and Chiang 2008).

DHA (22:6, n-3) is a long chain Ω-3 PUFA with 22 carbons and 6 double bonds (Stillwell and Wassall 2003), present in fish oil, brain and retina from mammals and marine products and is beneficial for health (Horrocks and Yeo 1999; Guesnet and Alessandri 2011). DHA can be converted into the bioactive compounds including D-series resolvins, protectinD1 (PD1) / neuroprotectin D1 (NPD1) and maresins via COX2 / aspirin / 5-LOX or 15-LOX / 5-LOX pathway (Spite et al. 2014).

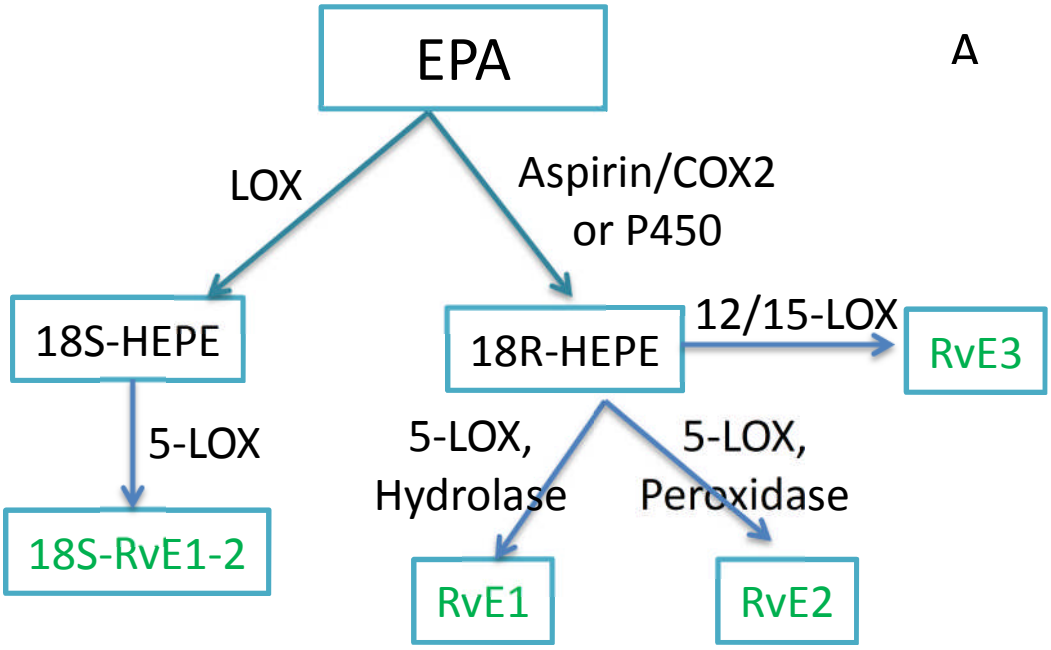
15-LOX or 5-LOX is a highly structural conserved member of the arachidonate lipoxygenases, having two functional domains (β-barrel domain and the catalytic domain) and 5-LOX is involved in leukotriene biosynthesis (Peters-Golden and Brock 2003).

In the 15-LOX / 5-LOX pathway, DHA is firstly converted into 17S-H(p)DHA /17S-HDHA via 15-LOX and is rapidly metabolized to 7S(8)-epoxy-17S-HDHA and 4S (5)-epoxy-17S-HDHA or 16S,17S-epoxide- protectin by enzymatic epoxidation (see fig 1.10B). Finally, those precursors are metabolized into their corresponding bioactive compounds such as RvD1-4 via 5-LOX, RvD 5-6 via peroxidase and PD1 / NPD1 via 15-LOX and hydrolase (Hong et al. 2003; Sun et al. 2007; Kohli and Levy 2009; Serhan and Petasis 2011).

COX2 is an inducible enzyme expressed at a low level under normal conditions, but it can be rapidly induced by various stimuli such as inflammatory cytokines, growth factors and bacterial endotoxins (Smith et al. 1996). COX2 has four functional domains including amino-terminal hydrophobic signal peptide, the dimerization domain,

the membrane-binding domain and the catalytic domain which is composed of peroxidase and cyclooxygenase active sites (Chandrasekharan and Simmons 2004). In the presence of aspirin, COX2 is acetylated at serine 530 within the catalytic site , which inhibits the cyclooxygenase activity but has little effects on peroxidase activity, indicating the seperate function and physical property of the two active sites (Smith et al. 1996). In addition, COX_2 homodimer was asymmetric acetylated by aspirin and has various activity on oxygenation of arachidonic acid, EPA and DHA (Sharma et al. 2010). DHA is converted to 17R-H(p)DHA/ 17R-HDHA (also named 17(R)HDoHE) via aspirin / COX2 or cytochrome P450, and then produces aspirin triggered D-series resolvins (AT-RvD1-4) via 5-LOX (Hong et al. 2003; Sun et al. 2007; Kohli and Levy 2009). In addition, DHA is also converted into 13S, 14S-epoxy-maresin via 12-lipoxygenase (12-LOX) and maresin 1 via a new epoxide hydrolase in human neutrophils and macrophages (Dalli et al. 2013; Abdulnour et al. 2014).

EPA is the other main Ω-3 poly-unsaturated fatty acid beneficial for fetal development, healthy aging, cardiovascular and neurodegenerative diseases (Swanson et al. 2012) and is the precursor for the bio- active E-series resolvins. Similar to DHA, EPA is converted into 18R-hydroperoxy-EPE (18-HpEPE), the precursor of the E-series resolvins, via aspirin / COX2 or CYP450 and then biosynthesizes bioactive RvE1-2 via 5-LOX and subsequent enzymatic epoxidation or RvE3 via 12-LOX / 15-LOX. In addition, EPA could be converted into 18S-RvE1 and 18S-RvE2 via 5-LOX (Serhan 2004; Oh et al. 2011; Serhan and Petasis 2011; Isobe et al. 2012; Buckley et al. 2014). The metabolic pathways for resolvins, protectins and maresin from DHA and EPA are summarized in **Fig 1.10.**


EPA
A
LOX
Aspirin/COX2
or P450
18S-HEPE
18R-HEPE
12/15-LOX
RvE3
5-LOX
5-LOX,
Hydrolase
5-LOX,
Peroxidase
18S-RvE1-2
RvE1
RvE2

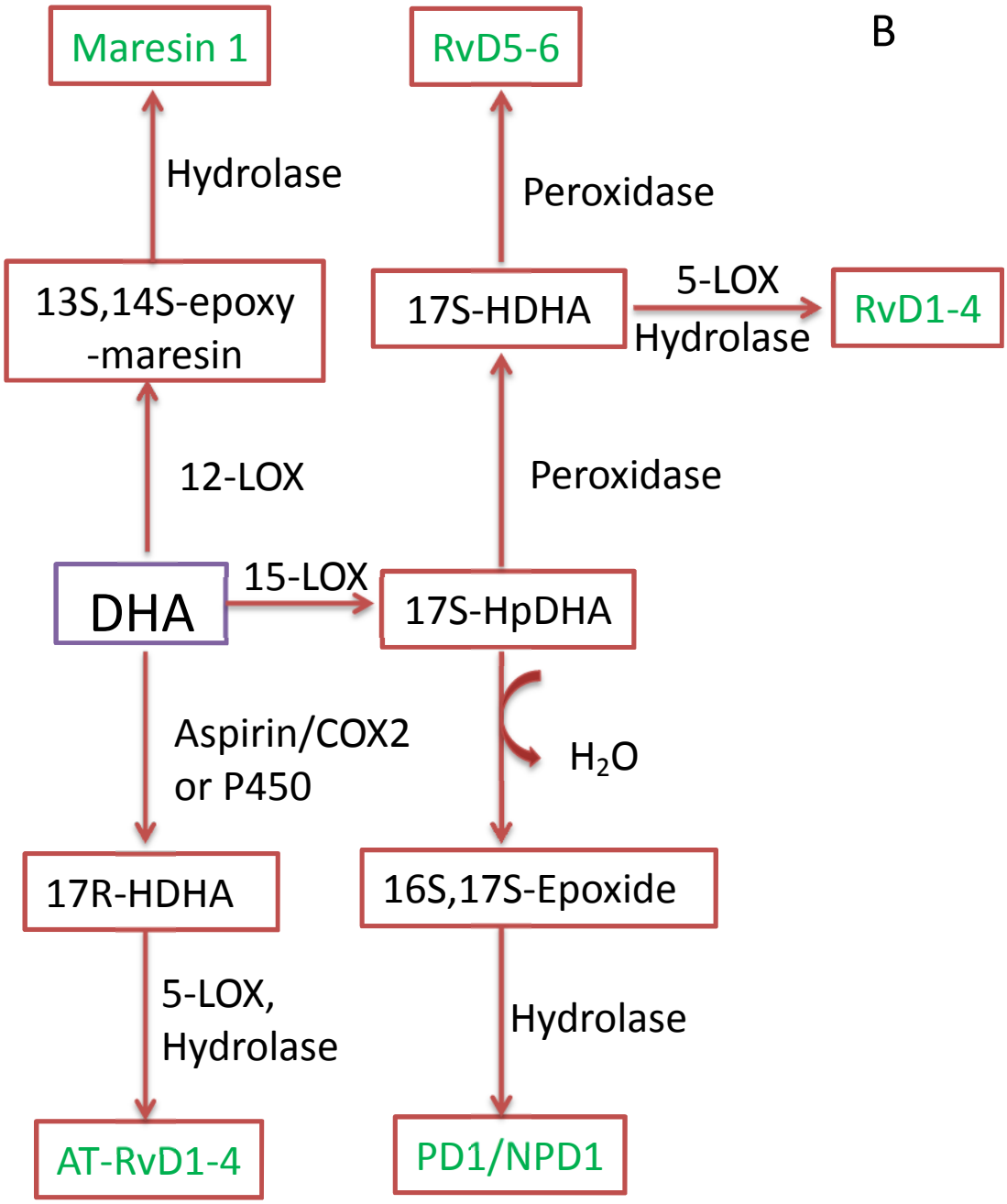


Fig 1.10 Biosynthesis pathways of resolvins, protectins and maresin 1 from EPA and DHA. E-series resolvins are produced from EPA via various metabolic enzymes (A). D-series resolvins, PD1/NPD1 and maresin 1 are generated from DHA via respective metabolic enzymes (B).

1.5.2 Resolvin receptor system

The biological functions of resolvins are mediated via their specific receptors, which belong to the GPCR superfamily (Cash et al. 2014). To date, four resolvin receptors have been identified, which are ALX and GPR32 for the D-series resolvins (Krishnamoorthy et al. 2010; Chiang et al. 2012; Dalli et al. 2013) and ChemR23 (Arita et al. 2005) and leukotriene B_4 receptor (BLT_1) (Arita et al. 2007) for the E-series resolvins.

ALX is a formyl peptide receptor involved in chemotaxis of PMNs or phagocytes in bacterial infected tissues or *Escherichia coli* cultures respectively via binding N-formyl methionyl peptide (Chiang et al. 2006) and was first identified in promyelocytic lineages (HL60 cells) and human neutrophils (Fiore et al. 1994). Later on, mouse ALX was cloned and studied in Chinese hamster ovary (CHO) cells (Takano et al. 1997) and rat ALX was cloned from peripheral leukocytes, which shows 74% and 84% homology of amino-acid sequences with human and mouse ALX respectively (Chiang et al. 2003). Currently, human GPR32 has been cloned (Marchese et al. 1998) and related pseudogene has been identified on chromosome 19 by RefSeq. ALX and GPR32 are expressed in leukocytes (neutrophils, monocytes/macrophages) (Devchand et al. 2003; Chiang et al. 2005), neurons and glial cells (Serhan et al. 2002; Abdelmoaty et al. 2013). ALX can modulate inflammatory arthritis via lipoxin A_4 (Sodin-Semrl et al. 2000; Fiore et al. 2005). The function of GPR32 is less understood as there are few reports on this receptor.

ChemR23 was first cloned and expressed in human monocyte-derived dendritic cells and macrophages (Samson et al. 1998). ChemR23 and BLT_1 receptors are expressed by leukocytes (neutrophils, eosinophils, monocytes/macrophages, dendritic cells) (Yokomizo et al. 1997; Uddin and Levy 2011), neurons (Xu et al. 2010) and glial cells (Croitoru-Lamoury et al. 2003; Connor et al. 2007). ChemR23 is also expressed by chondrocytes from OA patients and in macrophages and fibroblast-like synoviocytes in synovia from rheumatoid arthritis (RA) and OA patients (Berg et al. 2010; Kaneko et al. 2011).

1.5.3 The anti-inflammatory and pro-resolving roles of resolvins

As described above, resolvins and precursors were first discovered in inflammatory exudates and were shown to inhibit human polymorphonuclear leukocytes (PMNs) infiltration and microglial cytokine (IL1β) in dermal inflammation and peritonitis (Serhan et al. 2000; Serhan et al. 2002). Later on, the anti-inflammatory and pro-resolving actions of resolvins were reported in a number of studies.

RvD1 can directly bind to ALX and GPR32 to enhance macrophage phagocytosis (Krishnamoorthy et al. 2010) as well as limit recruitment of human PMNs and reduce prostaglandins and leukotrienes levels in murine peritonitis (Norling et al. 2012). Pre-treatment of RvD1 (intraperitoneal injection (i.p.), 1μg/kg, 5μg/kg) and post-treatment of RvD1 (intravenous, i.v, 5μg/kg) have been shown to reduce lipopoly-saccharide (LPS)-induced acute lung injury in mice and rats respectively (Wang et al. 2011; Wang et al. 2014). In addition, RvD1 acted to down-regulate the expression of TNFα, IL6, COX2 and iNOS, and inhibited MAPK and NFκB signalling (Wang et al. 2011) and increased activity of Na, K-ATPase and ALX/cAMP/PI3K signalling (Wang et al. 2014). RvD1 reduced LPS-induced TNFα in murine macrophage-like RAW 264.7 cells via inhibition of NFκB, zymosan-A-induced TNFα in peritonitis (Lee et al. 2013), and leukocyteinfiltration and outcome of peritonitis (Recchiuti et al. 2014). RvD1 reduced LPS-induced expression of COX2, PGE_2 and PGD_2 in lung fibroblasts (Wu et al. 2013) and attenuated LPS-induced expression of TNFα, IL1β, and iNOS. In addition, it inhibited activation of MAPK and NFκB in microglia from rats (Xu et al. 2013) and down-regulated superoxide dismutase-1 (SOD-1)-induced expression of TNFα and IL6 in spinal microglia from amyotrophic lateral sclerosis (ALS) patients (Liu et al. 2012).

Some other related actions of RvD1 have been shown in various models for diseas-es. RvD1 promoted IL4-induced alternative activation of murine microglia (cell line BV-2), phosphorylation of signal transducer and activators of transcription 6 (STAT6) and peroxisome proliferator-activated receptor gamma (PPARγ) activation (Li et al. 2014), reduces myofibroblasts proliferation in obstructed kidney with unilateral ure-teric obstruction (UUO) and inhibits pERK1/2 and p-AKT (protein kinase B) (Qu et al. 2012). It also has renoprotective effects on podocytes in adriamycin-induced nephropathy in a mouse model via improvement of proteinuria, glomerulosclerosis and tubulointerstitial fibrosis (Zhang et al. 2013). RvD1 is also reported to inhibit

transforming growth factor beta 1 (TGFβ1)-induced epithelial-mesenchymal-transition (EMT) in A549 lung cancer cells via ALX and GPR32. Moreover, RvD1 and RvD2 inhibited TGFβ1-induced invasion and migration of cancer cells (Lee et al. 2013). RvD1 and RvD2 enhance human macrophage efferocytosis to apoptotic osteoblasts (Mccauley et al. 2014) and decrease expression of TNFα, IL1β, IL6 and IL12 in adipose tissue from an obese mouse model (Claria et al. 2012).

Similar to RvD1, RvD2 has been shown to decrease neutrophil recruitment in microbial peritonitis, inhibit cytokines (TNFα,IL1β, IL6, IL10, IL17, IL23 MIP2) and lipid mediators (PGE_2 and LTB_4) in mouse model of microbial sepsis and enhance macrophage phagocytosis (Spite et al. 2009). Different from other D-series resolvins, RvD3 is produced in the late resolution phase and can inhibit neutrophil infiltration in peritonitis model and TNFα-induced dermal inflammation at a very low concentration (10ng/mouse) (Dalli et al. 2013). RvD3 and AT-RvD3 decrease levels of IL6, MCP1, PGD2, leukotriene B_4 (LTB_4) and thromboxane B_2 (TxB_2), up-regulate IL10 expression and enhance macrophage phagocytosis (Dalli et al. 2013). In *E. coli* inoculated mice, RvD5 enhanced macrophage phagocytosis via GPR32, which promoted bacterial clearance (Chiang et al. 2012).

AT-RvD1 inhibits LPS-induced levels for TNFα, IL6, COX2, MCP1 via ALX in bone marrow-derived macrophages (Bento et al. 2011) , decreases expression of IL6 in a mouse model for surgery-induced cognitive decline and reduces release of TNFα and superoxide production in LPS stimulated macrophages (Terrando et al. 2013).

In the mouse lung injury model, AT-RvD1 reduced lung vascular permeability, neutrophils, inflammatory cytokines (TNFα, IL6), and chemokines in the bronchoalveolar lavage fluid. Moreover, AT-RvD1 inhibited NFκB activation and secretion of TNFα and IL6 from immunoglobin (IG) stimulated alveolar macrophages and neutrophils (Tang et al. 2014). In addition, AT-RvD1 improved astrocytic morphology and prevented surgery-induced synaptic transmission and plasticity (Terrando et al. 2013). The anti-inflammatory and pro-resolving actions of D-series resolvins are summarized in **Table 1.2.**

Table 1.2 The anti-inflammatory and pro-resolving actions of D-series resolvins in various models

Lipid Mediator	Species /Model	Dose/ route	Action(s)	Ref
17(R) HDHA	Mouse/colitis	0.1, 0.3, 1μg/mouse (i.v.)	Reduces TNFα, IL1β, MIP2, NFκB	(Bento et al. 2011)
RvD1	Macrophage	10nM	Enhances macrophage phagocytosis via ALX and GPR32	(Krishnamoorthy et al. 2010)
	Mouse/LPS/lung injury	1μg/kg, 5μg/kg (i.p)	Reduces lung injury Inhibits TNFα, IL6, COX2 and iNOS, Inhibits MAPK and NFκB	(Wang et al. 2011)
	Rat/LPS/lung injury	5μg/kg (i.v)	Promotes alveolar fluid clearance Increases Na, K-ATPase activation via ALX/cAMP/PI3K	(Wang et al. 2014)
	Macrophages/LPS	50nM	Reduces TNFα via inhibition of NFκB Enhances phagocytosis	(Lee et al. 2013)
	Mouse/peritonitis	300ng/mouse (i.p)	Reduces TNFα	
	Microglia/LPS	10, 100nM	Reduces TNFα, IL1β, and iNOS Inhibits MAPK and NFκB	(Xu et al. 2013)
	Microglia/SOD1	26.6nM	Reduces TNFα, IL6	(Liu et al. 2012)
	Microglia	1,10,100nM 100nM	Increases microglia activation, Enhances STAT6 and PPRγ activation	(Li et al. 2014)
	Human macrophages	0.1-100nM	Enhances macrophage efferocytosis of apoptotic osteoblasts	(Mccauley et al. 2014)
	Mouse/peritonitis	1-100ng (oral) 0.1-100nM	Reduces leukocytes infiltration Limits recruitment of human PMNs via ALX and GPR32	(Recchiuti et al. 2014) (Norling et al. 2012)
	Mouse/peritonitis	0.1-10ng (i.v)	Decreases prostaglandin and leukotrienes via ALX	
	Mouse/UUO	4ng/g(i.v)	Inhibits fibroblast proliferation	(Qu et al. 2012)
	Mouse/nephropathy	4ng/g(i.p)	Renoprotective on podocytes	(Zhang et al. 2013)
	Cancer cells/TGF1β	1,10,100,200 n M	Inhibits EMT via ALX and GPR32	(Lee et al. 2013)

Continued

			Inhibits cell invasion and migration	
	Fibroblasts/LPS	10, 50,100nM	Decreases COX2, PGE2 and PGD2	(Wu et al. 2013)
	Mouse/high-fat model	10nM	Reduces TNFα, IL1β, IL6 and IL12	(Claria et al. 2012)
RvD2	Mouse/colitis	0.1, 0.3, 1μg/mouse (i.v.)	Reduces TNFa, IL1β, MIP2, NFκB	(Bento et al. 2011)
	Human macrophage	0.1-100nM	Enhances macrophage efferocytosis of apoptotic osteoblasts	(Mccauley et al. 2014)
	Mouse/ peritonitis	10-10000pg/mouse	Reduces leukocytes infiltration	(Spite et al. 2009)
	Mouse/microbial sepsis	100ng/mouse (i.v)	Decreases TNFα, IL1β, IL6, IL10, IL17, IL23, MIP2, PGE2 and LTB4 Enhances macrophage phagocytosis	
	Cancer cell/TGF1β	1,10,100,200 n M	Inhibits cell invasion and migration	(Lee et al. 2013)
	Mouse/high-fat model	10nM	Reduces TNFα, IL1β,IL6 and IL12	(Claria et al. 2012)
RvD3	Mouse/peritonitis	10ng/mouse (i.v)	Inhibits neutrophil infiltration Enhances macrophage phagocytosis Decreases IL6, MCP1, PGD2, LTB4, TxB2	(Dalli et al. 2013)
RvD5	Mouse/bacterial infection	0.01-100nM	Increases macrophage phagocytosis	(Chiang et al. 2012)
AT-RvD1	Macrophage/LPS	300μM	Reduces TNFα, IL6, COX2, MCP1 via ALX	(Bento et al. 2011)
	Mouse/colitis	0.1, 0.3, 1μg/mice (i.v.)	Reduces TNFα, IL1β, MIP2, NFκB	
	Mouse/lung injury	500ng/mouse (i.v)	Reduces lung injury Decrease TNFα, IL6, and NFκB activation	(Tang et al. 2014)
	Mouse/surgical model	100ng/mouse (i.p)	Reduces release of IL6	(Terrando et al. 2013)
	Macrophage/LPS	50nM	Decreases release of TNFα	
	Mouse/high-fat model	10nM	Reduces TNFα, IL1β, IL6 and IL12	(Claria et al. 2012)
AT-RvD3	Mouse/peritonitis	10ng/mouse (i.v)	Inhibits neutrophil infiltration Enhances macrophage phagocytosis Decreases IL6, MCP1, PGD2, LTB4 and TxB2	(Dalli et al. 2013)

Table 1.2 Anti-inflammatory and pro-resolving roles of D-series resolvins in various models and cell types. Abbreviation: 17(R)HDHA: 17(R)-hydroxy docosahexaenoic acid; RvD1-3,5: resolvin D 1-3,5; AT-RvD1,3: aspirin-triggered RvD1,3. i.p : intraperitoneal , i.v: intra-venous.

RvE1 has been reported to enhance macrophage phagocytosis in peritonitis and limits leukocyte recruitment in the TNFα-induced inflammatory air-pouch model, peritonitis model and 2,4,6-trinitrobenzene sulfonic acid (TNBS) induce colitis (Arita et al. 2005; Arita et al. 2005; Arita et al. 2006; Schwab et al. 2007). RvE1 increases phosphorylation of MAPK in human blood leukocytes, down-regulates expression of IL12 in dendritic cells , inhibits NFκB activation in HEK-chemR23 cells via ChemR23 (Arita et al. 2005), inhibits expression of TNFα, IL12, COX2 and iNOS in colitis (Arita et al. 2005) and reduces TNFα expression in peritonitis (Arita et al. 2006). It has been reported that RvE1 reduces PMN infiltration in a BLT_1-dependent manner (Arita et al. 2007), promotes macrophage phagocytosis and reduces neutrophil infiltration and pro-inflammatory cytokines (TNFα,IL1β and IL6) in peritonitis (Oh et al. 2011). In a rabbit periodontitis model, RvE1 protects inflammation (ligation and gingivalis)-induced bone loss and tissue damage (Hasturk et al. 2006). RvE1 reduces L-selectin and CD18 expression on both PMNs and monocytes which are critical for early leukocyte activation and transmigration of neutrophils respectively, and inhibits ADP and thromboxane receptor-stimulated platelet aggregation (Dona et al. 2008). In the asthma, RvE1 attenuates airway hyperresponsiveness, inhibits asthmatic inflammation and down-regulates IL13 expression (Aoki et al. 2008). RvE1 reduces myofibroblasts proliferation and macrophage infiltration in the obstructed kidney of mouse model and primary renal fibroblasts and these effects are mediated by ChemR23 (Qu et al. 2012). RvE1 was also reported to modulate bone resorption and bone formation (see 4.1.2).

Similar to RvE1, RvE2 is generated from 18-HEPE via 5-LOX in human neutrophils and is revealed to inhibit PMNs infiltration in peritonitis (Tjonahen et al. 2006). RvE2 also prevents chemotaxis of neutrophils, enhances macrophage phagocytosis and IL10 and interacts with BLT_1 and ChemR23 in human leukocytes (Oh et al. 2012). RvE3 was identified recently in eosinophils and was biosynthesized via 12/15-LOX and was shown to inhibit neutrophil infiltration in peritonitis and chemotaxis of PMNs from murine bone marrow (Isobe et al. 2012). The anti-inflammatory and pro-resolving actions of E-series resolvins are summarized in **Table 1.3.**

Table 1.3 The anti-inflammatory and pro-resolving actions of E-series resolvins in various models

Lipid Mediator	Species /Model	Dose/ route	Action(s)	Ref
RvE1	Mouse/peritonitis/air pouch	100ng/mouse (i.v)	Limits leukocytes recruitment	(Arita et al. 2005)
	Dendritic cells	1μg/ml	Decreases IL12	
	Lukocytes/HEK cells	100nM	Inhibits NFκB and increases MAPK	
	Mouse/peritonitis/colitis	1μg/mouse (i.p)	Prevents leukocytes infiltration	(Arita et al. 2005)
	Mouse/colitis	1μg/mouse (i.p)	Decreases TNFα, IL12, COX2 and iNOS	
	Mouse/peritonitis	100ng/mouse (i.v)	Inhibits PMNs infiltration and TNFα	(Arita et al. 2006)
	Rabbit/periodontitis	4μg/topical	Attenuates tissue damage and bone loss	(Hasturk et al. 2006)
	Mouse/peritonitis	1μg/mouse (i.p)	Inhibits leukocytes infiltration Enhances macrophage phagocytosis	(Schwab et al. 2007)
	Mouse/peritonitis	100ng,1μg (i.v)	Reduces PMNs infiltration via BLT1	(Arita et al. 2007)
	Human blood samples	30nM	Decreases L-selectin and CD18	(Dona et al. 2008)
	Human blood samples	100nM	Inhibits platelet aggregation	
	Mouse/asthma	1μg/mouse (i.p)	Attenuates airway hyperresponsiveness Inhibits airway inflammation and IL13	(Aoki et al. 2008)
	Mouse/peritonitis/air pouch	20,100ng(i.v)	Decreases PMNs infiltration	(Oh et al. 2011)
	Human macrophages	1,10nM	Increases macrophage phagocytosis Reduces TNFα,IL1β and IL6	
	Mouse/UUO,	4ng/g(i.v)	Inhibits fibroblast proliferation	(Qu et al. 2012)
	Renal fibroblasts	0.25-10ng/ml	Inhibits fibroblast proliferation via ChemR23	
18S-RvE1	Mouse/peritonitis/air pouch	20ng,100ng (i.v)	Decreases PMNs infiltration	(Oh et al. 2011)
	Human macrophages	1,10nM	Increases macrophage phagocytosis	
RvE2	Mouse/peritonitis	1, 10,100ng (i.v)	Reduces PMNs infiltration	(Tjonahen et al. 2006)
	Human leukocytes	10nM	Prevents neutrophil chemotaxis	(Oh et al. 2012)
	Human macrophage	1, 10,100nM	Enhances macrophage phagocytosis and IL10	
	Human whole blood	30nM	Decreases CD18 in leukocytes (integrin)	
RvE3	Mouse/peritonitis	10ng (i.v)	Inhibits neutrophil infiltration	(Isobe et al. 2012)
	PMNs	10nM	Prevents PMNs chemotaxis	

1.5.4 Actions of resolvins and other anti-inflammatory mediators on pain modulation

In addition to anti-inflammatory roles, anti-nociceptive and anti-hyperalgesic actions of resolvins are emerging and have been investigated in various pain models (Ji et al. 2011).

Pre-treatment with 17(R)HDoHE (i.p.), aspirin-triggered resolvin D precursor, attenuates both mechanical and thermal hyperalgesia in adjuvant-induced arthritis (AIA). Post-treatment with 17(R)HDoHE (i.p.) reduced mechanical hyperalgesia, inhibited activation of NF-kB and COX-2 in the DRG and the spinal cord and decreased mRNA level of COX2 in spinal cord (Lima-Garcia et al. 2011). Single dosing of 17(R) HDoHE (i.p) only decreased mechanical hyperalgesia at day 14 and didn't attenuate pain behaviour at day 35. Repeated administration of 17(R)HDoHE (i.p.) (once a day for 5 days or AT-RvD1 (i.p.) for 3 days) attenuated joint stiffness and mechanical hyperalgesia and down-regulated expression of TNFα and IL1β in the hindpaw (Lima-Garcia et al. 2011).

Intradermal pre-treatment with RvD1 attenuated TRP-mediated and formalin-induced pain (hind paw licking/flicking or flinching), prevented mechanical and thermal pain in CFA model and inhibited responses to TRPA1, TRPV3 and TRPV4 in (human embryonic kidney) HEK293T cells and DRG neurons (Bang et al. 2010). Spinal pre-treatment or post-treatment with RvD1 reduced the mechanical allodynia in the incision surgery model and skin/muscle incision and retraction (SMIR) model (Huang et al. 2011). RvD1 was reported to attenuate mechanical allodynia in chronic pancreatitis (CP) and inhibit expression of pNR1 (subunit of NMDA receptor), pNR2B, TNFα, IL1β and IL-6 in the spinal cord (Feng et al. 2012). AT-RvD1 attenuated TRPV3-mediated pain behaviour and reversed thermal hypersensitivity in the CFA model and farnesyl pyrophosphate (FPP)-induced model (Bang et al. 2012). Intrathecal injection of AT-RvD1 reduced mechanical hypersensitivity in carrageenan model and TNFα release in cerebrospinal fluid (CSF) from the model or cultured astrocytes (Abdelmoaty et al. 2013). Single or repeated administration of AT-RvD1 and RvD2 decreased mechanical allodynia and thermal hypersensitivity in reserpine-induced fibromyalgia-like model with these effects associated with increased levels of dopamine in the cortex and 5-HT in the thalamus (Klein et al. 2014). Spinal administration of RvD2 inhibited capsaicin and formalin-induced spontaneous pain and reduced

heat hyperalgesia and mechanical allodynia in carrageenan and CFA-induced models (Park et al. 2011). RvD2 was reported to block TRPV1/A1 agonist-induced currents in DRG neurons and reversed C-fibre-induced LTP in the spinal cord after electrical stimulation of sciatic nerve (Park et al. 2011).

Xu and colleagues conducted a series of experiments investigating anti-hyperalgesic effects of RvE1 on inflammatory, post-operative and neuropathic pain. They revealed intrathecal pre-treatment with RvE1 reduced formalin-induced second phase of pain behaviour via ChemR23 and post-treatment with RvE1 attenuated heat hyperalgesia in CFA and carrageenan models (Xu et al. 2010). Intraplantar or spinal administration of RvE1 attenuated formalin-induced and capsaicin-induced spontaneous pain and SNL-induced neuropathic pain. RvE1 reduced paw edema, neutrophil infiltration and expression of TNFα, IL1β, IL6, MCP1 and MIP1α in the hindpaws from a carrageenan model. Further experiments showed RvE1 attenuated inflammatory pain by blocking TRPV1, TNFα and ERK phosphorylation (Xu et al. 2010). In the same study, RvD1 was reported to attenuate inflammatory pain in carrageenan and CFA models and incision-induced post-operative pain by intrathecal and intraplantar administration (Xu et al. 2010). Pre-treatment with RvE1 (i.t) attenuated nerve injury-induced mechanical allodynia and inhibited microglia activation and TNFα expression in the spinal cord in the CCI model. Post-treatment with RvE1 reversed mechanical allodynia and thermal hyperalgesia in the SNL model. RvE also reversed LPS-induced microgliosis in a dose-dependent manner and TNFα release in cultured microglia (Xu et al. 2012).

Some other lipid mediators from Ω-3 PUFAs such as NPD1 (Park et al. 2011; Xu et al. 2013) and maresin1 (Serhan et al. 2012) or arachidonic acid derived lipoxins (Hu et al. 2012; Wang et al. 2014) also show potential anti-nociceptive and anti-hyperalgesic effects on pain modulation. The anti-nociceptive and anti-hyperalgesic actions of resolvins are summarized in **Table 1.4**.

Table 1.4 The anti-nociceptive and anti-hyperalgesic actions of resolvins in various models

Lipid Mediator	Species /Model	Dose/ route	Action(s)	Ref
17(R)HDoHE	Rat/AIA	300ng (i.p)	Attenuates mechanical and thermal hyperagesia	(Lima-Garcia et al. 2011)
	Day 3, single	300ng (i.p)	Attenuates mechanical hyperalgesia	
			Inhibits activation of NFκB and COX2 in spinal cord and DRG	
			Decreases m RNA level of COX2 in spinal cord	
	Day 14, single	600,900 (i.p)	Attenuates mechanical hyperalgesia	
	Day 3, repeat	300ng (i.p)	Attenuates joint stiffness and mechanical hyperalgesia	
			Decreases TNFα and IL1β in the hindpaw	
AT-RvD1	Day 3, repeat	100ng (i.p)	Attenuates joint stiffness and mechanical hyperalgesia	(Lima-Garcia et al. 2011)
			Decreases TNFα and IL1β in the hindpaw	
	Mouse/CFA/FPP	30μM (i.d)	Inhibits thermal hypersensitivity	(Bang et al. 2012)
	Keratinocytes	3μm	Inhibits TRPV3 activity	
	Rat/carrageenan	3,300ng (i.t)	Reverse mechanical allodynia	(Abdelmoaty et al. 2013)
		300ng (i.t)	Decrease TNFα in CSF	
	Astrocytes/LPS/INFγ	500nM	Reduces TNFα release	
	hAstrocytes/TNFα	265nM	Inhibits ERK activation	
	Mouse/fibromyalgia	300ng (i.v)	Inhibits mechanical allodynia	(Klein et al. 2014)
			Attenuates thermal hyperalgesia	
	Mouse/fibromyalgia	80ng (i.t)	Inhibits mechanical allodynia and thermal hyperalgesia	

Lipid Mediator	Species /Model	Dose/ route	Action(s)	Ref
RvD1	Mouse/carrageenan/CFA	20ng (i.t)	Inhibits Inflammatory pain	(Xu et al. 2010)
	Mouse/incision	20ng (i.pl.)	Attenuates post-operative pain	
	Mouse/ TRP-mediated	20ng (i.d)	Attenuates licking/flicking or flinching	(Bang et al. 2010)
	Mouse/formalin	20ng (i.d)	Attenuates licking/flicking or flinching	
	Mouse/CFA	20ng (i.d)	Attenuates mechanical and thermal pain	
	HEK293T/neurons	100-1000nM	Inhibits TRPA1, TRPV3 and TRPV4 channels	
	Rat/ incision surgery	20ng (i.t)	Attenuates mechanical allodynia and hyperalgesia	(Huang et al. 2011)
	Rat/SMIR	40ng (i.t)	Attenuates mechanical allodynia and hyperalgesia	
	Rat/CP	100ng/kg (i.t)	Inhibits mechanical allodynia	(Feng et al. 2012)
		10, 500ng (i.t)	Reduces phosphorylation of NMDA receptor subunits	
		10, 500ng (i.t)	Down-regulates TNFα, IL1β and IL6	
RvD2	Mouse/capsaicin/mustard oil	10ng (i.pl)	Attenuates spontaneous pain	(Park et al. 2011)
	Mouse/formalin	0.01, 0.1, 1ng (i.t)	Inhibits the second phase pain	
	Mouse/carrageenan/CFA	10ng (i.t)	Attenuates heat hyperalgesia and mechanical allodynia	
	Mouse/Capsaicin/ AITC	0.05,0.1ng/ml	Inhibits TRPA1/TRPV1 currents in DRG neurons	
	Mouse/sciatic never	10ng (i.t)	Reverse C-fibre-induced LTP	
	Mouse/fibromyalgia	300ng (i.v)	Inhibits mechanical allodynia Attenuates thermal hyperalgesia	(Klein et al. 2014)
	Mouse/fibromyalgia	40ng (i.t)	Inhibits mechanical allodynia Attenuates thermal hyperalgesia	

Lipid Mediator	Species /Model	Dose/ route	Action(s)	Ref
RvE1	Mouse/ formalin	1ng (i.t)	Reduces inflammatory pain via ChemR23	(Xu et al. 2010)
	Mouse/carrageenan	20ng (i.pl.)	Attenuates heat hyperalgesia	
			Reduces paw edema and neutrophil infiltration	
			Down-regulates TNFα, IL1β, IL6, MCP1 and MIP1α.	
	Mouse/CFA	1,3,10 ng (i.t)	Attenuates heat hyperalgesia	
		10, 30,100 ng (i.t)	Inhibits mechanical allodynia	
	Mouse/formalin/capsaicin	20ng (i.pl.)	Reduces spontaneous pain	
			Blocks TRPV1 and TNFα signalling	
	TNFα-induced allodynia	10ng (i.t)	Inhibits ERK phosphorylation	
	Mouse/SNL	10ng (i.t)	Decreases neuropathic pain	
	Mouse/CCI	100ng (i.t)	Reduces mechanical allodynia	(Xu et al. 2012)
			Inhibits microglia activation and TNFα expression	
	Mouse/SNL	100ng (i.t)	Attenuates mechanical allodynia and thermal hyepralgesia	
	Microliga/LPS	1, 10,100ng	Prevents microgliosis and TNFα release	

Table 1.4 The anti-nociceptive and anti-hyperalgesic roles of resolvins in various models. Abbreviation: 17(R)HDoHE: 17(R)-hydroxy docosahexaenoic acid; RvD1-2: resolvin D 1-2; AT-RvD1: aspirin-triggered RvD1. i.p : intraperitoneal , i.v: intravenous, i.d: intradermal, i.pl: intraplantar

1.6 Hypotheses and aims

The anti-nociceptive and anti-hyperalgesic actions of resolvins have been studied in various pain models; however, the potential analgesic effects of resolvins in models of OA pain have yet to be described. This thesis aims to investigate the therapeutic potential of 17(R)HDoHE to treat OA pain in two pre-clinical models and to investigate the underlying mechanisms of action.

The hypotheses tested are:

- The MIA procedure and MNX surgery can produce established pain behaviour and joint histopathology reminiscent of clinical OA.
- Resolvin receptors (ChemR23 and ALX) are expressed by the key structures in the knee joint likely to contribute to OA pain (synovia and bone) and levels of expression are altered in models of OA. Expression of the resolvin receptors is associated with expression of inflammatory cytokines and metabolic enzymes in the knee joint in these two OA models.
- Systemic administration of 17(R)HDoHE can block OA pain in these two models and the analgesic effects of 17(R)HDoHE on OA pain are mediated by effects on inflammatory cytokines and/or metabolic enzymes.
- The resolvin receptors and the key metabolic enzymes are expressed in human synovia and bone from OA sufferers and expression levels of the receptors are related to levels of inflammatory cytokines and the metabolic enzymes.

2 Materials and methods

2.1 Introduction

The animal experiments were performed on male Sprague-Dawley rats (130-150g) purchased from Charles River (Margate, Kent, UK) under the guidance of the Animals (Scientific Procedures) Act 1986 and guiding principles for behavioural laboratory animal science (Home Office, UK). Research on human samples was approved by generic ethics for the University Hospitals Nottingham (UHN) Trust Bio-bank, Research & Innovation (RSCH448). Human synovia and bone from joints were collected from OA patients undergoing total knee replacement (TKR) at the City Hospital, Nottingham and femoral heads were collected from trauma patients at UHN, Queen's Medical Centre campus.

2.2 Animal models of OA pain

Two models of OA, MIA (a chemical which induces chondrocyte death and cartilage degeneration, see 1.4.2) and MNX model (a surgery with transection of medial meniscus) were used in this study.

2.2.1 Anaesthesia and procedure preparation

Animals were anaesthetized by inhalation of isoflurane (3% for induction and 2.5% for maintenance) delivered in 1L/min O_2 (Abbott, Kent, UK) before MIA injection or MNX induction. Isoflurane was maintained at 2.5% once the rat was areflexic which was confirmed by pinching the toe of the hind paw and no response was seen. The rat was laid in the supine position and both hind legs were shaved from the ankle to groin and diameters of both knees were measured. The entire left hind-leg was cleaned using cotton wool soaked in 4% chlorhexidine solution (Ecolab, Leeds, UK). EMLA Cream (provided by BSU, University of Nottingham) was used as a local anaesthetic for MNX or sham surgery. Rats then either underwent MIA injection or MNX surgery.

2.2.2 Intra-articular injection of MIA to the knee joint of rats

The procedure for intra-articular injection of MIA is based on Bove's work on the MIA model (Bove et al. 2003) and our previous studies (Sagar et al. 2011). MIA solution (Sigma U.K.) was made with sterile 0.9% saline (20 mg /ml Fannin, Ireland) and 50µl of MIA solution was injected (0 .5 ml insulin syringe (VWR international, Leicester, UK) into the rat's left knee joint of rat. The rat was anaesthetized (see 2.2.1), and

placed on autoclaved blue tissue paper on a heating pad. Hands were cleaned with alcohol to minimise bacterial transfer. The left ankle of the rat was held between the thumb and middle finger of the left hand and was extended away from the body to increase the space between the femoral and tibial bone. The groove between the femur and tibial was found and was pressed with the edge of a needle to make a mark for injection. A syringe with MIA (1mg/50µl) or saline (50µl) was placed perpendicular to the knee and injected to the joint cavity (1/3-1/2 of the needle was inserted into the space to avoid injecting through the cavity and loss of solution). The needle was gently removed from the joint and blood (if any) was cleaned with cotton wool. After injection, the rat was weighed and moved to a new cage on a heating pad for recovery. Rats were checked at 1, 2 and 4 hours after the procedure and then transferred back to the holding room or were kept overnight in the recovery room. Health assessments were conducted for 3 consecutive days post MIA-injection; these included body weight, joint swelling, limping, movement, hydration and evidence of pain/suffering.

2.2.3 Medial meniscus transection of knee joint of rats

The MNX surgery was performed by my colleagues based on the method previously described (Janusz et al. 2002; Mapp et al. 2008; Mapp et al. 2013). All instruments were autoclaved before surgery. Rats were anaesthetized (see 2.2.1) and transferred to a surgical working station in a supine position. Animals were covered by a Buster Op-Cover (Buster, Germany) with a suitable hole to expose the left knee joint. The skin of the medial knee joint was cut using small scissors and the superficial connective tissue layer was cauterised with a cauterizer. The medial collateral ligament was then located and removed with iris scissors. The meniscus was then transected, also with iris scissors. After the meniscus was removed, the superficial layer of connective tissue was sutured with Ethicon coated 8/0 Vicryl (Johnson &Johnson, Diegem, Belgium) and closed with interrupted stitches using Ethicon coated 4/0 Vicryl (Johnson &Johnson, Diegem, Belgium) or closed with skin clips (9mm, Cell Point Scientific, Gathersburg, USA). The suture knots were buried under the skin to minimize the risk of the rats opening their wound.

Sham surgery was performed with the same protocol as MNX surgery but without transection of the meniscus.

After surgery, rats were weighed and moved to a new cage and placed on a heating pad for recovery. They were checked at 1, 2 and 4 hours post-surgery and moved back to the holding room. Health assessments were conducted for 3 consecutive days post-surgery with regard to body weight, joint swelling, surgical wounds, limping and general behaviour. The diagram of the surgery is summarized in **Fig 2.1**.

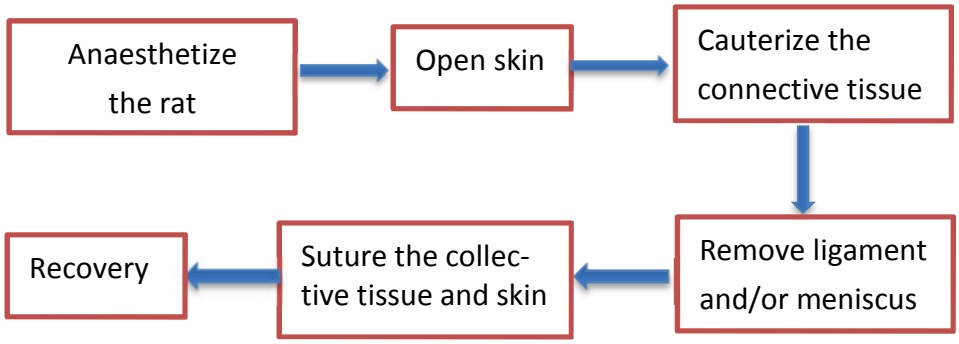


Fig 2.1 The diagram of simplified procedure for MNX or sham surgery.

2.3 Pain behaviour testing

Weight bearing (WB) and paw withdrawal thresholds (PWT) of hindpaws were tested to measure pain behaviour in the MIA and MNX models of osteoarthritis. Rats were habituated to the apparatus for WB and PWT a day before model induction. The baseline behaviour was tested on the day before MIA injection or MNX surgery. Pain behaviour was then tested from day 3 post-model induction until the end of the study. All pain behaviour testing was performed in the morning and rats were habituated to the room for 30 min prior to behavioural assessment to minimize stress and circadian variations.

2.3.1 Weight bearing and paw withdrawal threshold

Weight bearing was tested as previously described using an Incapacitance Tester (LintonInstrumentation, Norfolk, UK) (Pomonis et al. 2005; Sagar et al. 2011). Rats were placed in a perspex chamber (Medical Engineering Unit, University of Nottingham) with their two hindpaws on transducer pads and their two forepaws on the slanted wall of the chamber, as shown in **Fig2.2 A**. Three readings, which recorded weight applied each pad for 3s, were made. One or two more recordings were made if there were large differences in the first three readings. The mean of weight bearing differences (injured vs non-injured limb) was used for data analysis.

Paw withdrawal thresholds were tested using vonFrey hair (Linton Instrumentation, Norfolk, UK) stimulation with different forces (in grams) of filaments. Rats were

placed in a plexiglass chamber with 5 compartments and a wire mesh floor (**Fig2.2 B**) and were habituated for 5-10 minutes after weight bearing testing. After the rats had acclimatised to the chamber (as indicated by sitting still and no longer engaging in exploratory behaviour) , vonFrey hairs were applied to the central plantar surface of the hindpaw. The testing force of stimulation was 1, 1.4, 2, 4, 6, 8, 10, 15 and 26g. Briefly, stimulation started at 4g and 3 different sites of the central plantar surface of the hindpaw were stimulated at 3s interval between each stimulation. A stimulation force was then increased to 6g or decreased to 2g depending on whether there was a withdrawal response to the 4g hair. The lowest force which elicited a paw withdrawal was recorded as threshold. If no response were observed up to 26g, it was recorded as > 26g; higher forces were not used in order to prevent tissue damage. All the instruments and the chamber were cleaned with 20% ethanol after behavioural testing.

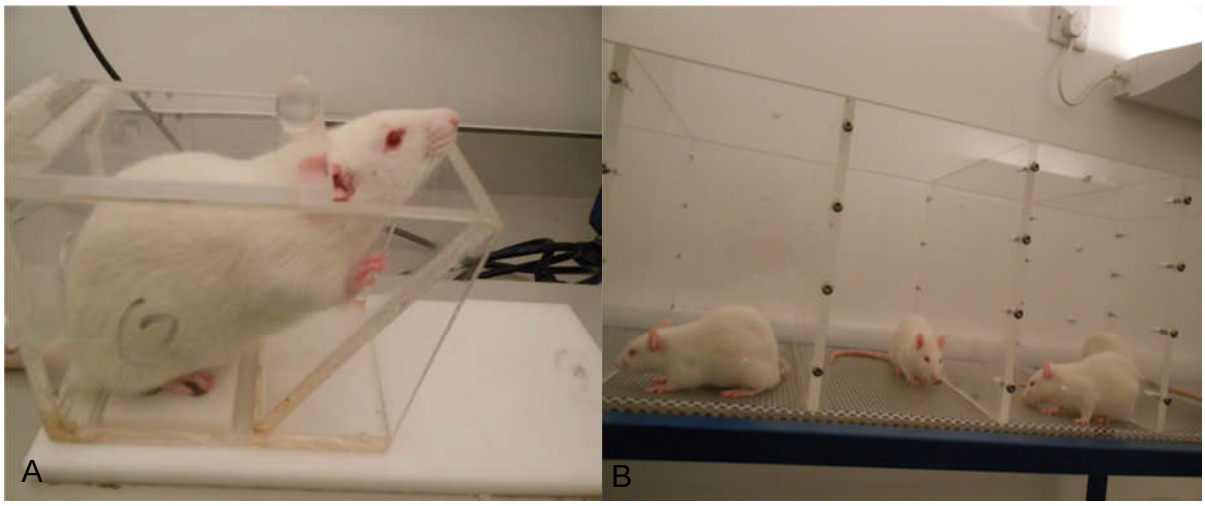


Fig 2.2 Pain behavioural testing for OA. Weight bearing was measured by an incapcitance tester (A) and paw withdrawal threshold was tested in a plexiglass chamber by vonFrey monofilaments (B).

2.3.2 Intraperitoneal drug administration

17(R)HDoHE is a oxygenated derivative from DHA via aspirin / COX2 and is the precursor of AT-RvD (Serhan et al. 2002). The drug was bought from Cayman Chemical, USA and the structure is shown in **Fig 2.3**:

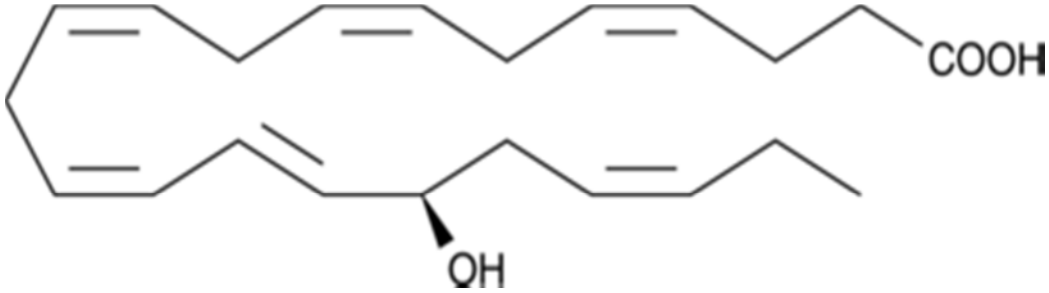


Fig 2.3 The structure of 17(R) HDoHE.

17(R) HDoHE was made up from the stock (100µg / 1ml in ethanol) by diluting in normal sterile saline to give a concentration of 1ng per µl, 300 µl of which was then intra-peritoneally injected. Vehicle injection consisted of a 1% ethanol solution in saline. To minimise potential subjective bias, drugs were administered in a blinded fashion. Rats were habituated to i.p injection every other day for two weeks by injection of 300µl of 0.9% saline with a 1ml syringe (25G needle, BD microlance, Louth, Ireland). The drug or vehicle was administered by intra-peritoneal (i.p) injection on day 14 post-MIA injection or MNX surgery.

2.3.3 Data analysis

The behaviour data were analysed by GraphPad Prism Version 5/6 (GraphPad Software Inc., USA) and presented as mean±S.E.M (standard error of mean). Pain behavioural data in different groups were analysed by two-way ANOVA with Bonferroni's post-hoc test with $p<0.05$ indicating statistical significance.

2.4 Tissue collection

Tissues were collected at the end of each study. Spinal cords, DRGs, synovia and blood were freshly collected and knee joints were fixed in 10% neutral-buffered formalin.

2.4.1 Reagents

Heparin sodium

Liquid nitrogen,

10% neutral buffered formalin**:**

100ml methanal

900ml double distilled water

4g NaH_2PO_4

6.5g Na_2HPO_4

2.4.2 Euthanasia

Rats were sacrificed by a Schedule 1 technique after pain behaviour testing at the end of the study. Rats were placed in an acrylic chamber and carbon dioxide delivered at 2L/min for 6 min or until respiration ceased (Coenen et al. 1995; Smith and Harrap 1997). Death was confirmed by checking the corneal reflex.

2.4.3 Blood, synovia and femorotibial joints

Blood (approximately 4ml) was collected in a container with 50µl (50 units) of heparin sodium and was gently shaken up and down to mix blood and heparin and then was immediately transferred onto wet ice to minimize the coagulation and post-mortem changes. After tissue collection, each container of blood was divided into two 2 ml polypropylene tubes and centrifuged (at 4°C) for 10 min at 10000 g to separate plasma from blood cells. The supernatant layer was transferred to new 2ml polypropylene tubes and stored at -80°C.

The skin over the knee joint was opened with a scalpel, the synovium with patella and infra-fat pad was gently removed from the femorotibial joint and then the patella was cut. The synovium was isolated from the infra-fat pad, placed in a cryo-vial and immediately snap frozen in liquid nitrogen. The femorotibial knee joint was cut with a bone clipper and the muscles were trimmed from the joint to minimize the soft tissue which could slow fixation. Each joint was fixed in a tube containing about 20 ml of 10%

neutral-buffered formalin for 48 h and decalcified with diamonoethanetetra-acetic acid disodium salt dehydrate (EDTA, Fisher scientific, UK) solution for 4-6 weeks for joint histology (see 2.5). Both ipsilateral and contralateral synovia and knee joints were taken.

2.4.4 Spinal cord and DRG

The spinal cord was taken immediately after blood collection based on previous studies (Woodhams et al. 2012) but was split into four parts (ipsi-dorsal, ipsi-ventral, contra-dorsal and contra-ventral side). Rats were placed in the prone position and the skin was opened from the midline of the back with a scalpel. Muscles covering the spine and spinous processes were removed with rongeurs. The vertebral lamina was carefully removed from the caudal (bottom) to rostral (top) terminals and the bone was trimmed to expose the spinal cord which was excised with small sharp scissors. The spinal cord was gently lifted away from the column with fine forceps and the dorsal roots on the two sides were carefully cut. Spinal cord was placed on a metal plate with dorsal side upward. Dura and vessels around the spinal cord were removed and the lumber section cord (L3-5) was cut away. The cord was flipped over to make the ventral side uppermost with the ipsilateral cord on the right-handed side and contralateral side on the left-handed side. The cord was split into ipsi- and contra- lateral sides along the midline with a sharp scalpel and then ipsi- and contralateral sides were split into dorsal and ventral parts respectively. The four quadrants of spinal cord were placed into cryo-vials and immediately snap frozen in liquid nitrogen. DRGs (L3-5) were removed under a dissecting microscope immediately after the spinal cord was taken as described in previous studies (Wood et al. 1988; Sagar et al. 2005). Briefly, spinal nerves and roots were cleaned under microscope to expose the DRGs with conjunctival scissors and fine forceps. DRGs were placed in cryo-vials and snap frozen in liquid nitrogen immediately.

All the fresh tissues were kept at -80°C for long-term storage and were used for Taqman RT-qPCR (see2.8). Formalin-fixed knee joints were used for joint histology (see 2.5 - 2.7).

2.5 Knee joint processing

2.5.1 EDTA decalcification and wax embedding

Joint processing was conducted as previously described (Mapp et al. 2013). Knee joints were fixed in neutral-buffered formalin for 48 h after tissue collection (see 2.4.3). Formalin buffer was replaced with EDTA buffer and tubes were placed on a shaker to agitate the solution and aid with decalcifying the joints. EDTA buffer was changed twice a week for 4-6 weeks. The decalcification of joints was confirmed by splitting the joint into anterior and posterior sections along the lateral collateral ligament but was split in sagittal transection. If joints were fully decalcified, splitting was easily achieved. Joints were placed in printed plastic cassettes with unique code for each joint. Joint samples were sent to King's Mill Hospital, Nottingham, where they were dehydrated and incubated overnight before paraffin wax embedding.

Briefly, knee joints in cassettes were placed in a warming molten wax module of a Tissue-Tek® TEC® 5 EME2 Tissue Embedding Console system (Sakura Finetek Europe, the Netherlands) to incubate the samples and metal moulds were heated in the other module of the Tissue-Tek to clean them for embedding. The temperature was controlled at 62°C. The empty mould was filled with melting wax and placed on a hotplate. Knee joints were placed in the middle of the mould with the flat side facing down. Joints were cooled down on a cold plate for a few seconds to fix the sample and then the mould was covered with the lid of the plastic cassette. The whole block of knee joint was cooled down on the Cryo Module of Tissue-Tek to tightly embed the sample. After the blocks were completely cold, they were taken out from moulds with a spatula and were kept at room temperature.

2.5.2 Sectioning knee joints with a microtome

The joints were cut based on previous studies (Gerwin et al. 2010). Joint sections from medial and lateral parts were cut in coronal transection. All the medial blocks of joints were cut on a rotary microtome (Slee cut 4060, Slee Medical GmbH, Mainz, Germany) based at the Advanced Microscopy Unit (AMU), University of Nottingham. Briefly, each block was fixed on the rack of the microtome and adjusted to make it parallel to the edge of blade. Wax was removed from the block until tissue appeared. The first 500µm of tissue was removed from the top to get a suitable transection of the joint. Several consecutive sections were cut and floated on a surface of warm

water in a paraffin section mounting bath. Sections were mounted on 3-aminoproplytriethoxysilane (APES)-coated slides and heated on a thermal plate at 40°C. 30 sections were cut from each block at 5 levels with 6 sections per level and 200µm intervals between each level. The slides were kept at room temperature for future use.

2.6 Haematoxylin and eosin staining

H&E staining was used to enable morphological assessment of the joint sections. Haematoxylin stains the nuclei blue and eosin stains the cytoplasm pink because of their respective affinities for basophilic and eosinophilic structures (Fischer et al. 2008; Cardiff et al. 2014). I selected 4 sections of joint with good quality of structure from 4 different levels in 30 sections (5 levels, 6 sections per level) from each block for staining. The staining was performed at AMU.

2.6.1 Haematoxylin&eosin staining

Knee joint slides were placed on a staining rack and sections were dewaxed by xylene (5 min) and a series of descending concentrations of industrial methylated spirits (IMS) (100%, 100%, 90%, 70% and 50%, 30s for each concentration) and rehydrated in tap water for 30s. Sections were stained by Harris Haematoxylin for 5 min and the rack was gently lifted up-and-down to ensure full contact with staining solution. Sections were washed under running tap water until the water was clear. Then sections were dipped in acid alcohol for 5-10 s to remove excess staining and washed again with tap water. Sections were placed in Scott's tap water for 15s and washed with normal tap water. The sections were viewed under a microscope to confirm the presence of blue dye. After that, the sections were stained with 1% eosin for 5min and washed with running tap water until clear. The sections were dehydrated with a series of ascending concentrations of IMS (50%, 70%, 90%, 100% and 100%, 10s for each concentration) and were placed in fresh xylene twice (2min each) to remove residual IMS. A drop of dibutyl phthalate in xylene (DPX) was added on a coverslip and the coverslip was placed on top of section/slide. The slides were placed in a fume hood for 4-6 hours or overnight to evaporate xylene and dry the sections.

2.7 Histological scoring for H&E stained knee joint sections

The scoring was blinded and performed under an upright microscope (Leica DB4000B, Germany) with 25 and 100 magnifications at AMU.

2.7.1 Histological scoring system

As described above (2.6), I selected and scored 4 sections from each stained sample. The chondropathy, synovial inflammation, chondrocyte appearance (proliferation, nuclear cloning and loss of chondrocyte) and osteochondral junction integrity (vascular channels/mm length of medial tibial plateau) for each section were scored. The chondropathy of medial tibial plateau, which indicates cartilage damage in the joint was assessed by the Janusz scoring system (Janusz 2002). Briefly, cartilage damage of the medial tibial plateau was scored on a 0 to 5 scale as follows:

0 = Cartilage of normal appearance

1 = Minimal fibrillation in superficial zone only

2 = Mild, extends into the upper middle zone

3 = Moderate, well into the middle zone

4 = Marked, into the deep zone but not to the tidemark

5 = Severe, full thickness degeneration to the tidemark

The proportion of involved damage of the tibial plate was assessed by 1/3, 2/3 or 3/3, so the total chondropathy score was cartilage score multiplied by the length of involvement of the tibial plate (1, 2 or 3).

Synovial inflammation, chondrocytes appearance and ostechondral junction integrity were scored based on previous studies (Mapp et al. 2008; Mapp et al. 2013). Briefly, synovial inflammation was evaluated by the thickness of cellular layers of synovia and was scored on a scale of 0 to 3 as follows:

0 Lining cell layer 1-2 cells thick

1 Lining cell layer 3-5 cells thick

2 Lining cell layer 6-8 cells thick and/or mild increase in cellularity.

3 Lining cell layer > 9 cells thick and/or severe increase in cellularity.

Chondrocytes appearance was scored on cloning and death of chondrocytes in the cartilage as follows:

0 = No chondrocyte cloning observed (normal appearance and density)

1 = Diffuse hypercellularity of chondrocytes.

2 = Chondrocyte cloning (2 instances of single cloned chondrocyte with 4 nuclei)

3 = Chondrocyte hypocellularity.

Integrity of the osteochondral junction was quantifying by counting the number of vascular channels breaching the osteochondral junction (the interface between cartilage and subchondral bone) and dividing the length of the medial tibial plateau. Images were taken under a microscope at different magnifications after the scoring. The simplified procedure for knee joint processing is shown in **Fig 2.4**.

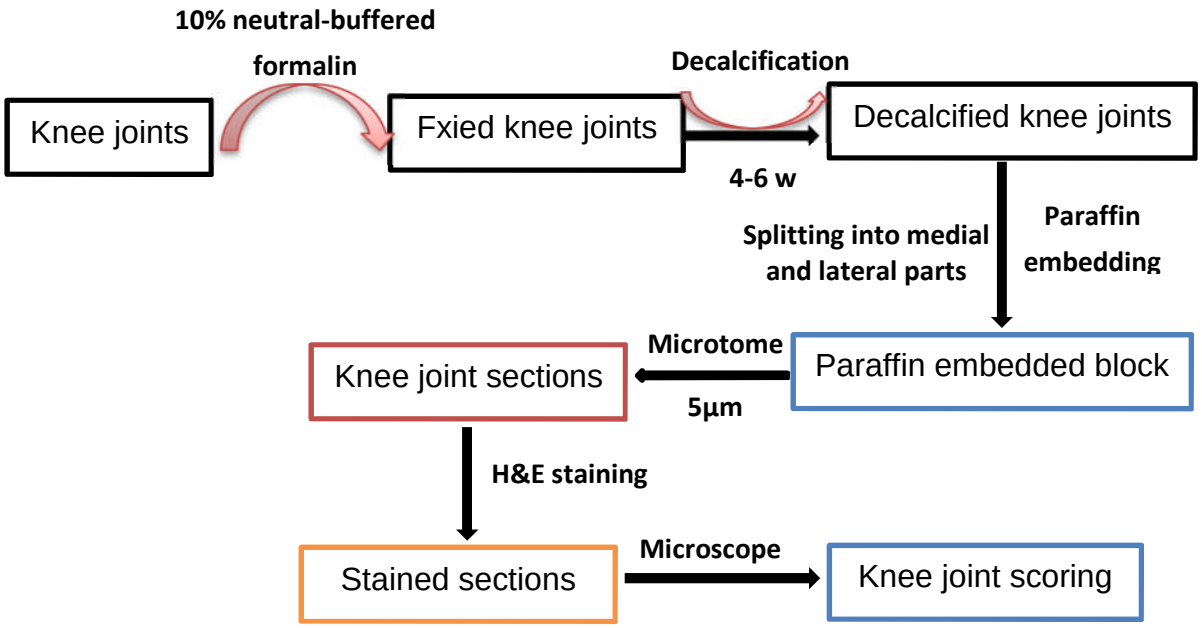


Fig 2.4 The key procedures for histopathological assessment of ipsilateral knee joints. Knee joints were fixed in 10% neutral-buffered formalin and decalcified for 4-6 weeks. After the decalcification, knee joints were split in to medial and lateral parts and embedded in paraffin. Medial tibial plateau of ipsilateral knee joints were cut by microtome, stained with H&E and scored under the microscope.

2.7.2 Data analysis

Data were analysed by Graphpad Prism 6 and are presented as mean±S.E.M.

Histological scoring for different groups was analysed by one-way ANOVA with Bonfferoni's post-hoc test and $p<0.05$ was considered statistically significant.

2.8 Gene expression quantified by Taqman quantitative Real-Time Polymerase Chain Reaction (RT-qPCR)

2.8.1 Principles of Taqman quantitative RT-qPCR

RT-PCR is a complex reaction system involving RNA extraction, biosynthesis of cDNA and amplification of cDNA and the reaction system includes essential components such as dNTP, primers; probes, DNA polymerase, reverse transcriptase and RNA inhibitor (see details in 2.8.3-2.8.9) (Ohan and Heikkila 1993). There are different fluorescent based systems available, such as SYBR®Green (Carl T. Wittwer et al. 1997), TaqMan® (Pamelam. Holland et al. 1991), Scorpions (Whitcombe et al. 1999) and Molecular Beacon (Sanjay Tyagi and Kramer 1996), which can detect production of DNA, cDNA or RNA in real time.

Thermus aquaticus (Taq) DNA polymerase is a thermostable enzyme which has 5´ - 3´ exonuclease activity and can cleave probes into small fragments (Pamelam. Holland et al. 1991). The Taqman probe is a specific sequence of nucleotides which has a fluorescent reporter dye (6-carboxyfluorescein, FAM) at the 5' end and quencher dye (6-carboxytetramethylrhodamine, TAMRA) at the 3' end and can hybridize with its target. The primer and probe bind with the target sequence when annealing. Taq DNA polymerase is activated and binds to the primer to extend the template of the target sequence. When the complex attaches to the probe, the polymerase cleaves the probe which allows the extension to continue to the end and release the fluorescent reporter from the probe. In this way, the fluorescent signals accumulate and can be detected at a cycle threshold (C_T value) (**Fig 2.5**). As PCR cycles continue, the number of copies of target sequence exponentially increase after the PCR product is first detected and finally the PCR reaction reaches a plateau stage when the assay components are limiting (Arya et al. 2005).

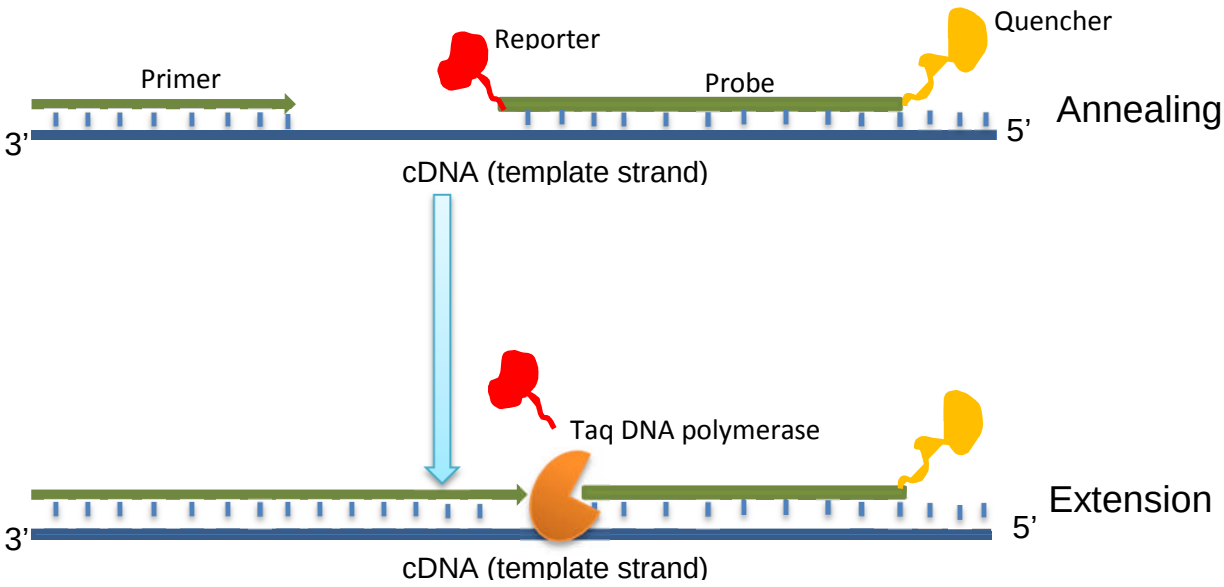


Fig 2.5 Principle of Taqman RT-qPCR. Both the primer and probe anneal to the target sequence but no fluorescence is detected when the probe is intact. Then Taq DNA polymerase binds to the primer and start to extend the template. When the polymerase attaches to the probe, the 5' →3' exonuclease cleaves the reporter from the probe and releases the signal fragment. As PCR cycles continue, the intensity of the fluorescent signals is accumulated and can be detected at a threshold cycle. The reaction reaches a plateau stage and fluorescent signals are not increased.

2.8.2 Design of primers and probes

Expression levels of resolvin receptors (ChemR23 and ALX), inflammatory cytokines, and their metabolic enzymes were quantified by Taqman RT-qPCR in synovia and spinal cords from rats and synovia, tibial plate, femoral condyles and femoral heads from human subjects. The design of primers and probes was based on published mRNA sequences (found at NCBI) and Primer Express® software v 3.0. As the genome consists of coding and non-coding sequences, designed sequences of primers and probes were generated from the coding sequences spanning over exon-exon junctions. Minor groove binding (MGB) probes were employed when the regular probes could not be designed. The MGB probes, which can bind with complementary DNA at an enhanced melting temperature (Kutyavin et al. 2000), are more specific, shorter and more efficient sequences than regular designed probes. Primers and probes were designed according to some general rules such as appropriate length of bases (18-30 bp), optimal melting temperatures for primers (58-60°C) and probes (69-70°C), percentage of GC content (30-80%) and avoiding primer-dimer

formation. When designing the primers and probe of a target gene, the mRNA sequence from NCBI was copied into Primer Express3 software, and exon-exon junctions were found in the sequence. After the primers and probes were designed, the sequences were “blasted” at NCBI to confirm that they were 100% specific to the target genes. The primers and regular probes were synthesized by Eurofins MWG Operon (Germany). MGB probes were synthesized by Applied Biosystems (Cheshire, UK). The specific sequences of primers and probes for rat (**Table 2.1**) and human (**Table 2.2**) are shown below:

Target gene	Forward Primer Sequence	Reverse Primer Sequence	Probe sequence
β-actin	CGTCCACACCCGCCG	CACAGCCTGGATGGCTACGT	TGAGACCTTCAACACCCCAGCCATG
ChemR23*	AGGACCTACCCTCGAGTTCTATTCT	CGTAGATGCTGGAGTCGTTGTAA	TCCAAAGAGATGGAGTACGA
ALX	CTTGGACCGCTGCATTTGT	CCTTCCTAGCCAGGCTCACA	CAGTCTGGGCTCAGAACCACCGC
TNFα	GCAGGAGAAAGTCAGCCTCCT	TACTACCAGGGCTTGAGCTCA	AGAGCCCTTGCCCTAAGGACACCCCT
IL1β	CACCTCTCAAGCAGAGCACAG	GGGTTCCATGGTGAAGTCAAC	TGTCCCGACCATTGCTGTTTCCTAGG
IL6*	CAGAGCAATACTGAAACCCTAGTTCA	CCATTAGGAGAGCATTGGAAGTTG	TTCAAACAAGAGATAAAAGACTC
IL10	GAAGCTGAAGACCCTCTGGATACA	CCTTTGTCTTGGAGCTTATTAAAATCA	CGCTGTCATCGATTTCTCCCCTGTGA
5-LOX*	TGGTGTCTGAGGTGTTCGGTAT	GGCAATGGTGAACCTCACATG	CCCTTTTCAAGCTGCTG
FLAP	CCCCACTTTCCTTGTGGTACTC	TGCCTCACGAACAGATACATCAG	AGCCAAGTCCCCGCCGCCT
15-LOX	TGATGCCTGATGGACAACTCTT	CCGAGGGCGTGAAAATAGG	CCATAGCCATCCAGCTTGAACTTCCCA
COX2	GCACAAATATGATGTTCGCA	CCTCGCTTCTGATCTGTCTTGA	TCTTTGCCCAGCACTTCACTCATCAGTTT
EPHX2	GCCAGTTTGAACACTCCATTAATG	TTGAAAACTGGGATCGATCTGA	CACCAAATCCTGAGGTGTCCCCCA
CYP2J2	GCAATGGATACCACTTTGAATGG	CCCTGTGCAGTGCAGTTAGGT	CCACCTGCCAAAGGGTACCATGGTTCT
mPGES1	GCGAACTGGGCCAGAACA	GGCCTACCTGGGCAAAATG	CCCCGGAGCGAATGCGTGG
iNOS	CCCAGAGTCTCTAGACCTCAACAAA	GCCCTCGAAGGTGAGTTGAA	AAGTCCAGCCGCACCACCCTCC
GFAP	TGGCCACCAGTAACATGCAA	CAGTTGGCGGCGATAGTCAT	CAGACGTTGCTTCCCGCAACGC
TRPV1	TCAAAGACCCAGAGACAGGAAAG	CTGTCTTCCGGGCAACGT	AAAAGCCATGCTCAATCTGCACAATGG

Table 2.1 Primer and probe sequences of target genes from rat were designed for Taqman RT-PCR by Primer Express 3. The sequences for primer and regular probe were synthesized by Eurofins MWG Operon. MGB probes were synthesized by Applied Biosystems. The probes with"*" are MGB probes. EPHX2: soluble epoxide hydrolase 2, CYP2J2: cytochrome P450, family 2, subfamily J, polypeptide 2

Target gene	Forward Primer Sequence	Reverse Primer Sequence	Probe sequence
hβ-actin	CCTGGCACCCAGCACAAT	GCCGATCCACACGGAGTACT	ATCAAGATCATTGCTCCTCCTGAGCGC
hChemR23	CATGCCTGGCTCTGTCTTCA	TGCAGCTGTTGGCAATGG	TTGCCCCTGGCCACTGCCCT
hALX	ACGAGCTCCCTGGCCTTCT	GGAGGTCTTGGCCCACAAAG	CAACAGCTGCCTCAACCCCATGC
hTNFα	CCCAGGGACCTCTCTCTAATCA	GGTTTGCTACAACATGGGCTACA	CTCTGGCCCAGGCAGTCAGATCATCT
hIL6	CTGCAGAAAAAGGCAAAGAATCTAG	CGTCAGCAGGCTGGCATT	TGCAATAACCACCCCTGACCCAACC
h5-LOX	GAGAGCAAAGAAGACATCCCCTACT	GGCCGTGAACGTCCTGAT	TTCTACCGGGACGACGGGCTCC
h15-LOX1	GGCAAGGAGACAGAACTCAAGGT	GAACCAGGCGTCGTCCTTAA	TGTGAAACTGCGCAAACGGCACC
h15-LOX2	CACCTGCTGCACTCACATCTG	CGATGCCTGTGGACTTGAAGA	CACCCTGGCTACCCTGCGTCAGC
hCOX2	GCCCTTCCTCCTGTGCC	AATCAGGAAGCTGCTTTTTAC	ATGATTGCCCGACTCCCTTGGGGTGT

Table 2.2 Primer and probe sequences of human target genes were designed for Taqman RT-PCR using Primer Express 3 software and were biosynthesized by Eurofins MWG Operon.

2.8.3 Reagents

RNA extraction: Trizol (TRI) reagent (Sigma, UK), 1% sodium dodecylsulfate (Fisher Scientific, UK), liquid nitrogen, bromo-3-chloropropane (BCP, Sigma, UK), isopropanol (Fisher Scientific, UK), sodium acetate (2M, pH=4), ethanol absolute (Sigma, UK), HPLC water (Fisher Scientific, UK), RNeasy Mini Kit (Qiagen, Netherlands).

Reverse transcription: Random Primers, 500µg/ml, 20µg (Promega, USA), deoxyribonucleotide triphosphate (dNTPs) mix (100mM each of dATP, dCTP, dGTP, dTTP, Promega, USA), AfintyScript MultipleTemperature Reverse Transcriptase 200 µl, 100mM dithiothreithol (DTT, 800µl),10xAffinityscript RT buffer 1ml (Agilent Technology, USA), RNaseOUT™ Recombinant Ribonuclease inhibitor, 5000 Units 40U/µl (Invitrogen, Life Technology, USA).

Pre-amplification: 20xTris-EDTA buffer (200mM Tris-Cl, 20mM EDTA, pH 7.5, Invitrogen, US). Primers (see Table 2.1 and 2.2). Taqman®Fast Universal PCR Master Mix (2x) (Applied Biosystems by Life Technology, USA)

RT-qPCR: Primers and probes (see Table 2.1 and 2.2), Taqman®Fast Universal PCR Master Mix (2x), HPLC water, cDNA samples.

2.8.4 Total RNA extraction for rat synovia and spinal cord

Total RNA was extracted from samples with TRI reagent which is a monophasic lysis reagent that dissolves the biological material and denatures protein (Rio et al. 2010). I followed the manufacturer's instructions modified on the basis of previous studies conducted within the group (Burston et al. 2013). Frozen tissue (synovium or spinal cord) was homogenized in cold TRI reagent (1ml for synovia and 2ml for spinal cord) and incubated at room temperature for 5 min to fully digest the nucleoprotein mixture. BCP (0.2 ml for each 1 ml of TRI reagent) was added to isolate RNA from DNA and protein. The samples were mixed by vigorous vortexing and centrifuged at 10000g for 15 min at 4°C. After centrifuging, the solution was separated into three phases with the aqueous phase containing RNA on the top, DNA at the interface and protein in the lower organic phase. The supernatant layer containing RNA was transferred in to fresh collection tubes and 0.25 NaAC (2M, pH4) and 0.7ml isopropanol were added and mixed thoroughly. The mixed solution containing RNA was precipitated at -20°C for at least 30 min. Protein in the organic phase was stored at -80°C for future

use. Precipitated RNA was pelleted by centrifugation at 17000g for 15min at 4°C. Pellets were washed twice with 70% ethanol and centrifuged again for 10 min at 10000g. Ethanol was removed by pipetting and the remaining residues of ethanol were evaporated under the filament lamp. Pellets were solubilized in 50µl of HPLC water or RNase-free water. The extracted RNA samples were heated at 65°C for 5 min to aid solubilizaiton. RNA samples were kept at -80°C for long-term storage (**Fig 2.6**). The concentration and purity of RNA samples were measured using Nanodrop™ 2000 spectrophotometer (Thermo Scientific, UK). The normal spectral absorbance ratio of 260/280 for RNA is 1.8-2.0 and ratio of 260/230 is 1.8-2.2. The higher and lower ratios indicate protein or chemical contamination. RNA samples were purified with an RNeasy Mini Kit if the purity of samples was outside the normal ranges mentioned above.

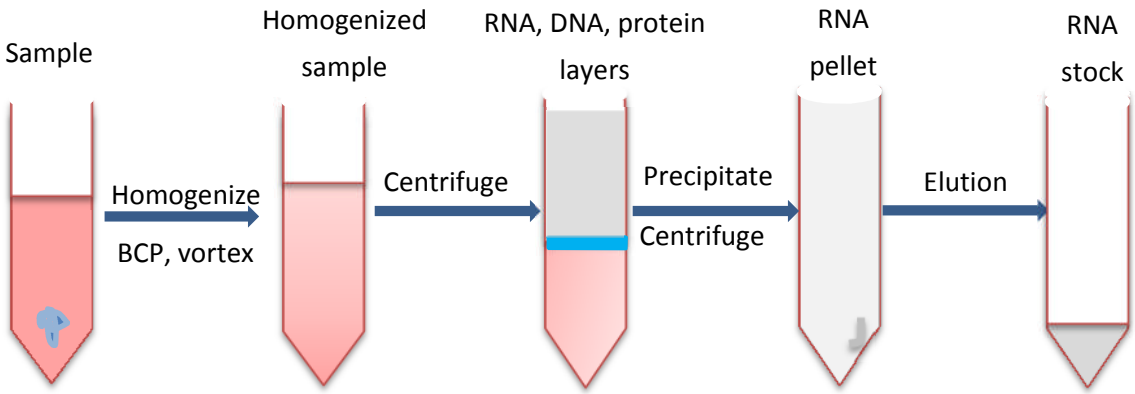


Fig 2.6 Procedure of RNA extraction from tissues. Samples were homogenized in cold TRI reagent, vortexed with BCP and centrifuged at 10000g for 15 min at 4°C. The mixed solution was subsequently separated into three layers with RNA, DNA and protein from top to bottom. RNA was precipitated in sodium acetate and isopropanol at -20°C for at least 30min and centrifuged at 17000g for 15 min at 4°C. Pellets were collected in 50µl of HPLC water after washing twice with 70% ethanol and stored at -80°C for long-term storage.

2.8.5 RNA purification

RNA samples were cleaned with RNeasy Mini Kit, performed according to the manufacturer's instruction. Briefly, sample volume was adjusted to 100µl with RNase-free water. RNeasy Lysis Buffer (RLT, 350 µl per sample, β-mercaptoethanol 10µl/ml of β-ME was added before using) and 250 µl of ethanol (96%-100%) was added to the diluted RNA and mixed thoroughly. The RNA solution (700µl) was cleaned up and RNA was collected in 50µl RNase-free water following the instructions. The concentration and purity of RNA samples were measured by Nanodrop™ 2000 spectrophotometer.

2.8.6 Reverse transcription with AffinityScript Reverse Transcriptase

RNA samples were reverse transcribed with AffinityScript Reverse Transcriptase following the manufacturer's instructions with slight modification. Random primer and dNTP were diluted to 0.1µg/µl and 10mM respectively as working solution. 500 ng of RNA was used for reverse transcription. 19.5 µl of solution for reverse transcript reaction was made (1µl of random primer, 1µl dNTP, 500 ng RNA and appropriate volumes of HPLC water) to anneal primer to RNA at 65°C for 5 min and cool on ice for 1min. 25.5 µl of solution was used to synthesize cDNA (3µl of 10xAffinityscript RT buffer, 1 µl DTT, 1µl AffintyScript MultipleTemperature Reverse Transcriptase, 1µl RNaseOUT and 19.5 µl mixed RNA solution). cDNA synthesis was conducted as follows: 25°C for 10min to extend primers, 50°C for 60 min to synthesize cDNA and 70°C for 15 min to terminate the reaction. cDNA samples were centrifuged for seconds at 4°C to clean the drops on the wall of tubes and stored at -20°C or used directly for Taqman RT-qPCR analysis. The scheme for cDNA synthesis is shown below (**Fig 2.7**):

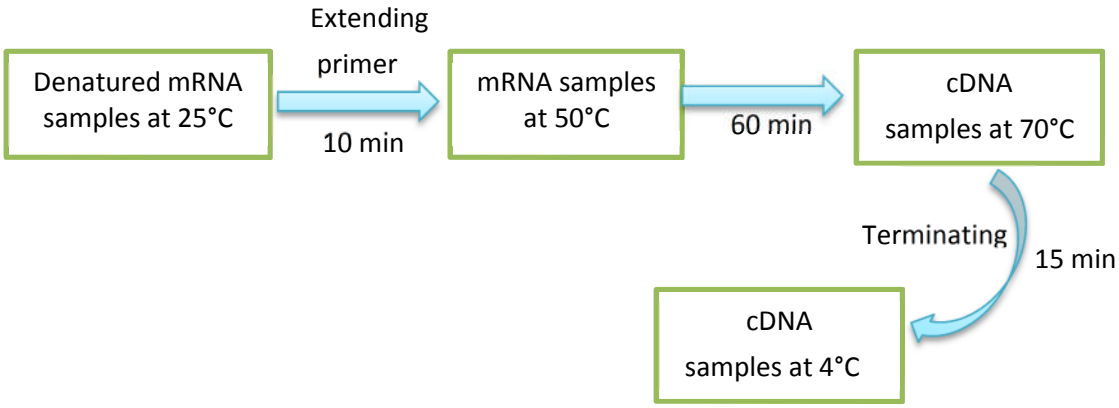


Fig 2.7 cDNA synthesis. Primer was annealed to mRNA samples and extended at 25°C for 10min. cDNA samples were synthesized at 50°C for 60 min. The reaction was terminated at 70°C for 15 min and cDNA samples were kept at 4°C after termination of the reaction.

2.8.7 cDNA pre-amplification

Synthesized cDNA was pre-amplified because expression levels of some of target genes were very low. Briefly, working solutions of forward primers and reverse primers from each gene were diluted 1 in 50 inTris-EDTA buffer (10mM Tris-Cl, 1mM EDTA) to make a primer pool. 5µl of cDNA sample was added to 5µl of primer mixture and 10µl of Taqman® Fast Universal PCR Master Mix. Samples were pre-amplified for 3 or 8 cycles and the reaction sequence was as below:

1) 95°C 10 min to activate Hot-start Taq DNA polymerase

2) 95°C 15 s for denaturation of cDNA

3) 60°C 4 min for extension of template

4) Step 2 and 3 repeated for 3 or 8 cycles

5) 4°C holding after the reaction was stopped.

Pre-amplified cDNA was stored at -20°C.

2.8.8 qRT-PCR for gene expression

2.8.8.1 Standard curve and sample preparation

As the cDNA template amplifies in an exponential way, the quantity of PCR production could be calculated by $\log_{10}$ transformation as the template is based on 10-fold serial dilutions to generate a linear plot. Standard curves were used to determine the initial starting amount of unknown sample and the efficiency of PCR by diluting in sequential concentrations. The C_T value of unknown sample was compared with the standard curve to determine the relative quantity of the sample. The slope of the standard curve reflects the efficiency of PCR reaction and the correlation co-efficient (R^2) measures how the standard curve fits the data. A PCR efficiency of 100% corresponds to a slope of –3.32, as determined by the following equation: Efficiency = $10^{(-1/slope)}$–1(Ginzinger 2002). A slope of the standard curve between -3.2~ -3.6 and R^2 >0.95 were considered as acceptable criteria for data analysis.

The stock for the standard curve was made from a mixture of each cDNA sample (5µl) to a common pool and vortexing. The standard curve was made from the superneat and diluted in 5 sequential concentrations of points to neat, 1: 3, 1:9, 1:27 and 1:81 with autoclaved HPLC water (**Fig 2.8**). The standard curve and sam-

ple/unknown cDNA were normally freshly made up and freeze-thawed no more than twice to avoid degradation of cDNA.

slope	-3.403533395
intercept	21.07608158
r2	-0.998081885

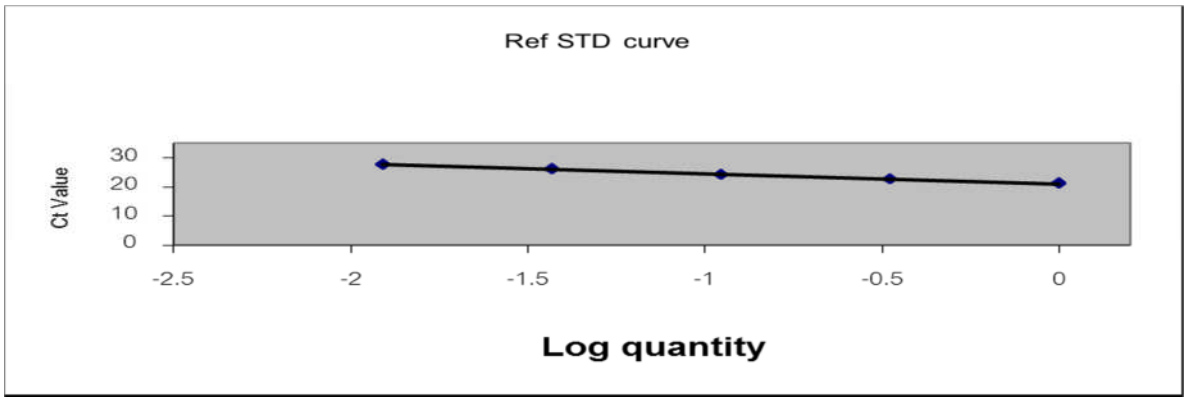


Fig 2.8 Standard curve of β-actin expression in synovia of saline-treated rats. The slope of the curve is -3.40 and R^2 is -0.998 which indicates high efficiency and fidelity of PCR amplification.

2.8.9 RT-qPCR Assay

RT-qPCR of cDNA samples was performed in a sealed 96-well optical plate using the Applied Biosystem StepOnePlus Real-Time PCR system (Applied Biosystems, US) . Each cDNA sample was run in triplicate and each of PCR reaction well contained 6.5 µl of Fast Master Mix, 0.375µl of each forward and reverse primer, 0.25 µl of probe, 2.5 µl of HPLC water and 3 µl of test sample. 10µl Fast Master Mix and 3µl of cDNA sample were added into each well of 96-well plate. The plate was sealed with optical film and spun with a mini plate spinner (Labnet international, Inc, USA) to collect any drops of sample on the well wall. The PCR reaction was started at 95°C for 20s to activate hot-start Taq DNA polymerase and 40 cycles of 95°C for 1s and 60°C for 20s to denature, anneal and extend cDNA template. A flow diagram of the PCR reaction scheme is shown in **Fig 2.9**.

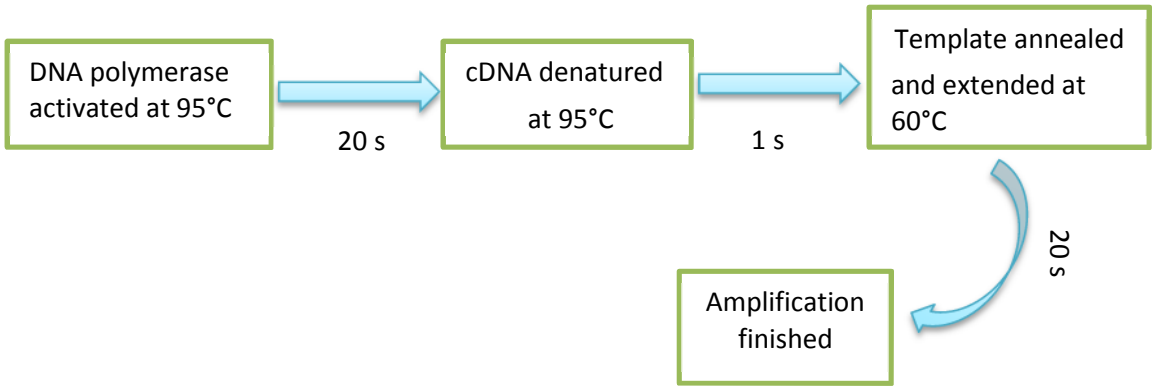


Fig 2.9 PCR reaction. Taqman DNA polymerase was activated at 95°C for 20s. 40 cycles of PCR reaction was performed at 95°C for 1s to denature cDNA templates and 60°C for 20s to extend the templates. The amplification finished after 40 cycles.

2.8.10 Data analysis

Data were analysed by GraphPad Prism Version 6 and presented as mean± S.E.M. Gene expression levels in multiple groups were analysed by one-way ANOVA with Bonferroni's multiple comparison post-hoc testing for parametric data and with a Kruskal-Wallis test with Dunn's multiple comparison post-hoc testing for non-parametric data. Data for two groups were analysed by unpaired t-test for parametric data and Mann-Whitney test for non-parametric data. To investigate correlation between expression levels of target genes or pain behaviour with resolvin receptors (ChemR23 and ALX), XY analysis was used. Gene expression level and weight bearing difference are continuous variables and paw withdrawal threshold is discrete variable. To investigate how close the correlation between the variables would be, we expected that the expression levels of resolvin receptors (ChemR23 and ALX) will have a linear correlation with pain behaviour and target genes, so linear regression has been used for the correlation analysis. Pearson correlation analysis is sensitive to linear correlation between two variables and the co-efficient indicates strength of the correlation, therefore, was used for parametric data with normal distribution. Spearman correlation was used for non-parametric data to measure the extent of tendency between resolvin receptors and other genes or pain behaviour. $p < 0.05$ was taken as statistically significant.

2.9 Human tissue samples

2.9.1 Sample collection

The research project on clinical samples was approved by generic ethics for the Trust Biobank, Research & Innovation, University of Nottingham. A letter of support was issued to allow access to operating theatres in NHS hospitals for fresh tissue collection (ASCH 448, see Appendices). Human synovia, femoral condyles and tibial plateau were taken from OA patients undergoing total knee replacement (TKR) at the City Hospital. Femoral heads from trauma patients were harvested at the Queen's Medical Centre campus. Human samples were immediately placed on dry ice and were stored at -80°C in the Bio-bank. Sample details were registered in the Bio-bank database with individual barcodes for tracking. Samples were transported to the laboratory on dry ice for experiments.

2.9.2 Tissue homogenizing

Human synovia were taken on dry ice and placed in a 5 ml container containing 3ml of TRI Reagent. Synovia were roughly homogenized with a large Ultra Turrax probe and finely homogenized with a small probe in 4 ml of TRI reagent. Samples were then incubated at room temperature for 5min. 800µl of BCP was added to each container and mixed by vortexing. Samples were centrifuged at 10000g for 15min at 4°C. and the following steps of RNA extraction and RT-PCR were conducted as previously described (see 2.8.4-2.8.9).

For bone tissue, the middle part of the medial tibial plateau from OA patients was taken to investigate expression levels of genes of interest as OA damage is more frequently observed in that compartment (Miyazaki et al. 2002; Mundermann et al. 2005). Bone fractions of femoral heads from trauma patients were used as controls for OA patient. As bone is a highly dense connective tissue and it is much more difficult to homogenize directly than synovium or spinal cord, a slightly modified procedure was employed (C. Reno et al. 1997). Bone tissue (medial tibial plateau or femoral head) was placed on dry ice and tissue from the middle part of medial tibial plateau or bone fractions from femoral head (about 200 mg per sample) were removed using a bone clipper. Frozen tissue was pulverized in a mortar containing liquid nitrogen and transferred to a 5 ml container. 3 ml of TRI Reagent was added to the container and the tissue was homogenized as described above. The remaining steps

for RNA extraction and RT-PCR were performed as previously described (section 2.8.4-2.8.9).

2.9.3 Data analysis

Data were analysed by GraphPad Prism Version 6 and presented as mean± S.E.M. Expression of ChemR23 and ALX were compared by un-paired t-test. Comparisons of gene expression in OA and trauma patient tissues were compared by un-paired t-test or Mann-Whitney t-test. Correlations were analysed by XY analysis with the Pearson correlation for parametric data and Spearman for non-parametric correlation.

3 MIA-induced and MNX-induced models of osteoarthritis

3.1 Introduction

3.1.1 Translational relevance of rodent models of OA pain

Animal models of OA are widely used to mimic the pathology, pathogenesis and clinical symptoms associated with human OA, and to investigate potential therapeutics for the treatment of OA in pre-clinical studies. The models of OA are either experimentally-induced or spontaneous in various species of animals. The experimentally-induced models are conducted by surgery or chemical agents while spontaneous models occur naturally or arise as a result of genetic modification (Bendele 2001; Lampropoulou-Adamidou et al. 2014). Two osteoarthritic models are widely used to investigate pathogenesis and mechanisms underlying pain behaviour associated with OA. Firstly, the MIA-induced model of OA pain. This model is associated with changes in weight distribution (weight bearing asymmetry) and a lowering of hind-paw withdrawal threshold; these effects are dose-dependent and last for at least 10 weeks post model induction (Bove et al. 2003; Combe et al. 2004). The types of pain exhibited in this model mimic the reported pain on standing and changes in pressure pain thresholds seen in human OA (Kosek and Ordeberg 2000; Hendiani et al. 2003). Secondly, the MNX-induced model of OA pain, which leads to pathological changes to the structure and instability of the joint. The MNX model is associated with osteoarthritic lesion of medial tibial plateau; the severity and area of cartilage damage increases over time (Janusz et al. 2002). Subchondral and synovial angiogenesis, synovial inflammation and osteophytes, as well as damage to articular cartilage have been demonstrated in this model (Mapp et al. 2008). In addition, proteoglycan loss and cartilage fibrillation have been demonstrated in the MNX model (Mapp et al. 2010), consistent with some of the histological features of human OA (Aigner et al. 2001; Hardy et al. 2002; Fuerst et al. 2009). This model mimics the tears to different compartments of medial meniscus and popliteal pain in knee OA patients (Kamimura et al. 2014). Although the broader histological features of joint damage are similar in the two models, the MNX model has higher inflammatory and osteophytes scores, compared to the MIA model (Mapp et al. 2013).

3.1.2 Resolvins and resolvin receptor system

As described in 1.5.2, there are two series of the resolvins (D-series and E-series). D-series resolvins act at ALX and GPR32 receptors whereas E series resolvins act at ChemR23 and BLT_1 receptors (Serhan et al. 2011; Im 2012). These receptors are GPCR expressed by immune cells (Cash et al. 2014), neurons, glial cells (Ji et al. 2011), chondrocytes (Berg et al. 2010) and synoviocytes (Kaneko et al. 2011). D-series receptors (ALX and GPR32) were identified using radio-labelled RvD1 binding and actin polymerization assays (Krishnamoorthy et al. 2010). ChemR23 was identified using a radio-ligand binding assay and demonstrated high expression in human monocytes (Arita et al. 2005). The resolvins have receptor-mediated anti-inflammatory and anti-nociceptive effects, regulating inflammatory cytokines and various pro-inflammatory signalling pathways (Ji et al. 2011). There has been considerable work on the anti-inflammatory properties of the resolvins (Serhan et al. 2008; Spite and Serhan 2010; Serhan 2014). However, there have been fewer studies of their analgesic properties.

RvE1 attenuated inflammatory and neuropathic pain and the effects were mediated by ChemR23 (Xu et al. 2010) . RvD1 reduced pain behaviour in inflammatory models, however, the receptor mediating this effect was not demonstrated (Xu et al. 2010). The precursor of the resolvin D-series and aspirin-triggered RvD1 have been shown to attenuate hyperalgesia in a model of adjuvant-induced arthritis (Lima-Garcia et al. 2011). Mechanisms underlying these effects were suggested to be a reduction in TNFα and IL1β at peripheral sites and blockade of COX2 and NF-κB signaling at central sites (Lima-Garcia et al. 2011).

As presented in the general introduction, inflammation is one of the major pathological features of OA and multiple mediators are involved in pathogenesis and progression of OA. Resolvins play broad anti-inflammatory and anti-nociceptive roles by targeting multiple mechanisms in various inflammatory and neuropathic models of pain (Sungjae Yoo et al. 2013). To date, the resolvin receptor system has not been characterized in models of OA and potential effects on pain have yet to be reported.

3.1.3 Aim

To determine whether the expression of resolvin receptors in synovia and spinal cord is altered in models of OA pain and whether expression of resolvin receptors is correlated with pain behaviour, expression of inflammatory cytokines and metabolic enzymes.

3.1.4 Objectives

- To confirm the extent of pain behaviour and joint histology in both models of OA
- To characterize the expression of ChemR23 and ALX in synovial and spinal cord tissues from the OA models
- To quantify gene expression levels of inflammatory cytokines and metabolic enzymes relevant to the resolvin system in synovia and spinal cord.
- To investigate potential correlations between the expression of resolvin receptors and pain behaviour, and expression of inflammatory cytokines and their respective metabolic enzymes in synovia and spinal cord

3.2 Methods

The induction of models has been discussed in the general methods (2.2-2.3). Sprague Dawley male rats (130-150g) were habituated for environment and equipment before model induction and behavioural test.

3.2.1 Comparison of pain behaviour between two models of osteoarthritis

To compare the pain behaviour between MIA and MNX model, four groups were used in this study, namely MIA (1mg/50µl) group, saline (50µl) group, MNX surgery group and sham surgery group. Two separate studies were undertaken for the work described in this Chapter. In the first study, 6 rats per group were used to measure pain behaviour from day 0 until day 28 post model induction and pain behaviour was tested twice a week. The reason that I chose day 28 as the end point is based on previous studies. Pain behaviour is maximal at day 28 and there is evidence of spinal sensitization, such as increased glial fibrillary acidic protein (GFAP) immunofluorescence, which indicates activation of astrocytes in the MIA model (Sagar et al. 2010; Sagar et al. 2011). MNX-induced pain behaviour and joint histopathology are also known to be established, compared to sham control at day 28 (Mapp et al. 2013). Weight bearing asymmetry was tested from day 0 until day 28. At the end of the study, knee joints were harvested for histological analysis. The protocol is simplified as below (**Fig 3.1**):

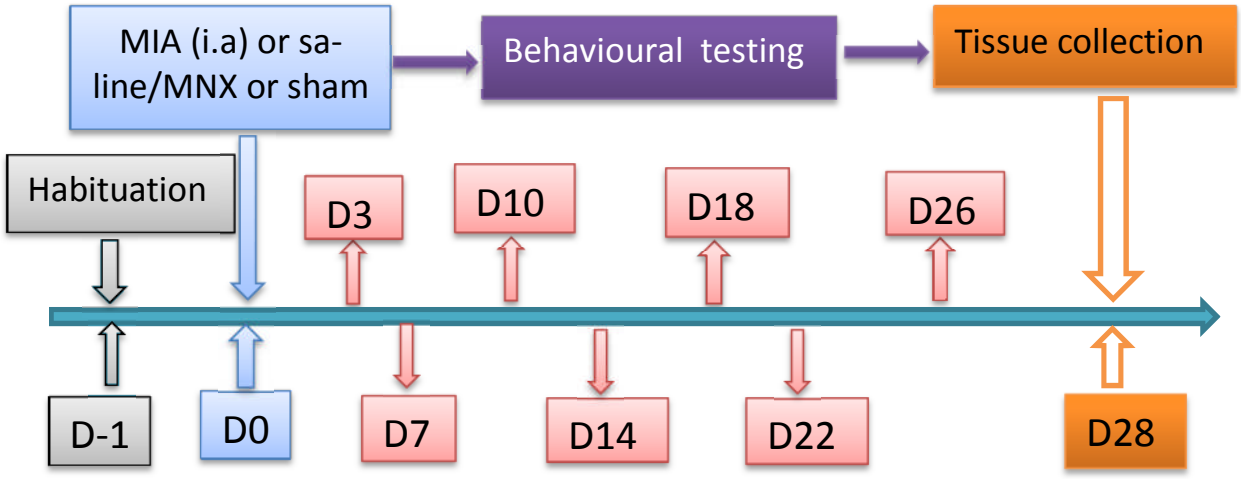


Fig 3.1 Flow-chart of study 1. Baseline values of behaviour (weight bearing and paw withdrawal threshold) were tested before surgery at day 0 and pain behaviour was tested twice a week from day 3 onwards until day 28. Tissues were collected at day 28 for joint histology.

A second study using the MIA and MNX was performed to allow the collection of fresh synovia and spinal cords (quadrant) for gene expression studies. For practical reasons in this study, tissues were collected at day 35 using a slightly extended protocol to that described in Figure 3.1.

3.2.2 Assessment of histopathology of knee joint

The general methods have been detailed in Chapter 2 (see 2.5-2.7). Briefly, joints were fixed in 10% neutral-buffered formalin and decalcified twice a week for 4-6 weeks. Knee joints were embedded in paraffin and cut into 5μm sections.

To assess joint histopathology in these two OA models, joint sections with clear structures of femoral condyle and medial tibial plateau qualified for H&E staining. Furthermore, 4 sections selected from different levels in each block were used for assessing histological scoring for each rat. Janusz's scoring system (Janusz et al. 2002) was used to score chondropathy, chondrocyte appearance and osteochondral junction integrity of medial tibial plateau in the ipsilateral knee joint. Synovial inflammation was also scored according to methods described in Chapter 2 (see 2.7.1).

3.2.3 Quantification of genes of interest in synovia and spinal cord

The synovia were dissected from the patella and infra-fat pad and were snap frozen in liquid nitrogen. Ipsilateral synovia were used for the quantification of gene expression. Spinal cord was split into ipsi-dorsal, ipsi-ventral, contra-dorsal and contra-ventral quadrants. The ipsi-dorsal part receives sensory inputs from the knee joint and is the region involved in the integration and modulation of noxious inputs, and was therefore used for gene expression studies. Tissues were homogenized and RNA was extracted by TRI reagent lysis buffer. Expression levels for resolvin receptors (ChemR23 and ALX), inflammatory cytokines and metabolic enzymes (see Table 2.1) were quantified by Taqman RT-qPCR as described in Chapter 2 (see 2.8).

3.2.4 Data analysis

Data were analysed with Graphpad Prism 6 and are presented as mean±S.E.M. Pain behavioural data (WB and PWT) were analysed by two-way ANOVA with Bonferronni's post-hoc test. Histological scoring was analysed by one-way ANOVA with Bonferroni's post-hoc test or Kruskal Wallis with Dunn's post-hoc test for non-parametric data. Gene expression levels were analysed by un-paired t-test (parametric data) or Mann-Whitney (non-parametric data) test. Correlation of gene expression of resolvin receptors and pain behaviour or genes of interest was performed with Pearson (parametric data) or Spearman (non-parametric data) test.

3.3 Results

3.3.1 The MIA and MNX-induced models of OA produce comparable pain behaviour

Behavioural assessment revealed that weight bearing asymmetry (**Fig 3.2 A**) and a lowering of the hindpaw withdrawal threshold (**Fig 3.2 B**) started as early as day 3 in the MIA model and day 7 in the MNX model, compared to their respective controls. The weight bearing difference increased and paw withdrawal threshold decreased with time and both measures of pain behaviour were maintained until the end of study in both models. There was no significant difference between the magnitudes of the pain behaviour exhibited by the MIA and MNX models.

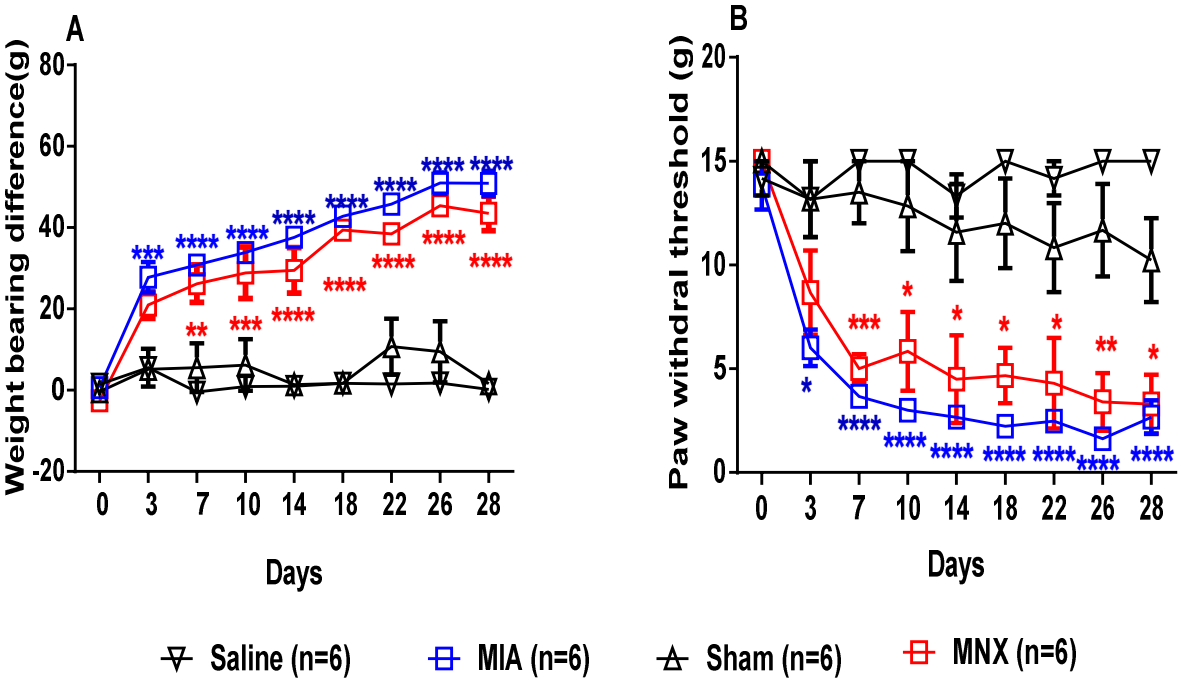


Fig 3.2 Intra-articular injection of MIA (1mg/50µl) increased weight bearing difference (A) and decreased paw withdrawal threshold of ipsilateral joint (B) from day 3 post model induction until day 28 in MIA rats, compared to saline treated rats. Medial meniscus transection (MNX) increased weight bearing difference (A) and reduced paw withdrawal threshold (B) in the MNX model, compared to sham control from day 7 post model induction until day 28. Data are mean±SEM (n= 6 rats per group) analyzed by two-way ANOVA with Bonferroni's post-hoc test (*p<0.05, **p<0.01, ***p<0.001, ****p<0.001).

3.3.2 Histological changes to the knee joint in the MIA and MNX models of OA pain

Histological scores for chondropathy (**Fig 3.3 A**), synovial inflammation (**Fig 3.3 B**) and chondrocyte appearance (**Fig 3.3 C**) were significantly higher in MIA rats compared to saline treated-rats. Vascular breaching across the osteochondral junction (**Fig 3.3 D**) appeared higher in MIA model compared to saline controls, but significance was not reached. MNX surgery resulted in significantly higher score for chondrocyte appearance compared to sham surgery (**Fig 3.3 C**) as well as apparently greater chondropathy (**Fig 3.3 A**), synovial inflammation (**Fig 3.3 B**) and vascular channels (**Fig 3.3 D**) although significance was not reached. There was no significant difference in joint histology between the two models.

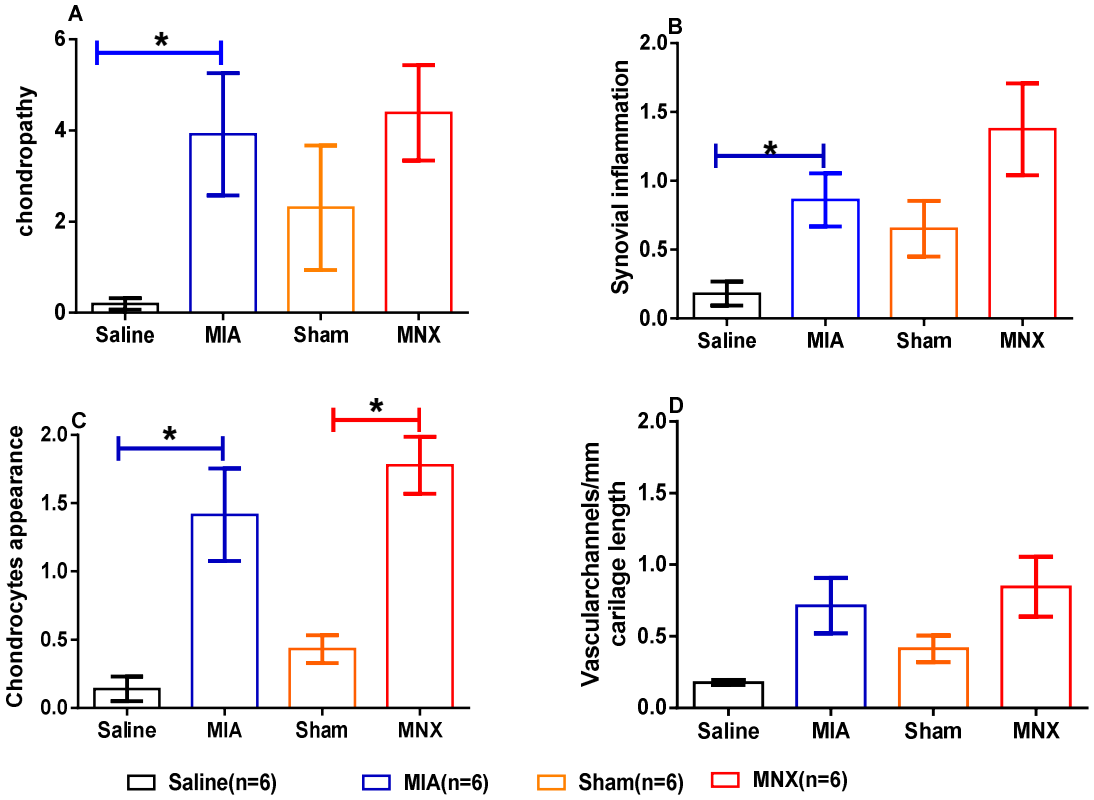


Fig 3.3 Histological assessment for knee joints was shown in MIA and MNX rats compared to the respective controls. Chondropathy score (A), synovial inflammation score (B), chondrocytes appearance score (C) and vascular breaching across osteochondral junction (D). Data are mean±S.E.M (n= 6) analysed with Kruskal-Wallis (A, B, C, non-parametric data) or one-way ANOVA with Bonferroni's post-hoc test (D, parametric data) (*$p<0.05$ vs saline or sham surgery).

H&E staining revealed intact structures of knee joints for both saline (**3.4 A**) and sham surgery (**3.4 C**) treated rats. Cartilage degeneration and hypocellularity of chondrocytes were evident in the MIA and MNX models (**3.4 B, D**).

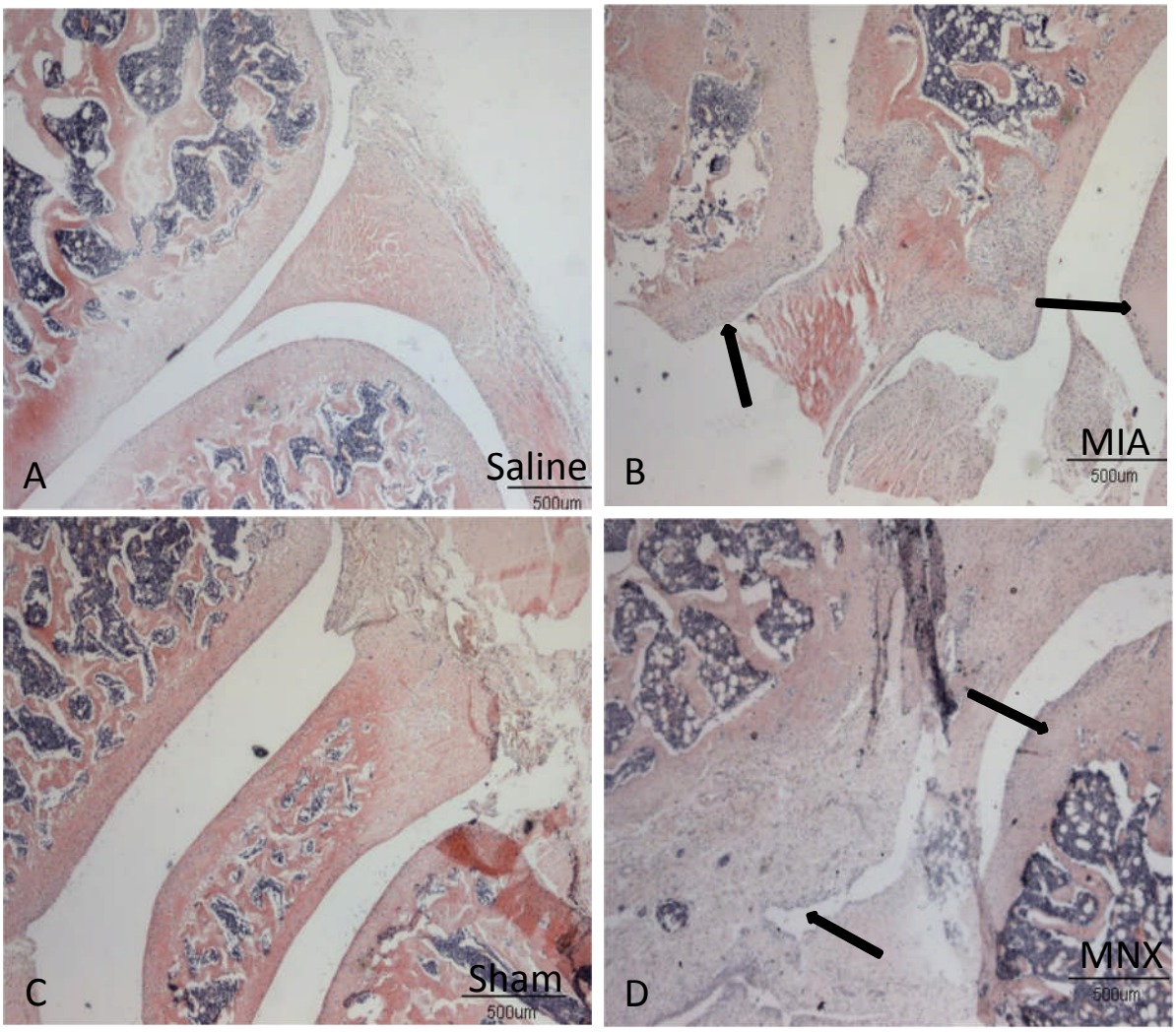


Fig 3.4 H&E staining revealed chondropathy and hypocellularity of chondrocyte in models of OA compared to the respective controls. Cartilage damage and hypocellularity of chondrocyte (black arrows) was present in MIA (B) and MNX (D) rats, compared to their respective controls, saline (A) and sham(C). Scale bar equals 500µm.

The synovia from saline (**Fig 3.5 A**) or sham (**Fig 3.5 C**) treated rats were only 1-2 cells thick. The synovia from MIA (**Fig 3.5 B**) or MNX (**Fig 3.5 D**) rats were composed of many layers of cells (hyperplasia).

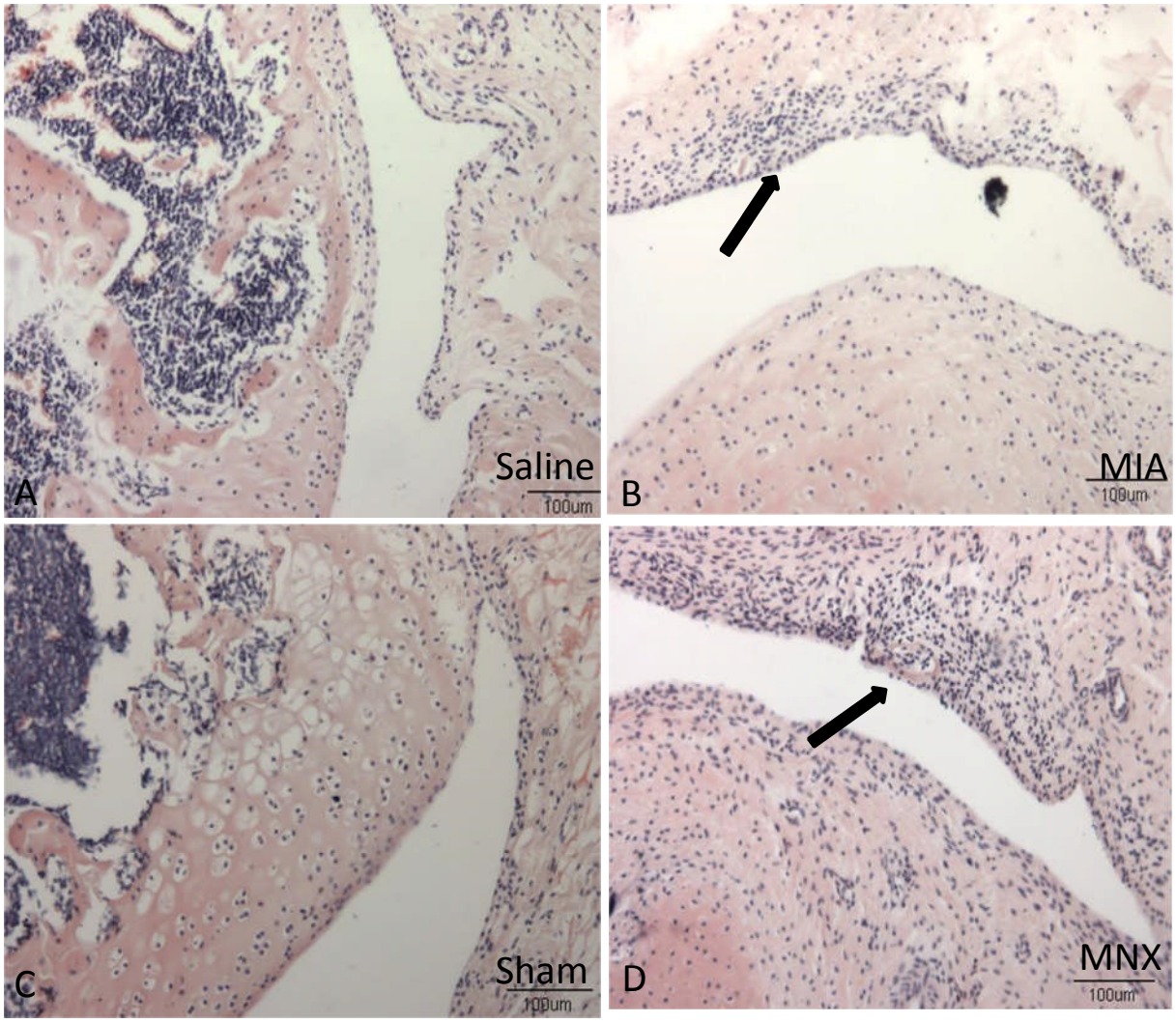


Fig 3.5 Comparison of the extent of synovial inflammation in MIA and MNX rats, versus respective controls. HE staining revealed a greater number of cells (black arrows) within the synovia in MIA (B) and MNX (D) compared to, saline (A) and sham (C). Scale bar is 100µm.

3.3.3 MIA associated behaviour and synovial gene expression

3.3.3.1 Pain behaviour in the MIA model

Similar to the data shown in 3.3.1, intra-articular injection of MIA resulted in robust pain behaviour (**3.6 A, B**) until day 35 post model induction.

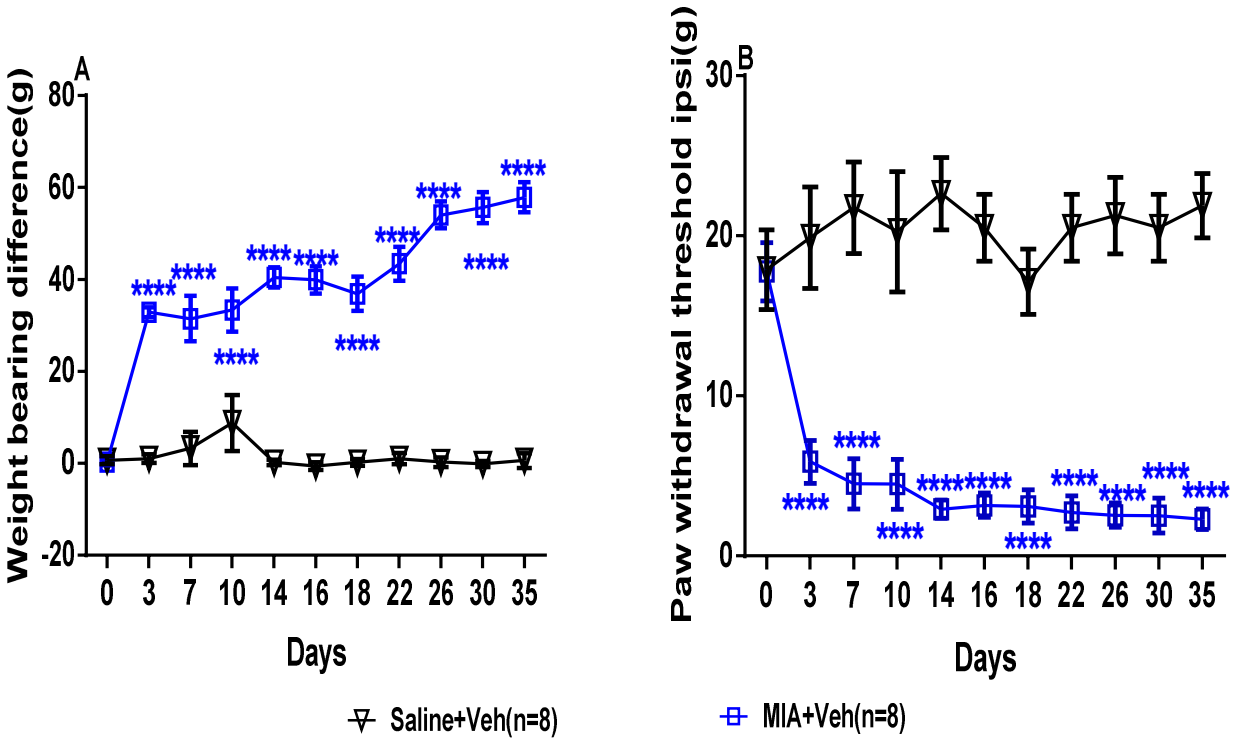


Fig 3.6 Intra-articular injection of MIA (1mg/50µl) was associated with an increase in weight bearing difference up to day 35 (A) and a lowering of the hindpaw withdrawal threshold (B) compared to saline injected control group. Data are mean ± S.E.M (n= 8), analyzed by two-way ANOVA with Bonferroni ‘s post-hoc test (****p<0.0001).

3.3.3.2 Expression of resolvin receptors, cytokines and enzymes in the MIA model of OA pain

The levels of gene expression of ChemR23 and ALX, some key inflammatory cytokines and associated metabolic enzymes were quantified in synovia from the models. As showed in **Fig 3.7**, the expression level of ChemR23 was significantly lower in synovia from MIA treated rats, compared to saline treated-rats (**Fig 3.7A**) Expression of ALX was comparable between the two groups of rats (**Fig 3.7B**).

There was no significant difference in the gene expression of the cytokines studied in MIA and saline treated rats (**Fig 3.8**). With regards to the enzymes, expression of 15-LOX was significantly lower in synovia from MIA-treated rats compared, to saline-treated rats (**Fig 3.9B**). There were no other differences in the expression of the other enzymes studied (**Fig 3.9**).

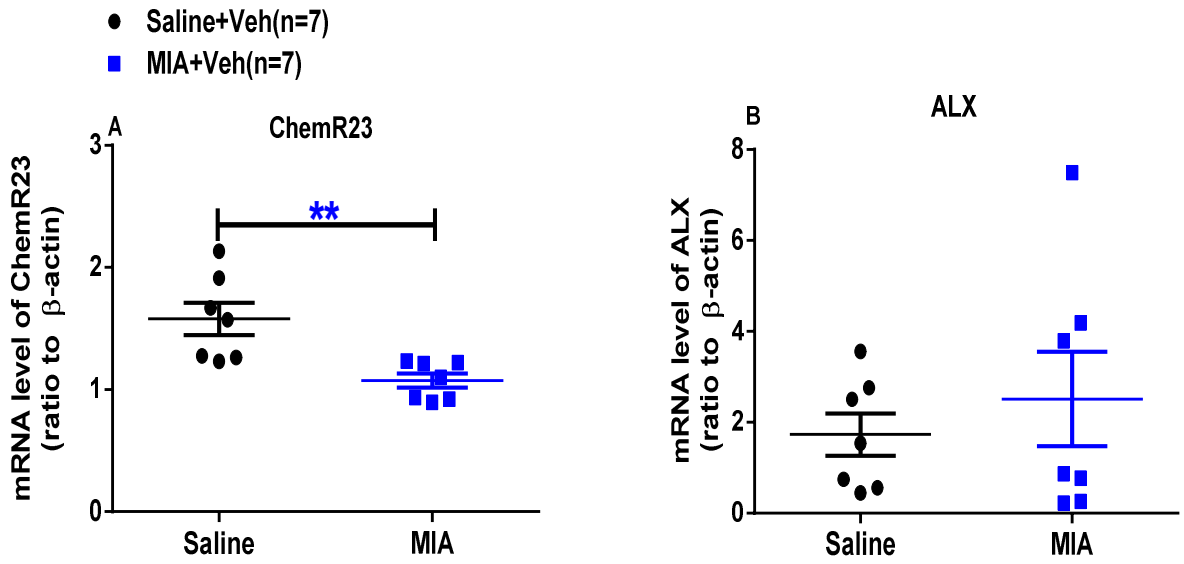


Fig 3.7 Gene expression of ChemR23 (A) was significantly lower in the MIA synovia, compared to control (saline) at 35 days following model induction. There was no difference in ALX (B) expression between synovia from MIA and saline treated rats. Data areindividual data points as a scatterplot, with mean ± S.E.M indicated by the bars, analysed with an unpaired t-test, (**p<0.01).

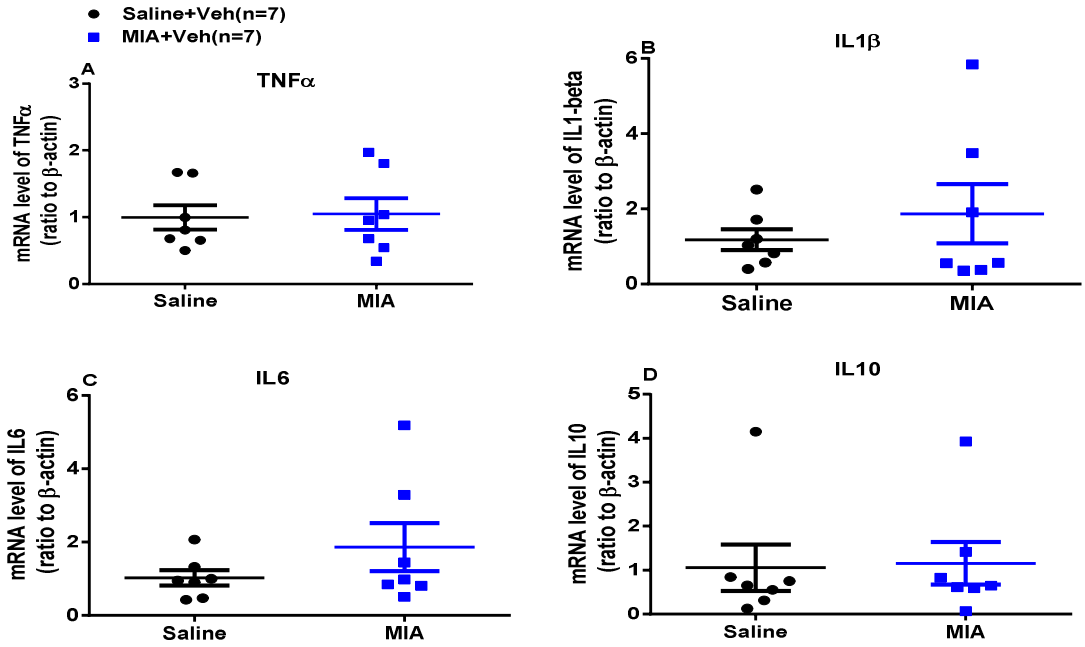


Fig 3.8 Gene expression of selected cytokines (TNFα, IL1β, IL6 and IL10) in the synovia of MIA rats at day 35 following model induction compared to saline treated rats. Data are individual data points as a scatter plot, with mean±S.E.M (n= 7) analysed with un-paired t-test or Mann-Whitney t-test.

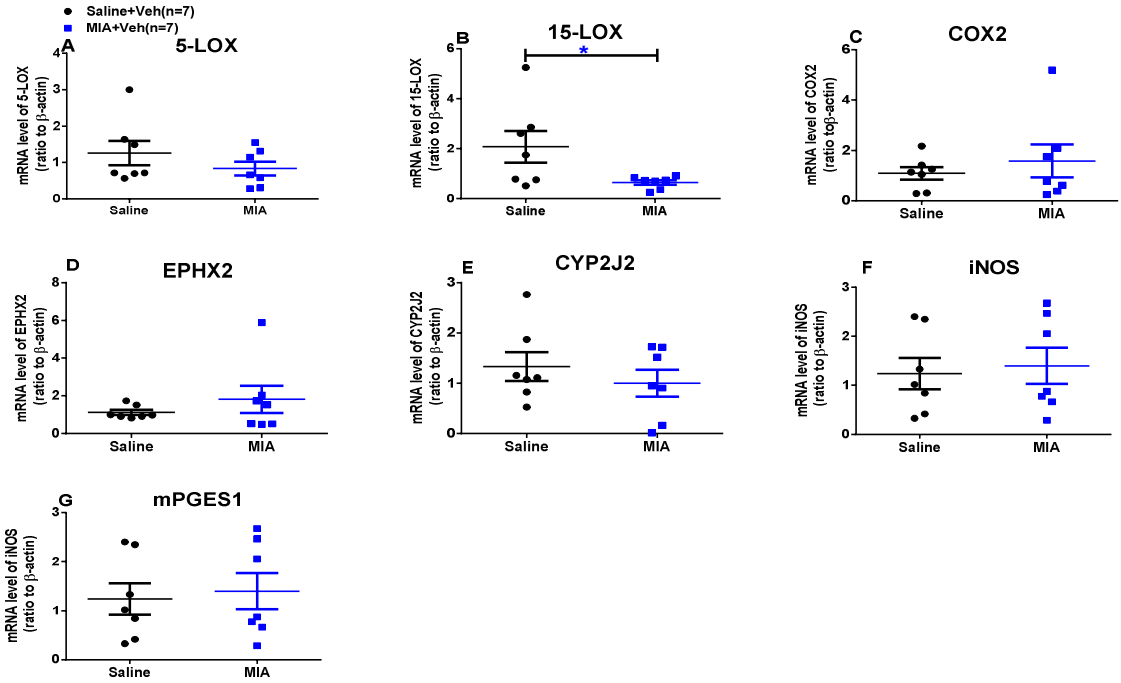


Fig 3.9 Expression levels of enzymes in synovia from the MIA model at day 35 post model induction compared to saline controls. 5-LOX(A),15-LOX (B) , COX_2(C), $EPHX_2$(D), CYP_2J_2(E), iNOS(F) and m $PGES_1$ (G). Data are individual data points as a scatter plot, with mean±S.E.M analysed with an unpaired t-test or Mann-Whitney t-test (*p<0.05).

3.3.4 MNX associated behaviour and synovial gene expression

3.3.4.1 Pain behaviour in the MNX model

The development of pain behaviour in the MNX rats, compared to sham controls was studied up to day 35 post-surgery. Significant weight bearing difference was evident at day 18 post MNX surgery and lasted until the end of the study (**Fig3.10A**). The paw withdrawal threshold appeared lower in the MNX model, but was not significant compared to sham controls (**Fig3.10B**). Some of the sham surgical rats also showed pain behaviour over the time-course of the study.

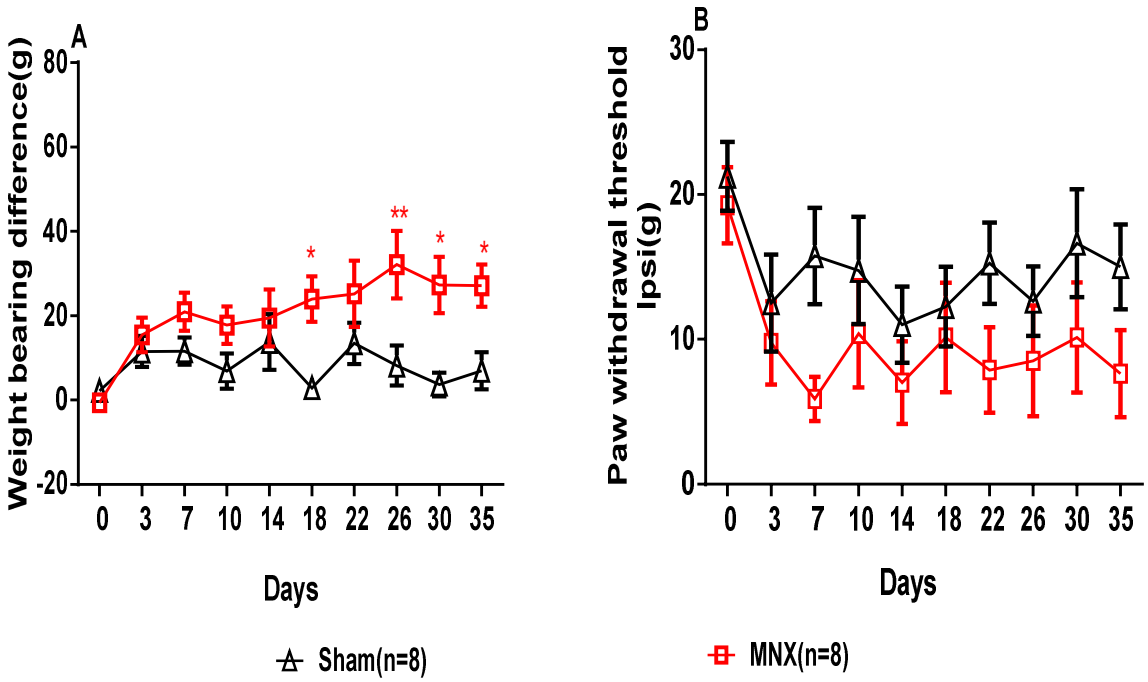


Fig 3.10 Medial meniscus transection (MNX) significantly increased weight bearing difference (A) compared to sham control from day 18 until day 35 post model induction. Data are mean±S.E.M analyzed by two-way ANOVA with Bonferroni 's post-hoc test (*p<0.05, **p<0.01).

3.3.4.2 Expression of resolvin receptors, cytokines and key enzymes in the synovia of MNX model

The expression of mRNA encoding ChemR23 and ALX, inflammatory cytokines and associated metabolic enzymes was quantified in synovia of MNX rats. Gene expression was normalized to the geometric mean of β-actin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in the MNX model and sham control as β-actin was not consistent across the two groups based on data analysis for β-actin. There was no significant difference in the expression of ChemR23 (**Fig 3.11A**) between the two groups.

Expression of ALX was significantly lower in MNX rats compared to sham surgical rats (**Fig 3.11B**). TNFα and IL1β expression was significantly lower in MNX synovia (**Fig 3.12A, Fig3.12B**), compared to sham control. Gene expression of COX2, EPHX2 and mPGES1 was also significantly lower in MNX synovia, compared to sham control. There were no significant differences in the other genes studied (**Fig 3.13**).

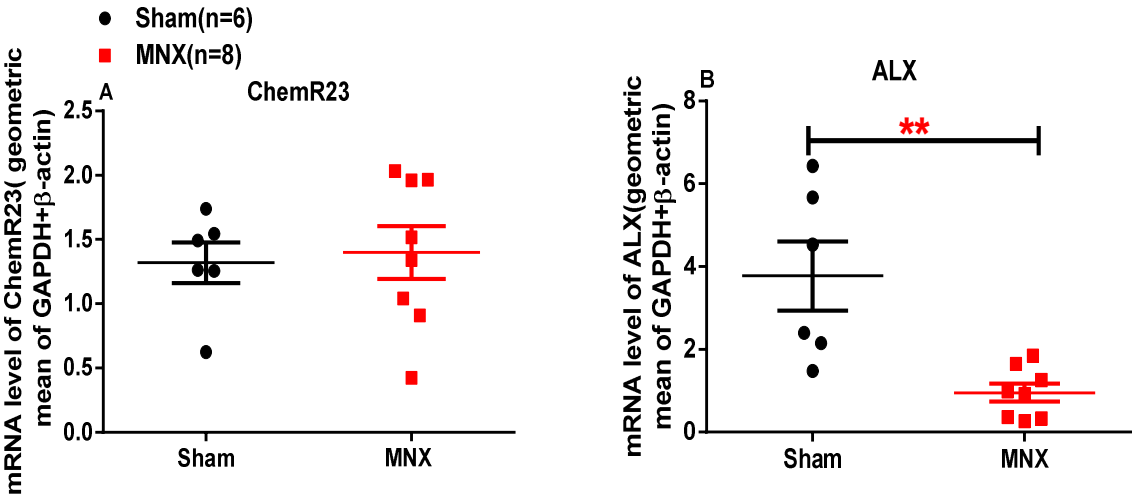


Fig 3.11 Gene expression of ChemR23 (A) and ALX (B) in the synovia from MNX rats at 35 days following model induction compared to sham controls. Data are individual data points as a scatter plot, with mean ± S.E.M, analysed with an unpaired t-test (*p<0.05, **p<0.01).

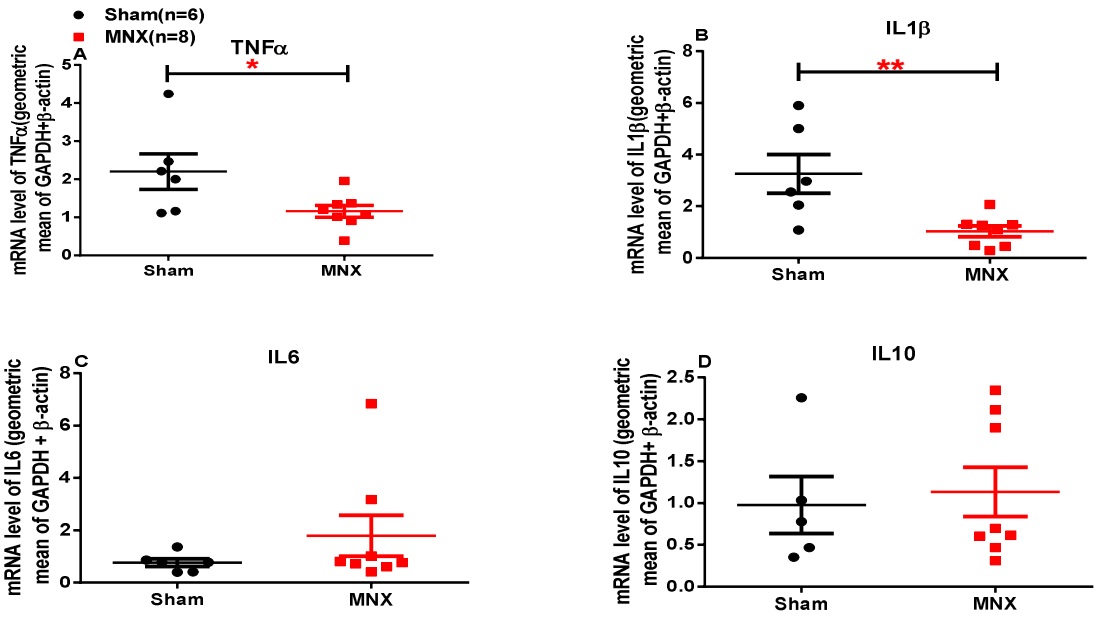


Fig 3.12 Gene expression of cytokines (TNFα, IL1β, IL6 and IL10) in synovia from MNX rats at day 35 post model induction compared to sham controls. TNFα (A), IL1β (B), IL6 (C) and IL10 (D). Data are individual data points as a scatter plot, with mean±S.E.M (n= 5- 8) analysed with unpaired t-test or Mann-Whitney t-test. (*p<0.05, **p<0.01).

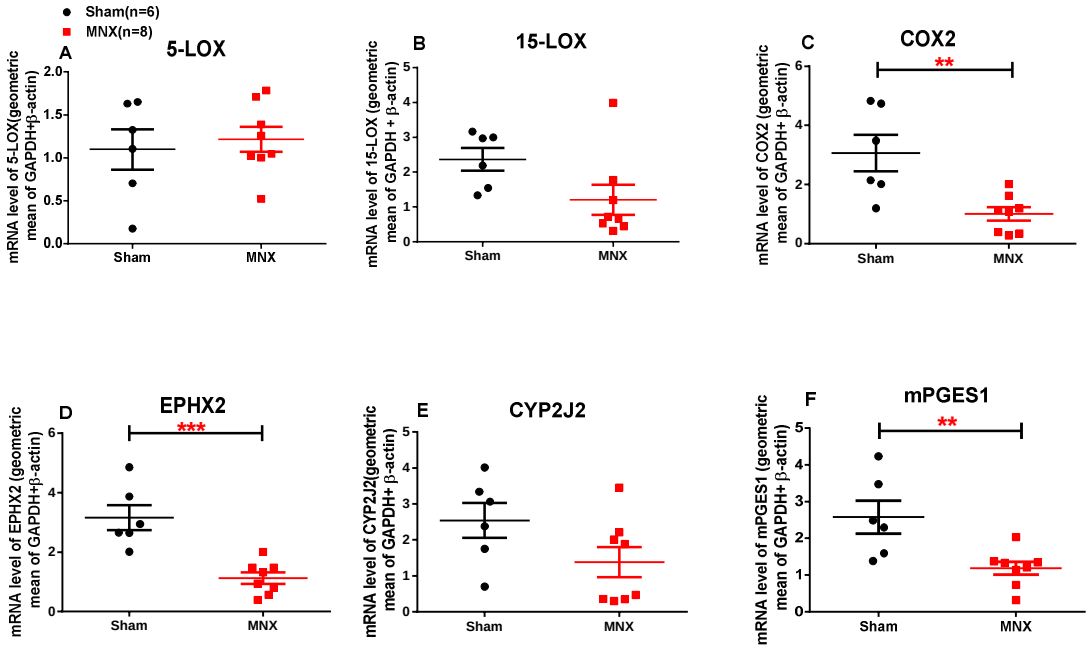


Fig 3.13 Gene expression of enzymes in synovia from MNX rats at day 35 post model Induction compared to sham controls. 5-LOX(A),15-LOX (B) , COX_2(C), $EPHX_2$(D), CYP_2J_2(E), iNOS(F) and m $PGES_1$ (G). Data are individual data points as a scatter plot, with mean±S.E.M (n= 6-8) analysed with unpaired t-test or Mann-Whitney t-test. (**p<0.01, ***p<0.001).

3.3.5 The relative expression of ChemR23 and ALX in synovia of the two models

To obtain a clearer picture of the relative contribution of the resolvin receptors in synovia in the models of OA pain, expression levels of these two receptors were compared for each condition **(Fig3.14)**. It was evident that the relative expression of the two receptors in synovia was comparable for all treatments, apart from the sham surgery group which had a significantly higher expression of ALX compared to ChemR23 (**Fig3.14C**).

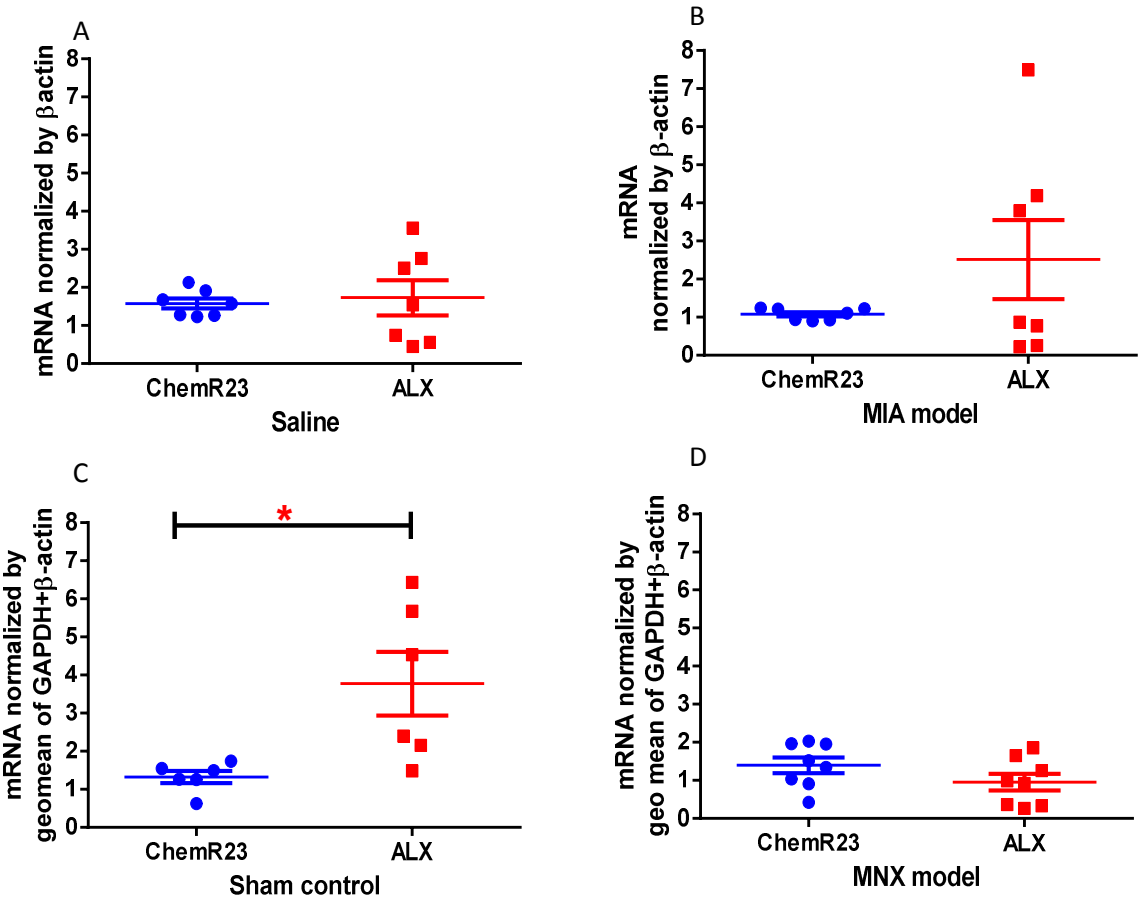


Fig 3.14 Comparison of gene expression for ChemR23 and ALX in the models of OA and control groups at day 35. Data are presented as individual data points as a scatter plot, with mean ± S.E.M, analysed with unpaired t-test (*p<0.05).

3.3.6 Correlation of resolvin receptors with genes of interest in synovia from the two models of OA

To investigate whether the expression pattern of the two resolvin receptors in synovia is associated with expression of inflammatory cytokines and their respective metabolic enzymes, correlation analysis was undertaken in MIA (**Table 3.1**) and MNX (**Table 3.2**) rats. Expression of ChemR23 in the synovia was not correlated with the expression of any of the genes of interest in the synovia from MIA or saline treated rats. There was a significantly positive correlation of ALX expression with TNFα, IL1β and COX2 in MIA-treated rats and ALX with IL1β and COX2 expression in saline-treated rats. There was a significantly negative correlation of ALX and 5-LOX expression in MIA rats. Gene expression of ChemR23 in the synovia was not correlated with the expression of any genes of interest in the synovia from MNX or sham surgical rats. There was a significantly positive correlation of expression of ALX with TNFα, IL1β, COX2, EPHX2 and mPGES1 in synovia of both MNX and sham surgical controls.

Treatment	Genes	TNFα	IL1β	IL6	IL10	5-LOX	15-LOX	COX2	EPHX2	CYP2J2	iNOS	mPGES1
Saline	ChemR23	r=0.09 p=0.84	r=-0.22 p=0.63	r=0.46 p=0.30	r=-0.04 p=0.96	r=0.60 p=0.17	r=-0.27 p=0.56	r=0.02 p=0.97	r=0.21 p=0.66	r=0.60 p=0.17	r=0.36 p=0.42	r=0.38 p=0.10
	ALX	r=0.43 p=0.33	r=0.83 p=**0.02***	r=0.07 p=0.89	r=0.50 p=0.23	r=-0.14 p=0.78	r=0.00 p=0.99	r=0.77 p=**0.04***	r=0.68 p=0.11	r=0.68 p=0.11	r=0.39 p=0.39	r=0.49 p=0.27
MIA	ChemR23	r=0.35 p=0.44	r=0.19 p=0.71	r=0.68 p=0.11	r=0.42 p=0.40	r=0.17 p=0.71	r=0.20 p=0.67	r=0.55 p=0.25	r=0.26 p=0.61	r=-0.07 p=0.88	r=0.30 p=0.52	r=0.71 p=0.07
	ALX	r=0.88 p=**0.01***	r=0.82 p=**0.03***	r=0.25 p=0.59	r=0.65 p=0.12	r=-0.79 p=**0.04***	r=-0.21 p=0.65	r=0.84 p=**0.04***	r=0.72 p=0.11	r=-0.09 p=0.84	r=0.60 p=0.15	r=0.60 p=0.15

Table 3.1 Correlation of ChemR23 and ALX expression with inflammatory cytokines (TNFα, IL1β, IL6 and IL10) and associated metabolic enzymes (5-LOX, 15-LOX, COX2, $EPHX_2$, CYP_2J_2, iNOS and mPGES1) in the synovia from the MIA model and saline control. Data were analysed with Pearson or Spearman correlation (n=7, r is the co-efficient of correlation, *p<0.05).

Groups	Genes	TNFα	IL1β	IL6	IL10	5-LOX	15-LOX	COX2	EPHX2	CYP2J2	mPGES1
Sham	ChemR23	r=0.55 p=0.25	r=0.34 p=0.51	r=0.47 p=0.35	r=-0.77 p=0.13	r=0.63 p=0.18	r=0.29 p=0.57	r=0.34 p=0.50	r=-0.02 p=0.98	r=-0.19 p=0.72	r=0.44 p=0.39
	ALX	r=0.86 p=**0.03***	r=0.94 p=**0.02***	r=0.30 p=0.56	r=0.09 p=0.88	r=-0.13 p=0.81	r=-0.19 p=0.71	r=0.99 p=**0.02** **x10^{-2}***	r=0.81 p=**0.05***	r=-0.12 p=0.82	r=0.90 p=**0.01***
MNX	ChemR23	r=0.56 p=0.15	r=0.33 p=0.43	r=0.57 p=0.15	r=0.10 p=0.84	r=0.52 p=0.19	r=0.56 p=0.15	r=0.02 p=0.97	r=0.21 p=0.63	r=0.15 p=0.73	r=0.72 p=0.05*
	ALX	r=0.84 p=**0.01***	r=0.95 p=**0.04** **x10^{-2}***	r=0.12 p=0.79	r=-0.12 p=0.79	r=0.43 p=0.29	r=0.52 p=0.19	r=0.96 p=**0.02** **x10^{-2}***	r=0.91 p=**0.15** **x10^{-2}***	r=-0.25 p=0.56	r=0.73 p=**0.04***

Table 3.2 Correlation of ChemR23 and ALX expression with inflammatory cytokines (TNFα, IL1β, IL6 and IL10) and associated metabolic enzymes (5-LOX, 15-LOX, COX2, $EPHX_2$, CYP_2J_2, iNOS and mPGES1) in the synovia from the MNX model and sham control. Data were analyzed with Pearson or Spearman correlation (n=7, r is the co-efficient of correlation, *p<0.05).

3.3.7 Correlation of ChemR23 and ALX with pain behaviour in models of OA

Correlation of the synovial expression of ChemR23 and ALX with pain behaviour (WB and PWT) in the MIA model (day14 and day 35) and MNX model (Day 35) was determined.

Expression of ChemR23 in the synovia was negatively correlated with weight bearing difference at both 14 days (**Fig3.15A)** and 35 days **(Fig3.16A**) following MIA induction. In parallel, expression of ChemR23 in the synovia was positively correlated with paw withdrawal thresholds at both time-points (**Fig3.15B, Fig3.16B**) in the MIA model. These data demonstrate that less weight bearing difference and higher paw withdrawal threshold (less pain) are associated with higher expression of ChemR23 in the synovia from the MIA model at both day 14 and day 35. There was no correlation of ALX expression in the synovia with pain behaviour at either time-point in the MIA model (**Fig3.15C, D and Fig3.16C, D**).

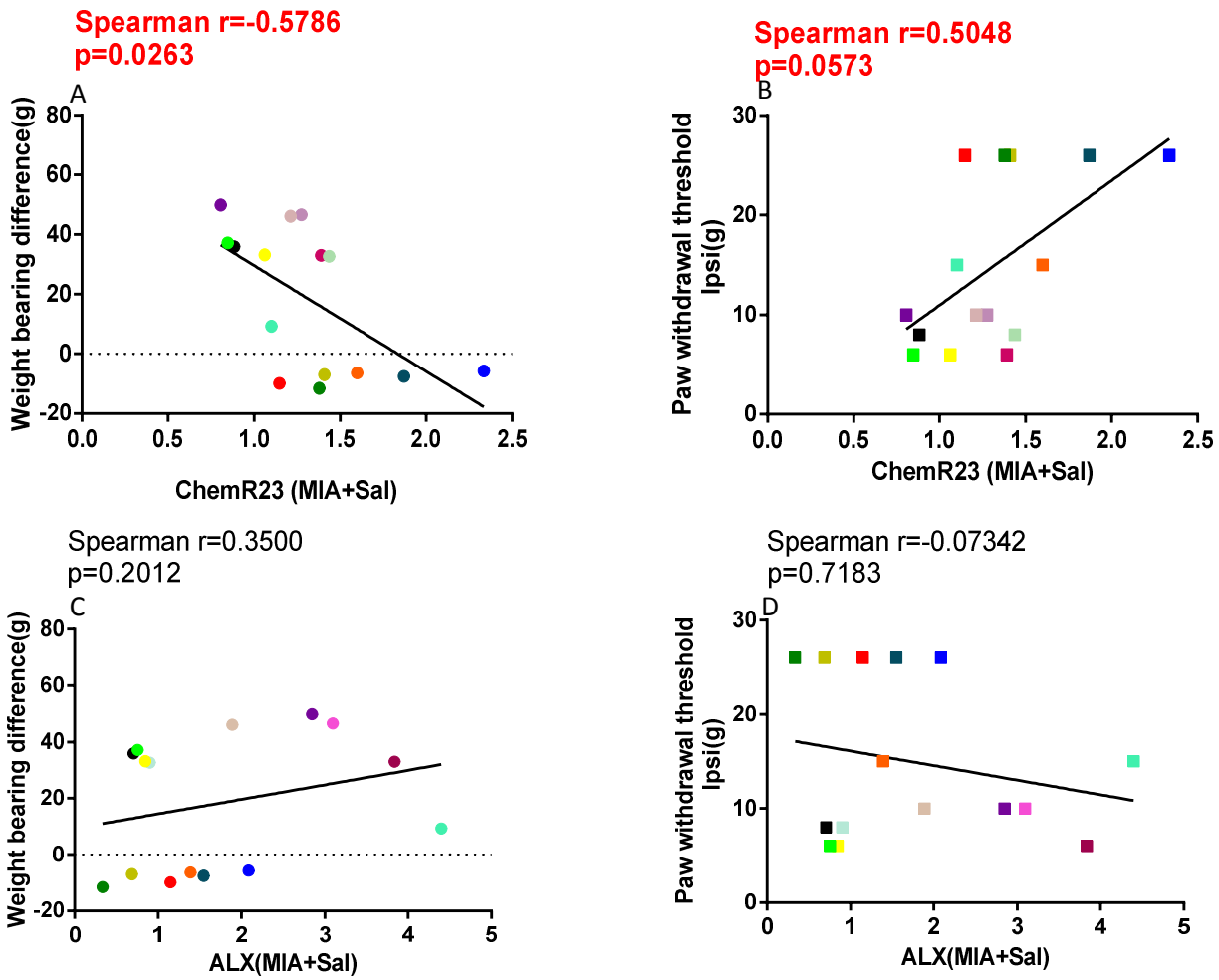


Fig 3.15 The correlation of mRNA level of ChemR23/ALX with weight bearing (A/C) and paw withdrawal threshold (B/D) in the synovia in the MIA model and saline control at day 14. The maximal force for von Frey hair was 26g. Data were analysed with Spearman

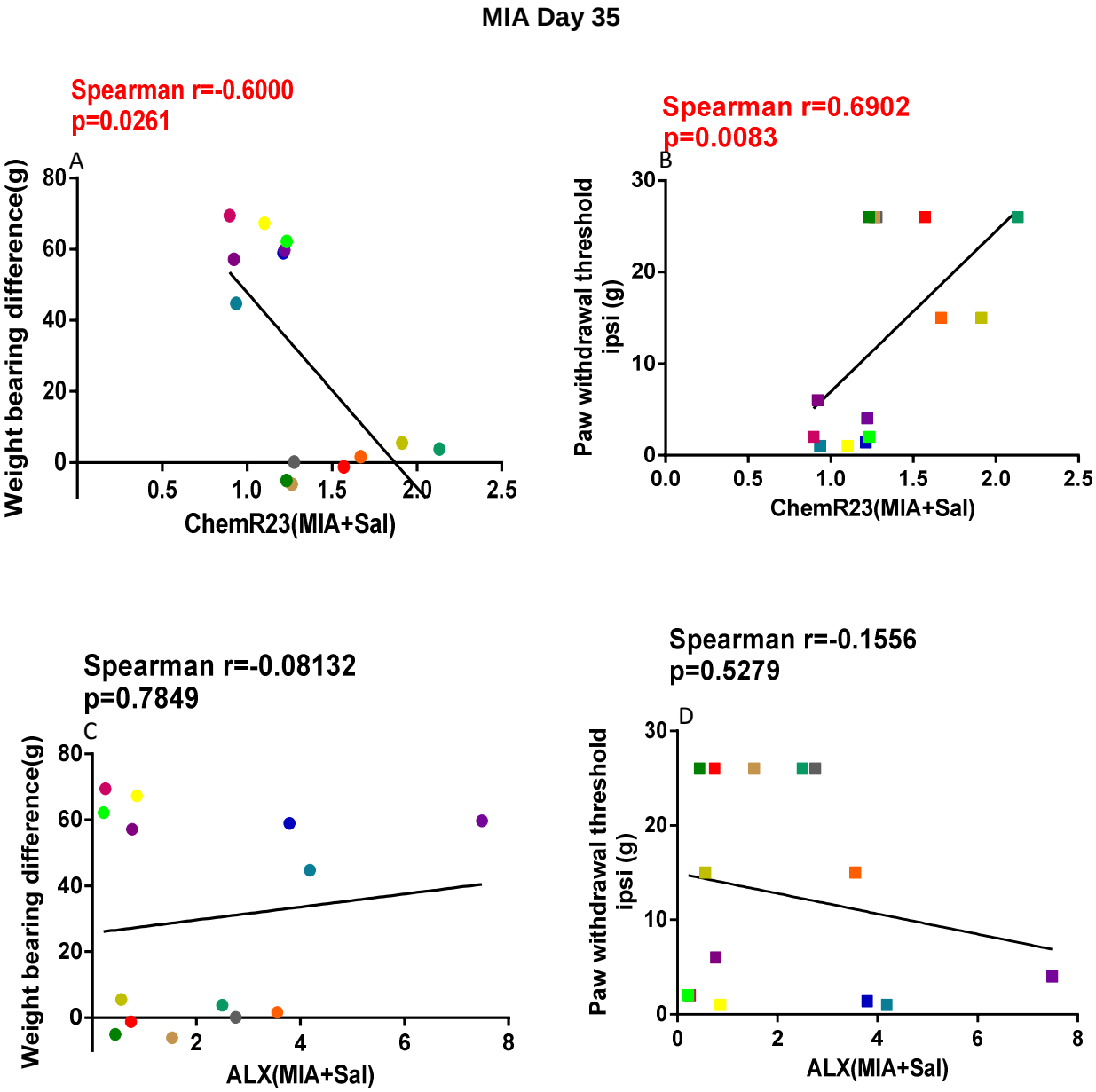


Fig 3.16 The correlation of mRNA level of ChemR23/ALX with weight bearing (A/C) and paw withdrawal threshold (B/D) in the synovia in the MIA model and saline control at day 35. The maximal force for von Frey hair was 26g. Data were analysed by Spearman correlation.

In the MNX model, expression levels of ChemR23 in the synovia were negatively correlated with paw withdrawal threshold (**Fig 3.17B**) but not correlated with weight bearing difference (**Fig 3.17A**). There was no correlation between the expression of ALX in the synovia and either measure of pain behaviour.

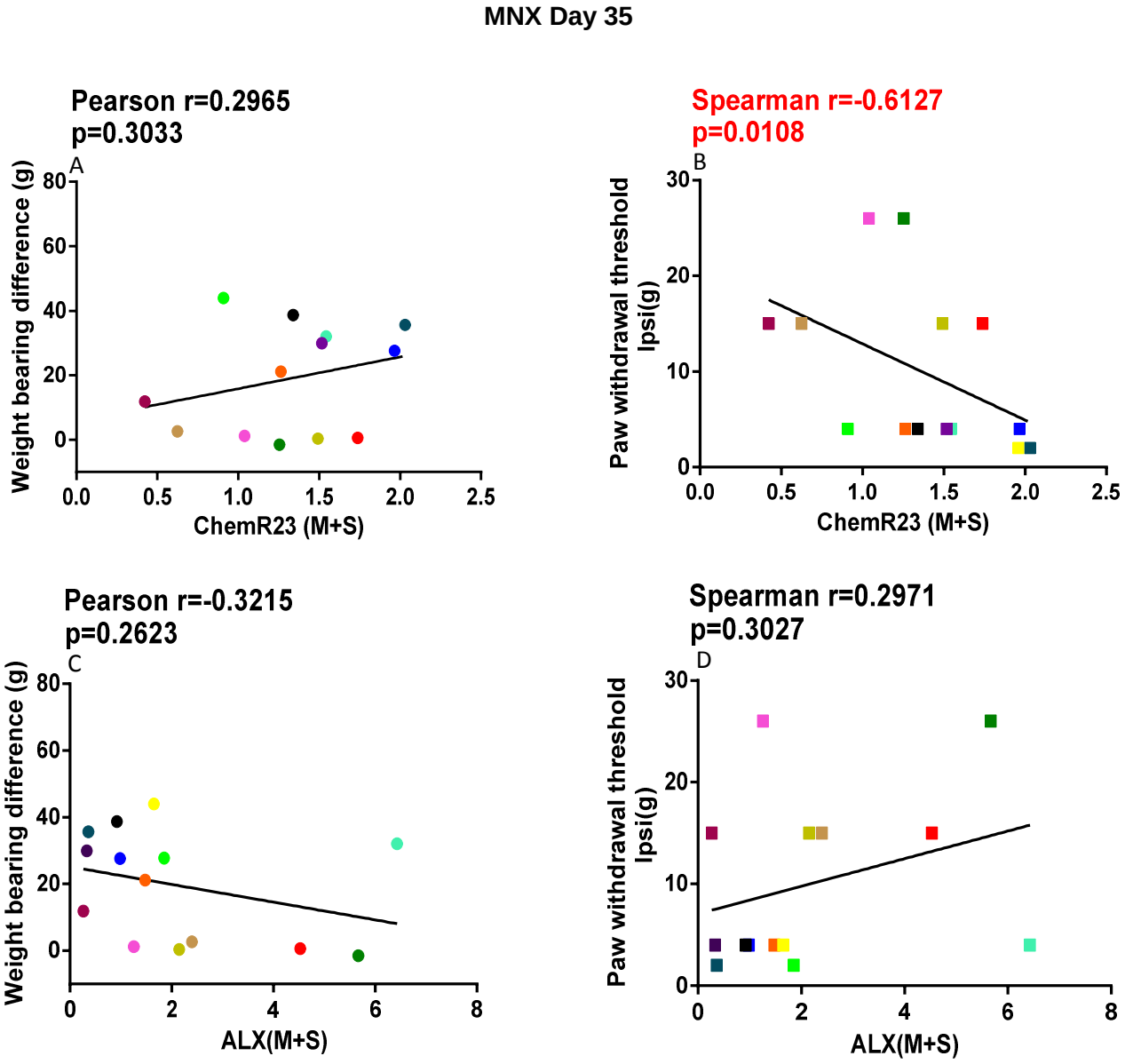


Fig 3.17 The correlation of mRNA level of ChemR23/ALX with weight bearing (A/C) and paw withdrawal threshold (B/D) in the synovia in the MNX model and sham control at day 35. The maximal force for von Frey hair was 26g. Data were analysed by Pearson or Spearman correlation.

3.3.8 Expression levels of target genes in spinal cord from the MIA model

Gene expression of ChemR23 and ALX, inflammatory cytokines and associated metabolic enzymes was quantified in the ipsilateral dorsal horn quadrant of the spinal cord from MIA rats at day 35 post model induction. As showed in **Fig 3.18,** expression level of ChemR23 in the ipsilateral dorsal horn quadrant was significantly higher in MIA rats, compared to saline treated-rats (**Fig 3.18A**) Expression levels of ALX in the ipsilateral dorsal horn quadrant of MIA rats were comparable to levels in saline treated rats (**Fig 3.18B**).

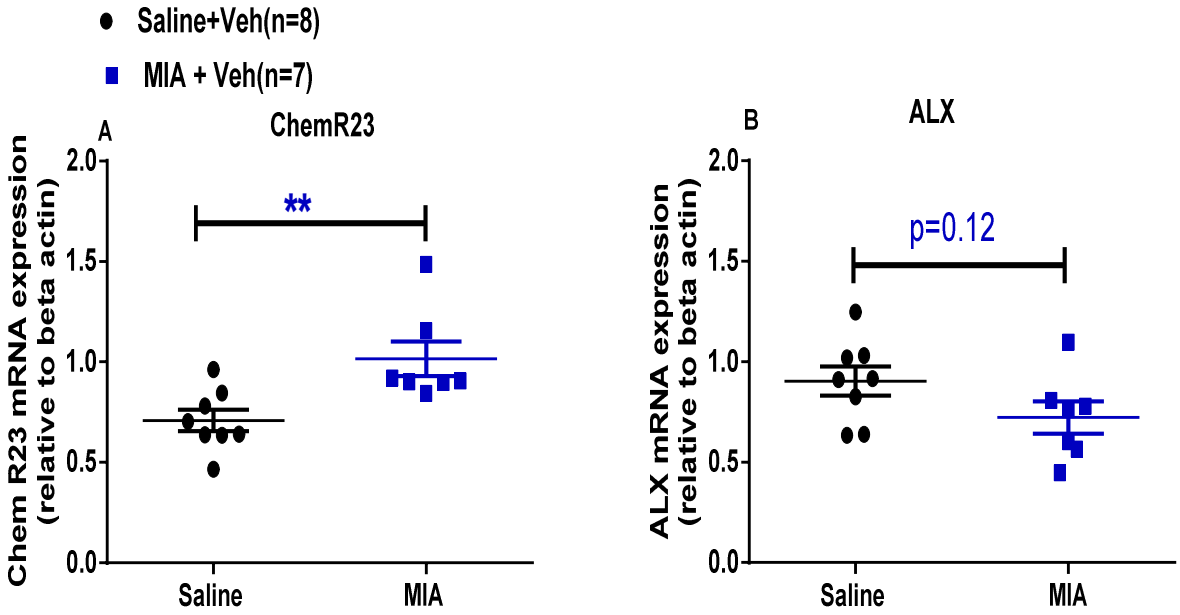


Fig 3.18 Gene expression of ChemR23 (A) and ALX (B) in the ipsilateral dorsal quadrant of the spinal cord from MIA or saline-treated rats at 35 days following model induction. Data are individual data points as a scatterplot, with mean ± S.E.M, analysed with Mann-Whitney test (A) and unpaired t-test (B) (**p<0.01)

There was no significant difference in the expression of selected cytokines in the ipsilateral dorsal horn quadrant between MIA and saline treated rats (**Fig 3.19**). Expression of 15-LOX had a trend towards a decrease in the ipsilateral dorsal horn quadrant of MIA-treated rats, compared to saline controls (**Fig 3.20C**). There was a trend towards an increase in the expression of COX2 (**Fig 3.20D**) in the ipsilateral dorsal horn quadrant of MIA rats, compared to saline controls.

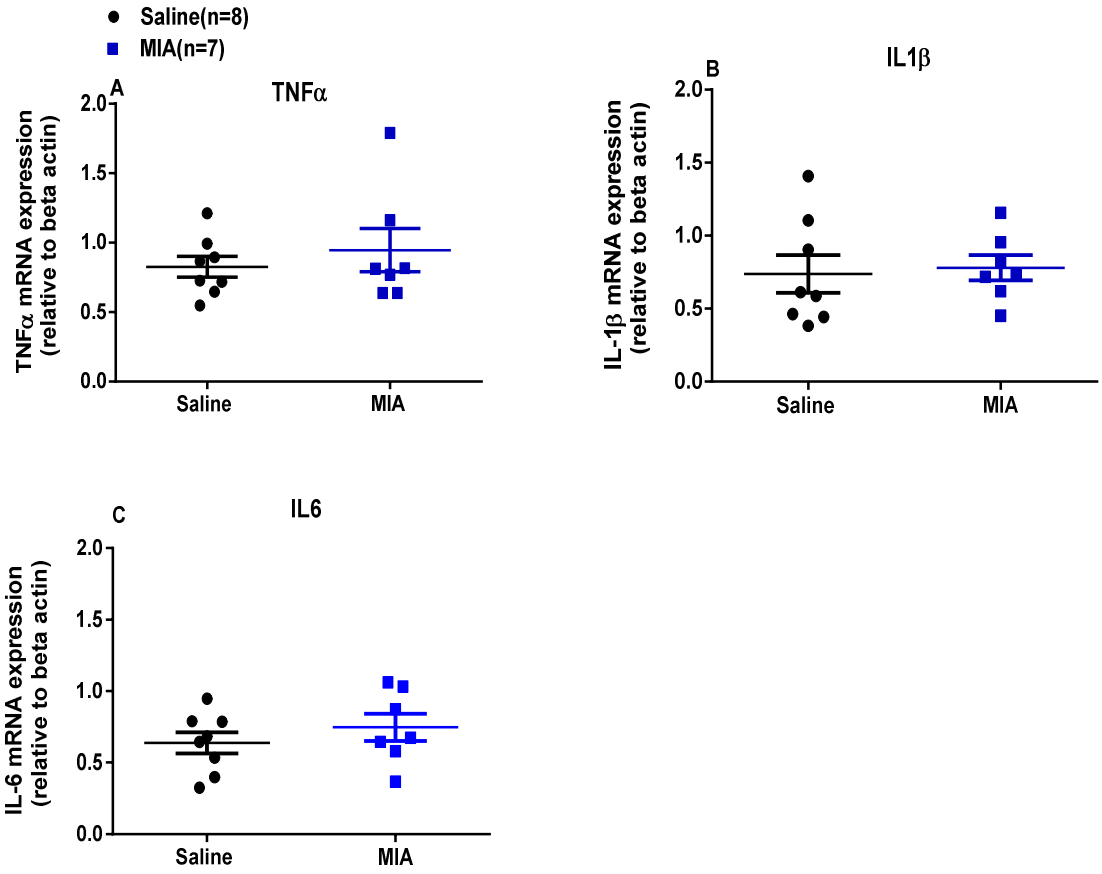


Fig 3.19 Gene expression of selected cytokines in the ipsilateral dorsal of the spinal cord from MIA rats at day 35 post model induction. TNFα (A), Ilβ (B) and IL6 (C). Data are individual data points as a scatterplot, with mean±S.E.M (n= 7-8) analysed with un-paired t-test.

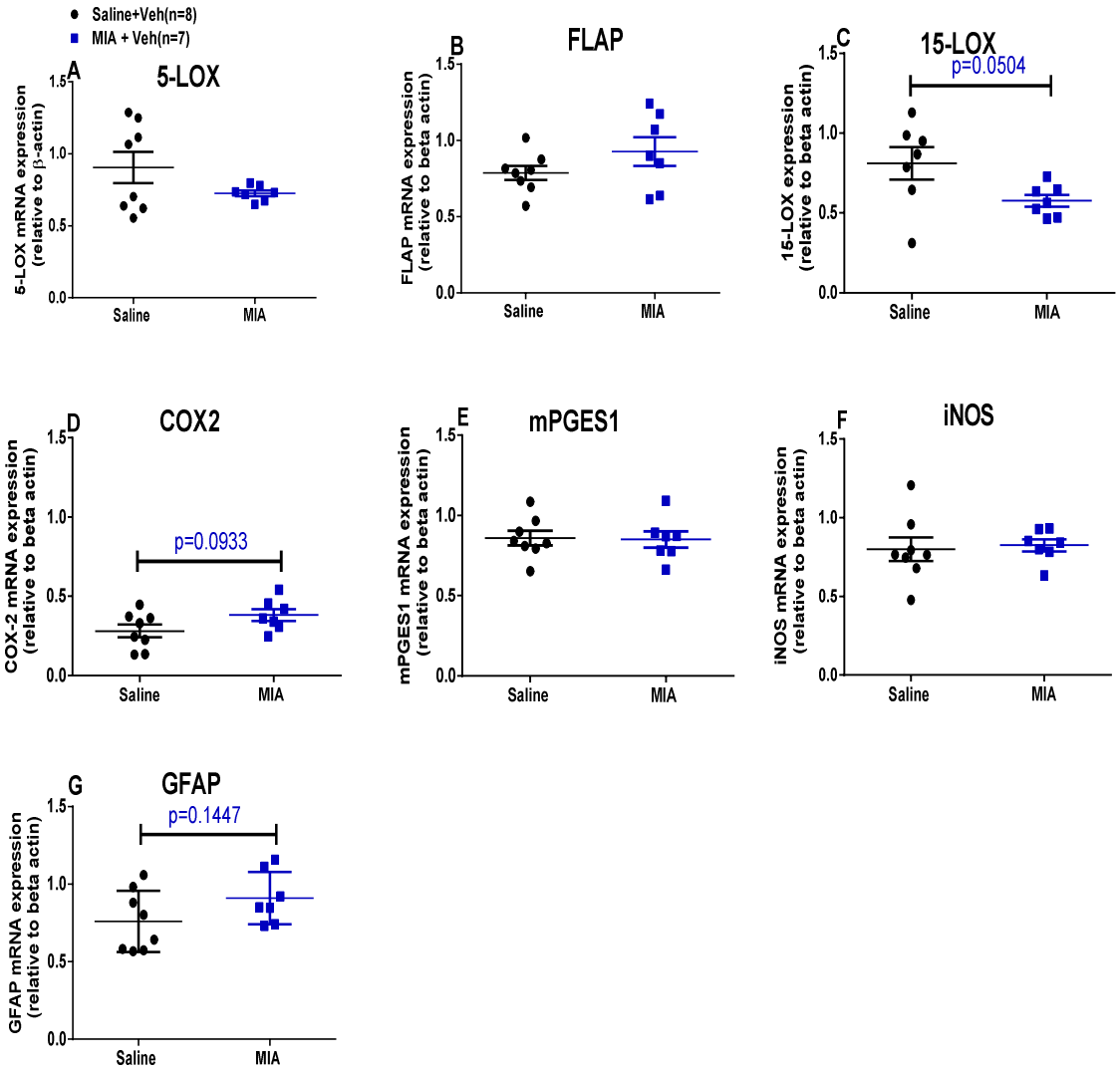


Fig 3.20 Gene expression of enzymes in the ipsilateral dorsal quadrant of the spinal cord from MIA rats at day 35 post model induction. 5-LOX (A), FALP (B), 15-LOX (C), COX_2 (D), m PGES1 (E), iNOS (F) and GFAP (G). Data are individual data points as a scatterplot, with mean±S.E.M analysed with unpaired t-test or Mann-Whitney t-test. (*p<0.05)

3.3.9 Expression levels of target genes in spinal cord from the MNX model

Gene expression of ChemR23 and ALX, inflammatory cytokines and associated metabolic enzymes was quantified in ipsilateral dorsal horn quadrant of the spinal cord in MNX rats and sham controls. There was no significant difference in the spinal expression of ChemR23 or ALX between the two groups (**Fig 3.21A, Fig 3.21B**).

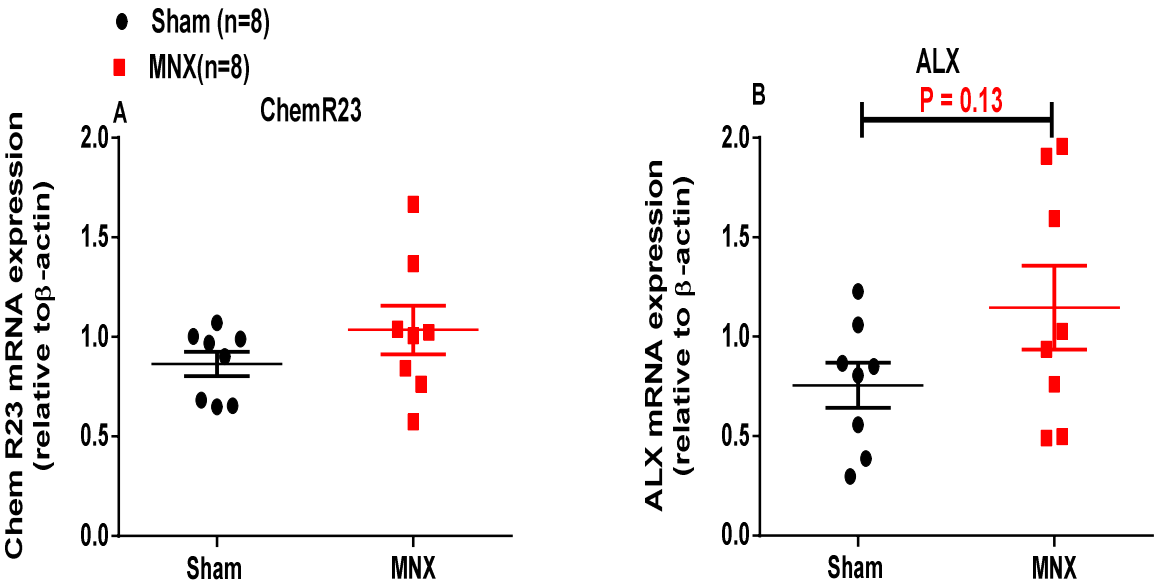


Fig 3.21 Gene expression of ChemR23 (A) and ALX (B) in the spinal cord from MNX and sham-operated rats at 35 days following model induction. Data are individual data points as a scatterplot, with mean ± S.E.M, analysed with unpaired t-test.

For the majority of the genes of interest, there was no difference in the levels of cytokines between the two groups (**Fig 3.22A, B, and C**). Expression of IL10 was higher in the ipsilateral quadrant of the dorsal horn of the spinal cord in MNX rats, compared to sham controls (**Fig 3.22D**). Gene expression of FLAP was significantly higher in the ipsilateral dorsal quadrant of the spinal cord in MNX rats, compared to sham controls (**Fig3.23B**).

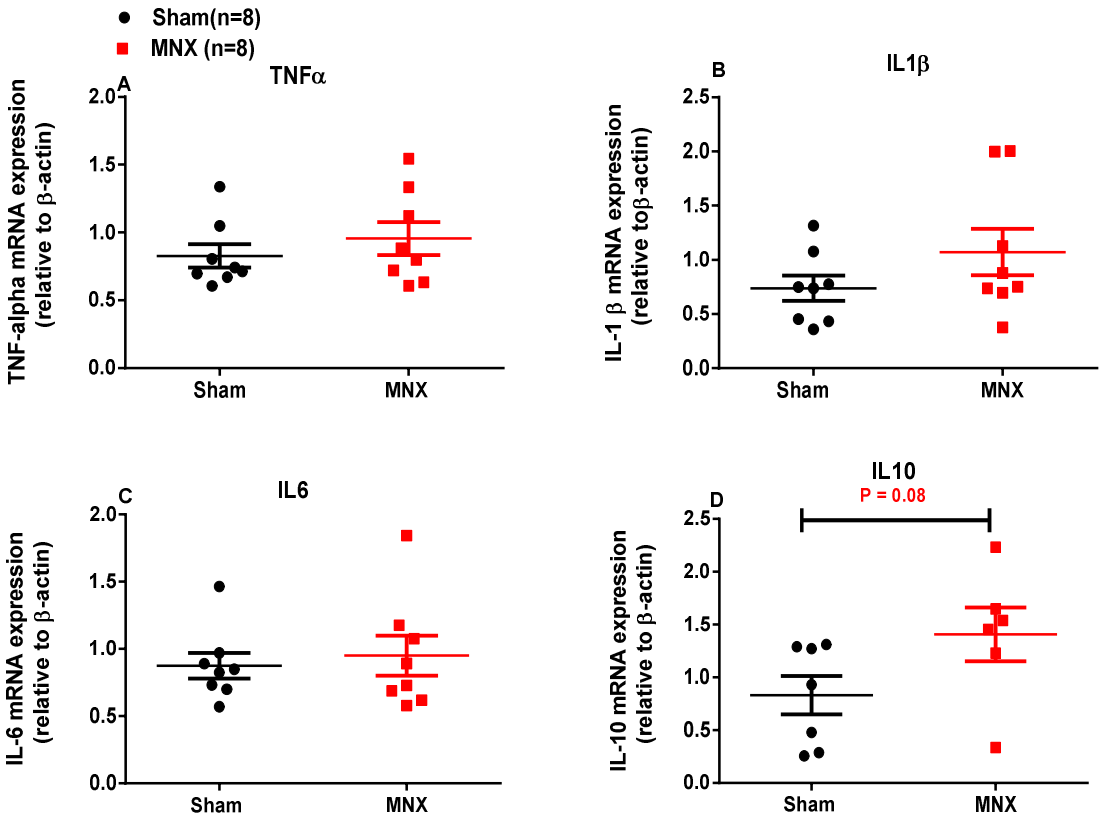


Fig 3.22 Gene expression of cytokines in spinal cord from MNX or sham-operated rats at day 35 post model induction. TNFα (A), Iiβ (B), IL6 (C) and IL10 (D). Data are individual data points as a scatterplot, with mean±S.E.M (n= 6-8) analysed with un-paired t-test or Mann-Whitney t-test.

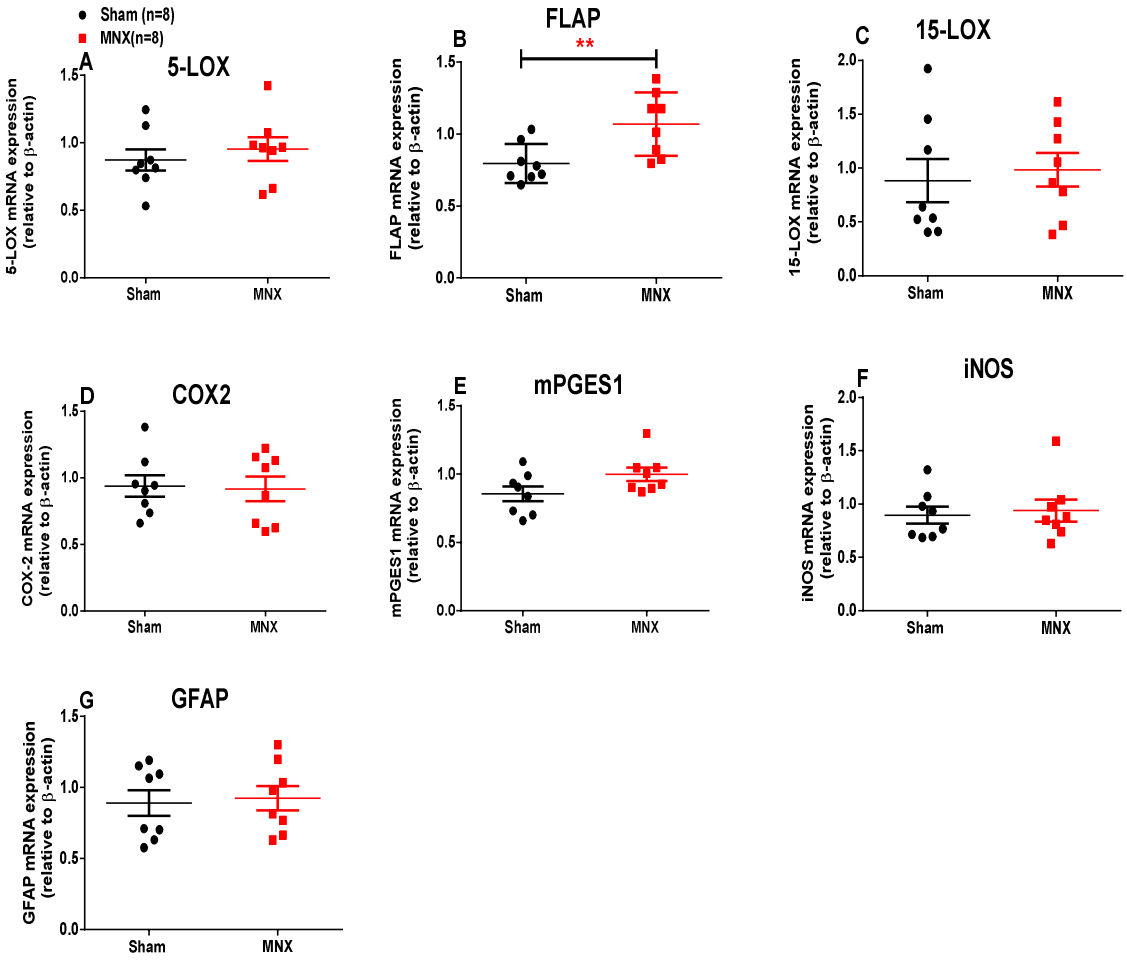


Fig 3.23 Gene expression of selected enzymes in spinal cord from MNX or sham-operated rats at day 35 post model induction, compared to controls. 5-LOX (A), FALP (B), 15-LOX (C), COX_2 (D), m PGES1 (E), iNOS (F) and GFAP (G). Data are individual data points as a scatterplot, with mean±S.E.M (n= 7-8) analysed with unpaired t-test or Mann-Whitney t-test (*p<0.05, **p<0.01).

3.3.10 The relative expression of ChemR23 and ALX in spinal cord of the two models

To obtain a clearer picture of the relative contribution of the resolvin receptors in spinal cord in the models of OA pain, expression levels of these two receptors were compared for each condition **(Fig3.24)**. It was evident ALX expression was significantly higher in saline rats (**Fig3.24A**) but lower in MIA rats (**Fig3.24B**), compared to ChemR23. There was no difference in the expression of the two receptors in the MNX model and sham control.

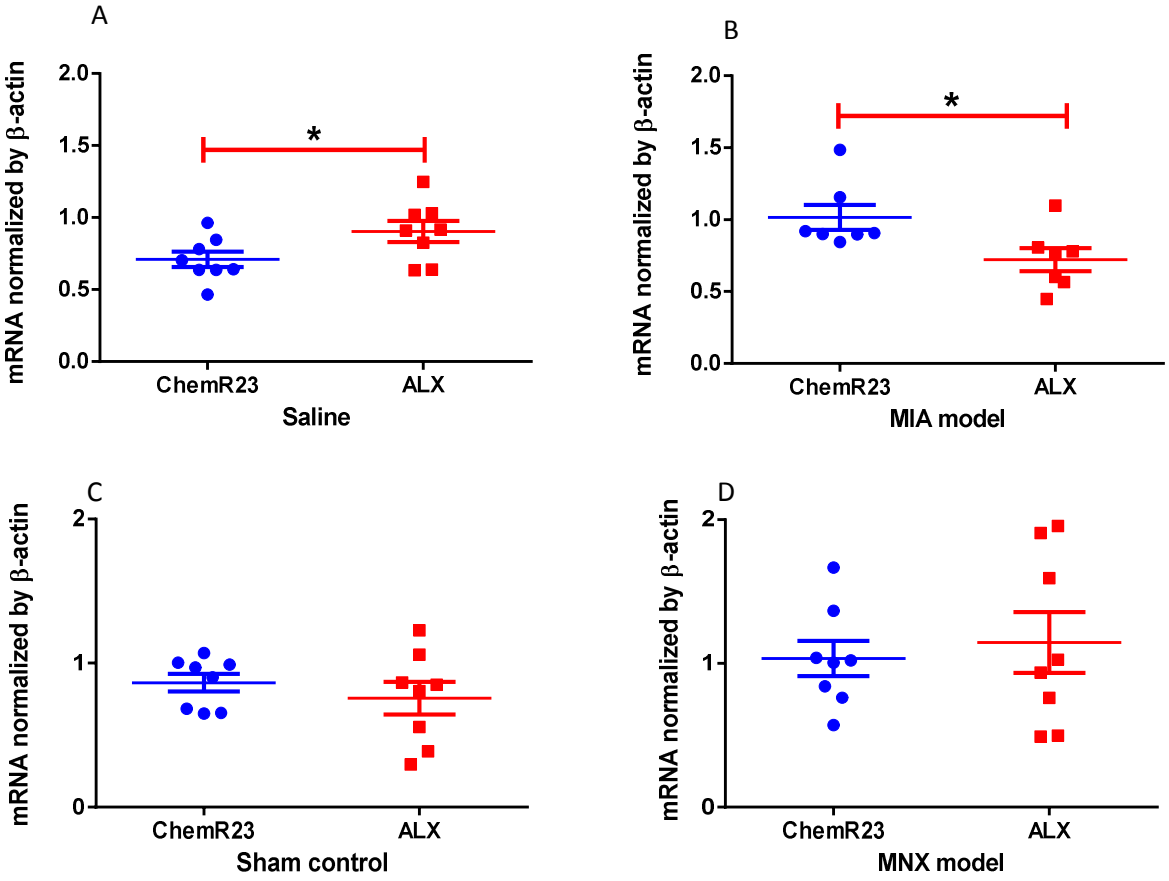


Fig 3.24 Comparison of ChemR23 and ALX in ipsilateral dorsal quadrant of the spinal cord in the models of OA and control groups at day 35. Saline (A), MIA (B), Sham (C) and MNX (D). Data are individual data points as a scatterplot, with mean ± S.E.M, analysed with unpaired t-test (A, C, D) or Mann-Whitney test (B). (*$p<0.05$)

3.3.11 Correlation of resolvin receptors with genes of interest in the spinal cord from the two models of OA

To investigate whether there is an association between the expression pattern of the two resolvin receptors in the spinal cord and the expression of inflammatory cytokines and their respective metabolic enzymes, a correlation analysis was undertaken in MIA (**Table 3.3**) and MNX (**Table 3.4**) rats. There was no correlation of ChemR23 expression with in flammatory cytokines and metabolic enzymes in the spinal cord of the MIA model. There was a negative correlation of ALX with IL6 and a week negative correlation with COX2 but a weak positive correlation between the ALX and 15-LOX in the spinal cord from MIA-treated rats which were not shown in saline treated rats. Gene expression of ChemR23 in the spinal cord of sham rats was negatively correlated with 5-LOX but positively correlated with COX2. Expression of ALX was positively correlated with IL1β and 5-LOX. There was no correlation between ChemR23 expression with other genes in the spinal cord of the MNX model while ALX expression was positively correlated with IL1β.

Treatment	Genes	TNFα	IL1β	IL6	5-LOX	FLAP	15-LOX	COX2	iNOS	mPGES1	GFAP
Saline	ChemR23	r=0.03 p=0.94	r=0.16 p=0.71	r=0.03 p=0.94	r=0.35 p=0.39	r=0.50 p=0.21	r=0.67 p=0.08	r=-0.02 p=0.96	r=-0.21 p=0.62	r=-0.18 p=0.67	r=-0.03 p=0.94
	ALX	r=0.88 p=**0.41 x10^{-2}***	r=0.52 p=0.18	r=0.59 p=0.12	r=-0.16 p=0.71	r=-0.29 p=0.48	r=-0.14 p=0.75	r=0.43 p=0.28	r=-0.26 p=0.53	r=0.32 p=0.45	r=-0.22 p=0.60
MIA	ChemR23	r=0.14 p=0.78	r=0.71 p=0.09	r=0.43 p=0.35	r=0.21 p=0.66	r=-0.18 p=0.72	r=0.29 p=0.56	r=0.14 p=0.78	r=0.14 p=0.78	r=0.18 p=0.71	r=-0.43 p=0.35
	ALX	r=-0.43 p=0.35	r=0.36 p=0.43	r=-0.87 p=**0.01***	r=-0.27 p=0.57	r=-0.08 p=0.89	r=0.70 p=**0.08**	r=-0.66 p=0.10	r=0.04 p=0.94	r=-0.14 p=0.77	r=-0.54 p=0.21

Table 3.3 Correlation of ChemR23 and ALX expression with inflammatory cytokines (TNFα, IL1β and IL6) and associated metabolic enzymes (5-LOX, FLAP, 15-LOX, COX2, iNOS, mPGES1 and GFAP) in the ipsi-dorsal spinal cord from the MIA model and saline control. Data were analysed with Pearson or Spearman correlation (n=7-8, r is the co-efficient of correlation, *p<0.05).

Groups	Genes	TNFα	IL1β	IL6	IL10	5-LOX	15-LOX	COX2	mPGES1	GFAP	TRPV1
Sham	ChemR23	r=0.03 p=0.95	r=0.06 p=0.89	r=0.28 p=0.50	r=0.11 p=0.82	r=-0.77 p=**0.02***	r=0.10 p=0.84	r=0.76 p=**0.03***	r=-0.09 p=0.84	r=-0.66 p=**0.07**	r=0.10 p=0.84
	ALX	r=0.39 p=0.34	r=0.72 p=**0.04***	r=0.46 p=0.25	r=0.62 p=0.14	r=0.75 p=**0.03**	r=-0.19 p=0.66	r=0.02 p=0.97	r=0.33 p=0.43	r=0.58 p=0.13	r=0.60 p=0.13
MNX	ChemR23	r=0.32 p=0.44	r=0.34 p=0.41	r=0.27 p=0.51	r=0.48 p=0.28	r=0.21 p=0.63	r=0.48 p=0.22	r=0.63 p=0.09	r=0.10 p=0.81	r=-0.29 p=0.49	r=0.17 p=0.69
	ALX	r=0.57 p=0.14	r=0.91 p=**0.20 x10^{-2}***	r=0.42 p=0.30	r=-0.25 p=0.60	r=-0.39 p=0.34	r=-0.13 p=0.76	r=0.44 p=0.27	r=0.58 p=0.13	r=0.15 p=0.73	r=0.04 p=0.93

Table 3.4 Correlation of ChemR23 and ALX expression with inflammatory cytokines (TNFα, IL1β and IL6) and associated meta-bolic enzymes (5-LOX, FLAP, 15-LOX, COX2, iNOS, mPGES1, GFAP and TRPV1) in the ipsi-dorsal spinal cord from the MNX model and sham control. Data were analyzed with Pearson or Spearman correlation (n=7-8, r is the co-efficient of correlation, *p<0.05).

3.3.12 Correlation of ChemR23 and ALX with pain behaviour in models of OA

Correlation of the expression of ChemR23 and ALX in the ipsilateral dorsal quadrant of the spinal cord with pain behaviour (WB and PWT) in the MIA model (day14 and day 35) and MNX model (Day 35) was determined. There was no correlation between ChemR23 expression with pain behaviour at day 14 post MIA induction (**Fig3.25A; Fig3.25B**). Expression of ALX was positively correlated with WB (**Fig3.25C**) and negatively correlated with PWT (**Fig3.25D**) at day 14 MIA model. At the later time-point (day 35), expression of ChemR23 in the spinal cord was positively correlated with WB difference (**Fig3.26A**) and negatively correlated with PWT (**Fig3.26B**). Expression of ALX was positively correlated with PWT (**Fig3.26D**) but not WB difference (**Fig3.26.C**) at day 35 in the MIA model. These data suggest that more pain was associated with higher ALX expression at early time-point (day 14) but lower ALX expression at late time-point (day 35) in the spinal cord from MIA model. Furthermore, greater pain behaviour was associated with higher ChemR23 expression at late time point (day 35) in the spinal cord from MIA model.

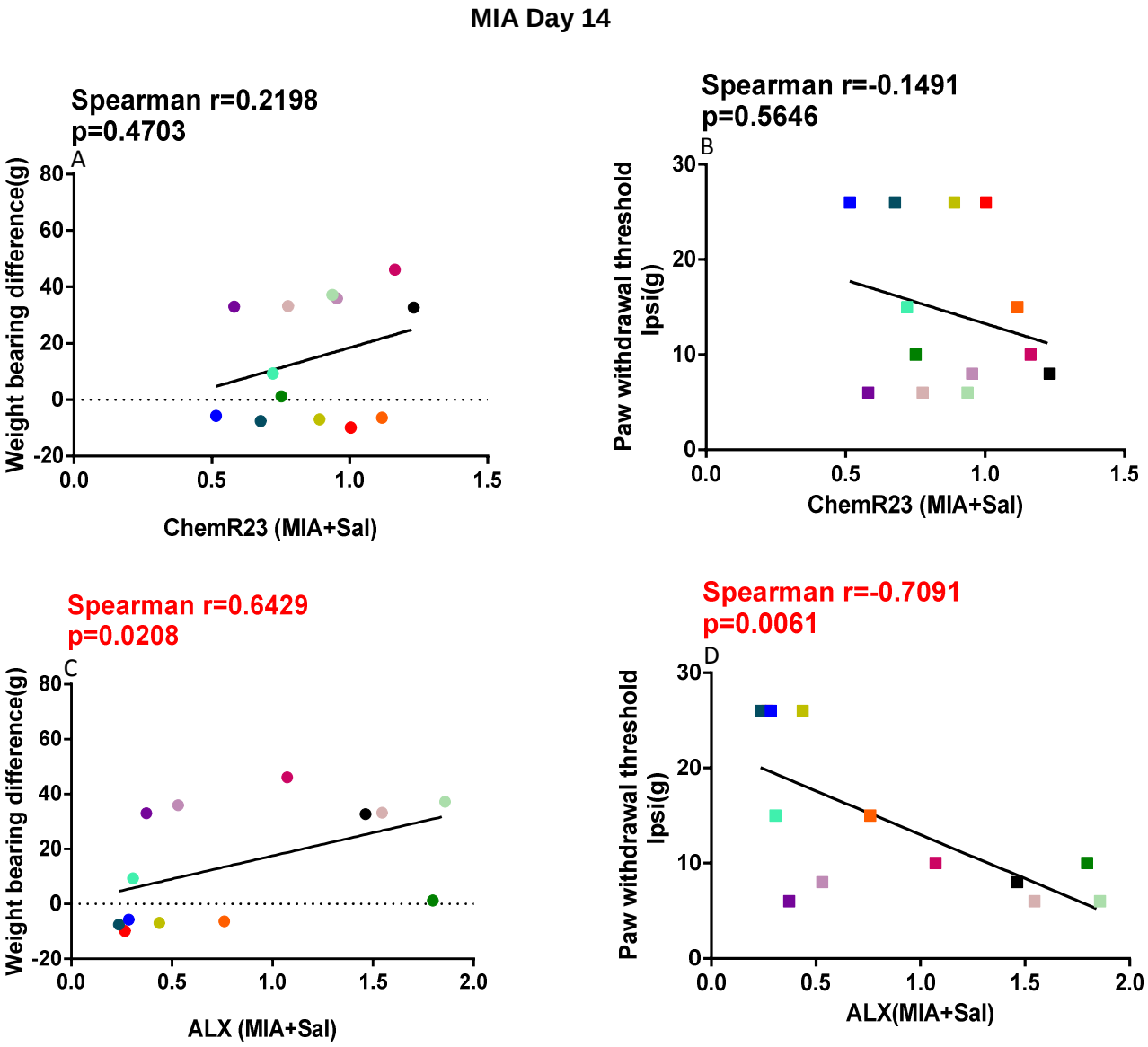


Fig 3.25 The correlation of mRNA level of ChemR23/ALX with weight bearing (A/C) and paw withdrawal threshold (B/D) in the ipsi-dorsal spinal cord in the MIA model and saline control at day 14. The maximal force for von Frey hair was 26g. Data were analysed with Spearman correlation.

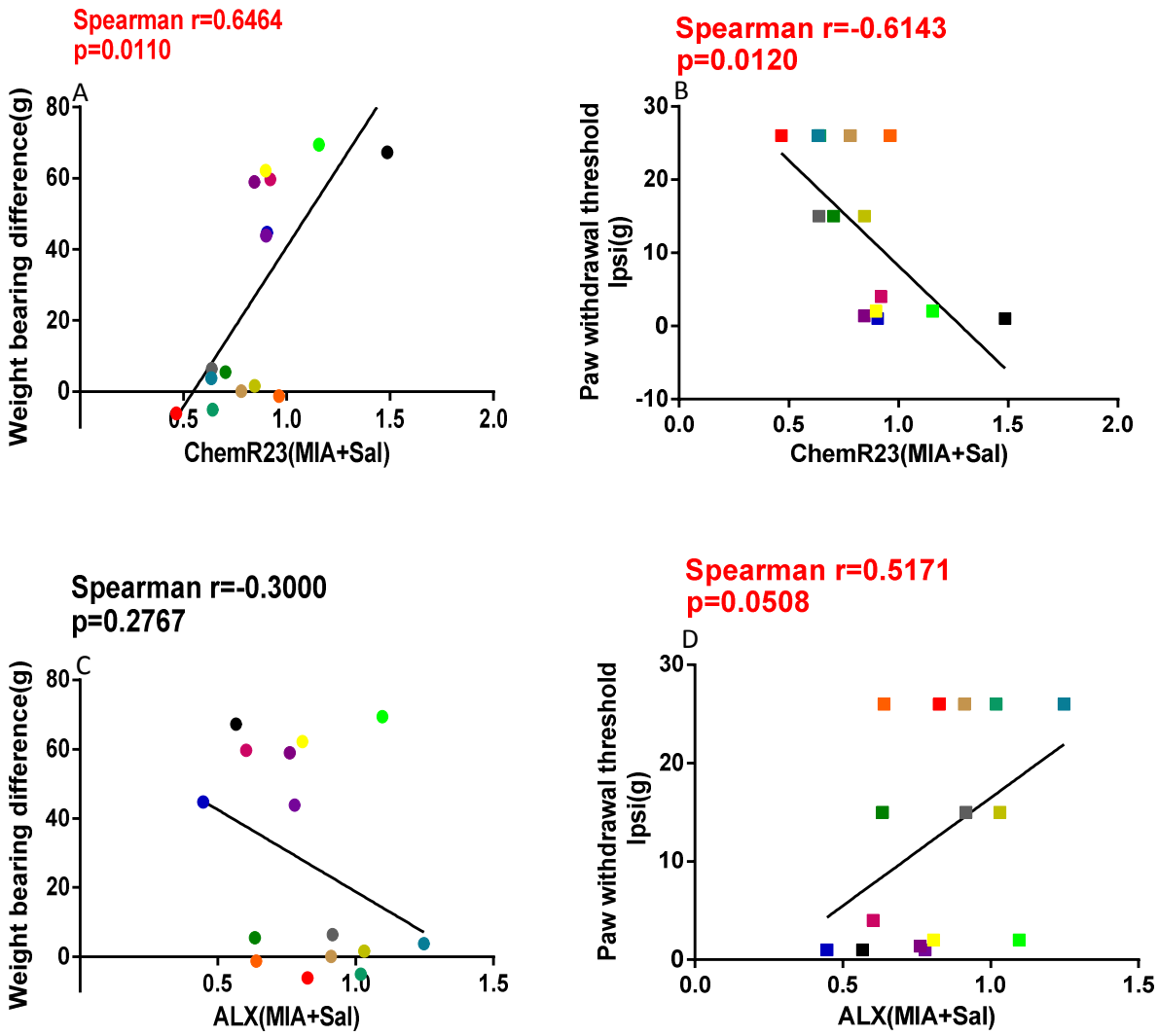


Fig 3.26 The correlation of mRNA level of ChemR23/ALX with weight bearing (A/C) and paw withdrawal threshold (B/D) in the ipsi-dorsal spinal cord in the MIA model and saline control at day 35. The maximal force for von Frey hair was 26g. Data were analysed by Spearman correlation.

In the MNX model, there was no correlation between the expression of ChemR23 or ALX with either WB or PWT at day 35 in the ipsilateral dorsal quadrant of spinal cord from the MNX model (**Fig3.27**).

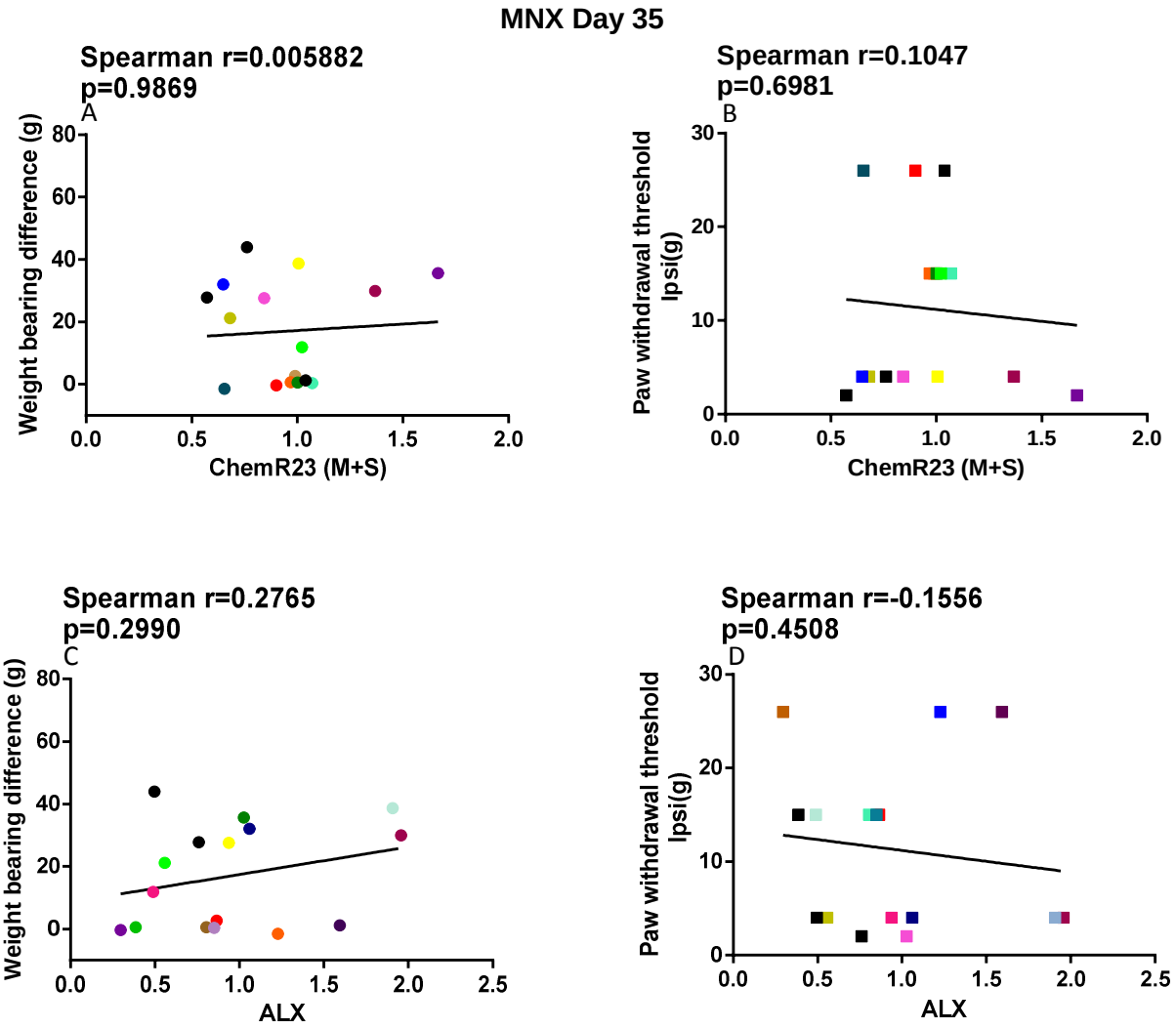


Fig 3.27 The correlation of mRNA level of ChemR23/ALX with weight bearing (A/C) and paw withdrawal threshold (B/D) in the ipsi-dorsal spinal cord in the MNX model and sham control at day 35. The maximal force for von Frey hair was 26g. Data were analysed by Spearman's correlation.

3.3.13 Summary of results

- Pain behaviour was established and joint damage developed in both MIA and MNX-induced models of OA.
- Expression of ChemR23 and 15-LOX were significantly lower in synovia from the MIA model, at a time-point of established pain behaviour, compared to controls.
- Expression of ALX, TNFα, IL1β COX2, EPHX2 and mPGES1 were significantly lower in synovia from the MNX model compared to sham control.
- There was no significant difference between ChemR23 and ALX expression in synovia from the MIA and MNX models.
- There was no correlation of expression of ChemR23 with genes of interest in synovia from either the MIA or MNX model.
- Expression of ALX was positively correlated with expression of TNFα and negatively correlated with expression of 5-LOX in the synovia from MIA-treated rats but not saline-treated rats. ALX expression was positively correlated with TNFα, IL1β, COX2, EPHX2 and mPGES1 in synovia from both sham and MNX rats.
- Higher synovial expression of ChemR23 was correlated with lower pain behaviours in the MIA model at both early (day 14) and late (day 35) time-points; this was not the case for the MNX model. There was no correlation between the expression of ALX in the synovia and pain behaviour in either model.
- Gene expression of ChemR23 was increased but 15-LOX was decreased in the ipsilateral dorsal horn quadrant of the spinal cord in MIA rats, compared to saline treated rats. These effects were not seen in the MNX model. However, FLAP expression was significantly higher in the ipsilateral dorsal horn quadrant of the spinal cord from the MNX model, compared to sham control.
- Expression level of ALX was modestly higher than ChemR23 in the ipsilateral spinal cord of saline treated rats but modestly lower in MIA-treated rats. These differences were not evident in the MNX model.
- Greater levels of pain behaviour were associated with higher spinal cord ALX expression at day 14 in the MIA model, but the converse was true at day 35.
- Greater levels of pain behaviour were associated with higher spinal cord ChemR23 expression at day 35 in the MIA model.

- There were no correlations between spinal expression of resolvin receptors and pain behaviour in the MNX model.
- There was a negative correlation of ALX with IL6 and a week negative correlation with COX2 but a weak positive correlation between the ALX and 15-LOX in the spinal cord from MIA-treated rats which were not shown in saline treated rats. These correlations were not shown in saline treated rats. It is noteworthy that these correlations were not seen in the MNX rats.
- There was a positive correlation between ALX expression in the spinal cord with IL1β in both the MNX model and sham control.

3.4 Discussion

3.4.1 Pain behaviour was established and joint pathology was shown in both the MIA and MNX models.

Comparison of the MIA and MNX models showed pain behaviour (WB and PWT) was established in both models and joint histology was comparable. However, the progression of pain behaviour and pathology of OA were slightly different between these two models. Pain behaviour was evident at very early time point (day 3) in the MIA studies presented in this thesis, which is consistent with previous studies (Beyreuther et al. 2007). The behavioural as well as the histopathological data presented in this Chapter are also consistent with earlier studies using this model (Bove et al. 2003; Sagar et al. 2010; Stevenson et al. 2011). Whereas, pain behaviour developed at later time points in the MNX model, compared to the MIA model. In the first MNX study, both weight bearing asymmetry and lower paw withdrawal thresholds were evident at day 7 and were maintained until the end of study at day 28. However, in the second MNX study, weight bearing asymmetry was observed at day 18 and no significant change in paw withdraw thresholds were evident compared to sham controls. This variable extent of pain behaviour exhibited by the MNX model was also reported in previous studies (Janusz et al. 2002; Mapp et al. 2008; Mapp et al. 2010; Mapp et al. 2013), which seems to be related to the extent of pain behaviour exhibited by the sham control. Indeed, the sham control underwent surgery that removed medial collateral ligament from the joint, which may change the microenvironment and stability of joint (Jung et al. 2009; Guilak 2011). In addition, the surgery itself can produce various levels of nerve damage, which could contribute to the post-operative pain evident in the sham control (Bove et al. 2006). These factors need to be taken into consideration when interpreting the gene expression studies reported in the synovia from this model.

The development of joint pathology including chondropathy, synovial inflammation, chondrocyte appearance and vascular channel breaching was comparable between the two models of OA pain. Chondropathy score, synovial inflammation and chondrocyte appearance were significantly higher in MIA treated rats, compared to saline controls. In the MNX model, only chondrocyte appearance was shown to be significantly higher compared to sham control. The non-significant joint histopathology for chondropathy score, inflammation score and vascular breaching osteochondral junc-

tion in the MNX rats may mainly be masked by joint damage in the sham control as the medial collateral ligament was removed. The number of rats per group may lead to an under powered study resulting in non- significant results. This was also shown in a previous study which indicated the higher chondropathy score and vascular breaching in both MIA and MNX models but significance was not reached, compared to their respective controls (Mapp et al. 2013). The variation between MIA and MNX models may indicate different phenotypes and mechanisms in initiation and pathogenesis of OA.

3.4.2 Resolvin receptors were altered in the two models of OA

Herein, I demonstrated for the first time that the expression of ChemR23 was altered in both synovia and spinal cord in the MIA model and ALX expression was altered in the synovia from the MNX model, compared to their respective controls.

The expression of ChemR23 was significantly lower in the synovia and higher in the spinal cord in the MIA model at Day 35, compared to saline controls. Furthermore, lower synovial expression of ChemR23 was correlated with higher pain behaviour in the MIA model at both early (day14) and late (day 35) time-points and this was not shown in the MNX model. This finding suggests that a loss of ChemR23 in the synovia may contribute to pain behaviour in the MIA model, which is consistent with a previous study that found RvE1 reduced carrageenan-induced pain via ChemR23 and knockdown of ChemR23 in the hindpaw blocked the anti-nociceptive action of RvE1 (Xu et al. 2010). These findings suggest a protective role of ChemR23 on pain at the initial injury site. Given the amount of change occurring in the synovia of the sham control, it isn't surprising that this was not replicated in the MNX model. Higher expression of ChemR23 in the spinal cord was associated with more severe pain behaviour in the MIA model at day 35. This may be attributed to the increased activation of glial cells and increased neuronal responses that express ChemR23 in spinal cord (Ji et al. 2011).

The expression of ALX was significantly lower in the synovia and had a trend to be higher in the spinal cord in the MNX model at Day 35, compared to sham control. Expression of ALX had a trend to be lower in the spinal cord of MIA rats, compared to saline treated rats at day 35.

Moreover, higher spinal expression of ALX was associated with more pain at day 14 and less pain at day 35 in the MIA model. These findings may suggest that different cell-types express ALX at different time-points in the spinal cord of the MIA model as microglia are reported to be activated at earlier time-points and astrocytes activated at a late time-point at day 28 (Sagar et al. 2011). The expression of ALX increased in the spinal cord at the early time (day 14) as pain developed and loss of ALX in the spinal cord was associated with more pain at late time-point (day 35) in the MIA model which may indicate protective role of ALX in this model

There was no correlation between spinal expression of resolvin receptors and pain behaviour in the MNX model. Maybe this isn’t surprising as this model does not show clear-cut central sensitization of pain.

A scheme to summarise the expression of ChemR23 and ALX and their correlations with pain behaviour in the two models is shown in **Fig 3.28**.

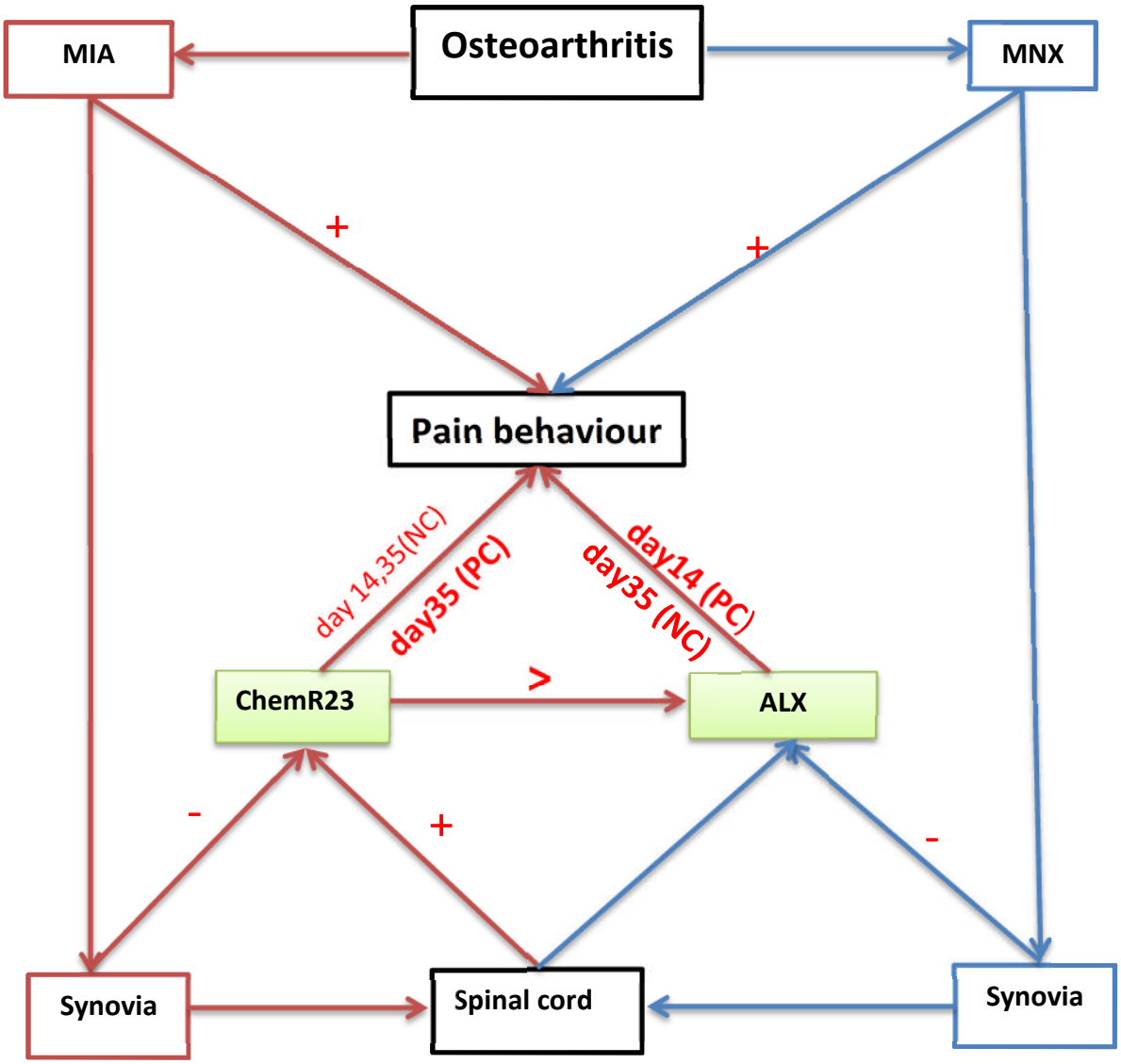


Fig 3.28 Gene expression of resolvin receptors in the synovia and spinal cord and their correlation with pain behaviour were shown in the MIA (magenta arrows) and MNX (blue arrows) models. The red text shows correlation of resolvin receptors with pain behaviour in the synovia and the red bold characters show correlation of resolvin receptors with pain behaviour in the spinal cord. '+' /increase, '-' /decrease. PC / positive correlation, NC / negative correlation, '>' / greater

3.4.3 Inflammatory cytokines and metabolic enzymes in synovia and spinal cord of the two models of OA

In the current study, we didn't find a significant difference in inflammatory cytokine expression in both synovia and spinal cord in the MIA (1mg/50μL) rats compared to saline-treated rats. This was also reported in a previous study that no significant difference in inflammatory cytokines (IL-1β, IL6, MCP1, IL10, etc.) expression was observed in serum of MIA- induced (50μg/5μl) OA (Bowles et al. 2014). In another study, only IL15 was up-regulated in early OA and there was no difference in TNFα, IL1β, IL2, IL6 and IL21 in synovial fluid between early OA and late OA (Scanzello et al. 2009). However, more studies have shown up-regulation of inflammatory cytokines in the OA models and human OA. Synovial inflammatory cytokines (TNFα, IL1β), COX2 and NF-κB were significantly increased in early stage compared to end stage in OA patients (Benito et al. 2005). Lambert et al. has shown increased IL6, IL8 and chemokines in the synovial membrane from OA patients (Lambert et al. 2014). Lee et al. has reported the increase of IL1β, IL6, IL15, and iNOS in cartilage of OA by intra-articular injection of MIA (3mg/50μl) (Lee et al. 2013). In the MNX model, expression of TNFα and IL1β, were significantly lower in the synovia, compared to sham controls. The inconsistent findings may indicate tissue specific and time-dependent inflammation at different phases of OA. It may also indicate lower inflammation in these two models of osteoarthritis. In addition, the sham surgery also induced pain behaviour and inflammation at the joints which may mask the up-regulation of inflammatory genes expected to be observed in the MNX model. The limitation for the MNX model is the expression of housekeeping genes (β-actin and GAPDH) was lower than sham control in the synovia which may also affect the normalization of target genes.

I further demonstrated that the expression of 15-LOX was significantly lower in the synovia of the MIA model and lower expression of COX2, mPGES1 and EPHX2 in the MNX model, compared to respective controls. The findings in the MNX model were not consistent with previous studies on metabolic enzymes in OA. COX_2 was up-regulated in OA-affected cartilage and meniscus, synovia and osteophytes of osteoarthritis by incubation of IL1β (Amin et al. 1997; Hardy et al. 2002). IL1β stimulated significant increase in COX2, $mPGES_1$ and iNOS expression in cartilage and synovial tissue of OA (Kojima et al. 2005; Alvarez-Soria et al. 2008). However, the

up-regulation of these enzymes in OA was induced by exogenous adding IL1β, which is slightly different from the current study.

In the spinal cord, I found higher expression of COX_2 in the MIA model although significance was not reached. This indicates activation of astrocytes and central sensitization of pain in the model which is consistent with previous studies (Sagar et al. 2011). The expression of 15-LOX was significantly lower in the spinal cord of MIA rats, compared to saline control, indicating a protective role of 15-LOX in OA as 15-LOX metabolites inhibit IL1β-induced MMP1 and MMP13 in chondrocytes and type II collagen degradation cleavage from OA patients (Chabane et al. 2009).

Spinal expression of FLAP was significantly higher in the MNX model, compared to sham control. The highly expressed FLAP in the spinal cord of the MNX model may support the evidence that 5-LOX metabolites are involved in the inflammatory process in OA or RA (Bonnet et al. 1995) and that FLAP is also required for the biosynthesis of leukotriene, indicating its pro-inflammatory property (Dixon et al. 1990).

3.4.4 Resolvin receptors (ChemR23 and ALX) were associated with inflammatory cytokines and metabolic enzymes in these two models of OA

There was no correlation between ChemR23 expression and inflammatory cytokines or metabolic enzymes in both synovia and spinal cord in either MIA-induced or MNX-induced model. These findings suggest there is dissociation between the relationship of ChemR23 with pain behaviour versus inflammatory signalling in the MIA model. Previous studies revealed RvE1 inhibited the inflammatory cytokines such as TNFα, IL1β, IL6 and MCP1 in inflammatory models of pain (Xu et al. 2010) or inflammatory cytokines and metabolic enzymes in inflammatory diseases or tissues (Arita et al. 2005; Schwab et al. 2007). My data may suggest that RvE1 inhibits pain behaviour via other signalling pathways such as ion channels or inhibition of ERK mediated glutamate release and activation of NMDA receptor (Xu et al. 2010) as glutamate/NMDA receptor is an essential component on pain transmission and central sensitization of pain (Latremoliere and Woolf 2009).

Expression of ALX was positively correlated with TNFα, IL1β, and COX2 and negatively correlated with 5-LOX in synovia from MIA rats which was not shown in saline treated rats. In the MNX model, expression of ALX was significantly positive correlated with TNFα, IL1β, COX2 and mPGES1 in synovia from both MNX model and

sham control. These findings indicate that synovial expression of ALX was increased in association with the inflammatory response, which may reflect the infiltration of macrophages and fibroblast-like synoviocytes, which express ALX (Fiore et al. 2005).

Spinal expression of ALX was negatively correlated with IL6 and had a weak positive and negative correlation with 15-LOX and COX2 respectively in the MIA model at day 35. This may indicate the protective role of ALX in pain behaviour via inhibiting inflammatory signalling in the spinal cord in this model. This was evident in previous studies in which RvD1 reduced mechanical allodynia via inhibiting generation of TNFα, IL1β and IL6 in a model of chronic pancreatitis (Quan et al. 2012) and RvD1/RvD2 dampened the inflammatory cytokines in obesity-induced adipose inflammation (Claria et al. 2012). There was no correlation of expression of ALX with other genes in the spinal cord in the MNX model except a positive correlation with IL1β. The correlation of ChemR23 or ALX expression with inflammatory cytokines and metabolic enzymes in the two models are summarized in **Fig 3.29**

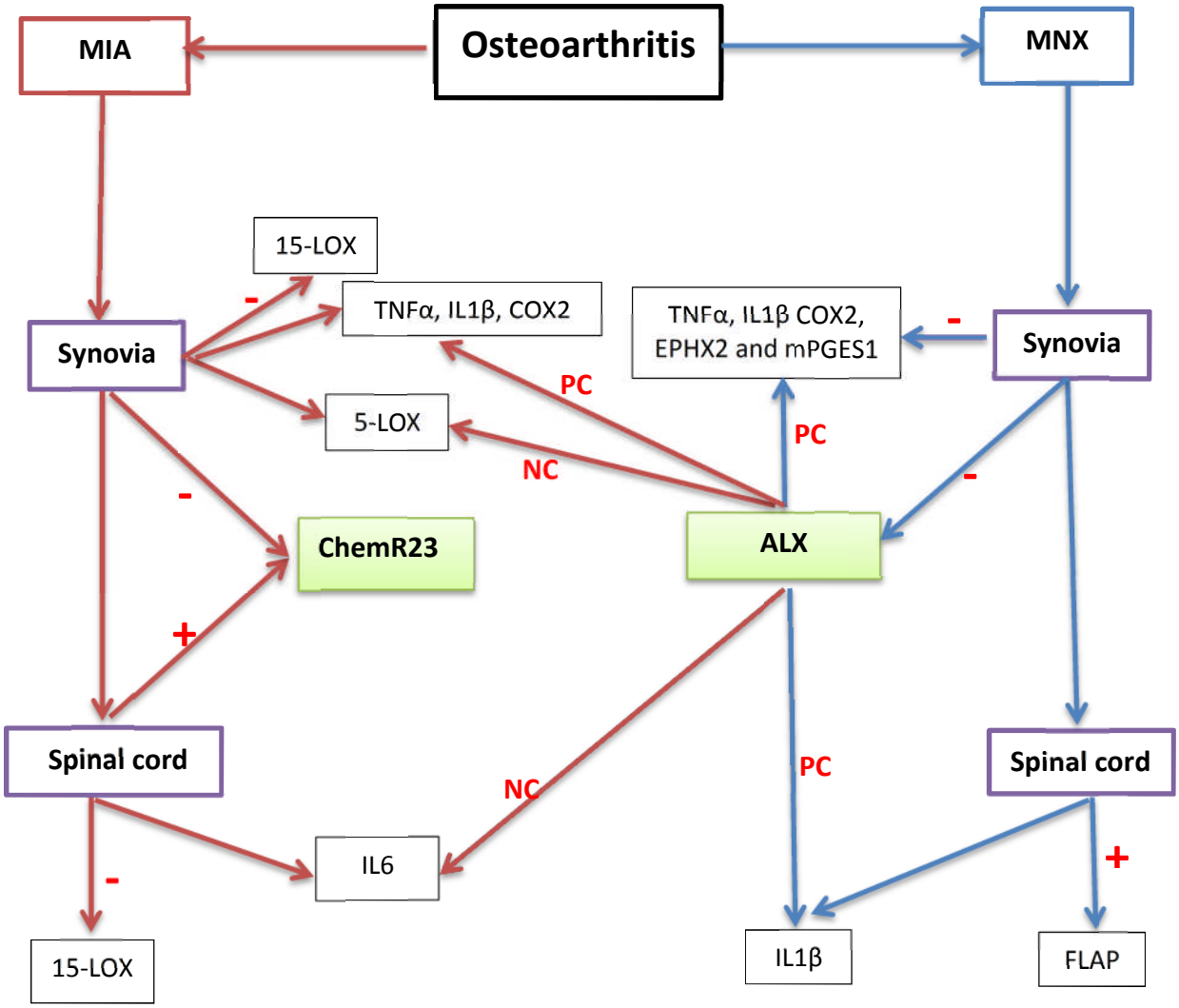


Fig 3.29 Gene expressionof resolvin receptors, inflammatory cytokines and metabolic enzymes in the synovia and spinal cord and correlation of the resolvin receptors with these genes were shown in the MIA (magenta arrows) and MNX (blue arrows) models. '+' / increase, '-' /decrease, PC /positive correlation, NC /negative correlation.

3.5 Conclusion

Pain behaviour and joint pathology were established in both MIA and MNX-induced models of OA. However, pain behaviour developed much quicker and more severely in the MIA model than in the MNX model which may indicate two different phenotypes and pathological signs of OA.

The expression of ChemR23 was altered in synovia and spinal cord in the MIA model and ALX expression was altered in the synovia from MNX model. Correlation of ChemR23 and ALX with pain behaviour in the MIA model suggests a potential important role of resolvin receptors/ligands in OA pain. Furthermore, this correlation may be associated with various cell types expressing the two receptors and may be mediated via various molecular signalling pathways.

Lower expression of 15-LOX in both synovia and spinal cord in the MIA model indicates active roles of these metabolic enzymes in modulation of osteoarthritic pain.

Correlation of ALX expression with inflammatory cytokines and associated metabolic enzymes in the MIA model may suggest the action of ALX on osteoarthritic pain via interaction with inflammatory signals and metabolic enzymes. However, the specific mechanisms of resolvins and their receptors on osteoarthritic pain require further investigation.

4 Therapeutic effects of a resolvin precursor in the MIA and MNX model

4.1 Introduction

As shown in Chapter 3, expression of the resolvin receptors (ChemR23 and ALX) and some associated enzymes were altered in the synovia and spinal cord from the MIA and MNX models of OA pain. In this Chapter, I have investigated the effects of exogenously augmenting the resolvin system on OA joint pathology and pain behaviour in the MIA and MNX-induced models.

4.1.1 Anti-inflammatory and pro-resolving roles of resolvins

As presented in the general introduction (Chapter 1.5.3), resolvins play essential roles in curtailing the inflammation; this section briefly introduces the identification of resolvins and their anti-inflammatory roles. The structure of RvD1 was identified by Serhan's group in 2007, they demonstrated anti-inflammatory properties of RvD1, such as preventing transmigration and infiltration of neutrophils (Sun et al. 2007). RvD1 was shown to play anti-inflammatory roles (macrophage phagocytosis and inhibition of neutrophil recruitment) and decreased inflammatory mediators (PGD_2 and LBT_4) via FPR2 /ALX (Norling et al. 2012). Next, they identified the stereochemical structure of RvD2 and showed that RvD2 can inhibit neutrophil infiltration, increase phagocytosis of macrophage and down-regulate inflammatory mediators including TNFα,IL1β,IL6, IL10, IL17, IL23 and PGE_2 in microbial sepsis (Spite et al. 2009). More recently, the stereochemical assignment of RvD3 was confirmed and similar anti-inflammatory roles, such as inhibition of neutrophil infiltration, promoting phagocytosis of macrophage and modulating inflammatory cytokines (IL6 , MCP1and IL10) were demonstrated (Dalli et al. 2013). Furthermore, effects of RvD3 on the phagocytosis of macrophage were mediated by GPR32 (Dalli et al. 2013). RvD5 has been shown to resolve *E. coli* infections by enhancing macrophage phagocytosis via GPR32 and down-regulation of NF-kB and TNFα signalling (Chiang et al. 2012).

E-series resolvins also have established anti-inflammatory and pro-resolving roles in various inflammatory models. RvE1 was produced in inflammatory exudates after treatment with Ω-3 PUFA and aspirin via COX2 and 5-LOX pathway, and was shown to limit transmigration and infiltration of human polymorphonuclear leukocytes (neutrophils) but it was not defined as RvE1 at that time (Serhan et al. 2000). RvE1 attenuates 2, 4, 6-trinitrobenzene sulfonic acid-induced colitis by reducing leukocytes

infiltration and down-regulating some inflammatory mediators such as TNFα, IL12, COX2 and iNOS (Arita et al. 2005). RvE2 was identified in human neutrophils, generated by the 5-LOX pathway during the biosynthesis of RvE1 (Tjonahen et al. 2006) and can inhibit infiltration of PMNs (neutrophils) in murine peritonitis (Tjonahen et al. 2006). A further study showed RvE2 enhanced phagocytosis of human macrophages and the production of anti-inflammatory cytokines such as IL10 via its GPCRs (BLT_1 and ChemR23) and decreased expression of integrin (CD18) in human PMN and macrophage (Oh et al. 2012). RvE3 has been identified in eosinophils, rather than neutrophil or macrophage, generated via 12/15-LOX pathway and can prevent neutrophil chemotaxis (Isobe et al. 2012). Collectively, these studies have shown that resolvins play anti-inflammatory or pro-resolving roles. Interestingly, resolvins have also been shown to have anti-nociceptive roles in various models of pain (see 4.1.2).

4.1.2 Analgesic effects of resolvins in models of pain

The analgesic effects of resolvins were first reported in inflammatory models of pain. Both RvE1 and RvD1 attenuated inflammatory pain in mouse models of inflammatory pain (formalin, carrageenan and CFA models) following intra-plantar and intrathecal routes of administration (Xu et al. 2010). RvE1 also reduced capsaicin and TNFα-induced spontaneous pain and pain hypersensitivity in mice (Xu et al. 2010). This study demonstrated a role for ChemR23 in mediating the anti-nociceptive effects of the resolvins, which results in inactivation of ERK in pre- and post-synaptic neurons (Xu et al. 2010). Concurrently, the anti-nociceptive effects of RvD1 were shown to be mediated via actions at TRP channels on sensory nerves (Bang et al. 2010; Bang et al. 2010; Bang et al. 2012). RvD1 attenuated acute pain responses, as well as mechanical and thermal hypersensitivity and inhibited expression of TRPA1, TRPV3 and TRPV4 in DRG neurons (Bang et al. 2010). RvD1 was shown to reduce acute nociceptive processing via an action at TRPV3 (Bang et al. 2012). Preemptive spinal administration of RvD1 reduced post-operative pain (mechanical allodynia and hyperalgesia) in the skin/muscle incision and retraction model, but only at early stages of the model (Huang et al. 2011). Intrathecal injection of RvD1 attenuated chronic pancreatitis-induced mechanical allodynia in a dose-dependent manner, an effect which was associated with inhibition of phosphorylation of NMDA receptors and inhibition of expression of TNFα,IL1β and IL6 in neurons or microglia in the spi-

nal dorsal horn (Feng et al. 2012). More recently, spinal administration of RvD1 was reported to reduce mechanical hypersensitivity in the carrageenan model of inflammatory pain, which was associated with the inhibition of TNFα in spinal astrocytes (Abdelmoaty et al. 2013). Intra-plantar injection of RvD2 was also shown to relieve capsaicin-induced spontaneous pain, formalin, carrageenan and adjuvant-induced inflammatory pain via inhibition of TRPA1 and TRPV1 (Park et al. 2011). Acute intravenous and intrathecal administration of RvD2 and AT-RvD1 decreased the mechanical allodynia and thermal nociception in a model of fibromyalgia (Klein et al. 2014). Spinal administration of RvE1 partially attenuated nerve injured pain (CCI and SNL) by inhibiting activation of microglia and expression of TNFα in the spinal dorsal horn (Xu et al. 2012).

On top of the anti-inflammatory and anti-nociceptive roles, RvE1 decreases bone resorption by inhibiting osteoclast growth and differentiation, these effects were mediated by the BLT_1 receptor and were associated with inhibition of receptor activator of NF-kB ligand-induced nuclear translocation (Herrera et al. 2008). A further study showed RvE1 decreased osteoclast formation at the late stage of osteoclast maturation, but not differentiation of early osteoclast precursor (Zhu et al. 2013). In addition, RvE1 inhibited the fusion of osteoclast precursor, which is a critical step for osteoclast differentiation (Zhu et al. 2013). In addition to inhibiting bone resorption, RvE1 enhanced bone formation in a uniform craniotomy and *in vitro* study of primary osteoblasts demonstrated that bone-protective effects of RvE1 were mediated via increased expression of OPG from osteoblasts (Gao et al. 2013).

The ability of the resolvins to modulate pain behaviour and neuronal responses in models of inflammatory pain, coupled with the known effects on bone resorption, makes this system an attractive target for the treatment of OA pain.

The major limitation of the resolvin system is that the active molecules are rapidly metabolized (Serhan and Petasis 2011) and therefore unstable even when given via a direct route ofadministration, e.g. intraplantar or intrathecal administration. However, a previous study revealed that systemic administration (intraperitoneal injection) of the D-series precursor of resolvin, 17(R) HDoHE, reversed the mechanical hyperalgesia in adjuvant-induced arthritis, following either pre-treatment or post-treatment (Lima-Garcia et al. 2011). These analgesic effects were associated with an inhibition of NF-κB and COX2 signalling in the DRG and spinal cord in this model of arthritis

(Lima-Garcia et al. 2011). Based on this previous study, the aim of the work in this Chapter was to determine the potential analgesic effects of systemic administration of 17(R) HDoHE on OA pain behaviour and the mechanisms of action.

4.1.3 Objectives

- To investigate the effects of acute versus chronic administration of 17(R)HDoHE on MIA-induced pain behaviour and joint pathology.
- To determine the effects of 17(R)HDoHE treatment on the expression of resolvin receptors, inflammatory cytokines and metabolic enzymes in the synovia and spinal cord in the MIA model.
- To determine whether discontinuation of treatment with 17(R)HDoHE has long- lasting analgesic effects in the MIA model of OA pain and to effects on gene expression of resolvin receptors, inflammatory cytokines and metabolic enzymes in the synovia in the MIA model

4.2 **Methods**

The general methods for model induction, joint histological assessment and RT-PCR have been described in Chapter 2. Sprague-Dawley male rats (130-150g) were habituated for environment and equipment before model induction and pain behavioural test. They were also habituated for intra-peritoneal injection with sterile saline for 2 weeks before drug administration.

4.2.1 Acute drug administration and pain behavioural testing in the MIA model

To investigate the acute effects of 17(R) HDoHE in the model, three groups (MIA+17(R)HDoHE, MIA+vehicle (1% ethanol in saline) and saline+vehicle (1% ethanol in saline) were used (n=8 per group). 17(R) HDoHE was administered systemically at 300ng per rat by intra-peritoneal injection, based on previous published work (Lima-Garcia et al. 2011). The working solution of drug was diluted from the stock (100µg/1ml) into 1ng/1µl with sterile saline (1% ethanol). Baseline behaviour (WB and PWT) was tested at day 0, before intra-articular injection of MIA (1mg/50µl) injection. Pain behaviour was then tested twice a week from day 3 until day 14. 17(R)HDoHE (300ng/300µl) or vehicle was injected i.p. on day 14, pain behaviour was measured at 1, 2, 4 and 6 hours post drug administration. Tissues (synovia, quadrant spinal cords, and knee joints) were immediately harvested after the final behavioural test on day 14 (**Fig 4.1**)

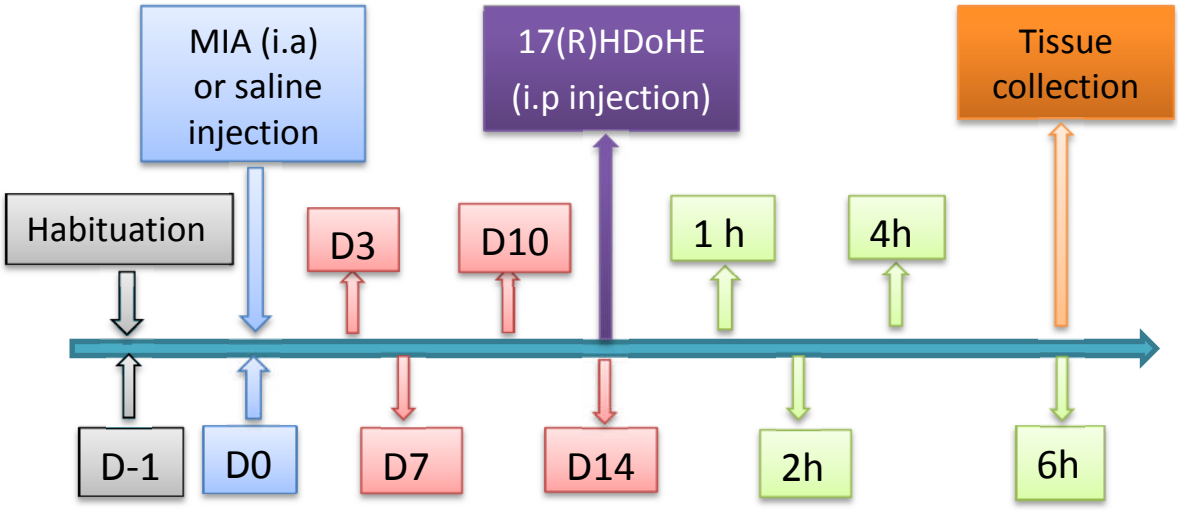


Fig 4.1 Rats were habituated a day before model induction. Baseline of behaviour (WB and PWT) was tested before MIA injection on day 0 and pain behaviour was then tested twice a week. 17(R HDoHE was administrated (i.p.) on day 14 and pain behaviour was measured at 1, 2, 4 and 6 hours post drug administration. Tissues were collected at 6 hours for subsequent gene expression studies.

4.2.2 Repeated drug administration and pain behavioural testing in the MIA model

To observe the potential chronic effects of 17(R)HDoHE in MIA and MNX-induced osteoarthritic pain, the following groups of rats were used (MIA+17(R)HDoHE, MIA+vehicle-1% ethanol in saline, (n=6 per group), (MNX+17(R)HDoHE, MNX+vehicle, sham+ 17(R)HDoHE and sham+vehicle, n=8 per group).

Baseline pain behaviour (WB and PWT) was tested at day 0 before MIA (1mg/50µl) injection or MNX surgery. Pain behaviour was tested twice a week from day 3 -14 17(R) HDoHE (300ng/300µl per rat, i.p. injection) was administrated every other day from day 14 until day 28. Pain behaviour was tested every four days until day 28 post-drug administration and tissues were immediately harvested after the final behavioural test on day 28 (**Fig 4.2**) :

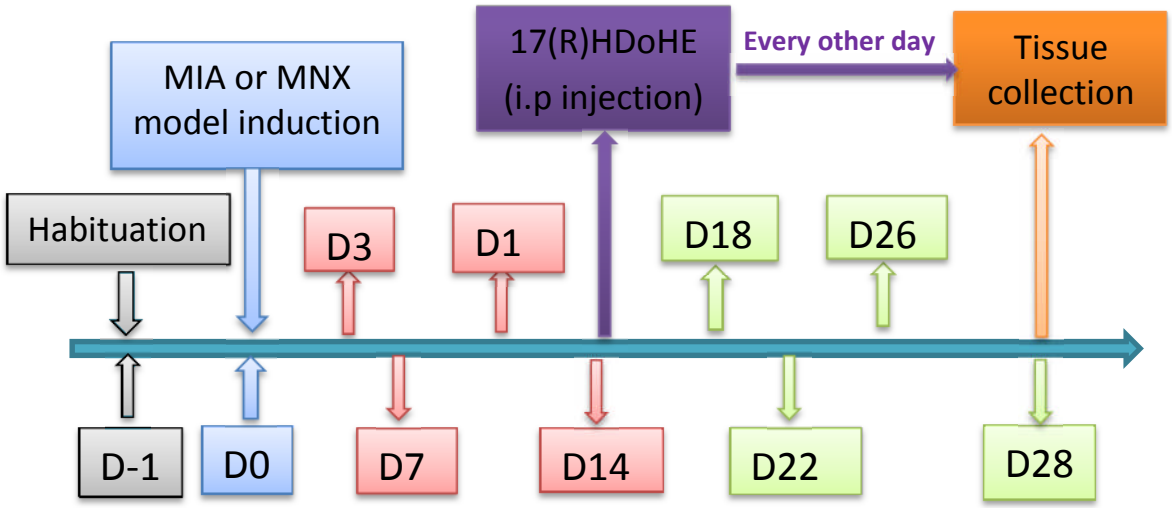


Fig 4.2 Rats were habituated before model induction. Baseline behaviour (WB and PWT) was tested before MIA injection or MNX surgery (day 0) and then twice a week from day 3 - 14. 17(R) HDoHE was administered (i.p. injection) every other day and pain behaviour was measured every 4 days post drug administration. Tissues were collected at day 28 for joint pathology.

4.2.3 Discontinuous drug administration and pain behavioural testing in the MIA model

To investigated the discontinuous treatment with 17(R)HDoHE in the MIA model, MIA+17(R)HDoHE, MIA+vehicle and saline+vehicle(n=8 per group) were used. For these studies, an extended protocol until day 35 was used to ensure adequate time to determine the potential duration of the effect following treatment cessation. Pain behavioural testing prior to drug administration was conducted as described in 4.2.2. 17(R) HDoHE (300ng/300µl per rat) was administrated (i.p injection) every other day from day 14 – 22. Following treatment on day 22, drug treatment was terminated; pain behaviour was then tested for a further 2 weeks until day 35. Tissues were immediately harvested after the final behavioural test at day 35.

4.3 **Results**

4.3.1 Effects of acute administration of 17(R) HDoHE in MIA-induced pain behaviour and gene expression

Acute injection of 17(R) HDoHE transiently attenuated established MIA-induced pain behaviour (weight bearing asymmetry (**Fig 4.3 A**) and lowered hindpaw withdrawal thresholds (Fig **4.3B**)). Inhibitory effects of 17(R) HDoHE were significant at 1 hour and were maintained until 6 hours post-drug administration.

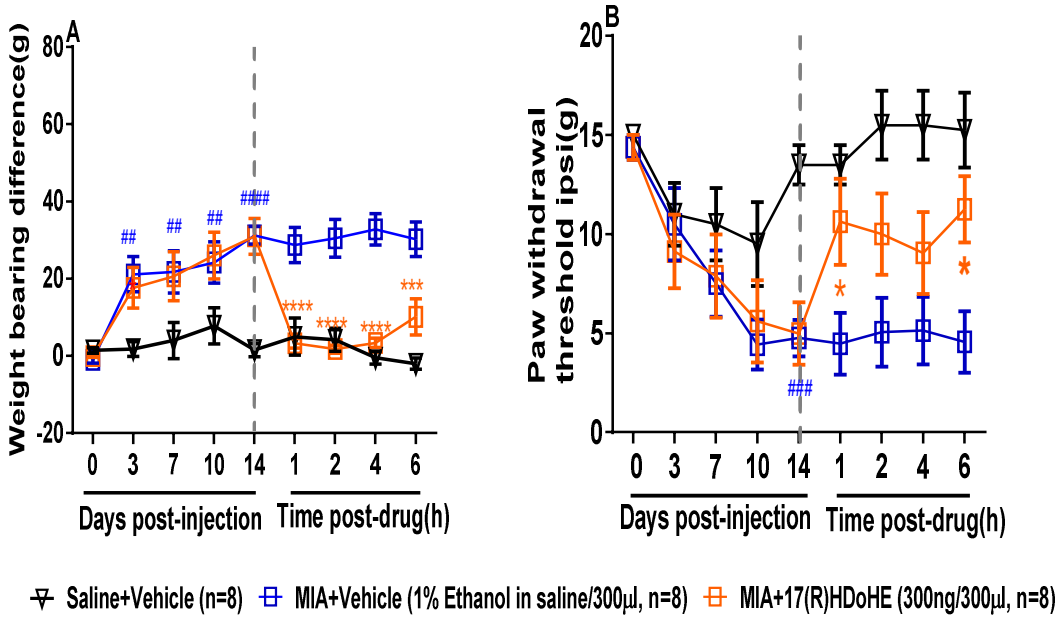


Fig.4.3 Intra-articular injection of MIA resulted in significant pain behaviour at 14 days post model induction. Acute administration of 17(R)HDoHE attenuated pain behaviour in the MIA model, compared to the effect of vehicle. Data are mean ± S.E.M (n= 8) analyzed with two-way ANOVA and Bonferroni's post-hoc test (*/$^{\#}$p<0.05, **/$^{\#\#}$ p<0.01, ***p<0.001, ****$^{/\#\#\#\#}$ p<0.001 vs control),' # 'MIA+17(R)HDoHE vs saline+vehicle, '*' MIA+17(R)HDoHE vs MIA+vehicle.

4.3.1.1 Effects of 17(R) HDoHE on synovial expression of resolvin receptors, inflammatory cytokines and enzymes in the MIA model

The levels of gene expression of ChemR23 and ALX, some inflammatory cytokines and associated metabolic enzymes were quantified in synovia from acute vehicle MIA and acute 17(R) HDoHE MIA rats at day 14. Expression of ChemR23 was significantly lower in the synovia from MIA vehicle rats and 17(R) HDoHE treated MIA rats, compared to saline treated-rats (**Fig 4.4A**), there was no difference between the two MIA groups. There was no significant difference in the synovial expression of ALX between any groups (**Fig 4.4B**).

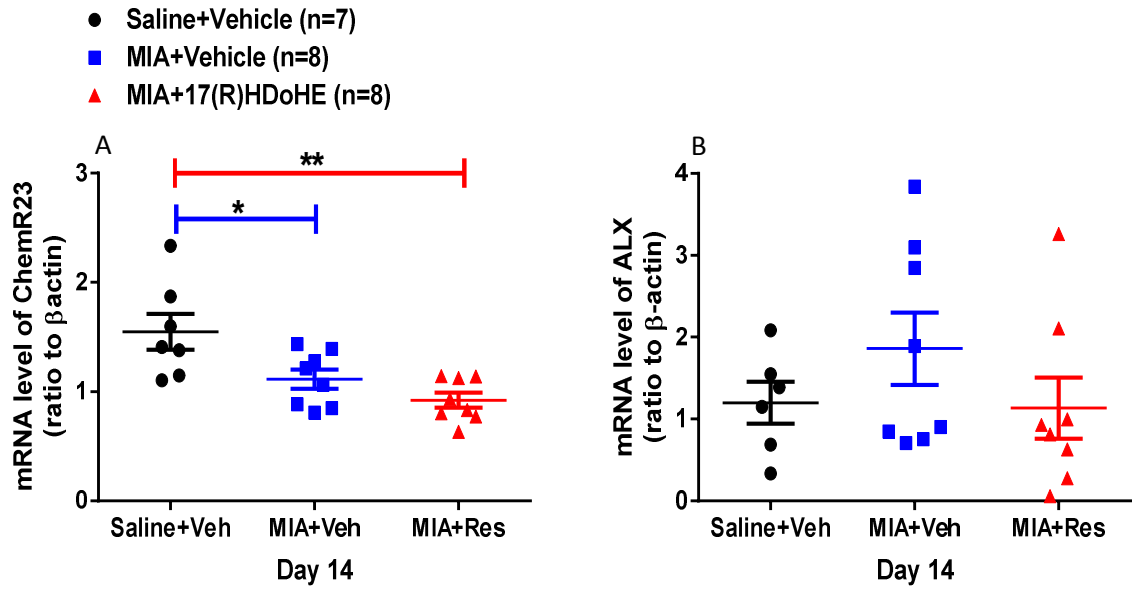


Fig. 4.4 Expression levels of resolvin receptors in synovia at day 14 following intra-articular injection of MIA or saline, and the effect of acute 17(R) HDoHE treatment. ChemR23 (A) and ALX (B). Data were analyzed by one-way ANOVA with Bonferroni's post-hoc, P*< 0.05, P**< 0.05 (n=7-8 per group).

Acute administration of 17(R)HDoHE significantly upregulated the expression of IL6 (**Fig 4.5C**) in the synovia of MIA rats, compared to vehicle. None of the other inflammatory genes in the synovium were altered by 17(R)HDoHE (**Fig 4.5**).

Expression of the enzyme 5-LOX was significantly down-regulated by 17(R) HDoHE, compared to vehicle (**Fig 4.6A**), in the MIA model. Acute 17(R) HDoHE did not alter the expression of any other genes studied (**Fig 4.6**).

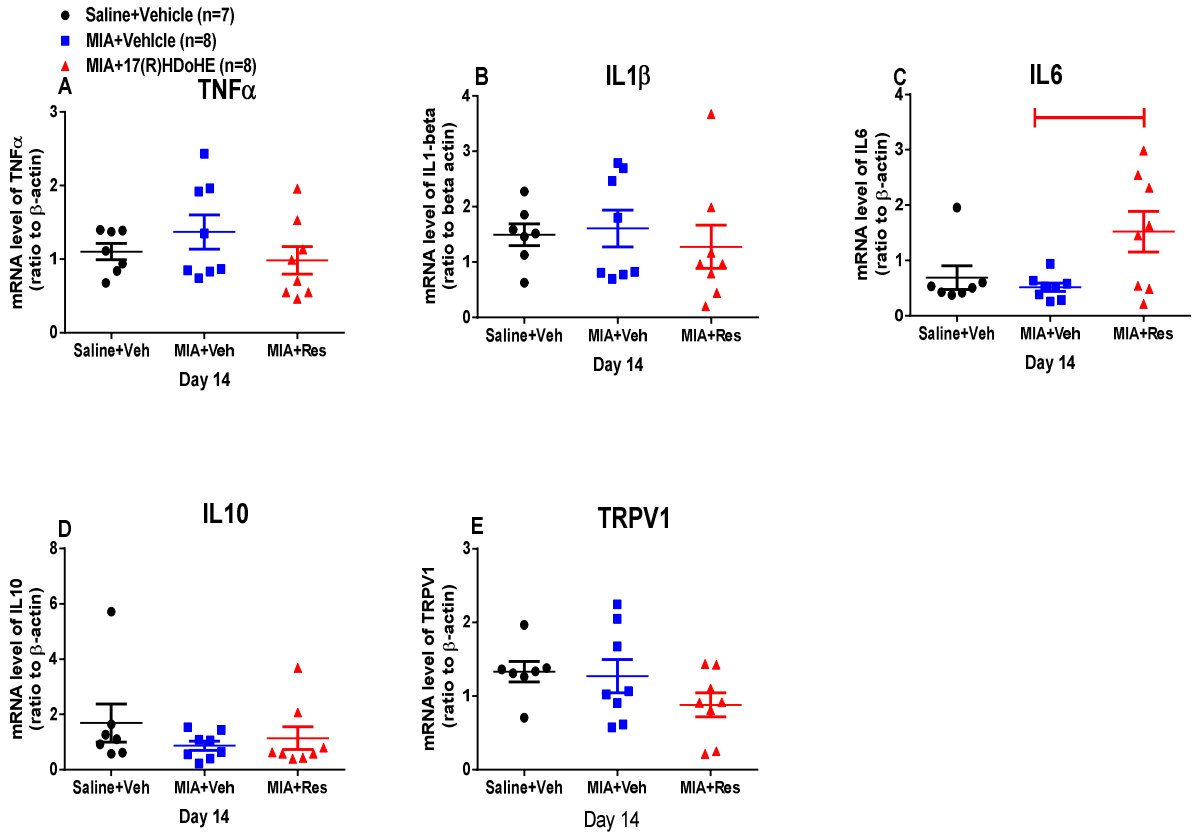


Fig.4.5 Expression levels of cytokines in the synovia at 14 days following intra-articular injection of MIA or saline, and the effect of acute 17(R)HDoHE treatment in the MIA model. Data were analyzed by one-way ANOVA with Bonferroni's post-hoc test (n=7-8 per group).

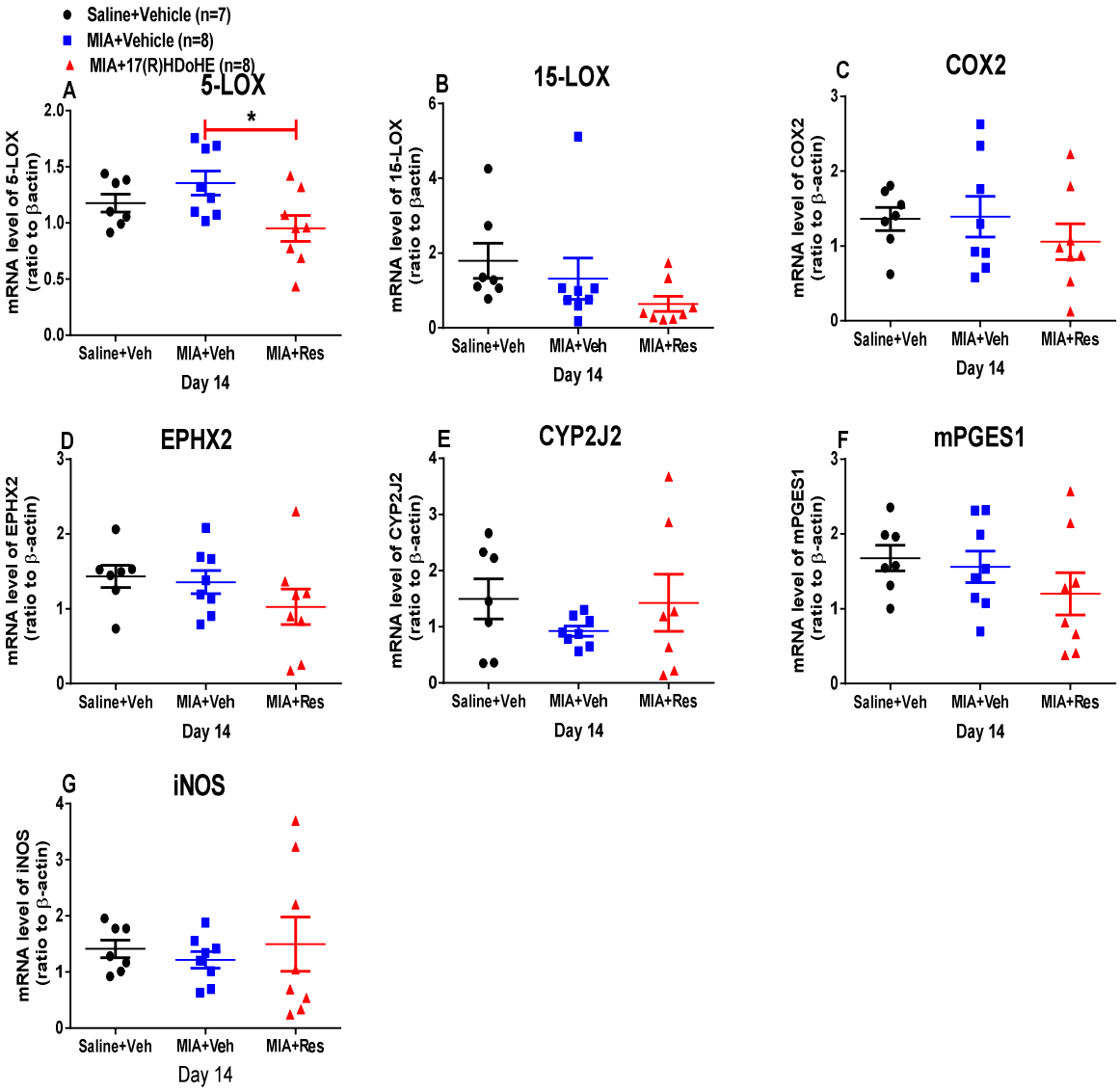


Fig. 4.6 Expression levels of metabolic enzymes in the synovia at 14 days following intra-articular injection of MIA or saline, and the effect of acute 17(R) HDoHE treatment. Data were analyzed by one-way ANOVA with Bonferroni's post-hoc test (n=7-8 per group).

4.3.1.2 Gene expression of resolvin receptors, inflammatory cytokines and enzymes in the spinal cord in the MIA model

Gene expression of ChemR23 and ALX, inflammatory cytokines and associated metabolic enzymes was quantified in ipsilateral quadrants of the spinal cord in rats that received intra-articular injection of MIA (14 day), saline and MIA rats receiving acute treatment with 17(R)HDoHE. There was no difference in the expression of ChemR23 in the ipsilateral dorsal horn of spinal cord between the three groups (**Fig 4.7A**). By contrast, spinal expression of ALX (**Fig 4.7B**) appeared higher in the MIA+vehicle group, compared to saline group, acute 17(R) HDoHE reduced this increase. There was no significant difference in the gene expression of selected cytokines (**Fig 4.8**) or metabolic enzymes (**Fig 4.9**) in the spinal cord between the three groups.

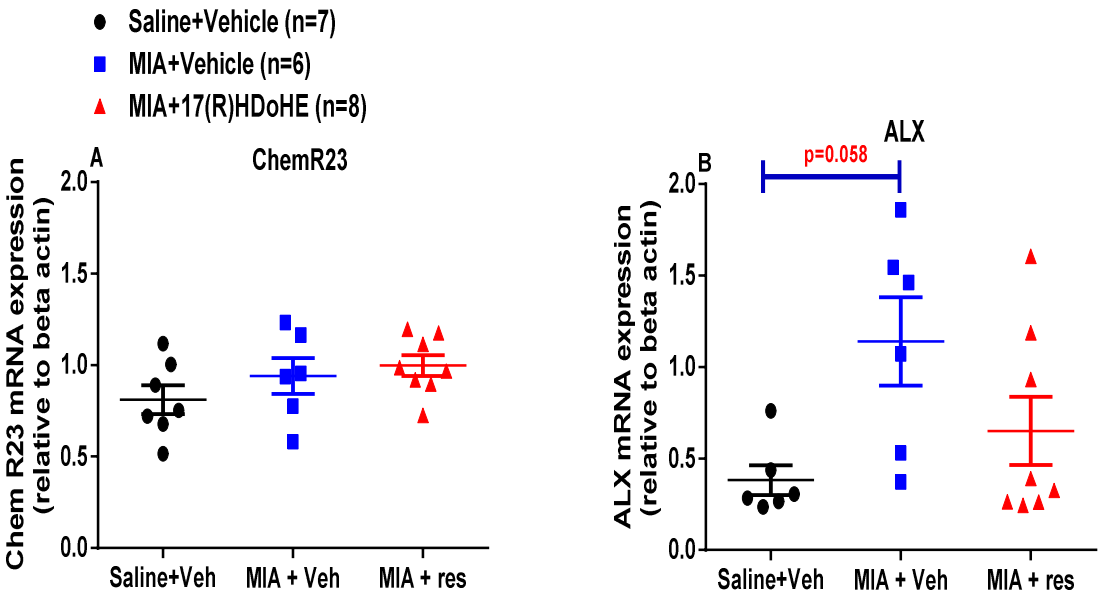


Fig. 4.7 Expression levels of resolvin receptors in quadrants of spinal cord of MIA vehicle, saline vehicle and MIA 17(R)HDoHE treated rats at day 14. Data were analyzed by one-way ANOVA with Kruskal-Wallis with Dunn's post-hoc test (n=6-8 per group).

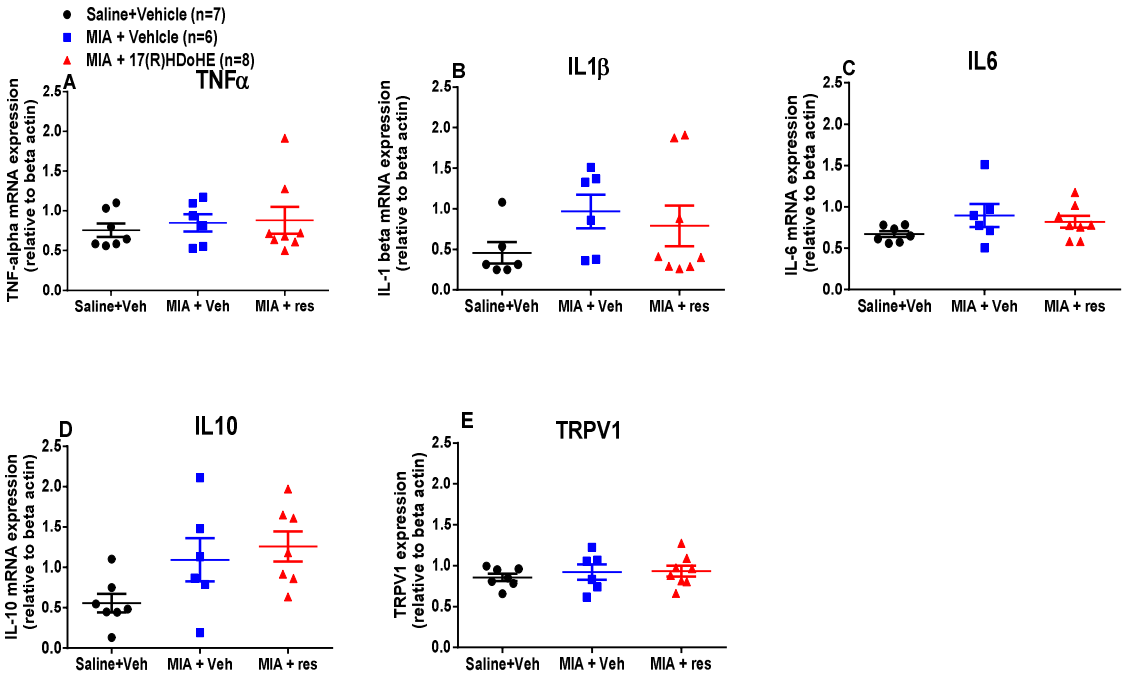


Fig.4.8 Expression levels of selected cytokines in quadrants of spinal cord. Data were analyzed by Kruskal-Wallis with Dunn's post-hoc test (n=6-8 per group).

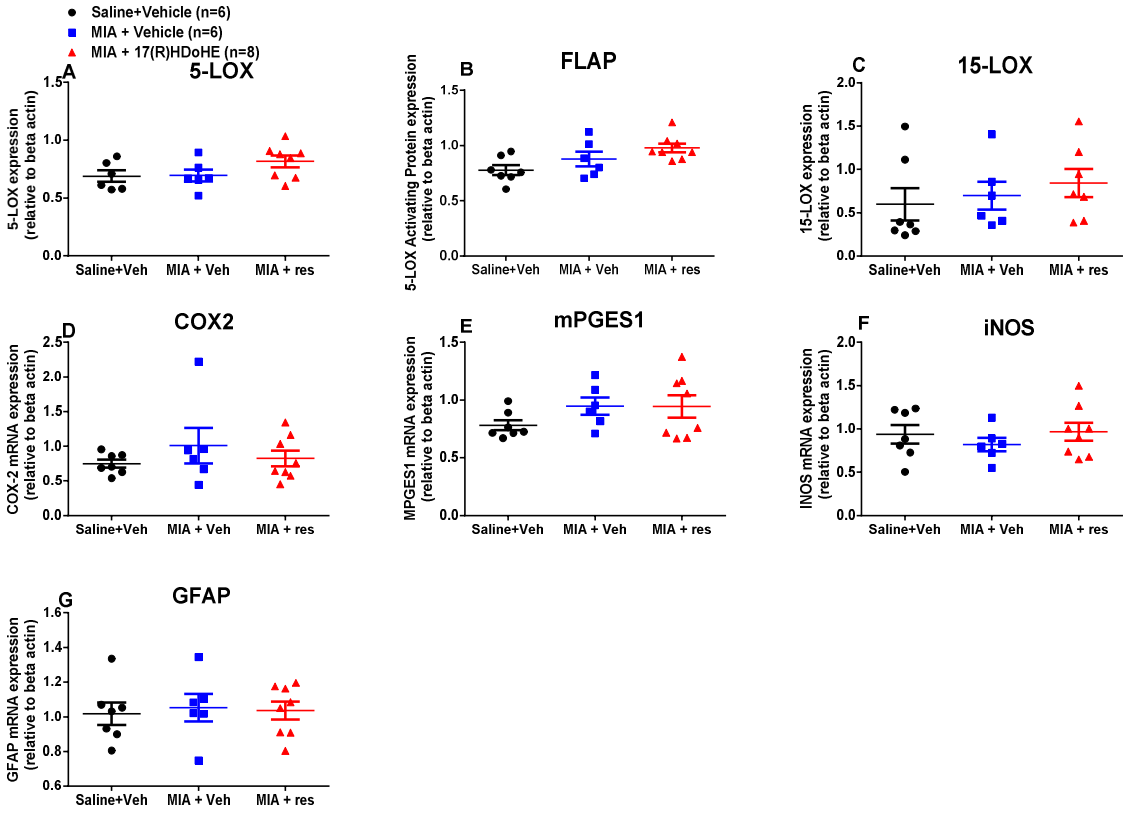


Fig.4.9 Expression levels of metabolic enzymes in quadrants of spinal cord. Data were analyzed by Kruskal-Wallis with Dunn's post-hoc test (n=6-8 per group).

4.3.2 Analgesic effects of chronic 17(R) HDoHE administration in the MIA model

Chronic treatment with 17(R)HDoHE (300ng/300µl every other day from day 14 until day 28 by i.p injection) significantly reversed OA pain behaviour in MIA rats, as evidenced by a significant inhibition of weight bearing asymmetry (Fig **4.10A**) and lowered of hindpaw withdrawal thresholds (**Fig 4.10B**), compared to vehicle treatment in MIA rats.

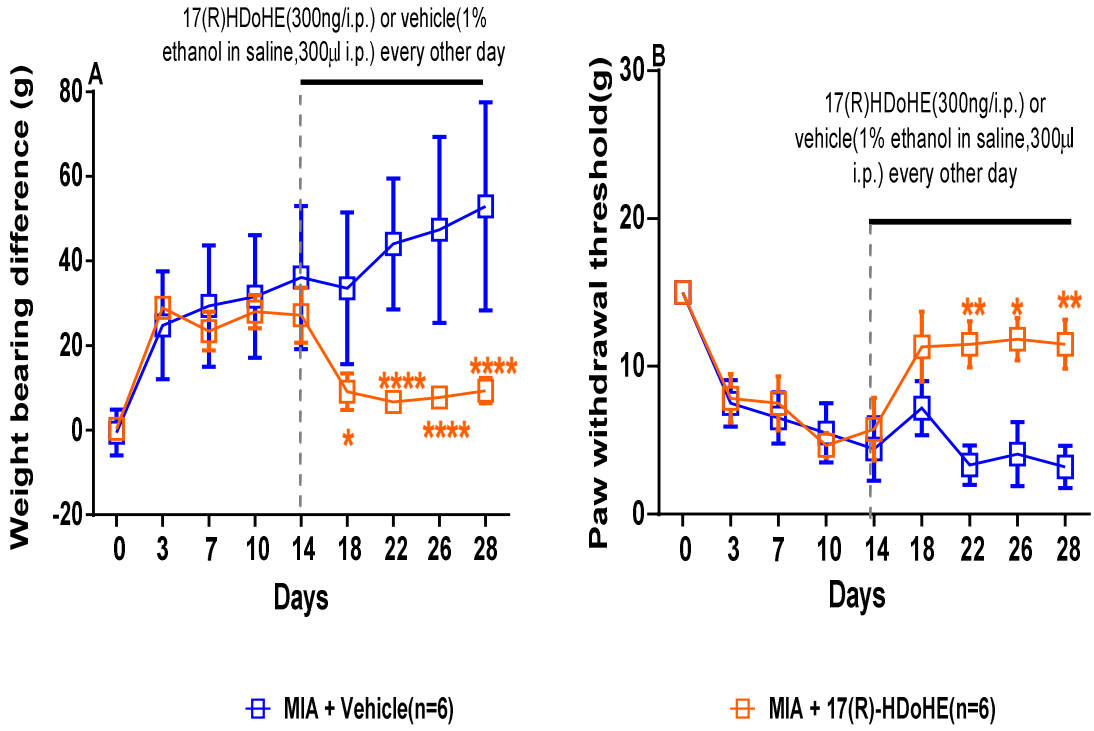


Fig.4.10 Intra-articular injection of MIA resulted in significant increases in weight bearing difference and lowered hindpaw withdrawal thresholds. Data are mean ± S.E.M, analyzed by two-way ANOVA with Bonferroni's post-hoc test, *p<0.05, **p<0.01, ****p<0.001 vs control.

4.3.2.1 Chronic treatment with 17(R)HDoHE did not alter joint pathology in the MIA model

Histological scores for chondropathy (**Fig 4.11 A**), synovial inflammation (**Fig 4.11B**), chondrocytes appearance (**Fig 4.11 C**) and vascular channel breaching of the osteochondral junction (**Fig 4.11 D**) were significantly higher in MIA rats, compared to saline treated-rats. Overall, 17(R) HDoHE treatment did not significantly alter the pathological scores of the joints from MIA rats, (**Fig 4.11 A**, **Fig 4.11 B**).

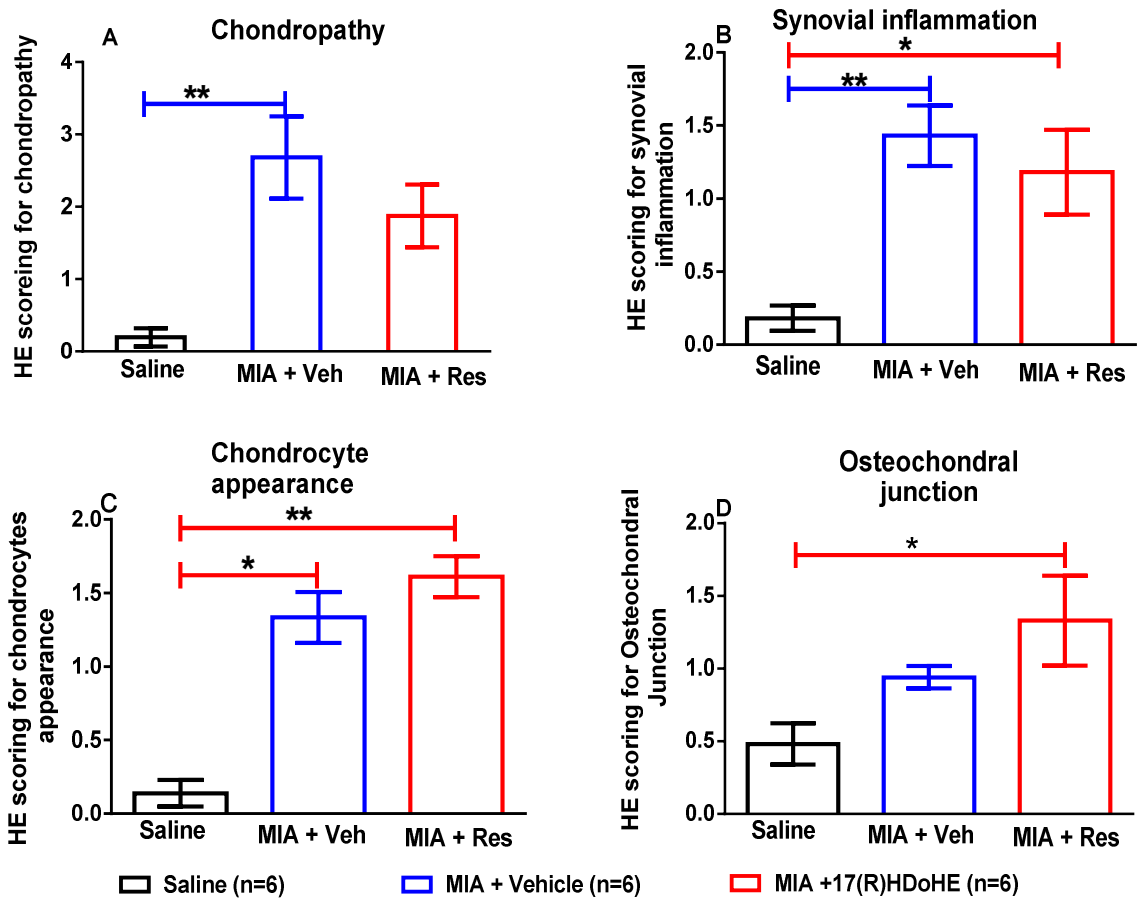


Fig.4.11 Intra-articular injection of MIA resulted in significant joint pathology at 28 days, compared to saline injected rats. Chondropathy score (A), Synovial inflammation (B), Chondrocyte appearance (C) and Vascular channels across osteochondral junction (D) in MIA treated rats. Data are mean ±S .E.M, analysed with One-way ANOVA with Bonferroni's post-hot test for parametric data(B and D) or Kruskal-Wallis with Dunn's post-hoc test for non- parametric data(A and C) (*p<0.05, **p<0.01).

H&E staining revealed intact structures of knee joints in saline treated rats (**Fig 4.12 A**). Cartilage damage was evident in MIA rats (**Fig 4.12B**) and 17(R) HDoHE did not attenuate the damage (**Fig 4.12C**). The synovium was composed of a single cellular layer in saline treated rat (**Fig 4.12D**) but numbers of infiltrated cells increased in the synovium of MIA treated rat (**Fig 4.12E**).

17(R) HDoHE treatment from day 14 until day 28 post model induction did not alter joint pathology.

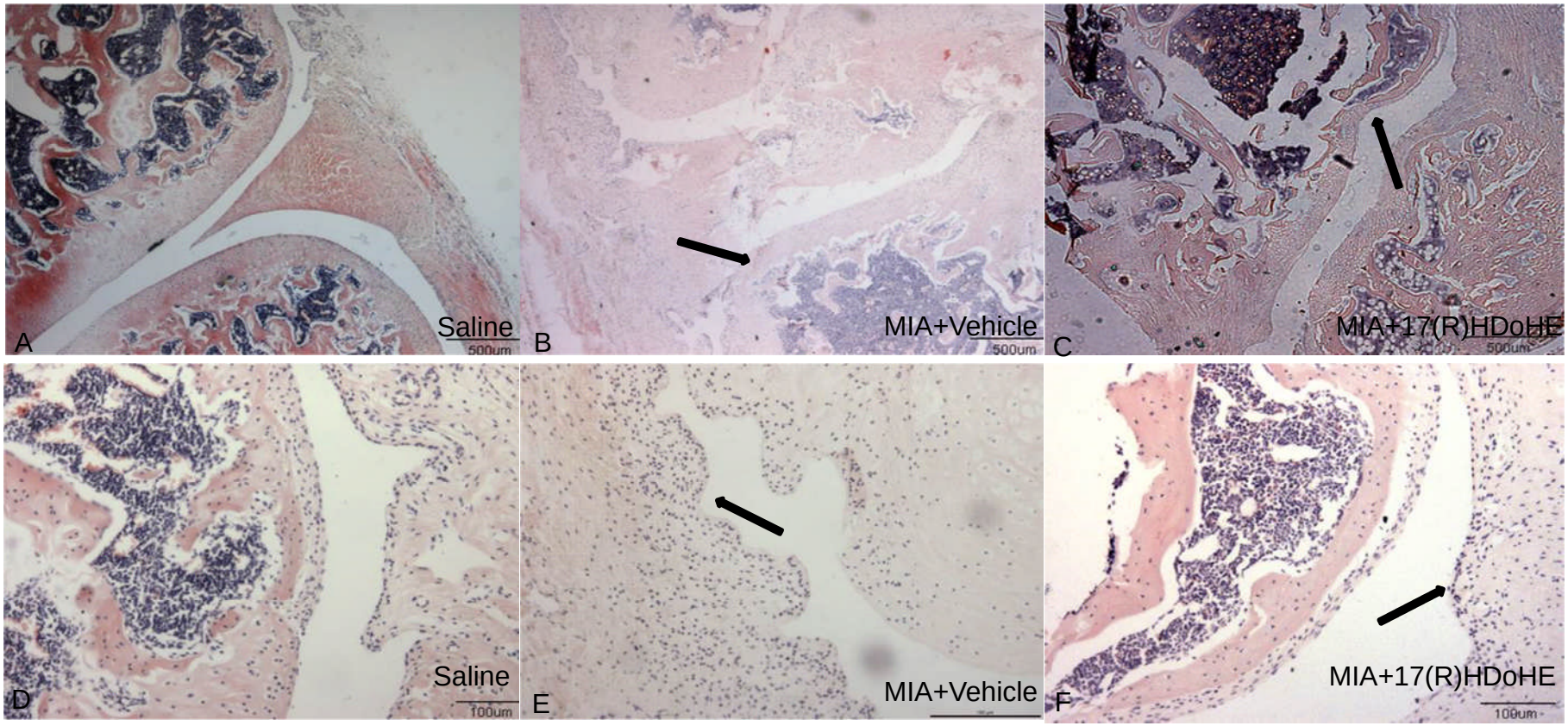


Fig 4.12 Joint histology for saline, MIA with vehicle and MIA plus 17(R) HDoHE treated rats at day 28. Cartilage damage: Saline (A), MIA+vehicle(B) and MIA+17(R)HDoHE (C); synovial inflammation: saline (D), MIA+vehicle (E) and MIA+ 17(R)HDoHE (F). Black arrows indicate chondropathy and synovial inflammation, scale bar : 500µm, 100µm,150µm for MIA+vehicle.

4.3.3 Effects of 17(R)HDoHE in the surgical medial meniscal transection model

MNX resulted in a significant increase in weight bearing difference (**Fig 4.13A**). Although hindpaw withdrawal thresholds appeared lower in MNX rats changes were not significant, compared to sham controls. Chronic treatment with 17(R)HDoHE (300ng/300µl every other day from day 14 until day 28 by i.p injection) significantly attenuated weight bearing difference in the MNX model (**Fig 4.13A**),

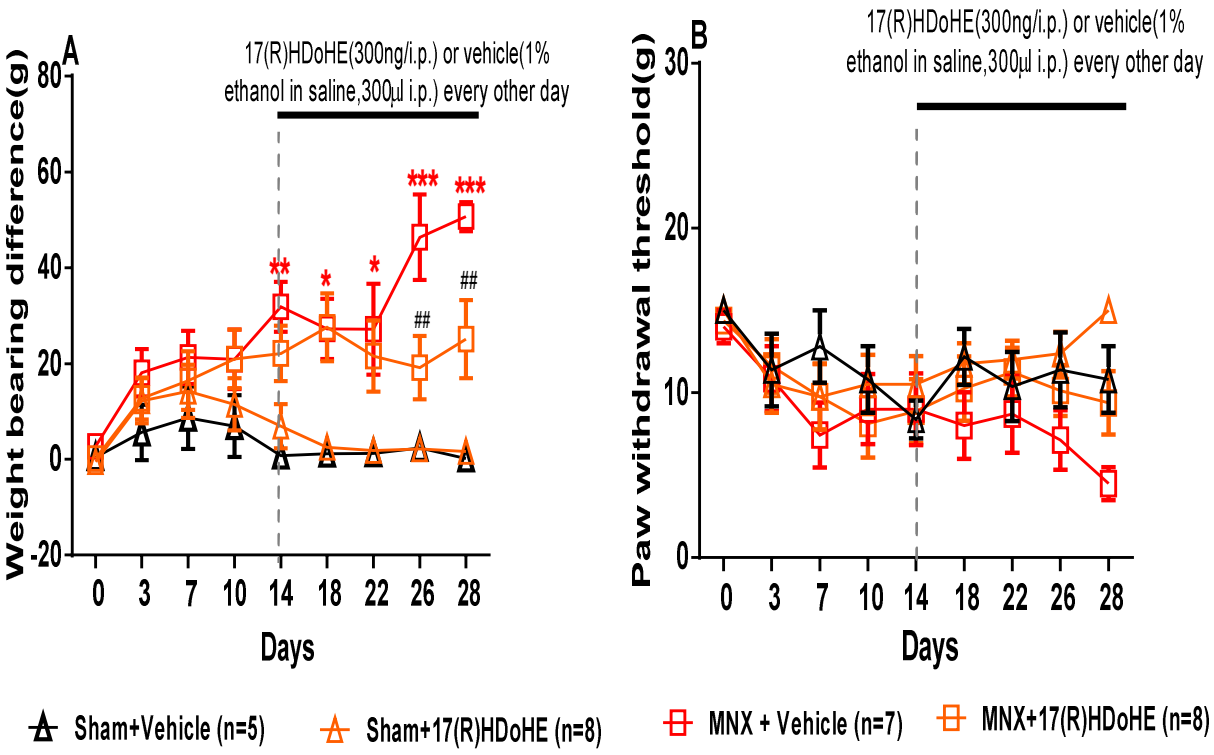


Fig 4.13 Medial meniscal transection (MNX) resulted in a significant increase in weight bearing difference, compared to sham control. Data are mean±S.E.M and were analyzed by two-way ANOVA with Bonferroni's post-hoc (*p<0.05, **p<0.01, ***p<0.001, vs sham vehicle, ##p<0.01 vs MNX vehicle, n= 5-8 per group)

4.3.3.1 Chronic treatment with 17(R) HDoHE did not alter MNX-induced joint pathology

Histological scores for chondropathy (**Fig 4.14 A**) and chondrocytes appearance (**Fig 4.14 C**) were significantly higher in MNX rats compared to sham rats.

17(R) HDoHE treatment did not significantly alter the MNX-induced chondropathy score or chondrocyte appearance score.

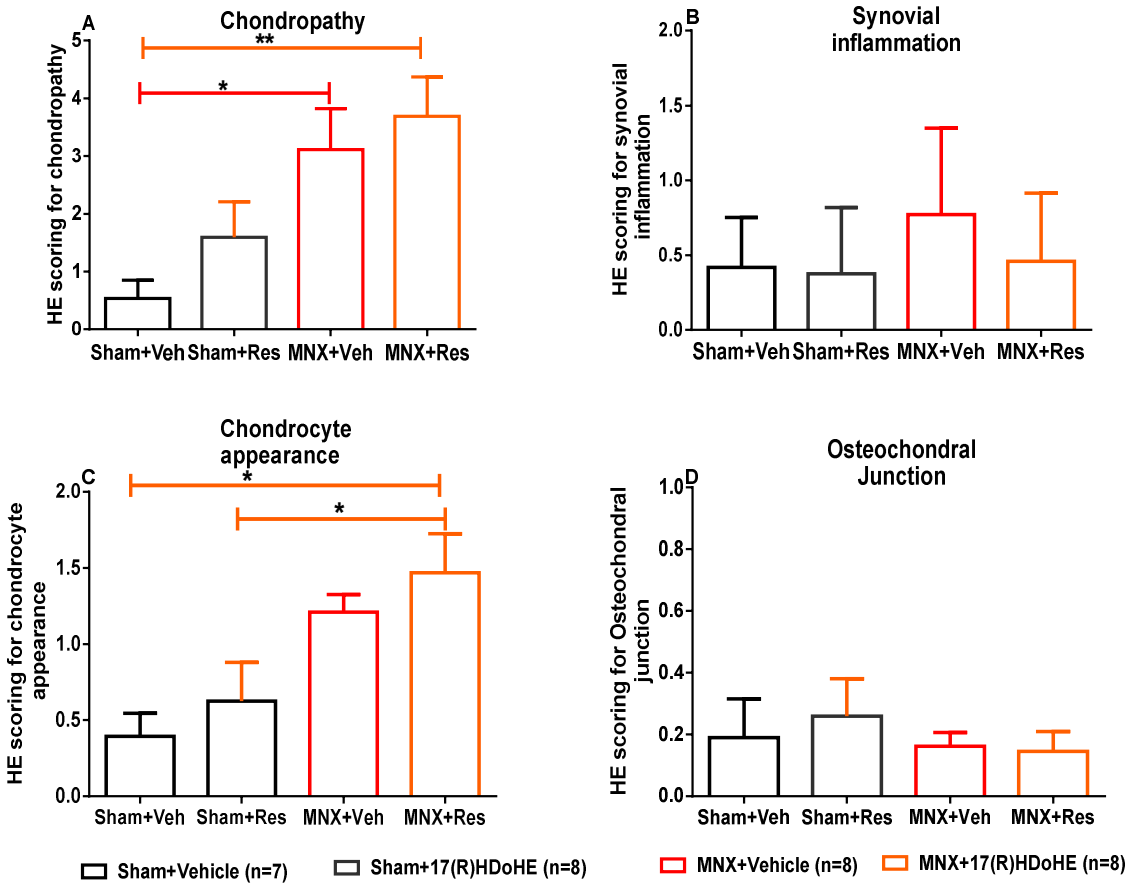


Fig.4.14 Histopathological assessment for knee joint in the MNX model and sham control and respective treatment with 17(R)HDoHE day 28. Chondropathy (A), synovial inflammation (B), chondrocyte appearance (C) and osteochondral junction (D). Data are mean±S.E.M analysed by one-way ANOVA with Bonferroni's post-hoc test (parametric data, B) or Kruskal Wallis with Dunn's test (non-parametric data, A, C, D) (*p<0.05, **p<0.01 vs controls, n= 7-8 per group).

H&E staining revealed intact structures of knee joint in sham treated rats (**Fig 4.15 A**). Cartilage damage was evident in MNX rats (**Fig 4.15 C**) and 17(R) HDoHE did not alter this damage (**Fig 4.15D**).

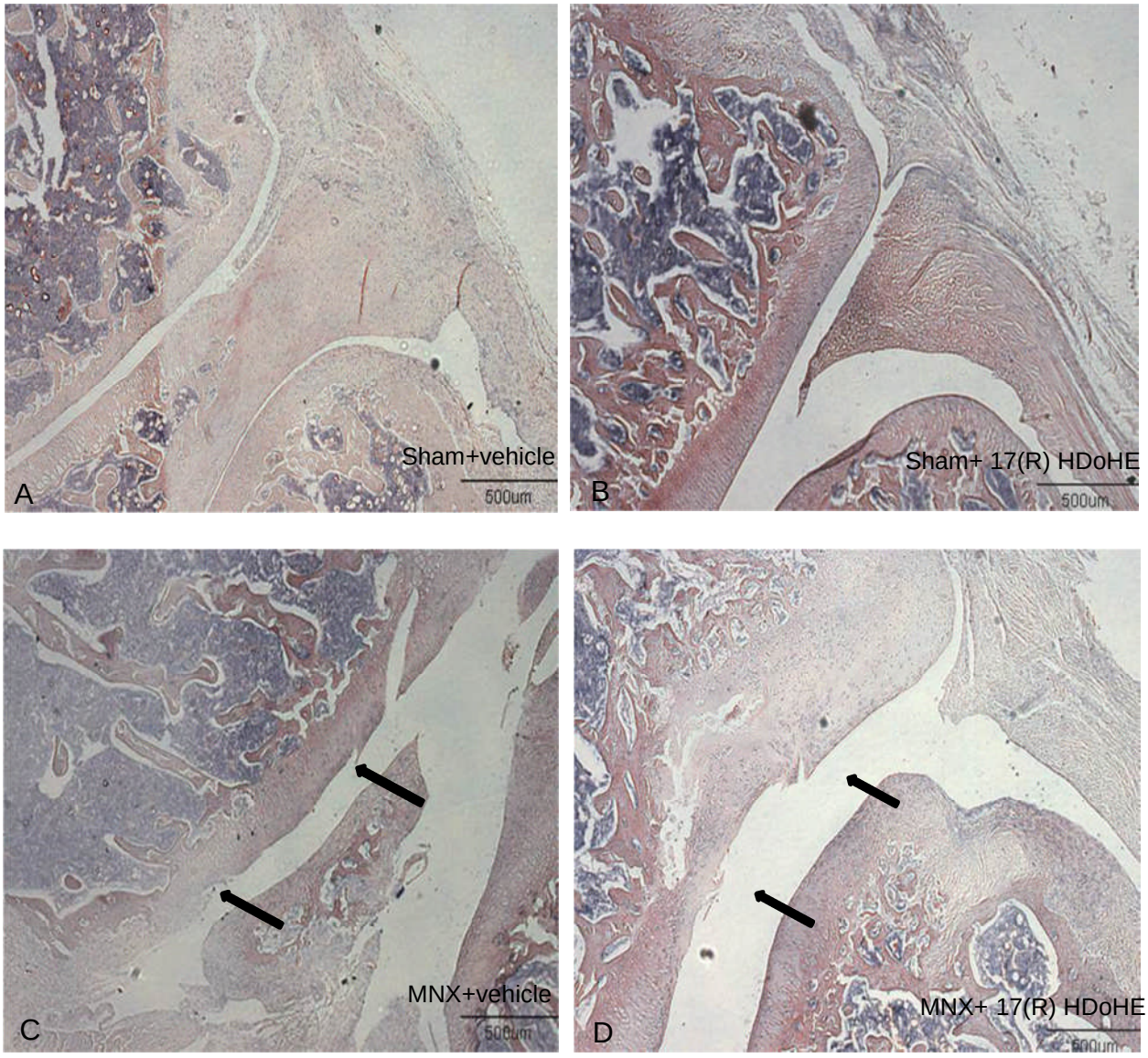


Fig.4.15 Joint histology for 17(R) HDoHE treated MNX rats and respective controls. Black arrows indicate the damage and scale bar was 500µm.

The synovium was a single cellular layer in the sham vehicle group (**Fig 4.16A**), numbers of cells were increased in the synovim of MNX rat, indicative of synovial inflammation (**Fig 4.16C**). 17(R)HDoHE treatment from day 14 until day 28 post model induction did not alter synovial inflammation (**Fig 4.16D**).

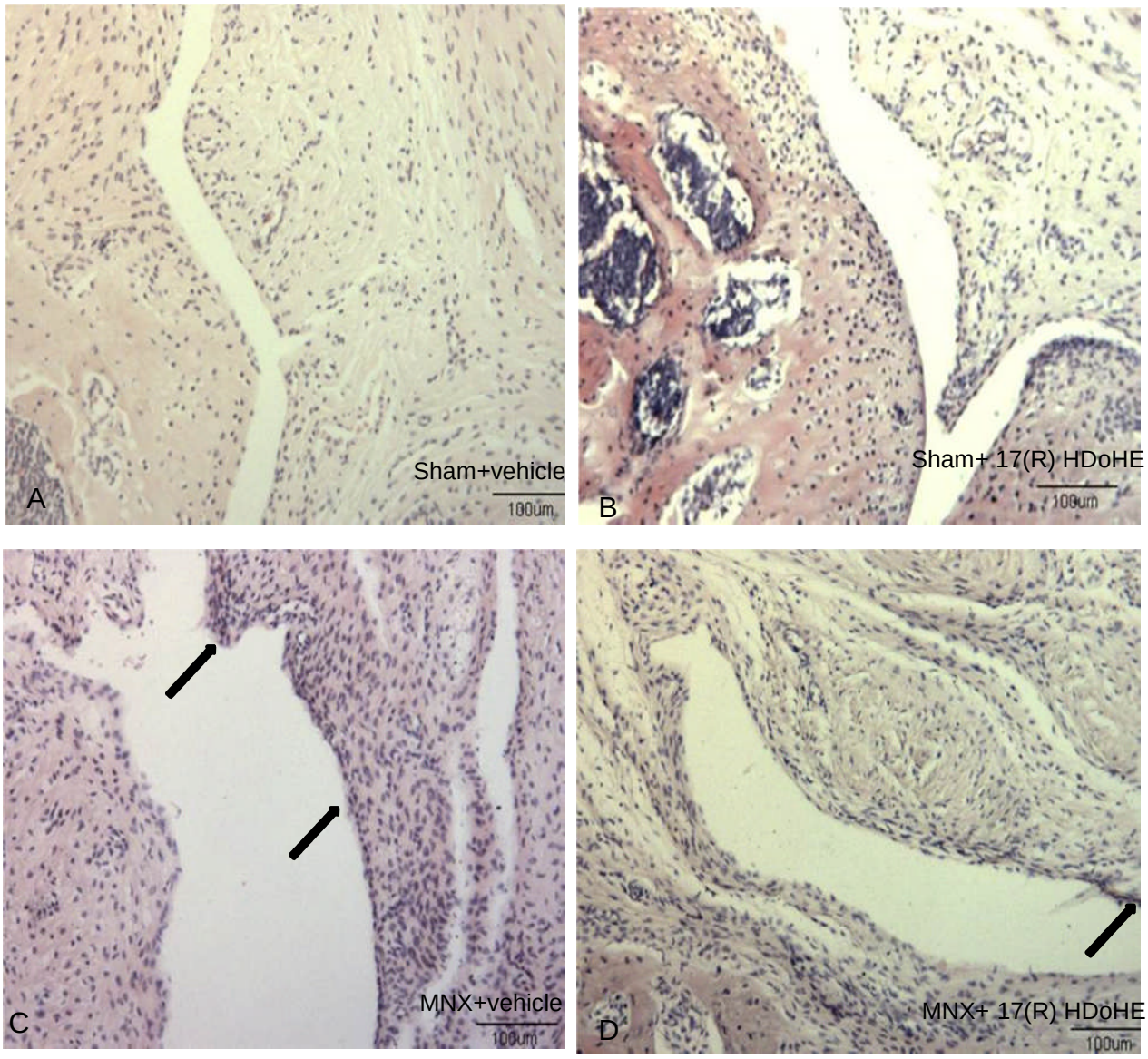


Fig.4.16 Representative sections of synovial inflammation in 17(R) HDoHE treated MNX rats and respective controls (Sham vehicle (A), sham 17(R) HDoHE (B), MNX vehicle (C) and MNX 17(R)HDoHE (D). Black arrows indicate synovial inflammation and scale bar was 100µm.

4.3.4 Analgesic effects of discontinuous 17(R)HDoHE administration in the MIA model

To investigate the effect of discontinuous analgesic treatment with 17(R)HDoHE, treatment was given from day 14-22 and MIA-induced pain behaviour was quantified until day 35. Pain behaviour was established in the model before drug administration and 17(R) HDoHE attenuated both weight bearing difference (**Fig 4.17A**) and paw withdrawal threshold (**Fig 4.17B**) from day 16 to day 22. Once treatment was ceased, this inhibitory effect gradually wore off. Pain behaviour returned back to the levels in MI vehicle group at day 35 (**Fig 4.17**).

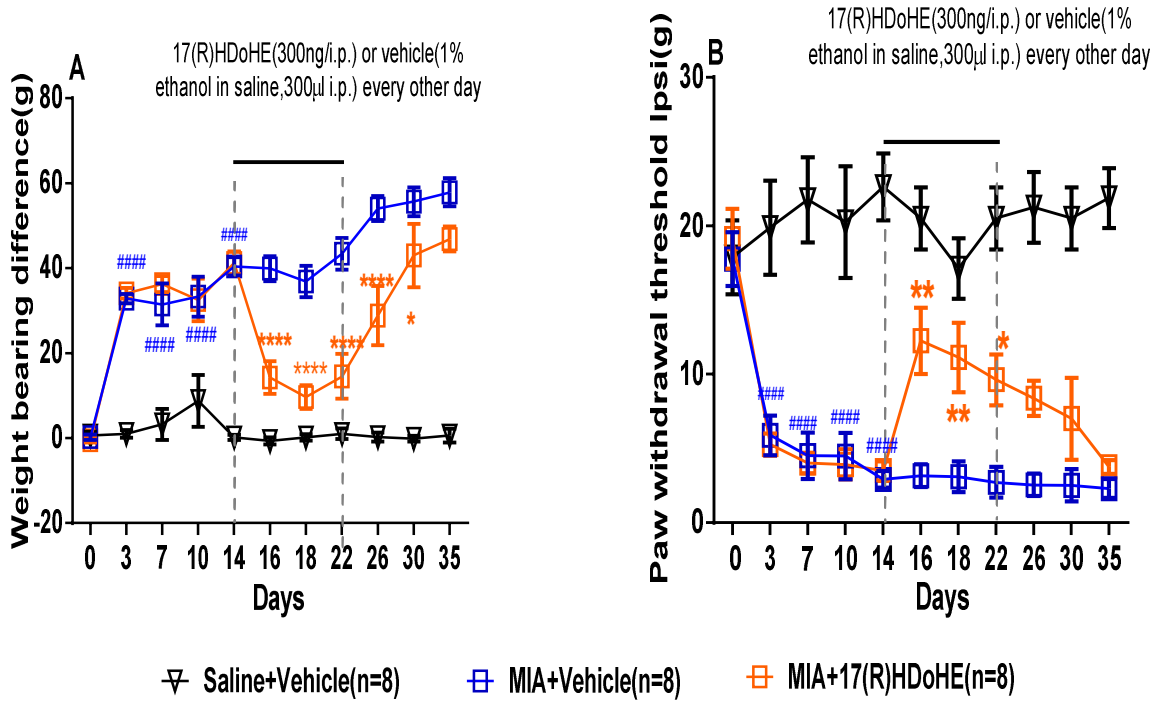


Fig 4.17 Effects of discontinuous administration of 17(R)HDoHE in the MIA model from day 14 until day 35. Data are mean±S.E.M (n= 8 per group) analyzed by two-way ANOVA with Bonferroni's post-hoc test, $^{\#\#}$ p<0.01, $^{\#\#\#}$p<0.001 compared to saline vehicle and *p<0.05, **p<0.01, ***p<0.001 compared to MIA vehicle.

4.3.4.1 Gene expression of resolvin receptors, cytokines and enzymes in synovia and effects of discontinuous treatment with 17(R) HDoHE

At day 35, expression of ChemR23 was significantly lower in the synovia from MIA rats, compared to saline vehicle rats (**Table 4.1**). There was no significant difference in levels of gene expression between the MIA + 17(R) HDoHE group and MIA vehicle group at day 35 after termination of the drug treatment (**Table 4.1**). 17(R) HDoHE did not modulate expression level of any of the enzymes studied in the MIA model, compared to the saline group (**Table 4.2**).

Groups	ChemR23	ALX	TNFα	IL1β	IL6	IL10
Sal veh	1.58±0.13	1.73±0.46	1.00±0.18	1.18±0.28	1.02±0.21	1.06±0.52
MIA veh	1.07±0.06**	2.51±1.03	1.05±0.24	1.87±0.79	1.87±0.66	1.16±0.49
MIA 17(R)HDoHE	1.10±0.10	1.40±0.43	0.78±0.14	1.20±0.35	0.98±0.13	1.17±0.60

Table 4.1 Gene expression of resolvin receptors (ChemR23 and ALX) and inflammatory cytokines (TNFα, IL1β, IL6 and IL10) in the synovia from saline control, the MIA model and discontinuous treatment with 17(R)HDoHE in the MIA model at day 35.

Groups	5-LOX	15-LOX	COX2	EPHX2	CYP2J2	mPGES1	iNOS
Sal veh	1.26±0.33	2.08±0.63	1.09±0.25	1.13±0.13	1.33±0.28	1.06±0.08	1.24±0.32
MIA veh	0.83±0.19	0.65±0.09	1.58±0.66	1.82±0.72	1.00±0.27	1.21±0.15	1.40±0.37
MIA 17(R)HDoHE	0.82±0.19	0.83±0.18	1.51±0.43	1.04±0.17	1.67±0.37	0.98±0.13	1.65±0.41

Table 4.2 Gene expression of metabolic enzymes (5-LOX, 15-LOX, COX2, EPHX2, CYP2J2, mPGES1 and iNOS) in the synovia from saline control, the MIA model and discontinuous treatment with 17(R)HDoHE in the MIA model at day 35.

4.3.5 Summary of results

- Acute injection of 17(R)HDoHE transiently attenuated pain behaviour in the MIA model at day 14, compared to the effect of vehicle.
- Expression of ChemR23 in the synovia was significantly lower in MIA rats at day 14 and day 35, compared to saline controls.
- Acute injection of 17(R)HDoHE was associated with a significant increase in the expression of IL6 and down-regulation of 5-LOX expression in the synovia of MIA model at day 14, compared to vehicle control.
- Chronic 17(R) HDoHE produced a sustained attenuation of OA pain behaviour in the MIA model. Chronic 17(R)HDoHE produced a sustained attenuation of weight bearing asymmetry in the MNX model.
- Chronic treatment with 17(R)HDoHE did not significantly alter MIA or MNX - induced joint pathology.
- The effects of 17(R)HDoHE on MIA-induced weight bearing asymmetry were maintained for up to a week following cessation of treatment, but then pain behaviour returned to levels comparable to those exhibited in MIA vehicle rats.

4.4 Discussion

In the current Chapter, I have demonstrated the analgesic effects of a precursor of resolvin D-series (17(R)HDoHE) in a series of studies on MIA and MNX-induced OA pain and investigated the underlying mechanisms of the action.

Firstly, I demonstrated 17(R)HDoHE had robust analgesic effects on OA pain behaviour in the MIA and MNX-induced models following systemic administration. Acute administration of 17(R)HDoHE significantly increased the expression of IL6 and down-regulated the expression of 5-LOX in the synovia of MIA rats, compared to vehicle control. These findings may suggest the acute inhibitory effects of 17(R)HDoHE on OA pain via a peripheral site of action.

17(R)HDoHE produced a sustained analgesic effect in the MIA model during drug administration and this effect was maintained for a week after drug cessation, which may indicate transient effects of the drug to OA pain. The mechanisms of chronic inhibitory effects of 17(R)HDoHE to treat OA pain are still unclear as joint pathology was not significantly improved by the compound.

4.4.1 The acute analgesic effects of 17(R)HDoHE in the MIA model

Acute administration of 17(R)HDoHE showed a transient effect to attenuate pain behaviour (WB and PWT) in the MIA model. Weight bearing asymmetry was reduced by 17(R)HDoHE from 1h until 6h post drug administration. Ipsilateral paw withdrawal threshold was significantly reversed by 17(R)HDoHE at 1h and 6 h in the model. The acute effects of 17(R)HDoHE showed here in the MIA model are consistent with a previous study in the AIA model (Lima-Garcia et al. 2011). Concurrently, I found the up-regulation of IL6 in the synovia after treatment with 17(R)HDoHE, compared to vehicle control. The enhanced expression of IL6 here may indicate its anti-inflammatory role in the acute inflammation and OA which has been shown in some previous studies (Van De Loo et al. 1997; Xing et al. 1998). IL6 can induce the synthesis of TIMP1 in chondrocytes, fibroblasts and synoviocytes (Lotz and Pa 1996) and was positively correlated with proteoglycan synthesis, but it was also increased in OA synovial fluid which may also act on chondrocytes (Venn et al. 1993). This may indicate dual roles of IL6 in the pathogenesis of OA (Venn et al. 1993; Lotz and Pa 1996; Westacott and Sharif 1996). 17(R)HDoHE also reduced the expression of 5-LOX in the synovia from MIA rats, compared to MIA vehicle rats, suggesting the

effects of 17(R)HDoHE on pain behaviour may involve the inhibition of 5-LOX, which is an essential enzyme for the metabolism of arachidonic acid to down-stream pro-inflammatory lipid mediators such as leukotrienes (Burnett and Levy 2012) and is over expressed in synovial tissues promoting progress of inflammatory arthritis (Gheorghe et al. 2009; Lin et al. 2014). No alteration of the inflammatory cytokines and associated metabolic enzymes was observed in the spinal cord after treatment with 17(R)HDoHE. This may indicate that the effects of this intervention on pain are predominantly mediated by peripheral mechanisms at the primary site of injury. It is possible that the effects are mediated via actions on ion channels, including the TRP channels (Bang et al. 2010). Nevertheless, it also could be that 17(R)HDoHE reduced the pain behaviour via inhibition of central components such as neuronal hyperexcitability (Xu et al. 2010) or activation of glial cells (Xu et al. 2012)

4.4.2 Aanalgesic effects of chronic 17(R)HDoHE administration in the MIA and MNX model

My studies further revealed 17(R)HDoHE produced a sustained analgesic effect in both MIA and MNX models following repeated administration.

17(R)HDoHE reduced weight bearing asymmetry in both the MIA and MNX model. This may further suggest 17(R)HDoHE attenuated OA pain in peripheral sites and by peripheral mechanisms as evident in 4.4.1 and some previous studies of the effects of the resolvin receptor (Xu et al. 2010) on inflammatory signalling (Lima-Garcia et al. 2011; Feng et al. 2012; Abdelmoaty et al. 2013) and TRP channels (Bang et al. 2010; Bang et al. 2010; Bang et al. 2012). However, the inhibitory effects of 17(R)HDoHE on the ipsilateral hindpaw withdrawal thresholds also indicate an effective central sensitization of pain at the distal sites in the MIA model but less so in the MNX model. This observation in the MNX model may be due to the sham surgery which also had lowered the hindpaw withdrawal threshold, which may mask the analgesic effects of the 17(R)HDoHE on this type of pain behaviour in this model.

In the joint histological assessment, repeated administration of 17(R)HDoHE failed to improve the joint histopathology in both the MIA and MNX models. This may suggest the analgesic effects of 17(R)HDoHE are not overly involved in bone remodelling in the joint and may be more contributed by other peripheral and central components.

4.4.3 Effects of 17(R)HDoHE by discontinuous administration in the MIA model

I also investigated the potential maintenance of analgesic effects of 17(R)HDoHE in the MIA model with a discontinuous treatment protocol. 17(R)HDoHE attenuated the pain behaviour (WB and PWT) during drug administration, which was consistent with previous studies in the MIA model. 17(R)HDoHE produced a sustained analgesic effect for a week after termination of treatment and then pain behaviour returned to the level of vehicle-treated MIA rats at day 35 (two weeks after cessation of 17(R)HDoHE). This was different from the traditional analgesic drugs such as opioids which often exhibit anti-nociceptive tolerance after repeated administration (Collett 1998).

Given that the effects of the intervention had ceased by the time when tissues were collected, it is not surprising that I observed no effect of this intervention on levels of inflammatory cytokines and enzymes. Expression of IL6 was decreased in the current study but up-regulated in the acute administration of 17(R)HDoHE in the MIA model. This further demonstrated the dual role of IL6 in inflammation (Kaplanski et al. 2003) with an anti-inflammatory role in acute inflammation (Xing et al. 1998) and pro-inflammatory properties in the late stage of inflammation (Scheller et al. 2006). As 17(R)HDoHE failed to show significant improvement of joint pathology in previous chronic studies and pain behaviour was worn off at the end of this study, joint pathology was not assessed here. This study further demonstrated that the analgesic effects of 17(R)HDoHE were transient in the MIA model but mechanisms for the inhibition of pain behaviour need further investigation.

The limitations of the studies were to demonstrate the specific mechanisms, especially the central mechanisms on pain control. Some of the mechanisms such as blocking spinal synaptic plasticity or microglial activity may contribute to the modulation of pain behaviour. As previous studies have shown spinal administration of RvE1 can block the activity of TRPV1 and TNF-α-evoked NMDA receptor hyperactivity in the spinal cord (Xu et al. 2010) and prevent neuropathic pain by blocking microglial singling in the spinal cord (Xu et al. 2012). It may also be possible that 17(R)HDoHE modulates bone resorption and bone formation to improve OA pain, as RvE1 has been shown to decrease bone resorption by inhibiting osteoclast growth and differentiation (Herrera et al. 2008) and decrease osteoclast formation (Zhu et al. 2013). On the other hand, RvE1 enhanced bone formation and expression of OPG from os-

teoblasts (Gao et al. 2013). However, understanding of the anti-nociceptive roles of resolvins on pain still needs further investigation.

5 Expression levels of target genes in synovia and knee joints of human OA.

5.1 Introduction

Currently, no investigation of resolvins on OA has been reported. However, the effects of Ω-3 PUFAs, which are the precursors to resolvins and other lipid mediators, on OA or RA have been shown in some previous studies in human samples and animal models. This may help us understand the association of resolvins with human OA better.

Supplementation with Ω-3 PUFAs decreased IL1-induced release of proteoglycan and blocked proteolytic activity of collagenase and aggrecanase in human cartilage from osteoarthritis (Curtis et al. 2002). In addition, Ω-3 PUFAs inhibited IL1-induced mRNA expression of 5-LOX, COX2, FLAP, TNFα, IL1β, MMP3, MMP13, and ADAMTS-4 in cultured cartilage from human OA (Curtis et al. 2002). A meta-analysis revealed supplementation with Ω-3 PUFAs for 3–4 months attenuated joint pain, stiffness of joint and number of painful joints in RA (Goldberg and Katz 2007). DHA and EPA, two Ω-3 PUFAs, inhibited IL1β-induced degradation of bovine cartilage (Wann et al. 2010). Supplementation of Ω-3 PUFAs also improved histopathological scores of right tibial plateau, increased pro-MMP9 and decreased MMP2 activation in guinea pig model of spontaneous OA (Knott et al. 2011). Endogenous Ω-3 PUFAs were increased in the cartilage of DMM-induced osteoarthritis compared to sham controls in wild-type mice. In addition, DMM mice transfected with fat-1gene, which encodes a Ω-3 PUFAs desaturase and converts Ω-6 PUFAs to Ω-3 PUFAs, have lower ratio of Ω-6 PUFAs /Ω-3 PUFAs (Huang et al. 2014). Moreover, the endogenously increased Ω-3 PUFAs and decreased Ω-6 PUFAs reduced formation of osteophyte and cartilage damage, inhibited expression of MMP-13 and ADAMTS-5 and decreased chondrocytes number in the DMM model (Huang et al. 2014).

Ω-3 PUFAs are beneficial for various diseases such as cardiovascular diseases (Lavie et al. 2009), inflammatory diseases including arthritis (Calder 2006) and cancer (Rose and Connolly 1999). Lipid mediators derived from Ω-3 PUFAs were reported in various tissues in both healthy people and patients with RA. A previous study has identified profiles of pro-resolving and anti-inflammatory lipid mediators such as lipoxins, resolvins, protectins and maresins in serum and lymphoid tissues

from 100 healthy volunteers by liquid chromatograph- mass spectrometry (LC-MS). Furthermore, levels of RvD1, RvD2, RvD3, MaR1, and NPD1/PD1 were quantified within their bioactive ranges (Colas et al. 2014). In the serum and plasma of healthy volunteers given Ω-3 PUFAs for 3 weeks, the resolvin precursors, RvD1 and RvD2 were identified and quantified and levels of RvD1 and RvD2 were within the biological range which showed anti-inflammatory action (Mas et al. 2012). Supplementation with Ω-3 PUFAs for 7 days significantly increased levels of RvE1, 18R/S-HEPE, 17R/S-HDHA, and 14R/S-HDHA in the plasma of healthy individuals (Barden et al. 2014). Lipidomic screening by LC-MS identified and quantified the levels of RvD5, maresin 1 and lipoxin A_4 in the synovial fluid from RA patients (Giera et al. 2012). Hydroxy-derivatives from PUFAs, RvD5, maresin 1 and lipoxinA_4 were detected in synovial fluid of human OA (Giera et al. 2012). In addition, metabolites from other PUFAs and metabolic enzymes are also involved in human OA. Expression of 15-LOX1 and 15-LOX2 were firstly identified in the chondrocytes of human OA. 13(S)-hydroxy octadecadienoic acid (13-HODE) and 15(S) - hydroxyeicosatetraenoic acids (15-HETE), which were derived from LA and AA via 15-LOX, inhibited TNFα-induced, IL-1β-induced and IL17-induced protein and mRNA levels of MMP-1 and MMP-13 in chondrocytes from OA patients (Chabane et al. 2009).

As we have shown robust (sgnificant) analgesic effects of 17(R) HDoHE in a series of animal experiments on osteoarthritic pain in the MIA model and weight bearing in the MNX model and investigated the underlying mechanisms (see Chapter 4). Furthermore, expression of ChemR23 was negatively associated with pain behaviour in the synovia of MIA model, which indicated the possibility of aloss of ChemR23 induced severe pain behaviour. Correlation between expression of ALX and pain behaviour was also shown in the spinal cord in the MIA model at different time points (positive correlation at day 14 and negative correlation at day 35). Expression of ALX was positively correlated with TNFα and negatively with 5-LOX in the synovia of MIA model. In addition, expression of ALX was negatively correlated with expression of COX2 and IL6 and positively correlated with 15-LOX in the spinal cord in MIA model. Therefore, it is of interest to see how the resolvin receptor system might be altered in human OA to seek clinical evidence for the association between resolvins, resolvin receptors and inflammatory cytokines or their associated metabolic enzymes in OA patients.

5.2 Aim and objectives

Aim:

To investigate the expression of the resolvin receptor system in human OA tissues.

Objectives:

- To compare expression levels of ChemR23 and ALX in synovia and medial tibial plateau from OA patients.
- To identify the potential for correlation between the two resolvin receptors with inflammatory cytokines and their metabolic enzymes in synovia and medial tibial plateau OA patients.
- To investigate the potential for correlation between expression of target genes between synovia and medial tibial plateau from OA patients.
- To compare gene expression of resolvin receptors, inflammatory cytokines and metabolic enzymes between the medial tibial plateau from human OA and femoral heads from trauma patients.

5.3 Methods

In the current study, 16 OA patients with TKR and 5 trauma patients were recruited (The ethic approval letter and personal research passport are attached in the appendices of the thesis and consenting forms were kept by the bio-bank at the City Hospital, University of Nottingham). A patient who was diagnosed with spondyloepiphyseal dysplasia was excluded later. Synovia from 15 OA patients, fresh medial tibial plateau from 14 OA patients and fresh femoral heads from 5 trauma patients were used to quantify gene expression of resolvin receptors, inflammatory cytokines and associated metabolic enzymes. The general methods for sample collection, sample processing and quantification of gene expression have been detailed in Chapter 2 (see 2.8 and 2.9).

To make an objective assessment on the correlation between expression of resolvin receptors (ChemR23 and ALX) and genes of interest, some risk factors for osteoarthritis such as body mass index (BMI) and age which may affect expression levels of target genes were evaluated as well (Manek et al. 2003; Rai et al. 2013; Rai et al. 2014).

Patients' data are summarized in **Table 5.1 and Table 5.2**.

Biobank ID	Sex	Age	TKR	BMI	Collected Date
13/005449	M	66	Left knee	34.7	19/11/2013
13/005465*****	F	69	Right Knee	32.4	19/11/2013
13/005243	M	55	Left knee	35.8	05/11/2013
13/05469	M	67	Right Knee	24.5	21/11/2013
13/005268	F	79	Right Knee	30.9	03/12/2013
13/003012	F	67	Left knee	40.1	17/12/2013
14/0000349	M	65	Left knee	36.9	28/01/2014
14/000776	F	64	Left knee	37.3	25/02/2014
14/000778	F	68	Right Knee	26.5	25/02/2014
14/000845	F	51	Right Knee	37.7	04/03/2014
14/001134	M	60	Right Knee	N/A	25/03/2014
14/001241	F	58	Left knee	37.8	01/04/2014
14/001347	F	72	Left knee	31.1	08/04/2014
14/001354	F	82	Right Knee	25.7	08/04/2014
14/001632	M	70	Left knee	27.5	06/05/2014
14/001638	M	79	Right Knee	36.1	06/05/2014

Table 5.1: The basic information for OA patients investigated in the study. BMI Categories: Underweight ≤18.5; Normal weight = 18.5–24.9; Overweight = 25–29.9 and Obesity = 30 or greater. '*'indicates the excluded non-OA sample. M/male, F/female.

Biobank ID	Sex	Age	Tissue collected	BMI	Collected Date
14/001104	F	-	femoral head	22.3	21/03/2014
14/001165	M	-	femoral head	-	27/03/2014
14/001242	F	-	femoral head	-	01/04/2014
14/001384	F	-	femoral head	15.8	11/04/2014
14/001423	M	-	femoral head	-	16/04/2014

Table 5.2: The basic information for trauma patients investigated in the study. BMI Categories: Underweight ≤18.5; Normal weight = 18.5–24.9; Overweight = 25–29.9 and Obesity = 30 or greater. '-'indicates as not available.

5.4 Results

5.4.1 mRNA levels of ChemR23 and ALX in the synovia and medial tibial plateau from OA patients

Gene expression of ChemR23 was significantly higher than ALX in synovia, but not in the medial tibial plateau in OA patients (**Fig 5.1A, Fig 5.1B**).

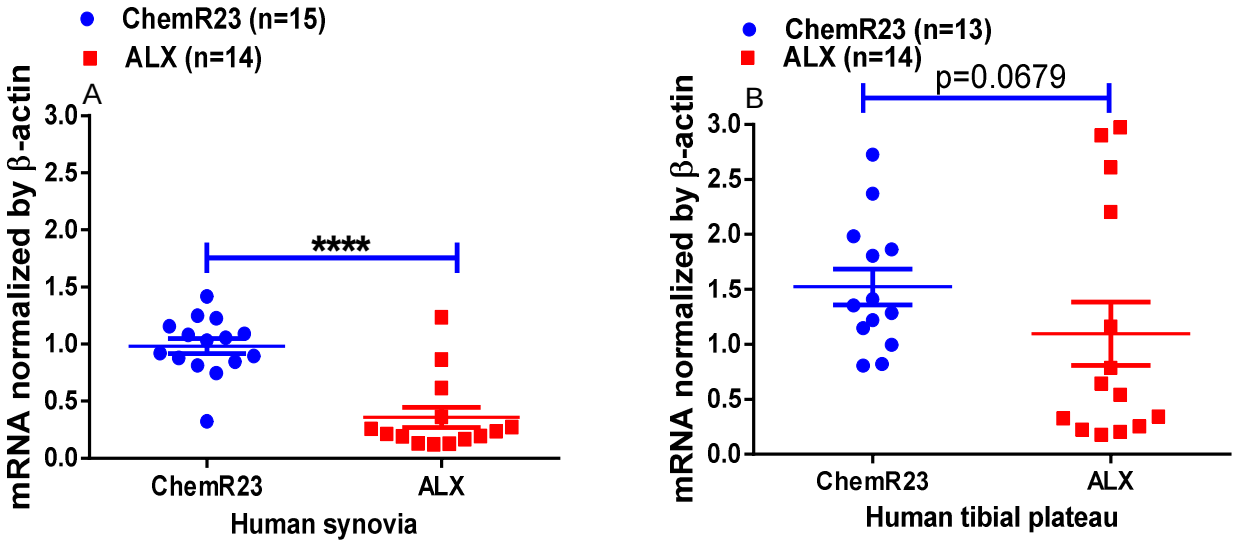


Fig 5.1 mRNA levels of resolvin receptors in the synovia and medial tibial plateau from OA patients. Data are mean ± S.E.M, analyzed with Mann-Whitney t-test (****p< 0.0001).

5.4.2 Correlation of resolvin receptors with genes of interest in the synovia of OA patients

5.4.2.1 mRNA levels of genes of interest in the synovia

Gene expression of resolvin receptors, inflammatory cytokines and associated metabolic enzymes in the synovia from OA patients was summarized in the **Table 5.3**.

Genes/sample	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
ChemR23	0.90	0.88	1.25	1.06	1.08	0.85	1.42	1.09	1.03	0.33	0.81	0.75	1.23	0.92	1.16
ALX	0.13	0.21	/	0.27	0.13	0.36	0.17	0.20	0.26	1.24	0.62	0.12	0.87	0.19	0.24
TNFα	0.70	0.85	0.85	0.88	1.56	0.97	1.11	1.50	1.76	1.04	0.88	1.62	0.83	0.93	1.36
IL6	0.45	0.41	2.55	0.90	0.74	2.86	0.55	0.59	0.77	1.98	1.21	0.69	1.15	1.15	0.61
5-LOX	0.81	1.01	1.45	1.34	0.85	0.66	1.10	0.82	1.09	0.33	0.47	0.57	0.86	0.99	1.10
15-LOX1	0.07	0.09	/	0.25	0.02	0.15	0.11	0.10	0.13	0.68	0.28	0.06	0.80	0.05	0.04
15-LOX2	0.68	0.23	0.42	0.77	0.47	0.77	0.23	0.96	1.02		0.60	1.52	/	1.59	0.59
COX2	1.11	0.75	0.91	1.13	1.03	0.91	1.11	1.15	0.90	0.50	0.94	0.94	0.90	0.90	0.92

Table 5.3: mRNA levels of target genes (normalized to β-actin) in the synovia of OA patients. Synovial samples from 15 OA patients with TKR were used to quantify the expression levels of target genes. '/' was the excluded outlier with Grubb's test.

5.4.2.2 Correlation of resolvin receptors with genes of interest in the synovia of OA patients

There was a positive correlation between gene expression of ChemR23 and 5-LOX (**Fig 5.2D**).

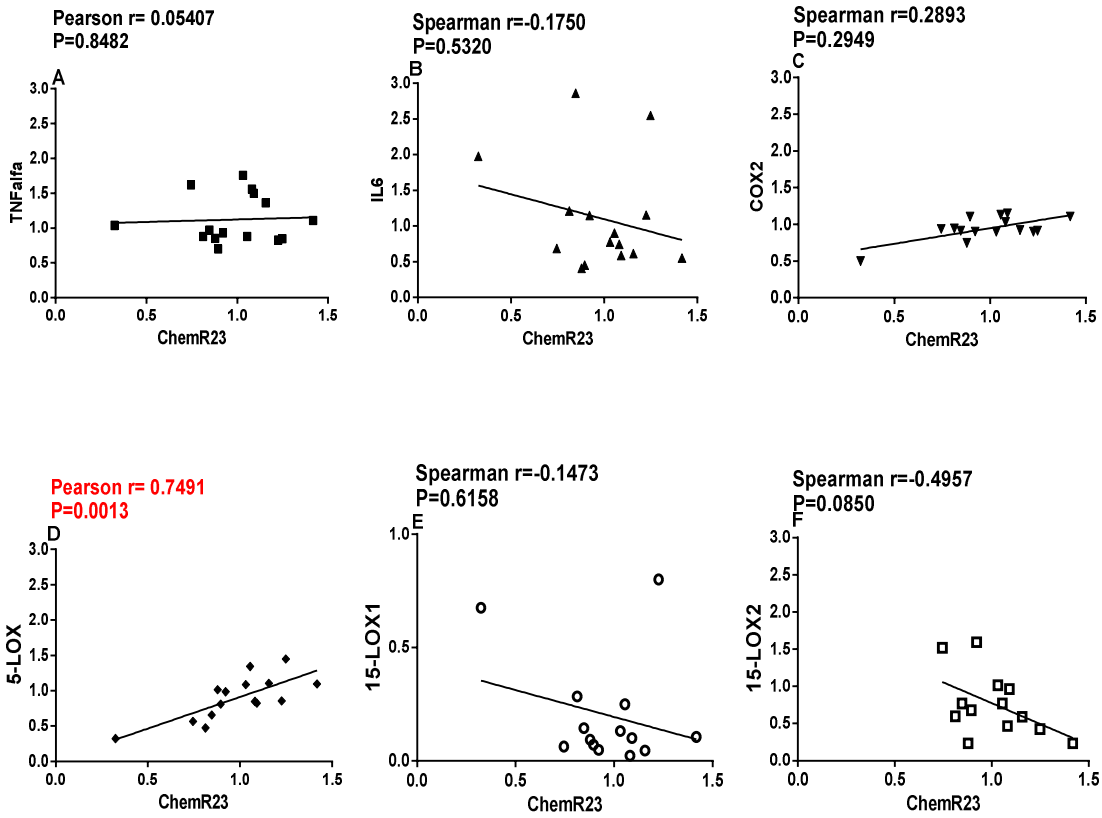


Fig 5.2 Correlation of expression of ChemR23 with inflammatory cytokines (TNFα and IL6) and associated enzymes (COX2, 5-LOX, 15-LOX1 and 15-LOX2) in the synovia from OA patients (n=14-15).

Expression of ALX was positively correlated with IL6 (**Fig 5.3B**) and 15-LOX1 (**Fig 5.3E**).

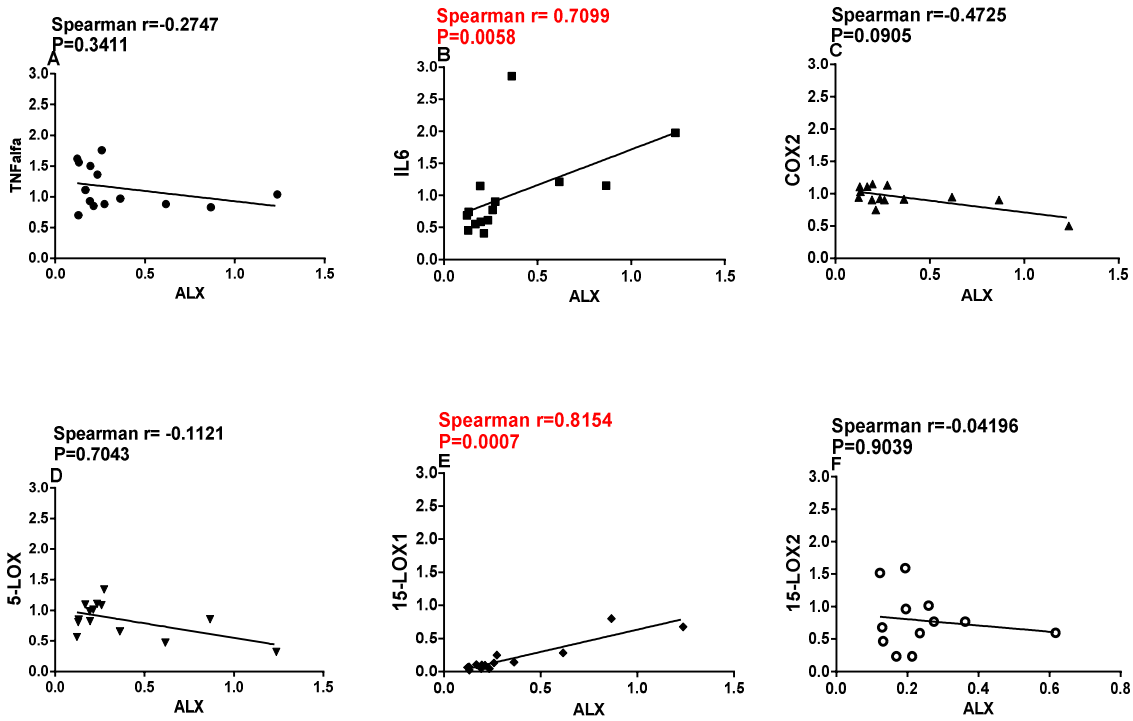


Fig 5.3 Correlation of ALX expression with inflammatory cytokines and enzymes in the synovia of OA patients (n=14-15).

5.4.2.3 Correlation of BMI and age with genes of interest in the synovia

Correlation analysis of BMI and age with selected target genes is shown in **Table 5.4**.

There was no correlation of BMI or age with resolvin receptors and any other genes in the synovia in OA patients

Factors	ChemR23	ALX	TNFα	IL6	COX2	5-LOX	15-LOX1	15-LOX2
BMI	r=-0.45 p=0.11	r=0.06 p=0.85	r=-0.60x10^{-2} p=0.98	r=-0.12 p=0.68	r=0.01 p=0.98	r=-0.35 p=0.22	r=-0.11 p=0.72	r=0.05 p=0.89
Age	r=0.06 p=0.82	r=-0.20 p=0.49	r=-0.16 p=0.56	r=0.17 p=0.55	r=0.22 p=0.42	r=0.10 p=0.74	r=-0.04 p=0.89	r=0.47 p=0.10

Table 5.4: Correlation of BMI/age with expression of resolvin receptors (ChemR23 and ALX), inflammatory cytokines (TNFα and IL6) and metabolic enzymes (COX2, 5-LOX, 15-LOX1 and 15-LOX2) in the synovia from human OA.

5.4.3 Correlation of resolvin receptors with genes of interest in the medial tibial plateau of OA patients

5.4.3.1 mRNA levels of genes of interest in the medial tibial plateau

Gene expression of resolvin receptors, inflammatory cytokines and associated metabolic enzymes in the medial tibial plateau from OA patients was quantified in **Table 5.5**.

Genes/sample	1	2	3	4	5	6	7	8	9	10	11	12	13	14
ChemR23	2.73	2.37	1.15	1.86	1.35	0.81	1.81	4.42	0.82	1.98	1.22	1.29	1.41	1.00
ALX	2.61	2.20	0.25	0.54	0.79	0.18	1.16	2.90	0.22	2.97	0.33	0.34	0.64	0.20
TNFα	0.46	1.60	1.16	1.13	0.65	0.57	1.28	0.70	0.81	0.44	0.78	1.24	1.02	0.45
IL6	$0.08x\ 10^{-1}$	$0.09x\ 10^{-1}$	$0.09x\ 10^{-1}$	$0.08x\ 10^{-1}$	$0.06x\ 10^{-1}$	$0.07x\ 10^{-1}$	$0.13x\ 10^{-1}$	$0.27x\ 10^{-1}$	$0.03x\ 10^{-1}$	$0.21x\ 10^{-1}$	$0.07x\ 10^{-1}$	$0.24x\ 10^{-1}$	$0.05x\ 10^{-1}$	$0.05x\ 10^{-1}$
5-LOX	0.40	1.74	0.75	0.86	0.29	0.38	1.14	3.20	0.43	0.46	0.56	1.21	0.90	0.43
15-LOX1	2.26	2.34	0.41	0.50	1.57	0.79	0.95	2.69	0.25	1.20	0.73	0.19	0.46	0.91
15-LOX2	0.18	0.10	0.92	0.40	0.49	0.66	0.69	0.36	2.60	2.31	0.23	0.55	0.94	2.68
COX2	0.11	0.01	0.06	0.04	0.10	0.12	0.02	0.05	0.02	0.03	0.02	0.08	0.02	0.04

Table 5.5: mRNA levels of target genes (normalized to β-actin) in the medial tibial plateau of OA patients. Medial tibial plateau from 14 OA patients with TKR were used to quantify expression levels of target genes.

5.4.3.2 Correlation of resolvin receptors with genes of interest in medial tibial plateau

Expression of ChemR23 was positively correlated with IL6 (**Fig 5.4B**), 5-LOX (**Fig 5.4D**) and 15-LOX1 (**Fig 5.4E**) and negatively correlated with 15-LOX2 (**Fig 5.3C**)

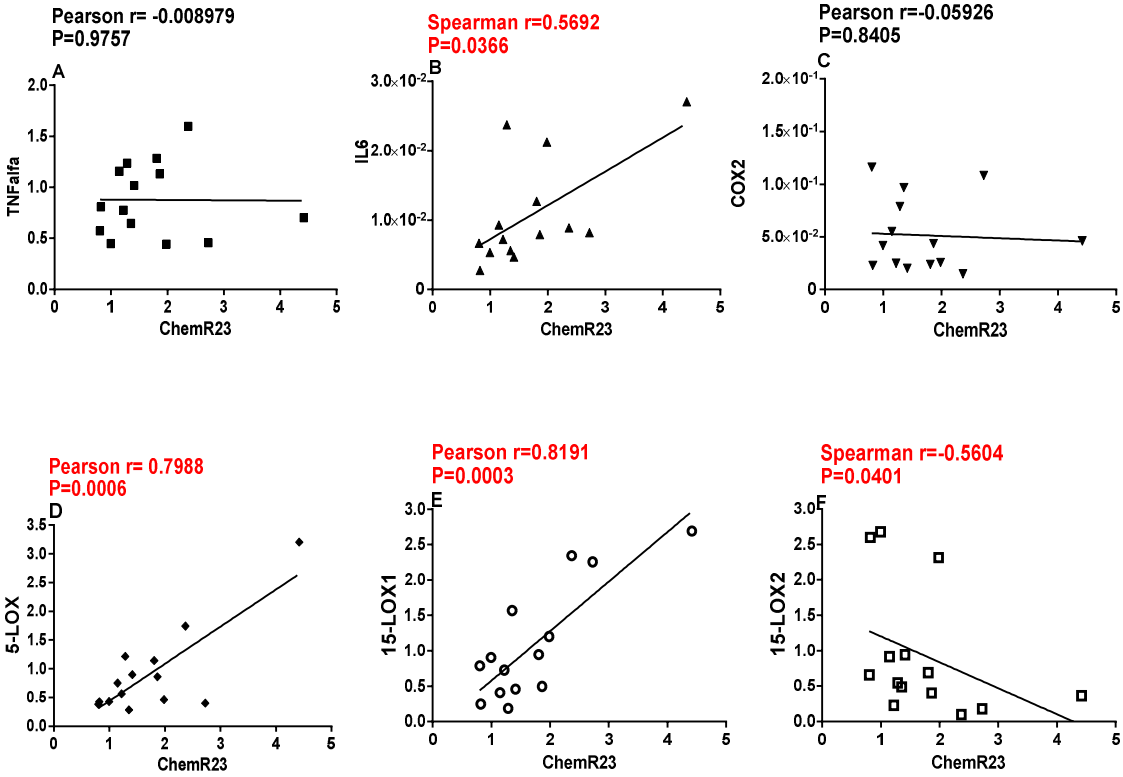


Fig 5.4 Correlation analysis of ChemR23 expression with inflammatory cytokines and enzymes in the medial tibial plateau of OA patients (n=14).

Expression of ALX was positively correlated with IL6 (**Fig 5.5B**) and 15-LOX1 (**Fig 5.5E**).

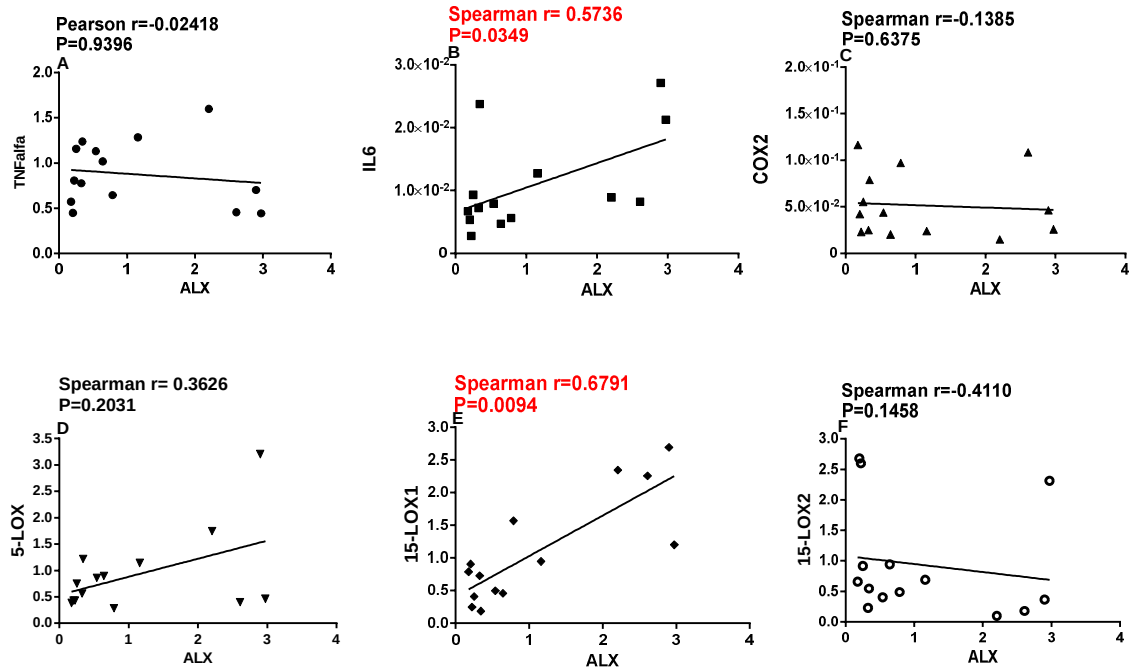


Fig 5.5 Correlation analysis of ALX expression with inflammatory cytokines and enzymes in medial tibial plateau of OA patients (n=14).

5.4.3.3 Correlation of BMI and age with genes of interest in the medial tibial plateau

Correlation analysis of BMI and age with expression of selected target genes in the medial tibial plateau from OA patient is shown in **Table 5.6**. BMI was negatively correlated with expression of TNFα, 5-LOX and 15-LOX1 but not correlated with resolvin receptors and any other genes. Age showed a negative correlation with the expression of ChemR23 and 15-LOX1 but not with ALX and any other genes.

Factors	ChemR23	ALX	TNFα	IL6	COX2	5-LOX	15-LOX1	15-LOX2
BMI	r=-0.41 p=0.17	r=0.44 p=0.14	r=-0.76 p=**0.34x 10^{-2}***	r=-0.24 p=0.43	r=0.31 p=0.31	r=-0.70 p=**0.01***	r=-0.75 p=0.42 **x10-2***	r=0.22 p=0.47
Age	r=-0.53 p=0.052*	r=-0.43 p=0.12	r=-0.08 p=0.78	r=-0.17 p=0.54	r=0.15 p=0.61	r=-0.43 p=0.12	r=-0.64 p=**0.01***	r=0.14 p=0.63

Table 5.6 Correlation of BMI/age with expression of resolvin receptors (ChemR23 and ALX), inflammatory cytokines (TNFα and IL6) and metabolic enzymes (COX2, 5-LOX, 15-LOX1 and 15-LOX2) in the medial tibial plateau from human OA.

5.4.3.4 Correlation of the expression of target genes between synovia and medial tibial plateau in human OA

There was no significant correlation of expression of the target genes between the two tissues with a weak trend of positive correlation for 15-LOX2 (**Fig 5.6H**).

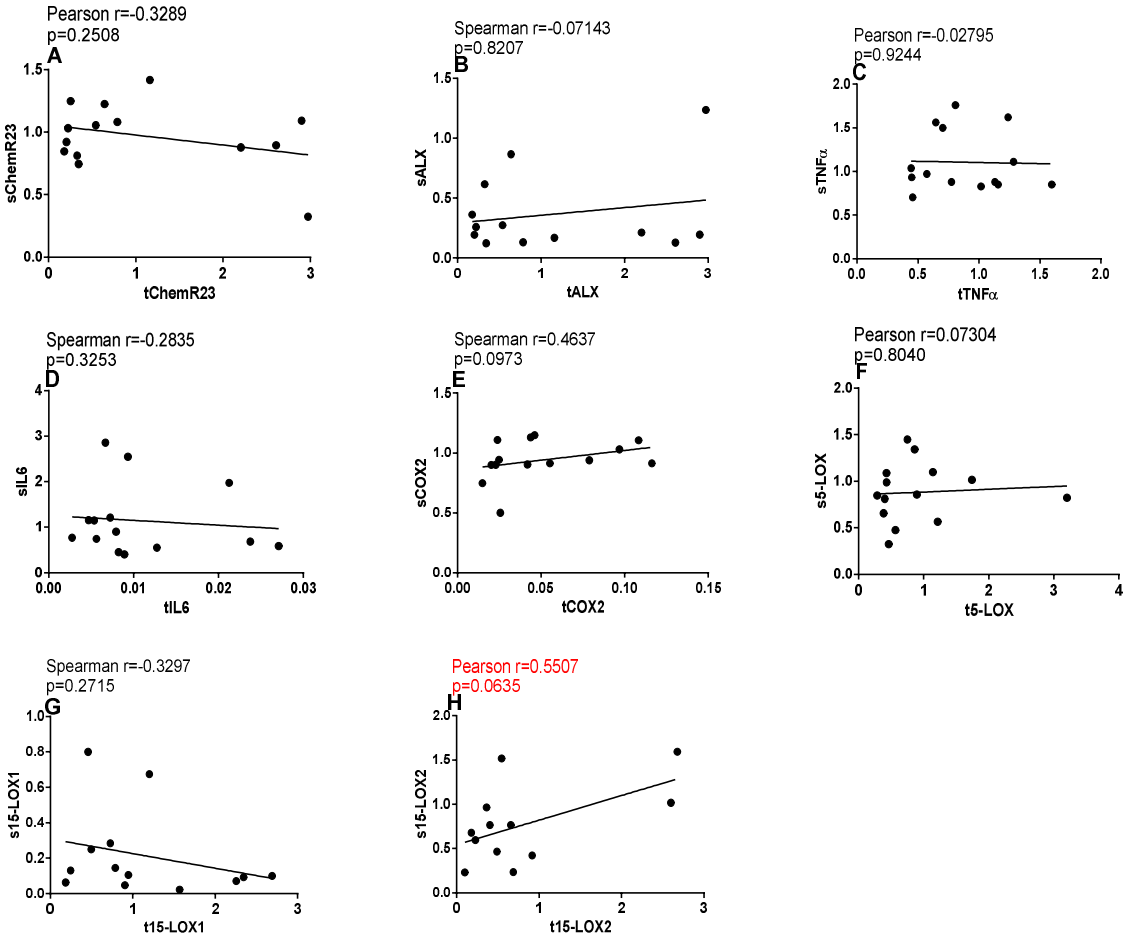


Fig 5.6 Correlation analysis of gene expression between synovia and tibial plateau in human OA (n=13-14).

5.4.4 Comparison of gene expression of resolvin receptors and other genes in OA patients and trauma patients

Expression of ALX (**Fig 5.7B**), TNFα (**Fig 5.7C**), IL6 (**Fig 5.7D**), 5-LOX **(Fig 5.7E)** and COX2 (**Fig 5.7H**) in medial tibial plateau were significantly lower from OA patients, compared to trauma patients which may indicate more inflammation in trauma patients.

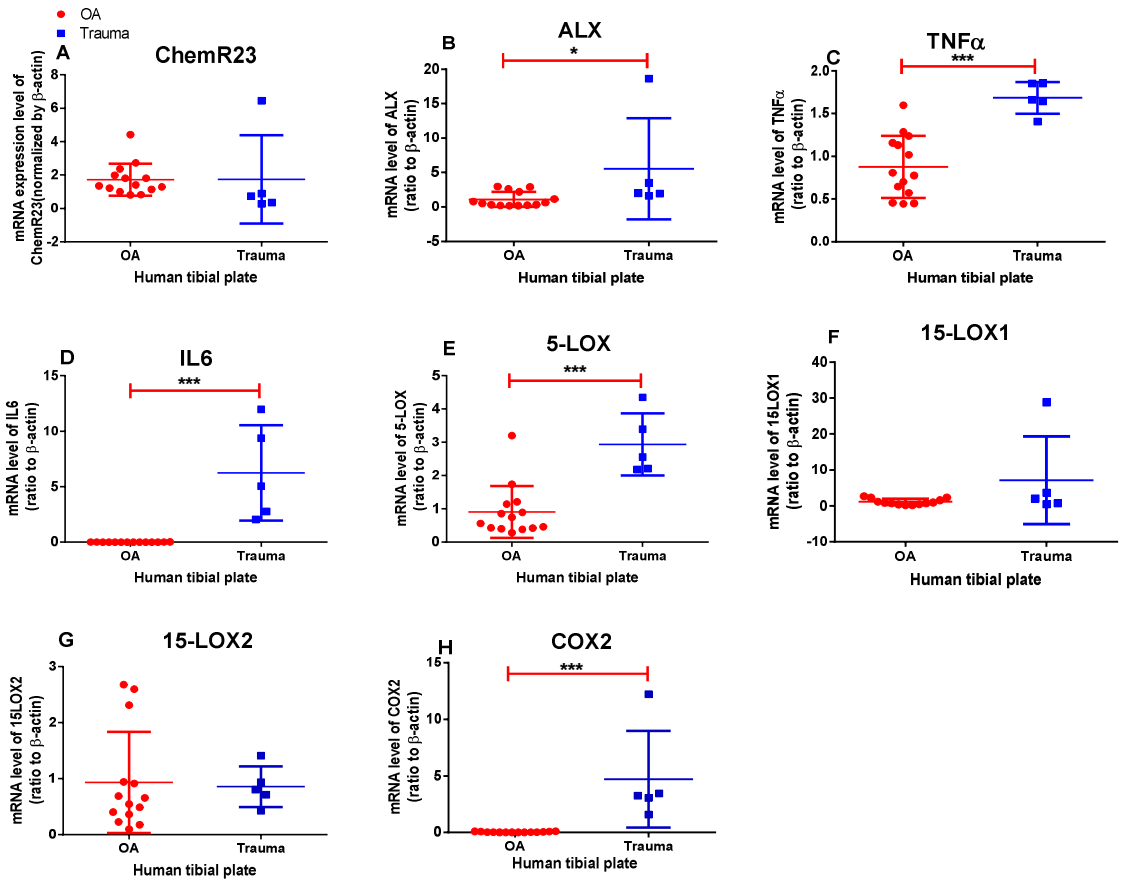


Fig 5.7 Expression levels of genes of interest in the medial tibial plateau from OA and femoral heads from non-OA patients with trauma. Data are presented as mean±S.E.M and analyzed with Mann-Whitney t-test（*p<0.05, ***p<0.001).

To sum up, correlation of expression of resolvin receptors with associated metabolic enzymes (5-LOX, 15-LOX and COX2) and inflammatory cytokine (IL6) has been demonstrated in the synovia and tibial plateau in OA patients. These findings indicate potential protective roles of resolvins/resolvin receptors and their metabolic enzymes in OA, however, specific mechanisms on this need investigation in depth.

5.4.5 Summary of results

- Resolvin receptors, inflammatory cytokines and metabolic enzymes were quantified in both synovia and medial tibial plateau from OA patients.
- Expression of ChemR23 was significantly higher than ALX in synovia of OA patients.
- Expression of ChemR23 was positively correlated with 5-LOX in both synovia and medial tibial plateau from OA patients. Expression of ChemR23 showed a positive correlation with IL6 and 15-LOX1 and a negative correlation with 15-LOX2 in the medial tibial plateau but not in the synovia.
- Expression of ALX was positively correlated with IL6 and 15-LOX1 in both synovia and medial tibial plateau in OA patients.
- There was no correlation of BMI and age with genes of interest in the synovia. BMI showed a negative correlation with the expression of TNFα, 5-LOX and 15-LOX1 and age was negatively correlated with the expression of 15-LOX1 in the medial tibial plateau in OA patients.
- There was no significant correlation of gene expression between synovia and medial tibial plateau in OA patients.
- Expression of ALX, TNFα, IL6, 5-LOX and COX2 were significantly lower in the medial tibial plateau of OA patients, compared to femoral heads from trauma patients.

5.5 Discussion and conclusion

The correlation between resolvin receptors and inflammatory cytokines and metabolic enzymes are summarized in **Fig 5.8**.

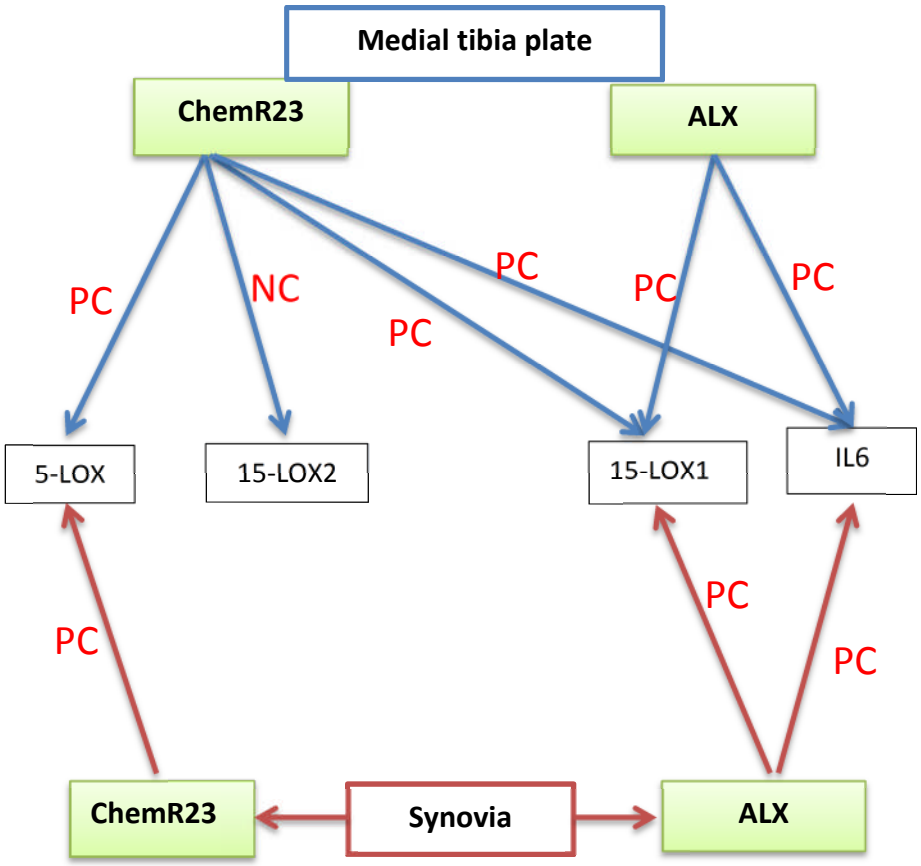


Fig 5.8 Correlation between expression of resolvin receptors and inflammatory cytokines and metabolic enzymes are shown in synovia (red) and medial tibial plateau (blue) from OA patients. positive correlation/PC, negative correlation/NC.

The expression of ChemR23 shown in the current study is consistent with the expression in articular chondrocytes from OA patients and synoviocytes in the synovia from RA and OA patients demonstrated in previous studies (Berg et al. 2010; Kaneko et al. 2011). Expression of ALX has been shown in synovial fibroblasts from healthy individuals (Sodin-Semrl et al. 2000) and may modulate joint inflammation (Fiore et al. 2005). Herein, I reported for the first time the expression of ALX in both synovia and tibial plateau from OA patients. Expression of ChemR23 was significant-

ly higher than ALX in both synovia and medial tibial plateau in OA patients, and this was not evident in synovia from the OA models.

Higher expression of ChemR23 was associated with greater expression of 5-LOX mRNA in both synovia and medial tibial plateau, but lower expression of 15-LOX2 mRNA in the medial tibial plateau of OA patients. There was a similar trend for ChemR23, 15-LOX1 and IL6 expression in the tibial plateau. This pattern was true for ALX as well in both synovia and tibial plateau from OA patients. These findings suggest an association between bioactive resolvins and these enzymes in OA patients which is consistent with the evidence that 15-LOX1 and 5-LOX are key metabolic enzymes involved in the generation of resolvins from up-stream precursors (Recchiuti and Serhan 2012; Spite et al. 2014). In addition, the positive correlation of ChemR23 and ALX with 15-LOX1 may suggest a protective role of resolvins in human OA as 15-LOX has chondroprotective effects in cultured chondrocytes from OA patients (Chabane et al. 2009).

Previous studies have reported higher expression of COX2 in cultured synovia, meniscus and osteophytes from OA patients (Hardy et al. 2002) and the COX2 is up-regulated by TNFα or IL1β in chondrocytes from OA patients (Kojima et al. 2004). My study demonstrated that higher expression of ALX was associated with higher expression of 15-LOX1 and had a trend towards lower expression of COX2 in the synovia from OA patients. These findings are consistent with ALX and its corresponding ligands (Resolvin D) having anti-inflammatory properties, which have been demonstrated in previous studies of the effects of resolvins in inflammatory models.

Positive correlation of ChemR23 expression with 5-LOX and this change was associated with variable expression of FLAP (activator of 5-LOX) (Maxis et al. 2006). In addition, the positive correlation of ChemR23 and ALX with IL6 in the tibial plateau supports the view that IL6 has anti-inflammatory roles in human OA (Van De Loo et al. 1997; Xing et al. 1998).

There was no correlation between BMI / age and the target genes in the synovia.

BMI was negatively correlated with TNFα, 5-LOX and 15-LOX1 expression and age was negatively correlated with 15-LOX1 in the medial tibial plateau expression from OA patients. Although obesity is associated with pathogenesis of OA (Grotle et al. 2008), BMI alone does not indicate the relevance of obesity to OA or the levels of adipocytokines that may contribute to OA (Sowers and Karvonen-Gutierrez 2010).

As no correlation between the expression of resolvin receptors and BMI or age has been found and resolvin receptors were positively correlated with 5-LOX and 15-LOX1in expression levels, this may suggest that the correlation between resolvin receptors and the metabolic enzymes is not affected by BMI or age in human OA (Blagojevic et al. 2010).

As for previous clinical studies, it was difficult to get control synovial samples. I determined whether the resolvin receptors, inflammatory cytokines and metabolic enzymes were altered in medial tibial plateau from human OA and bone fractions from femoral heads in trauma patients used as controls. There was lower expression of ALX, TNFα, IL6, 5-LOX and COX2 in the medial tibial plateau from OA patients, compared to femoral heads from trauma patients. This finding may suggest elevated levels of inflammation following bone fracture and suggests that this is not a good control for these studies of OA tissue as the systemic inflammation and large amount of immune cells flushing through injured joints in trauma patients (Giannoudis 2003; Lenz et al. 2007). It may also suggest low inflammation in the medial tibial plateau (cartilage and subchondral bone) at the late stage of OA patients (Benito et al. 2005).

To sum up, the findings demonstrated in the clinical samples from OA patients support the further investigation of the anti-inflammatory and protective roles of resolvin receptors/ligands in human OA.

6 Discussion, limitations and future directions

6.1 General discussion

This thesis reported evidence of alterations in the resolvin receptor system (ChemR23 in the MIA model and ALX in synovia of the MNX model), correlation of the receptors with pain behaviour in the MIA model and correlation of the ALX with inflammatory cytokines and metabolic enzymes in the two preclinical models of OA. Translational studies also provided evidence that the resolvin receptors were associated with inflammatory cytokines and metabolic enzymes in clinical samples from OA patients. A series of drug intervention studies demonstrated therapeutic effects of 17(R)HDoHE on OA pain in preclinical models and the underlying mechanisms of the actions have been studied.

Administration of single dose of 17(R)HDoHE attenuated established pain behaviour in the MIA model while repeated administration of 17(R)HDoHE reversed pain behaviour in both the MIA and MNX models. The analgesic effects of 17(R)HDoHE were maintained for a week after cessation in the MIA model. These effects of 17(R)HDoHE on pain behaviour may be mediated by actions of the resolvin receptors on inflammatory cytokines or metabolic enzymes. Significant associations between the expression of the resolvin receptors and expression of inflammatory cytokines and metabolic enzymes were demonstrated in the human OA samples.

Pain is a cardinal symptom in OA that limits the motion of joints and the main reason for patients to seek treatment (Hunter et al. 2013). Pain behaviour and joint histopathology have been revealed in the MIA (Guingamp et al. 1997), MNX (Bove et al. 2006) and DMM (Malfait et al. 2010) models of OA.

This thesis has demonstrated that pain behaviour and joint histopathology developed in both MIA and MNX-induced models. In the MIA model, pain behaviour was initiated at day 3 and maintained until day 28 and day 35 in two separate studies post model induction, which supports a rapid development pain in this model of OA (Beyreuther et al. 2007). Histological assessment in this study confirmed joint damage including chondropathy, synovial inflammation, chondrocyte appearance and osteochondral junction integrity in the model, which is consistent with previous reports (Bove et al. 2003; Sagar et al. 2010; Stevenson et al. 2011). In the MNX model,

pain behaviour developed at a late time point and joint histology also revealed damage, which has been shown in previous studies (Mapp et al. 2010; Mapp et al. 2013).

Inflammation is an essential component leading to pathogenesis of OA (Scanzello and Goldring 2012; Sokolove and Lepus 2013) and resolvins shows tremendous actions on resolving inflammation (Serhan et al. 2008; Serhan and Petasis 2011). Therefore, determining the potential alteration of resolvin receptors, inflammatory cytokines and the key metabolic enzymes involved in OA is of great interest to elucidate inflammatory mechanisms underlying OA and find new drugs to treat OA pain.

My data revealed that expression of ChemR23 and 15-LOX was lower in the synovia from the MIA model. In addition, synovial expression of ChemR23 was negatively correlated with pain behaviour at both time points (day 14 and day 35), supporting a role of ChemR23/15-LOX in the modulation of OA pain behaviour via a peripheral site of action (Xu et al. 2010). In the spinal cord, ChemR23 expression was higher in the ipsilateral quadrant of the dorsal horn of the spinal cord in the MIA model. In addtion, this increase in expression was positively correlated with pain behaviour, suggesting that increased numbers of activated neurons or glial cells express this receptor in the model of OA pain (Xu et al. 2012). Spinal ALX was positively correlated with pain behaviour at early time point (day 14) but negatively at later time point (day 35), indicating the potential for a phenotype shift of glial cells, expressing this receptor at different stages of the model (Svensson et al. 2007; Sagar et al. 2011; Abdelmoaty et al. 2013). Expression of inflammatory cytokines (TNFα, IL1β and IL6) was not significantly altered in either the synovia or spinal cord in the MIA model. However, expression of 15-LOX in the dorsal horn of the spinal cord was lower in the MIA model, compared to control rats. Moreover, spinal ALX expression was negatively correlated with IL6 in the spinal cord. These data suggest a contribution of ALX/15-LOX mediated anti-inflammatory effects on pain modulation in the spinal cord in this model (Svensson et al. 2007).

In the MNX model, ALX, TNFα, IL1β, COX2 and mPGES1 were expressed at a lower level in the synovia and FLAP expressed at a higher level in the spinal cord, compared to the sham control group. There was a positive correlation between ALX expression and these genes in the synovia. Expression of ChemR23 was not altered in this model. These data suggest that despite the resolving intervention attenuating pain behaviour in the MNX model, there is limited evidence for associations with the

pro-inflammatory pathways in this model. Resolvins have been reported to show anti-nociceptive and anti-hyperalgesic actions on pain modulation (Ji et al. 2011; Sungjae Yoo et al. 2013). I found the robust analgesic effects of 17(R) HDoHE on pain behaviour by systemic administration in a series of studies in two models of OA pain.

My data are consistent with a previous study using an inflammatory arthritis model (Lima-Garcia et al. 2011). In the acute treatment study, 17(R)HDoHE decreased expression of 5-LOX and up-regulated IL6 expression. There was also a trend towards down-regulation of pro-inflammatory cytokines and metabolic enzymes. These findings may indicate anti-inflammatory role (s) of 17(R)HDoHE on pain modulation. The analgesic effects of 17(R)HDoHE were maintained by repeated administration for two weeks, indicating no obvious tolerance to the effects of the compound, which is a huge advantage compared to opioids (Dumas and Pollack 2008). As discussed in 4.4.2, 17(R)HDoHE didn't attenuate mechanical allodynia in the MNX model. However, the sham control may mask the therapeutic effects of the drug as this type of pain behaviour was exhibited by the sham rats. Repeated administration of 17(R)HDoHE did not alter joint histopathology in either the MIA or the MNX model, may be indicating more central components governing the chronic effects of the compound on OA pain.

I demonstrated the prescence of mRNA for ChemR23 and ALX in both synovia and medial tibial plateau in OA patients. Expression of ChemR23 was higher than ALX. Resolvin receptors are expressed by synovial fibroblasts (Kaneko et al. 2011; Eisinger et al. 2012), chondrocytes (Berg et al. 2010), osteoclasts (Herrera et al. 2008) and osteoblasts (Gao et al. 2013). Key inflammatory enzymes such as COX2, 15-LOX and 5-LOX are also expressed in synovial fibroblasts and chondrocytes in OA or RA patients (Chabane et al. 2009; Gheorghe et al. 2009; Chen et al. 2010).

Expression of ChemR23 was positively correlated with 5-LOX in both synovia and tibial plateau, positively correlated with 15-LOX1 and IL6 and negatively correlated with 15-LOX2 in tibial plateau. These data support that E series resolvins are produced via the 15LOX1/5-LOX pathway (Isobe et al. 2012; Lee and Surh 2012).

Expression of ALX was positively correlated with 15-LOX1 and IL6 in both synovia and medial tibial plateau, indicating production of D-series resolvins via 15-LOX/5-LOX pathway (Kohli and Levy 2009; Spite et al. 2014). mRNA levels for ALX, TNFα,

IL6, 5-LOX and COX2 were significantly lower in medial tibial plateau from OA patients compared to neck of femoral heads from trauma patients. These data suggest more inflammatory cells infiltrated to injury sites in trauma patients (Giannoudis 2003; Lenz et al. 2007) and inflammation is lower in the tibial plateau from OA patients. Samples from trauma patients may not be good controls for OA patients.

6.2 Limitations and future directions

Limitations for the studies in the thesis have been discussed here based on the data analysis and discussion described in the previous chapters.

Comparison of the two OA models indicated that pain behaviour was more variable in the MNX model than the MIA model. This may be due to the pain behaviour exhibited by the sham rats as a result of the removal of the anterior lateral ligaments in the joints. This procedure on rats may mask the effects of MNX surgery on pain behaviour. Nevertheless, it is important that the control rats experience the same surgery procedure as MNX surgery except meniscus transection. In addition, the environment for behavioural testing, individual tolerance of rats to the surgery and pain behaviour itself may also contribute to the variability in this model, which is inevitable. A way to address this issue is that a naive control group is used to see how pain develops in sham surgery rats. In gene expression studies using the sham and MNX rats, synovial expression of inflammatory cytokines (TNFα and IL1β) and metabolic enzymes (COX2 and mPGES1) was higher in sham rats compared to MNX rats. These differences may be due to different expression levels of reference genes (β-actin and GAPDH), which appeared about two fold higher in sham rats compared to MNX rats although significance was not reached. Even with the geometric mean of both genes used as reference, it seems that expression of these two genes was not stable in this surgical model. Other reference genes such as 18S and 28S rRNA (Vandesompele et al. 2002) may be used or inflammatory markers should be used as reference gene for inflammatory conditions (Qureshi et al. 2012) in future.

Expression of some of the target genes, such as ALX in the spinal cord of the MIA and MNX model and COX2 in the spinal cord of the MIA model was close to significant differences compared to controls. This suggests that the number of rats per group was not higher enough for statistical power and needs to be increased in future studies.

For the chronic studies of the effects of 17(R)HDoHE in both the MIA and MNX model, joint histopathology was not improved. However, the activity of osteoclasts or osteoblasts in knee joints was not assessed in these two studies. Future studies should investigate potential effects of the resolvin percursor on activity of osteoclasts or osteoblasts as resolvins are known to inhibit bone resorption (Herrera et al. 2008; Zhu et al. 2013) and promote bone formation (Gao et al. 2013). In addition, the spinal mechanisms of the therapeutic effects of resolvin precursor are not established as well. Therefore, activation of glial cells and neuronal activity could be investigated in OA pain in future studies.

The anti-nociceptive and anti-hyperalgesic roles of the bio-active resolvins (RvD1, RvD2, RvE1 and AT-RvD1) have been reported in recent years as described in previous chapters. However, there is no report on resolvins and other pro-resolving lipid mediators such as neuropeptide D and maresin to treat OA pain. There is a huge gap to fill to understand therapeutic effects of these pro-resolving lipid mediators to treat OA pain and the underlying mechanisms.

From a pharmacodynamic aspect, a series of drug intervention studies could be conducted to investigate the therapeutic effects of down-stream bioactive mediators or their analogues on pain behaviour or the effects of compounds on responses of sensory neurons in the central nerve system in the models of OA pain by peripheral and spinal administration. At the cellular level, the activities of these ligands and their cognate receptors (many of them un-identified), the structure of receptors and binding sites in OA system are unknown. Future studies on these aspects could be conducted with multi-disciplinary cooperation to investigate enzyme-activity, identification of cognate receptors and analyze the crystal structure of the receptors with multiple laboratory techniques. The antagonists for these receptors are few and may also be designed to investigate the supressing actions in OA and OA pain. In addition, interaction between the pro-resolving lipid mediators and some other receptors or ion channels, which may contribute to therapeutic effects of the compounds in OA pain, is also expected to investigate for future studies.

From a pharmacokinetic aspect, how the resolvins and other pro-resolving lipid mediators are distributed and metabolized in organs and the specific tissues and the anti-nociceptive and anti-hyperalgesic roles of these mediators in various pain models remain unclear. The levels of resolvin precursors, the bio-active resolvins and

other pro-resolving lipid mediators in serum, synovial fluids, spinal cords or DRGs from naïve rodents or model-induced rodents could be measured with liquid chromatgraphy- mass spectrometry (LC-MS) eventhough the half-lives of these mediators are relatively short (Shimizu 2009). The measurement of levels of these mediators in naïve and model-induced rodents may suggest tissue-specific biomarkers for OA, which helps to find novel therapeutic targets for OA treatment. The metabolic pathways for these mediators have previously been discussed in inflammatory status (Serhan et al. 2008; Serhan and Petasis 2011), however, the activity and modulation of the metabolic enzymes such as COX2, 5-LOX and 15-LOX are not well established in peripheral tissues and spinal cord. Future studies on the metabolic pathways for fatty acids, resolvins and other lipid mediators in OA pain and OA system are of great interest in future studies.

In a broad view, the specific mechanisms on anti-nociceptive and anti-hyperalgesic roles of the pro-resolving lipid mediators are yet to be elucidated. At a molecular and genetic level, genetic tools and bioinformatic methods are novel emerging techiques and hot-spots applied in the biomedical researches including pain and osteoarthritis. Transgenic mouse models are widely used to learn the function of specific genes in dieseases. It is of great interest to manipulate genes encoding the receptors for resolvins and other lipid mediators to observe whether the analgesic effects are affected. Recently, the genome-wide screening for heat nociception in drosophila identified α2δ3 as a specific gene for transmission of thermal nociceptive signals from thalamus to the sensory cortex (Neely et al. 2010) , three loci such as rs2651899, rs10166942(TRPM8) and rs11172113 (LRP1) for migraine (Chasman et al. 2011) and rs6976 within the 3'UTR of GLT8D1 on chromosome 3 and rs11177 in the third exon of GNL3 for osteoarthritis (Consortium and Collaborators 2012). The large-scale single-cell RNA sequencing of DRG cells has identified different types of sensory neurons such as low-threshold mechnoreceptive, thermosensitive, itch sensitive, mechnosensitive and nociceptive neurons expressing the specific markers and genes (Usoskin et al. 2015). Future studies focusing on identification and manipulation of specific genes or markers (receptors, ion channels) that are responsible for transduction, transmission and modulation of OA pain are fascinating directions to explore. Optogenetics is currently a novel technique for activating signalling molecules such as GPCRs and receptor tyrosine kinases (Karunarathne et al. 2015) or

manipulation of neuronal activity that may modulate animal behaviour (Miesenbock 2011). Optogenetic stimulation has been shown to decrease the activity of inhibitory neurons in anterior cingulate cortex and attenuate formalin induced pain behaviour in Thy1-ChR2-YFP transgenic mouse model (Gu et al. 2015). It also attenuated mechanical and thermal hyperalgesia via control of ChR2-eYFP transfected neurons pre-frontal cortex in neuropathic pain (Lee et al. 2015). Siuda et al developed a Gi-coupled, optically sensitive, muopioid-like receptor which mimics mu-opioid signaling dynamics including t he canonical intracellular G protein signaling mechanisms and controls opioid signalling and behaviour by photostimulation (Siuda et al. 2015). Optogenetic tools also have been shown to inhibit the activity of subunits in GPCRs, thus inhibit G protein signalling and control down–stream cell signalling (O'neill and Gautam 2014). The optogenetic tools could be applied to manipulate resolvin receptors, other GPCRs for neurotransmitters and lipid mediators and sensory neuronal activity in OA pain, which is well deserved and highly expected with great advantage for future studies.

The neuro-immune interaction is another attractive direction for pain study. The immune system play active role in pain processing and modulation (Ren and Dubner 2010) as well as OA (Haseeb and Haqqi 2013), therefore, should be a good direction to study functions of the system in OA pain. The signalling pathways, functions and molecular mechanisms for specific neurotransmitters, channels and receptors in various immune cells, glial cells and neurons are still not well understood. Signalling pathways for pain transduction, transmission and modulation and the interaction between immune cells, glial cells and neurons are interesting for future studies. Furthurmore, neural circuits for pain modulation are much less known as well and are a big gap to fill in future. In the clinical study, we revealed a negative correlation for BMI with the expression of TNFα, 5-LOX and 15-LOX1 and age with 15-LOX1 however, these correlations do not affect correlations between resolvin receptors with target genes. However, this couldn’t preclude that the correlation between resolvin receptors and inflammatory cytokines and metabolic enzymes may be affected by some other factors such as drug use or case history for individual OA patient. In fact, 3 OA patients used aspirin before the surgery. A major limitation for this clinical study was the lack of control synovia from healthy volunteers or patients without OA. With these controls in future studies, we can determine how the resolvin receptor

system is altered in human OA. Another limitation for this study is the bone fractions from femoral heads of trauma patients may not be a good control for OA patients when studying inflammatory states as trauma may cause great primary and systemic inflammation as discussed before (see 5.5). Therefore, selecting a better control for samples from OA patients may be better considered in future studies. In molecular biology, the limitation of the thesis is that I didn't measure the alteraltion of protein levels of resolvin receptors and other target genes. I have tried to confirm the expression of resolvin receptors in spinal cord with immunofluorescence and I indeed observed receptor-positive cells in the spinal cord, however, the antibodies for the receptors didn't work very well. This work continues to be conducted by our colleague in the lab.

To sum up, although limitations are exhibited in these studies, this thesis demonstrated robust analgesic effects of 17(R)HDoHE on treatment of OA pain in a series of preclinical models and determined the alteration of ChemR23 and correlation of ALX with pain behaviour in the MIA model and alteration of ALX in the MNX model. Possible mechanisms of pain modulation by the resolvin D precursor have been studied and correlation of the resolvin receptors with inflammatory cytokines and metabolic enzymes were shown in human OA. The findings in this thesis provide evidence to explore resolvins as novel potential therapeutics to treat OA pain.

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8 APPENDICES

Reagents:
10% neutral buffered formalin:

100ml methanal

900ml double distilled water

4g NaH_2PO_4

6.5g Na_2HPO_4

Neutral buffered formalin was made by mixing 100ml methanal (formaldehyde solution, Sigma, UK) with 900ml double distilled water containing 4g NaH_2PO_4 (Fisher Scientific, UK) and 6.5g Na_2HPO_4 (Fisher Scientific, UK).

EDTA solution (10% w/v):

Distilled water (2.5L),

Trizma®Base (3.35g, Sigma life science, UK),

10% w/v EDTA (250g),

NaOH (pellets, 25g, Fisher scientific, UK)

7.5% w/v Poly (vinylpyrrolidinone) (PVP, 187.5g, Sigma, UK). EDTA solution (10% w/v) was made via the following method: pellets of sodium hydroxide were first added to distilled water and dissolved using a magnetic stirrer and then the rest of reagents were added until dissolved. The pH was adjusted to 7 and the solution stored at room temperature.

Letter of access for clinical samples and Research Passport

Nottingham University Hospitals NHS
NHS Trust

Research & Innovation
Nottingham Integrated Clinical Research Centre
C Floor, South Block
QMC Campus
Derby Road
Nottingham
NG7 2UH

Tel: 0115 970 9049

www.nuhrise.org

RSCH 448

24/10/2013

Mr Junting Huang
4 Kenilworth Road
Beeston
Nottingham
NG9 2HX

Dear Mr Huang

Re: Letter of Access for Research

Study Title: Investigating Receptors in Osteoarthiritis

Study utilizing Nottingham Health Science Biobank, no R&I Ref Required.

PLEASE NOTE If study opens to active patient recruitment, R&I must review the study and issue approval

This letter confirms your right of access to conduct research through **Nottingham University Hospitals** for the purpose and on the terms and conditions set out below. This right of access commences on **24/10/2013** until **30/09/2014** unless terminated earlier in accordance with the clauses below.

You have a right of access to conduct such research as confirmed in writing in the letter of permission for research from this NHS organisation. Please note that you cannot start the research until the Principal Investigator for the research project has received a letter from us giving permission to conduct the project.

The information supplied about your role in research at **Nottingham University Hospitals** has been reviewed and you do not require an honorary research contract with this NHS organisation. We are satisfied that such pre-engagement checks as we consider necessary have been carried out.

You are considered to be a legal visitor to **Nottingham University Hospitals** premises. You are not entitled to any form of payment or access to other benefits provided by this NHS organisation to employees and this letter does not give rise to any other relationship between you and this NHS organisation, in particular that of an employee.

While undertaking research through **Nottingham University Hospitals** you will remain accountable to your employer **The University of Nottingham** but you are required to follow the reasonable instructions of **Mr William Monoghan** in this NHS organisation or those given on her/his behalf in relation to the terms of this right of access.

Where any third party claim is made, whether or not legal proceedings are issued, arising out of or in connection with your right of access, you are required to co-operate fully with any investigation by this NHS organisation in connection with any such claim and to give all such assistance as may reasonably be required regarding the conduct of any legal proceedings.

You must act in accordance with **Nottingham University Hospitals** policies and procedures, which are available to you upon request, and the Research Governance Framework.

You are required to co-operate with **Nottingham University Hospitals** in discharging its duties under the Health and Safety at Work etc Act 1974 and other health and safety legislation and to take reasonable care for the health and safety of yourself and others while on **Nottingham University Hospitals** premises. You must observe the same standards of care and propriety in dealing with patients, staff, visitors, equipment and premises as is expected of any other contract holder and you must act appropriately, responsibly and professionally at all times.

If you have a physical or mental health condition or disability which may affect your research role and which might require special adjustments to your role, if you have not already done so, you must notify your employer and the Trust **R&I office** prior to commencing your research role at the Trust.

You are required to ensure that all information regarding patients or staff remains secure and *strictly confidential* at all times. You must ensure that you understand and comply with the requirements of the NHS Confidentiality Code of Practice (http://www.dh.gov.uk/assetRoot/04/06/92/54/04069254.pdf) and the Data Protection Act 1998. Furthermore you should be aware that under the Act, unauthorised disclosure of information is an offence and such disclosures may lead to prosecution.

You should ensure that, where you are issued with an identity or security card, a bleep number, email or library account, keys or protective clothing, these are returned upon termination of this arrangement. Please also ensure that while on the premises you wear your ID badge at all times, or are able to prove your identity if challenged. Please note that this NHS organisation accepts no responsibility for damage to or loss of personal property.

We may terminate your right to attend at any time either by giving seven days' written notice to you or immediately without any notice if you are in breach of any of the terms or conditions described in this letter or if you commit any act that we reasonably consider to amount to serious misconduct or to be disruptive and/or prejudicial to the interests and/or business of this NHS organisation or if you are convicted of any criminal offence. You must not undertake regulated activity if you are barred from such work. If you are barred from working with adults or children this letter of access is immediately terminated. Your employer will immediately withdraw you from undertaking this or any other regulated activity and you MUST stop undertaking any regulated activity immediately.

Your substantive employer is responsible for your conduct during this research project and may in the circumstances described above instigate disciplinary action against you.

Nottingham University Hospitals will not indemnify you against any liability incurred as a result of any breach of confidentiality or breach of the Data Protection Act 1998. Any breach of the Data Protection Act 1998 may result in legal action against you and/or your substantive employer.

If your current role or involvement in research changes, or any of the information provided in your Research Passport changes, you must inform your employer through their normal procedures. You must also inform your nominated manager in this NHS organisation.

Yours sincerely

Alison Steel
Senior Research Manager

cc: HR department of the substantive employer

RSCH 448

Research Passport Application Form – Version 2 04/03/10

Please refer to the guidance notes before completing the form.

Section 1 - Details of Researcher
To be completed by Researcher

1.	Surname: Huang	Prof☐ Dr☐ Mr☒ Mrs☐
	Forename(s): Junting	Miss ☐ Ms☐ Other☐
	Home Address: 4 Kenilworth Road, Beeston, Nottingham, NG9 2HX	
	Work Tel: 01158230154 Mobile: 07435504168 Email: mbxjh2@nottingham.ac.uk	
2.	Date of birth: 30/04/1983	Gender: Male ☒ Female ☐
	Ethnicity: Chinese	National Insurance number: n/a
3.	Professional registration details (if applicable): PhD student, registration to University of Nottingham N/A ☐	
4.	Employer: or place of study: School of Life Sciences, Queen's Medical Centre THE UNIVERSITY OF NOTTINGHAM	
	Work Address/Place of Study: School of Life Sciences, Queen's Medical Centre THE UNIVERSITY OF NOTTINGHAM.	
	Post or status held: PhD student	

Section 2 - Details of Research
To be completed by Researcher

5.	What type of Research Passport do you need? Project-specific ☒ Multi-project ☐			
	If you will be conducting one project only please complete the details below. If you anticipate that you will be undertaking more than one project at any one time, please give details in the Appendix.			
	Project Title: Investigation of inflammatory mechanisms in models of Osteoarthritic Pain			
	Project Start Date: 01/10/2011 End Date: 30/09/2014			
	Proposed start and end date of three-year Research Passport: Start Date: 09/09/13 End Date: 08/09/16			
	NHS organisation(s):	Dept(s):	Proposed research activities:	Manager in NHS organisation:
	NUH	Orthopaedics	Investigating receptors in osteoarthritis	Mr Will Monaghan

Section 3 – Declaration by Researcher
To be completed by Researcher

6.	Have you ever been refused an honorary research contract?	No
	Have you ever had an honorary research contract revoked?	No
	If yes to either question, please give details:	

I consent to the information provided as part of this Research Passport and attached documents being used, recorded and stored by authorised staff of the NHS organisations where I will be conducting research.
For researchers undertaking regulated activity as from July 2010, and mandatory as from November 2010: I understand that the information I have provided may be used by my employer and the NHS to access the ISA on-line service to receive updates on my ISA-registration status.

Signed: Junting Huang		Date: 16/08/2013

When Sections 1-3 have been completed, the researcher should forward the form to the appropriate person to complete Section 4.

Section 4 - Suitability of Researcher
To be completed by researcher's substantive employer, e.g. line manager, or academic supervisor

7.a	Will this person's research activity mean that they may be undertaking regulated activity (please use the Research Passport algorithm to make this judgement)	Yes No ☒
7. b	I am satisfied that the above named individual is suitably trained and experienced to undertake the duties associated with the research activities outlined in this Research Passport form.	
	Signed: Brigitte E. Scammell	Date: 02/09/13
	Name: Prof BE Scammell	Job Title: Orthopaedic Consultant
	Department and Organisation: Trauma and Orthopaedics	Managerial responsibility for the applicant: Professor V Chapman, PhD supervisor
	Address: Orthopaedics, Trauma and Sports Medicine, C Floor, West Block, Queen's Medical Centre, Nottingham, NG7 2UH	
	Tel No:01823 1115	
	Email: b.scammell@nottingham.ac.uk	

When Section 4 has been completed, the researcher should forward the form to the appropriate person to complete Section 5.

Section 5 - Pre-engagement checks
To be completed by the HR department of the researcher's substantive employer or registry at place of study

8.	Does this individual's research involve Regulated Activity:	☐ Yes x No
	For Regulated Activity: **To be completed for RP applications supported by enhanced CRB disclosures certificates issued between 12th October 2009 and 25th July 2010 only** If yes to the above, has the individual been checked against ISA barred lists for vulnerable adults and / or children, as appropriate and have you received confirmation via the CRB disclosure that the person is not barred from working with children or vulnerable adults? *(NB individuals who are barred from working with children or vulnerable adults must not undertake a regulated activity within the NHS, and you must not submit a Research Passport form in such cases)*	Checked against ISA Vulnerable Adults List? Yes ☐ No ☐ N/A ☒ Checked against ISA Children's List? Yes ☐ No ☐ N/A ☒
	For Regulated Activity: **To be completed for RP applications supported by enhanced CRB disclosures certificates issued after 26th July 2010 only** If yes to the above, can you confirm that you have registered the individual with the ISA as their employer / place of study, and that you will continue to monitor the registration status of this individual and withdraw them immediately from any regulated activity should their registration status change. **NB ISA registration is mandatory from November 2010 for researchers undertaking regulated activity**	ISA Registered for Vulnerable Adults? Yes ☐ No ☐ N/A ☒ ISA Registered for Children? Yes ☐ No ☐ N/A ☒

	Can you confirm that a clear criminal record disclosure has been obtained for the above-named individual, with no subsequent reports from the individual of changes to this record? *NB for Regulated Activity this must be an enhanced CRB. For non-regulated activity, ensure the CRB is at the mandated level*	Yes ☒ No ☐ N/A ☐
	If yes, please provide details of the clear disclosure Date of disclosure: 25th SEPT 2013 Type of disclosure: ENHANCED CERTIFICATE Organisation that requested disclosure: THE UNIVERSITY OF NOTTINGHAM CRB Disclosure Reference No. 001419542486. Researcher's ISA Unique ID: n/a.	
9.	Have the pre-engagement checks described below been carried out with regard to the above-named individual?	
	▪ Employment/student screening: o ID with photograph o two references o verification of permission to work/study in the UK o exploration of any gaps in employment	Yes ☒ No ☐ Yes ☒ No ☐ Yes ☒ No ☐ Yes ☒ No ☐
	▪ Evidence of current professional registration	Yes ☐ No ☐ N/A ☒
	▪ Evidence of qualifications	Yes ☒ No ☐
	▪ Occupational health screening / clearance	Yes ☒ No ☐
	Is the named individual on a fixed term contract or is the contract end imminent? Please indicate current contract end-date	Yes Date: ~~08/09/16~~ 30th September 2014
	Signed: VSedman	Date: 22/10/2013
	Name: VICTORIA SEDMAN	Job Title: RESEARCHER TRAINING & DEVELOPMENT MANAGER
	Organisation: THE UNIVERSITY OF NOTTINGHAM	Department: THE GRADUATE SCHOOL, RGS
	Address: THE UNIVERSITY OF NOTTINGHAM, UNIVERSITY PARK, NOTTINGHAM NG7 2RD	
	Tel No: (0115) 84 68583	Email: victoria.sedman@nottingham.ac.uk.
Please return the form to the researcher.		
Section 6 - Instructions to applicants *To be completed by Researcher*		
Please indicate which of the following documents are attached to this Research Passport:		
Current curriculum vitae, including details of qualifications, training and professional registration (please use the template C.V. at http://www.rdforum.nhs.uk/docs/template_cv.doc)		Yes ☒ No ☐
Researcher's copy of criminal record disclosure: Disclosures issued before 26th July 2010 only: Criminal record disclosure includes confirmation of check against the appropriate Barred List(s) Disclosures issued after 26th July 2010 only: Criminal record disclosure confirms appropriate ISA registration. NB where appropriate, ISA registration is mandatory after November 2010.		Yes ☒ No ☐ N/A ☐ Yes ☐ No ☐ N/A ☐ Yes ☐ No ☐ N/A ☐
Evidence of occupational health screening / clearance		Yes ☒ No ☐ N/A ☐
Appendix		Appendix numbers: N/A ☒

Please send the completed form and original documents to the Lead R&D office. The completed form and original documents will be returned to you. This package of documents will be used to validate your completed Research Passport form. You may then, and where relevant, provide the Research Passport to other NHS organisations.

You must inform all NHS organisations that have received this Research Passport of any changes to the information supplied above. Failure to do so may result in withdrawal of your honorary research contract or letter of access. As part of the quality control procedures for the Research Passport, random checks on the accuracy of the information held on this Research Passport may be made.

Section 7
This section should be completed by HR in the Lead NHS organisation, only if additional checks are undertaken

The following additional checks have been completed:	
Having confirmed that the necessary additional pre-engagement checks have been completed, I am satisfied that the above named researcher is suitable to carry out the duties associated with their research activity outlined in this Research Passport.	
Signed:	Date:
Name:	Job Title:
Organisation:	Department:
Email:	

Section 8 - For Office Use Only

This section should be completed by the NHS R&D office that received the initial application. The NHS R&D office must countersign and date retained photocopies of the documents. The grey section must be completed before the form is returned to the applicant.

CV reviewed?	Yes ☑ No ☐	Training?	Yes ☑ No ☐
Evidence of qualifications?	Yes ☑ No ☐	Appendix pages reviewed?	Numbers:
Professional registration details reviewed?	Yes ☐ No ☐ N/A ☑	Occupational health clearance reviewed?	Yes ☑ No ☐ N/A ☐
Criminal record disclosure reviewed?	Yes ☑ No ☐ N/A ☐	Date of disclosure:	CRB Disclosure Certificate No: 001419542486
For Research Passport applications submitted after 26th July 2010 only: Confirmation that HEI have subscribed their interest in this individual via the ISA on-line monitoring service, (where appropriate) and have agreed to withdraw the individual immediately, should the individual's ISA registration status change **NB ISA registration, where appropriate, is mandatory from November 2010**			Yes ☑ No ☐ N/A ☐
Enter Electronic Staff Record Number (if issued):			
Confirmation of valid Research Passport: Project specific ☑ Three-year ☐ Other End date ☐			
Signed:	A. S	Date: 30/10/2013	
Name:	ALISON STEEL		
NHS Organisation Name and contact details	QMC - RESEARCH & INNOVATION NOTTINGHAM UNIVERSITY HOSPITALS		
Date Honorary Research Contract/letter of access issued *(delete as appropriate)*			