A CLINICAL STUDY EXPLORING HIP AND KNEE OSTEOARTHRITIS PAIN TRANSMISSION USING CEREBROSPINAL FLUID

By

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Background: Osteoarthritis (OA) reduces quality of life and affects more than 40% of older adults but the molecular mediation of OA pain in the dorsal horn is yet to be explored clinically. Sufferers may experience central sensitisation and some may have an increased risk of persistent post-arthroplasty pain.

The study explored two hypotheses.

1. There would be significant differences in pro-inflammatory cytokines (interleukin [IL]-1β IL-2, IL-6, IL-7, IL-8, IL-12, interferon [IFN]-γ and tumour necrosis factor [TNF]-α), excitatory amino acids (glutamate, aspartate, arginine, citrulline, serine and glutamine), anti-inflammatory cytokines (IL-4, IL-5, IL-10 and IL-13) and inhibitory amino acids (glycine and gamma-amino-butyric acid [GABA]) when participants with OA were compared with pain free controls after adjustment is made for the influence of age, gender and psychological distress (total Hospital Anxiety and Depression [HAD] score).
2. Concentrations of these amino acids and cytokines would differ significantly when a group of OA participants who have no pain at rest (0PAR) were compared with a group who did have pain at rest (PAR) after adjustment for age, gender and total HAD score (HADS-T).

Method: After ethical approval people having elective primary hip or knee arthroplasty (OA group) or urological surgery (pain-free controls) were recruited on the day of surgery. Pain at rest (PAR), pain on movement (POM) (0-10 numerical rating scale), and HADS data was collected pre-operatively. A 2ml sample of cerebrospinal fluid (CSF) was aspirated by an independent anaesthetist prior to spinal anaesthesia. Filtered CSF was snap frozen and then stored at -80°C. Samples thawed on ice were then subjected to High Pressure Liquid Chromatography for assay in duplicate of amino acids and high-sensitivity multiplex bead array assay in duplicate for the cytokines.

Differences in amino acids and cytokines between the groups were explored using ANCOVA adjusting for age, gender and total HAD score (HADS-T). Where residuals were not normally distributed a boot-strapping technique was used.

Binary logistic regression was conducting using a forward step-wise approach with age, gender and HADS-T as part of the initial model. The regression analyses explored the predictor variables for membership of the OA group, membership of the PAR group, and having psychological distress (HADS-T≥12). A multiple linear regression was conducted to determine the predictor variables that influenced HADS-T.

Results: A total of 21 control (75% male) and 59 OA (46% male) participants were eligible for the statistical analysis. Median pain on movement in the OA group was 6 and 59% of this group had pain at rest (PAR≥1). 57% (n=34) of the OA group had psychological distress (HADS-T ≥12) compared with 19% (n=4) of the pain free group (Fischer's exact test p=0.006).

Serine, leucine, valine, and TNFα were significantly higher in the OA group ($p<0.05$) than the control group. IL-12 was significantly lower in the OA group ($p<0.05$). Aspartate and IFNγ were both significantly lower in the PAR group than the 0PAR group ($p<0.05$).

Membership of the OA group was predicted by being male, increasing HADS-T, serine and leucine and decreasing GABA. Membership of the PAR group was predicted by decreases in aspartate and IFNγ. Having psychological distress (HADS-T≥12) was predicted by increasing POM and decreasing serine.

Discussion: Significant differences were identified for serine and TNFα in the CSF of the OA/control therefore the null hypotheses was rejected. Although there were significant differences between the 0PAR and PAR groups these were not in the direction expected and not in keeping with the hypothesis therefore the null hypothesis was retained.

The findings in this study indicate an unexpected role for IL-12 and IFNγ; it is possible that these cytokines have an anti-inflammatory influence centrally in OA pain.

This study offers support to the theory that central sensitisation is part of OA pain signalling at the level of the dorsal horn. Further, psychological distress is an integral part of the OA experience. The findings are in agreement with other published studies which model a three-way influence between peripheral disease activity, depression and pain.

The study provides a preliminary but broad over-view of the pain of OA in the clinical setting. It demonstrates the complexity and utility of this kind of study and provides a platform for further work.

Declaration of authenticity:

I, Amelia Swift, designed this study, prepared for and gained ethical and Research and Development approval. I recruited participants for this study and collected data in the majority of cases. A small number of participants were recruited by Dr. Katarina Kos, Research Fellow University of Warwick and in those cases she was also responsible for data collection and CSF processing until the point of storage in the -80 freezer.

CSF samples were taken by anaesthetic staff employed by Heart of England NHS Foundation Trust and the research took place on the premises of the same. I processed the majority of samples myself (see above) and was responsible for their safe storage from the start of the study until 2003 when I transferred employment from the Trust to the University of Birmingham, thereafter the samples became the responsibility of Dr. Barbara Hoggart, Heart of England NHS Trust, who was the principle investigator for this study.

HPLC analysis was undertaken by Naomi Langman with supervision from Dr Kevin Whitehead at the University of Birmingham. I was responsible for transcription of data from the HPLC output and had supervision in this from Dr. Whitehead.

Multiplex bead array assay for cytokines was undertaken by me with close supervision from Dr. Alison Harte, University of Warwick and her staff. Cytokine assay took place at the University of Warwick.

I designed and undertook the statistical analysis of the data in this study with the supervisory support of Dr. Roger Holder and Dr. Linda Nichols from the University of Birmingham. Bootstrap analysis undertaken by Dr. Nichols.

Dedication

For Georgia, Callum and Dodo

Acknowledgements

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Dr Barbara Hoggart and Professor Norman Bowery conceived of the initial study from which this work grew, and secured the funding that made it possible from Pfizer pharmaceuticals; many thanks to them. Thank you also to Karen Kilbride who helped me to create original illustrations.

I also owe a huge debt of gratitude to my husband Callum who picked up the pieces, supported and believed in me and took the lead in caring for our daughter, Georgia. Many thanks also to the friends and family who have waited and waited and waited for an end to this work.

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GLOSSARY

Aδ fibre	Relatively large myelinated fast pain fibre
Acute pain	pain of a short duration related to damaged tissues
Aggrecan	A large proteoglycan: in aggregated form along with type II collagen provides structure to cartilage and gives it strength
Agonism	Ligand binding to a receptor that leads to activity
Algesic	Endogenous substance capable of producing pain
Allodynia[1]	Pain due to a stimulus that does not normally evoke pain
Angiogenesis	The growth of new blood vessels from old
Anhedonia	The inability to experience pleasure
Antagonism	Ligand binding to a receptor that prevents activity
Anxiety[2]	A negative mood state characterised by bodily symptoms of physical tension and apprehension about the future
Astrocytes	Non-neuronal cells whose chief function is the support of neuronal cells and maintenance of homeostasis in the CNS.
Box rating scale	A visual pain rating scale that presents 0-10 range of numbers to the patient in a horizontal table. 0 denotes no pain and 10 the most pain the person can imagine.
C-fibre	Unmyelinated, small and slow conducting pain fibres
C-reactive protein	A protein found in the blood whose levels increase in response to inflammation
Catastrophisation	The tendency to focus on, and even exaggerate the negative threat value of a painful stimulus or experience
Central sensitisation[3]	The increased synaptic efficacy established in somatosensory neurons in the dorsal horn of the spinal cord following intense peripheral noxious stimuli, tissue injury or nerve damage. This heightened synaptic transmission leads to a reduction in pain threshold, an amplification of pain responses and a spread of pain sensitivity to non-injured areas

[1] International Association for the Study of Pain (1994)

[2] Barlow and Durand (2009)

[3] Ji *et al.* (2003)

Chemokine	Small cytokine proteins that function as chemoattractants.
Chondrocytes	Cartilage cells
Chondropathy	A disease of the cartilage often graded 0-5
Clinical Studies	Research studies involving human participants as distinct from animal studies
Crepitus	A feeling or sound of grating, crackling or popping often experienced by people with OA
Cytokine	A protein whose primary function is inter and intracellular signalling
Depression[4]	Depression is a common mental disorder that presents with depressed mood, loss of interest or pleasure, feelings of guilt or low self-worth, disturbed sleep or appetite, low energy, and poor concentration.
Dorsal horn	The posterior portion of the spinal cord that contains grey matter
Dorsal root ganglion	A collection of cell bodies of primary afferent (sensory) fibres that lies just outside the dorsal horn
Erythrocyte sedimentation rate	The speed with which red blood cells settle in a sample of blood left to stand. An indicator of inflammation with faster speeds indicating greater inflammation
Glia	Non-neuronal cells that have homeostatic function. Include astrocytes, microglia and oligodendrocytes
Hypoalgesia[1]	Diminished pain in response to a normally painful stimulus
Hyperalgesia[1]	An increased response to a stimulus which is normally painful
Incidence	The frequency that new cases appear over a period of time. Expressed as the number of new cases as the numerator and the population at risk as the denominator.
Interneuron	A local cell found within the spine forming connections with a high number of other cells but with not projection to the brain, also called a relay neuron
Large neutral amino acids	Amino acids that compete with tryptophan to cross the blood brain barrier (leucine, isoleucine, valine, tyrosine and phenylalanine)

[4] World Health Organisation (2010)

Matrix metalloprotease (MMP)	Enzymes that are involved in degradation of extracellular matrices, cleavage of membrane-bound receptors and induction of cytokines
Neuron	An electrically excitable cell that transmits information electrically and communicates with other cells chemically
Neuropathic pain[1]	Pain initiated or caused by a primary lesion or dysfunction in the nervous system
Neuropathy[1]	A disturbance of function or pathological change in a nerve: in one nerve, mononeuropathy; in several nerves, mononeuropathy multiplex; if diffuse and bilateral, polyneuropathy
Neurotoxicity	Damage to neurons (in this context this is often being caused by over-stimulation with glutamate)
Nociception	The sensory process that translates noxious stimuli into signals that will ultimately be interpreted by the brain as the perception of pain
Nociceptor[1]	A receptor preferentially sensitive to a noxious stimulus or to a stimulus which would become noxious if prolonged
Noradrenergic	Pertaining to the activity of noradrenalin
Noxious stimulus[1]	A noxious stimulus is one which is damaging to normal tissues
Number needed to treat	A measure of the effectiveness of an intervention. In analgesia studies it is common to see this expressed as the number of people who need to be treated before 1 person will achieve at least 50% pain relief. An NNT of 1 means everyone will get 50% pain relief, an NNT of 10 means that 1 in 10 people treated will achieve 50% or better pain relief, everyone else will achieve less than this
Osteophytes	Bony projections that form along a joint space as a result of damage to the joint's surface
Pain[1]	An unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage
Pain threshold[1]	The least experience of pain which a subject can recognise
Pain tolerance[1]	The greatest level of pain which a subject is prepared to tolerate
Pathological pain	Pain that does not serve a protective function
Physiological pain	Pain that serves a protective function
Prevalence	The amount of a disease in a population at a given time

Projection neuron	A neuron that projects an axon from the spinal cord to the brain/brainstem
Proteases	Enzyme that breaks down proteins
Self-efficacy	Self-belief relating to the ability to perform a specified function
Serotonergic	Pertaining to the activity of 5-HT
Subchondral bone	The layer of bone just below the cartilage
Subchondral bone sclerosis	Increased density of thickening of the subchondral bone
Synovitis	Inflammation of the synovial membrane
Thermal threshold	Temperature required to evoke pain
Wide dynamic range neuron/cell	Cells that respond to a wide range of stimuli and alter their output according to the frequency of the input
Wind-up	Repeated stimulation of C-fibres leads to summation of potentials in wide dynamic range neurons and this translates to sensitisation causing hyperalgesia and allodynia that reverts to normal over a relatively short period of time once the stimulus is removed

ABBREVIATIONS

5-HIAA	5-hydroxyindole acetic acid
5-HT	5-hydroxytryptamine also known as serotonin
5-HTT	5-HT transporter
ACTH	adrenocorticotrophic hormone
AIDS	Acquired Immune Deficiency Syndrome
AMPA(R)	α-amino-hydroxy-5-methyl-4-isoxazoleproprionic acid (receptor)
ANOVA	Analysis of variance
ANCOVA	Analysis of covariance
ATP	Adenosine triphosophate
BDNF	Brain derived neurotrophic factor
Ca+	calcium ion
CaM	Calmodulin
Ca+CaM	Calcium calmodulin
CFA	Complete Freund's Adjuvant
CCL2	a chemokine also known as monocyte chemoattractant protein 1 (MCP-1)
CCR2	the receptor for CCL2/MCP-1
cGMP	Cyclic guanosine 3,5-monophosphate
CGRP	Calcitonin gene related peptide
CNS	Central nervous system
COX-1/2	Cyclo-oxygenase enzyme 1 or 2
CRP	C-reactive protein
CSF	Cerebrospinal fluid
DLPT	dorsolateral pontine tegmentum
EAAT	Excitatory amino acid transporter
ECF	Extracellular fluid
ELISA	Enzyme Linked Immunosorbent Assay
EP2R	Prostaglandin E2 receptor
ESR	Erythrocyte Sedimentation Rate
GABA	Gamma amino butyric acid
GFAP	Glial fibrillary acidic protein
H+	Hydrogen ion
HADS	Hospital Anxiety and Depression Scale
HPA	hypothalamic-pituitary-adrenal (axis)
HPLC	High pressure liquid chromatography
HIV	Human Immunodeficiency Virus
IASP	International Association for the Study of Pain
ICD-10	International Classification of Diseases -10 (1990, World Health Organisation)
K+	Potassium ion
KCC2	Potassium and chloride ion transporter
HRQoL	Health related quality of life
MANOVA	Multivariate analysis of variance
MCP-1	Monocyte chemoattractant protein-1, a chemokine also known as CCL2

mRNA	memory ribonucleic acid
NA	noradrenalin
Na+	sodium ion
NK-1	Neurokinin-1 receptor, ligand = Substance P
NMDA(R)	N-methyl-D-aspartate (receptor)
NNT	Number needed to treat
NRM	Nucleus Raphe Magnus
NSAIDs	Non-steroidal anti-inflammatory drugs
NO	Nitric oxide
NOS	Nitric oxide synthase
OA	Osteoarthritis
OPA/3-MPA	ortho-phthaldiahyde/3-mercaptoproprionic acid
PAF	Primary afferent fibre
PAG	Periaqueductal grey
PGE2	Prostaglandin E2
QA	Quinolinic acid
RA	Rheumatoid Arthritis
RVM	Rostroventromedial medulla
SD	Standard deviation
sGC	soluble guanylyl cyclase
SP	Substance P
SPSS	Statistical Package for Social Sciences
TENS	Transcutaneous Electrical Nerve Stimulation
UK	United Kingdom
US	United States of America
VAS	Visual Analogue Scale
VGLUT	Glutamate transporter

Chapter 1

1.1 Introduction

Osteoarthritis is a prevalent chronic joint condition that affects synovial joints and can lead to pain, functional limitation and reduced quality of life. It affects joints with weight-bearing cartilage, sites where trauma has occurred and also the smaller joints including fingers, toes and ankles (NICE 2008; Badley and Tennant 1992: 494; Felson et al. 1987).

OA is thought to affect approximately 41% of those aged over 65 years in the United Kingdom (UK) (Dawson et al. 2004). Approximately 25-37% of those diagnosed with OA describe their condition as mild, 47-56% as moderate and 16-19% as severe (Bushmakin et al. 2011; Sadosky et al. 2010). The subjective severity level of the OA correlates with self-reported pain severity and functional impairment and demonstrates that within the cohort of people diagnosed with OA there is a wide variation in severity and impact. Much of the research in OA focuses on those at the more severe end of the spectrum and this may lead to over-statement of the impact of the condition generally. However, people with this disease often experience poor quality of life (Hirvonen et al. 2006) and many with more severe forms suffer from co-morbid depression (Rosemann et al. 2007b).

The pain of OA is assumed to be predominantly nociceptive, meaning that noxious stimulation created within the joint results in perception of pain. The pain may be partially managed using a combination of paracetamol, anti-inflammatory and weak opioid medication.

In addition to medication, pain relief and improved function can be facilitated by exercise, physiotherapy, acupuncture, Transcutaneous Nerve Stimulation (TENS), heat or cold and natraceuticals. Some, whose disease affects the larger joints such as shoulder, elbow, knee or hip, who do not find sufficient relief in this medical management approach will undergo joint replacement surgery, which can help to alleviate pain and improve function. However, a number of people continue to suffer an unacceptable level of pain for months or years after surgery (Wylde et al. 2011a; Puolakka et al. 2010; Lundblad et al. 2008; Singh et al. 2008; Hawker et al. 1998; Bourne et al. 1994). The reasons for this continued pain following surgery include intra-operative nerve damage (Brown et al. 2008), malalignment of the implant (Thompson et al. 2011; Czurda et al. 2010), heterotopic bone formation (Neal 2003), and infection (Parvizi et al. 2010). In addition to these factors it has been hypothesised recently that some cases of long-term post-operative pain are related to persistent central sensitisation (Wylde et al. 2011a).

Central sensitisation describes a form of synaptic plasticity where specific patterns of pain signals (nociceptive inputs) reduce the threshold of secondary dorsal horn neurons and alter their relationship with non-nociceptive sensory fibres. This leads to a situation where sub-threshold stimuli of a noxious type and non-noxious sensory signals create action potentials that are read as pain. The relationship of this phenomenon and persistent pain in the post-operative arthroplasty patient has been suggested by Lundblad *et al.* (2008) who found that the presence of pre-operative rest pain and lower pre-operative pressure-pain thresholds was associated with an increased risk of persistent post-operative pain. Wylde *et al.* (2011a) suggest that increased risk of persistent post-operative pain following arthroplasty is related to an increased 'vulnerability' to pain, i.e. sensitisation. This is based on their findings that

persistent pain is associated with the presence of pains elsewhere in the body in addition to the joint pain. Pain severity in the replaced joint was observed to increase with the number of pain sites elsewhere. There is also evidence of the presence of central sensitisation in OA from experimental studies on human participants (Arendt-Nielsen et al. 2010; Imamura et al. 2008; Lundblad et al. 2008; Bajaj et al. 2001), the pain sufferers' use of neuropathic pain descriptors (Hochman et al. 2010) and the efficacy of centrally acting pain relievers (Duloxetine) in OA (Chappell et al. 2009) . This has implications for the management of pre-operative OA pain but also for the risk and management of some forms of persistent post-surgical pain.

A number of studies exploring persistent post-surgical pain have identified depression as a risk factor (Hinrichs-Rocker et al. 2009). This finding extends to OA joint replacement pain. Wylde *et al.* (2011a) found that patients with major depression were 1.3 times more likely to suffer worse persistent post-arthroplasty pain. The reported lack of correlation between reported pain and radiographic severity of OA (Bedson and Croft 2008) may be partially explained by depression given some of the neurobiological interactions between pain and depression. Kim *et al* (2011a) identify an increased likelihood of moderate grade OA changes causing pain when depression is also present. This and the increased prevalence of depression in the OA population compared with the general population (Axford et al. 2010; Rosemann et al. 2007b) suggests that depression is an important covariate for pain in OA and perhaps a risk factor for persistent post-surgical pain in this group.

The clinical study of OA pain has focused on the peripheral joint. In order to fully explore central sensitisation as a factor in OA pain it is necessary to consider the transmission of pain

signals at the level of the dorsal horn, the seat of central sensitisation. Animal models of OA pain have been used to demonstrate the likelihood of central sensitisation at the level of the spinal cord in OA (Im et al. 2010; Schaible et al. 2009) but there are very few clinical studies of pain at this level. Lundborg *et al* (2010) found that interleukin 1-beta (IL-1β), IL-8 and Glial cell-line Derived Neurotrophic Factor (GDNF) were higher in the cerebrospinal fluid (CSF) of OA participants than controls in their study. Buvanendran *et al* (2006) found that IL-6 was upregulated in the CSF following arthroplasty surgery. These studies demonstrate that pro-inflammatory responses can be detected in the CSF of OA patients.

The present study aims to explore a number of amino acid and cytokine transmitters putatively involved in the dorsal horn transmission of osteoarthritis (OA) pain with consideration given to anxiety and depression. It will be the first time that OA pain has been characterised using CSF in a clinical study and will provide important insight into the mechanism of this pain.

1.2 Epidemiology, diagnosis and impact of osteoarthritis

The National Collaborating Centre for Chronic Conditions (2008: 3), in their guidance for the National Institute for Health and Clinical Excellence (NICE) define OA as:

… ' *a metabolically active, dynamic process that involves all joint tissues (cartilage, bone, synovium/capsule, ligaments and muscle). Key pathological changes include localised loss of articular (hyaline) cartilage and remodelling of adjacent bone with new bone formation (osteophyte) at the joint margins. This combination of tissue loss and new tissue synthesis supports the view of osteoarthritis as the repair process of synovial joints'.*

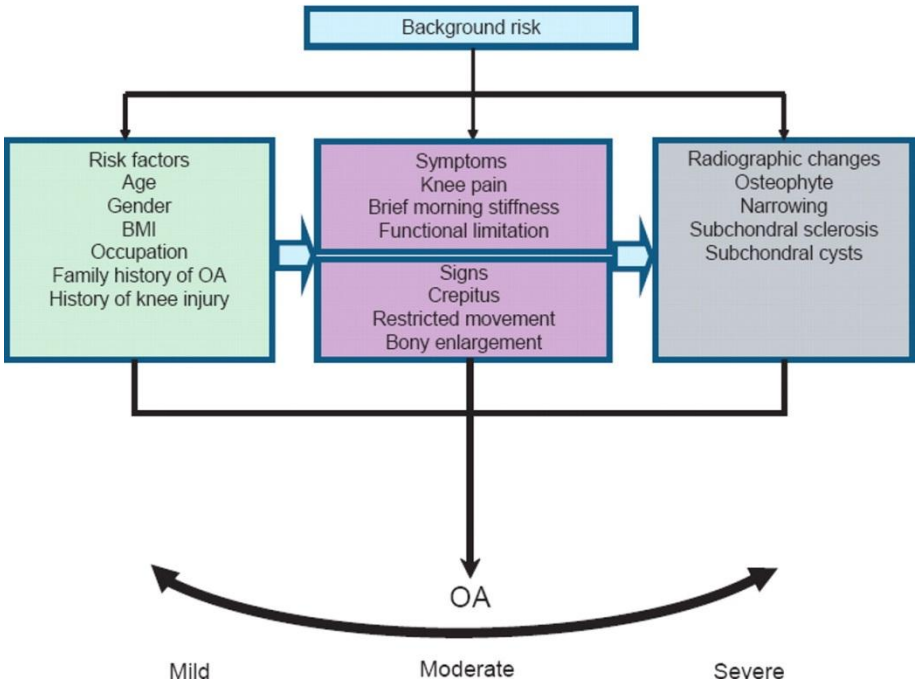
OA is a common condition that can be disabling. Much of the published research focuses on those people with OA who have sought medical help and it is therefore unclear whether the global impact of OA is as great as this data suggests. However, what is apparent is that for many, OA is a painful and disabling condition that has an impact on the ability to live a full and satisfying life.

1.2.1 Diagnosis of OA

The reported prevalence of hip and knee OA varies according to the criteria used to diagnose it. It is difficult to compare prevalence between populations and across studies because the diagnostic and sampling criteria vary broadly. A recent EULAR systematic review (Zhang et al. 2010a) states knee OA can be diagnosed with confidence using a detailed clinical interview and physical assessment of the sufferer (Figure 1-1). The population prevalence and personal risk factors (see 1.4) are taken into account along with symptoms of persistent pain, stiffness and functional limitation and physical signs such as crepitus, bony enlargement and restricted movement.

Radiographic evidence is often used in addition to the clinical interview as part of the diagnostic process and as a means of grading the severity of OA. One of the most commonly used grading tools for radiographic changes is the Kellgren-Lawrence (K-L) scale (Box 1-1). Changes can be graded from 0 (no radiographic evidence of OA) to 4 (severe OA) (Figure 1-2). A cut-off point of grade 2 - definitely present but of minimal severity (Kellgren and Lawrence 1957) is often used to define cases. Other criteria including pain and joint space width are also used in studies and this can lead to differences in the rates of diagnosis.

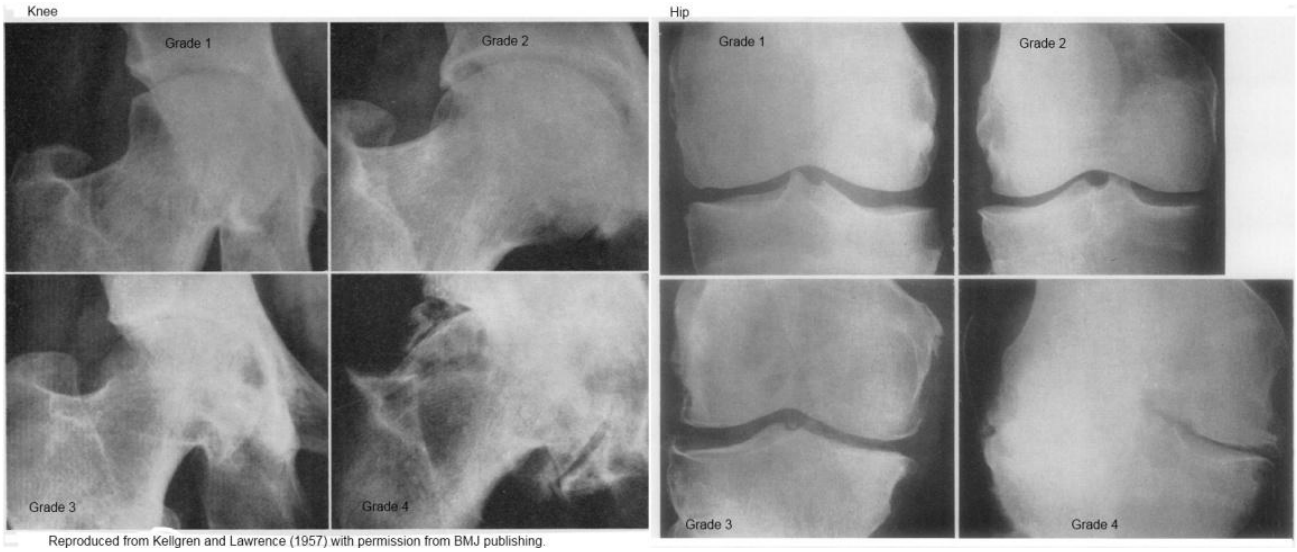
Figure 1-1: Diagnosis of knee OA (EULAR)



Box 1-1: The Kellgren-Lawrence Scale: joint changes that can be observed in OA (Kellgren and Lawrence 1957: 494)

Osteophytes on the joint margins or tibial spines; Periarticular ossicles (mainly seen in fingers); Narrowing of the joint cartilage associated with sclerosis of subchondral bone; Small pseudocystic areas with sclerotic walls usually situated in the subchondral bone Altered shape of bone ends, particularly the head of the femur.

Figure 1-2: Kellgren Lawrence radiographic grading of hip and knee OA



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1.2.1.1 Discrepancy between pain and radiographic observations of joint structures

There are a small number of studies that have identified associations between self-reported OA symptoms such as pain and radiographic changes such as joint space width and osteophyte development (Fukui et al. 2010; Neogi et al. 2009b; Cicuttini et al. 1996). However, a far greater number of studies report poor or no correlation between the severity of OA changes seen on X-ray and OA symptoms (Astephen Wilson et al. 2011; Bedson and Croft 2008; Dieppe et al. 2005; Hannan et al. 2000; Dieppe et al. 1997; Claessens et al. 1990).

There are a number of reasons reported for the discrepancies between x-ray findings and symptom reports. First is the view of the knee used in the diagnosis. The sensitivity of radiologic OA detection can be improved to 70% or more by using views of the three different knee compartments (Peat et al. 2007; McAlindon et al. 1992) but many studies do not include the patello-femoral joint view (Bedson and Croft 2008).

Varying definitions of pain are used in different studies. One may define OA pain as 'ever having an episode of pain lasting 15 day' while another uses 'any knee pain in the last month'. Studies also differ in their definition of OA, which might or might not include functional difficulty or disability as well as pain. Different K-L grades are also used to define OA in different studies. Finally study samples vary in terms of age range, ethnicity and other confounding factors (Bedson and Croft 2008). Neogi *et al*. (2009b) have addressed a number of these discrepancies by comparing pain and structural changes in the same person and therefore removing a great deal of the confounding noise typical of many of the previous studies. Overall they find that more significant x-ray changes are associated with symptoms of OA but the relationship is less strong in early OA.

A further reason for the discrepancy between radiographic findings and OA symptoms is that the x-rays provide only limited information. Pain and dysfunction from the OA joint is not only related to those structures that can be directly or indirectly assessed by x-ray. The use of magnetic resonance imaging (MRI) has enabled closer examination of a number of joint structures thought to be responsible for pain. In a systematic review of 5 cohort studies and 17 cross-sectional studies Yusuf *et al*. (2011) found a moderate level of evidence that bone marrow lesions and effusions/synovitis are associated with pain. There was limited evidence that ligamentous abnormalities were associated with pain, conflicting evidence that cartilage defects, meniscal tears and bone attrition were associated with pain and limited evidence that osteophytes and subchondral cysts were not associated with pain.

The part played by other physical, social and psychological factors in the experience of pain for the individual also has an influence over the apparently disproportionate amount of pain and disability experienced by some people with relatively minor observable OA changes. As will be seen in the next section a number of different factors, biological, social and psychological, influence the development and the impact of OA. Additionally, this study aims to explore the mechanism of pain in this disease and will demonstrate that central sensitisation plays an important role. Central sensitisation has the potential to cause a disconnection between the painful stimulus and the degree of pain experienced by the individual and this will be more fully explored in both Chapter 3 and the discussion.

1.3 Prevalence of OA

A systematic review of hip OA (Dagenais et al. 2009) examined 23 studies from Europe (n=25), North America (n=6), Asia (n=4), Africa (n=2), the Caribbean (n=1) and the Middle

East (n=1). They report mean prevalence ranging from 1.4% in Asia to 10% in Europe. Knee OA is thought to be more highly prevalent but perhaps due to the variety of aetiologies systematic reviews are lacking. A large cohort study in Holland that excluded people with developmental dysplasia, previous knee surgery and knee replacement, or amputation found that 12.1% of men and 142% of women had radiographic OA (≥K-L 2) (Laxafoss et al. 2010).

Prevalence of OA increases linearly with age in both genders (Kopec et al. 2007; Hart et al. 2002; Zhang et al. 2001a) with peak prevalence for women in their 60s and in men in their 70s (Rossignol et al. 2003). A Dutch study of a random sample of adults found a prevalence of knee OA to be approximately 11-12% in the 50-59 age group rising to more than 20% in those aged over 70 years (van Saase et al. 1989).

When women are compared to men OA of most joints is more prevalent in men under 50 years of age, but after the age of fifty the prevalence is greater in women than men for most joints (Jordan et al. 2007; Kopec et al. 2007; Jacobsen et al. 2004; Felson et al. 1987). Van Saase *et al.* (1989) did not see a gender difference in OA of most joints except for the hip and knee in those aged more than 65 years when the prevalence in women exceeded that in men.

In addition to the personal burden that OA causes, which will be explored in more depth below, this disease has an impact on the economy and health expenditure, which creates a further imperative to develop the most effective and efficient management strategies possible. The amount of years of productive life lost as a result of osteoarthritis in the year 2000 was 3,048, 000 years in the western and developing world (Reginster and Khaltaev 2002). To place this in context, this compares to a figure of 2,690,000 years for HIV and AIDS and 9 042 000 years for ischaemic heart disease. In 1997 an economic evaluation of five

industrialised nations (UK, US, Canada, Australia and France) estimated that between 1 and 2.5% of the gross national product was being spent on OA management (March and Bachmeier 1997). The use of 'managed care' in the US allows examination of a breakdown of that expenditure and shows that approximately one third is used for medication management, primarily pain-relieving drugs, and one half of the total is used to service the 5% of the US OA population who require arthroplasty (Bitton 2009).

1.4 Risk factors for symptomatic OA

There are a number of factors that increase the likelihood of developing symptomatic OA. Non-modifiable factors include age, gender and ethnicity, while others such as obesity, activity level and employment are modifiable.

1.4.1 Age as a risk factor for OA

The prevalence of OA increases with age because exposure to other factors has a cumulative effect and because changes associated with ageing increase the impact of factors such as load-bearing and obesity. One of the main characteristics of OA is deterioration in the cartilage of the joint; the health of the cartilage depends on the function of cartilage cells (chondrocytes) which deteriorates with ageing leading to a loss of ability of the cartilage to maintain and repair itself (Grogan and D'Lima 2010; Loeser 2009). This deterioration is observable in most people over 50 years of age, but it is far more pronounced in people with OA (Yamada et al. 2002).

1.4.2 Gender as a risk factor for OA

Differences in prevalence according to gender are due to a number of factors including differences in cartilage health (Hanna et al. 2009), a tendency for misalignment of the joint (varus-valgus laxity) (van der Esch et al. 2007) and congenital or developmental deformities (Barros et al. 2010; Doherty et al. 2008; Ganz et al. 2008; Jacobsen 2006; Jacobsen and Sonne-Holm 2005). Some of these skeletal malformations are associated with genetic mutations (Li et al. 2007). Genetic polymorphisms can also increase risk of OA via abnormalities in pathways involved in joint health such as apoptosis, inflammation and bone morphogenetic protein signalling (Valdes and Spector 2010) and a number of studies have established that there is a clear degree of heritability involved in the development of symptomatic and radiographic OA (Zhai et al. 2007; Neame et al. 2004; Page et al. 2003).

1.4.3 Ethnicity as a risk factor for OA

There are ethnic variations in prevalence of OA with Asians, African-Americans, American Indian women and non-white Hispanic women reported to have higher rates of knee OA and lower rates of hip OA than Caucasians in the US population (Wright et al. 2008; Jordan et al. 2007; Zhang et al. 2001a; Hoaglund et al. 1995). Some of the ethnic variation is related to genetic polymorphisms (Nakamura et al. 2007) but many of the studies suggesting a genetic cause for these differences are underpowered (Zintzaras et al. 2010). The research in this area will require large sample sizes because there is a great deal of interaction between ethnicity and other risk factors such as obesity, education, and socioeconomic status.

1.4.4 Occupation as a risk factor for OA

The risk of both hip and knee OA is increased by a number of occupational factors including for knee OA kneeling, lifting, crawling and heavy work while standing, and for hip OA bending, twisting and reaching even after joint injury has been controlled for (Allen et al. 2010; Rytter et al. 2009). It is possible that these factors contribute to the gender differences in prevalence of knee and hip OA due to differences in gender bias for load-bearing and heavy manual work, lorry driving and agricultural work, all associated with increased risk of OA (Rossignol et al. 2003).

1.4.5 Injury as a risk factor for OA

Knee injury is also associated with a much greater risk of developing knee OA at an earlier age. People who have experienced anterior cruciate ligament injury or a meniscal tear are much more likely to develop symptomatic and radiographic OA than their peers (Roos 2005; Lohmander et al. 2004; Gelber et al. 2000).

1.4.6 Obesity as a risk factor for OA

Obesity is a major risk factor for development of OA and is associated with higher incidence of OA requiring joint replacement (Turley et al. 2006; Flugsrud et al. 2006; Holmberg et al. 2005; Oliveria et al. 1999). Obesity increases the load managed by the weight-bearing joints (Messier et al. 2005), which leads to the development of abnormalities in the regeneration and structure of the cartilage (Gosset et al. 2006) and increased bone density (Nunez et al. 2007; Taguchi et al. 2007) leading to a higher rate of cartilage breakdown. Obesity is also associated with increased OA in small non-load-bearing joints like the fingers (Kalichman and Kobyliansky 2009; Grotle et al. 2008). This suggests that obesity also influences OA

humorally and there is growing evidence that leptin, an adipokine produced by white adipocyte fat cells, is involved (Vuolteenaho et al. 2009; Zhang et al. 1994). The link between obesity and OA appears to be established in early life (Karlson et al. 2003) and the increased prevalence of childhood obesity as well as that in the older population suggests that the personal and economic burden of OA will increase.

1.4.7 Socioeconomic status as a risk factor for OA

Lower socioeconomic status has been associated with increased risk of radiographic OA (Callahan et al. 2010; Hannan et al. 1992) and symptomatic OA (Odutola and Ward 2005; Thumboo et al. 2002) and this is likely to be mediated via other factors associated with poor socioeconomic status including obesity, occupation, physical activity, and in women access to hormone replacement therapy (Roskam et al. 2010; Seppanen-Nuijten et al. 2009; Wardle et al. 2002; Hannan et al. 1992).

1.5 The effect of OA on the individual

Many OA sufferers cope well with their condition and require little medical or emotional support. Approximately 25-37% of people describe their condition as mild but 16-19% describe it as severe (Bushmakin et al. 2011; Sadosky et al. 2010). It is likely that the latter group are the people whom the majority of research exploring the effects of the condition focuses upon.

1.5.1 OA is associated with poorer quality of life

One of the main ways in which the impact of a condition is measured and communicated is through evaluations of health-related quality of life (HRQoL). A number of tools are

available which attempt to quantify the effects of a disorder on an individual's satisfaction with life. The tools often include component measures of general health, physical function and symptoms, role function, mental well-being, emotional functioning, cognitive functioning and pain (Fayers and Machin 2007). It is reported that the HRQoL of people with moderate to severe OA is poorer than healthy normal controls (Rabenda et al. 2007; Cook et al. 2007; Lastowiecka et al. 2006; Jakobsson and Hallberg 2006; Hirvonen et al. 2006; Salaffi et al. 2005; Ackerman et al. 2005). In other words OA leads to a variety of effects that make their life less enjoyable and more challenging. These effects could be broadly divided into physical, social and psychological and are observable from the time that the person first presents to their general practitioner (Rosemann et al. 2007c; Roux et al. 2005; Birrell et al. 2000).

1.5.2 OA is associated with pain and stiffness

One of the most commonly reported physical symptoms of OA is joint pain, which tends to be localised to the affected joint sometimes radiating to the nearby tissues. Pain may be accompanied by swelling, stiffness, and loss of mobility (Martel-Pelletier 2004). Stiffness, which adds greatly to discomfort and disability, usually follows a period of inactivity and as such can be particularly troublesome on wakening when the normal activities of bathing and dressing can be significantly disrupted.

1.5.3 OA is associated with functional limitation

OA is often associated with functional limitation (Ling et al. 2003; Guccione et al. 1994) and together with pain this is one of the major factors that determine the likelihood of joint replacement being offered (Gossec et al. 2011). The relationship between functional

restriction and objective indicators of OA is weak because it is mediated by the working model that the sufferer builds about their condition. Perceptions and cognitions about cause, likely duration and outcome all influence current behaviour and treatment outcomes so that a person who believes their OA to have more serious consequences is likely to be less functionally active (Orbell et al. 1998; Hampson et al. 1994). A person's function will also be influenced by a number of psychological factors such as learned helplessness, self-efficacy and mood (Botha-Scheepers et al. 2006). Function is itself a construct composed of a number of different components including activity, participation and health. In terms of health, people with hip OA have been demonstrated to have lower scores on the physical functioning and role functioning sub-scales of the SF36, lower range of movement of the joint, lower knee muscle extensor strength and shorter 6 minute walking distance than matched controls (Rydevik et al. 2010). Similarly people with knee OA have been found to have reduced muscle strength, reduced flexibility and functional restrictions (Swank et al. 2011).

1.5.4 OA is associated with increased risk of falls

Muscle weakness and joint instability in people with lower limb OA increases the risk of falls (Yakhdani et al. 2010). A Canadian study of community-dwelling older adults who had more than 6 month history of OA found that approximately 45% of them had experienced a fall and 77% occasional or frequent near-falls (Arnold and Faulkner 2007). Concern or fear about falling is a significant risk factor that predicts balance and number of falls even when other factors such as medication use, use of walking aids and other factors are controlled for (Delbaere et al. 2009; Arnold and Faulkner 2007). Fear of falling, or catastrophic thoughts about the consequences of falling leads to a loss of mobility (Delbaere et al. 2009;

Bialoszewski et al. 2008). This is of concern generally and in particularly for people who suffer from OA where this will further decrease lower limb strength.

1.5.5 OA is associated with fatigue

People with OA also have a higher than normal incidence of fatigue, in fact a similar incidence to that seen in Rheumatoid Arthritis (RA) (Wolfe and Michaud 2004; Currey et al. 2003; Wolfe et al. 1996). Fatigue in people with OA tends to escalate during the day and can have more of a negative impact on physical activity than pain (Murphy et al. 2008). OA sufferers who complain of fatigue suggest it is like 'coming up against a brick wall' (Power et al. 2008: 4). The factors that have a reciprocal relationship with fatigue include sleep, depression, stressful events, older age, pain, pain medication, disability, physical function and social interactions (Murphy and Smith 2010; Stebbings et al. 2010; Murphy et al. 2008; Currey et al. 2003; Wolfe et al. 1996).

1.5.6 OA is associated with lower levels of social participation

Despite pain fatigue and functional restrictions knee and hip OA does not appear to lead directly to work loss through sick leave or early retirement in those of working age (Bieleman et al. 2011). However, OA does lead to difficulty achieving satisfactory participation in a variety of social roles, and this causes people with OA upset and dissatisfaction (Wilkie and Peat 2008; Gignac et al. 2008).

1.5.7 OA is associated with increased risk of depression

The prevalence of depression among patients who seek help with their OA is relatively high with approximately twenty per cent of people with OA in the primary care setting having

moderate to severe depression (e.g. Rosemann et al. 2007a) in comparison to a rate in the general population of approximately three per cent (Singleton 2001; Jenkins et al. 1997). One of the factors that influence people with OA to seek medical help is co-morbid depression (Dexter and Brandt 1994). Variance in depression among those with OA is explained largely by the level of perceived pain, social isolation, and physical limitation and these factors in turn relate to other risk factors for symptomatic OA including a high body mass index (Rosemann et al. 2008).

Depression is linked with reduced functional ability and activity in people with pain. The more physically disabled a person is by their pain the more likely they are to suffer from concurrent depression (McIlvane et al. 2007; Chou 2007; Mossey and Gallagher 2004; Williamson and Schulz 1992). The relationship between depression and functional ability is bidirectional: treatment of depression has been shown to reduce disability in pain patients (Lin et al. 2006; Lin et al. 2003) and improving activity levels through exercise programmes has improved the patients mood as well their pain (Lim et al. 2005).

There has been a long-standing observation that certain disorders involving inflammation have a tendency to be associated with depression, for example cardiovascular disease (Lippi et al. 2009; Halaris 2009; Panagiotakos et al. 2004), type 2 diabetes (Pickup 2004) and rheumatoid arthritis (Kojima et al. 2009). They share symptoms including sleep disruption, low mood, fatigue, and appetite changes and there is a high level of co-morbidity between painful disorders and depression. The relationship can be partially but not fully explained by the psychological burden of living with the disease or disorder (Pollak and Yirmiya 2002). There is a great deal of compelling evidence that shared neurochemistry is equally

responsible. The degree of inflammation in most OA cases is thought to be minor but evidence that some people have a greater inflammatory involvement may increase the likelihood of co-morbid depression.

1.5.8 OA is not associated with increased risk of anxiety

There is a positive correlation between anxiety and pain (Loncar et al. 2006; Velikova et al. 1995; Smedstad et al. 1995; Casten et al. 1995; Walsh 1993; Linton and Gotestam 1985) but anxiety is no more prevalent in the OA population than the general population (approximately 24%) (Kessler et al. 2003; Memel et al. 2000; Regier et al. 1988). One recent study of 54 consecutive OA patients attending a rheumatology clinic identified a prevalence of 31% using psychiatric diagnostic criteria (Axford et al. 2010), which suggests that anxiety is not a particular problem in this cohort as those attending such a clinic are likely to represent the more severely affected.

Anxiety in OA is associated with poorer function (Scopaz et al. 2009), which is mediated by fear-avoidance behaviour. Fear-avoidance behaviour describes how the fear of pain leads to the avoidance of activities or behaviours that the person believes will trigger pain. It was first described in the 1980s (Lethem et al. 1983) and is known to be associated with disuse, disability and hypervigilance (Lethem et al. 1983; Slade et al. 1983). Trait anxiety and anxiety-sensitivity (a heightened fear of anxiety-related symptoms) are both associated with pain-avoidance behaviour (Ocanez et al. 2010; Newcomer et al. 2010). This behaviour accounts for up to 40% of the variance in functional level in people with OA (Heuts et al. 2004).

Chapter 2 The pathophysiology and pain of OA

The traditional view of OA has been that it is a disease of wear and tear and in comparison to other forms of arthritis the degree of inflammation is thought to be low.

The focus for OA pathophysiology has been the cartilage but it is now clear that the whole joint is involved in the development and progression of OA. The pain of OA is initiated as a consequence of the production of a number of inflammatory mediators generated as a result of the changes within the joint.

2.1 Bone, cartilage and synovium as major components of the OA joint

2.1.1 Subchondral bone in the pathophysiology of OA

There is debate about whether bone changes lead to or are caused by changes to the cartilage (Martel-Pelletier et al. 2007) and although the debate continues there is evidence that subchondral bone attrition occurs before cartilage loss in the same sub-region (Roemer et al. 2009; Neogi et al. 2009a). Bone changes occur early in the development of OA before they can be detected radiographically (Hutton et al. 1986) and include increased thickness of the subchondral bone plate, alterations in the structure of the trabecular bone, and the development of osteophytes and cysts (Goldring and Goldring 2010a).

Bone is continually remodelled via the activities of osteoblasts, which construct new bone matrix and osteoclasts, which degrade it. Alterations in the structure of subchondral bone vary according to the stage of OA and the site of the bone (Goldring and Goldring 2010b). In early OA it is reported that an increased rate of resorption leads to a reduction of trabecular

thickness and an increase in the size of the trabecular spaces (Martel-Pelletier et al. 2007). The hardness of trabecular bone, one of its key qualities, is reduced in severe OA (Dall'Ara et al. 2011) and this likely results from alterations in its architecture and also its composition, with a reduced mineral content and an increased water content (Lories and Luyten 2011).

Bright areas visualised on MRI scans of subchondral bone represent a variety of alterations in the bone marrow including oedema, fibrosis and necrosis (Tanamas et al. 2010b). Collectively these changes are termed bone marrow lesions (BMLs). The cause of BMLs is poorly understood but they may be a response to an acute inflammatory event, oedema or contusion and gradually become replaced by more permanent bone marrow remodelling (Wildi et al. 2010). They are associated with cartilage loss (Hunter et al. 2006;Felson et al. 2003) and correlate with OA severity (Wildi et al. 2010;Tanamas et al. 2010b). There is debate about whether their presence is associated with pain in OA with some studies observing a relationship (Ip et al. 2011; Lo et al. 2009; Felson et al. 2007; Felson et al. 2001) and others not (Davies-Tuck et al. 2009; Kornaat et al. 2007).

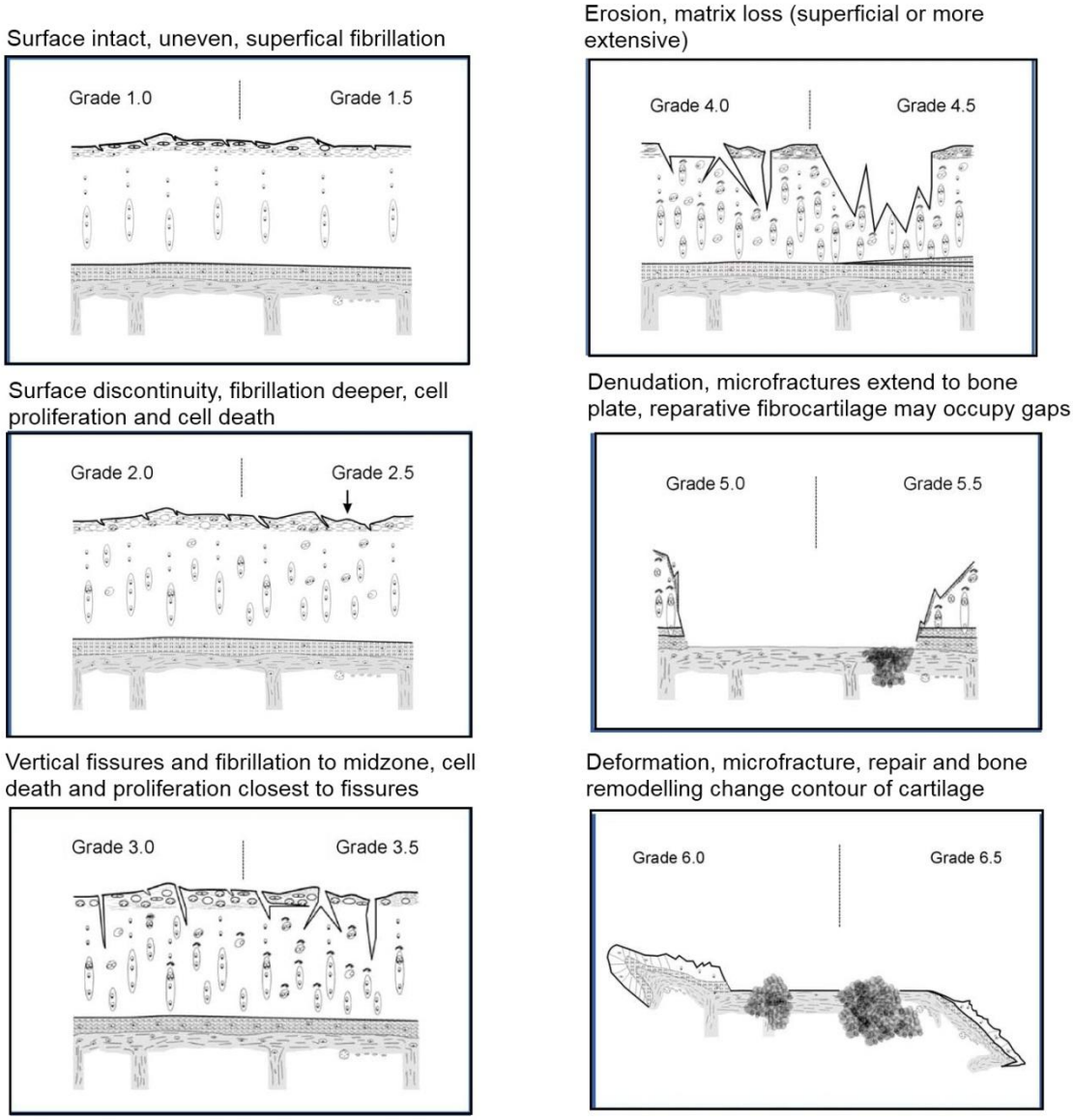
BMLs are often associated with subchondral bone cysts. These are thought to be formed following necrosis caused by collision between opposing joint surfaces, and are more likely to occur where there is significant cartilage erosion (Marra et al. 2008). Most progress but some also regress and they are associated with reduced cartilage volume and an increased risk of knee replacement (Tanamas et al. 2010a).

2.1.2 The cartilage in the pathophysiology of OA

In health the cartilage maintains a balance between synthesis and breakdown. In early OA there is an increase in cartilage synthesis (Pritzker et al. 2006) but later this balance tips in favour of degeneration (Goldring et al. 2006; Mandelbaum et al. 2005). Histological examination of cartilage shows focal changes associated with OA (Mankin et al. 1971) as opposed to the more widespread changes associated with rheumatoid arthritis. Radiographic and MRI studies of the cartilage show changes over time that seem to be associated with severity or progress of OA (Eckstein et al. 2011; Cibere et al. 2011; Hanna et al. 2009; Eckstein et al. 2009; Amin et al. 2009; Eckstein et al. 2006; Amin et al. 2005). The Osteoarthritis Research Society International (OARSI) have developed a grading system for histopathological changes (Figure 2-1) (Pritzker et al. 2006) which also serves to provide a visual representation of the longitudinal change of the cartilage.

Cartilage breakdown is catalysed by matrix metalloproteases (MMPs) and in healthy cartilage this activity is balanced by Tissue Inhibitors of MMPs (TIMPs). The expression of MMPs is upregulated by pro-inflammatory cytokines so that there is a imbalance in favour of MMPs over TIMPs (Hegemann et al. 2003). Pro-inflammatory cytokines also interfere with the synthetic activities of chondrocytes (Fernandes et al. 2002) and thus contribute to the destruction of the cartilage.

Figure 2-1: OARSI stages of the deterioration of articular cartilage in OA



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2.1.3 The synovium in the pathophysiology of OA

It is thought that breakdown products from the cartilage matrix are released into the synovial fluid and detected by the synovium, which leads to an inflammatory reaction (Pelletier et al. 2008). Synovitis can occur early in the course of OA and may not be clinically detectable.

According to arthroscopic studies it occurs in up to 50% of people with knee OA (Ayral et al. 2005). Moderate to severe synovitis is associated with increased WOMAC pain score (Guermazi et al. 2008). This degree of synovitis can be clinically detectable in many people due to the production of heat, swelling and effusion (Sellam and Berenbaum 2010; Benito et al. 2005). The symptoms and signs of synovitis tend to worsen along with the radiological progression of OA (D'Agostino et al. 2005; Ledingham et al. 1995). The severity of synovitis correlates with the degree of cartilage damage (Ayral et al. 2005).

Similar to rheumatoid arthritis (RA) macrophages are activated in the inflamed OA synovium producing pro-inflammatory cytokines and vascular endothelial growth factor (VEGF) (Benito et al. 2005; Farahat et al. 1993). The levels of pro-inflammatory cytokines are lower in OA than in RA however, and unlike the situation in RA the use of anti-TNFα therapy does not prevent the expression of IL-1β (Bondeson et al. 2006). Despite synovial inflammation being a localised condition in OA increased levels of IL-6 and C-reactive protein have been found in the circulation of patients with active synovitis (Pearle et al. 2007).

2.1.4 Angiogenesis in the pathophysiology of OA

Cartilage is normally avascular and is supplied with nutrients and oxygen by the synovium and subchondral bone (Martel-Pelletier, Lajeunesse, Reboul, & Pelletier 2007). There is consistent evidence that in OA, blood vessels penetrate the tidemark between the calcified and non-calcified cartilage from the subchondral bone to vascularise the cartilage (Walsh et al. 2007).

Angiogenesis of the cartilage from the subchondral bone is facilitated by the activity of the MMPs, which degrade the matrix creating space for new blood vessels and enhance processes relating to the endothelium of the blood vessels (Rundhaug 2005). The vascular channels formed also contain sensory and sympathetic nerve fibres and these may contribute to the pain of OA (Suri et al. 2007).

Epiphyseal growth involves the ossification of cartilage, with apoptosis of hypertrophic chondrocytes providing a structure that can be mineralised. This process is inhibited in normal cartilage because of the absence of blood vessels. The invasion of blood vessels and the production of angiogenic factors in OA can lead to the ossification of the cartilage (Babarina et al. 2001). This process contributes to the thinning of the articular cartilage.

Angiogenesis is also important in the development of osteophytes, bony spurs that form at joint margins by the same process of endochondral ossification (Moskowitz et al. 1981). Osteophytes are thought to contribute to the pain of OA (Neogi *et al.* 2009; Lanyon et al. 1998) as a combination of hypoxia, compressive forces and inflammation sensitise the sensory nerves that grow alongside the new blood vessels (Ashraf 2008).

Synovitis and angiogenesis have a reciprocal relationship where the activity of one influences the activity of the other (Ashraf and Walsh 2008; Afuwape et al. 2003). Inhibition of angiogenesis inhibits synovitis (Afuwape et al. 2003), and can disrupt its progression from an acute to a chronic condition (Ashraf et al. 2010). Synovitis stimulates angiogenesis by the secretion of VEGF from macrophages, angiogenic factors from endothelial cells and fibroblasts, and the presence of pro-angiogenic inflammation-induced hypoxia, (Bonnet and Walsh 2005).

Newly formed blood vessels contribute to oedema; inflammatory mediators are attracted to and can reach the site, and there may be difficulty modulating inflammation due to deficiency in neural and peptide regulatory factors (Bonnet and Walsh 2005). The angiogenesis of the synovium therefore contributes to the inflammatory process, and facilitates it progression from an acute state to a chronic state.

2.1.5 Pannus in OA

Often people with OA will also have localised patches of pannus-like tissue, a thin layer of tissue over-laying the articular surface (Shibakawa et al. 2003). Shibakawa *et al.* (2003) characterise the pannus-like tissue as being either vascular or fibrous in character. It produces catabolic factors such as IL-1β and MMPs and therefore contributes to the destruction of the cartilage. Its origins are not clear. In RA the pannus originates from the synovial membrane but this is not the case in OA and it is felt that it probably originates from either the mesenchymal cells or from the cartilage itself, (Yuan et al. 2004; Shibakawa et al. 2003).

2.2 Pain in OA

2.2.1 Nociception in the joint

Nociception is the sensory process that translates noxious (an actual or potentially damaging) stimuli into signals that will ultimately be interpreted by the brain as the perception of pain. The instruments of nociception are sensory nerve endings, also known as nociceptors.

There are two main types of sensory fibre that terminate in nociceptors, the small unmyelinated C fibre and the fast myelinated Aδ fibre. The C-fibre has a relatively slow transmission speed (2 m s^{-1}) and can be subdivided into polymodal nociceptors that respond to

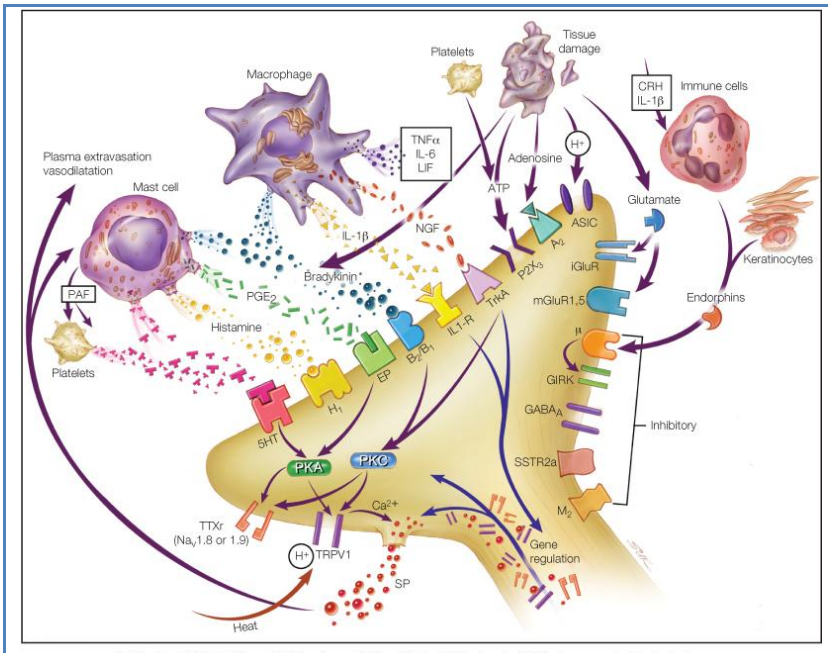
a number of different stimuli, and those that respond to a single stimulus type for example chemical, thermal or mechanical. A proportion of C fibres are called 'silent' because they don't respond to noxious stimuli unless there is inflammation or tissue damage present. C-fibres are found in the synovial layer, fibrous capsule, fat, ligaments, periosteum and menisci contain terminals of Aδ and C fibres (Schaible 2006) and the infrapatellar fat pad (Dye et al. 1998). Normal cartilage does not contain nociceptors but the process of angiogenesis in OA is accompanied by neurogenesis so that cartilage becomes responsive to noxious stimuli (Suri et al. 2007).

The Aδ fibres (20 m s^{-1}) are responsible for the transmission of short, sharp sensation, for example noxious pinch. Aδ fibres are found in the same tissues as C-fibres. A third type of sensory fibre called the Aβ fibre, is not involved in pain transmission in normal conditions but detects information about non-noxious touch, vibration and pressure (Millan 1999). The fibrous capsule of the joint, ligaments, menisci and periosteum contain thick myelinated Aβ nerve endings.

There are a number of conditions that will lead to initiation of pain signals from within the synovial joint. Movement outside the normal range activates a number of Aβ, Aδ and C fibres that are sensitive to movement and/or pressure (Schaible 2006; Schaible and Grubb 1993). The sensitivity of these fibres increases in inflammatory conditions and they show increased responses in the normal working range of the joint and some spontaneous activity when the joint is at rest (Grigg et al. 1986; Coggeshall et al. 1983). This leads to hyperalgesia in the joint (Grubb et al. 1991).

Synovial inflammation leads to plasma extravasation and this increases the volume of synovial fluid, thus increasing intra-articular pressure. Increased pressure correlates positively with increased pain (Goddard and Gosling 1988). Goddard and Gosling demonstrated that medial rotation or extension of the hip, which are associated with increased pain in people with OA, causes an elevation of intra-articular pressure.

Figure 2-2: Chemical mediators of pain in the periphery



Reprinted including text with Permission: Potential peripheral mediators of peripheral sensitization after inflammation. Inflammation leads to the release of numerous chemicals from mast cells, macrophages, immune cells, and injured cells that may act directly or indirectly to alter the sensitivity of peripheral nerve terminals. ASIC, acid-sensing ion channel; CRH, corticotrophin-releasing hormone; GIRK, G-protein-coupled inward rectifying potassium channel; iGluR, ionotropic glutamate receptor; IL-1β, interleukin-1β; IL-6, interleukin-6; mGluR, metabotropic glutamate receptor; NGF, nerve growth factor; PAF, platelet-activating factor; PGE2, prostaglandin E2; PKA, protein kinase A; PKC, protein kinase C; SSTR2A, somatostatin receptor 2A; TNF-α, tumour necrosis factor-α; TrkA, tyrosine kinase receptor A; TTXr, tetrodotoxin-resistant sodium channel; μ, mu-opioid receptor; M2 muscarinic receptor; 5HT, serotonin; LIF, leukemia inhibitory factor. (Artwork by Ian Suk, Johns Hopkins University, adapted from Woolf & Costigan 1999.)

2.2.1.1 Initiation of pain in the joint

The process of cartilage breakdown is driven by the production of a number of pro-inflammatory and excitatory mediators. Each of these acts as a ligand in conjunction with

specific receptors expressed on the surface of sensory nerve fibres and immune cells (Figure 2-2).

2.2.1.2 Inflammatory mediators in the pathophysiology of OA

Chondrocytes, mononuclear cells, osteoblasts, synoviocytes and adipocytes of the infrapatellar fat pad can all produce the pro-inflammatory cytokines interleukin 1-beta (IL-1β), interleukin-6 (IL-6) and tumour necrosis factor alpha (TNFα). In OA the local concentration of these cytokines increases in synovial fluid, synovial membrane, (Sellam and Berenbaum 2010) subchondral bone, (Lisignoli et al. 1999) cartilage (Saklatvala 2007) and muscles (Levinger et al. 2011).

IL-1β and TNFα receptor expression is increased on fibroblasts and chondrocytes (Fernandes et al. 2002; Alaaeddine et al. 1997; Sadouk et al. 1995) (Martel-Pelletier et al. 1992). This makes these cells in OA more sensitive to IL-1β and TNFα activation thus enhancing production of MMPs (Goldring and Berenbaum 2004; Martel-Pelletier et al. 1992).

IL-1β and TNFα are also involved in the production of nitric oxide (NO) and prostaglandin E2 (PGE2) (El Mansouri et al. 2011). NO plays a role in suppression of the synthesis of the matrix (Taskiran et al. 1994), inhibits chondrocyte proliferation and induces apoptosis of chondrocytes (Blanco and Lotz 1995; Blanco et al. 1995). PGE2 is one of the major mediators involved in destruction of the cartilage and the progression of OA (Li et al. 2009; Martel-Pelletier et al. 2003; Hardy et al. 2002).

IL-6 is produced in low levels by normal chondrocytes but its production is upregulated by IL-1β, TNFα (Bender et al. 1990; Guerne et al. 1990; Guerne et al. 1989) and PGE2 (Wang et

al. 2010). IL-6 and its soluble receptor (sIL-6R) are also produced by the infrapatellar fat pad (Distel et al. 2009). IL-6 and sIL-6R reduce proteoglycan synthesis and stimulates the production of NO in the cartilage (Guerne et al. 1999).

2.2.1.3 Anti-inflammatory mediators in the pathophysiology of OA

A number of other cytokines are involved in the maintenance of the cartilage. Chondrocytes respond to the process of degradation by increasing the synthesis of matrix components and releasing anti-inflammatory cytokines (IL-4, IL-10, IL-13). These anti-inflammatory cytokines can reduce TNFα-induced PGE2 release (Alaaeddine et al. 1999). IL-4 can down-regulate the expression of pro-inflammatory cytokines (Martel-Pelletier et al. 1999) and their downstream products such as MMPs and NO (Fernandes et al. 2002; Nishisaka et al. 2001; Nemoto et al. 1997). However, soluble IL-4 receptor (sIL-4R) is found in increased levels in the serum of people with OA and it is thought that the increased ratio of sIL-4R to IL-4 reduces the availability of IL-4 to chondrocytes and therefore inhibits its normally protective function (Silvestri et al. 2006).

2.2.2 Osteoarthritis as an inflammatory disease

Having explored the peripheral mechanism of OA it is clear that there are inflammatory processes involved. It seems clear that OA is an inflammatory arthritis but in degree it differs greatly from a systemic inflammatory disease such as Rheumatoid Arthritis (RA) and examination of serum and synovial fluid supports this.

Measures of systemic inflammation such as C-reactive protein tend to be much lower in OA than Rheumatoid Arthritis (RA) (Mahmoud et al. 2005; Sitton et al. 1987) although the

correlation of this much-relied upon inflammatory with disease activity in RA is thought by some to be weak (Keenan et al. 2008).

Other markers of inflammatory processes found in the serum and synovial fluid of people with OA and RA also suggest a much more active and systemic inflammatory process is present in RA compared with OA. Comparison of serum in the two diseases has identified higher levels in RA of nitrates and nitrates (Ersoy et al. 2002), advanced glycation end (AGE) product pentosidine (Chen et al. 1999), adipocytokines such as resistin and leptin (Ibrahim et al. 2008), Tumour Necrosis Factor alpha (TNFα) and matrix metalloprotease-3 (Mahmoud et al. 2005). In synovial fluid RA patients have higher levels of pro-inflammatory cytokines such as IL-1β, IL-6, TNFα, IFNγ (Kokebie et al. 2011; Manicourt et al. 2000;Schlaak et al. 1996), adipocytokines (Ibrahim et al. 2008), matrix metalloproteases 1 and 3 (Mahmoud et al. 2005) and VEGF (Biswas et al. 2011).

There are similarities between the pathophysiology of RA and OA such as the increased expression of pro-inflammatory cytokines in both diseases and an increased immune response (Biswas et al. 2011; Leheita et al. 2005) as well as broadly similar histopathological changes in the synovium in OA and RA without effusion (Baeten et al. 2000).

2.2.3 Primary afferent fibre terminations in the dorsal horn

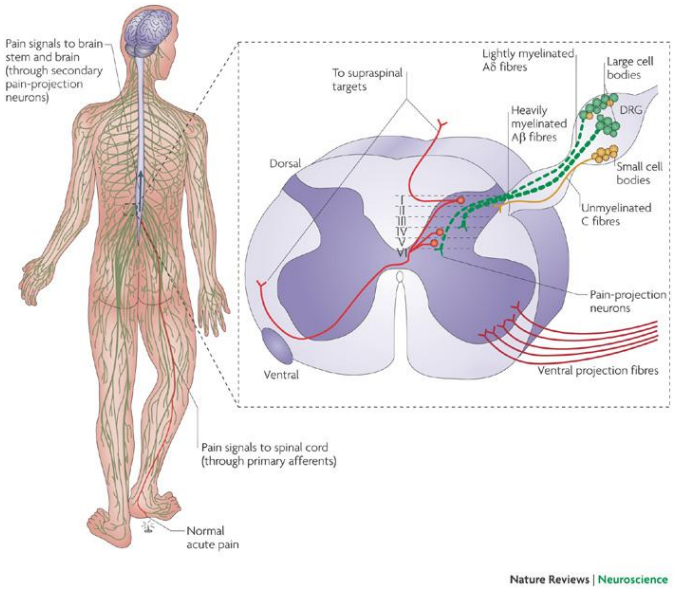
The central endings of the primary afferent fibres lie within the laminae or layers found within the spinal cord. Although there is overlap at the edges of each the structure and function of each lamina are different (Table 2.1,and Figure 2-3).

Table 2.1: Dorsal horn nociceptive neuron types and termination within the dorsal horn

Type of neuron	Location in dorsal horn laminae (L)	Stimulus type that initiates response	Primary afferent terminations	Receptive field
Nociceptive neurons	L1, LII Also LV, LVI, LVII, LX	High intensity peripheral stimuli	Aδ, C	Limited
Nonspecific nociceptive neurons (Wide Dynamic Range)	Primarily LV Some LI , LII	High and low intensity peripheral stimuli	Aβ, Aδ,C	Variable but can be extensive. Activity greatest when centre of field stimulated.

The locations of the terminals of the primary afferents is highly organised: C and Aδ fibres terminate largely in lamina I and II and some in V, while Aβ fibres terminate largely in the deeper laminae (Willis and Coggeshall 2004) (Figure 2-3).

Figure 2-3: Sensory nerve pathways and terminations in the dorsal horn



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The location of the receptive fields of the primary afferents translates to the location of their terminals within the dorsal horn so that a somatotopic map is created. The arbors of the central terminals also run rostrocaudally and are able to excite hundreds of neurons each (Woolf and Salter 2006).

2.2.3.1 Classes of dorsal horn neurons

DH neurons can be divided into three classes: projection neurons, propriospinal neurons and local interneurons (Willis and Coggeshall 2004). Projection neurons transfer information from the DH to the brain and also can initiate descending facilitation and inhibition. Propriospinal neurons communicate between spinal segments and have a role in reflex responses as well as movement. Interneurons are localised neurons whose axons travel only a short distance within the spinal cord and may be either excitatory or inhibitory.

Projection neurons can be categorised as nociceptive specific or non-specific nociceptive cells. The latter are more commonly known as wide dynamic range cells. Nociceptive specific cells are located primarily in lamina I (LI) and to a lesser extent in LII, VI, VII and IX. These cells respond to high intensity stimuli and have a limited receptive field. The wide dynamic range (WDR) cells are located predominantly in LV and to a lesser extent in LI and LII and respond to stimuli of varying intensities. The WDR cells increase the frequency with which they fire according to the intensity of the stimulus they receive and therefore can code stimulus intensity.

The WDR cells receive input from non-nociceptive A-β fibres as well as input from nociceptive C and A-δ fibres. Their receptive fields are much larger than the nociceptive-

specific cells and their response varies according to the origin of the stimulus, being greatest when the stimulus originates close the centre of the receptive field and decreasing as the stimulus moves closer to the outer edges (Calvino and Grilo 2006). The receptive fields of WDR cells overlap to some extent and both innocuous and noxious stimuli can activate more than one WDR. However, tactile (mechanical, such as touch) stimuli activate a limited number of neurones; those whose receptive field centres are close to the site of stimulation. Noxious stimuli activate more WDR cells, those whose receptive field centres are close to the site of stimuli and also those whose receptive field fringes overlap with the site of stimulation (LeBars 2002).

Inflammation leads to an increase in the size of the receptive field via two mechanisms. Primary hyperalgesia is the production of algesic substances local to the site of inflammation. These substances activate silent nociceptors and also lower the activation threshold of nociceptors creating a situation where sub-threshold stimuli can lead to the generation of action potentials. This leads to an increased barrage of afferent impulses reaching the dorsal horn cells which creates an amplification process.

2.2.3.2 Synaptic transmission of pain signals in the dorsal horn

Depolarisation of the primary afferent fibre leads to the release of glutamate and other neurotransmitters. Glutamate binds with and activates ion channel α-amino-hydroxy-5-methyl-4-isoxazoleproprionic acid (AMPA) receptors. This causes a sub-threshold change in amplitude that can summate leading to the generation of an action potential (Woolf and Salter 2006). AMPA receptors are also found on the primary afferent fibres and modulate the release of glutamate from the primary afferent fibres (Lu et al. 2002; Ferreira and Lorenzetti

1994). AMPA receptors also contribute to the plasticity of the dorsal horn and long-term potentiation (Lee et al. 2003) both by alteration of their structure and their quantity as a result of synaptic activity.

Intense pain or burst firing of primary afferent fibres leads to the release of other transmitters such as neuropeptides and growth factors including Substance P, Calcitonin Gene Related Peptide (CGRP), and Brain Derived Neurotrophic Factor (BDNF), from the primary afferent fibres (Benarroch 2010). These transmitters lead to slow post-synaptic depolarisations lasting tens of seconds (Yaksh 2006). The result is an increase in intracellular calcium caused by summation of trains of signals from primary afferent fibres and leads to release of the magnesium ion block in the N-methyl-D-aspartate (NMDA) receptor ion channel.

The NMDA receptor can be activated by glutamate providing a suitable co-agonist is present. The co-agonist can be either glycine (Johnson and Ascher 1987) or D-serine (Wolosker 2007; Schell et al. 1997; Schell et al. 1995; Kleckner and Dingledine 1988). Serine is produced by activated glial cells (Halassa and Haydon 2010). Animal studies have shown that increasing the amount of either serine or glycine in the extracellular fluid enhances NMDA receptor activity (Zhang et al. 2001b; Wood 1995).

Activation of the NMDA receptor leads to a prolonged excitatory post-synaptic potential in comparison to the transient potentials mediated by non-NMDA receptors like the AMPA receptor (Mori and Mishina 1995). It also leads to a greater increase in intracellular calcium levels. The consequences of increased intracellular calcium levels include the production of nitric oxide (NO), and prostaglandin, molecules that contribute to enhanced release of glutamate from the primary afferent fibre, an amplification mechanism.

NO is created from arginine and this process also generates the by-product citrulline (Garthwaite 1991). NO is a gaseous molecule that is synthesised on demand and cannot be stored in the cell. NO diffuses to its site of action in the primary afferent fibre where it leads to increased release of glutamate from the primary afferent fibre (Vetter et al. 2001; Kawamata and Omote 1999).

Prostaglandins are produced as a consequence of increased intracellular Ca^{2+} and presence of extracellular cyclo-oxygenase (cox) enzymes (Yang et al. 1996). The process is modulated by glial uptake of glutamate from the synaptic cleft (Svensson et al. 2003).

An increase in CSF prostaglandins, especially PGE2 has been demonstrated to follow peripheral inflammation in some animal models, a process that appears to be dependent on central induction of cox-2 from spinal neurons by interleukin 1 beta (IL-1β) (Hosl et al. 2006; Samad et al. 2001) in a process that is driven by both neural and humoral communication from the periphery (Samad et al. 2001).

Prostaglandins contribute to an increase in pain in two ways. Firstly activation of the PGE2 receptor on projection neurons disrupts the inhibitory action of glycine at the glycine receptor (Reinold et al. 2005; Zeilhofer 2005; Harvey et al. 2004). Secondly activation of prostaglandin EP receptors, which are expressed in primary afferent fibres (Bar et al. 2004) increase influx of Ca^{2+} into the primary afferent fibre and facilitate the release of neurotransmitters like glutamate thus amplifying pain transmission.

2.2.4 Glia and cytokines in pain and mood

Relatively recently it has been discovered that transmission of pain signals does not just involve primary afferent fibres, interneurons and projection neurons. The term the tri-partite synapse refers to an evolution in understanding of synaptic transmission and we now appreciate that glial cells form a third and important component of synaptic transmission (Halassa et al. 2007; Nadkarni and Jung 2007).

Glial cells comprise approximately 70% of the cell population in the central nervous system (Inoue and Tsuda 2009). There are two types of glial cells found within the dorsal horn, astrocytes and microglia. Astrocytes perform a supportive role to the nerve cells and closely associate with synapses communicating with those cells by gap junctions (McMahon and Malcangio 2009). Microglia are the resident macrophages of the CNS and make up about 10% of the glial population (Stoll and Jander 1999). In physiological conditions glial cells are vital to homeostasis but they can become activated by afferent pain signalling and contribute to the amplification and maintenance of pain.

2.2.4.1 Activated glial cells as modulators of pathological pain

Glia become activated in response to peripheral nerve or tissue injury via activation of a number of cell surface receptors including AMPA and NMDA (glutamate), and NK1 (Substance P) (Abbadie et al. 2009; Milligan and Watkins 2009; Tsuda et al. 2009). The activation of these receptors leads to an excitatory response with the release of glutamate, ATP, Tumour Necrosis Factor-α (TNFα), IL-1β (interleukin 1-β), IL-6 (interleukin-6), NO, prostaglandin, and Brain Derived Neurotropic Factor (BDNF). In this way glial cells contribute to the amplification and facilitation of pain in pathological conditions.

Serine and GABA are also produced by glia (Lee and Soltesz 2011; Rao et al. 2003). Their release from the glia is enhanced by agonism of non-NMDA glutamate receptors (Schell et al. 1995), in other words, the release of serine and GABA from glia can be stimulated by the glutamate increasing levels of extracellular glutamate.

2.2.4.2 Pro-inflammatory cytokines and pain signalling

Cytokines are large peptides that have a key role in the regulation of the inflammatory response; their major function is inter and intracellular communication (Cunha and Ferreira 2003). The three main pro-inflammatory cytokines IL-1β, IL-6 and TNFα have well documented pronociceptive effects within the dorsal horn. Other pro-inflammatory cytokines include IL-2, IL7, IL-8 and IL-12; although there is less published about this latter group in terms of dorsal horn activity their role is beginning to be defined.

IL-1β enhances NMDA receptor phosphorylation (Zhang et al. 2008a; Guo et al. 2007) and calcium influx via that receptor (Kawasaki et al. 2008; Viviani et al. 2003). IL-1β also suppresses GABA and glycine inhibitory post-synaptic currents (Kawasaki et al. 2008) and so this cytokine facilitates pain both by enhancing the excitatory process and also by disinhibition. Increased IL-1β in an animal model of inflammatory pain (Complete Freund's Adjuvant to the rat hind paw) is mediated by both humoral and neural mechanisms (Samad et al. 2001; Cartmell et al. 2000) and is associated with an increase in cox-2 (Samad et al. 2001) (see also 2.2.4.4).

IL-2 is released from activated glia (Labuzek et al. 2005) and is thought to have a biphasic effect, at lower doses producing a thermal anti-nociception but at higher doses producing mechanical and thermal hyperalgesia (Cata et al. 2008).

IL-6 has both anti- and pro-inflammatory functions. It is involved in the production of IL-1 receptor antagonist, a soluble antagonist that inhibits the activity of IL-1 and as a result of this IL-6 has an anti-inflammatory action (Jordan et al. 1995). Following nerve injury IL-6 has an inhibitory action on primary afferent fibres (Flatters et al. 2003). IL-6 also has a pro-inflammatory function via the induction of IL-1β and TNFα, increasing microglial activation and increasing cox-2 mRNA (Schoeniger-Skinner et al. 2007).

IL-8 is produced by activated astrocytes in response to pro-inflammatory stimuli (Meeuwsen et al. 2003). In animal models of discogenic pain its release from macrophages within the disc has been identified as a key pro-inflammatory process and independent from TNFα in terms of the cytokine cascade. This means that ameliorating the effect of IL-8 would require it to be targeted specifically (Takada et al. 2012; Kim et al. 2011b).

Interferon-γ (IFNγ) activates microglia in the spinal cord (Tsuda et al. 2009) and promotes the expression of other pro-inflammatory cytokines from the glial cells (Dong et al. 2001;Satoh and Kuroda 2001). IFNγ has been demonstrated to enhance pain response to stimulus via disinhibition of GABA receptors (Vikman et al. 2007; Vikman et al. 2003).

TNFα is expressed by activated astrocytes and microglia (Shen et al. 2009; DeLeo et al. 2000). Two TNFα receptors (TNFR1 and TNFR2) have been located in the dorsal horn. TNFR1 are found on DRG neurons and also non-neuronal cells, while TNFR2 are found

exclusively on non-neuronal cells (Li et al. 2004). Both receptors are upregulated after nerve injury (Schafers et al. 2003). TNFR1 activation leads to an upregulation in IL-6 in the spinal cord and DRG (Lee et al. 2009).

TNFα released by glial cells acts on post-synaptic TNF receptors leading to an increase in the expression of AMPA receptors, (Stellwagen et al. 2005; Beattie et al. 2002) which will lead to an increase in post-synaptic cell permeability to calcium. Stellwagen et al also demonstrated that TNFα decreases the post-synaptic expression of $GABA_A$ receptors and this further shifts the excitatory/inhibitory balance in favour of excitation.

Recent evidence has shown the microglial production of nitric oxide and TNFα can be inhibited by use of a selective serotonin inhibitor via IFNγ inhibition (Horikawa et al. 2010).

2.2.4.3 Anti-inflammatory cytokines and pain signalling

IL-10 is an anti-inflammatory cytokine that has the capacity to reverse the excitatory effects of the pro-inflammatory cytokines. Intrathecal quisqualic acid produces an excitotoxic injury in rats which is accompanied by increases in TNFα and IL-1β and pain behaviour; systemic injection of IL-10 given within 30 minutes of the intrathecal injection prevents or delays much of the process (Plunkett et al. 2001).

The ability of IL-10 seemingly to halt some of the damage and changes caused in neurogenic insults has led to investigations into the potential therapeutic effects of this cytokine (Lau et al. 2012; Sloane et al. 2009; Zhou et al. 2008; Milligan et al. 2006).

Less work has been published on the roles of other anti-inflammatory cytokines such as IL-4 and IL-5 in the CNS but IL-4 has also been demonstrated to have the ability to reverse behavioural and biochemical manifestations of neuropathic pain (Hao et al. 2006).

2.2.4.4 Neural and humoral activation of dorsal horn glia in inflammatory conditions

IL-1β, IL-6 and TNFα concentrations in the cerebrospinal fluid and markers of glial activation are noted in animal models of inflammatory pain (Guo et al. 2007; Raghavendra et al. 2004; Bao et al. 2001). Guo *et al* established that glial activation could be prevented by local anaesthetic block of neuronal input and proposed that inflammatory activation of glia is due to neuronal signalling via the primary afferent fibres. This mechanism is supported by the observation that the onset of fever is rapid (10-12min) and the transcription and production of cytokines from immune cells takes up to 30min. A quick response is mediated by the vagus nerve (Blatteis et al. 2005) which through its wide innervation is able to transmit immune information to the central nervous system and vice versa (Eskandari et al. 2003; Hosoi et al. 2002).

In addition to the neuronal signalling of peripheral inflammation there is also a humoral route. A number of different mechanisms have been proposed for this. The process of active transport of cytokines across the blood brain barrier (BBB) has been supported by the findings of several studies in which radio-labelled cytokines including IL-1α, IL-1β, IL-6 and TNFα were tracked across the blood brain barrier (Banks et al. 1994; Gutierrez et al. 1994; Gutierrez et al. 1993; Banks and Kastin 1992; Banks et al. 1991; Banks and Kastin 1991; Banks et al. 1989).

The second mechanism is activation of receptors on the endothelial walls of capillaries forming the blood brain barrier. The cells of the BBB respond to immune cell-mediated activation or lipopolysaccharide by releasing cytokine. This process can be initiated from either direction (Verma et al. 2006). The circumventricular organs (CVO), which include the pineal gland and posterior pituitary are located close to the ventricles and do not have a complete blood brain barrier (Gross 1992). This enables the direct secretion of hormones into the blood stream (Peruzzo et al. 2000). Any cytokines crossing here from the blood stream cannot proceed to the brain due to the protection of specialised cells called tanycytes (*ibid)* located in the ventricular walls. The cytokines instead interact with neural projections connecting these areas with other parts of the brain (Banks and Erickson 2010).

The consequence of these interactions of cytokines with receptors in these regions would be the release of prostaglandins, cytokines and other mediators into the central nervous system using a mechanism whereby a relatively small quantity of blood borne cytokine could be amplified to provide a strong signal.

While there is evidence to support all these mechanisms of communication there is also evidence that suggests that their reliance on blood borne cytokine communication is unlikely to be the predominant method of communication.

Maier et al. (1998) propose that the predominant communication pathway between the immune response and the central nervous system is via the vagus nerve. The vagus innervates widely including lymph nodes and tissues involved in the immune response, it also terminates in the nucleus tractus solitarius in the brainstem, an area that is highly involved in the 'sick response'. Sub-diaphragmatic vagotomy has been used to demonstrate that this is a key

pathway for immune-brain communication and Maier *et al*. point out that cytokines are also known to signal via the activation of other nerves.

2.2.5 Supraspinal modulation of pain and links with mood

Pain signals are not only subject to modulation from within the dorsal horn but are also influenced by feedback via the descending pathways. Descending influences on pain are both facilitatory and inhibitory and the degree of pain experienced depends on the balance between the two. Many of the substrates and pathways involved in supraspinal facilitation and inhibition of pain are also involved in the mediation of anxiety, depression and stress and therefore this section will combine a discussion of the processes of both to highlight where interaction occurs.

There are three key brainstem centres that are of concern for descending modulation of pain; the periaqueductal grey (PAG), the rostroventral medulla (RVM) and the locus coeruleus (LC). Persistent tissue injury leads to excitation of the descending modulatory systems, both facilitatory and inhibitory (Miki et al. 2002).

The PAG is situated in the midbrain. It receives nociceptive input and facilitates pain-related behaviours, cardiovascular changes (Dostrovsky and Craig 2006), anti-nociceptive pain modulation (Millan 2002) and opioid tolerance (Tortorici et al. 2009; Tortorici et al. 2001). The PAG receives inputs from a number of supraspinal sites including the hypothalamus, limbic system and amygdala, and the anterior cingulate cortex. These connections facilitate the integration of autonomic, affective and cognitive information with nociceptive information (Millan 2002).

The PAG projects to the RVM and the LC. The RVM also receives extensive input from other supraspinal structures enabling integrate autonomic and sensory information (Millan 2002). The RVM is rich in serotonergic cells and projects direct to the dorsal horn. The LC is rich in noradrenergic cells and also projects to the dorsal horn.

2.2.5.1 Pain, mood and 5-hydoxytryptamine

The RVM contains the Nucleus Raphe Magnus (NRM), an area rich in serotonergic (5-HT producing) cells (Hentall et al. 2006). The cells contained within this structure include so-called On-cells, off-cells and neutral cells. The on-cells have a facilitatory effect on pain processing via descending pathways connecting with the dorsal horn, and their activity can be depressed by opioids. Off-cells have an inhibitory effect. Active discharge of RVM on-cells is required to sustain allodynia and injection of local anaesthetic into this area leads to a reversal of these symptoms (Ossipov et al. 2000).

5-HT fibres project to the dorsal horn from the RVM and synapse with neurons within the dorsal horn, predominantly in the superficial laminae (Mason 2001; Calejesan et al. 1998; Aimone et al. 1987) and via secondary connections have influence over the cells in the deeper dorsal horn (Heinricher et al. 2009). 5-HT contributes to pain inhibition via activation of spinal receptors on inhibitory interneurons leading to the release of gamma amino butyric acid (GABA).

Increasing the availability of 5-HT by use of re-uptake inhibitors such as the tricyclic antidepressants and serotonin and noradrenaline re-uptake inhibitors has a long-recognised role in the management of pain, initially focused on neuropathic pain (Saarto and Wiffen

2007) but now also being explored in other types of pain including OA (Citrome and Weiss-Citrome 2012). The use of these drugs is associated both with pain relief and alleviation of low mood (Demyttenaere et al. 2012; Hegerl et al. 2012).

The role of 5-HT in anxiety, stress and depression is mediated via the hypothalamic pituitary adrenal (HPA) axis. Acute stress is associated with analgesia because it increases activity of 5-HT neurons (Abbott et al. 1986). However, stress can also have the opposite effect due to corticotropic releasing factor mediated inhibition of the 5-HT producing neurons (Kirby et al. 2008; Price et al. 2002; Kirby et al. 1995).

The level of 5-HT in the CNS is dependent on the level of tryptophan, from which it is synthesised. Tryptophan is actively transported across the blood brain barrier in a quantity dependent on its serum concentration relative to the concentration of other large neutral amino acids in the blood that compete for the same transport system (Frazer et al. 1999). These amino acids, (including tyrosine, phenylalanine, valine, leucine and isoleucine), outnumber tryptophan by approximately 8:1 (Baumann 1985). The ratio of tryptophan to these competing amino acids has been shown to be lower in severe depression than in normal subjects or those with minor depression (Maes et al. 1993). A reduced ratio will lead to reduction in the central availability of tryptophan for metabolism to serotonin. This has led to some efforts to use tryptophan supplementation as a pain-relieving agent with equivocal results (Ceccherelli et al. 1991; Ekblom et al. 1991; Stockstill et al. 1989; Seltzer et al. 1982).

Another way in which 5-HT availability is reduced in the CNS is by pro-inflammatory cytokine mediated switch from the manufacture of 5-HT from tryptophan to the manufacture instead of quinolinic acid (QA) (Muller and Schwarz 2007). One of the factors that is known

to influence this process is depression – which is associated with both an increase in the concentrations of pro-inflammatory cytokines and the preference for the kynurenine pathway (leading to the production of QA) (Laugeray et al. 2010; Swardfager et al. 2009; Miller et al. 2008; Myint et al. 2007). Increased levels of cytokine-induced QA are associated with higher levels of depression (Raison et al. 2010). Pro-inflammatory cytokines also enhance 5-HT transporter function whereas IL-4, an anti-inflammatory cytokine, decreases 5-HT uptake (Mossner et al. 2001) and so cytokine activity in the CNS can affect 5-HT availability in a number of ways.

2.2.5.2 Pain, mood and noradrenaline

The locus coeruleus (LC) is part of the pons and produces noradrenalin (NA) in response activation by acute pain signalling from the dorsal horn (Jones 1991). It connects extensively to other supraspinal regions and receives input from the PAG. Descending NA pathways to the dorsal horn from the LC reduce pain (Jones and Gebhart 1988) by activating α1 receptors on interneurons to release GABA and glycine (Gassner et al. 2009; Baba et al. 2000). NA also acts by volume transmission on α2 adrenoreceptors, which are found mainly on terminals of primary afferent fibres and projection neurons mainly in laminas I and II of the dorsal horn (Pertovaara 2006; Millan 2002; North and Yoshimura 1984). NA leads to pain reduction via α2 adrenoreceptors by hyper-polarisation (North and Yoshimura 1984), depressing the release of Substance P (Kuraishi et al. 1985) and glutamate (Pan et al. 2002; Ueda et al. 1995).

Stress and depression increase levels of corticotrophin releasing factor (CRF) in the CNS (Hauger et al. 2009) including the LC and this stimulates increased production of noradrenalin (NA) released into the cortex (Curtis et al. 1997). NA in turn stimulates the increased release

of CRF (Chrousos 1998). This suggests that stress will lead to an increase in NA and this has been demonstrated in combat veterans with post-traumatic stress disorder who have higher levels of CSF NA than healthy normal controls (Geracioti, Jr. et al. 2001) and triggering increased stress in these individuals led to an increase in CSF NA (Geracioti, Jr. et al. 2008).

Wong et al. (2000) found CSF NA was elevated around the clock and was closely associated with hypercortisolism in people with melancholic depression. Others have found no difference in CSF NA between people with depression and normal controls (Placidi et al. 2001; Geracioti, Jr. et al. 1997a; Gjerris et al. 1987b). Acute pain is associated with an increase in CSF NA (Eisenach et al. 1996) but not low back pain (Hyyppa et al. 1985) or trigeminal facial pain (Bouckoms et al. 1992). There has been one report of CSF NA being elevated in chronic pain which correlated with the duration of the pain but did not correlate with pain intensity or depression (Strittmatter et al. 2005).

The interpretation of NA levels is complicated. Although NA has an inhibitory effect via its interaction with primary afferent fibres and interneurons in the dorsal horn, and therefore increased CSF levels of NA might suggest pain inhibition, it can have a facilitatory effect via α1 adrenoreceptors in the dorsal reticular nucleus, a part of the brain that has a significant role in the facilitation of pain (Martins et al. 2010).

2.2.5.3 Pain, mood, GABA and glycine

Interneurons are intrinsic cells within the central nervous system and within the dorsal horn are described as being excitatory cells (containing glutamate) or inhibitory (containing gamma-amino-butyric acid (GABA) and/or glycine) (Todd and Spike 1993). The knowledge

that has accumulated to date about interneurons has mainly been able to detail the inhibitory role. Approximately 30% of the interneurons in lamina I and II and 45% in lamina III have been shown to contain GABA and are therefore inhibitory (Todd and Sullivan 1990) and it has been assumed that all interneurons that are not GABAergic are likely to be glutamatergic (Todd and Spike 1993).

In addition to 5-HT and NA evoked release of GABA and glycine there is also evidence that Brain Derived Neurotrophic Factor (BDNF) released from activated glia also stimulates the release of these two inhibitory modulators (Bardoni et al. 2007). BDNF can also lead to a reversal in polarity of the GABA and glycine receptors so that in certain conditions (such as those produced by neuropathic pain) GABA and glycine have an excitatory effect (Molinaro et al. 2008; Coull et al. 2003).

Glycine and GABA are often co-localised and released in the same vesicles from spinal cord interneurones (Jonas et al. 1998) which synapse pre and post-synaptically with neurons in the dorsal horn. Although the two transmitters are often co-released the position of their receptors suggests that one or other tends to take the predominant inhibitory role dependent on the location (Todd and Koeber 2006). GABAergic interneurons are located throughout the dorsal horn and although glycine is often co-localised with GABA cells positive for this neurotransmitter tend to be denser in the deeper laminae (III-IV) (Todd and Lochhead 1990).

Glycinergic interneurons play a part in the tonic inhibition of pain input through the dorsal horn demonstrated by the emergence of pain if their inhibitory function is blocked (Sorkin and Puig 1996;Sivilotti and Woolf 1994). Prostaglandins facilitate pain in this way in the

dorsal horn by interfering with glycine receptors and therefore causing disinhibition (Reinold et al. 2005; Zeilhofer 2005).

There have been a number of studies that record a significantly lower level of CSF GABA in patients with depression in comparison with a variety of control groups (Vieira et al. 2006; Gerner et al. 1984; Kasa et al. 1982; Gerner and Hare 1981; Gold et al. 1980). Other studies have failed to find a significant difference (Roy et al. 1991; Post et al. 1980; Zimmer et al. 1980) but have observed a trend towards a reduction of GABA.

2.3 Summary

Osteoarthritis, up until the relatively recent past, has been considered a disease of wear and tear because of a focus on the deterioration of load bearing cartilage. The pathophysiology of OA is being revealed as more complex than this, involving both degeneration and regeneration of tissues including but not exclusive to cartilage, bone, synovium and fat. The cartilage, which in health is avascular and without innervation is changed radically by the disease process and together with changes in subchondral bone helps to explain the persistent pain that develops in OA and seems to get worse over time for many people.

OA in the periphery involves a number of inflammatory processes like those seen in diseases where inflammation is considered a characteristic – for example Rheumatoid Arthritis. The degree of inflammation is greater in Rheumatoid Arthritis but many of the processes are the same. The release of cytokines systemically from inflammatory pains plays as important role as peripheral pain is communicated to the dorsal horn not just neurally but also humorally. Raised levels of pro-inflammatory cytokines communicate with the CNS by active transport

across the blood brain barrier or by interaction with endothelial cells to stimulate induction of cytokines within the CNS. Although this dual mechanism of communication exists it is felt that the dominant form of communication is neural.

Pain signals reaching the dorsal horn trigger the release of excitatory amino acids and neuropeptides that diffuse across the synaptic cleft and create excitatory potentials in second order neurons and interneurons. Summation of post-synaptic potentials can lead to the development of a suprathreshold potential in the second order neuron which then transmits an action potential to higher structures. In severe or prolonged pain the short term activity of glutamate is accompanied by longer term post-synaptic potentials created by the neuropeptides, Substance P and CGRP. Summation to threshold potential is more easily achieved due to the longevity of each potential and sustained potential enables an increased concentration of intracellular calcium ions which leads to a number of plastic changes in the post-synaptic cells.

Nitric oxide is produced from arginine creating the by-product citrulline (Guo et al. 2007). The increase in intracellular calcium also releases a magnesium ion from the NMDA receptor channel and thus makes this available for activation. Nitric oxide produced in the post-synaptic neuron diffuses to the primary afferent fibre and leads to an increased release of glutamate and other excitatory transmitters (Bar et al. 2004;Vetter et al. 2001;Kawamata and Omote 1999). Formation of peroxynitrite, (produced in a reaction between superoxide and nitric oxide) results from NMDA receptor activation and this inactivates both the glial glutamate transporter and glutamine synthetase (Chen et al. 2010) so that synaptic glutamate concentrations increase. This triggers an increased release of serine (Halassa and Haydon

2010), IL-β, IL-6 and TNFα from glia. Serine acts as a co-agonist with glutamate at the NMDA receptor leading to a further increase of intracellular Ca^{2+}, which facilitates the release of arachidonic acid from the cell walls of the post-synaptic neurons. Increased levels of serine increase NMDA receptor activity (Zhang et al. 2001b).

Synaptic glutamate is taken up from the synapse into glia where it is transformed to glutamine and then transported back to the primary afferent fibre and interneurons where it can be converted back to glutamate or GABA.

Pro-inflammatory cytokines such as IL-1β, IL-2, IL-6, IL-7, IL-8, IL-12, IFNγ and TNFα work in a coordinated fashion with a degree of redundancy to activate of glial cells, stimulate the release of further pro-inflammatory molecules including cytokines and prostaglandin, up-regulate AMPA receptors, enhance NMDA phosphorylation and disinhibit GABA and glycine mediated responses. The anti-inflammatory cytokines can reverse the actions of the pro-inflammatory cytokines but less has been written about the concentrations of these molecules in pain models.

The evidence suggests that central sensitisation will be marked by increased levels of the pro-inflammatory cytokines, and excitatory amino acids. It is also likely that this pain state will be accompanied by a reduction in the concentration of inhibitory molecules such as GABA, glycine and the anti-inflammatory cytokines.

Pain signals are conducted from the dorsal horn to the brain stem and then the cortex. Here arousal, awareness, and physiological response to pain are initiated. Simultaneously there is an integration of emotion and pain and a bidirectional influence can be seen. The descending

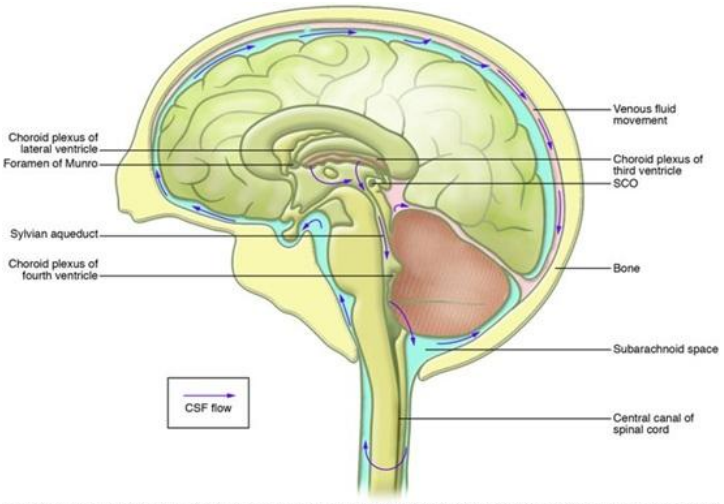
pathways from the brain-stem to the dorsal horn provide a means of both facilitation and inhibition of pain signaling and are influenced by the incoming pain signals and by supraspinal mood-related changes. The descending pathways originating in the RVM and LC influence the pain experience both by facilitation and inhibition of pain signaling pathways in the dorsal horn. Noradrenaline and 5-HT produce direct effects on neurons as well as stimulating the release of GABA and glycine. RVM cells demonstrate plasticity in response to ongoing stimulation and these changes are important in creating an imbalance between facilitation and inhibition in favour of facilitation in ongoing pain conditions. Input from the RVM is also necessary to maintain allodynia. The PAG and RVM are also subject to modulation from cytokines and other transmitters associated with stress and depression and so these conditions have an impact on the descending modulation of pain.

Chapter 3 The use of cerebrospinal fluid for the study of pain in OA

Cerebrospinal fluid is used by many researchers to explore diseases that affect the central nervous system. In this section the anatomy of CSF flow will be described and the evidence for is use in clinical studies will be critically explored. A number of factors that suggest caution is required in interpreting results will be identified.

Cerebrospinal fluid (CSF) is formed in the choroid plexus of the lateral, third and fourth ventricles. It flows via apertures in the fourth ventricle into the arachnoid space between the pia mater and the arachnoid mater. Here it circulates around the brain and spinal cord (Figure 3-1).

Figure 3-1: CSF production and circulation



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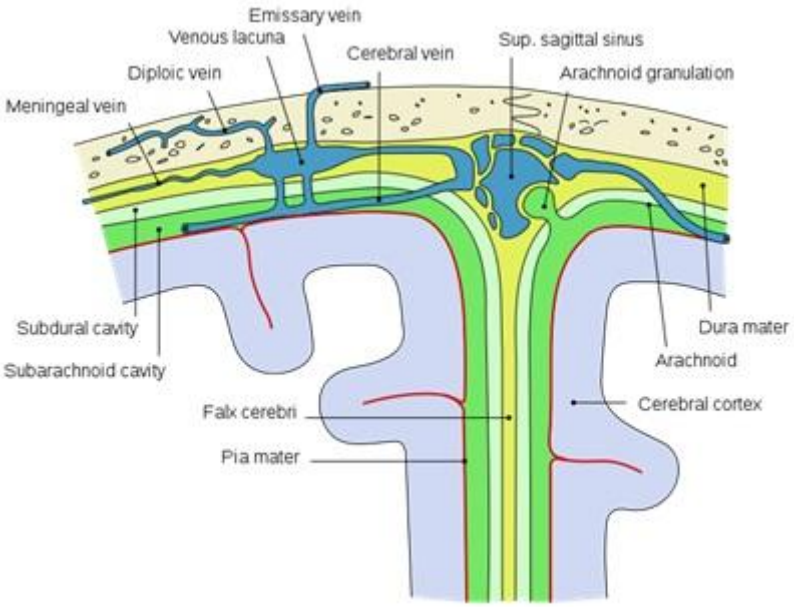
It is adsorbed into the venous circulation via collections of arachnoid villi named arachnoid granulations. These are finger-like projections of arachnoid mater that protrude into the fibrous dura mater and extend into the superior sagittal sinus (Figure 3-2).

Figure 3-2: the relationship between arachnoid villi and the superior sagittal sinus (source Wikipedia Commons)



Approximately 140 ml of CSF fills the ventricles, the spinal canal and the subarachnoid spaces (Brown et al. 2004). Constant production of CSF ensures that the total volume is replaced approximately four times per day (Wright 1978).

CSF has several functions. It is thought that is provides a cushioning effect for the brain (Laterra et al. 1999) and reduces its effective weight by approximately 60% (Segal 1993). By displacement, the CSF can mitigate the effects of increased intracranial pressure (Laterra et al. 1999).

CSF also provides a source of fluid to the brain in the event of dehydration and is a major transport system between neuronal structures for nutrients, hormones, and transmitters (Laterra et al. 1999; Johanson 1999). CSF is in constant communication with interstitial fluid in the brain and the spinal cord and therefore it is thought that lumbar fluid contains compounds that have been exchanged at the level of the brain and the spinal cord itself. It acts

as a drain or sink for the central nervous system, diluting and removing the products of metabolism and synaptic activity (Segal 1993). These functions of CSF have led to it being used to study a variety of disorders in the belief that it reflects the synaptic concentrations in neurotransmitters.

3.1 Precedent for the use of CSF in clinical studies

A wide range of studies have used CSF to reflect the activity of amino acids and cytokines in the brain and spinal cord in painful conditions and affective disorders – although never as comorbid conditions (Table 3.1). Critical appraisal of these studies reveals that practices have changed considerably since the earliest studies. Over time research has moved on from determination whether a substance of interest is present to identifying changes in concentrations of substances related to the presence or absence of a condition. Improvement of ethical standards has also made it increasingly difficult to recruit participants specifically to have CSF aspirated without any clinical reason to do so.

Although some studies have continued to access CSF from small numbers of healthy normal control participants it is far more common for control samples to come from people who are having spinal anaesthesia or lumbar puncture for clinical reasons (Table 3.2).

Control conditions in one study are often used as cases in other studies. An example of this can be seen in Alexander's studies of complex regional pain syndrome (CRPS) (2007; 2005) where the control participants had painful conditions such as spondylolisthesis and radiculopathy, and neurodegenerative disorders such as amyotrophic lateral sclerosis (ALS).

These conditions are likely to produce significant changes in amino acid concentrations including glutamate (Shaw et al. 1995; Perry et al. 1990).

Table 3.1: Clinical studies using CSF to explore painful conditions or affective disorders

Depression and/or anxiety	Berrettini *et al* (1986; 1983; 1982); Bertilsson *et al* (1982); Bridges *et al* (1976): Frye *et al* (2006); Geracioti *et al* (1997b); Gerner *et al* (1984); Gerner and Hare (1981); Gjerris *et al* (1988; 1987a; 1987b; 1987c); Gold *et al* (1980); Goodnick *et al* (1980); Honig *et al* (1988): Kasa *et al* (1982); Levine *et al* (2000; 1999); Roy *et al* (1991; 1988)
CRPS	Alexander *et al* (2005); Alexander *et al* (2007); Munts *et al* (2008)
Idiopathic pain, functional pain	Almay *et al* (1987); Jorgensen *et al* (1993)
Migraine or headaches	Bach *et al* (1992); Bo *et al* (2009); Gallai *et al* (2003); Langemark *et al* (1995); Martinez *et al* (2012; 1993a; 1993b); McGale *et al* (1977); Rozen *et al* (2007); Sarchielli *et al* (2001); Sarchielli and Gallai (2004); Vieira *et al* (2006); Welch *et al* (1975)
Facial pain	Bouckoms *et al* (1993)
Back pain (idiopathic, disc herniation)	Brisby *et al* (2002); Chan *et al* (1992); Garseth *et al* (2002); Hyyppa *et al* (1985); Kimura *et al* (1999); McGale *et al* (1977)
Neuropathic pain (diabetic; post-herpetic)	Backonja *et al* (2008); Chan *et al* (1992); Kikuchi *et al* (1999); Kotani *et al* (2004; 2000): Ludwig *et al* (2008); Mertens (2000)
Painful labour	Eisenach *et al* (1996); Hsu *et al* (2001); Olofsson *et al* (1997)
Fibromyalgia	Kadetoff *et al* (2012); Larson *et al* (2000): Legangneux *et al* (2001); Peres *et al* (2004); Sarchielli *et al* (2007a; 2007b)
Osteoarthritis	Lundborg *et al* (2010);

Table 3.2: Control groups used in clinical amino acid and cytokine studies of CSF

Healthy volunteers	Backonja *et al* (2008); Jorgensen *et al* (1993); Kimura *et al* (2001); Larson *et al* (2000); Olofsson *et al* (1997)
Neurological tests - no abnormality detected	Bo *et al* (2009), Gallai *et al* (2003), Garseth *et al* (2002); Peres *et al* (2004)
Non-inflammatory neurological symptoms	Kadetoff *et al* (2012)
Painless polyneuropathy	Ludwig *et al* (2008); Chan *et al* (1992)
Gynaecological or urological surgery	Lundborg *et al* (2012)
OA	Munts *et al* (2008); Garseth *et al* (2002)
Peripheral neuropathy	Alexander *et al* (2005; 2007)
Spondylolisthesis	Alexander *et al* (2005; 2007)
ALS	Alexander *et al* (2005; 2007)
Radiculopathy	Alexander *et al* (2005; 2007), Garseth *et al* (2002)
Pain free surgical patients	Kimura *et al* (1999); Olofsson *et al* (1997)
Elective C-section	Eisenach *et al* (1996); Hsu *et al* (2001)

3.2 Representation of pain and affective disorder by lumbar CSF

Where stipulated (and in some cases it could be inferred from other information) it was clear that the lumbar spine was the chosen level for access to spinal fluid in all the studies retrieved. The lumbar spine is considered to provide the most accessible route by which CSF can be collected.

Research into affective disorders has a focus on the release and activity of neurotransmitters in the brain but CSF from the lumbar spine is used in the expectation that its constituents will reflect what is happening in the brain because of its circulatory. As CSF flows from the brain, around the spinal cord and back to the brain, it will collect substances from extracellular fluid; there is a free-flow of substances between the two fluid compartments partly dependent on diffusion gradients (Veening and Barendregt 2010). Indeed, one of the ways that signalling takes place in the brain and spinal cord is via volume transmission using the CSF and extracellular fluid (ECF) as a transport system which results both from diffusion of substances into the CSF and from release of substances from dendritic terminals that lie within the CSF (Vigh-Teichmann and Vigh 1989). CSF contacting neurons also exist throughout the spine lying close to the central canal (Vigh *et al.* 2004). It is possible that bioactive substances in the CSF can diffuse into the spinal cord and also activate receptors on the spinal CSF contacting neurons. These neurons are GABAergic and express $P2X_2$ (ATP) receptors, which led Stoeckel *et al.* to suggest a role in autonomic function. Although there is a great deal more to be discovered about these neurons it remains a theoretical possibility that volume transmission from the brain stem to the spinal cord may modulate activity in addition to the more direct influence of the descending pathways.

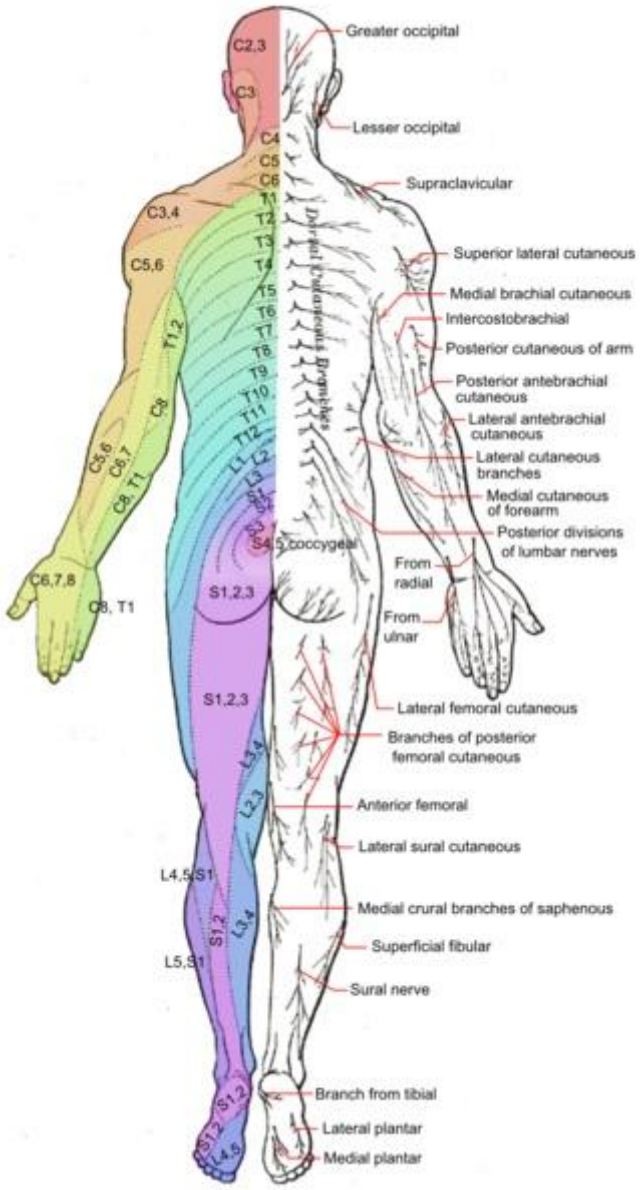
This mixing of substances of interest can be both a help and a hindrance. On the one hand lumbar CSF will reflect changes in concentrations of substances due to affective disorder and pain – even when that pain may have been communicated to segments of the spinal cord some distance from the lumbar puncture. In the case of lower limb surgery the primary afferent fibres from the knee enter the spinal cord at between L3 and S2, and those from the hips at L2 and L4 (Figure 3-3). There is some overlap in the areas served by each dorsal root with each

dermatome being fed by three dorsal roots (Bear et al. 2001). On entry to the dorsal horn the primary afferent fibres branch and travel a short distance both caudally and rostrally in the zone of Lissauer before they synapse with the dorsal horn. In this way neuronal communication from the hips and knees takes place close to the site of CSF collection. However, there is no guarantee that the changes in the concentrations of substances that are being observed are related to the phenomenon being studied – hence the need for as good, matched and healthy a control group as possible.

Despite the mixing effect suggested by the 'free' exchange of many substances between CSF and ECF, there are known rostro-caudal gradients for some amino acids and monoamines as demonstrated for GABA and taurine (Crawford et al. 1988; Perschak et al. 1987; Grove et al. 1982a; Bohlen et al. 1979). Reverse gradients have been observed for other amino acids and monoamine metabolites (Gjerris et al. 1988; Crawford et al. 1988). This creates yet more noise for the interpretation of CSF constituents.

In addition to a gradient between ventricular, cervical and lumbar CSF experiments have also demonstrated concentration gradients in serial measurements taken from the same subjects (Hill et al. 1999a; Blennow et al. 1993). Thus, in order to make comparisons between subjects or groups of subjects the CSF being assayed must be drawn from the same anatomical site and be of a similar volume, for example the first 2 ml drawn.

Figure 3-3: The dermatomes of the human body (source Wikipedia Commons)



Finally, there is also potentially an effect on CSF constituents of performing the lumbar puncture. The procedure begins with the injection of local anaesthesia to the cutaneous tissues and muscles prior to the insertion of a needle into the epidural space, through the dura mater and into the sub-dural space. It is anecdotally reported that anticipation of lumbar puncture is anxiety-provoking. Although there is no direct evidence to support this, research exploring the use of sedatives with spinal anaesthesia provides indirect evidence (Kestin et al.

1990). There is however, a body of evidence that the action of lumbar puncture produces a physiological stress response in terms of elevation of adrenocorticotrophic hormone (ACTH) and cortisol in the plasma of subjects who have undergone a lumbar puncture (Lerner et al. 2000; Geracioti, Jr. et al. 1999; Petrie et al. 1999; Hill et al. 1999b; Geracioti, Jr. et al. 1997c; Chappell et al. 1994; Breier et al. 1988). The main feature of this stress response is activation of the hypothalamic-pituitary-adrenal axis (HPA axis). One of the results of HPA axis activation is an increase in NA release into the dorsal horn, leading to an increase in GABA and glycine (Baba et al. 2000). Thus it is possible that the activity of performing a lumbar puncture will lead to increased GABA and glycine in the samples taken.

3.3 Contamination of CSF

Collection of CSF requires the disruption of tissues in the back and this causes some bleeding. In addition the epidural space contains a venous plexus (Harrison 1999; Parkin and Harrison 1985) and it is therefore possible that CSF aspirated from a needle puncture may be contaminated with cells and plasma. Although CSF contains proteins the protein concentration of blood is between 200 and 400 times higher and proteases in whole blood can cause protein degradation in CSF (You et al. 2005), which in turn affects the concentration of analytes. In order to prevent blood contamination of CSF it is necessary to either filter or centrifuge the sample immediately after collection. A number of studies use a centrifuge at 4°C and 3000G for 10-15 minutes either before or after sample storage to remove cellular material from their CSF samples (Alexander et al. 2007; Alexander et al. 2005; Peres et al. 2004; Sarchielli et al. 2001). An alternative method is to use a small filter (e.g. 0.22 micrometre) (Larson et al. 2000). This size of filter is fine enough to remove particulate

matter such as red blood cells and bacteria and enzymes greater than 20 000KDa (Millipore 2012).

3.4 Storage of CSF

The conditions in which the CSF is stored and how it is processed can affect the level of amino acids. Grove *et al.* (1982a) report 'normal' concentrations of GABA taken from a variety of studies to be between 87 picomole (pmol) ml^{-1} and 534 pmol ml^{-1} . This wide variation is partly because GABA does vary between individuals (Hare et al. 1981; Bohlen et al. 1978) but also because enzymatic activity in the sample degrades GABA conjugates such as homocarnosine to their its constituents (Grove et al. 1982b).

Freezing the samples at -20^{o}C or -80^{o}C may arrest the process of degradation but it begins again as soon as the sample is thawed (Grove et al. 1982a). Abbott *et al.* (1981) describe large increases in GABA concentration relating to freeze-thaw cycles, proposing that this is a result of the process of freezing and thawing but Ferraro *et al.* (Ferraro et al. 1983) found no increase in GABA in a similar experiment. Both Grove *et al.* (Grove et al. 1982a) and Ferraro *et al.* (1983) suggest that it is the assay technique employed by Abbott *et al.* (1981) that led to the GABA increase.

Acid precipitation has been used on CSF samples to arrest enzymatic degradation of the sample and to precipitate out protein that would otherwise reduce the efficiency of the assay process. Acids such as sulphosalicylic acid (SSA) and trichloroacetic acid (TCA) are used and these have been blamed for hydrolysis of GABA conjugates leading to falsely high GABA concentrations (Perry et al. 1982; Grossman et al. 1980). This effect has not been

observed by others using acid precipitation (Ferraro et al. 1983; Grove et al. 1982b). However, Grossman *et al.* (1980) assayed homocarnosine and GABA in a standard acidified solution of homocarnosine that was divided into two aliquots, one stored at -20^{o}C and one left at room temperature. GABA and histidine increased by up to 22-fold in the sample stored at -20^{o}C and by 1.7 fold in the sample stored at room temperature. This suggests that the combination of storage temperature and acidification does have an effect on degradation of homocarnosine but there is no explanation as to why lower temperature would increase the degradation.

Ferraro *et al.* (1983) hypothesise that the large increase in GABA observed by Perry *et al.* (1982) is related to the length of time that the CSF sample was subjected to temperatures above room temperature during the assay technique rather than hydrolysis of homocarnosine by acidification. However, Ferraro *et al.* (1983) identify a positive correlation between GABA concentration and the concentration of SSA added to the sample. Similarly glutamate is known to increase in acidified samples (Csernansky et al. 1996), an effect that can be counteracted when the acidified sample is subjected to immediate neutralisation (Aliprandi et al. 2002; Ferrarese et al. 1993).

There is a great deal of evidence to support the relationship between GABA increase and sample temperature. Samples left at room temperature for up to forty-eight hours yielded an increase in GABA of between 400 and 900% of the original value (Grossman et al. 1980; Bohlen et al. 1978). Similarly glutamate increases by as much as 50% as a result of degradation of glutamine at room temperature (Ferrarese et al. 1993). However, when Perry *et al.* (1982) added radiolabeled homocarnosine to CSF and allowed the sample to stand at room

temperature for four hours in an attempt to experimentally replicate this finding, they did not find any radiolabeled GABA. The likely reason for this is that the degradation takes place slowly. Storage of samples at -70^{o}C, whether treated or untreated, arrests enzymatic degradation. Glutamate stability has been demonstrated for up to two years (Espey et al. 2002) and GABA stability of untreated samples has been demonstrated at temperatures of -20^{o}C and -80^{o}C for up to eleven months (Grossman et al. 1980). However, acidified samples stored at -20^{o}C were not stable.

3.5 Risks to participants of CSF withdrawal in clinical studies

There are a number of risks associated with lumbar puncture including post-dural puncture headache, infection or damage to neural structures (see below). These risks were explained to the potential participants by the anaesthetists who were taking consent for their interventions.

3.5.1 Post-dural puncture headache

The incidence of post-dural puncture headache for women in labour, a group said to have the highest risk (Gosch et al. 2005), is thought to be between 1.5 and 11% (Choi et al. 2003). The risk of inducing a post-dural puncture headache increases with female gender and young age but the most important factors are the outer diameter of the needle used and the shape of the bevel (Halpern and Preston 1994). The incidence of headache varies according to the type of needle used, and the position of the needle as it is introduced through the dura. Larger gauge needles are associated with a higher incidence of headache (Turnbull and Shepherd 2003; Choi et al. 2003; Halpern and Preston 1994) but there is a lower limit on gauge because needles smaller than 29 Gauge are said to lead to failure of the anaesthetic (Turnbull and Shepherd 2003). Hatfalvi (1995) advocates an angled approach to the dura rather than

perpendicular and observed that following this advice reduced the rate of dural punctures he caused to zero.

Post dural puncture headache is attributed to continued leakage of CSF from the flap made in the collagenous dura mater by the passage of the needle. The rate of loss of CSF through the perforation is said to be greater than the rate of production of CSF (0.084 – 4.5 ml s^{-1} compared with 0.35 ml min^{-1}) (Turnbull and Shepherd 2003). Two theories are proposed for the genesis of a headache because of this persistent leakage. One theory is that the lowering the CSF pressure causes a tension on neural structures, which produces pain. The alternative theory is that the volume of the blood, CSF and brain is maintained as a constant, therefore loss of CSF will lead to venous dilation to maintain the volume of the system and this produces headache (Turnbull and Shepherd 2003). The headache is reported to worsen when the person sits or stands and reduces in intensity when the person lies down, as the pressure of the CSF reduces.

Dural puncture headache is not inevitable and the removal of up to 34 ml of CSF at a time from patients in a study where 342 subjects underwent a total of 428 lumbar punctures led to headache in only 0.97% of cases (Peskind et al. 2005). There was no relationship between the volume of CSF taken and the likelihood of post-dural puncture headache. The headache was also not affected by the position of the patient during the lumbar puncture, nor by the length of time that the patient spent recumbent after the procedure. This information can be provided to reassure potential participants.

3.5.2 Neurological complications and infection

Introduction of a needle into the subdural space can cause direct trauma to the nerve roots and this will be experienced by the patient as paraesthesia, numbness, weakness or persistent pain. Reports of the incidence of these effects vary and sometimes attribution is difficult when positioning, application of dressings and casts and surgery itself might have contributed to post-operative complications (Horlocker et al. 1997). The most up-to-date evidence available is from Horlocker et al. (1997) whose retrospective review of more than 4500 spinal anaesthetic procedures identified an incidence of 0.13% for persistent paraesthesia.

Infection following spinal anaesthesia is often reported in case studies (Huang et al. 2005;Donnelly et al. 1998;Schmutzhard et al. 1986). A review reports the incidence as between 1 in 1 000 and 1 in 100 000 (Grewal et al. 2006); it is considerably less than this in the Horlocker series.

3.6 Summary

CSF is widely used to explore diseases and processes that affect or are signalled via the central nervous system. CSF circulation arguably creates a fluid that represents activity at all levels of the spinal cord and brain but there is limited evidence available to substantiate this. The collection of CSF in clinical studies relies upon collection from the lumbar spine. There is some evidence that collection of fluid from other levels of the spinal cord or from the ventricles themselves may provide different results. However, the use of well-matched control participants can help the interpretation of the data collected provided that known confounding factors are addressed. The collection of CSF in clinical trials carries some risks for participants and in the UK at least participants are recruited from populations that are

already allowing access to the subdural space for a clinical purpose. This has the effect of creating potential populations of control participants but does not facilitate good matching with cases.

Storage and processing of CSF can also have an influence over the results obtained in assays. CSF samples must be frozen after collection and either before or after freezing they should be subject to filtration to remove particulate contamination by red blood cells and enzymes that may degrade the substrates of interest. In addition to filtration some researchers also use acid additives to precipitate out proteins but this has been associated with alteration in glutamate and GABA levels. In order to prevent degradation of samples prior to analysis they must be stored at low temperatures (<20°C) and must not be left at room temperature during the analysis process.

Chapter 4 The management of osteoarthritis

A number of guidelines have been produced that serve to bring together myriad research findings and perspectives on the most appropriate and effective management of OA. The Osteoarthritis Research International (OARSI) group was formed in 2005 with the purpose of arriving at an international multidisciplinary consensus agreement for recommended management of OA (Zhang et al. 2008b). There followed a systematic review of existing guidelines and the development, and subsequent updating of evidence based management guidelines (Zhang et al. 2010b).

The EULAR (European League Against Rheumatism) Standing Committee for International Clinical Studies including Therapeutic Trials (ESCISIT) has also published consensus statements on the management of knee osteoarthritis (Jordan et al. 2003) based on expert opinion and systematic review of the evidence. These guidelines recommend an individualised and multifaceted approach to OA management. In 2008 the National Institute for Clinical Excellence (NICE) also published evidence based guidelines for the management of OA (NICE 2008). NICE (2008) recognise that each individual will have their own preferences in terms of how to adopt recommended practices such as following an exercise programme and managing their weight. It is important that the individual has sufficient information to enable them to understand their condition, to follow healthy behaviours and to provide informed consent when that is appropriate. The NICE guidance is exceptional in the attention given within the document to concordance and pragmatism. Although there are some differences in the material reviewed to produce the guidelines and in some of the detail, broadly all the guidelines make similar recommendations (Table 4.1)

Table 4.1: Summary of guidelines for the management of OA

Strategy		*Aim*	*Comments*
Education/information	✓	Improve concordance. Improve healthy behaviour. Facilitate self-management. Facilitate informed consent.	Information is only helpful when it is wanted and delivered in a usable way to the individual. Education can be delivered individually or in groups, face to face or by telephone. Education for carers/partners can also be useful.
Exercise	✓	Improve muscle strength and aerobic fitness. Improve pain, function and gait. Improve post-operative recovery time.	Can be delivered in a group or solo, at home or in a health care setting. Uncertainty about utility of water based exercise.
Weight reduction	?	Decrease joint loading. Decrease systemic inflammatory markers. General health benefits.	Limited evidence of benefit of weight loss for slowing progression of OA or decreasing pain. Combined with exercise weight loss improves pain.
TENS	?	Pain relief Improvement in knee stiffness.	Some benefit in the short term has been demonstrated. Requires good patient education.
Acupuncture	?	Pain relief.	Some short term benefits. Electro-acupuncture is not recommended by NICE.
Electromagnetic therapy	X	Pain relief.	Not recommended, no proven benefit.
Glucosamine	X	Pain relief. Reduction in stiffness.	Modest benefit over placebo in knee pain. Relatively safe but costly. Not recommended.

Chondroitin sulphate	?	Reduced disease progression	Poor evidence but possible benefit
Anti-resorptive bone acting agents (e.g.	X	Reduced disease progression	No evidence of benefit in OA.
Paracetamol	✓	Pain relief	Recommended. Relatively safe profile.
Topical NSAIDS	✓	Pain relief	Recommended after or in addition to Paracetamol. Better safety profile than oral NSAIDS
Oral NSAIDS or cox-2 selective anti-inflammatories.	✓	Pain relief	Recommended if Paracetamol ± topical NSAIDs is not sufficient. Caution required due to side effects. Proton pump inhibitors recommended to be used in conjunction with NSAIDS.
Opioids	✓	Pain relief	Can be very helpful but benefits often limited by side effects. Some concerns over tolerance and addiction with long term use.
Intra-articular steroids	? X	Pain relief Reduced stiffness	Good short term relief but less beneficial over the longer term and probable diminishing benefits over the long-term.
Intra-articular hyaluronic acid	X X X	Pain relief Improved physical function Reduced stiffness	Not recommended although there is a small benefit and further research is called for.
Arthroscopic lavage/debridement	X X X	Pain relief Improved physical function Reduced stiffness	Not recommended unless the person has a history of mechanical locking.

4.1 Non-pharmacological management of OA pain

Transcutaneous Electrical Nerve Stimulation (TENS) is thought to be an effective pain reliever in osteoarthritis (Aiyejusunle et al. 2007; Bjordal et al. 2007) and can be an effective adjunct to physiotherapy, adding to improvements in physical function (Cheing and Hui-Chan 2004). However, the strength of evidence for TENS efficacy in OA is poor and more robust studies are called for (Rutjes et al. 2009).

Acupuncture is also helpful in pain management and facilitating a functional improvement when used alone or in conjunction with other therapies such as TENS (Itoh et al. 2008) but again there are studies that demonstrate that acupuncture is no better than placebo (Suarez-Almazor et al. 2010) or that the benefits are small but clinically insignificant (Manheimer et al. 2010).

The OARSI guidelines for the management of OA based on systematic review (Zhang et al. 2010b) gives acupuncture a number needed to treat of 4 (95% confidence interval 3 to 9) meaning that for every four people treated with acupuncture one will obtain symptomatic relief. The NNT for TENS was calculated to be 2 (95% confidence interval 1 to 5). Although the evidence for both TENS and acupuncture is equivocal both are included in management guidelines for OA.

Medical management of OA also includes exercise therapy in the form of aerobic, water-based or strengthening exercises and all have been shown to be beneficial in terms of pain relief and also improved function (Pisters et al. 2010a; Pisters et al. 2010b; Hernandez-Molina et al. 2008; Bartels et al. 2007; Roddy et al. 2005). Physical activity can also help to preserve

muscle strength, which can have a positive influence on post-operative recovery in those who have arthroplasty (Topp et al. 2009; Roder et al. 2007). In conjunction with this people with OA are encouraged to reduce weight if this is appropriate. A reduction of 5% in body weight can reduce physical disability and sometimes but not always pain (Christensen et al. 2007).

4.2 Pharmacological management of OA pain

The guidelines for the management of OA pain (NICE 2008;Zhang et al. 2005;Jordan et al. 2003) (Table 4.1) advise that all patients use paracetamol or topical NSAIDS as the first choice analgesic; the rationale for this is that it these drugs are relatively safe and have proven efficacy (Towheed et al. 2006; Wegman et al. 2004; Mason et al. 2004). The benefits of topical NSAIDS are well established and adverse events and risks associated with these preparations are smaller than their oral counterparts but there is less evidence of their benefit beyond a few weeks use (NICE 2008).

Oral NSAIDs are the second line recommendation for the medical management of OA. Concerns about the of NSAIDs safety (see below) have led to their use being restricted. It is estimated that approximately 10-20% of all patients who take NSAIDS experience dyspepsia and the risk of serious gastric complications (Wolfe et al. 1999). The development of cyclo-oxygenase-2 selective anti-inflammatories was initially heralded as a means to increase the number of people who could benefit from the pain relieving effects of NSAIDS due to the lower incidence of gastro-intestinal effects with these drugs (Laine et al. 2007;Hawkey et al. 2004; Hawkey and Skelly 2002). However, there is an increased risk of cardiovascular events with non-selective NSAIDs especially when used in the long-term (Cannon et al. 2006; McGettigan and Henry 2006; Hernandez-Diaz et al. 2006; Garcia Rodriguez and Gonzalez-

Perez 2005). Known risk factors for adverse events include older age, previous history of gastric bleeding and concurrent use of other anti-inflammatory drugs including low-dose aspirin (Lanas et al. 2010; Gonzalez et al. 2010). Unfortunately people who suffer from symptomatic OA often fall into the higher risk categories for either gastric or cardiovascular risk or both. There is also evidence that the anti-platelet effects of low dose aspirin are disrupted by some NSAIDS such as ibuprofen and naproxen (White 2007), and this suggests that the use of NSAIDs by those who are in the high risks groups needs careful management. The guidelines recommend that proton pump inhibitors are prescribed with NSAIDS and cox-2 inhibitors (NICE 2008).

Tramadol is considered a 'weak' opioid and is recommended for OA pain management (Schug 2007; Zhang et al. 2005; Jordan et al. 2003). Its use has been somewhat limited by the side effects of nausea, vomiting, dizziness, somnolence and changes in bowel habits (NICE 2008; Avouac et al. 2007; Babul et al. 2004) but slow titration to the optimal dose has been shown to limit the incidence of side effects and improve compliance (Ruoff 1999; Petrone et al. 1999). Tramadol seems to be effective for the partial relief of OA pain with up to 12.5% pain relief reported in a meta-analysis of studies relating to OA pain (Cepeda et al. 2007). One study has also demonstrated a concurrent improvement in pain-related sleep disturbance in people with OA (Florete et al. 2008).

The use of 'strong' opioids such as morphine in the control of long-term non-malignant pain is relatively newly explored. There is widespread acceptance that these drugs can be useful in the control of pain when Paracetamol and NSAIDS are insufficient, but there is also a fear

about prescribing them because of the potential for dependency, tolerance and addiction (Belgrade et al. 2006) as well as a relatively high incidence of side effects.

The side effects of long-term opioid use include constipation, drowsiness, difficulty in concentrating, lack of energy and nausea and are generally dose dependent (Wirz et al. 2008; Holzer 2008; Giacomuzzi et al. 2005; Kerr et al. 1991). Side effects and risks of opioid use may be increased in older people who have co-morbid conditions and may be taking a number of medications (Smith and Bruckenthal 2010). Side effects limit the usefulness of opioids for long-term management of pain, decreasing the maximum tolerable dose and contributing to reduction in quality of life (Manchikanti et al. 2009; Noble et al. 2008; Jensen et al. 2006).

Both the British Pain Society (BPS) and the European Federation of Chapters (EFIC) of the International Association for the Study of Pain (IASP) recognise the risk to pain sufferers of addiction in the long-term opioid use and have prepared guidelines for clinicians (British Pain Society 2005; Kalso et al. 2003), which advise careful assessment and close monitoring while recognising that opioids can be useful.

Long term use of opioids is also limited by tolerance; the need for escalating doses of the drug to achieve a constant effect. There is evidence that the development of tolerance begins almost immediately that morphine is given (Snijdelaar et al. 2004). However, a number of studies have observed gradual and acceptable increases in morphine dosage, which are not explained by the development of tolerance (Jensen et al. 2006; Cowan et al. 2002; Roth et al. 2000; Portenoy and Foley 1986). In long-term users of opioids it has been discovered that reducing, stopping or switching the drug, with the necessary physical and psychological

support leads to an improvement in pain (Aurilio et al. 2009; Mitra 2008; Baron and McDonald 2006).

It has also been reported that people with co-morbid pain and anxiety or depression get less analgesic benefit from opioids than others (Riley and Hastie 2008). These factors taken together demonstrate that while opioids have a place in the long term management of OA pain they are not a panacea and their usefulness is tempered by some limitations and side effects.

4.3 Joint replacement for OA

4.3.1 Criteria for joint replacement

The NICE guidelines (2008) state that approximately 120 000 hip and knee replacements are carried out each year for people whose pain and loss of function prevents them from carrying out everyday activities and disrupts their sleep. The main criteria for surgery for which there appears to be a consensus are pain, loss of function and radiographic changes (Toronto Arthroplasty Research Writing Group 2011; Dreinhofer et al. 2006; National Institutes for Health 2004; National Institutes for Health 1995). The threshold at which surgery is considered varies considerably with some believing for example that the presence of rest pain and night pain being present on at least one day per week was sufficient indication for surgery and others believing that these symptoms had to be troublesome most nights of the week (Dreinhofer et al. 2006). In the same survey more physicians than surgeons considered functional restrictions such as climbing stairs and putting on socks relevant. Walking distance was important to both physicians and surgeons but the level of restriction was different with surgeons tending to define this as <1km and physicians as <0.5km.

4.3.2 Outcomes and expectations of surgery

Many people with OA have high expectations of joint replacement surgery. Most expect that they will have no pain or a significant improvement in pain after their initial recovery period (Mannion et al. 2009; Lingard et al. 2006; Mancuso et al. 1997) as well as improvements in walking, psychological state and ability to perform activities (Mannion et al. 2009; Mancuso et al. 1997). Having a number of realistic expectations from surgery is associated with an increased likelihood of improvement post-operatively (Judge et al. 2011; Becker et al. 2011). The sort of expectations that have this positive effect are functional (e.g. walking further, household chores, exercise, driving, normal/improved sex life), or pain-related (e.g. having less pain, being pain free, being able to perform certain activities without pain).

Improvement after surgery is often measured in terms of pain relief and functional ability. Equally important is the patient's satisfaction with the surgery outcome and it is recommended that the outcome from surgery takes this into consideration (Becker et al. 2011). Additionally patients have concerns about participation issues such as their relationships with others, the part they are able to play in their community and specific issues that are not covered in the most commonly used outcome measures like ability to drive (Alviar et al. 2011).

4.3.3 Factors influencing outcome of total hip or knee arthroplasty

The surgery is considered relatively low risk with a relatively brief recovery period of 6-12 weeks and the prospect of returning to moderate levels of activity such as playing golf, tennis and swimming (NICE 2008). The majority of published studies with follow up ranging from a few months to many years document functional improvements following both hip and knee

replacement surgery (Becker et al. 2011; Xie et al. 2010; Thomasson et al. 2009). However, there are number of factors reported that can affect functional outcome including age, gender, obesity, and social deprivation.

4.3.3.1 The effect of age on outcome of hip or knee arthroplasty

Performing surgery on younger and fitter patients can carry a risk that due to higher post-operative activity levels there will be an increased risk of revision being required but survival of the prosthesis without revision generally seems high and many studies report greater than 90% survival without revision at 8+ years (de Kam et al. 2010; Ritter et al. 2007; Duffy et al. 2007; Crowder et al. 2005). On the other hand, operating on older, less fit people who have greater functional restrictions and more pain as measured by the Western Ontario and McMasters Universities Osteoarthritis Index (WOMAC) (Bellamy *et al.* 1988) is associated with poorer outcomes in terms of pain and function (Chang *et al.* 2010). Determining the influence of old age on outcome of surgery is complex because the presence of co-morbidities that can affect outcome measures such as walking restriction (Thomasson et al. 2009). In patient's aged 61-74 years the gain in Quality Adjusted Life Years (QALYs) was found to be an average of 4.59 giving a cost per QALY of 1795 Euros in a European study (Krummenauer *et al.* 2009), the same cost/QALY as pre-hospital thrombolysis therapy administered by paramedics for STEMI patients (Scuffham and Tippett 2008)

4.3.3.2 The effect of gender on outcome of arthroplasty

There are reports that women tend to have poorer outcomes than men (Singh et al. 2008;Holtzman et al. 2002) with speculation that this may be mediated by greater levels of arthritis helplessness (Gandhi *et al.* 2009), worse pain and function pre-operatively (Holtzman

et al. 2002) or anatomical differences between the genders (Johnson *et al.* 2011). The evidence suggests that women improve as much as men post-operatively when pre-operative differences are taken into account (Johnson et al. 2011; Lavernia et al. 2011; Lin et al. 2009; Gandhi et al. 2009).

4.3.3.3 The effect of obesity on outcome of arthroplasty

Obesity represents an increased risk for the need for arthroplasty (Bourne et al. 2007; Harms et al. 2007; de Guia et al. 2006). There is a great deal of published research about whether obesity affects the outcome of hip and knee replacements with both neutral and negative findings being abundant. A recent meta-analysis (Haverkamp et al. 2011) of fifteen studies of hip arthroplasty found that septic (but not aseptic) loosening was more common in the obese patient, as was early infection and venous thromboembolism. Dislocation was also more common in obese people. This meta-analysis was unable to draw any conclusions about whether there was any difference in terms of pain, function or participation.

The outcomes seem to be similar in total knee arthroplasty; patients are reported to make the same or greater gains than those of normal weight in terms of function (Rajgopal et al. 2008; Hamoui et al. 2006) and quality of life (McQueen et al. 2007). Post-operative infection has been reported as being a higher risk in the morbidly obese (Jarvenpaa et al. 2010; Namba et al. 2005) and also not occurring with any greater frequency than on those of normal weight (Amin et al. 2006). Further there is some suggestion that although eventual outcomes, or improvements are the same for both normal weight and obese patients those gains are less efficient and come at a higher clinical cost for the obese patient who may require more drugs and more therapy service input (Vincent and Vincent 2008; Vincent et al. 2007).

4.3.3.4 Socioeconomic factors affecting outcome of arthroplasty

People who are socially deprived tend to undergo arthroplasty earlier than those who are not. They also have a greater number of co-morbidities and worse symptoms (Clement et al. 2011; Davis et al. 2006; Ackerman et al. 2005). The suggestion is that this group of people is probably under-represented in terms of the numbers being offered surgery. There is debate about whether social deprivation is associated with poorer outcome of surgery. Clement *et* al (2011) found that the deprived had a worse outcome in terms of pain and function and are less satisfied with the outcome of their surgery when compared with their more affluent peers. However, Davis *et al.* (2008) found that there was no difference in outcome, in fact their gains appeared to be greater because they began with worse pre-operative WOMAC scores and ended up with similar scores at the two year follow up point.

4.3.3.5 The effect of delaying surgery

There is conflicting evidence about whether people once listed for surgery experience any significant deterioration in pain or function. Some studies report observable deterioration over a 6 to 9 month period (Desmeules et al. 2010; Chakravarty et al. 2005) while others do not (Hoogeboom et al. 2009; Kelly et al. 2001).

Economic benefits of shorter waiting time for surgery have been noted (Saleh et al. 1999;Saleh et al. 1997). However, historically the operation has been delayed for as long as possible (Crawford and Murray 1997), probably due to a perception that prostheses have a limited life span. The durability of hip and knee replacements is said to be improving due to increased understanding of the mechanical and patient-related factors that affect it (Walker et al. 2010). Survivorship of the unrevised total knee prosthesis is currently greater than 80% at

15-17 years (Parratte et al. 2010; Rand et al. 2003) and up to 90% for total hip replacements (Callaghan et al. 2008) although there are a number of factors that can affect this including the prosthesis used and the manner of the replacement.

Delaying surgery can mean that the OA sufferer is in poorer general health and has greater pain and functional limitations. As a result of this delayed surgery is often associated with poorer post-operative outcomes (Montin et al. 2008; Holtzman et al. 2002; Fortin et al. 2002).

4.3.4 Pain outcome after joint replacement

Surgical success rates are cited as 80% by some but Edwards *et al.* (2009) comment that this is rarely defined as complete pain relief. A number of studies exploring outcome of joint replacement suggest that the weight of evidence of excellent pain relief is so great that there is no longer any need to demonstrate this benefit (Thomasson et al. 2009), but outcomes are not so overwhelmingly positive as that statement suggests. Although most people report a clinically significant improvement in pain (Montin et al. 2008; McMurray et al. 2005;Fortin et al. 2002; Jones et al. 2000) there is a relatively high prevalence of persistent pain after surgery.

4.3.4.1 Persistent post-operative pain resulting from malalignment of the implant

Malalignment of the implant in knee arthroplasty can also lead to persistent post-operative pain and it is estimated that this occurs in up to 10% of primary surgeries (Mason et al. 2007). Revision surgery is often able to correct the malalignment and this tends to resolve the pain also (Pietsch and Hofmann 2011).

4.3.4.2 Persistent post-operative pain resulting from infection in the joint

Infection can lead to failure of the joint replacement and pain and it is estimated that this happens in between 0.4 and 2% of total knee replacements (Garvin and Konigsberg 2011). Reports of revision surgery for failure of total hip replacement suggest that the cause of the failure is infection in between 8 and 15% of cases (Jafari et al. 2010; Bozic et al. 2009; Ogino et al. 2008). It is difficult to diagnose and treat (Kalore et al. 2011) and a significant proportion of those affected continue to have pain once treatment is completed (Wang et al. 2002).

4.3.4.3 Persistent post-surgical pain due to aseptic loosening of the prosthesis

Pathological changes in the bone remnants following arthroplasty are thought to lead to loosening of the implant due to pseudoarthrosis, collapsed osteonecrosis, cement socket de-bonding and bone-cement loosening (Zustin et al. 2010).

Osteolysis is relatively common problem following total joint replacement. The process is caused by wear on the replaced surfaces creating particulate material which potentially leads to bone loss and prosthetic loosening requiring revision surgery (Mall et al. 2011). Osteolysis is preceded by synovial inflammation but not all synovial inflammation predicts osteolysis.

4.3.4.4 Persistent post-operative pain resulting from intra-operative or post-operative nerve damage

Intra-operative nerve damage is thought to account for up to 7.6% of cases of persistent post-surgical pain (Brown et al. 2008). The causes include direct nerve injury, significant leg lengthening, improper retractor placement, cement extrusion, patient positioning, and post-

operative haematoma (Zwolak et al. 2011; Brown et al. 2008; Pritchett 2004; Maloney and Keeney 2004; Johanson et al. 1983). Risk factors for nerve damage include the complexity or difficulty of the operation, the surgical approach taken, bilateral procedures, tourniquet time, rheumatoid arthritis, continuous local anaesthetic infusion via indwelling catheter and younger age (Jacob et al. 2011; Feibel et al. 2009; Eggli et al. 1999; van der Linde and Tonino 1997; Weale et al. 1996; Navarro et al. 1995). Approximately 80% of people who suffer a nerve injury will experience continued functional problems, and one of the predictors of continued problems is the presence of neurogenic pain (Edwards et al. 1987; Johanson et al. 1983). However, recovery is possible and can be spontaneous (Andrews et al. 2008) or it can be aided by surgery (Ducic et al. 2010).

4.3.4.5 Persistent post-surgical pain due to increased innervation of tissues

It has been shown that people who have anterior knee pain following arthroplasty have increased sensory innervation of the infrapatellar fat pad (IPFP) compared to people with OA knee (Lehner et al. 2008). This study demonstrated that the number of sympathetic fibres did not increase at the same time and this led to an alteration in the ratio of sensory to sympathetic fibres away from the usual 1:1 to 8:1 in favour of the sensory fibres. This suggests that sympathetically driven inhibition of the sensory fibres will be less and Lehner and colleagues suggest that this imbalance could lead to increased sensitivity, potentially contributing to an increased risk of persistent post-operative pain.

4.3.4.6 Persistent pain due to persistent sources of inflammation

Replacement of an osteoarthritic joint does not necessarily remove all sources of nociception from it. Although osteophytes are removed the infrapatellar fat pad may be retained. Leaving

the infrapatellar fat pad seems to be weakly associated with having continued pain in the knee at five years (Meneghini et al. 2007). Hyper-innervation by sensory fibres in the infrapatellar fat pad has been observed in the post-operative patient and is thought to cause an imbalance between the analgesic effects of the sympathetic innervation and the algesic activity of the sensory fibres (Lehner et al. 2008). This imbalance doesn't exist in the pre-operative arthroplasty patient.

Synovial inflammation has been observed in 39% of post-operative patients at between 1 and 3 years follow up but the presence or degree of this did not correlate with pain (Cooper et al. 2010).

4.4 Summary and rationale for the present study

OA is diagnosed using a combination of subjective reporting of pain and physical symptoms, clinical examination and X-ray. The prevalence in the UK population is difficult to define due to the range of different criteria used in epidemiological studies but it is a common disorder that often has physical, social and psychological consequences for the sufferer. The risk factors for OA include non-modifiable variables such as congenital joint deformity, ethnicity and gender, as well as modifiable variables such as obesity, and occupation. It is assumed that due to the increase in longevity and obesity in the population that the economic and personal burden of OA will continue to rise over time.

Many people are able to self-manage OA without support from health professionals. However, many people with OA tend to have a poorer quality of life than their healthy

counterparts. They suffer from pain, stiffness, functional impairment, depression, fatigue and increased risk of falls. One of the more significant effects of OA for the individual is the loss of social participation they experience and one of the important measures of the outcome of various management strategies from the sufferer's perspective is whether there is recovery of satisfying participation for the individual. Health professionals tend to measure treatment outcome more in terms of pain and functional restriction.

There are a number of evidence based guidelines available for OA management. The main guidance in the United Kingdom comes from the National Institute for Health and Clinical Excellence (NICE 2008). All guidance including NICE recommend individualised treatment aimed at maximising concordance with self-management strategies including education, exercise aimed at muscle strengthening and aerobic fitness and the use of Paracetamol and topical NSAIDS as a first line. Although the evidence is weaker to support weight loss this is also recommended. More invasive treatments such as joint injections have short term benefit for those who pain causes more lifestyle disruption and more than 100, 000 people per year will be offered joint replacement surgery.

Although most people report satisfaction post-operatively a significant proportion of people experience chronic pain after the surgery. There are a number of reasons for this continued pain and it is suspected that a small proportion is due to persistence in central sensitisation. Previous research suggests that pain at rest may be an indicator of the likelihood that this may affect an individual. Further investigation of this facet of OA pain is warranted.

Work on central sensitisation has begun to explore the role of non-neuronal cells and the immune activation and the results suggest that these relatively unexplored pathways are

important. There is very little published about spinal transmission of OA pain and together with the potential for persistent central sensitisation to be an important factor in continued post-operative pain this justifies exploration.

There are limitations in the current explorations of human pain mechanisms. One of the primary limitations is that many clinical studies examine pain in isolation of its affective component. Depression, anxiety and pain share common neurochemistry, and it accepted that there is an influential reciprocal relationship between them. This study will be one of the first to try to take into consideration the affective dimension of pain when exploring the transmission of pain at the level of the spinal cord. It is hoped that this will allow a more meaningful interpretation of the data in order that it translates better to the real, but rather complicated clinical picture of pain in humans.

The need for a better understanding of OA pain in order to improve knowledge and choices for health professional and OA sufferers, the search for newer pharmaceutical options, the need to bring nociceptive pain into the tripartite framework, the need to consider the influence of affect on pain transmission and the increasing prevalence of OA due to the ageing population are components of the main aim of this study: to investigate the transmission of OA pain at the level of the spinal cord.

4.5 Aims and Hypotheses

The aim of this study is to explore the mechanism of pain transmission at the level of the spinal cord in patients with hip and knee OA. To this end a group of amino acids and cytokines were selected for assay (Table 4.2) from the cerebrospinal fluid of patients who were undergoing surgical joint replacement (arthroplasty). The cytokine choices were partially driven by the availability of a high-sensitivity multiplex kit.

Table 4.2: Amino acids and cytokines selected for assay

Excitatory amino acids	Inhibitory amino acids	Neutral Amino acids	Pro-inflammatory cytokines	Anti-inflammatory cytokines
Glutamate	GABA	Leucine	IL-1β	IL-4
Aspartate	Glycine	Isoleucine	IL-2	IL-5
Arginine		Phenylalanine	IL-6	IL-10
Citrulline		Tyrosine	IL-7	IL-13
Serine		Tryptophan	IL-8	
Glutamine		Valine	IL-12	
			IFNγ	
			TNFα	

A comparison group of people having spinal anaesthesia who do not have pain had the same measurements and samples taken. The purpose of this exploration is to improve our understanding of the mechanism of OA pain in order to improve pain relief in this population.

4.5.1 Hypothesis

The study has two broad hypotheses.

Hypothesis 1:

The levels of locally produced amino acids and cytokines found in pre-operative cerebrospinal fluid samples from symptomatic osteoarthritis cases (OA) differ from those in pain-free controls (control) after taking into consideration the influence of age, gender and psychological distress (total HAD score).

Hypothesis 2:

The levels of locally produced amino acids and cytokines found in pre-operative cerebrospinal fluid samples from OA participants with pain at rest (PAR) will differ from those who have no pain at rest (0PAR) after taking into consideration the influence of age, gender and psychological distress.

These hypotheses can be broken down into a number of theoretically driven aims based on the suggested roles of the amino acids and cytokines being studied.

A1: To identify a significant increase in IL-1β, IL-2, IL-6, IL-7, IL-8, IL-12, IFNγ and TNFα concentrations in the OA group in comparison to the control group.

A2: To identify a significant increase in IL-1β, IL-2, IL-6, IL-7, IL-8, IL-12, IFNγ and TNFα concentrations in the PAR group in comparison with the 0PAR group.

A3: To determine whether differences in IL-1β, IL-2, IL-6, IL-7, IL-8, IL-12, IFNγ and TNFα persist between these groups after adjusting for the age, gender, and psychological distress.

B1: To identify a significant decrease in IL-4, IL-5, IL-10 and IL-13 concentration in OA cases in comparison with control cases.

B2: To identify a significant decrease in IL-4, IL-5, IL-10 and IL-13 concentration in PAR cases in comparison with 0PAR cases.

B2: To determine whether differences of IL-4, IL-5, IL-10 and IL-13 persist between these groups after adjusting for the potential confounders of age, gender, and psychological distress.

C1: To identify a significant increase in glutamate, aspartate, arginine, citrulline, glycine, serine and glutamine in the OA group in comparison with controls.

C2: To identify a significant increase in glutamate, aspartate, arginine, citrulline, glycine, serine and glutamine in the PAR group in comparison with the 0PAR group.

C3: To identify whether differences in glutamate, aspartate, arginine, citrulline, glycine, serine and glutamine between these groups persist after adjusting for age, gender, and psychological distress.

D1: To identify a significant decrease in GABA and glycine concentrations when the OA group is compared with controls.

D2: To identify a significant decrease in GABA and glycine concentrations when the PAR group is compared with the 0PAR group.

D3: To determine whether differences in GABA and glycine in these groups persist after adjusting for age, gender, and psychological distress.

Chapter 5 Method

5.1 Ethical approval and governance

At the time of the inception of this study multiple Local Research Ethics Committees (LREC) were involved in the approval process. Approval was granted by Solihull LREC, South Birmingham LREC and East Birmingham LREC (Appendix 1). Research and Development approval was sought and obtained from Solihull and Heartlands NHS Trust and the University Hospitals Birmingham NHS Trust.

An amendment was made to the study after initial approval (Appendix 1). This broadened the inclusion criteria and altered the recruitment process (Figure 5-1 and Figure 5-2).

5.2 The sample

This study used a sample of convenience. Patients who were having surgery under spinal anaesthesia were approached on the morning of their surgery. Two broad categories of patients were considered for eligibility. The first group were patients having replacement of knee or hip joints, diagnosed with osteoarthritis using radiographic, pain and disability criteria. The second group were patients having a spinal anaesthetic for surgery for a disorder unlikely to be characterised by the presence of pain. The largest group of patients who were likely to fall into this category were those having transurethral prostate or bladder surgery.

5.2.1 Feasibility

At the time of recruitment for this study the NHS Trust had 15 elective orthopaedic lists per week and 4 elective urological lists (Appendix 2). Three urologists and 4 orthopaedic surgeons agreed after face to face meetings that the research nurse could approach and recruit patients from their theatre lists for the study. A senior Consultant anaesthetist was responsible for recruiting colleagues to the study for the purpose of collection of CSF during the anaesthetic induction procedure. Verbal consent was obtained from all anaesthetists responsible for orthopaedic and urology lists. This allowed collection of CSF samples at between 9 and 10 orthopaedic lists and 4 urology lists per week. The maximum numbers of samples that it would be possible to collect per week were estimated to be 10 OA and 10 control participants. It was anticipated that sample collection could continue for 12 months providing an estimated maximum number of 400 samples for each group (40 weeks collection x 10 samples).

5.2.2 Sample size and power calculation

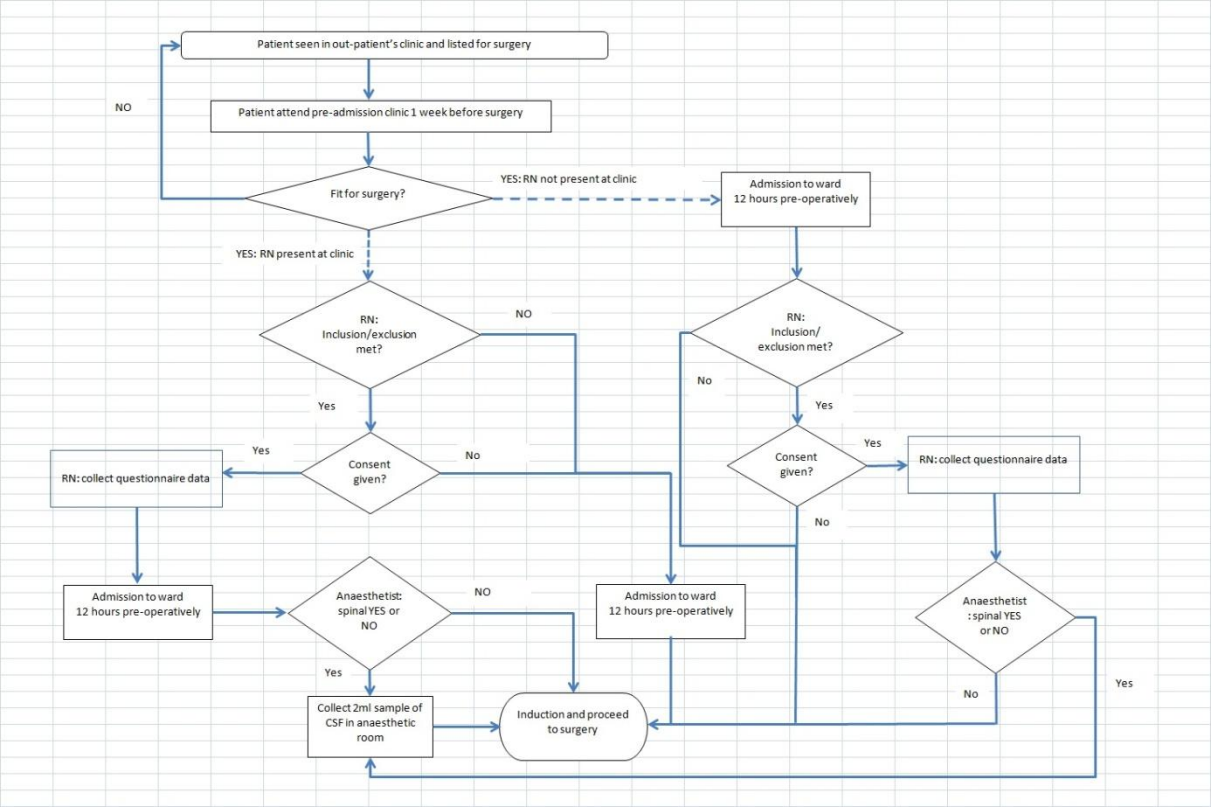
A search was conducted to find any study where any of the amino acid and cytokine levels in the CSF were compared between a pain group and a control group, without imposing strict requirements on the composition of the control group or specifying the type of pain. There was a wide variability in the levels reported and many studies had control subjects who were likely to have altered levels of these analytes. It was therefore concluded that a prospective sample size calculation was not possible. The study therefore set out to recruit as many participants as possible and use retrospective calculations to determine the power achieved.

5.2.3 Recruitment

Participants were initially recruited from the elective orthopaedic waiting list via the admissions clinic where they were seen a week before their planned arthroplasty (Figure 5-1). After this method was trialled for a four week period two major barriers to recruitment were discovered. The pre-admission clinic did not have an anaesthetist present and the research nurse (AS) was not qualified to discuss fully the risks and benefits of a spinal anaesthetic with the potential participant. Raising the possibility of a spinal anaesthetic with potential participants raised their anxiety levels and there was no effective procedure available to alleviate this anxiety before the patient left the clinic. This was felt to be an unnecessary and unforeseen stress for the patient.

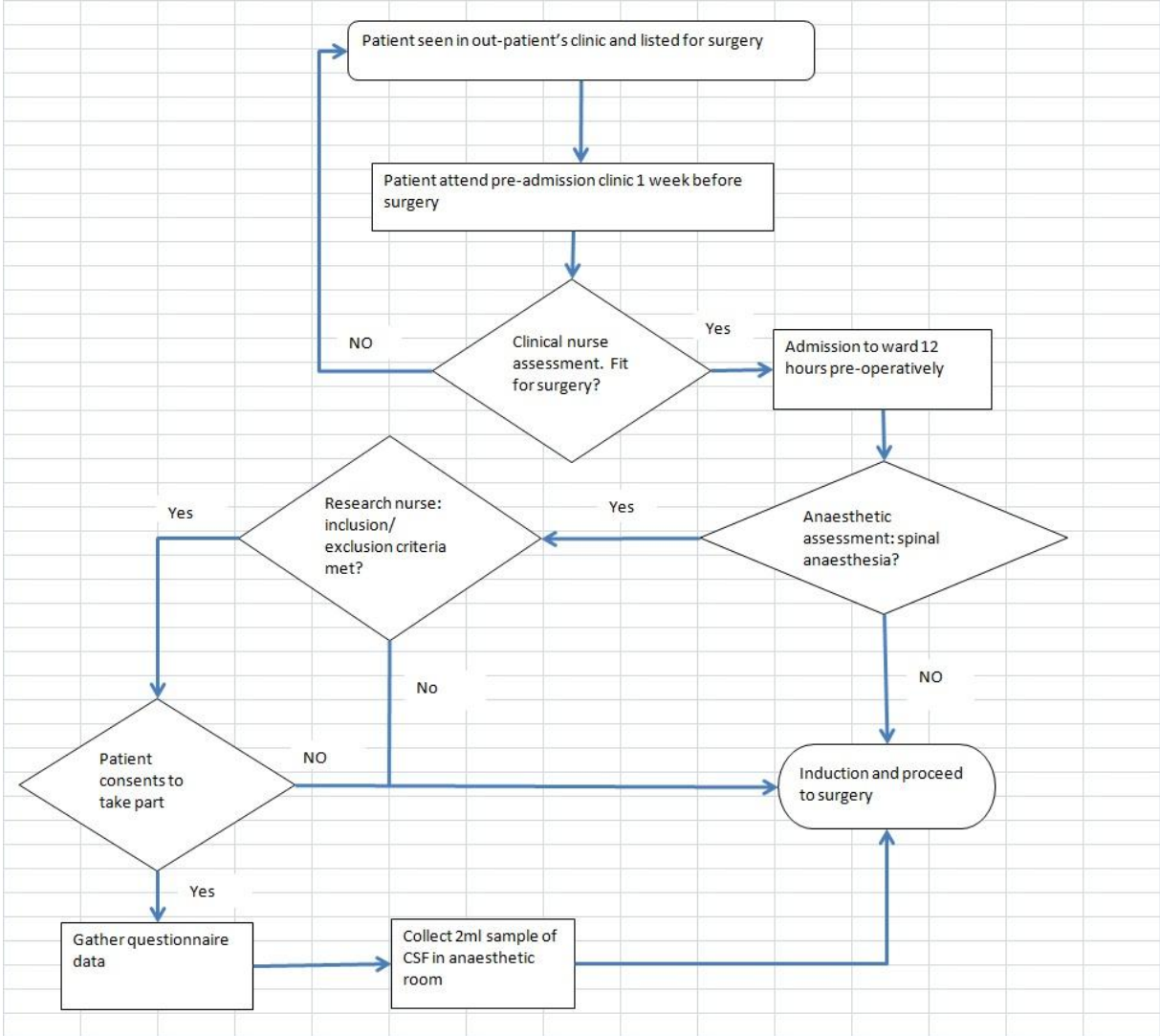
The second difficulty was that participants who consented to take part in the study were given an operation date that was subject to change at short notice, and once admitted the participant may not agree to or be suitable for a spinal anaesthetic. This meant that a large number of participants were recruited at pre-assessment but did not participate in the study.

Figure 5-1: Recruitment process number 1



A change was made to the recruitment process and approved by the LREC (Appendix 1) meaning that patients were recruited to the study on the day of their surgery after they had provided informed consent for a spinal anaesthetic with an independent anaesthetist (Figure 5-2).

Figure 5-2: Recruitment process number 2



5.3 Inclusion and exclusion criteria

Inclusion and exclusion criteria were established following a literature search exploring the published changes in amino acid levels in human CSF (Appendix 6). This literature search was conducted using the initial search terms 'amino acid' and (CSF or spinal or dorsal not GM-CSF) with a limit of human studies. A series of searches were conducted following this utilising the names of diseases, disorders and their synonyms and generic descriptors of these diseases (e.g. Parkinson's or neurodegen*) where * is a truncation symbol. In this way a

general picture was developed of the disorders and diseases where changes in amino acids in lumbar cerebrospinal fluid had been detected.

5.3.1 Inclusion criteria

a) Age > 50 years
b) Women should be post-menopause
c) Able to provide informed consent
d) Consented to surgery with spinal anaesthesia
e) For the OA recruits:
    i. diagnosis of OA in medical notes by orthopaedic team based on a history of pain, function and radiographic evidence.
    ii. Predominant pain in hip or knee
    iii. Listed for primary arthroplasty of the hip or knee
f) For the control recruits:
    i. Pain free
    ii. No diagnosed arthritis or inflammatory condition (see exclusion criteria)

5.3.2 Exclusion criteria

a) Pain other than OA pain (including for example, headache, rheumatoid arthritis, post-herpetic neuralgia, neuropathic pain including diabetic neuropathy, low back pain).
b) Recurrent intermittent pain e.g. migraine, chronic tension headache.
c) Neurodegenerative disorder (including for example Parkinson's disease, Motor Neurone Disease, Multiple Sclerosis, Alzheimer's disease).

d) Anorexia (eating disorder)

e) Alcoholism

f) Psychiatric disorder other than anxiety or depression (e.g. schizophrenia)

g) Head trauma or cerebrovascular accident within 6 months.

h) Current inflammatory disorder other than osteoarthritis (e.g. active (painful) gout)

i) Active cancer within past 6 months

j) Pregnancy

k) Current investigations for neurological disorder.

l) Use of immunosuppressants.

m) Use of steroid (excluding use of steroid inhalers for COPD or asthma less than once per day).

5.4 Measurement tools and data collection

Demographic data was collected from the medical records and the participant with cross-verification being used to establish key information such as diagnosis (Appendix 5). The data collected were:

Demographic data
Age in years
Sex (M/F)
Ethnicity (classified according to the Office for National Statistics ethnicity codes (2001).
Past and current medical history
Based on a search of the admission records for the participant
Grouping of this data was completed prior to entry into the SPSS database
Cardiovascular disease including ischaemic heart disease and hypertension
Endocrine disorder excluding diabetes mellitus
Diabetes mellitus
Renal disorders
Respiratory disorders
Previous major surgery

Previous minor surgery including urological procedures
Cancer or haematological disorders (an exclusion criterion)
Rheumatological disorder including fibromyalgia (an exclusion criterion)
Neurological or neuromuscular disorders (an exclusion criterion)
Operative procedure planned

5.4.1 Medication

A complete list of the participant's current medication was recorded. This list formed the basis for a more complete discussion of the participant's use of medication.

Name of medication, herbal remedy, homeopathic remedy or supplement
Dose and daily total used in past week
Usual dosing schedule (regular/as required)
Date medication stopped if appropriate

5.4.2 Pain intensity

There are a number of ways of measuring pain in research studies. Much osteoarthritis research uses the Western Ontario and McMasters University Osteoarthritis Index (WOMAC) (Bellamy et al. 1988) which is a multi-dimensional tool that allows the measurement of pain, stiffness and physical function. Pain is measured using a Likert scale or visual analogue scale for the intensity of pain evoked by walking, using stairs, sitting or lying and standing. The pain score is generated by summing all the pain items, which gives a number between 0 and 20 if using the Likert scale. This approach creates an intensity score that takes into consideration the fact that pain intensity in OA is related to activities undertaken. However, one of the requirements in the current study to differentiate between pain at rest and pain on movement and there have been no studies using the WOMAC in this way.

Participants were asked to rate their current pain using a 0-10 numerical rating scale with a visual box-scale to support this (Figure 5-3). Both the numerical rating scale and the 11 point

box scale have been demonstrated to have validity in measuring a single construct of subjective pain intensity with an Eigenvalue of 0.90 (Jensen et al. 1986). It is also a scale that is easy for participants to understand, and it is easy to administer (Herr et al. 2004).

The 11-point box rating scale (Figure 5-3) does not have the same sensitivity to detect change in pain intensity as tools such as the Visual Analogue Scale (VAS) or the 101-point numerical rating scale (Jensen and Karoly 2010) but this is not a requirement in the present study as the pain score is of greater interest that a change in pain as a result of treatment or disease progression.

Figure 5-3: Box rating scale

0	1	2	3	4	5	6	7	8	9	10

No pain Worst pain imaginable

Participants were asked to score their current pain intensity at rest and then to estimate their pain on movement. Evidence suggests that recall of recent pain is accurate in people with OA (Allen et al. 2010) and using recall was considered preferable to asking the participant to evoke pain. The participants were then asked to rate their average pain in the past three months and their average pain in the last twelve months using the same scale in order to assess stability of pain or otherwise. Finally the participant was asked to state the site of their worst OA pain and give an estimation of its duration.

Control participants were asked if they had any pain now or any pain in the last week. If they had more than one brief episode of acute pain within the past week, or a history of headaches or migraines they were ruled out from the study.

5.4.3 Screening for neuropathic pain

In participants with diabetes the Michigan Neuropathy Screening Instrument (Feldman et al. 1994) was used to rule out peripheral diabetic neuropathy. This instrument consists of 15 questions about the person's medical history that would indicate increased risk for peripheral neuropathy designed to determine whether the person is experiencing allodynia, paraesthesia or anaesthesia in the feet. This is followed up by a physical examination where the physical appearance of the feet is noted including any ulceration and then vibration sense, ankle reflexes and monofilament tests are conducted. High scores in this latter section are reserved for a decrease or absence of the expected perception with a maximum of 10 points available. Inter-rater reliability is good (Bax et al. 1996). The instrument has good sensitivity (65%) and specificity (83%) and overall has a diagnostic accuracy of 80% (Moghtaderi et al. 2006). Formal diagnosis of diabetic neuropathy would also require electrophysiological testing but this instrument is accurate for the purposes of excluding potential cases from this study.

5.4.4 Anxiety and depression measurement

Anxiety and depression are complex constructs, and somatic symptoms co-exist with emotional, for example insomnia or hypersomnia, and loss of appetite. These symptoms are often features of co-morbid physical illness and this can cause an increase in the number of false positives picked up by psychological assessment tools applied to a physically ill population (Snaith 1987; Kutner et al. 1985; Steuer et al. 1980). The development of the

Hospital Anxiety and Depression Scale (HADS) (Zigmond and Snaith 1983) was a response to this as it aims to limit the use of these ambiguous items and so is generally thought to be appropriate for use in hospitalised populations. It also does not use symptoms that are associated with severe psychopathology and is therefore thought to be more acceptable to a general population and more sensitive to less severe forms of anxiety and distress (Herrmann 1997).

The HADS is a fourteen-item scale (Table 5.1) in which respondents identify a level of agreement with 7 items related to feelings or depression and 7 items relating to feelings of anxiety. The responses are ordinal and are recorded numerically with values between 0 and 3. The maximum score for both anxiety and depression is twenty-one. The questionnaire can be self-administered as it was in the present study and takes minutes to complete. The ease of understanding of the items, and the speed of completion were key requirements in the present study as the time for data collection prior to transfer to the theatre department was relatively brief. The data returned by the responses is ordinal in nature due to the properties of the Likert-scale used (Svensson 2001).

Table 5.1: HADS items

Anxiety item		Depression item	
A1	I feel tense or wound up	D1	I still enjoy the things I used to enjoy
A2	I get a sort of frightened feeling as if something awful is about to happen to me.	D2	I can laugh and see the funny side of things
A3	Worrying thoughts go through my mind	D3	I feel cheerful
A4	I can sit at ease and feel relaxed	D4	I feel as if I am slowed down
A5	I get a sort of frightened feeling like 'butterflies' in the stomach	D5	I have lost interest in my appearance
A6	I feel restless as if I have to be on the move	D6	I look forward with enjoyment to things
A7	I get sudden feelings of panic	D7	I can enjoy a good book or radio or TV programme

5.4.4.1 The factor structure of the HADS

The HADS aims to differentiate between patients with mild (normal) anxiety and depression, those where anxiety and depression are on the borderline in terms of requiring treatment and those for whom anxiety and depression requires further assessment and perhaps treatment.

Numerous studies have explored the factor structure of the HADS. A review by Bjelland et al. (2002) examined nineteen of these studies. Eleven of the nineteen studies that reported a factor analysis of HADS data identified a two factor structure in samples of people with a psychiatric disorder or depression (2 studies), cancer (4 studies), HIV infection (1 study) and in the general population (3 studies). Five studies identified a three factor structure in samples of people with cancer (2 studies), dermatological conditions (1 study) and from the general population (3 studies). Two studies identified a four factor structure in samples with end stage renal disease and in a sample from the general population. On the whole the factors identified were consistent with the sub-scales of anxiety and depression but there was a consistent finding that one of the anxiety items ('I can sit at ease and feel relaxed') had a relatively low anxiety loading (<0.6) and some loadings on the depression subscale (>0.45). In Rasch analysis of the HADS this item has also proved to fit poorly in a two factor solution (Gibbons et al. 2011; Pallant and Tennant 2007; Pallant and Bailey 2005).

Despite the high number of studies exploring the HADS factor structure using principle components analysis (PCA) in a variety of populations, there is an argument that this is inappropriate due to the underlying level of measurement of the data. The HADS returns a numerical score but this is obtained from ordinal data and therefore it has been suggested recently that it is more appropriate to explore the psychometric properties of the scale using

the Rasch model (Pallant and Tennant 2007). Using this model Pallant and Tennant (2007) examined the HADS responses of 296 people with musculoskeletal problems who were attending an outpatient rehabilitation programme. Their analysis supported the use of the 14-item HADS scale as a global measure of psychological distress although they note that not all items fit the model well. Their work is echoed by Gibbons *et al* (2011) who also find support for a unidimensional solution to the HADS in their study of people with motor neurone disease. Both studies identify A6: I fell restless as if I have to be on the move as a poor fit whatever solution. Pallant and Tennant (2007) retain all the items in their solution but Gibbons *et al* (2011) remove D5: I have lost interest in my appearance and find a better fit.

One of the hypothesised reasons for the poor fit of items in Gibbons *et al* (2011) is that their cohort of participants interpret the meaning of the item differently because of the functional effects of motor neurone disease – thus in their exploration of the HADS as a two factor scale they found that 'I feel slowed down' was a poor fit and suggested that this item is more about physical function than mental fatigue. There is potential for this to be a confounding factor in the present study.

Pallant and Tennant (2007) recommend a cut-off point indicating psychological distress of 12 using the total HADs score (HADS-T). They found that this was able to capture all participants who score >8 for anxiety, >8 for depression or >8 for both which is the cut-off point for individual factors recommended by Bjelland *et al.* (2002). Pallant and Tennant (2007) offers the most appropriate guidance for the current study due to the similarity of the participant populations and therefore the HADS data will be used as a single measure of psychological distress.

5.4.4.2 Completion of the questionnaire

Participants were encouraged to complete the HADS questionnaire themselves and when necessary the investigator read the items and responses to the participant. Clarification of items was given when asked for; usually this consisted of reminding the participant that the items related to current mood.

5.4.4.3 Scoring the HADS and data management

Raw scores for each HADS item were entered into the SPSS database. Anxiety and depression scores and a total HADS (HADS-T) was created from the raw data and a categorical variable was also created to represent no psychological distress (HADS-T≤11), and psychological distress (HADS-T ≥12).

5.5 CSF collection, storage and processing

This study will use the contents of cerebrospinal fluid taken from the lumbar region as an indicator of pain and mood related processes taking part in the dorsal horn and the brain.

Prior to commencement of the anaesthetic procedure the research nurse (AS) prepared a work space and equipment on the anaesthetic room bench. The equipment required was brought to the anaesthetic room in an adapted tool box and comprised:

- Dewar flask (1L) containing 500ml liquid nitrogen
- Sterile syringes (2ml, 5ml)
- Sterile 0.22 micrometer Millipore filter (Millex-GS, Millipore)
- Sterile flat bottomed screw top 5ml container (NHS supplies)

- Pipette (Pipet-lite Standard 20-200µl; Anachem)
- Sterile pipette tips 200 µl (Plastibrand, Sigma Aldrich)
- Sterile Eppendorf 0.2ml PCR tubes (Sigma Aldrich)
- Permanent marker pen (Sharpie)
- Forceps

5.5.1 The lumbar puncture

The research nurse (AS) assisted the participant to adopt one of two preferred positions for the dural puncture. The first is a side-lying posture where the participants back is parallel to the edge of the theatre trolley and his or her knees are drawn up towards the chest and head drawn towards the knees, with the aim of 'opening' the spaces between lumbar vertebrae. The alternative posture is seated on the theatre trolley. The participant's knees are drawn up towards the chest with the aid of a stool for his or her feet to rest upon and he or she is asked to bow the head towards the knees. In both postures the participant was asked to try to 'push out' the lower spine as if creating a greater curvature.

5.5.1.1 Equipment

- Clean dressing trolley
- Sterile 'epidural pack'
- Spinal needle
- Sterile 5ml syringe x 2
- Sterile gloves
- Chlorhexidine/alcohol solution

Once the patient was comfortably positioned and minimally supported by the research nurse the anaesthetist cleansed the skin around the lumbar spine with a chlorhexidine/alcohol solution (2% chlorhexidine gluconate and 70% isopropyl alcohol) and allowed to dry while final preparations were made to the sterile field and equipment.

The anaesthetist injected a maximum of 5ml of 10mg ml^{-1} local anaesthetic (1% lignocaine) into the subcutaneous tissues, muscles and ligaments of the lumbar region at the level of L4/5 or L3/4. A 25 gauge spinal needle (Smiths Medical, Ashford, Kent, UK) was then advanced through the skin and subcutaneous tissue to access the sub-dural space using the loss of resistance technique to establish correct placement (Tran et al. 2009;Hoffmann et al. 1999). Once the needle was judged to be in the subdural space at lumbar region and spinal fluid was seen to leak from the needle under gravity a 5ml syringe was attached to the spinal needle and gently pressure applied to aspirate up to 2ml of spinal fluid. Once the fluid was collected an operating department practitioner took over from AS in caring for the patient and AS moved to the anaesthetic room work bench to process the CSF sample.

After the anaesthetist had collected 2ml of CSF the researcher filtered this immediately through a 0.22 micrometer filter (Millex-GS; Millipore, Watford, UK) into a sterile receptacle to remove cells from the sample. The CSF was then divided into 200 microlitre (µl) aliquots using a pipette (Pipet-lite Standard 20-200µl; Anachem, Luton, UK) in propylene Eppendorf safe-lock 0.5ml tubes (Sigma-Aldrich, Dorset, UK). Each tube was labelled using indelible ink with the participant's unique sequential numeric identifier. The tubes were sealed and then immersed in liquid nitrogen until boiling stopped in order to rapidly freeze the contents.

The tubes were then transported to the freezer room a short distance away still inside the Dewar flask.

Once in the freezer room a storage box with sufficient capacity was removed from the minus 80°C freezer (Nalgene cryobox (10x 10), Sigma-Aldrich) and the labelled tubes checked for intact seal and labelling and placed inside sequentially. The lid of the box was labelled with the corresponding participant ID and then the box placed flat into the freezer.

The tubes were then stored at -80ºC freezer until analysis. Storage durations ranged from a minimum of 1 month to a maximum of 18 months. The freezer was checked daily to ensure a stable temperature was maintained throughout the storage period.

5.6 Laboratory analysis of amino acids

Assay of amino acids was carried out using the method described below by Naomi Langman under the guidance of Dr. Kevin Whitehead at the University of Birmingham.

Single aliquots from each participant were placed on ice to thaw to room temperature prior to the assay procedure.

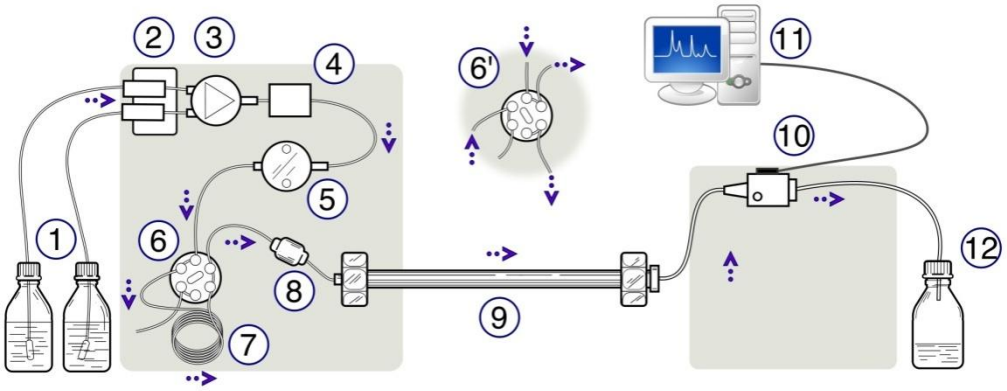
5.6.1 Reverse phase high pressure liquid chromatography

High pressure liquid chromatography (HPLC) is a method for separation of components in a mixture (Figure 5-4). The process relies upon the fact that each component will have a different degree of affinity for a specifically selected solvent in which they are contained (the mobile phase) and for the medium through which this solvent is passed (the stationary phase).

Each component will therefore take a slightly different length of time to pass through a column, and is detected upon leaving the column.

This technique was used to separate the selected amino acids in CSF samples. Before being injected into the column and pumped through the system the CSF was combined with an agent that alters the component properties in such a way that they can be detected as they leave the column; this is called the derivatising agent. In this case the amino acids are derivatised with ortho-phthaldiahyde/3-mercaptoproprionic acid (OPA/3-MPA). This method of derivatisation renders the amino acids into highly fluorescent components (Jones and Gilligan 1983) so that they may be detected using fluorescence, which is a highly sensitive method. This method was developed by colleagues to improve the efficiency and rapidity of HPLC analysis of amino acids in biological fluids (Devall et al. 2007).

Figure 5-4: HPLC schematic



Wikipedia commons: http://en.wikipedia.org/wiki/File:HPLC_apparatus.svg

The derivatising agent was prepared by mixing 975 µl of incomplete o-pthaldialdehyde reagent (1mg ml^{-1} Sigma UK) in potassium borate buffer (pH 10.4, Sigma, Dorset, UK) with 25 µl of 10% (v/v) 3-mercaptopropinonic acid (Sigma-Aldrich) in methanol (Fischer Scientific, Leicester, UK). Exactly 3µl of the derivatising agent was mixed three times with 10 µl of thawed cerebrospinal fluid and allowed to react for 60 seconds. Derivatisation was carried out automatically by a CMA/280 (CMA/Microdialysis Sweden) refrigerated autosampler (8 ºC) incorporating a 20 µl injection loop.

The mobile phase consisted of :

A: HPLC grade deionised water (18MΩ resistivity, Elga Water Systems UK),

B: 95% methanol and 5% 100mM sodium acetate buffer (pH 6.95)

C: 5% methanol.

A tertiary gradient elution profile was used (Table 5.2) which was programmed to run for 17min.

Table 5.2: Changes in the mobile phase in the HypersilTM ODS column during a 17 min chromatographic run.

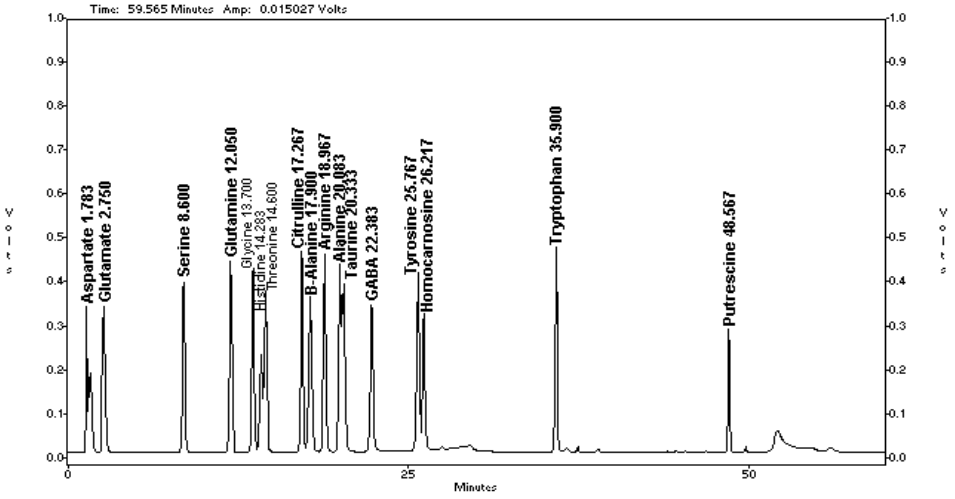
Time	A	B	C
0	48	2	50
27	20	30	50
47	0	70	30
48	0	100	0
50	0	100	0
51	48	2	50
60	48	2	50

The mobile phase was pumped by a PM-80 twin-reciprocating pump through a LC-26A vacuum degasser (both BAS Technicol UK) a 2ml min^{-1}. Derivatisation and sample injection

(20 µl) were automated by a CMA/200 (CMA/Microdialysis Sweden) refrigerated autosampler (8 ºC) incorporating a 20 µl injection loop. The separation was performed using a HypersilTM octadecyl siliane (ODS) 5 µm particle size reverse phase analytical column (3.0 mm x 150 mm; Chrompack UK) maintained at 30ºC. Fluorescence was detected by a CMA/280 fluorescence detector (maximum excitation 340-360 nm; maximum emission 495nm; CMA/Microdialysis Sweden). The amino acid peaks were identified on the basis of retention times with the data being collected and analysed using EZChrom software (Aston Scientific, UK). Quantification was made with reference to a three-point calibration curve.

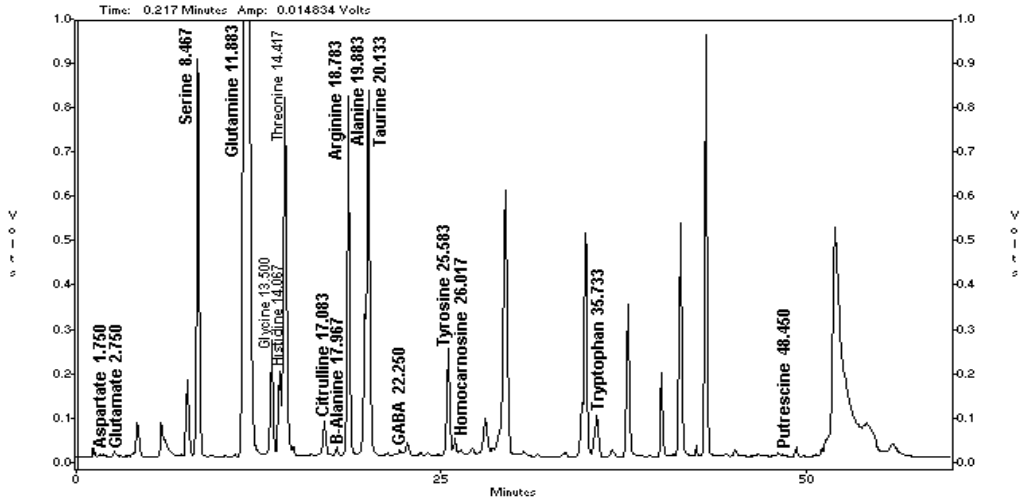
Standard curves were created from the injection of 10 µL of prepared standard solutions containing 20 pmol, 100 pmol and 200 pmol of the amino acids; arginine, aspartate, citrulline, GABA, glutamate, glutamine, glycine, isoleucine, leucine, phenylalanine, serine, tryptophan, tyrosine and valine. These solutions were prepared from stock solutions of the amino acids containing 200 µM (Sigma-Aldrich) in water and stored at -80ºC. Standard curves were forced through the origin for all amino acids except for glycine and serine where consideration was made for background levels that are present in detectable amounts in HPLC grade water. A typical chromatograph of the standards can be seen in Figure 5-5.

Figure 5-5: Typical chromatogram produced from a range of standards using the HypersilTM ODS column (courtesy of N. Langman).



All samples were in range of the sensitivity settings except for glutamine therefore a sample was diluted 10-fold to allow glutamine assay. A typical CSF chromatograph can be seen in Figure 5-6 which illustrates that glutamine is off-scale.

Figure 5-6: Example CSF chromatograph using HypersilTM ODS column (courtesy of N. Langman).



5.7 Laboratory analysis of cytokines

Assay of cytokines was carried out by A. Swift with the guidance and assistance of Dr Alison Hart at the University of Warwick.

Multiplex microbead array assay is a relatively new technology. It is often compared with Enzyme Linked Immunosorbent Assay (ELISA) because both assays use an antibody to capture a soluble ligand and detection of this paired complex with a second reporter antibody (Elshal and McCoy 2006). Multiplex differs in that it uses fluorescence detection and can be used for multiple analytes (Kellar and Douglass 2003). Up to 100 different analytes can be detected simultaneously as a result of the technology that uses a different red and near infra-red spectral 'address' for each of the bead populations used in each assay. At the time of this assay a newly developed high-sensitivity cytokine kit had been developed by Luminex, allowing the simultaneous assay of 13 cytokines. It is important to guard against cross-reactivity within the analytes being investigated and therefore an 'off-the-shelf' kit was chosen rather than creating a bespoke array of beads.

CSF levels of the chosen cytokines were determined by a high sensitivity 13-plex panel (LINCOplex HSCYTO-60SK, Luminex) on undiluted samples using Bioplex software (Bio-Rad, California). This kit simultaneously quantifies GM-CSF, IFNγ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12(p70), IL-13 and TNFα.

The manufacturer's literature stated that the minimum detectable concentration for each cytokine was:

IL-1β	0.06 pg ml^{-1}
IL-2	0.16 pg ml^{-1}
IL-4	0.13 pg ml^{-1}
IL-5	0.01 pg ml^{-1}
IL-6	0.1 pg ml^{-1}
IL-7	0.12 pg ml^{-1}
IL-8	0.11 pg mL^{-1}
IL-10	0.15 pg ml^{-1}
IL-12(p70)	0.11 pg ml^{-1}
IL-13	0.48 pg ml^{-1}
IFNγ	0.29 pg ml^{-1}
GM-CSF	0.46 pg ml^{-1}
TNFα	0.05 pg ml^{-1}

The kit was stored as recommended at 4 ºC prior to use and before the analysis began was placed on the laboratory bench to reach room temperature (21ºC). Single 200 µl aliquots of the samples to be assayed were thawed on ice prior to the procedure beginning.

Two 96-well plates were prepared. The wash buffer (10 mM phosphate buffered saline [PBS], 0.05% Proclin, 0.05% polyethylene sorbitan monolaurate [Tween 20], pH 7.4), and standards (containing a cocktail of mixed lyophilized cytokines) were prepared according the manufacturer's instructions (all from Linco Research, Luminex). 200 µl of wash buffer was added to each well of the plate, which was then sealed and shaken for 10 min on a plate shaker at room temperature. A vacuum of 100 mmHg was applied to the plate to remove the excess wash buffer.

The bottle containing the prepared beads was provided in the kit and was sonicated for 30 seconds and held on a vortex for a further 60 s; 25 µl of the bead mix was then added to each

well of the plate with intermittent shaking of the bottle to maintain mixing. A 100 mmHg vacuum was again applied to the plate to remove the liquid from the well plate.

The next step was to add 50 µl of control solution or standard to each well according to a plan documented on a Well Map Worksheet provided with the kit. The 2000 pg ml^{-1} standard was prepared by reconstituting one vial of the standard with 250 µl de-ionised water producing a solution of 2000 pg ml^{-1} for each analyte. Six polypropylene microcentrifuge tubes (Sigma UK) were pre-labelled with 400, 80, 16, 3.2, 0.64 and 0.13 pg ml-1 and 200 µl of assay buffer (50 mM PBS, 25 mM ehtylenediaminetetraaceticacid [EDTA], 0.08% sodium azide, 0.05% Tween 20, 1% bovine serum albumin [BSA], Lincoplex) was added to each tube. A serial dilution was then conducted beginning by adding 50 µl of the 2000 pg ml^{-1} standard to the 400 pg ml^{-1} vial and mixing thorough. The process was continued until then final dilution creating 0.13pg ml^{-1} tube was mixed. Assay buffer was used for the 0 pg ml^{-1} standard.

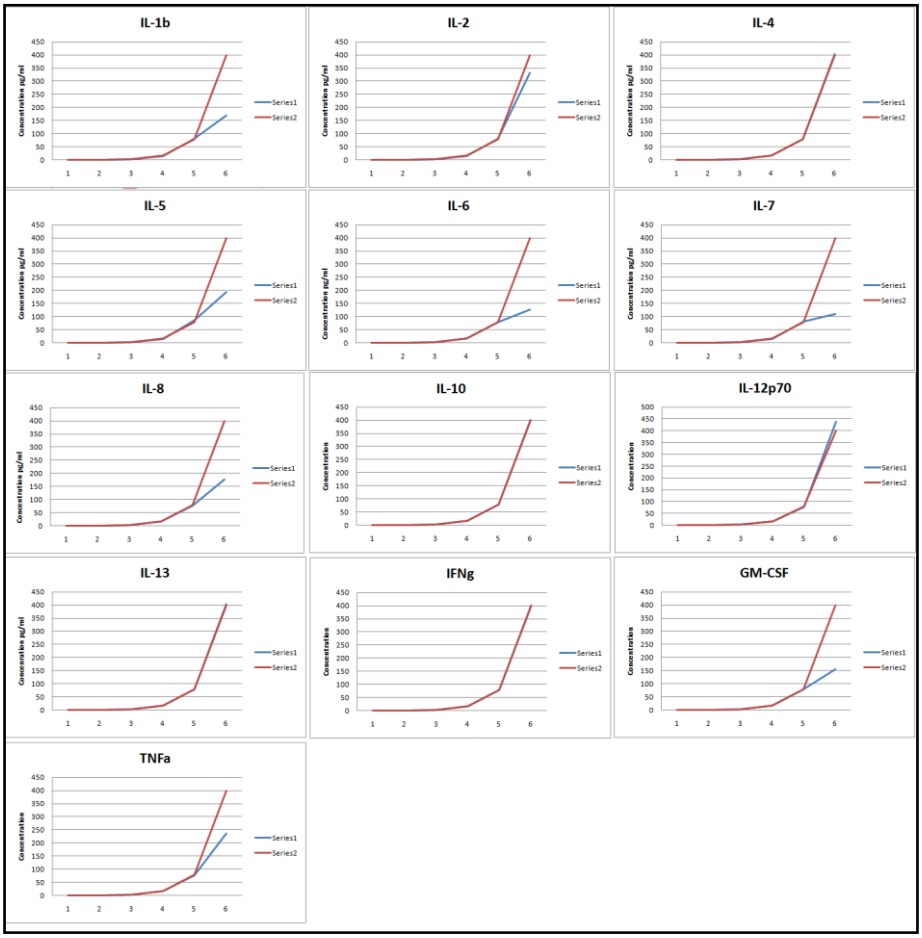
50 µl of assay buffer was added to each well on the plate. Each sample was then centrifuged in turn before 50 µl was added to the appropriate well in the plate (the samples were added to both plates to create duplicates). The plates were then sealed and incubated overnight on a plate shaker at 4 ºC.

The following morning a 100 mmHg vacuum was applied to each plate to remove excess fluid. The detection antibodies were allowed to warm to room temperature and 50 µl was added to each well. The plates were sealed and allowed to incubate on a plate shaker for one hour at room temperature. Following this 50 µl Streptavidin-Phycoerythrin was added to prepare the samples for detection. The lid was replaced and sealed and the plates shaken for a further 30 minutes. A vacuum was once again applied to remove the liquid. Well wash

buffer (200 µl) was added to each well and removed by vacuum two times. Finally 100 µl of sheath fluid (used to suspend the bead reagent prior to introduction to the analyser: composed of phosphate buffered saline with ProClin®300 and sodium azide (<0.1%) preservatives) was added to each well and the plate sealed and shaken on the plate shaker for a further five minutes. The plates were then placed in the BioPlex analyser using the settings advised by the manufacturer of the bead kit.

The calibration data (Figure 5-7) demonstrates that for most of the cytokines there was a high degree of accuracy in the lower ranges but in some cases this was lost in the upper ranges.

Figure 5-7: Standard curves for cytokine multiplex assay (produced by A Swift).



5.8 Statistical analysis

All data was coded and entered into SPSS version 18.0. The data was checked against original paper records before preliminary data exploration was conducted. All data analysis unless otherwise specified will be undertaken using the SPSS programme.

5.8.1 Analytical subgroupings

The differences in amino acids and cytokines as specified in the hypotheses will be assessed between the osteoarthritis group and the control group (Table 5.3). Subgroups will be formed from the OA group to create a group who have no pain at rest (0PAR) and those who have pain at rest≥1 (PAR).

Table 5.3: Amino acid and cytokine groupings

Excitatory amino acids	Inhibitory amino acids	Neutral Amino acids	Pro-inflammatory cytokines	Anti-inflammatory cytokines
Glutamate	GABA	Leucine	IL-1β	IL-4
Aspartate	Glycine	Isoleucine	IL-2	IL-5
Arginine		Phenylalanine	IL-6	IL-10
Citrulline		Tyrosine	IL-7	IL-13
Serine		Tryptophan	IL-8	
Glutamine		Valine	IL-12	
			IFNγ	
			TNFα	

5.8.2 Descriptive data and preliminary data exploration

Demographic data will be presented as frequencies and percentages with comparisons between groups being made using chi squared tests or when fewer than 5 cases are expected to occupy one or more cells, Fisher's exact test.

Data relating to the medication and medical history of the participants will be provided in order to give as thorough a picture as possible of the sample but this data will not be utilitised in any of the analyses due to small group sizes.

POM, PAR and HADS are considered ordinal level measurements and therefore comparisons of these scores between groups and sub-groups will be made using non-parametric statistical tests. Mann-Whitney U test will be used to compare pain scores between groups and Friedman will be used to explore differences in estimated pain on movement at the three different time points requested of the participants.

Missing values and outliers in the amino acid and cytokine data will be identified prior to further analysis. Outliers and extreme values can be problematic in a number of statistical analyses. Outliers and extreme values in the amino acid and cytokine data will be identified by visually inspecting box-plots. Outliers are defined as data points that lay more than 1.5 but less than 3 interquartile ranges (IQR) from the median. Extreme values are defined as those data points more than 3 IQR from the median. These values will be checked against paper records and corrected where appropriate. Outliers will not be removed from the dataset as a matter of course but residuals will be inspected during regression analysis to determine whether any of the outlying cases have undue influence.

Prior to data analysis retrospective power calculations for amino acids and cytokines will be performed using the online power calculation software from Decision Support Systems (2011).

5.8.3 Univariate analysis of amino acid and cytokine concentrations

This section of the results chapter will explore univariate relationships between the amino acids and cytokines (the independent variables) of the control and OA groups, and between the same variables in the PAR and 0PAR groups. In keeping with the hypothesis of the study that differences between groups will persist after adjustments are made for age, gender and HADS-T these statistical analysis of the differences between groups will take these into account. It was hoped to use medication as a further covariate but the sample sizes once groups have been created would preclude this.

Normality of this data will be tested using a combination of visual inspection of histograms and Kolmogorov-Smirnov tests. In the case that amino acids and cytokines are distributed normally summary data for these variables will be presented in the form of mean and standard deviation. In the event that the amino acids and cytokines are not normally distributed summary data will be presented as median and interquartile range.

ANCOVA will be used to identify differences in amino acids and cytokines in each group (control/OA and PAR/0PAR). In the event that data residuals are not distributed the ANCOVA will be supported by bootstrap analysis. This involves the repetitive sampling of data points to construct a distribution of F statistic values from which the significance of the original F statistic can be identified and can be used to over-come non-normality of the data

set (Tabachnick and Fidell 2001). Bootstrap analysis if necessary will be conducted using STATA with programming by Linda Nichols, University of Birmingham.

A p value of less than 0.05 will be used as a cut-off point for the identification of results that are significant. Bland and Altman (1995) suggest that in cases where a number of statistical tests are being carried out the p value should be adjusted to take into consideration the fact that each test is unlikely to be completely independent of the others. Following this reasoning the acceptable p value for each set of tests conducted should be 0.05 divided by the number of tests being conducted. Perneger (1998) argues that this rule should only be applied if the study were looking for a significant difference in all of the variables – a universal error rate. He goes on to explain that the original Bonferroni adjustment was intended as a decision making aid relating to quality assurance and is therefore not relevant to the application it has found in inferential statistics. Further he states that the reader can apply his or her own interpretation of the selected p value and that using too stringent a p value will increase the risk of potentially important results being missed.

Decisions about the significance of results also need to take into consideration the theoretical or clinical relevance of the result. This is an exploratory study and therefore has been little information published about the analytes in osteoarthritis pain and none in humans. It is therefore considered appropriate to retain an individual p value of 0.05 for each test but to view each result in its clinical and theoretical context.

5.8.4 Regression analysis

Binary logistic regression analyses will be performed in order to determine those variables that most usefully predict

a) membership of the OA group,
b) membership of the PAR group
c) membership of the HADS-T ≥12 (psychological distress) group.

The selection of binary logistic regression as opposed to multiple linear regression for the OA/control and PAR/0PAR is because they are naturally dichotomous groups. Participants either have pain as a result of OA or do not, and either have pain at rest or do not. Determination of the cut-off point to signify the presence or absence of psychological distress is less exact, and although Pallant and Tennant (2007) recommend a score of 12 as representative of the likely presence of psychological distress it is possible that this cut off point could differ dependent on the population being studied. Therefore, as well as exploring the presence or absence psychological distress with a binary logistic regression, HADS-T will also be explored using a linear regression technique.

The sample size dictates the number of independent variables (IV) that can be safely entered into the regression equation. Tabachnick and Fiddell (2001) use the equation

where *m* = the number of IV. Therefore, if this regression was to include all the possible IVs the sample size would be . If the regression included between 6 and 8 independent variables the required sample size would be between 98 and 114. A number of

criteria will be applied to the IVs in order to determine which are most appropriate for the analysis, which will have the secondary and beneficial effect of reducing their number.

The criteria applied to the independent variables will include inspection of the number of missing values for each independent variable in order to maximise the number of cases that will be entered into regression analysis.

In addition to this correlations between independent variables will be inspected in order to identify strong relationships. Pearson correlations will be used in the event that raw data is normally distributed or Spearman correlation in the event that raw data is not normally distributed. The amino acids and cytokines fall in natural groupings (Table 5.3), which were determined before the study and are based on research evidence identifying their primary role in pain signalling. Correlation data will be presented in these groupings and plotted diagrammatically in order to enable consideration of correlations between pairs of independent variables within their own group as well as between groups.

The value of r that will trigger further inspection of the correlation between two independent variables will take into consideration the theoretical biological relationship between the two as well as the number of cases entered into the test. Cohen (1988) described some 'rule of thumb' guidelines for the interpretation of strength of association with 0.1 being considered small, 0.3 being medium and greater than 0.5 being large. However, Cohen and subsequently others (e.g. De Vaus 2002) explained that these guidelines did not consider the sample size (small samples often produce higher correlation coefficients), nor the clinical or theoretical relationship between two variables. In this study all correlations greater than r=0.4 at a level of $p<0.05$ will be inspected. A criteria of $r > 0.8$, $p<0.05$, will be used to denote a strong

relationship between a pair of independent variables: in pairs where this occurs consideration will be given to removal of one of the pair from entry into the regression model to reduce the risk of redundancy in the analysis (Pallant 2007; Tabachnick and Fidell 2001).

Correlation coefficients will be used to determine whether the large neutral amino acid group should be summed to act as a single predictor, or whether one of this group appears to be pivotal and can be used alone. Summation has been used to create a composite large neutral amino acid score (ΣLNAA) in research investigating depression, where the amino acids competing with tryptophan to cross the blood brain barrier have been simply summed prior to statistical analysis (Moreno et al. 2010; Porter et al. 2005; Capuron et al. 2002).

Multicollinearity can reduce the trustworthiness of the *b* coefficients, (revealed as large *b* standard errors) and can make it difficult to interpret the importance of an individual predictor. It is therefore important to assess the model for multicollinearity and this can be conducted within the regression analysis by examination of the tolerance and variance inflation factor (VIF). Multicollinearity will be identified if any IV has a tolerance of less than 0.1 and VIF of greater than 10 (Pallant 2007: 155; Field 2009: 224).

Logistic regression will begin with an initial model comprised of age, gender and HADS-T (plus the constant). The next step will use a forward step-wise process to add selected predictor variables. Selection of the most appropriate variable(s) will be based the most significant score statistic using an entry criterion of p=0.15. This is recommended as it makes it less likely that important variable will be excluded (Bendel and Afifi 1977). The process also removes variables if they contribute less to the model than the most recently entered

variable. The criteria for removal will be that the independent variable contribution is not significant at the 0.10 level.

The forward stepwise method is a form of selection of variables by purely statistical criteria and although it is criticised for this reason it can be helpful in the initial stages of building a model as it can be used to eliminate variables that are superfluous (Tabachnick and Fidell 2001).

The model produced by binary logistic regression will be assessed using a number of criteria (Field 2009).

a) Percentage correct classification of cases: the percentage of cases correctly classified by the model. The cut-off for classification will be the default SPSS setting of 50%.
b) Model prediction better than initial model (containing only the constant): this is assessed by a Goodness of fit test, which returns a χ^2 value. Significance of $p<0.05$ indicates that the model containing the selected independent variables performs better than the initial model.
c) Hosmer and Lemeshow 'poorness of fit': This statistic indicates whether the model performs better than the initial model and returns a χ^2 value. A p greater than 0.05 indicates that the model does perform better than the initial model.
d) The Wald statistic: this calculates the statistical significance of each of the coefficients in the model. A p value of less than 0.05 identifies the predictor variable as making a significant contribution to the model.
e) The odds ratio: an indication the change in odds caused by a unit change in the predictor variable. A positive odds ratio indicates that membership of the group

becomes more likely as the value of the predictor variable increases. A negative value indicates that the likelihood of group membership decreases as the value of the predictor variable increases.

f) Nagelkerke R^2: This value gives an indication of how much variance in the outcome variable is explained by the model.

Following the regression process it is important to check that influential or unusual cases have not unduly affected the outcome. Detection of outliers that bias the model will be performed by examination of the standardised residuals (residuals divided by their estimated standard deviation). There should be no standardised residuals with an absolute value>3.29, less than 1% of cases should have a standardised residual absolute value >2.58 and less than 5% of cases should have a standardised residual absolute value >1.96 (Field 2009). The standardised residuals for outlying cases will be inspected via the case-wise listings produced by SPSS as part of the regression process (Burns and Burns 2009).

The potential influence of individual cases will be assessed by examining Cook's distance (D_i), DFBeta and leverage. Cook's distance measures the effect of deleting an individual observation on the regression coefficient. McDonald's guidelines (2002) will be used to evaluate Cook's distance (Table 5.4). These guidelines are based on the assumption that there are more than 15 cases.

Table 5.4: McDonald's guidance on Cook's Distance for influential cases in regression analysis

Cut-off point for influential case (D_i)	Number of predictors including the intercept
>0.7	1
>0.8	2
>0.85	>3

DFBeta similarly shows how much a *b* coefficient would change if the case were dropped from the regression. A standardised DFBeta of greater than 1 is suggested to indicate a case with unusual influence (Field 2009). High leverage is a term used to describe an outlying data point that is very far away from the mean and has a large residual. The result of a value with a high leverage is that it can pull the regression line toward it and therefore have undue influence over the outcome. SPSS calculates a version of leverage where the maximum value is equal to 1 and would mean that the case has complete influence over the prediction (Field 2009). It is suggested that cases with leverage of 3 to 5 times the average should be investigated. Average leverage is calculated — where κ is the number of predictors and n the sample size (Field 2009).

In the multiple linear regression (for HADS-T) the R^2 value will be given as in indication of how much variability of HADS-T is accounted for by the predictors. An F-ratio is used to determine whether the change in R^2 from the amount of variance explained by the initial model (containing only the constant) and the variance explained by the model is significantly different. An F-ratio with a p value of less than 0.05 indicates that the variance accounted for by the model significantly improves on the variance explained by the initial model.

An ANOVA is reported in the linear regression model which provides an indication of whether the model is better at predicting HADS-T than using the mean HADS-T as a 'best guess' (Field 2009: 236). Beta coefficients (*b*) will be reported along with their standard error. The t statistic calculated from the *b* indicates whether the predictor variable contributes significantly to the model and a value of $p<0.05$ will be used to indicate this is the case.

Multiple linear regression requires residuals to be uncorrelated and this is tested with the Durbin-Watson test. This test returns a value between 0 and 4. A value of 2 indicates the residuals are not correlated. The value returned by the Durbin-Watson test will be compared with the values given in the original paper (Durbin and Watson 1951) using a p value of 0.05. The upper and lower bounds of the critical value for d are given. When the value for the observed *d* is below the lower bound the test is significant (serial correlation has occurred), when it is above the upper bound it is not significant (serial correlation has not occurred). An observed value of *d* that lies between the upper and lower bound means that the test has been inconclusive.

Chapter 6 Results

6.1 Demographic and descriptive data

The total number of control participants entered into the analysis was 21 and the total number of OA participants was 59.

CSF samples were taken from 27 control cases and 67 OA cases on dates between 15 July 2003 and 2 March 2005. The number of patients who declined to participate in the study was not recorded. Two controls and 1 OA case had missing amino acid data and so were excluded from all analyses. A further three controls were excluded because they were having hip or knee surgery and although they did not admit to pain it was not possible to rule out a progressive or inflammatory process. Seven further OA cases were excluded because they were undergoing bladder surgery rather than hip or knee replacement, and one additional control because he had not received an all-clear notification with regard to previous bladder carcinoma. No data was retained regarding spoiled samples (i.e. contaminated with blood) but this was a rare occurrence and resulted in fewer than 5 participants being excluded from the study after consent.

Ninety-one per cent (n=19) of the control group were admitted for investigation or surgery of the bladder, and 9% (n=2) for transvaginal taping. In the OA group 34% (n=20) were admitted for primary total hip replacement, 43% (n=25) for primary total knee replacement and in 23% of cases (n=14) the joint being replaced was either hip or knee but was not recorded.

The age and gender of the OA participants in this study is similar the national demographic profile for hip and knee arthroplasty which comprises approximately 40% males with an average age of 69 years (Table 6.1).

Table 6.1: Age and gender of participants

	Control (n=21)	OA (n=59)	Statistic/probability
Male	16 (76%)	28 (47%)	[a]p= 0.020
Age: mean (SD)	67.1 (11.9) range 42-85	68.9 (11.2) range 45-88	[b]t_{78}=-0.5, p= 0.601

[a]Fisher's exact test; [b]independent *t* test

Most members of the OA group and some members of the control group took anti-inflammatory or analgesic medication (Table 6.2). Anti-inflammatory medications (and low dose aspirin) was stopped by participants at least 10 days before surgery.

Table 6.2: NSAID and analgesic use in OA and control participants

Medication type	Name	Control n(%)	OA n(%)
None		17 (81)	10 (17)
	Paracetamol	1 (3)	12 (20)
Combination	Coproxamol	1 (3)	7 (12)
	Codydramol	0 (0)	4 (7)
	Cocodamol	1 (3)	11 (19)
NSAID		1 (3)	31 (53)
Strong opioid	Tramadol	0 (0)	7 (12)
	Morphine	0 (0)	2 (3)

The past medical history and general medication use is shown in Table 6.3 and Table 6.4. Totals in these tables may exceed the total number of participants as participants can specify more than one condition or treatment.

Table 6.3: Past medical history of participants

		Control (n=21)	OA (n=59)
Cardiovascular	Hypertension	3	21
	Angina	1	5
	Previous stroke		1
	Previous MI		1
	Peripheral vascular disease	1	1
Respiratory	Asthma	1	9
	COPD	1	2
Endocrine	Diabetes type 1	1	
	Diabetes type 2	2	5
	Hypothyroid	1	2
	Hyperthyroid		1
Gastrointestinal	Peptic/duodenal ulcer	2	4
	Hiatus hernia	1	1
Skin	Psoriasis		1
Psychological	Depression	1	1
	Anxiety		1
	Psychosis	1	
Musculoskeletal	Gout	3	4
Previous surgery		13	43
Other	Previous opioid abuse		1

Table 6.4: General medication use

Drug	Control	OA
Aspirin 75mg	4 (19%)	9 (15%)
Antihypertensive	6 (28%)	33 (56%)
Proton pump Inhibitor	2 (10%)	10(17%)
Antiprotozoal (Quinnine)		2 (3%)
Indigestion	1(5%)	3 (5%)
Laxative		2 (3%)
Inhaled steroids	2 (10%)	10 (17%)
Antihistamine		1 (2%)
Bronchodilators	2 (10%)	8 (13%)
Hypnotics/anxiolytics		7 (12%)
Lipid regulation	4 (19%)	8 (14%)
Hypoglycaemic agent	1 (5%)	6 (10%)
Glaucoma		1 (2%)
Urinary retention/prostate	6 (29%)	3 (5%)
Thyroxine	1 (5%)	4 (7%)
Ferrous sulphate		2 (3%)
Antipsychotic		1 (2%)
Anticoagulant		2 (3%)
Bisphosphonate		4 (7%)
Antidepressant SSRI (Sertraline, paroxetine)		6 (10%)
Antidepressant SNRI (Venlafaxine)		3 (5%)
Antidepressant tricyclic (Amitriptyline, doxepin)	1 (5%)	1 (2%)
Allopurinol	2 (10%)	

6.1.1 Pain on movement (POM) and pain at rest (PAR)

The participants in the control group had zero POM and zero PAR. The distribution of POM and PAR scores in the OA group is not normal (Figure 6-1, Figure 6-2 and Table 6.5)

Figure 6-1: Distribution of POM scores

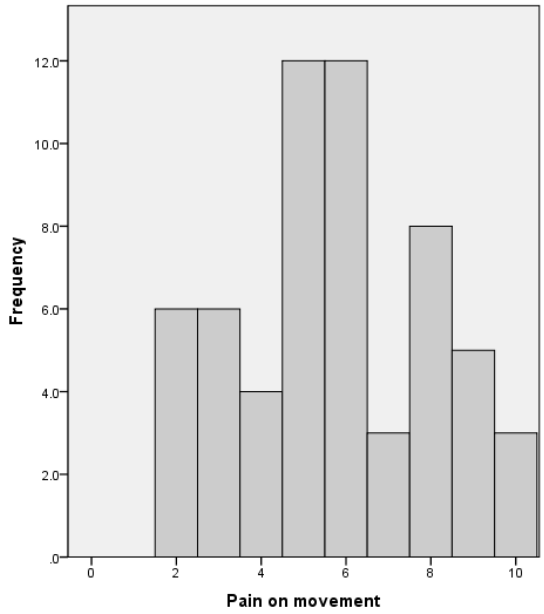


Figure 6-2: Distribution of PAR scores

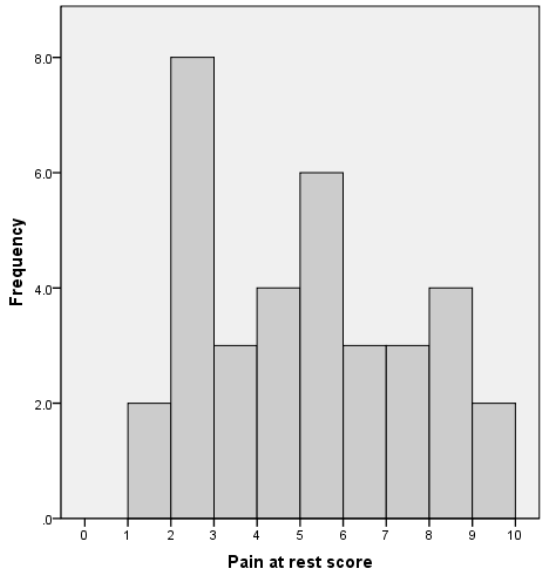


Table 6.5: Kolmogorov-Smirnov normality tests for POM and PAR in the OA group

	Statistic	Degrees of freedom	Significance
POM	0.131	59	p = 0.014
PAR	0.231	59	p = <0.001

The median POM in the OA group was stable in the 12 months leading up to the study (Table 6.6: = 3.83, p=0.147). The median PAR in the OA group was 2 (range 0-9, IQ range 5).

Table 6.6: Median pain on movement now, last three months and last 12 months

Pain on movement	Median (IQR)
Current	6(4)
Average last 3 months	6(3)
Average last 12 months	6 (3)

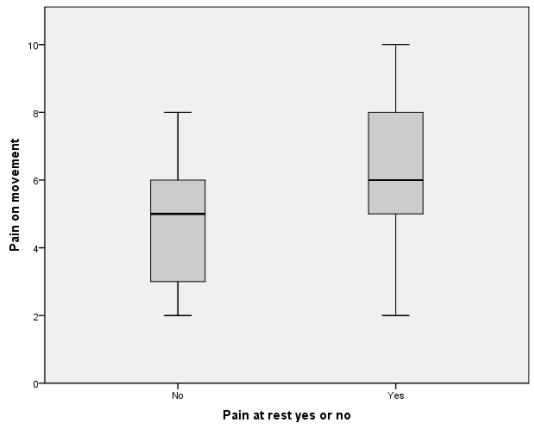
When the PAR/0PAR groups were compared there was no significant difference in gender, age, or proportions of hip or knee sites of pain (Table 6.7). POM was significantly higher in the PAR group (Table 6.7, and Figure 6-3).

Table 6.7: Gender and POM difference between PAR and 0PAR groups

	0PAR n(%)	PAR n(%)	Statistic
n(%)	24 (41)	35 (59)	
Male: n(%)	14 (58)	14 (40)	[a] =1.919, p=0.131
Age: Median (IQR)	72 (15)	67 (18)	[b] *U*=368.500, p=0.426
POM : Median (IQR)	5 (3)	6 (3)	[b] *U*=603, p=0.004
Hip: n(%)	6 (13)	14 (31)	[a] =0.336, p=0.258
Knee: n(%)	11 (24)	14 (31)	

[a]Chi-squared; [b] Mann-Whitney *U* test;

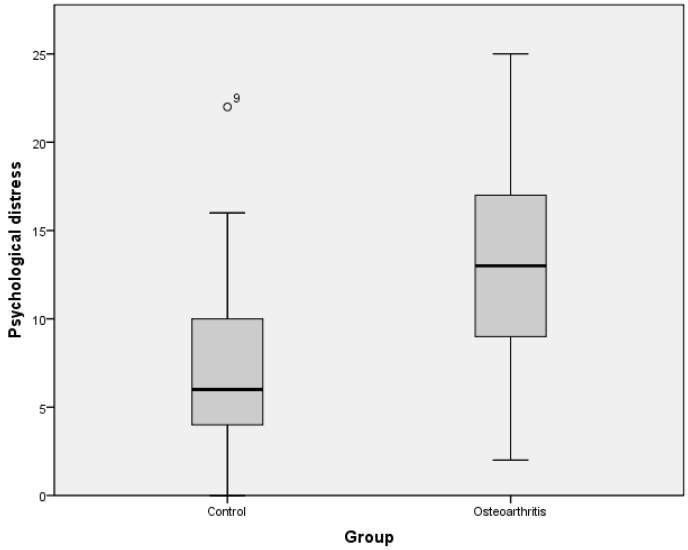
Figure 6-3: Box plots comparing median pain on movement between 0PAR and PAR groups.



6.1.2 Psychological distress (HADS-T)

HADS-T is significantly higher in the OA group than in the control group (Figure 6-4, and Table 6.8) despite the presence of a control outlier (HADS-T=22).

Figure 6-4: Box plot of Total HADS for control and OA group



There are a significantly greater proportion of participants with psychological distress (HADS-T≥12) in the OA group than the control group (Table 6.8).

Table 6.8: Differences in HADS-T≥12 between the control and the OA groups

	Control	OA	Statistic
Median (IQ)	6(7)	13 (8)	[a]*U*=854, p<0.001
Proportion HADS-T ≥12	n=4 (19%)	n=34 (57%)	[b]p=0.006

[a] Mann-Whitney *U* test; [b] Fisher's exact test

Median HADS-T does not differ in 0PAR and PAR groups (Figure 6-5 and Table 6.9). The proportion of OA participants who have psychological distress (HADS-T≥12) in the 0PAR and PAR groups is the same (Table 6.9).

Figure 6-5: Box plot of HADS-T scores for 0PAR and PAR groups

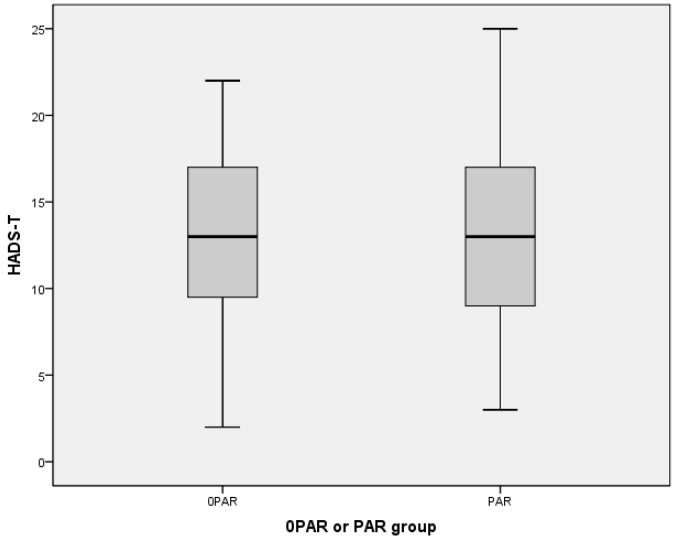


Table 6.9: Differences HADS-T between the 0PAR and PAR groups

	0PAR	PAR	Statistic
Median (IQR)	13 (8)	13 (8)	[a] *U*=380, p=0.858
Proportion HADS-T ≥12	n=15 (65%)	19 (56%)	[b] =0.497, p=0.335

[a] Mann-Whitney *U* test; [b] Chi-Squared test

6.2 Cytokine and amino acid data

6.2.1 Missing values for cytokines and amino acids

The whole sample was put forward for amino acid and cytokine assay. There are few missing cases in the amino acid data but many more in the cytokine data (Table 6.10).

Table 6.10: Missing cases and cases below the limit of detection (LOD) in the amino acid and cytokine data

	Control				OA			
	Missing cases		Cases below LOD		Missing cases		Cases below LOD	
	n	%	n	%	n	%	n	%
Glutamate	1	5	0	0	0	0	0	0
Aspartate	0	0	0	0	3	5	0	0
Arginine	0	0	0	0	1	2	0	0
Citrulline	0	0	0	0	0	0	0	0
Serine	0	0	0	0	0	0	0	0
Glutamine	1	5	0	0	1	2	0	0
GABA	0	0	0	0	0	0	0	0
Glycine	0	0	0	0	1	2	0	0
Tryptophan	0	0	0	0	0	0	0	0
Leucine	0	0	0	0	0	0	0	0
Isoleucine	0	0	0	0	0	0	0	0
Phenylalanine	0	0	0	0	1	2	0	0
Tyrosine	1	5	0	0	1	2	0	0
Valine	0	0	0	0	0	0	0	0
IL-1β	2	10	16	90	13	22	46	78
IL-2	4	20	1	5	20	34	2	3
IL-6	2	10	0	0	9	15	1	2
IL-7	2	10	1	5	9	15	4	7
IL-8	2	10	0	0	9	15	0	0
IL-12	2	10	9	43	11	19	27	46
IFNγ	2	10	8	38	11	19	21	36
TNFα	2	10	0	0	9	15	0	0
IL-4	2	10	8	38	10	17	38	64
IL-5	2	10	1	5	9	15	1	2
IL-10	2	10	0	0	10	17	0	0
IL-13	4	20	8	38	10	17	35	60

IL-1β will be excluded from all analyses as there were very few participants with concentrations of this cytokine above the limit of detection.

6.2.2 Cytokine and amino acid descriptive data

6.2.2.1 Retrospective power calculations for amino acid and cytokines

Retrospective power calculations were performed an all cytokines and amino acids using a significance level of 0.05 (Table 6.11 and Table 6.12). Serine, IL-12 and TNFα are adequately powered.

Table 6.11: Mean, SD and power calculation for amino acids in control and OA group

	Control			OA			
	n	mean	SD	n	mean	SD	power
Glutamate[a]	20	0.740	0.212	59	0.749	0.198	7%
Aspartate[a]	21	0.329	0.066	56	0.336	0.074	10.5%
Arginine[a]	21	19.564	5.986	58	18.367	3.753	21.6%
Citrulline[a]	21	2.741	0.959	59	2.711	0.825	6.2%
Serine[a]	21	18.841	5.502	59	22.601	4.888	85.85%
Glutamine[a]	20	408.667	149.517	58	485.511	141.136	64.3%
GABA[b]	21	99.233	29.780	59	103.355	34.929	13%
Glycine[a]	21	9.621	4.734	58	10.171	2.639	12.7%
Tryptophan[a]	21	0.921	0.303	59	0.980	0.301	44.6%
Leucine[a]	21	12.230	3.256	59	12.919	3.285	20.9%
Isoleucine[a]	21	4.507	1.153	59	4.478	1.192	6.6%
Phenylalanine[a]	21	8.146	1.493	58	8.424	1.637	17.1%
Tyrosine[a]	20	7.491	1.235	58	7.776	1.835	19%
Valine[a]	21	14.735	3.702	59	16.042	4.183	38.2%

[a]μmol l^{-1}; [b]pmol ml^{-1}

Table 6.12: Mean, SD and power calculation for cytokines in OA and control group

	Control			OA			
	n	Mean	SD	N	Mean	SD	Power
IL-2	16	0.036	0.038	36	0.043	0.042	15%
IL-6	19	3.282	1.557	48	3.847	2.184	32.1%
IL-7	18	0.183	0.088	45	0.160	0.097	22.8%
IL-8	19	24.214	8.863	49	25.344	9.515	11.8%
IL-12	10	0.031	0.040	21	0.013	0.013	>99.9%
IFNγ	11	0.100	0.115	26	0.088	0.082	13.1%
TNFα	19	0.103	0.086	49	0.175	0.103	>99.9%
IL-4	11	0.242	0.261	10	0.125	0.211	43.6%
IL-5	18	0.298	0.094	48	0.300	0.129	5.7%
IL-10	19	0.770	0.325	49	0.666	0.303	31%
IL-13	9	0.049	0.051	14	0.350	0.030	18.7%

All cytokines measured in pg ml^{-1}

The amino acids and cytokines are not normally distributed (Appendix 12 and Appendix 14). The median and interquartile range of each amino acid and cytokine in the OA and control groups, and the PAR and 0PAR groups is given in Table 6.13 and Table 6.14)

Table 6.13: Median values of amino acids (OA/control and PAR/0PAR)

	Median (IQR)			
	Control	OA	0PAR	PAR
Glutamate[a]	0.688 (0.310)	0.744 (0.238)	0.766 (0.225)	0.713 (0.246)
Aspartate[a]	0.337 (0.072)	0.331 (0.901)	0.379 (0.109)	0.308 (0.074)
Arginine[a]	18.388 (7.617)	17.789 (4.898)	17.615 (4.218)	18.232 (15.150)
Citrulline[a]	2.474 (1.149)	2.549 (1.085)	2.630 (1.191)	2.516 (0.903)
Serine[a]	16.394 (7.093)	22.601 (7.454)	20.982 (7.135)	22.058 (7.344)
Glutamine[a]	383.680 (190.271)	469.002 (133.492)	472.467 (112.085)	462.756 (146.226)
GABA[b]	99.368 (49.890)	102.922 (46.597)	104.353 (60.314)	100.119 (43.688)
Glycine[a]	8.167 (7.352)	9.976 (3.857)	9.648 (4.394)	10.322 (3.623)
Tryptophan[a]	1.026 (0.527)	0.936 (0.392)	0.945 (0.496)	0.936 (0.391)
Leucine[a]	12.240 (4.598)	11.972 (5.681)	12.437 (4.986)	11.688 (5.300)
Isoleucine[a]	4.560 (1.591)	4.388 (1.665)	4.539 (1.384)	4.254 (1.887)
Phenylalanine[a]	8.060 (1.502)	8.127 (2.475)	8.510 (2.985)	8.118 (1.930)
Tyrosine[a]	7.420 (1.457)	7.857 (2.112)	8.034 (2.327)	7.817 (1.939)
Valine[a]	14.169 (5.253)	15.294 (5.136)	16.242 (3.739)	14.585 (5.163)

[a] micromol l^{-1} (μmol l^{-1}); [b]picomole ml^{-1} (pmol ml^{-1})

Table 6.14: Median values of cytokines (OA/control and PAR/0PAR)

	Median (IQR)			
	Control	OA	0PAR	PAR
IL-6	3.040 (2.880)	3.355 (2.790)	3.140 (3.210)	3.555 (2.570)
IL-7	0.170 (0.140)	0.130 (0.120)	0.125 (0.150)	0.140 (0.110)
IL-8	22.960 (13.950)	22.850 (9.030)	23.350 (8.880)	24.520 (9.380)
IL-12	0.015 (0.040)	0.010 (0.010)	0.010 (0.030)	0.010 (0.010)
IFNγ	0.050 (0.160)	0.070 (0.100)	0.090 (0.070)	0.030 (0.090)
TNFα	0.060 (0.150)	0.168 (0.140)	0.185 (0.150)	0.140 (0.150)
IL-4	0.100 (0.400)	0.030 (0.150)	0.045 (0.070)	0.025 (0.430)
IL-5	0.285 (0.120)	0.275 (0.130)	0.270 (0.220)	0.285 (0.120)
IL-10	0.850 (0.520)	0.590 (0.410)	0.545 (0.450)	0.630 (0.370)
IL-13	0.030 (0.050)	0.025 (0.060)	0.020 (0.080)	0.030 (0.040)

All cytokines measured in pg ml^{-1}

6.2.3 Univariate analysis of amino acids and cytokines in control and OA groups

ANCOVA on the amino acids and cytokines adjusting for age, gender and HADS-T identify a significant difference for serine, valine, leucine (Table 6.15) and IL-12 and TNFα (Table 6.16).

Table 6.15: ANCOVA OA/control amino acids adjusted for age, gender and HADS-T

	ANCOVA[a]			Kolmogorov-Smirnov[b]		
	F statistic	Fdf	p	Statistic	df	p
Glutamate	0.406	1,70	0.526	0.103	75	0.349
Aspartate	0.291	1,68	0.591	0.065	73	0.892
Arginine	0.177	1,70	0.675	0.085	75	0.597
Citrulline	0.152	1,71	0.698	0.143	76	0.070
Serine[c]	11.303	1,71	0.001	0.079	76	0.670
Glutamine	2.149	1,70	0.124	0.116	75	0.220
GABA	0.621	1,71	0.433	0.073	75	0.773
Glycine	0.473	1,70	0.494	0.091	75	0.510
Tryptophan	0.007	1,710	0.935	0.055	76	0.964
Leucine[c,d]	5.143	1,71	0.026	0.161	76	0.029
Isoleucine	1.555	1,71	0.216	0.109	76	0.274
Phenylalanine	1.467	1,70	0.230	0.055	75	0.965
Tyrosine	1.767	1,69	0.188	0.102	74	0.366
Valine[c]	6.274	1,71	0.015	0.138	76	0.087

[a] ANCOVA data generated by SPSS; [b] Residuals generated by STATA
[c] Significant difference, $p<0.05$; [d] Residuals not normally distributed, $p<0.05$
These results have been generated excluding all cases that were missing or below the limit of detection.

The residuals for leucine were not normally distributed and therefore differences in this amino acid in the OA and control group were explored using a boot-strap technique. 1000

replications were carried out and the p value calculated for the F statistic was 0.001, confirming the significant difference for this amino acid between the OA and control groups.

Table 6.16: ANCOVA cytokines control/OA group adjusted for age, gender and HADS-T

	ANCOVA[a]			Kolmogorov-Smirnov[b]		
	F statistic	df	p	Statistic	df	p
IL-2	0.000	1,44	0.997	0.184	49	0.052
IL-6	1.462	1,58	0.232	0.122	63	0.254
IL-7	1.404	1,54	0.241	0.121	59	0.298
IL-8	0.882	1,59	0.351	0.086	64	0.674
IL-12[c]	4.887	1,24	0.037	0.227	29	0.067
IFNγ	0.001	1,30	0.981	0.199	35	0.089
TNFα[c]	5.326	1,59	0.025	0.076	64	0.810
IL-4	0.065	1,14	0.803	0.148	19	0.717
IL-5	0.196	1,57	0.660	0.111	62	0.370
IL-10	0.095	1,59	0.759	0.112	64	0.342
IL-13	0.793	1,17	0.386	0.140	22	0.706

[a] ANCOVA data generated by SPSS; [b] Residuals generated by STATA
[c] Significant difference, $p<0.05$; [d] Residuals not normally distributed, $p<0.05$
These results have been generated excluding all cases that were missing or below the limit of detection.

Inspection of median values for the amino acids and cytokines found to be significantly different in the OA and control groups shows that serine, leucine, valine and TNFα are higher in the OA group while IL-12 is lower (Table 6.13 and Table 6.14).

6.2.4 Univariate analysis of amino acids and cytokines in 0PAR and PAR groups

ANCOVA comparison of amino acids and cytokines in the PAR and 0PAR groups adjusting for age, gender and HADS-T reveals a significant difference in aspartate and IFNγ between the two groups (Table 6.17 and Table 6.18).

Table 6.17: ANCOVA for amino acids PAR 0PAR adjusting for age, gender and HADS-T

	ANCOVA[a]			Kolmogorov-Smirnov[b]		
	F statistic	df	p	Statistic	df	p
Glutamate	0.735	1,52	0.395	0.127	57	0.258
Aspartate[c]	10.057	1,49	0.003	0.094	54	0.672
Arginine	0.746	1,51	0.392	0.094	56	0.642
Citrulline	0.014	1,52	0.905	0.144	56	0.145
Serine	0.023	1,52	0.881	0.067	57	0.943
Glutamine	0.560	1,52	0.457	0.137	57	0.187
GABA	1.306	1,52	0.258	0.069	57	0.931
Glycine	0.492	1,51	0.486	0.087	56	0.740
Tryptophan	0.007	1,52	0.934	0.069	57	0.927
Leucine	0.009	1,52	0.924	0.138	57	0.182
Isoleucine	0.001	1,52	0.978	0.132	57	0.226
Phenylalanine	0.147	1,51	0.703	0.080	56	0.828
Tyrosine	0.638	1,51	0.428	0.097	57	0.612
Valine	0.047	1,52	0.829	0.160	57	0.080

[a] ANCOVA data generated by SPSS; [b] Residuals generated by STATA
[c] Significant difference, $p<0.05$; [d] Residuals not normally distributed, $p<0.05$
These results have been generated excluding all cases that were missing or below the limit of detection.

Inspection of the medians for aspartate and IFNγ reveals that both are present in lower concentration in the PAR group than the 0PAR group (Table 6.13 and Table 6.14).

Table 6.18: ANCOVA for cytokines PAR and 0PAR adjusting for age, gender and HADS-T

	ANCOVA[a]			Kolmogorov-Smirnov[b]		
	F statistic	df	p	Statistic	df	p
IL-2	0.000	1,30	0.993	0.164	35	0.233
IL-6	0.055	1,41	0.816	0.137	46	0.288
IL-7	0.013	1,38	0.908	0.123	43	0.460
IL-8	0.439	1,42	0.511	0.087	47	0.829
IL-12	4.142	1,15	0.060	0.099	20	0.979
IFNγ[c]	4.561	1,20	0.045	0.187	25	0.265
TNFα	0.616	1,42	0.437	0.061	47	0.992
IL-4	0.514	1,5	0.505	0.147	10	0.961
IL-5	0.776	1,41	0.383	0.087	46	0.838
IL-10	0.150	1,42	0.701	0.114	47	0.503
IL-13	0.066	1,9	0.803	0.196	14	0.542

[a] ANCOVA data generated by SPSS; [b] Residuals generated by STATA
[c] Significant difference, $p<0.05$; [d] Residuals not normally distributed, $p<0.05$
These results have been generated excluding all cases that were missing or below the limit of detection.

6.3 Regression analyses

Logistic regression analyses will be performed to explore the predictor variables that contribute most to membership of the OA group, the PAR group and psychological distress. Variables predicting HADS-T score will be evaluated using linear regression.

6.3.1 Selection of predictor variables.

One of the first considerations in this step of the process is to maximise the number of cases that can be entered into the regression analysis without losing representation of important facets of the pain signaling process. There are high percentages of missing values for IL-2, IL-4, IL-12, IL-13 and IFNγ. These variables will be omitted from the analysis and this will

be considered while selecting other predictor variables so that representation from the appropriate groups of analytes will be included.

Table 6.19 and Table 6.20 show all correlations between the independent variables. Colour coding helps to identify the strongest significant relationships (those above r=0.7 and r=0.8). These correlations can also be viewed diagrammatically (Figure 6-6 and Figure 6-7) and demonstrate correlations within and between each group of analytes.

Table 6.19: Control group correlations between amino acids and cytokines

	Age	HADS-T	Glutamate	Aspartate	Arginine	Citrulline	Serine	Glutamine	GABA	Glycine	Tryptophan	Leucine	Isoleucine	Phenylalanine	Tyrosine	Valine	Lysine	IL-2	IL-6	IL-7	IL-8	IL-12	IFNg	TNFa	IL-4	IL-5	IL-10
HADS-T	-.420																										
	.073																										
Glutamate	.279	-.156																									
	.233	.535																									
Aspartate	.120	-.129	.619**																								
	.603	.600	.004																								
Arginine	.022	-.287	.730**	.539*																							
	.925	.234	.000	.012																							
Citrulline	.488	-.599**	.354	.274	.553*																						
	.025	.007	.126	.230	.009																						
Serine	.093	-.430	.677**	.397	.705**	.362																					
	.688	.066	.001	.075	.000	.107																					
Glutamine	-.030	.174	.511*	.278	.152	-.030	.404																				
	.901	.491	.025	.236	.522	.899	.077																				
GABA	.333	.028	.774**	.527*	.614**	.221	.557**	.230																			
	.140	.911	.000	.014	.003	.336	.009	.329																			
Glycine	.362	-.262	.346	.253	.663**	.579**	.545*	.055	.437*																		
	.107	.278	.135	.268	.001	.006	.011	.818	.048																		
Tryptophan	-.106	.418	-.062	.091	-.354	-.196	-.267	.108	.022	-.373																	
	.646	.075	.797	.696	.115	.396	.242	.649	.924	.096																	
Leucine	.175	-.577**	.437	.391	.495*	.576**	.591**	.205	.322	.525*	-.172																
	.447	.010	.054	.080	.022	.006	.005	.386	.154	.015	.456																
Isoleucine	.067	-.552*	.207	.189	.314	.508*	.436*	.222	.013	.503*	-.127	.862**															
	.773	.014	.381	.411	.166	.019	.048	.347	.956	.020	.584	.000															
Phenylalanine	.158	-.308	.433	.176	.477*	.653**	.622**	.267	.396	.507*	.196	.699**	.640**														
	.493	.199	.057	.445	.029	.001	.003	.255	.076	.019	.395	.000	.002														
Tyrosine	-.071	.087	.410	.119	.334	.152	.493*	.387	.376	.192	.252	-.016	.018	.532*													
	.767	.732	.081	.617	.150	.524	.027	.102	.103	.417	.284	.948	.941	.016													
Valine	-.046	-.348	.434	.334	.397	.262	.589**	.206	.334	.377	.053	.865**	.812**	.664**	.205												
	.841	.144	.056	.139	.075	.251	.005	.384	.139	.092	.819	.000	.000	.001	.387												
Lysine	-.311	-.140	.219	.146	.308	.182	.490*	.300	.193	.147	.269	.618**	.560**	.742**	.424	.725**											
	.170	.567	.353	.527	.174	.431	.024	.199	.402	.525	.239	.003	.008	.000	.062	.000											
IL-2	.177	-.248	.063	-.066	-.018	.029	.272	.150	.233	.103	.201	.543*	.388	.432	.086	.616*	.504*										
	.513	.393	.822	.808	.947	.914	.308	.593	.385	.703	.457	.030	.138	.095	.762	.011	.046										
IL-6	-.001	-.159	-.263	-.473*	-.149	-.086	.047	-.056	.056	.015	-.039	.320	.290	.234	-.125	.394	.259	.739**									
	.996	.541	.293	.041	.541	.727	.848	.825	.819	.950	.874	.181	.229	.336	.622	.096	.285	.001									
IL-7	.076	-.114	-.078	-.312	-.124	-.067	-.065	-.215	.013	-.217	-.389	-.109	-.076	-.224	-.267	-.125	-.229	-.251	.253								
	.757	.663	.758	.193	.614	.786	.793	.392	.959	.373	.100	.656	.757	.355	.285	.609	.346	.348	.296								
IL-8	.205	-.340	.238	-.056	.245	.180	.195	-.129	.295	.108	-.245	.386	.143	.138	-.327	.294	.005	.642**	.608**	.115							
	.400	.182	.342	.821	.312	.460	.424	.611	.221	.660	.312	.103	.559	.574	.185	.222	.985	.007	.006	.640							
IL-12	.290	-.324	.008	-.036	.211	.224	.046	-.244	-.175	.323	-.569*	.090	.077	-.207	-.492*	-.086	-.355	-.366	-.114	.248	.104						
	.228	.205	.974	.884	.387	.356	.851	.329	.473	.177	.011	.715	.753	.395	.038	.727	.136	.163	.642	.305	.671						
IFNg	.145	.093	.051	-.380	-.028	.149	-.211	-.013	-.074	.123	-.312	-.156	-.064	-.128	-.402	-.368	-.458*	-.308	.086	.276	.296	.258					
	.553	.724	.841	.109	.908	.543	.385	.960	.765	.617	.194	.523	.793	.601	.098	.122	.048	.246	.726	.253	.218	.286					
TNFa	.176	-.075	.472*	.399	.344	.231	.232	.020	.632**	.202	.336	.397	.233	.368	-.071	.434	.241	.478	.287	.019	.568*	-.249	.031				
	.470	.776	.048	.091	.150	.341	.339	.936	.004	.408	.159	.093	.338	.121	.779	.063	.320	.061	.233	.938	.011	.304	.898				
IL-4	.277	-.367	.043	.072	.256	.268	.101	-.031	.026	.386	-.446	.029	.130	-.134	-.093	-.199	-.294	-.472*	-.187	.276	-.262	.472*	.136	-.210			
	.252	.148	.867	.768	.291	.267	.680	.903	.917	.102	.056	.907	.596	.585	.713	.413	.221	.065	.444	.253	.279	.041	.578	.389			
IL-5	.212	.202	.607**	.224	-.007	-.131	.146	.658**	.321	-.084	.071	.112	.083	.016	.017	.131	-.023	.172	-.115	-.056	.172	-.224	.276	.216	-.201		
	.383	.438	.008	.358	.977	.594	.551	.003	.181	.732	.773	.649	.736	.948	.948	.593	.924	.523	.638	.821	.481	.358	.252	.374	.409		
IL-10	.204	-.383	.388	.183	.317	.132	.352	.087	.513*	.042	-.269	.335	.005	.104	-.124	.204	.038	.545*	.487*	.164	.853**	-.060	.165	.541*	-.148	.194	
	.403	.129	.112	.454	.186	.589	.139	.730	.025	.864	.266	.160	.983	.671	.624	.402	.877	.029	.034	.503	.000	.807	.499	.017	.546	.426	
IL-13	-.094	-.117	-.019	-.263	.259	-.030	.050	-.332	.046	.082	-.511*	-.253	-.329	-.295	-.251	-.388	-.529*	-.321	.303	.265	.599*	.417	.528*	-.035	.292	-.235	.508*
	.720	.677	.943	.309	.315	.908	.848	.208	.860	.756	.036	.327	.198	.250	.349	.124	.029	.264	.238	.304	.011	.096	.029	.893	.256	.363	.037

Table 6.20: OA group correlations between amino acids and cytokines

	PAR	POM	Age	HADS-T	Glutamate	Aspartate	Arginine	Citrulline	Serine	Glutamine	GABA	Glycine	Tryptophan	Leucine	Isoleucine	Phenylalanine	Tyrosine	Valine	Lysine	IL-2	IL-6	IL-7	IL-8	IL-12	IFNg	TNFa	IL-4	IL-5	IL-10
POM	.612**																												
	.000																												
Age	-.153	.002																											
	.248	.990																											
HADS-T	.044	.069	-.156																										
	.748	.608	.247																										
Glutamate	-.143	.050	-.085	-.077																									
	.281	.708	.523	.569																									
Aspartate	-.367**	-.104	.086	-.090	.753**																								
	.005	.444	.527	.516	.000																								
Arginine	.086	.163	-.219	.030	.365**	.129																							
	.521	.222	.099	.826	.005	.348																							
Citrulline	-.041	.067	.156	-.119	.303*	.168	.391**																						
	.757	.615	.238	.379	.020	.216	.002																						
Serine	-.112	.052	.011	-.176	.120	-.177	.257	.359**																					
	.398	.694	.937	.191	.366	.191	.052	.005																					
Glutamine	-.147	-.214	-.017	-.064	.111	.024	.172	.309*	.501**																				
	.271	.106	.901	.636	.406	.864	.200	.018	.000																				
GABA	-.188	-.384**	-.288*	-.131	.417**	.346**	.309*	.281*	.256	.450**																			
	.155	.003	.027	.330	.001	.009	.018	.031	.050	.000																			
Glycine	.163	.264*	-.014	.032	.416**	.238	.391**	.521**	.353**	.242	.233																		
	.222	.045	.917	.815	.001	.080	.003	.000	.007	.070	.078																		
Tryptophan	-.069	-.156	.033	-.080	-.208	-.005	-.118	-.046	.174	.136	-.028	-.102																	
	.603	.237	.802	.553	.114	.972	.376	.731	.188	.310	.835	.445																	
Leucine	-.097	.044	.054	-.322*	.177	.137	.282*	.597**	.227	.057	.226	.352**	-.107																
	.465	.738	.684	.015	.179	.315	.032	.000	.083	.671	.086	.007	.420																
Isoleucine	-.146	.025	.090	-.186	.275*	.241	.255	.522**	.164	.065	.197	.350**	-.037	.905**															
	.269	.851	.497	.166	.035	.073	.054	.000	.214	.625	.135	.007	.784	.000															
Phenylalanine	-.197	-.142	.149	-.260	.183	.235	.274*	.700**	.288*	.399**	.331*	.351**	.070	.702**	.680**														
	.138	.289	.263	.053	.169	.084	.038	.000	.028	.002	.011	.007	.602	.000	.000														
Tyrosine	-.132	-.042	.125	-.280*	.213	.111	.317*	.405**	.435**	.342**	.185	.006	.138	.372**	.317*	.490**													
	.322	.753	.351	.037	.108	.420	.015	.002	.001	.009	.165	.967	.302	.004	.015	.000													
Valine	-.144	-.011	.038	-.239	.201	.179	.183	.546**	.270*	.037	.318*	.318*	.001	.891**	.842**	.627**	.337**												
	.276	.934	.777	.073	.127	.186	.170	.000	.038	.782	.014	.015	.997	.000	.000	.000	.010												
Lysine	-.051	-.169	-.027	-.207	.102	.205	.282*	.222	-.055	-.081	.142	.082	.058	.554**	.549**	.443**	.193	.557**											
	.704	.204	.843	.127	.445	.132	.033	.095	.681	.548	.287	.543	.663	.000	.000	.001	.150	.000											
IL-2	.081	.141	.111	.124	.159	.146	.107	.018	-.032	-.193	-.067	.149	-.164	.038	.065	-.061	-.155	.014	-.081										
	.634	.406	.512	.471	.349	.396	.529	.918	.853	.252	.693	.385	.333	.824	.702	.721	.359	.934	.637										
IL-6	.103	.071	-.161	.011	-.102	.023	.236	.202	.105	.101	.111	.090	-.094	.201	.072	.180	.052	.156	.192	.457**									
	.476	.625	.263	.939	.482	.876	.100	.159	.469	.492	.442	.538	.516	.161	.620	.211	.721	.278	.185	.004									
IL-7	.020	-.019	-.197	.320*	.041	.277	.107	-.070	-.197	-.144	.069	-.008	.094	-.045	.105	-.112	.023	.009	.052	.325*	-.045								
	.892	.893	.170	.027	.777	.057	.458	.630	.170	.324	.634	.956	.514	.754	.470	.438	.876	.952	.723	.050	.755								
IL-8	.043	.117	.084	-.021	.153	.148	.162	.263	.194	-.106	-.032	.174	-.354*	.140	.049	.181	.002	.067	.003	.485**	.386**	-.160							
	.767	.419	.560	.886	.289	.314	.260	.065	.176	.467	.824	.232	.012	.333	.736	.209	.989	.645	.983	.002	.006	.267							
IL-12	.260	-.014	-.176	.269	-.179	-.097	.060	-.199	-.060	-.127	.135	-.063	-.123	.004	-.031	-.170	-.091	.088	.058	.218	.124	.266	.075						
	.074	.927	.233	.071	.224	.522	.685	.176	.685	.394	.361	.673	.405	.978	.836	.247	.540	.552	.697	.208	.401	.068	.611						
IFNg	.236	.249	.054	.082	-.289*	-.160	-.137	.111	.157	-.199	-.196	.100	.003	.008	-.090	-.122	.078	.030	-.143	.031	.160	.190	.062	.237					
	.107	.088	.717	.587	.047	.282	.352	.453	.285	.179	.182	.504	.982	.957	.541	.408	.598	.838	.339	.857	.277	.196	.678	.113					
TNFa	-.262	-.236	.263	-.010	.404**	.249	.033	.279	.278	.105	.234	.219	-.352*	.148	.152	.234	.254	.154	-.070	.538**	-.084	-.017	.582**	-.024	.046				
	.066	.099	.065	.948	.004	.088	.823	.050	.050	.475	.102	.130	.012	.306	.293	.103	.075	.286	.634	.001	.563	.905	.000	.873	.758				
IL-4	.056	.109	-.138	-.046	.014	-.094	.221	.043	.095	.017	-.031	.090	.094	.107	.153	.128	.296*	.068	.109	-.111	-.270	.160	-.263	-.027	.111	-.116			
	.703	.457	.344	.757	.923	.529	.127	.771	.518	.906	.834	.545	.522	.465	.294	.380	.039	.641	.460	.519	.061	.271	.068	.858	.457	.428			
IL-5	.200	.132	-.046	.240	-.153	-.277	.041	-.090	.035	.129	-.085	-.110	-.127	-.184	-.205	-.245	-.057	-.242	-.385**	.427**	-.022	-.042	.186	.129	-.129	-.050	.077		
	.164	.362	.752	.100	.289	.057	.778	.535	.812	.376	.558	.453	.380	.201	.153	.086	.696	.090	.006	.008	.878	.770	.196	.383	.384	.732	.597		
IL-10	-.052	.060	.102	-.072	.254	.131	.173	.261	.243	-.029	.021	.238	-.480**	.225	.173	.268	.220	.168	.007	.433**	.105	-.098	.728**	.071	.060	.781**	-.024	-.026	
	.724	.680	.484	.630	.078	.382	.234	.070	.092	.843	.886	.103	.000	.119	.236	.062	.128	.250	.962	.008	.475	.504	.000	.635	.689	.000	.871	.858	
IL-13	-.060	-.031	.154	.190	.117	.097	.098	.335*	.197	-.031	.098	.257	-.209	.230	.199	.211	.121	.294*	-.013	.227	.096	.189	.261	.305*	.244	.417**	.114	-.228	.470**
	.681	.830	.291	.200	.422	.515	.503	.019	.175	.837	.504	.078	.150	.112	.171	.146	.409	.040	.929	.182	.511	.194	.070	.037	.099	.003	.441	.116	.001

Figure 6-6: Diagrammatic representation of correlations in control group

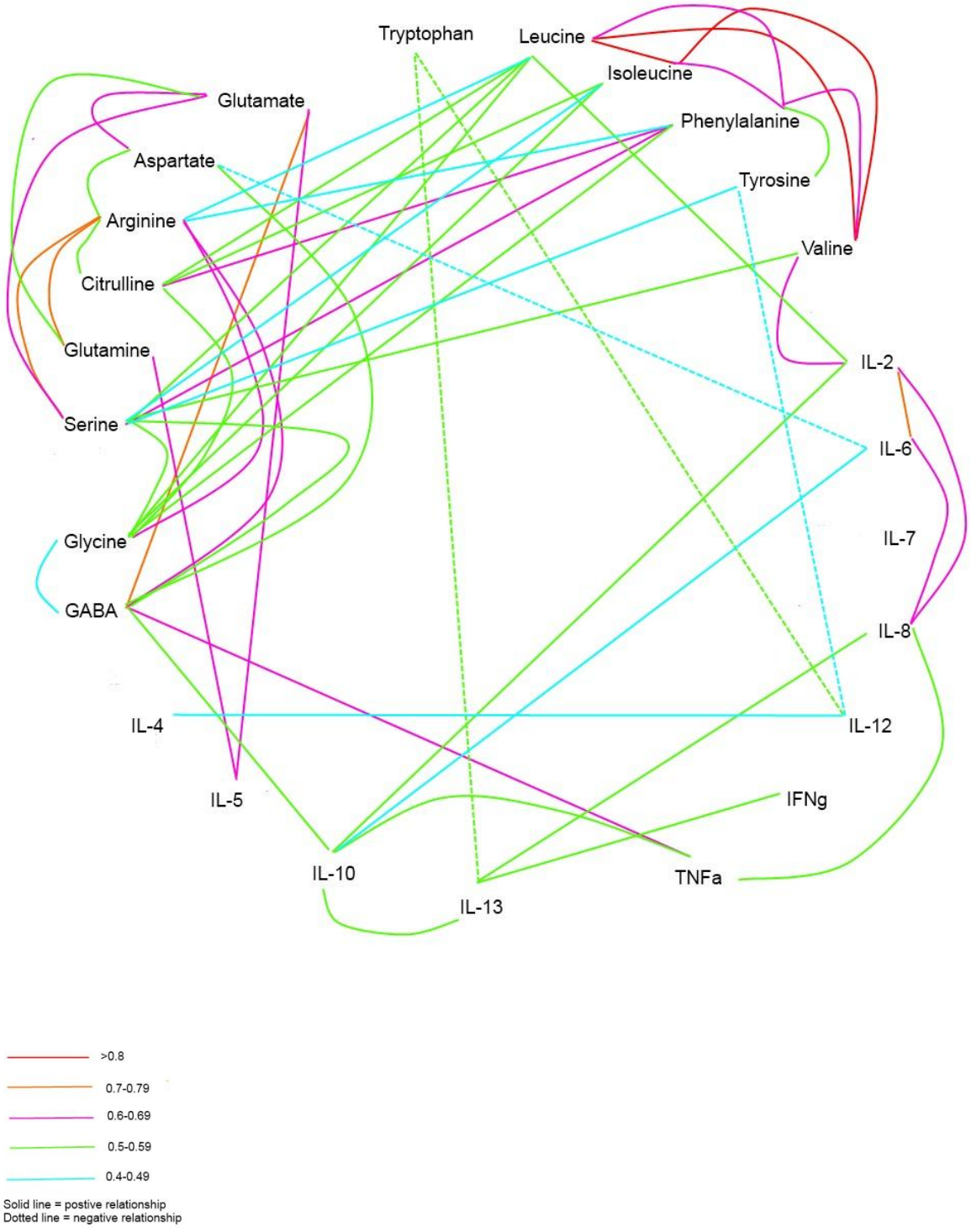
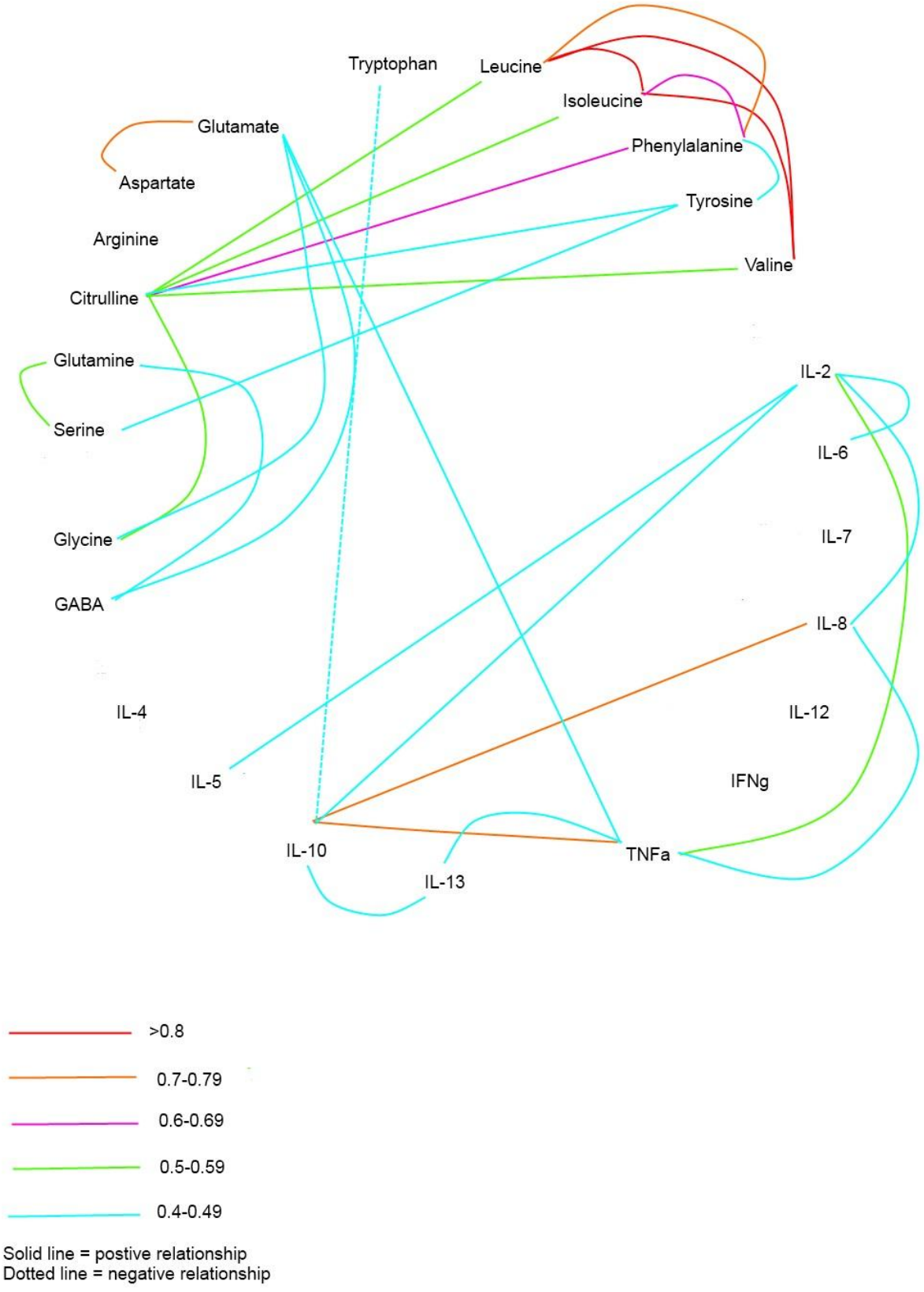


Figure 6-7: Diagrammatic representation of correlations in OA group



The diagrammatic representations of the correlations help to visualize the relationships within and between groups of analytes in this data set (for groups see Table 5.3). Within group relationships are denoted by connectors drawn across the group or around the outside of the group; between group connectors cross the central portion of the diagram. For example, in the control group it can be seen that there are more significant correlations within the excitatory amino acid group than there are in the OA group. Similarly it can be seen that some group members have a number of relationships with analytes within their own group (e.g. IL-4 in the control group) and that these relationships do not necessarily occur in the OA group. Other analytes have few relationships within their own group e.g. IL-12.

The members of the excitatory amino acid group have many significant associations both within and between groups. Serine and glutamine are selected from this group because they have already demonstrated a significant difference in concentration between the OA group and the control group. In addition citrulline will be selected from this group to represent the result of NMDAR activation and because it has a high number of associations between different groups.

TNFα will be selected from the pro-inflammatory cytokine group because it is significantly higher in the OA group and because it has a number of relationships with the excitatory amino acids.

The diagrammatic representations of the linear relationships illustrate relatively high number of correlations between the members of the large neutral amino acid group (leucine,

isoleucine, phenylalanine, tyrosine and valine). Valine and leucine will be selected as representatives for this group because they both differ significantly between the OA and control group and they both have strong associations with other members of their group.

IL-10 will be selected from the anti-inflammatory group and GABA from the inhibitory amino acid group due to their high number of associations with other variables and known involvement in the pain modulation. This process has led to the selection of a set of predictor variables

Box 6-1:Predictor variables for binary logistic regression OA/control

HADS-T, age, gender, serine, glutamine, citrulline, valine, leucine, TNFα, IL-10, GABA

6.3.2 Binary logistic regression: OA/control

HADS-T, age and gender were included in the initial model for the binary logistic regression of OA/control. All other variables were included from the second step onwards using a forward likelihood ratio stepwise method.

The model containing HADS-T, age, gender, serine, leucine and GABA (Table 6.21) correctly classified 94% of the OA cases and 56% of the control cases; overall the classification was correct in 84% of the cases.

The goodness of fit test suggests that this model performs better than the initial model that contained only the constant (= 24.910, p<0.00). Hosmer and Lemeshow poorness of fit test

also supports this model (=5.406, p=0.713). The model accounted for 56% (Nagelkerke R^2) of the variance.

Table 6.21: Binary logistic regression OA/control (n=62)

							Odds	95% C.I. for odds ratio	
		B	S.E.	Wald	df	p	ratio	Lower	Upper
Step 1	age	0.040	0.037	1.150	1	0.283	1.040	0.968	1.119
	gender	0.800	0.842	0.903	1	0.342	2.226	0.427	11.598
	HADS-T	0.244	0.086	8.071	1	0.004	1.277	1.079	1.511
	Serine	0.198	0.078	6.522	1	0.011	1.219	1.047	1.420
	Constant	-8.675	3.575	5.889	1	0.015	0.000		
Step 2	age	0.048	0.040	1.385	1	0.239	1.049	0.969	1.135
	gender	1.449	1.001	2.094	1	0.148	4.259	0.598	30.311
	HADS-T	0.294	0.098	8.945	1	0.003	1.342	1.107	1.627
	Serine	0.158	0.082	3.709	1	0.054	1.172	0.997	1.376
	Leucine	0.297	0.177	2.838	1	0.092	1.346	0.953	1.903
	Constant	-12.834	4.819	7.093	1	0.008	0.000		
Step 3	age	0.047	0.044	1.157	1	0.282	1.048	0.962	1.142
	gender	1.498	1.024	2.141	1	0.143	4.472	0.601	33.252
	HADS-T	0.315	0.101	9.649	1	0.002	1.371	1.123	1.672
	Serine	0.199	0.091	4.824	1	0.028	1.220	1.022	1.458
	Leucine	0.366	0.193	3.609	1	0.057	1.442	0.988	2.104
	GABA	-0.022	0.014	2.708	1	0.100	0.978	0.952	1.004
	Constant	-12.469	5.102	5.971	1	0.015	0.000		

This model suggests that being male, higher HADS-T, higher serine, higher leucine and lower GABA are predictive of belonging to the OA group at the level of $p<0.15$. HADS-T and serine are both significant contributors to the model at the level of $p<0.05$.

6.3.2.1 Assumption checks for binary logistic regression OA/control

There are no suggestions of multicollinearity within this data set (Table 6.22).

The case-wise listing produced in this regression analysis shows 4 cases are outliers with standardised residuals being > 2.58, which makes them significant at the 0.1 level (Table 6.23).

Table 6.22: Multicollinearity for regression analysis OA/control model 1

	Tolerance	VIF
Serine	0.623	1.605
Glutamine	0.752	1.330
Leucine	0.119	8.381
Valine	0.147	6.807
Citrulline	0.555	1.802
TNF alpha	0.407	2.454
IL-10	0.478	2.092
Age	0.763	1.310
HADS-T	0.652	1.533
GABA	0.993	1.007

[a] Multicollinearity = Tolerance <0.1 and VIF>10

Table 6.23: Outlying cases for logistic regression OA/Control

Case Number	Residual	Standardised residual
31	-0.919	-3.364
50	0.907	3.131
56	-0.878	-2.688
76	-0.939	-9.914

Examination of Cook's Distance suggests case number 56 may have undue influence over the model (Table 6.24). The average leverage for the regression is 0.079 (—— =——) and none of the outlying cases exceed the suggested cut-off point (0.238 to 0.395) (Table 6.24).

Table 6.24: Cook's Distance and leverage values for binary logistic regression OA/control

Case Number	Cooks Distance	Leverage value
31	0.931	0.076
50	0.742	0.070
56	1.714	0.192
76	0.906	0.056

DFBeta values for each of the outlying cases is below the value for concern of 1 (Table 6.25)

Table 6.25: DFBeta values binary logistic regression OA/control

Case Number	constant	age	gender	HADS-T	Serine
31	2.408	-0.006	-0.007	-0.029	-0.067
50	3.499	-0.018	-0.327	-0.077	-0.031
56	-1.601	0.029	-0.517	-0.051	0.025
76	3.587	-0.032	-0.001	-0.064	-0.025

6.3.3 Binary logistic regression: PAR/0PAR

A similar process to that undertaken for prediction of membership of the OA group was followed to determine which variables were most strongly associated with PAR in the OA group.

Age, HADS-T, and gender were entered into the regression as an initial model. Due the high numbers of missing values IL-2, IL-4, IL-12, and IL-13 were omitted from the model. Aspartate, and IFNγ were selected because they differ significantly in the 0PAR/PAR groups, however, IFNγ has a large number of missing values and this may create difficulties with the model due to the small number of cases. Serine, citrulline, TNFα, IL-10 and POM were selected because of their theoretical importance to the development of PAR. As in the previous model valine and leucine were chosen to represent the large neutral amino acids.

Box 6-2: Predictor variables for binary logistic regression PAR/0PAR

HADS-T, age, gender, aspartate, serine, glutamine, citrulline, valine, leucine, TNFα, IL-10, IFNγ, POM

In this model IFNγ was the first independent variable entered in the step-wise process but the large standard error, and the odds ratio/confidence intervals approaching zero suggest difficulty with convergence (Table 6.26).

Table 6.26: Binary logistic regression PAR/0PAR (n=24)

	B	S.E.	Wald	df	Sig.	Odds ratio	95% C.I. for odds ratio	
							Lower	Upper
age	-0.164	0.079	4.286	1	0.038	0.849	0.727	0.991
gender	2.037	1.392	2.141	1	0.143	7.666	0.501	117.348
HADS-T	0.114	0.109	1.096	1	0.295	1.121	0.905	1.387
IFNγ	-25.440	12.636	4.054	1	0.044	0.000[a]	0.000[a]	0.509
Constant	10.949	5.600	3.822	1	0.051	56885.398		

[a] Approaching zero

The model was repeated without IFNγ (Box 6-3).

Box 6-3: Variables in the equation logistic regression PAR/0PAR version 2

HADS-T, age, gender, aspartate, serine, glutamine, citrulline, valine, leucine, TNFα, IL-10, POM

The model produced in version 2 of the binary logistic regression for membership of the PAR group correctly classified 46% of the PAR group and 71% of the 0PAR group, the overall classification was 60%. Step 2 of this model reveals that there may be a failure to converge (indicated by the large confidence intervals and standard error relating to aspartate) (Table 6.27).

Table 6.27: Binary logistic regression PAR/0PAR version 2 (n=61)

								95% C.I.for EXP(B)	
		B	S.E.	Wald	df	Sig.	Exp(B)	Lower	Upper
Step 1	age	-0.012	0.038	0.103	1	0.748	0.988	0.918	1.064
	gender	0.991	0.769	1.660	1	0.198	2.694	0.597	12.163
	HADS-T	-0.005	0.068	0.005	1	0.945	0.995	0.871	1.138
	POM	0.730	0.210	12.119	1	0.000	2.074	1.375	3.128
	Constant	-3.539	3.157	1.257	1	0.262	0.029		
Step 2	age	-0.001	0.041	0.000	1	0.985	0.999	0.922	1.083
	gender	0.819	0.833	0.966	1	0.326	2.268	0.443	11.604
	HADS-T	0.014	0.073	0.039	1	0.843	1.015	0.879	1.171
	POM	0.773	0.223	12.036	1	0.001	2.166	1.400	3.352
	Aspartate	-13.611	6.212	4.801	1	0.028	0.000[a]	0.000[a]	0.238
	Constant	-0.212	3.715	0.003	1	0.954	0.809		

[a] approaching zero

The model (HADS-T, age, gender, aspartate, and POM) performs better than the constant-only model (Goodness of fit (= 19.622, p=0.001). Hosmer and Lemeshow poorness of fit test also supports this model (=4.681, p=0.699). The model accounted for 66% (Nagelkerke R^2) of the variance between the two groups.

This model suggests that increasing levels of aspartate and POM are associated with an increased likelihood of a person with OA having PAR.

6.3.3.1 Assumption checks for binary logistic regression PAR/0PAR

There is no sign of multicollinearity within the predictor variable group used in this model (Table 6.28).

Case-wise listing of outliers reveals two cases with standardised residuals greater than 2.58, making them significant at the 0.1 level (Table 6.29).

Table 6.28: Multicollinearity testing logistic regression PAR/0PAR

	Tolerance	VIF
Serine	0.720	1.388
Valine	0.156	6.402
Citrulline	0.555	1.803
TNFα	0.495	2.022
IL-10	0.464	2.155
Age	0.748	1.338
HADS-T	0.689	1.451
IFNγ	0.707	1.414
Aspartate	0.738	1.355
Leucine	0.112	8.926
POM	0.738	1.346

Multicollinearity =Tolerance <0.1 and VIF>10

Table 6.29: Outlying cases in logistic regression PAR/0PAR model

Case Number	Residual	Standardised residual
12	0.888	2.819
27	0.724	1.620
50	0.959	4.867
64	-0.843	-2.319

Examination of Cook's Distance suggests cases 27, 50 and 64 may have undue influence over the model (Table 6.30). The average leverage for the regression is 0.098 (—— =——) and cases 12 and 27 exceed the suggested cut-off point (0.196 to 0.294) (Table 6.30).

Table 6.30: Cook's Distance and leverage values for binary logistic regression PAR/0PAR

Case Number	Cooks Distance	Leverage value
12	0.811	0.654
27	1.518	0.268
50	1.777	0.043
64	1.099	0.185

Influential cases: Cook's D_i >1, Leverage > 2-3 times average leverage (—

DFBeta values for each of the outlying cases are acceptable for all variables except aspartate (Table 6.31). This suggests that these four cases are influential outliers with regard to this model.

Table 6.31: DFBeta values binary logistic regression PAR/0PAR

Case Number	constant	age	gender	HADS-T	POM	Aspartate
12	1.807	-0.017	-0.252	0.001	-0.149	1.546
27	4.922	-0.043	-0.094	-0.030	-0.151	-0.958
50	2.238	-0.017	0.0294	-0.057	-0.200	3.421
64	-3.149	0.030	-0.513	-0.013	0.001	3.744

6.3.3.2 Repetition of binary logistic regression PAR/0PAR after removal of influential cases

Repeating the logistic regression (using the enter method; Table 6.32) after the deletion of these four cases improves the classification of PAR from 46% to 88%, and classification of the 0PAR from 71% to 88%. The amount of variance the model accounts for increases from 66% to 76% (Nagelkerke R^2).

Table 6.32: Logistic regression PAR/0PAR after removal of influential cases

							Odds ratio	95% C.I. for Odds ratio	
		B	S.E.	Wald	df	p		Lower	Upper
Step 1	age	-0.019	0.044	0.192	1	0.661	0.981	0.901	1.069
	gender	1.581	0.903	3.063	1	0.080	4.858	0.827	28.522
	HADS-T	-0.007	0.072	0.008	1	0.927	0.993	0.862	1.145
	POM	0.873	0.271	10.346	1	0.001	2.393	1.406	4.072
	Constant	-4.154	3.740	1.234	1	0.267	0.016		
Step 2	age	-0.017	0.048	0.130	1	0.718	0.983	0.895	1.080
	gender	1.904	1.121	2.886	1	0.089	6.715	0.746	60.427
	HADS-T	0.035	.083	0.177	1	0.674	1.035	0.880	1.218
	POM	1.024	.319	10.275	1	0.001	2.783	1.488	5.204
	Aspartate	-21.274	8.500	6.264	1	0.012	0.000[a]	0.000[a]	0.010
	Constant	1.342	4.521	0.088	1	0.767	3.827		

This model suggests higher levels of POM and aspartate are associated with being a member of the PAR group.

6.3.4 Binary logistic regression whole group: presence or absence of psychological distress (total cohort)

Psychological distress was defined as a HADS-T score of 12 or greater. High numbers of missing values IL-2, IL-4, IL-12, and IL-13 led to these predictor variables not being entered into the model. HADS-T did not correlate with many of the amino acid and cytokine variables and so guidance for inclusion from examination of correlation coefficients was not helpful. Tryptophan and the large neutral amino acids have a strong theoretical relationship with depression and so the variable ΣLNAA was created by summation of leucine, isoleucine, phenylalanine, tyrosine and valine. POM, glutamate and GABA have been shown in other studies to vary with stress and depression and so both are included in the model. The hypothesised relationship between pain/central sensitisation and depression mediated by

cytokines led to the selection of TNFα and IL-10 as representatives of pro and anti-inflammatory processes in the dorsal horn being included in the model. The potential involvement of the NMDAR in depression and pain led to the selection of serine (Box 6-4).

Box 6-4: Predictor variables for binary logistic regression psychological distress

Age, gender, glutamate, GABA, TNFα, IL-10, serine, tryptophan, ΣLNAA, POM

Age and gender were made part of the basic model and then the other predictor variables were entered using a forward step-wise process. Serine and POM made a significant contribution to the model (Table 6.33).

Table 6.33: Logistic regression Psychological Distress (HADS-T ≥12)(n=63)

								95% C.I. for Odds ratio	
		B	S.E.	Wald	df	Sig.	Odds ratio	Lower	Upper
Step 1[a]	age	-0.014	0.025	.295	1	0.587	0.986	0.939	1.037
	gender	0.670	0.557	1.446	1	0.229	1.954	0.656	5.826
	POM	0.155	0.087	3.154	1	0.076	1.168	0.984	1.386
	Constant	0.053	1.797	.001	1	0.976	1.055		
Step 2[b]	age	-0.006	0.026	.054	1	0.816	0.994	0.944	1.047
	gender	0.666	0.579	1.323	1	0.250	1.946	0.626	6.051
	Serine	-0.129	0.061	4.452	1	0.035	0.879	0.779	0.991
	POM	0.237	0.104	5.166	1	0.023	1.267	1.033	1.554
	Constant	1.982	2.078	.910	1	0.340	7.256		

The model (age, gender, POM and serine) performs better than the constant-only model (Goodness of fit = 11.624, p<0.020). Hosmer and Lemeshow poorness of fit test also supports this model (=10.617, p=0.224). The model correctly predicted 70% of the

psychological distress cases and accounted for 23% (Nagelkerke r^2) of the variance between the two groups.

This model suggests decreasing levels of serine and increasing POM are associated with an increased likelihood of a person having a HADS-T score ≥12.

6.3.4.1 Assumption checks for binary logistic regression whole group: psychological distress

There is no sign of multicollinearity within the predictor variable group used in this model (Table 6.34).

Table 6.34: Multicollinearity testing logistic regression psychological distress

	Tolerance	VIF
Age	0.840	1.190
Gender	0.691	1.447
Glutamate	0.648	1.544
GABA	0.592	1.689
Serine	0.680	1.471
POM	0.668	1.498
TNFα	0.451	2.215
IL-10	0.416	2.404
Tryptophan	0.662	1.511
ΣLNAA	0.730	1.371

Multicollinearity =Tolerance <0.1 and VIF>10

Case-wise listing of outliers reveals two cases with standardised residuals approaching or greater than 2.58, making case 67 significant at the 0.1 level (Table 6.35).

Table 6.35: Outlying cases in logistic regression psychological distress model

Case Number	Residual	Standardised residual
55	-0.867	-2.551
67	-0.930	-3.643

Examination of Cook's Distance suggests neither case has undue influence over the model (Table 6.36). The average leverage for the regression is 0.079 (—— =——) and neither case exceeds the suggested cut-off point (0.238 to 0.396) (Table 6.36).

Table 6.36: Cook's Distance and leverage values for binary logistic regression psychological distress

Case Number	Cooks Distance	Leverage value
55	0.358	0.052
67	0.991	0.069

Influential cases: Cook's D_i >1, Leverage > 2-3 times average leverage (——

DFBeta values for each of the outlying cases is below the value for concern of 1 (Table 6.37).

Table 6.37: DFBeta values binary logistic regression psychological distress

Case Number	constant	age	gender	serine	POM
55	-0.520	0.002	-0.153	0.024	-0.041
67	-0.196	-0.009	-0.138	0.053	-0.066

There are no concerns raised by examination of the assumptions for the binary logistic regression of psychological distress in the whole group.

6.3.4.2 Assumption checks for binary logistic regression OA group: psychological distress

There is no sign of multicollinearity within the predictor variable group used in this model (Table 6.38).

Table 6.38: Multicollinearity testing logistic regression OA group psychological distress

	Tolerance	VIF
Age	0.671	1.489
Gender	0.708	1.413
Glutamate	0.590	1.696
GABA	0.515	1.943
Serine	0.697	1.435
POM	0.562	1.781
TNFα	0.212	4.712
IL-10	0.258	3.878
Tryptophan	0.637	1.571
ΣLNAA	0.548	1.826

Multicollinearity =Tolerance <0.1 and VIF>10

There were no outlying cases.

6.3.5 Multiple linear regression: HADS-T

HADS-T is a continuous variable, which can be dichotomised to indicate presence of absence of psychological distress. However, the cut-off point for psychological distress may differ from population to population and therefore a linear regression will be undertaken using this variable in its continuous form.

The independent variables entered into the linear regression were glutamate, GABA, TNFα, IL-10, serine, ΣLNAA, POM, gender and age: the same used in the logistic regression predicting presence of psychological distress (Box 6-4).

The model is able to predict HADS-T ($F_{5, 57}$=5.723, p<0.001) and accounts for 33% of the variance of HADS-T (R^2). Being female, younger, increasing pain, TNFα and decreasing serine are associated with an increasing HADS-T (Table 6.39).

Table 6.39: Linear regression HADS-T (n=60)

	B	SE	t	p	95% confidence interval for B	
Age	-0.118	0.061	-1.948	0.056	-0.0240	0.003
Gender	-2.885	1.339	-2.154	0.035	-5.567	-0.203
POM	0.640	0.213	3.009	0.004	0.214	1.065
Serine	-0.330	0.128	-2.575	0.013	-0.587	-0.073
TNFα	14.720	6.741	2.184	0.033	1.221	28.219

6.3.5.1 Assumption checks for linear regression HADS-T

There was no multicollinearity within the predictor variables (Table 6.40). The Durbin-Watson statistic was 2.275; with 5 predictor variables and n=60 bounds for the Dubin-Watson statistic are 1.41 to 1.77. The observed *d* is greater than this value and therefore there is no serial correlation between residuals.

There were no outlying cases with a standardised residual >2.5. Cook's Distance was below 1 for all cases (minimum <0.001 to maximum 0.120).

Table 6.40: Multicollinearity statistics for linear regression HADS-T

	Tolerance	VIF
Age	0.896	1.116
Gender	0.870	1.149
POM	0.822	1.216
Serine	0.844	1.185
TNFα	0.814	1.229

Multicollinearity =Tolerance <0.1 and VIF>10

6.4 Summary of results

A total of 59 cases and 21 controls were entered into the analysis. The age and gender of the OA group is similar to the national profile for hip and knee arthroplasty. The groups were similar in terms of age but the OA group contained a higher proportion of men than the control group. There was no gender or age difference when the PAR group was compared with the 0PAR group. The proportion of primary OA sites (hip: knee) was similar in the PAR and the 0PAR groups.

The median POM in the OA group was 6, and POM was higher in those participants who had PAR. The median PAR was 2 and 59% of the OA group had PAR.

The total HADS score and the proportion of participants with psychological distress (HADS-T ≥12) is significantly higher in the OA group than the control group. The proportion of participants with psychological distress in the PAR and 0PAR groups was similar.

There were a high number of cases with variables below the level of detection in the cytokine data and the study was underpowered for most of the amino acids and cytokines with the exception of serine and TNFα. The data was not normally distributed.

Comparison of concentrations of amino acids and cytokines in the OA and control groups identified significantly higher levels of serine, valine, leucine and TNFα in the OA group. IL-12 was significantly lower in the OA group than the control group. These differences were significant after adjustments were made for age, gender and HADS-T.

When amino acids and cytokines were compared in the PAR and 0PAR group aspartate and IFNγ were found to be significantly higher in the 0PAR group. These differences were significant after adjustments were made for age, gender and HADS-T.

Binary logistic regression analyses revealed that membership of the OA group was predicted by a model containing age, gender, HADS-T, serine, leucine and GABA. The model suggests that being male, higher HADS-T, higher serine, higher leucine and lower GABA are predictive of belonging to the OA group.

Membership of 0PAR/PAR groups was predicted by a model containing age, gender, HADS-T, POM, aspartate and IFNγ but IFNγ had a very low odds ratio and large confidence interval for the odds ratio. IFNγ was withdrawn from the model as it was felt that small numbers of cases with a value for this variable affected the convergence. The second version of the model containing age, gender, POM, HADS-T, and aspartate was also able to predict membership of the PAR group and increases in aspartate and POM made a significant contribution to the model.

Serine and POM were associated with an increased likelihood of membership of the psychological distress (HADS-T≥12) group. Increases in serine and POM made a significant contribution to this model. A multiple linear regression exploring HADS-T score identified being female, increasing POM, decreasing serine, and increasing TNFα as significant predictors of increasing HADS scores.

Chapter 7 Discussion

This study is first to report a concurrent exploration of amino acids, cytokines and mood in the CSF of people with OA. The study has identified a significant increase in serine and TNF in CSF and this can be interpreted to support the presence of central sensitisation in OA pain. This enables the null hypothesis, that there was no difference in amino acids and cytokines between the OA and control groups, to be rejected.

This study is one of the first to report the concentrations of a large number of amino acids and cytokines in human CSF and the first to do this in people with osteoarthritis. The findings are interesting both for the support they offer to the case for central sensitization in OA pain and also because there are differences between OA and control groups that are unexpected, such as the significantly lower level of IL-12 in the 0PAR group when compared with the PAR group. IL-12 has been regarded in neuropathic pain studies to have a pro-inflammatory role. It is possible that this reflects an anti-inflammatory role for this cytokine in central OA pain signaling and thus this reduced level represents and imbalance between pro- and anti-inflammatory activities in OA pain. An anti-inflammatory role has been attributed to this cytokine in an inflammatory model of the dorsal root ganglion (Xie et al. 2006a) but there are no reports of IL-12 in the CSF of clinical pain studies at present.

Increased levels of TNFα in the CSF in OA indicate glial activation, an important requirement for central sensitisation. This is supported by the observation of increased serine, a co-agonist of the NMDA receptor whose activation is also a requirement for central sensitisation. This study is one of very few to report on levels of serine in the CSF and as such adds important

information to our understanding of the role of this amino acid, so far only reported in animal studies.

As well as being elevated in the OA group when compared with the control group, increased serine was also a significant contributor to a regression model for membership of the OA group, and decreasing serine was a significant predictor for membership of the psychological distress group (HADS-T≥12).

There were minimal differences between the 0PAR and PAR groups in this study and the differences that were significant were not those expected. This means that the null hypothesis in respect of these groups cannot be rejected. Lower levels of IFNγ in the PAR group suggest increased anti-inflammatory activity in this sub-group. This does not fit in with the reports from other studies identifying pain at rest as a risk factor for persistent post-operative pain in arthroplasty patients (Wylde et al. 2011a; Lundblad et al. 2008). Those reports lead to the expectation that that pro-inflammatory cytokine levels would be elevated in the CSF of the PAR sub-group. It is interesting finding therefore that a group suggested by other research to have higher levels of central sensitisation should have lower levels of molecules that explain the sensitisation process. Equally anomalous is the finding in this study that there was no difference in the levels of HADS between the PAR and 0PAR groups. This finding is unexpected as one might expect a group troubled by spontaneous pain and rest pain to be more distressed by that experience than those whose pain tends to be stimulus-evoked. These findings call into question the meaning of pain at rest in OA.

Psychological distress was defined as having a HADS-T score greater than 12 and this was more prevalent in the OA group than the control group than the OA group. HADS score was also significantly higher in the OA group. This is an important finding which supports previous work demonstrating that OA is co-morbid with depression in many people and adds to this body of evidence.

The finding that increased TNFα is a predictor of psychological distress is in agreement with current neurobiological models of depression and pain, which identify raised CNS cytokine levels as part of the process. However, the regression model in the current study also identified that decreasing levels of serine and increasing levels of pain on movement were associated with also predictive of psychological distress and increasing HADS-T. This is an unexpected result as the most likely role for serine to play in the mediation of pain is as an NMDA receptor activator. Thus, in situations where increased pain, and increased psychological distress are present one might also expect increased serine. The anomaly is further underlined by the absence of linear correlation between serine and POM, PAR or TNFα.

The role of tryptophan metabolism and 5-HT are equally important in the development of depression and the OA group in the present study has elevated leucine and valine levels, two of the large neutral amino acids that compete with tryptophan for active transport across the blood brain barrier. This might represent a relative imbalance in tryptophan in depression in OA.

This chapter will present the evidence from the present study in support of the presence of central sensitisation in OA pain. A brief over-view of central sensitisation will provide background for the discussion of the findings of this study. Throughout the discussion the current findings, both positive and negative, will be explored in the context of clinical research. The findings will be related both to pain and to psychological distress or mood where this is relevant.

This chapter will begin by exploring the evidence for central sensitisation from the findings related to the pro-inflammatory cytokines with a focus on TNFα. Following on from this the role of the anti-inflammatory cytokines in OA will be discussed, including a novel proposed role for IL-12 as an anti-inflammatory agent in this study. At the end of the section discussing the role of cytokines the relationship between peripheral and central cytokine levels will be explored. This will be followed by a discussion of the role of excitatory and inhibitory amino acids with a focus on the role of serine, and the combined roles of glutamate, glutamine and GABA.

The notion of rest pain will then be explored with a particular focus on the role of IFNγ and HADS-T. Finally the discussion section will end with a review of what the results relating to tryptophan and the large neutral amino acids suggest about psychological distress in this cohort.

The chapter will end by discussing the implications of the findings for people who have OA pain, describing the limitations of the present study and addressing these in a plan for further work to continue to explore OA pain.

7.1 Support for central sensitisation in OA from the present study

Increased concentration of TNFα and serine in the OA group in comparison to the pain free control group can be interpreted to support central sensitisation as a mechanism involved in the pain experience in OA. This study also offers correlational support of central sensitisation from other cytokine and amino acid analytes. This result has implications for the accumulated knowledge to date on central sensitisation.

Central sensitisation is defined as 'increased responsiveness of nociceptive neurons in the central nervous system to their normal or subthreshold afferent input' (Merskey and Bogduk 2011). Most synaptic input to neurons is sub-threshold and has little or no effect on the post-synaptic cell either because the signal strength is too weak or because the inhibitory inputs to the membrane are sufficient to overcome the signal. These sub-threshold signals can cause action potentials however if there is an increase in pre-synaptic excitatory transmitter release, if the inhibitory processes are themselves inhibited (disinhibition) or if the excitability of the post-synaptic membrane is increased (Woolf 2011). These processes together contribute to the state of central sensitisation.

Central sensitisation features altered sensation in the form of hyperalgesia and allodynia. Following injury there is an increase in afferent signaling that leads to neurochemical changes. These changes create a heightened sensitivity in the dorsal horn such that subthreshold stimuli can evoke pain (hyperalgesia) as can innocuous stimuli (allodynia).

This is highly relevant as it is thought that these changes account for some of the discrepancy between radiographic findings and the severity of pain experienced by people with OA (Graven-Nielsen et al. 2012). In other words, people with OA experience disproportionate pain in comparison with the level of joint damage and inflammation that can be observed radiographically (see 1.2.1.1).

Movement in the normal range is painful in OA because of peripheral and central sensitisation. This lowers the threshold of nociceptive fibres in the joint so that normal range of movement and relatively innocuous mechanical stimulation can trigger action potentials. This is hyperalgesia and it has been demonstrated in OA by a reduction in pressure pain thresholds (Farrell et al. 2000; Graven-Nielsen et al. 2012; Imamura et al. 2008) and a heightened response to noxious muscle stimulation in the form of saline injection (Bajaj et al. 2001). Hyperalgesia of the joint can be explained partly by local sensitisation (primary sensitisation) but OA is also associated with alterations in sensitivity at sites distant to the diseased joint.

Centrally driven sensitisation also accounts for alterations in sensitivity and pain threshold that are found at sites distant to the affected joint (Bradley et al. 2004; Lundblad et al. 2008; Wylde et al. 2012). Lundblad et al (2008) assessed sensation and pain threshold in 69 pre-operative knee arthroplasty participants using electrical stimulation via electrodes grasped between the thumb and index finger. The OA participants had significantly higher sensation thresholds and significantly lower pain thresholds than an age and gender matched control group. The fact that these alterations in threshold were distant to the primary pain site demonstrates that the changes are centrally mediated.

Wylde *et al* (2012) performed quantitative sensory testing on 107 people with knee OA and 50 healthy controls and found that 71% of the OA participants had at least one somatosensory abnormality including reduced thermal and tactile thresholds as well as pressure hyperalgesia. Again, the sensory changes were distant to the primary pain site demonstrating that changes to sensitivity were central and had a widespread effect. These findings are felt to provide direct evidence that central sensitisation is an integral part of OA pain but until recently there has been no evidence from within the CNS to support these findings or explain how these changes are mediated in clinical OA pain.

Information about the processes and substrates involved in central sensitization originates mainly from *in vitro* and *in vivo* animal work and has been confirmed in humans mainly through studies of neuropathic pain conditions. Up until this point there has been little evidence of the central sensitization process in OA from a neurochemical perspective. The present study addresses this and provides support for the notion that central sensitization is an integral part of the OA pain experience.

7.1.1 Cytokine activity in the dorsal horn in OA pain

One of the main findings of the present study was that TNFα was significantly higher in the OA group than the control group and that IL-12 was lower. The production of TNFα is a key component of the central sensitisation process and indicates glial activation is part of the pain mechanism in this condition. There have been very few reports of IL-12 in pain-related studies prior to this one, and its role is less well defined than TNFα, but this study supports an anti-inflammatory role that together with the finding of increased TNFα in the OA group

suggests an imbalance between pro-inflammatory and anti-inflammatory activity in favour of pro-inflammation.

7.1.2 Pro-inflammatory cytokines in the CSF in OA pain

7.1.2.1 The role of CSF TNFα as a pro-inflammatory cytokine in OA pain

There are relatively few clinical studies of cytokines in the CSF and the present study is one of the few pain-related clinical studies to report TNFα levels (Table 7.1). This pro-inflammatory cytokine is reported variously as being undetectable, unchanged and higher in pain groups in comparison to pain-free controls in published studies. As a key molecule involved in the genesis of central sensitisation one might expect to find more consistent evidence of increased levels of TNFα in these studies, many of which involve pains where central sensitisation is a known mechanism.

TNFα was undetectable in participants with neuropathic pain (Backonja et al. 2008), migraine and headache (Bo et al. 2009) and post-operative OA patients who had been given anti-inflammatory medication (Buvanendran et al. 2005). It was no different to the control participants in studies investigating pre-operative OA (Lundborg et al. 2010), Complex Regional Pain Syndrome (CRPS) (Alexander et al. 2005), sciatica (Brisby et al. 2002), painful polyneuropathy (Ludwig et al. 2008) and post-herpetic neuralgia (Kikuchi et al. 1999). The only published clinical study demonstrating an increased level of TNFα found that it was elevated in people with persistent daily headache when compared with those who had chronic migraine (Rozen and Swidan 2007). This latter finding was explained by the research team as

resulting from the likely infective pathogenesis of new persistent daily headache, which thus was associated with central nervous system inflammation.

Backonja *et al* (2008) studied people with CRPS and diabetic neuropathy and hypothesised that pro-inflammatory cytokines would be found in higher levels in the CSF of the participants with pain than the pain-free controls but levels of TNFα were undetectable. They found instead that soluble TNF receptor (sTNFr) was significantly higher in participants with CRPS and diabetic neuropathy than in their healthy volunteers. Soluble TNF receptors are shed from membranes and compete with membrane bound receptors for TNFα and therefore block the activity of TNFα. TNFα and sTNFr levels are thought to closely reflect one another because the induction process is the same (Aderka 1996); the shedding of the sTNFr is stimulated by TNFα itself as well as other cytokines such as IL-1β, IL-2, IL-6 and IFNγ (Aderka 1996). Backonja *et al* (2008) suggested that elevated levels of sTNFr in the CSF indicate that TNFα was being locally produced despite it not being present in detectable levels. Thus other studies that fail to detect elevated levels of TNFα might find elevated levels of sTNFr.

Table 7.1: Clinical pain studies exploring cytokines in CSF of people with painful conditions

Author	Focus	Control groups	Molecules of interest	Summary of changes observed
Maier *et al* (2005)	Healthy volunteers (n=113)		IL-6, IL-8, IL-10	
Alexander *et al* (2005)	CRPS (n=24)	Mixed (pain and pain free) (n=16)	IL-1β, IL-6, TNFα	↑IL-6 =IL-1β, TNFα
Alexander *et al* (2007)	CRPS (n=22)	Mixed (pain and pain free) (n=31)	IL-4, IL-10, IL-6, IL-8, MCP-1, GFAP, nitrate, nitrite, glutamat3	↑IL-6 ↓IL-4, IL-10
Backonja *et al* (2008)	Post-traumatic neuralgia + CRPS (8), painful diabetic neuropathy (6)	Healthy volunteers (n=6)	IL-1β, IL-6, TNFα, sTNFr, IL-1ra, IL-8, IL-10, SP	↑sTNFr and ↓IL-10 in pain group TNFα undetectable
Bo *et al* (2009)	Migraine, headache (n=107)	Neurological diagnosis normal and no pain (n=20)	IL-1β, IL-1ra, IL-4, IL-10, TNFα, MCP1, TGFb1	IL-1β, TNFα undetectable in most samples
Brisby *et al* (2002)	Disc herniation + sciatica (n=39)		IL-1β, IL-6, IL-8, IFNγ, TNFα,	= IL-1β, IL-6, IFNγ, TNFα ↑IL-8 in 12 out of 39.
Buvanendran *et al* (2005)	OA: elective joint replacement (n=20)	Placebo (n=10)	PGE2, IL-1β, IL-6, IL-8, TNFα	IL-1β, TNFα were undetectable. Pre-operative Rofecoxib reduced CSF PGE2 and IL-6.

Author	Focus	Control groups	Molecules of interest	Summary of changes observed
Kadetoff *et al* (2012)	Fibromyalgia (n=15)	Non-inflammatory neurological symptoms (n=11)	IL-1β, IL-8	↑IL-8 =IL-1β
Kikuchi *et al* (1999)	Post-herpetic neuralgia (n=25)		IL-1β, IL-8, IL-6, TNFα	IL-8 decreased after intrathecal steroid. Other cytokines 'normal'.
Kotani *et al* (2004)	Post-herpetic neuralgia (n=277)		IL-8	IL-8 decreased with duration of pain
Ludwig *et al* (2008)	Painful polyneuropathy (n=18)	Painless polyneuropathy (n=18)	TNFα, IL-6	=IL-6, TNFα
Lundborg *et al* (2010)	Osteoarthritis (n=20)	Gynaecological or urological surgery (n=20)	GDNF, IL-1β, IL-6, TNFα, IL-8, IL-10	↑GDNF, IL-8, IL-1β. =TNFα, IL-6
Munts *et al* (2008)	CRPS with dystonia - women only (n=20)	TURP, knee replacement, general surgery (n=29)	IL-1β, IL-6,	=IL-1β, IL-6 and NO
Rozen & Swidan (2007)	Daily persistent headache (n=20)	Chronic migraine (n=16)	TNFα	↑TNFα
Sarchielli *et al* (2001)	Chronic migraine, Fibromyalgia (n=20)	Age and sex matched (n=20)	NGF, BDNF, glutamate	↑glutamate
Yeager *et al* (1999)	Post joint replacement surgery (n=19)	Healthy volunteers (n=6)	IL-6, IL-10	↑ IL6, = IL10

Backonja *et al.* speculate that rapid metabolism of TNFα makes it difficult to detect this cytokine but this does not explain why it is detected in other studies that explore similarly persistent pains including other CRPS studies (Alexander et al. 2005). It is also possible that TNFα was below the level of detection in a number of the clinical studies and yet it might still be present in sufficient concentration to have an effect. It is likely that a combination of these factors has led to a failure to detect increased levels of TNFα in the CSF of people with pain conditions where glial activation is known to be involved.

Lundborg *et al* (2010) is a particularly interesting study as it involves the same patient group as the present study. Their aim was to explore the concentrations of pro-inflammatory cytokines (IL-1β, IL-6, IL-8 and TNFα) as well as IL-10 and glial derived neurotrophic factor (GDNF) in the blood and the CSF. The main focus of the study was GDNF and they found that this was elevated in the CSF and reduced in the blood in the pain cases. GDNF has pro-algesic effects in inflammatory conditions (Fang et al. 2003) and analgesic effects in neuropathic (Leffler et al. 2002; Boucher et al. 2000). Lundborg *et al* (2010) conclude that the GDNF increase they observed was as a result of its production within the CNS and that is was acting in a pro-algesic fashion. They found that TNFα was present in similar levels in pain cases and controls while IL-1β and IL-8 were elevated in the CSF of the OA group. The lack of difference in TNFα in the Lundborg *et al.* study may be explained, as hypothesised by Backonja *et al* (2008) by an actual but unmeasured difference in sTNFr. Their finding of increased IL-1β and IL-8 supports a pro-inflammatory process in their OA sample.

7.1.2.2 The role of CSF IL-1β in OA pain

IL-1β was largely undetectable in the samples in the present study despite the high sensitivity of the assay. However, it is not unusual to fail to detect this cytokine. A number of clinical studies in which IL-1β was included for assay have similarly failed to detect it (Table 7.1) including in post-operative OA (Buvanendran et al. 2006), post-herpetic neuralgia (Kikuchi et al. 1999), spinal stenosis and disc degeneration (Scuderi et al. 2006), and chronic fatigue syndrome (Natelson et al. 2005). In studies where IL-1β has been detected no significant difference has been observed in its concentration when compared to the pain free control group, including CRPS (Munts et al. 2008; Alexander et al. 2005), sciatica (Brisby et al. 2002) and fibromyalgia (Lundborg et al. 2010). No studies have at present reported IL-1β to be increased in people with painful conditions.

The role of IL-1β in OA pain has been demonstrated in animal studies. Inhibition of IL-1β leads to an improvement in the condition of the osteoarthritic animal joint (Fiorentino et al. 2008) and Rheumatoid joint (Guo et al. 2007; Boyle et al. 2002).

The role of IL-1β in pain transmission may need to be inferred indirectly. IL-1β often correlates with IL-6 and TNFα because activation of glia results in the production of all the pro-inflammatory cytokines as a cascade (Milligan and Watkins 2009). This correlation has been reported in animal models of inflammatory arthritis (Bao et al. 2001; Sweitzer et al. 1999). Thus activity of IL-1β may be inferred from observing activity of other pro-inflammatory cytokines.

IL-1β also plays an important role in the induction of cox-2 and thus amplification of pain via centrally produced prostaglandins (Narita et al. 2008). Thus it may be possible to infer the involvement of IL-1β in a pain signaling cascade from observation of increased prostaglandin E2. Although it has been observed that PGE_2 is evoked in measurable quantities in an animal model of arthritis (Ebersberger et al. 1999; Sorkin and Moore 1996) there are no published clinical studies at present exploring PGE_2 in OA.

7.1.2.3 The role of CSF IL-6 and IL-8 in OA pain

In the present study there was no difference between IL-6 or IL-8 concentration in the OA group and the control group. Retrospective power calculation revealed that the study was underpowered for detecting a difference in either between the groups. There were moderate positive correlations between TNFα, IL-6 and IL-8 in the OA group although IL-6 and TNFα did not correlate in the control group. This is not unexpected given the usual pattern of release of pro-inflammatory cytokines and this suggests that with a better powered study an increase in IL-6 and/or IL-8 may have been seen.

The majority of clinical studies that have set out to explore CSF IL-6 levels have likewise not been able to detect a difference between their pain cases and the pain-free controls (Table 7.1). These studies include investigations of CRPS (Munts et al. 2008), polyneuropathy (Ludwig et al. 2008), and osteoarthritis (Lundborg et al. 2010). However, increased levels of IL-6 have been observed in the CSF of patients with CRPS (Alexander et al. 2007; Alexander et al. 2005). Increases in IL-8 have been observed in osteoarthritis (Lundborg et al. 2010), fibromyalgia (Kadetoff et al. 2012) and some patients with sciatica (Brisby et al. 2002).

All of these studies had similarly low numbers of participants (<30) and so it is unlikely that differences in power explain the lack of agreement in their results but given the variance of many cytokines and their involvement in multiple processes it is also quite likely that many of these clinical studies were underpowered.

It is interesting that in the Lundborg *et al* (2010) study there was a difference seen in IL-8 and IL-1 but not TNFα or IL-6. A pattern is emerging from this study and others that one or two key substrates are found to be significantly different between groups while other related molecules are not.

7.1.3 Anti-inflammatory cytokines in the CSF in OA pain

7.1.3.1 The role of CSF IL-12 as an anti-inflammatory cytokine in OA pain

A lower concentration of IL-12 was seen in the OA group in this study when compared with the control group. This seems an anomalous finding given that IL-12 is thought to have a pro-inflammatory role. Much of the work exploring IL-12 has a focus on CNS infection and there is very little published about this cytokine in relation to pain and what has been published suggests the role of IL-12 may differ dependent on the stimulus for its production.

IL-12 production is increased in the spinal cord following nerve injury in mice (Cao et al. 2009) and also from microglia *in vitro* stimulated simultaneously with lipopolysaccharides (LPS) and IFNγ (Aloisi et al. 1997). This latter study also demonstrated that IL-12 secretion could be down-regulated effectively by IL-10. However, in a model of dorsal root ganglion (DRG) inflammation (zymosan injection to the DRG)– designed to explore

how inflammation affects the DRG in the absence of nerve damage, Xie *et al* (2006b) categorise IL-12 as an anti-inflammatory cytokine along with IL-2 and report that it was significantly lower in the inflammation group.

The lower concentration of IL-12 in the OA group present study might therefore represent a reduction in anti-inflammatory activity. This is a novel interpretation for the function of this cytokine prompted by animal work in which both an anti-inflammatory (Xie et al. 2006a) and pro-inflammatory role (Cao et al. 2009; Aloisi et al. 1997). Dual effects of cytokines have been described in contexts other than pain, for example the pro-inflammatory and immunosuppressant activities of TNFα in animal models of Multiple Sclerosis (Kassiotis and Kollias 2001). There are two different explanations for the dual role observed in some cytokines. Activity can be dependent on other local factors (i.e. the presence of other cytokines) as illustrated by the lack of effect on beta-cells by IFNγ until in the presence of TNFα and IL-1β when IFNγ induces apoptosis (Gysemans et al. 2008). Activity may be dose dependent, as illustrated by the changing role of IL-1β in memory function in the hippocampus. At low and high doses IL-1β has a detrimental effect but there is a range within which increasing the level of IL-β above baseline levels improves memory in a water-maze task (Goshen et al. 2009).

The cytokines do work together as a network and the exploration of potential interdependences is not possible in the current data set but it worth exploring in the future.

7.1.3.2 The role of CSF IL-4, IL-5 and IL-10 in OA pain

There was no difference in the concentrations of anti-inflammatory cytokines between the OA group and the control group. However, IL-10 has a number of strong correlations with other anti-inflammatory and pro-inflammatory cytokines in the control and OA groups. This supports the idea that increasing pro-inflammatory activity causes an increase in anti-inflammatory activity designed to modulate the level of excitation.

Rather less has been published about the concentrations of anti-inflammatory cytokines in pain conditions in comparison with what has been written about the pro-inflammatory cytokines (Milligan et al. 2005) although their analgesic effect is an accepted fact (Uceyler and Sommer 2007; Milligan et al. 2005; Vale et al. 2003; Kanaan et al. 1998). IL-10 is a key modulator known to suppress the release of pro-inflammatory cytokines (Ding et al. 2003). This has led to interest in using IL-10 therapeutically for neuropathic pain (Soderquist et al. 2010) and to promote neuronal survival after spinal trauma (Zhou et al. 2009).

Clinical studies of people with CRPS (Alexander et al. 2007), diabetic and post-traumatic neuropathy (Backonja et al. 2008) and recovering from arthroplasty (Yeager et al. 1999) have reported low or unchanged levels of CSF IL-10. IL-4 and IL-10 mRNA and protein levels have been observed to be reduced in the blood of people with chronic widespread pain, many of whom had fibromyalgia (Uceyler et al. 2006). These findings suggest that lower levels of anti-inflammatory cytokines can contribute to pathological pain, perhaps not as an absolute but relative to the change in balance between excitation and inhibition. It would be interesting to follow up this idea in a post-hoc analysis, exploring the ratio of

pro and anti-inflammatory levels in the different groups identified in this study. It would be necessary to construct hypotheses that allowed for a potential dual role for certain cytokines such as IL-12.

7.1.4 Peripheral stimulation of cytokine activity in the dorsal horn

The increase in CSF TNFα seen in the present study can be attributed to increased activity in the primary afferent fibres as has already been outlined. However, increased CNS pro-inflammatory activity is also a response to communication from the periphery to the CNS via the blood stream and vagus nerve. Signaling pathways from the immune system to the CNS were identified following observation of the sick response: that peripheral immune activation causes activation of central nervous system responses mediating symptoms such as fatigue, fever, anorexia and sleepiness. The proof that the sick response is centrally mediated triggered by peripheral immune activation has been demonstrated by blocking the activity of peripheral cytokines, and by peripheral administration of cytokines (Maier et al. 1998; Kent et al. 1992).

Communication of peripheral immune activation to the CNS involves neural pathways (the vagus afferent and the glossopharyngeal nerve) and blood borne communication via interaction with, and active transport across the blood brain barrier (Dantzer 2001; Watkins et al. 1995). Thus the increased levels of pro-inflammatory cytokines observed in the periphery as a result of an inflammatory process will directly influence the levels of cytokines measured in the CNS.

There are increased levels of pro-inflammatory cytokines in the synovial fluid (SF) of people with OA (Kokebie et al. 2011; Goldring 2000;Sipe 1995). These changes in the levels of pro-inflammatory cytokines local to the site of the disease processes of OA are reflected in the serum where levels of acute phase proteins (C-reactive protein and serum amyloid A) (Sturmer et al. 2004) and pro-inflammatory cytokines (Petrovic-Rackov 2005) are also observed to be higher than in age matched controls. This demonstrates that there is a systemic pro-inflammatory cytokine stimulus in OA, which has the potential to initiate the immune-CNS communication discussed above. Thus, increased levels of TNFα observed in the present study are a consequence of direct pain signaling via primary afferent fibres but also may be related to increased peripheral levels of pro-inflammatory cytokines resulting from the inflammatory processes within the joint and also increased levels relating to depression in some of the group. This suggests that complete understanding of CSF levels of cytokines requires concurrent measurement of their levels in the synovial fluid and blood.

The sick response shares common symptoms with depression and it has been shown that TNFα and IL-6 are consistently elevated in the periphery of people with major depression (Dowlati et al. 2010). The sick response/peripheral immune activation also heightens pain sensitivity (Wiertelak et al. 1994; Watkins et al. 1994a; Watkins et al. 1994b). Thus there is an inter-relationship between pain, depression and the levels of peripheral and central cytokines. This is supported in the findings of the present study, which identify the predictive value of HADS for OA group membership and of TNFα for HAD score. These findings together with the increased prevalence of psychological distress in the OA group are in agreement with the inter-relationship between pain, cytokines and depression.

These findings support a combined role of peripheral afferent activity and an activation of the CNS by elevated peripheral cytokines as a result of OA or depression or both.

7.1.5 Amino acid activity in the dorsal horn in OA pain

The finding in this study that serine is elevated in the OA group provides considerable support for central sensitisation being a part of OA pain signaling. As well as the increased concentration of serine in the OA group of the present study there were a number of significant correlations between serine and glutamine and between glutamate, GABA and glutamine. These amino acids are all related to the process of pain signaling and recycling of glutamate (Struzynska and Sulkowski 2004).

7.1.5.1 The role of CSF serine as an excitatory amino acid in OA pain

Serine is released from activated glia as result of AMPA receptor activation (Mothet et al. 2005). It then acts as a co-agonist of the NMDA receptor, the activation of which is an integral part of the amplification processes in central sensitisation (Wolosker 2007). No published reports of CSF serine in clinical studies could be found: it has received more attention in animal studies. Serine has been found in elevated levels in the CSF following plantar incision in rats (Zahn et al. 2002), and acetic-acid induced visceral pain (Feng et al. 2003) but not in an animal model of neuropathic pain (intrathecal pertussis-toxin) (Wen et al. 2003).

The finding in the present study that TNFα and serine are higher in the OA group indicates NMDA receptor activation.

One of the effects of serine activity observed in animal studies is the development of dynamic allodynia. Usually light brushing of the skin or mucosa does not evoke pain. The fibres that transmit these sensations synapse deep within the dorsal horn with wide dynamic range cells. However, there are circumstances in which light brushing does evoke pain and this involves disinhibition of glycine receptors. This disinhibition is an outcome of prostaglandin activity, the production of which is stimulated by pro-inflammatory cytokines. The process of disinhibition and the development of allodynia are NMDA receptor and serine dependent (Miraucourt et al. 2011); degrading serine prior the experiment prevents the development of allodynia.

Thus, the finding that serine is elevated in OA pain might increase the risk of allodynia in this pain. Evidence of allodynia in OA has been seen in rats following intra-articular injection of monosodium iodoacetate (MIA) (Sagar et al. 2011; Stevenson et al. 2011), and partial medial meniscectomy in mice (Knights et al. 2012).

Quantitative sensory testing is a technique for the assessment of cutaneous sensory nerve function (Courtney et al. 2010), and is often used as a diagnostic tool (Geber et al. 2011). Standardised processes have been developed to reduce inter-rater reliability problems with this technique, and the standard test battery takes approximately one hour to administer per participant. The standard set of tests comprises assessment of normal thermal perception and pain thresholds, touch and vibration detection thresholds and mechanical pain sensitivity including thresholds for pin-prick and blunt pressure, stimulus-response functions for pin-prick sensitivity and dynamic mechanical allodynia and pain summation to repetitive pin-prick (Rolke et al. 2006a; Rolke et al. 2006b).

Clinical studies using quantitative sensory testing have not yet reported any evidence of allodynia in OA. However, most reports focus on pressure pain thresholds and hyperalgesia (Wylde et al. 2011a; Wylde et al. 2011b; Gwilym et al. 2009; Kosek and Ordeberg 2000a; Kosek and Ordeberg 2000b) and only one published report has documented specifically looking for allodynia in OA. Westermann *et al* (2011), provide the only published report where allodynia was specified as an outcome and they found no evidence of it in the hands of 20 people with OA pain.

This suggests that the presence of serine and TNFα in the CSF in OA is not sufficient of itself to trigger allodynia and that other factors (not present in this study) are also required.

The higher level of serine in the OA group in comparison to the control group can be interpreted as seen above by the known mechanism of central sensitisation. However, this study also identifies an unexpectedly inverse relationship between serine levels and HADS-T.

7.1.6 The role of CSF glutamate, GABA and glutamine in OA pain

One might expect that raised serine and TNFα in this study would be accompanied by a simultaneous increase in glutamate levels, and decrease in glutamine and GABA. This is because glutamate, glutamine and GABA are all affected by NMDA R activation and linked by a cycle of production and regulation (Struzynska and Sulkowski 2004; Schousboe et al. 1997). Although direct comparisons between groups of the concentrations of these amino acids did not reveal a statistically significant difference at the 0.05 level GABA does contribute to the regression model for membership of the OA group with

decreased levels of GABA being associated with an increased likelihood of belonging to the OA group.

Glutamate is cleared from the synaptic cleft by active transport into glia where it is transformed into glutamine in a process driven by glutamine synthetase (Arriza et al. 1994). Glutamine is then released into the synaptic cleft from the glia and is taken up by neuronal cells where it is converted to glutamate (in the primary afferent fibres) or GABA (in the GABAergic interneurons) (Liang et al. 2006). NMDA activation leads to the formation of peroxynitrite (produced in a reaction between superoxide and nitric oxide), which inactivates both the glial glutamate transporter and glutamine synthetase (Chen et al. 2010). Therefore synaptic glutamate levels rise because of inhibition of the transport systems and glutamine levels fall due to the decreased availability of glial glutamate and the inhibition of glutamine synthetase, which then affects the production of GABA (Chen et al. 2010). This outcome of this process is increased excitation due to increased levels of glutamate and also disinhibition due to decreased amount of GABA. The contribution of reduction in GABA to the membership of the OA group may be an indication that reduced inhibition plays a part in the pain mechanism in this pain.

Correlations between glutamate, glutamine and GABA in the present study can be explained by this mechanism but there was no change in levels of these amino acids in the OA group. Increased levels of glutamate have been reported in other clinical studies involving severe pain or presumed central sensitisation including headache or migraine (Sarchielli et al. 2007a; Peres et al. 2004; Castillo et al. 1995; Martinez et al. 1993b), fibromyalgia (Sarchielli et al. 2007a), CRPS (Alexander et al. 2007) and labour pain (Hsu

et al. 2001; Olofsson et al. 1997). Equally, some studies report no difference when a pain-free control group is compared with active labour (Sethuraman et al. 2006) and fibromyalgia (Larson et al. 2000).

One of the reasons offered for finding no significant difference in glutamate levels is provided by Larson *et al* (2000) who suggest that efficient reuptake of glutamate and subsequent transformation of this to glutamine masked increases in glutamate. However, they did find an increased level of glutamine and this they state demonstrates an increase in excitatory amino acid activity. In this study direct comparison of glutamine in the OA and control groups and the PAR/0PAR groups does not reveal a statistically significant difference. However the median levels of glutamine are higher in the OA group than the control group and in the 0PAR group than the PAR group. This non-significant difference is interesting as it matches Larson *et al's* explanation for lack of observable difference in the glutamate levels. Further, the observation that there is a non-significant reduction of glutamine in the PAR group in comparison to the 0PAR group matches with the unexpected finding in this study that other pro-inflammatory/excitatory mediators, IFNγ and aspartate are also reduced.

Fibromyalgia has been associated with increased CSF IL-8 (Kadetoff et al. 2012), a pro-inflammatory cytokine the presence of which indicates glial activation. Glial activation suggests NMDAR activation and that in turn increases the likelihood that the glutamate-glutamine cycle would be disrupted by peroxynitrite inhibition of glutamate transport and glutamine synthesis. Thus it could be expected that fibromyalgia would be accompanied by increased glutamate and decreased glutamine. Fibromyalgia is likewise associated with

a dysfunction of the inhibitory descending pathways (Mense 2000) and this also is likely to create elevated levels of glutamate.

In this study there was no significant difference observed in the concentration of GABA between the groups. There is very little that can be compared this finding. GABA is known to be upregulated in animal models of inflammatory pain (Castro-Lopes et al. 1995). GABA is produced in the dorsal horn by glial cells and interneurons. Glia continuously synthesise GABA and maintain a gradient between intracellular and extracellular concentrations by active transport (Lee et al. 2011). An increased release of GABA from the glia can be stimulated by glial uptake of synaptic glutamate (Heja et al. 2009) and by receptor agonism by glutamate, glycine and serine (Lee et al. 2011). GABA release from inhibitory interneurons is similarly triggered by glutamate (Kerchner et al. 2001) or 5-HT receptor agonism (Kawamata et al. 2003). The release of GABA in inflammatory pain has been associated with activation of presynaptic (primary afferent fibre) $GABA_A$ receptors, which create an action potential resulting in an antidromic signal to the peripheral terminals causing the release of Substance P and calcitonin gene related peptide (CGRP) which contribute to peripheral sensitisation, vasodilation and plasma extravasation (Kelley et al. 2008; Willis, Jr. 1999). This suggests that in theory GABA levels in the CSF should be increased in response to increased glutamatergic activity and in this study the increased level of serine in the CSF also provides a stimulus for GABA release.

There are no published clinical studies exploring GABA levels in clinical pain conditions except for one demonstrating that GABA release is increased in migraine with depression

but not in migraine alone (Vieira et al. 2006). GABA levels in the CSF are consistently reported to be reduced in depression in clinical studies (Gerner et al. 1984; Kasa et al. 1982; Gerner and Hare 1981). This suggests that understanding the mood of the participants might help in the interpretation of the levels of these amino acids and that levels of these amino acids should not be interpreted without that information in clinical studies.

In the present study GABA, glutamate and glutamine were not found to correlate with HADS-T, nor did GABA or glutamate remain in the regression model developed to predict the presence of psychological distress, nor the model developed to predict the level of HADS-T. HADS-T was higher in the OA group as was pain, which is associated with a decrease and an increase in GABA levels respectively. GABA may be one of the analytes in this study for which a more sophisticated and better powered analysis is required to appreciate the relative influences of each of the potential predictors for this amino acids expression.

7.1.7 Spontaneous pain in OA

Pain at rest was present in 59% of the participants with OA in this study. These participants did not differ from those who did not have pain at rest in terms of the primary joint where they had pain, their HADS, age or gender. When amino acids and cytokines were explored in the 0PAR and PAR groups the only differences were that the 0PAR group had higher levels of aspartate and IFNγ. Logistic regression showed that both these substrates contributed to the model predicting membership of the PAR group (lower levels

being predictive) but the confidence intervals were very large and that suggests a failure to converge.

7.1.8 The role of CSF IFNγ in spontaneous pain

Pain at rest is by definition present in the absence of movement or other external stimulus. True rest pain may be a form of spontaneous pain. Lundblad *et al.* (2008) associated the presence of pain at rest in their OA cohort with an increased risk of persistent post-operative pain. They explain that rest pain represents an extreme form of central sensitisation, which is resistant to extinction following the removal of the noxious input. The expectation that arises from this hypothesis is that the present study should detect increased levels of pro-inflammatory cytokines or serine in the PAR group, and this was not the case.

IFNγ is found in increased levels in the dorsal horn following nerve injury (Tanga et al. 2005), induced from microglia by the activity of IL-12 (Aloisi et al. 1997). The role of IFNγ therefore might be expected to relate to sensitisation of the dorsal horn and to play a role in spontaneous pain and that would lead to an expectation that this cytokine would be elevated in the PAR group. However, we have seen evidence in respect of IL-12 that the behaviour of that cytokine in neuropathic and inflammatory conditions is very different and that may provide an explanation for this result. Aspartate is an excitatory amino acid about which little documented except in the context of co-release with glutamate (Zahn et al. 2002). The fact that it is elevated alongside IFNγ in the 0PAR group suggests that both are acting in a pro-inflammatory role. That they are elevated in comparison to their concentration in the PAR group might be explained by a difference in the pain mechanism

of these two groups. Aspartate and IFNγ may be involved in transmission of stimulus-evoked pain but not in spontaneous pain. The fact that the study is under-powered may prevent detection of the cytokines that are important in spontaneous pain in the current study. However, there may also be heterogeneity within the PAR group which together with the small sample size will mask differences. The group may contain people with true spontaneous pain as well as some who do not. It would be appropriate to 'test' for rest pain in future studies to try and improve the reliability of this category so that comparisons can be made.

7.2 Psychological distress and OA pain

This study found that OA participants had higher HADS-T scores and a higher prevalence of psychological distress (HADS-T≥12) than those in the control group. This is in agreement with other studies that identify co-morbidity between OA and depression and cite it as one of the factors that contributes to poor quality of life in some people with OA (Rosemann et al. 2007b; Dexter and Brandt 1994).

This study also identified a relationship between the large neutral amino acids OA. An increase in leucine was one of the predictors for membership of the OA group and leucine and valine were both found to be significantly higher in that group. These results suggest that one of the mechanisms of depression in OA is via the tryptophan-serotonin pathway. This is strengthened by the finding of increased TNFα in the OA group because of the role that cytokines play in tryptophan metabolism (see below). However, this study did not reveal a direct relationship between the large neutral amino acids or tryptophan and HADS-T as might have been expected. Further, the idea that depression is more prevalent

because OA sufferers have pain is not supported by the finding that decreased serine is a significant predictor of increased HADS-T.

The neurobiological relationship between pain and depression is complex. The findings of this study are in agreement with current neurobiological models of depression that integrate the effects of cytokines, the HPA axis and monoamines. Increased levels of pro-inflammatory cytokines in the CNS lead to a switch in tryptophan catabolism from the production of 5-HT to the production of quinolinic acid, thus reducing the amount of 5-HT available for central signaling and enhancing depression (Laugeray et al. 2010; Muller and Schwarz 2007; Raison et al. 2006). The concurrent effect of this is a reduction of descending inhibition via the serotonergic pathways and because of this a decrease in inhibition of pain signaling. This is reinforced by the finding in this study that HAD score could be predicted by a combination of pain, and increased TNFα for the cohort when explored as a whole.

This study has demonstrated that OA is associated with increased pain, increased TNFα, increased HADS-T and increased serine but the regression analysis suggests that lower levels of serine are associated with an increased likelihood of having psychological distress or increasing HADS-T. This suggests a potential disconnect between the pain mechanism involving serine and the mechanism of psychological distress associated with OA. In support of this it is notable that another 'pain-related' amino acid, GABA is also absent from the regression models for HADS-T but is present in the OA model. OA-psychological distress seems to be more influenced by cytokine activity than it is related to other mediators of the pain process. The relationship between the disease process, pain

and psychological distress is mediated by many factors including stress, fatigue and catastrophising. This and the pleiotropic nature of many of the mediators being investigated in this study mean that this interesting finding cannot be fully explored but is potentially important in helping to understand the relationship between pain and depression.

The findings relating to HADS-T are therefore in agreement with the pain-related findings and generally support the contention at the outset of this study that it is important to take anxiety and depression into account in clinical pain studies.

7.3 Limitations

7.3.1 Sample size

There were fewer participants in this study than desirable. Although a power calculation suggests TNFα, serine and serine were adequately powered the remainder of the analytes were not. The lack of difference in some of the transmitters and modulators explored may be related to the fact that the true difference between the OA/control group and the PAR/0PAR groups of participants was likely to have been small and would therefore require a larger sample to demonstrate this.

7.3.2 Recruitment of sufficient numbers to the pain-free control group

It was difficult to recruit participants for the control group because the experience of pain was an exclusion criterion and this is a common feature of the lives of many people aged over 50 years. The present study is not alone in struggling to recruit large numbers of

pain-free control participants. Clinical pain studies that do include normal healthy controls generally obtain only low numbers (Backonja et al. 2008 (8); Steensberg et al. 2006 (24); Scuderi et al. 2006 (3); Natelson et al. 2005 (13); Stenlof et al. 2003 (10); Yeager et al. 1999 (6)). A favoured method of creating a larger control group is to recruit people who are being tested for possible neurological disorders, who subsequently were declared to have no detectable abnormality (Gallai et al. 2003 (20); Garseth et al. 2002 (31); Garseth et al. 2001 (31)). Other control groups are comprised of people with known pathology including painful neuropathy (Alexander et al. 2007 (16); Alexander et al. 2005 (31)), and general surgery patients without pain (Munts et al. 2008 (20); Kotani et al. 2004 (50)). The difficulties encountered necessitate the collection of samples over a much longer period of time.

A further limitation of this study relating to the eventual size of the control group was that the study involved a number of simultaneous processes (recruitment, data collection, sample collection, sample processing, assay work, and liaison with surgeons, anaesthetists, ward staff and participants). In this study these processes were all conducted by one person and this limited recruitment at times.

7.3.3 Recruitment issues relating to anaesthetic technique

Recruitment to this study was dependent on the participants being willing to have a spinal anaesthetic as part of their operative process. The choice of anaesthetic technique was entirely independent from the study; however, potential participants who were approached about the study prior to seeing an anaesthetist expressed worries not about the study but about spinal anaesthesia. Experience during the recruitment process suggests that it is

important to have an anaesthetist available to assess the patient prior to any discussion about participation in the study.

Resolution of this problem during the course of the study required the recruitment to be moved from a pre-admission clinic visit 7-10 days before surgery, to the day of surgery. This raises ethical concerns as it does not provide the participants with a sufficient 'cooling off' period, nor time to digest the written information given in support of the study. A better solution would be for the anaesthetic assessment to take place in the pre-admission clinic where it could be followed up where appropriate with an invitation to discuss the study with the research nurse.

The rate of use of spinal anaesthesia varied among anaesthetic staff and was generally low. This was entirely unanticipated and was exacerbated by the change of role of one of the most enthusiastic anaesthetists such that she no longer delivered anaesthetics at all. Future studies will need to ensure adequate support from anaesthetists and a more thorough feasibility study to calculate the expected number of spinal anaesthetics per week.

The surgeons were approached prior to ethical approval being sought and were generally supportive of the study but during the course of the study it became clear that some had concerns about the time it would take to take a CSF sample and give a spinal anaesthetic – this was particularly an issue for the participants in the control group who were generally in the operating theatre for a very short time thus increasing the 'turn-over' in the anaesthetic room. This may have had an influence on the likelihood of the anaesthetist to

offer a spinal anaesthetic as part of the procedure and this affected recruitment significantly.

7.3.4 Pain intensity in the OA group

It is probable that the lack of large numbers of people with moderate to severe pain in this study influenced the findings. Pain is a key indicator of the need for surgery and yet one fifth of the OA group were offered surgery with pain on movement scores of three or less on a scale of 0-10 with 0 being no pain and 10 being the worst the participant could imagine.

It will be important in future studies to collect samples over a longer period of time, to aim to recruit participants with a range of pre-operative pain intensities and to be able to analyse the data in pain subgroups.

7.3.5 Pain at rest

In this study pain at rest was assessed by asking the participant to score his or her pain following a period of inactivity and without moving. It was made clear to the participant that the pain score being sought was the amount of pain present without movement or other stimulus. It is possible that pain at rest scores may include score related to residual activity pain despite efforts to prevent this. It would be appropriate in future studies to explore pain scores in a more sophisticated way, and to consider quantitative sensory testing to enable distinctions to be drawn between different groups of OA sufferers.

7.3.6 Supporting information about impact of OA

Interpretation of the data in this study would be enhanced by a more detailed understanding of the impact that OA had on the recruits. The information that would be useful would come from more detailed diagnostic information about the degree of OA changes as well as greater information about pain interference and function. Many studies exploring osteoarthritis use the Western Ontario and McMaster's Universities Osteoarthritis Index (WOMAC) scale (Bellamy et al. 1988) to measure pain, stiffness and function and this would have been an appropriate tool to include in this study so that comparisons could be made with other OA studies and so that functional restriction could be examined as one of the covariates. It is quite likely that this would have affected some of the modulators as well as the level of depression and anxiety. The use of tools in this study was restricted by the limited amount of time for data collection prior to the participant being taken to theatre and therefore recruitment at pre-assessment where more time could be taken would be an advantage.

7.3.7 The incidence and degree of anxiety and depression

There were relatively few participants in this study with significant levels of psychological distress and this limited the ability to consider fully the effect that these affective disorders may have on the spinal transmission of pain signals. Anxiety and depression have a strong reciprocal relationship with pain and this exploration could have a significant impact on future management strategies. It is possible that self-selection for regional anaesthesia skewed the research population artificially in favour of normal anxiety and depression. Future studies may be able to rectify this.

7.3.7.1 Hospital Anxiety and Depression Scale

The HADS was used in this study because it has been validated, and in the clinical setting where time is at a premium it is quick and reliable to administer. However, it became apparent in the interpretation of the data that more sophisticated tools would have assisted greater insight and have been more informative.

7.3.8 Quantification of inflammation

One of the clearest limitations that emerged during data analysis was that there was no objective marker of inflammation for each participant. The lack of data regarding the presence or absence of ongoing active inflammation makes much of the interpretation of the data speculative. This study should be repeated on a larger scale in the future and identifying and collecting information about reliable inflammatory biomarkers will be essential. These should include a general measure of inflammation such as C-reactive protein in both the OA and the control group in addition to measures of pro and anti-inflammatory cytokines in the synovial fluid and plasma. This would have enabled the characterisation of the control group to be more detailed and exclusion from this group of those who had undeclared or unknown inflammatory processes that may have confounded the results. It would also have been very useful to be able to identify the OA participants who had a higher degree of peripheral inflammation in order to interpret the dorsal horn data.

7.3.9 The selection of amino acids and cytokines

The range of amino acids and cytokines assayed in this study was extensive by comparison with other human studies, in part made possible by the development of multiplex bead arrays. The early adoption of this technology had limitations in as much as the cytokine profile was 'off the shelf' and further work would benefit from a bespoke selection of cytokines that could more fully explore some of the questions left by this study. However, the complexity of relationships between the different substrates in this study, the influence of mood, the suggestion that there are different subtypes of OA pain, and the desire to have contributing information from some that weren't assayed (see below) means that future studies need greater numbers of participants to be able to properly characterise this pain.

It has become apparent by data analysis and interpretation in this study that 5-HT, NA and NO metabolites are important to the interpretation of the data. In addition it would have been useful to have data about Substance P, CGRP, cox-1, cox-2 and PGE2. This study provides a basis for a better informed choice of analytes to consider in the future.

7.3.10 Confounding factors

7.3.10.1 Participant's weight

There are some other factors that also influence cytokine and amino acid levels that would be useful to take into consideration. CSF IL-6 correlates negatively with body weight in obese people (Stenlof et al. 2003) and therefore this is a variable that needs to be taken into account.

7.3.10.2 Medication use and analgesia

The use of anti-depressant and analgesic drugs by a small number of participants may have had a limited impact on the results of the study and more rigorous method for the collection and integration of this data needs to be built into any future study. It may be appropriate to use antidepressant medication as an exclusion criteria. In this study the majority of OA participants who used analgesic medication did so on an ad hoc basis and the led to difficulty in generating precise data. Future studies may take advantage of the natural pause between pre admission and admission by asking patients to keep a pain and medication diary.

7.3.10.3 Effect of lumbar puncture on CSF constituents.

CSF is obtained from humans by the use of a lumbar puncture. It is anecdotally reported that anticipation of lumbar puncture is anxiety-provoking and produces a physiological stress response in terms of elevation of adrenocorticotrophic hormone (ACTH) and cortisol in the plasma of subjects who have undergone a lumbar puncture (Lerner et al. 2000; Geracioti, Jr. et al. 1999; Petrie et al. 1999; Hill et al. 1999b; Geracioti, Jr. et al. 1997c; Chappell et al. 1994; Breier et al. 1988). The main feature of the stress response is the hypothalamic-pituitary-adrenal axis (HPA axis). One of the results of HPA axis activation is an increase in NA release into the dorsal horn, leading to an increase in GABA and glycine (Baba et al. 2000). Thus it is possible that the activity of performing a lumbar puncture will lead to increased GABA and glycine in the samples taken.

7.3.11 Missing data and cytokine concentrations below the level of detection

This study contains bias introduced by the method used to deal with concentrations of cytokines that were below the level of detection (non-detects). Concentrations lower than the level of detection that are not reported by the assay software tend to fall within the same range as the background 'noise' of the assay and are considered to be inaccurate. However, if these are omitted from the data analysis the resulting outcomes will only include complete cases. This can reduce the data set to unmanageable levels and this is seen in logistic regression in the current study where numbers of cases that can be entered into the analysis are dramatically reduced because so many cases do not have complete data sets for the predictor variables of interest.

Leaving non-detect variables out of the analysis on a variable by variable basis will lead to the data set being unrepresentative of the expected range of concentrations of cytokines, which can be present in very small but still biologically active concentrations. Thus, omission of non-detectable values can lead to bias in interpretation of the results.

One of the methods of dealing with non-detects is to use a symbolic 'non-detect' term to exclude these data from the analysis, which is how the very low concentrations were dealt with in the current study (achieved by assigning a value tag in SPSS). This censoring of the data will have an effect on the analysis that is performed by skewing the findings of the study because only the higher values of the analytes will be included in the statistical analysis. An alternative method for dealing with non-detects is to replace them with a nominal constant value. This is a popular method which involves replacing the non-detects

with one half or ⎯ times the detection limit but Helsel (2009) describes this as a dangerous procedure. The reason, he explains, is that 'substituted values possess a pattern that is alien to the pattern of the original data' (p. 258).

When the non-detects are missing at random, determined by Little's MCAR (missing completely at random) test then a multiple imputation method can be used (White and Carlin 2010) using a maximum value of the limit of detection as given by the assay manufacturer. Prior to publication of the findings of this study the bias introduced by leaving the non-detect cases out of the analysis will be corrected by using multiple imputation to substitute non-detects with values below the limit of detection and then re-analysis to determine any changes to the outcome of the statistical tests.

7.4 Implications of this study for the management of OA pain

7.4.1 The potential for persistent pain after surgery

The findings of the present study support a process of central sensitisation in OA pain and have also identified a difference between people with OA rest pain and those who no pain at rest.

Wylde *et al* (2011a) speculate that depression and rest pain are suggestive of an underlying vulnerability to pain and are both factors that increase the risk of persistent post-operative pain. This study identifies psychological distress in the form of HADS-T as one of the predictors of pain at rest and therefore offers some support of Wylde *et al.*'s

findings. The implication for practice is that close attention needs to be paid to the psychological comorbidities associated with OA, more-so in those who have pain at rest.

Lundblad *et al* (2008) offer evidence to support the hypothesis that persistent post-operative pain can be predicted by the presence of higher levels of pain at rest before surgery. This idea of pain vulnerability is supported by work into the cortical processing of pain (Apkarian et al. 2009). There are differences in cortical processing for stimulus-evoked pain and spontaneous pain. Spontaneous pain has a higher emotional salience whereas stimulus evoked pain creates a greater levels of activity in regions associated with the sensory-discriminative aspect of pain. The present study does not provide direct and rigorous support for this hypothesis, possibly because of the small sample size. However, the findings suggest a tentative support because of the finding that there are difference between the PAR and 0PAR groups in terms of IFNγ and aspartate. Together with the support for Wylde *et al.*'s findings this suggests that closer attention needs to be paid to the PAR group in terms of research and perhaps in terms of ensuring effective pre-operative pain and mood management.

7.4.2 Pharmaceutical management of OA pain

Current management strategies for pain relief in OA are capable of ameliorating this type of pain in many cases. Combinations of capsaicin, NSAIDs, Paracetamol and opioids target appropriate parts of this pain pathway at the periphery, in the brain and in the dorsal horn. There were no findings in the present study that suggested that these measures are

not the most appropriate, indeed the findings tell a story of pain signalling that suggests that OA pain, very often, will be very responsive to these drugs.

The findings of the present study also support the use of anti-depressant medication for the management of OA pain. The rationale for this is that 5-HT driven inhibition of OA pain is likely to be diminished by the activity of pro-inflammatory cytokines in the CNS. A number of studies have been exploring the use of duloxetine, a selective serotonin and noradrenaline reuptake inhibitor approved for use for chronic musculoskeletal pain in OA pain, and have found this drug to be helpful in the relief of pain and improvement of function (Hochberg et al. 2012).

7.5 Future work

The findings from this study suggest that future exploration into the role of amino acid and cytokines in OA pain would be highly beneficial. Whilst there are some limitations, this work has added to the discussion about the underlying pain mechanisms of people with OA and contributed to our understanding of its central processing. It is important that this work is followed up by an adequately powered study exploring a range of amino acids and cytokines designed to confirm the present results and facilitate interpretation by the inclusion of other substrates such as $PGE_{2,}$ cox-2, 5-HT and its metabolites, nitrites and nitrates, the metabolites of noradrenaline, and soluble receptors of IL-β and TNFα. Furthermore these analytes should be assessed in the synovial fluid, plasma and CSF to

enable a more complete understanding of peripheral inflammation to be taken into consideration when CNS data is interpreted.

The same groups will be of interest in future studies (OA/control, PAR/0PAR) and need to be recruited in sufficient numbers to ensure the study is adequately powered to detect differences in the key independent variables. Using mean data from the present study it was calculated that detecting a significant difference ($p<0.05$) between two groups for IL-6, with a power of 80%, would require a sample of 173 in each group and for IL-8 the sample size required increases to 1039 in each group. Detecting a significant difference in IFNγ in a comparison of 0PAR and PAR would require a sample size of 88 in each group while a TNFα between the same groups would require a sample size of 5157 (SISA 2012). These are fairly crude calculations based on the observed mean and standard deviation in the present study and have not taken into consideration the skew of the data.

Recruitment of control participants for studies such as this are always difficult and there are two ways that this can be addressed in future studies. In the first instance the population from which the control sample is drawn can be enlarged to include participants undergoing lumbar puncture for neurological diagnostic purposes. Many of those who have these procedures have no abnormality detected either at the time or following subsequent tests. Providing sufficient follow up via medical records was conducted this group offers a further pool from which control participants can be drawn. The second consideration for sample collection is the use of a tissue bio-repository approach. This involves the consent of individuals for their tissues to be used in a number of studies. The

tissues are collected in standardized ways by hospital staff and stored in a University bio-repository where they can be accessed by a number of different research teams.

Measurement tools in a future study of this nature should include the WOMAC scale and more sophisticated measures of anxiety and depression such as the Beck Depression Inventory and the State-Trait Anxiety Inventory. Data interpretation in the present study would also have enhanced by additional information about the participant's weight (and in particular whether they were overweight) as well as information regarding hyperalgesia and allodynia that would be collected by quantitative sensory testing. In addition it allow more complete data interpretation if more complete data were available on general inflammatory conditions the participants and controls were experiencing and to this end a C-reactive protein assay should be incorporated into the data set.

It is probable that anti-depressant, anti-inflammatory and analgesic use will affect central and peripheral levels of amino acids and cytokines and therefore a future study will ensure a more rigorous collection of data about the participant's medication use. This could be collected using a diary and enable simultaneous collection of contemporaneous pain data.

A greater amount of time with the participant for discussion about the study and informed consent is necessary for future work. This would enable a more ethical approach to consent, and a greater amount of time for preliminary data collection. A future study will aim to recruit participants before admission for their surgery, and ensure an anaesthetic assessment takes place at the time of the pre-admission visit. This would also enable the

participant to be approached for recruitment in a more timely fashion and facilitate the collection of a more sophisticated data set.

One of the requirements for a future study to be successful would be an increased number of research staff. Recruitment, data collection, CSF processing and assay work need to be conducted simultaneously and therefore require a team of research staff.

One of the unanswered questions at the end of this study was whether any of the participants went on to experience persistent post-operative pain. It is theorectically possible to contact participants who took part in this study to determine whether they had residual pain and if so how long it lasted for. However, the value of doing this must be weighed against the ethics of returning to a participant who was assured at the time of the research that the study was a one-off and did not involve follow up. In this case, the small sample size of the present study, the amount of missing data, and the amount of time that has passed since samples were collected makes the potential benefit of tracing the individuals minimal. It is important that a future study includes patient follow up of 2 years to include quantitative sensory testing, and thorough examination of residual joint pain to allow full interpretation of the collected data.

This small sample size of this study is not unusual in clinical CSF work and it is suspected that other studies have similar challenges in recruiting sufficient numbers of participants to enable detection of relatively small differences in highly variable substances, the nature of which is involvement in myriad biological processes. The data itself is therefore precious and deserves closer scrutiny. One of the questions that were not addressed in this study

was whether OA pain is affected in anyway by the ratio of pro- to anti-inflammatory cytokines. This would be interesting to pursue in a post-hoc analysis. Similarly there are bidirectional influences on the levels of some of the analytes in this study e.g. GABA is under stimulatory and inhibitory conditions, and it would be interesting to investigate these influences using linear regression techniques.

Large sample sizes are necessary to investigate statistically the idea of interdependence between the cytokines. Some of the findings in the present study suggest that cytokines like IFNγ and IL-12 may have different roles depending on the local conditions, perhaps dependent on the levels of other cytokines. It is therefore proposed that following the conclusion of this study the idea of further statistical analysis will be discussed with expert colleagues. Although the heterogeneity of study populations suggests it might be difficult, it is proposed that the feasibility of a meta-analysis is discussed.

7.6 Conclusions

This study has added important new information to the knowledge we have of the spinal transmission of OA pain. Up until this point this knowledge has been accumulated, in the main, from animal studies using various models to represent inflammatory conditions including osteoarthritis. Very few clinical studies have reported amino acid or cytokine data for OA pain, and none has reported both in the same study. Despite the very likely relationships between pain, inflammation and depression, no clinical studies until the present study have sought to control for mood as a confounding variable.

The findings of this study support the involvement of central sensitisation in OA pain. However, while increased levels of TNFα and serine fit in with what is currently known about sensitized pain transmission in the dorsal horn other results from this study do not, and suggest that there is further work to be done. The role of IL-12 is interesting as it may be functioning in an anti-inflammatory capacity in OA pain. The potential for this and other modulators to have dual roles, and the known redundancy within the cytokine network suggests closer investigation of single and groups of pain-related mediators is appropriate.

The data generated in the study was not able to detect a clear and clinically relevant difference between those OA participants who suffer from pain at rest and those who do not. There is some interest in this group of people because of the increased risk they have of developing persistent pain following arthroplasty. This risk is very likely to relate to changes in the central mediation of pain and although some work to date has demonstrated these changes in relation to sensory thresholds there has been very limited exploration of changes in the dorsal horn that might provide explanation and potentially modifiable targets for novel therapies.

This study also supports previous findings that pain and depression are co-morbid for a number of people with OA. The findings of this study are in agreement with the current understanding of the relationships between peripheral inflammation, pain and depression in as much as the findings provide support for the role of pro-inflammatory cytokines and the serotonin pathway. However, there are anomalies in this data too; if pain has a direct influence over the development of depression irrespective of inflammation, one might

expect that serine would be one of the amino acids to demonstrate an association with depression or psychological distress. The fact that this amino acid does not have a linear relationship with HADS-T in this study but does have an association with OA, stimulates questions about the relationship between the mediation of pain and depression.

Psychological distress was identified in this study as a key variable that has an association with the pain of OA and is likely to influence changes in the concentrations of some of the amino acid and cytokine variables of interest.

The finding that OA pain involves central sensitisation is supported by the data in this study but some results are anomalous with this. Other results are more tentative and need to be treated with some caution, but they nevertheless contribute importantly to our knowledge of OA pain and contribute to the development of future studies. The study of OA is important; it affects a great many people and carries a significant personal and economic cost which is likely to increase as the demographic of the population continues to change. The work in this study provides important information about OA pain and provides a template and foundation for future work.

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Appendix 1 Ethical approval

Not available in the digital version of this thesis

Appendix 2 Theatre timetable

	Theatre 1	Theatre 2	Theatre 4
Mon am	Elective orthopaedic list Surgeon 1		Urology Surgeon 2 or 3
Mon pm			
Tues am	Elective orthopaedic list Surgeon 5	Elective orthopaedic list Surgeon 6	
Tues pm		Elective orthopaedic list Surgeon 7	
Weds am	Elective orthopaedic list Surgeon 9	Elective orthopaedic list Surgeon 10	Urology Surgeon 11
Weds pm			
Thurs am	Elective orthopaedic list Surgeon 14	Elective orthopaedic list Surgeon 15	
Thurs pm		Elective orthopaedic list Surgeon 16	
Fri am	Elective orthopaedic list Surgeon 15	Elective orthopaedic list Surgeon 18	
Fri pm	Elective orthopaedic list Alt wks – Surgeon 16	Elective orthopaedic list Surgeon 14	

Appendix 3 Patient Information

Title of Project: Neurotransmitters in the Spinal Cord

EXPLANATION

We would like to invite you to take part in a research study. This study is being funded by an academic grant from Pfizer. Before you decide if you would like to be involved it is important to understand why the research is being done and what it will involve. Please take as much time as you need to read the following information carefully and discuss it with friends, relatives and your doctor if you wish. We will write to your GP to explain the study should you decide to take part. Please ask us if there is anything that is not clear or if you would like more information. We appreciate you thinking about being in our study.

What is the purpose of the study?

Pain is a common experience but it is not well understood. The study is designed to help us understand what happens inside the body when a person experiences pain.

Neurotransmitters are chemicals that are involved in the transmission of pain signals. There are lots of different neurotransmitters involved in this process. The amounts of neurotransmitters are altered by pain and pain relieving drugs. In this study we will examine the amounts of neurotransmitters in the spinal cord fluid and relate it to your pain. We hope to find out if the amount of each neurotransmitter is affected by the type of pain you are experiencing.

Why have I been chosen?

You are being asked because you are experiencing one of the types of pain we are interested in studying and because you are having an operation that often involves the use of a spinal anaesthetic.

The neurotransmitters we are interested in are found in the spinal fluid. The operation you have come into hospital for is being performed under spinal anaesthetic. The decision for a spinal anaesthetic will be made by the anaesthetist after discussion with you. A spinal anaesthetic involves placing a needle into your back and injecting local anaesthetic into the cerebrospinal fluid (CSF). Correct placement of the needle is important. To ensure the needle is placed correctly the anaesthetist will check that spinal fluid flows freely from the needle. We would like to take this opportunity to collect a 2 ml volume of that spinal fluid. The collection of such a small volume does not harm you in any way. The anaesthetist will then inject the local anaesthetic as normal and this will provide the anaesthetic for your operation.

There is no difference in the way the anaesthetic procedure will be performed whether you take part in the study or not.

Collection of CSF from many selected patients will enable us to identify how neurotransmitter levels vary with differing types of pain.

Do I have to take part?

No, it is up to you to decide whether or not to take part. If you do decide to take part you will be asked to sign a consent form and we will ask you to keep this information sheet to refer to. If you decide to take part you are still free to withdraw from the study at any time without giving a reason. This will not affect your medical care.

What will happen to me if I take part?

You will have an opportunity to discuss the study with the research nurse. If you decide to take part she will ask you to sign a consent form. This form is used to show that you have been given information about the study and that you have understood that information and agree to take part.

The research nurse will then gather some information about your medical history from your notes. She will then visit you at a convenient time before your intervention to ask about your pain. If the pain you have is due to a type of neuropathy the research nurse will perform two tests on you in addition to asking you the questions. The tests are performed on an area of skin where you have the pain and on an area of skin from the opposite side of the body where you do not have any pain. The tests do not take long to do.

You will be visited by the anaesthetist before your intervention and will be asked to consent to the intervention and to the use of a spinal anaesthetic.

The anaesthetist will explain the procedure as it progresses. When he or she has entered the appropriate space with the spinal needle a 2 ml specimen of spinal fluid will be collected, the local anaesthetic will then be given. The research nurse will take the sample away to prepare and analyse.

What do I have to do?

This study will require you to give a sample of CSF and answer a few straightforward questions about your pain and about how you feel.

If I decide not to take part will my treatment be affected?

No. If you decide not to take part this will not affect your medical care. There are no penalties for deciding not to take part.

What are the risks of taking part?

There are risks associated with having a spinal anaesthetic. These are explained below. You can discuss these with the anaesthetist before the intervention takes place. The anaesthetic staff are trained to prevent or minimise the risks associated with providing anaesthesia using a spinal technique.

Risks: temporary fall in blood pressure, temporary headache, temporary change in heart rate or rhythm, urine retention, nausea or vomiting.

The total volume of cerebrospinal fluid in your head and bathing the spinal cord is approximately 130 millilitres. You produce 500 millilitres of this fluid each day. We will take a sample of 2 millilitres.

You are covered by the normal arrangements in the Trust whereby you are entitled to complain about any aspect of your care. There is no specific indemnity identified for the collection of the CSF.

What are the benefits of taking part?

You will receive no payment for taking part in this study. We hope the study will help in our understanding of pain and this may lead to developments of better pain control techniques in the future.

What will happen to the sample of cerebrospinal fluid (CSF)?

The sample of fluid will be analysed to determine the levels of neurotransmitters. The fluid (CSF) will not be used for any other purpose and will be destroyed at the end of the analysis.

Will my taking part in this study be kept confidential?

Records obtained while you take part in this study will be kept confidential. These records will need to be seen by the research team at Heartlands and Solihull Hospital as well as the research team at the University of Birmingham and their collaborators. By signing the consent form you agree to the information that is collected related to the study being used by this group of people.

What will happen to the results of the study?

The research will be reported in medical, nursing and scientific journals. It will also be discussed at conferences. You will not be identified in any discussions or publications related to the research.

Data protection act: What use will be made of data collected from this study?

Personal data: e.g. date of birth. This data will be collected and processed. This data may be shared within the research team but not in a form that would lead to your identification.

The research team will take steps to ensure that your personal data is protected.

By agreeing to take part in this study you agree to the transfer of your personal data to the research team.

Contact for further information.

Research Nurse: Amelia Williamson

0121 424 4329

If you have any problems with the conduct of this study you can telephone the secretary of the ethics committee who has considered this application, on 0121 424 0594, who will arrange for your worries to be investigated.

Appendix 4 Consent form

Title of study: Neurotransmitters in spinal cord study.

Patient name _____

Address _____

Date of Birth _____

Hospital number _____

Please read each statement carefully. If you agree with the statement please initial the appropriate line.

I have read the patient information sheet about this study _____

I have had an opportunity to discuss the study _____

I agree to take part in the study _____

I understand that I am free to withdraw at any time without penalty _____

I agree to a 2 ml sample of CSF being taken _____

I agree to the sample of CSF being analysed

to determine the levels of neurotransmitters _____

I agree to my GP being told about my participation _____

Name of patient	Signature of patient	Date
_____	_____	_____
Name of research nurse	Signature of research nurse	Date
_____	_____	_____

Appendix 5 Data collection tool

<table>
<tr><td rowspan="3">Patient Label</td><td>Patient code</td></tr>
<tr><td>Site</td></tr>
<tr><td>Date</td></tr>
<tr><td><u>Exclusion criteria</u>

Pain other than OA pain.
Neurodegenerative disorder
Anorexia
Psychiatric disorder
Head trauma or CVA
Inflammatory disorder
Active cancer within past 6 months
Pregnancy
Current neuro investigations
Immunosuppressants.
Steroids
<u>Inclusion criteria</u>
Age > 18 years
Able to consent
English language speaker</td><td><u>Procedure (tick)</u>

Joint replacement or revision

TURP/TURB

Other surgical procedure (given name below)

Lumbar puncture (diagnostic)

Lumbar puncture (therapeutic)

Other

_____</td></tr>
</table>

Pain Group	Ethnic group
Neuropathic	A White
Osteoarthritis	A1 British
Trauma	A2 Irish
Mixed OA and neuropathic	A3 Any other white
Mixed trauma and OA	B Mixed
Control (no pain)	B1 Mixed white/black Caribbean
Other (please state)	B2 Mixed white/black African
	B3 Mixed white/Asian
	B4 Any other mixed
	C Asian/Asian British
	C1 Indian
	C2 Pakistani
	C3 Bangladeshi
	C4 Any other Asian
	D Black/black British
	D1 Caribbean D2 African D3 Other
	E Chinese/other ethnic group
	E1 Chinese
	E2 Any other ethnic group

Medication	Daily dose	Start date	Stop date	Changes to dose in last 6 months

Past Medical History

Pain Rating Scales

Pain at REST

0	1	2	3	4	5	6	7	8	9	10

Pain on MOVEMENT

0	1	2	3	4	5	6	7	8	9	10

Average daily pain in last 3 months

0	1	2	3	4	5	6	7	8	9	10

Average daily pain in last 12 months

0	1	2	3	4	5	6	7	8	9	10

Primary pain Duration (trauma pain first)

days	months	years

Chronic pain duration

days	months	years

Site of pain

1	2	3

Hospital Anxiety and Depression Score

Clinicians know that emotions play an important part in most illnesses. This questionnaire is designed to help us measure feelings. Read each item and tick to box next to the response that comes closest to how you have been feeling in the last week. Don't take too long over your replies, your immediate reaction to each item will probably be more accurate that a long thought-out response.

I feel tense or 'wound-up'

Most of the time	3	☐
A lot of the time	2	☐
From time to time, occasionally	1	☐
Not at all	0	☐

I feel as if I am slowed down

Nearly all the time	3	☐
Very often	2	☐
Sometimes	1	☐
Not at all	0	☐

I still enjoy the things I used to enjoy

Definitely as much	0	☐
Not quite so much	1	☐
Only a little	2	☐
Hardly at all	3	☐

I get a sort of frightened feeling like 'butterflies' in the stomach

Not at all	0	☐
Only occasionally	1	☐
Quite often	2	☐
Very often	3	☐

I get a sort of frightened feeling as if something awful is about to happen

Very definitely and quite badly	3	☐
Yes, but not too badly	2	☐
A little but it doesn't worry me	1	☐
Not at all	0	☐

I have lost interest in my appearance

Definitely	3	☐
I don't take as much care as I should	2	☐
I may not take quite so much care	1	☐
I take just as much care as ever	0	☐

I can laugh and see the funny side of things

As much as I always could	0	☐
Not quite so much now	1	☐
Definitely not so much now	2	☐
Not at all	3	☐

I feel restless as if I have to be on the move

Very much indeed	3	☐
Quite a lot	2	☐
Not very much	1	☐
Not at all	0	☐

Worrying thoughts go through my mind

A great deal of the time	3	☐
A lot of the time	2	☐
From time to time but not too often	1	☐
Only occasionally	0	☐

I look forward with enjoyment to things

As much as I ever did	0	☐
Rather less than I used to	1	☐
Definitely less than I used to	2	☐
Hardly at all	3	☐

I feel cheerful

Not at all	3	☐
Not often	2	☐
Sometimes	1	☐
Most of the time	0	☐

I get sudden feelings of panic

Very often indeed	3	☐
Quite often	2	☐
Not very often	1	☐
Not at all	0	☐

I can sit at ease and feel relaxed

Definitely	0	☐
Usually	1	☐
Not often	2	☐
Not at all	3	☐

I can enjoy a good book or radio or TV programme

Often	0	☐
Sometimes	1	☐
Not often	2	☐
Seldom	3	☐

Score A (1+3+5+7+9+11+13) ☐

Score D (2+4+6+8+10+12+14) ☐

Michigan Neuropathy Screening Instrument Instructions

Instructions for all physical assessments: For all assessments, the foot should be warm (>30^{o}C)

Foot inspection

The feet are inspected for evidence of excessively dry skin, callous formation, fissures, frank ulceration or deformities. Deformities include flat feet, hammer toes, overlapping toes, halux valgus, joint subluxation, prominent metatarsal heads, medial convexity (Charcot foot) and amputation.

Vibration sense

Vibration sense should be performed with the great toe supported. Vibration sense will be tested bilaterally using a 128Hz tuning fork placed over the dorsum of the great toe on the bony prominence of the DIP joint. Patients, whose eyes are closed, will be asked to indicate when they can no longer sense the vibration from the vibrating tuning fork.

In general, the examiner should be able to feel vibration from the hand-held tuning fork for 5 seconds longer on his distal forefinger than the normal subject can at the great toe (e.g. the examiners DIP joint of the first finger versus the patient's toe). If the examiner feels vibration for 10 or more seconds on his or her finger, then vibration is considered decreased. A trial should be given when the tuning fork is not vibrating to be certain that the patient is responding to vibration and not pressure or some other clue. Vibration is scored as:
Present if the examiner senses the vibration on his or her finger for <10 seconds
Reduced if sensed for >10 seconds
Absent (no vibration detected)

Muscle stretch reflexes

The ankle reflexes will be examined using an appropriate reflex hammer (e.g. Trommer or Queen Square). The ankle reflexes should be elicited in the sitting position with the foot dependent and the patient relaxed. For the reflex, the foot should be passively positioned and the foot dorsiflexed slightly to obtain optimal stretch of the muscle. The Achilles tendon should be percussed directly. If the reflex is obtained it is graded as present. If the reflex is absent, the patient is asked to perform the Jendrassic manoeuvre (i.e. hooked fingers together pulling). Reflexes elicited with the Jendrassic manoeuvre alone are designated 'present with reinforcement'. If the reflex is absent, even in the face of the Jendrassic manoeuvre, the reflex is considered absent.

Michigan Neuropathy Screening Instrument

Appearance of feet		LEFT		RIGHT	
Normal		YES 0	NO 1	YES 0	NO 1
If not check all that apply					
	Deformities				
	Dry skin, callus				
	Infection				
	Fissure				
	Other (specify)				

Ulceration	Absent 0	Present 1	Absent 0	Present 1

Ankle reflexes					
RIGHT			LEFT		
Present 0	Present/ reinforcement 0.5	Absent 1	Present 0	Present/ reinforcement 0.5	Absent 1

Vibration perception at great toe (128Hz)					
Present 0	Decreased 0.5	Absent 1	Present 0	Decreased 0.5	Absent 1

Score _____/8

Appendix 6 Studies used in literature review for inclusion/exclusion criteria

Author	*Year*	*Focus*	*Control groups*	*Molecules of interest*
Abbot	*1982*	*Parkinson's*	*Diagnostic lumbar puncture normal*	*GABA*
Alexander	*2005*	*CRPS*	*Pain from spondylolisthesis, radiculopathy, peripheral neuropathy, also some with no pain*	*IL1B, IL6, TNFa*
Alexander	*2007*	*CRPS*	*Radiculopathy (13), ALS (9), peripheral neuropathy (3), spondylolisthesis (4), normal pressure hydrocephalus with shunt (2)*	*IL4, IL10, IL6, IL8, MCP1, GFAP, nitrate, nitrite, glutamate*
Altemus	*2004*	*Pregnancy*	*Control (normal)*	*MHPG, HVA, 5HIAA, GABA, Glu*
Araki	*1986*	*Parkinson's*		
Araki	*1988*	*Epilepsy*	*Headache*	
Asahara	*1996*	*Lumbar spondylosis*		*nitrate,. Nitrite*
Backonja	*2008*	*Post-traumatic neuralgia with features of CRPS (8) or painful diabetic neuropathy (6)*	*Healthy volunteers*	*IL1B, IL6, TNFa, sTNFr, IL1ra, IL8, IL10, SP*
Basun	*1990*	*Alzheimer's*	*Healthy volunteers*	
Belleroche	*1984*	*Motor neurone disease*	*Diagnostic myelography*	
Ben Menarchem	*1989*	*Healthy volunteers*		*GABA, homocarnosine, HVA, 5-HIAA,*
Berretini	*1986*	*Manic depression*		
Berretini	*1982*	*Bipolar affective disorder*	*Healthy controls*	
Berretini	*1983*	*Unipolar and biploar disorder*		
Bertislsson	*1982*	*Depressed*	*Healthy controls*	
Blennow	*1993*	*Neuropsychiatric patients*	*Healthy controls*	
Blin	*1994*	*ALS*	*Neurological disorders but not degenerative*	
Blin	*1994*	*ALS*	*Neurological disorders (not neurodegenerative)*	*asp, asgn, glu, ser, gln, tau, ala, tryp, val, phe, iso, leu*

Bo	*2009*	*Migraine, cervicogenic and tension headache*	*Neurological diagnosis normal and no pain*	*IL1b, IL1ra, IL4, IL10, TNFa, MCP1, TGFb1*
Bowers	*1980*	*Psychosis*	*Neurological controls*	
Bridges	*1976*	*Depression and anxiety*		
Brisby	*2002*	*Disc herniation and sciatica*		*IL1b, IL6, IL8, IFNg, TNFa,*
Buvanendran	*2006*	*OA for elective joint replacement, either cox inhibitor on day of surgery (10), or cox inhibitor on 4 days leading up to surgery (10)*	*Placebo*	*PGE2, IL1b, IL6, IL8, TNFa*
Carrieri	*1998*	*Multiple Sclerosis*	*Non inflammatory neurological diseases*	
Castillo	*1995*	*Acute CVA headache*	*Acute CVA no headache*	*Glu*
Castillo	*1995*	*CVA*		
Crawford	*1987*	*Newly diagnosed Untreated Epilepsy, chronic drug resistant epilepsy*	*control*	
Csernansky	*1996*	*Alzheimers*	*Control*	
de Jong	*1984*	*Untreated Parkinsons*	*Treated Parkinsons*	
Degrell	*1989*	*Dementia*	*Young neurotics*	
Devinsky	*1993*	*Epilepsy*		
Enna	*1977*	*Huntingtons or Parkinsons*	*Control*	
Espey	*2002*	*HIV dementia*		
Frye	*2006*	*Refractory affective disorder*	*Recruited from the community controls*	
Gallai	*2003*	*Chronic daily headache*	*diagnostic lumbar puncture - no CNS disease, no neurodegenerative disease*	*Glutamate, nitrate, cGMP, SP, CGRP, NKA*
Garseth	*2001*	*Polyradiculopathy (Guillain Barre) or active MS*	*Normal controls*	

Garseth	*2002*	*Low back pain or sciatica*	*Neurological diagnosis with NAD (18), elective orthopaedic surgery (13) - no pain*	*Glucose, lactate, pyruvate, citrate, glutamine, alanine, creatine, creatinine, inositol, formate, acetate*
gattaz	*1986*	*Schizophrenia before Haloperidol*	*Schizo after haloperidol*	
Geracioti	*1997*	*Depression*		
Gerner	*1981*	*Depression, anorexia, mania*	*Normal*	
Gerner	*1984*	*Depression, mania, schiziphrenia*	*Normal*	
Gjerris	*1987*	*Depression, dementia*	*controls*	
Gjessing	*1972*	*Neurological investigation and disorders*		*tau, asp, thre, ser, aspn, glu, gln, gly, ala, cit, val, leu, emth, iso, tyr, phe, tryp, orn, lys, his, homo, arg, etc.*
Gold	*1980*	*Depression, psychosis*	*Neurologic controls*	
Hagenfeldt	*1988*	*Healthy twins*		
Hagenfeldt	*1984*	*Healthy twins comparison with plasma*		
Hashimoto	*2005*	*Schizophrenia*	*Normal control*	*D and L Serine*
Heyes	*1991*	*HIV*		
Honig	*1988*	*Depression*		*Tau, aspn, thre, ser, glu, gln, gly. Ala, val, meth, iso, leu, tyr, phe, his, lys, arg, GABA*
Hsu	*2001*	*Labour pain Csection*	*No pain Csection*	
Kalviainen	*1993*	*Epilepsy*	*Treated epilepsy*	
Kasa	*1982*	*Depressive disorders*	*controls*	
Kikuchi	*1999*	*Post-herpetic neuralgia*		*IL1B, IL8, IL6, TNFa*
Kimura	*1999*	*Degenerative lumbar disease, fracture, appendicitis*	*Healthy volunteers (6), inguinal herniation (10), removal of pins/plate (5), prolapsed uterus (1), hallux valgus (1) , soft tissue tumour to leg (1) but all were pain-free neurologically healthy.*	*nitrate, nitrite*
Klivenyi	*1997*	*MS*	*Low back pain*	

Kotani	*2008*	*Post-herpetic neuralgia*		*IL-8*
Kuroda	*1982*	*Psychiatric and neurological diseases*		
Lakke	*1986*	*Head injury (41), various neurodegenerative disorders (22), Parkinsons (31)*	*disc herniation (38), caudography (8), spondylolisthesis (1), RhA (2)*	*Alanine, glycine, Abu, valine, leucine, Ile, Phenylalanine, Tyrosine, Serine, Histidine, Threonine, Methionine, Arginine, Ornithine, Lysine, Citrulline, Taurine, Cystine*
Larson	*2000*	*Fibromyalgia (primary (16), secondary = with another disorder such as RhA (9)) or musculoskeletal pain (10)*	*Healthy volunteers*	*arginine, asparagine, aspartate, citrulline, glutamine, glutamate, glycine, taurine*
Levine	*1999*	*Acute depression*		
Levine	*2000*	*Acute depression*	*Neurological diagnosis NAD*	*given as mole percent*
Ludwig	*2008*	*Painful polyneuropathy*	*Painless polyneuropathy*	*TNFa, IL6*
Ludwig	*2008*	*Painful polyneuropathy*	*Painless polyneuropathy*	
Maier	*2005*	*Healthy volunteers*		*IL6, IL8, IL10, sICAM, sTNFr, sE-selectin*
Manyam	*1988*	*Parkinson's untreated or untreated*	*Controls*	
Martinez	*1993*	*Migraine*	*Stress*	*Glutamate, aspartate.*
Martinez	*1993*	*Alzheimer's*		
McGale	*1977*	*Headache or back pain*		*Serine, taurine, Threonine, asparagine, glutamine, valine, glutamate, glycine, leucine, tyrosine, phenylalanine, lysine, histidine, arginine*
Meier	*1988*	*ALS*		
Mertens	*2000*	*Neuropathic pain*	*Spasticity*	*asparate, glutamate, GABA, glycine, alanine, threonine,*

Molina	*1997*	*Parkinson's*	*controls*	
Molina	*2005*	*Dementia*	*Healthy normal controls*	
Munts	*2008*	*CRPS with dystonia - women only*	*Controls (13 women, 16 men), spinal anaesthesia for TURP, total knee replacement, femeropopliteal bypass, vulvectomy and general surgery e.g. lipoma excision*	*IL1b, IL6, IP10, RANTES, C3, Clq, MBL, sICAM1, ET1, NO, Lactoferrin*
Nisijima	*1995*	*Neuroleptic malignant syndrome*	*normal age matched controls*	
Olofsson	*1997*	*Labour pain (16); Elective C Section (20); Post-menopausal*	*Healthy volunteers or post-menopausal gynae surgery (not combined for analysis)*	*nitrate, nitrite, aspartate, glutamate,*
Peres	*2004*	*Migraine with fibromyalgia (12) or without (8)*	*diagnostic lumbar puncture - no CNS disease, no neurodegenerative disease*	*Glutamate*
Perry	*1982*	*Huntington's*	*Neurological diagnosis NAD but some disc problems*	
Perry	*1990*	*ALS*	*Neurological and psychiatric disorders*	*taurine, aspartate, threonine, serine, glutamate, citrulline, isoleucine, leucine, tyrosine, arginine, glycine etc.*
Pomara	*1989*	*Spouses (F) of Alzheimer's patients*	*Spouses of normal men*	
Roy	*1988*	*Depression*	*controls*	
Roy	*1990*	*Abstinent Alcoholics*	*controls*	
Roy	*1991*	*Depression*	*controls*	
Rozen	*2007*	*Daily persistent headache*	*Chronic migraine*	*TNFa*
Sarchielli	*2007*	*Chronic migraine. Fibromyalgia*	*Age and sex matched*	*NGF, BDNF, glutamate*

Shaw	*1995*	*Motor neurone disease*	*disc herniation (11), cervical spondylosis (10) stenosis (2) radiculopathy (1) back pain (3) spastic paraparesis (1) tumours (1) syringomyelia (1) unstable bladder (1) ischaemic cord disorder (3)*	*asparate, threonine, serine, asparagine, glutamate, glutamine, glycine, citrulline, alanine, valine, methionine, AABA, isoleucine, leucine, tyrosine, phenylalanine, histidine, ornithine, lysine, arginine, cystine, tryptophan*
Stahel	*1998*	*Head trauma*		
Stoeck	*2006*	*CJD*		
Stover	*1999*	*Brain injury*	*Back pain*	
Van Sande	*1970*			
Van Sande	*1970*	*Neurological disorders*	*Normal*	*tau, asp, thre, ser, glu, leu, tyr, phe, orn,, lys, his, homo, arg, cit, gly. Ala, val, cys, met, ile*
Viera	*2006*	*Migraine with depression*	*Migraine without depression*	*GABA*
Welch	*1975*	*Migraine*	*Tension headache*	*GABA, Leu, val, gly*
Yao	*2003*	*Schizophrenia*		
Yeager	*1999*	*Post joint replacement surgery*	*Healthy volunteers*	*IL6, IL10*
Zimmer	*1980*	*Psychiatric patients before treatment*	*After medication treatment*	

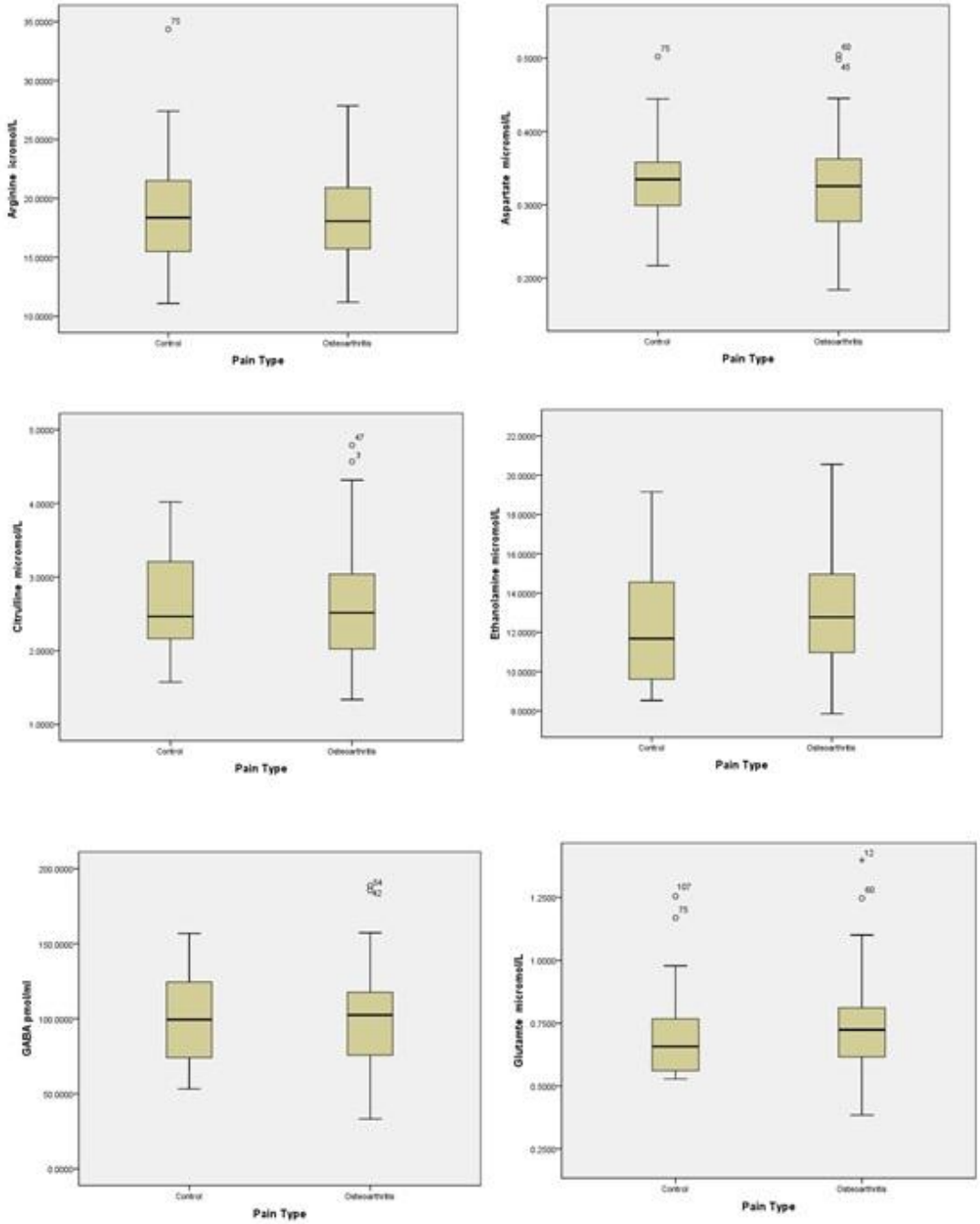
Appendix 7 Healthy normal control CSF amino acid concentrations from human studies

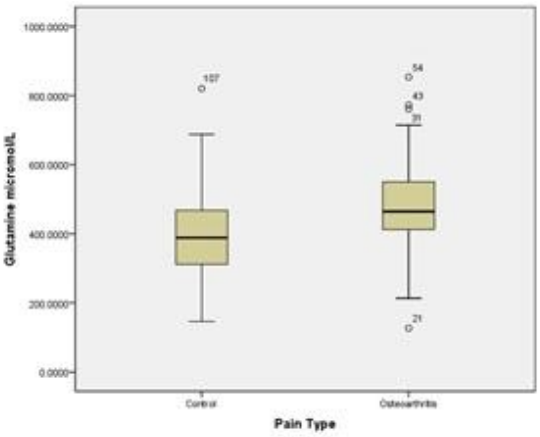
Substrate	Study	Number of control participants	Mean or median pg mL-1 ± SD
Glutamate	Frye et al 2006	16	0.59 ± 0.16
	Gallai et al 2003	20	0.31 ± 0.17
	Garseth et al 2000	25	0.55 ± 0.11
	Larson et al 2000	18	0.85 ± 0.25
	Mertens et al 2000	5	0.18 ±0.13
	Van Sande et al 1976	13	2.16 ± 1.96
Aspartate	Frye et al 2006	16	0.21 ± 0.19
	Larson et al 2000	18	3.71 ± 0.55
	Mertens et al 2000	5	0.05 ± 0.04
	Van Sande et al 1976	13	0.38 ± 0.36
Glutamine	Garseth et al 2001	25	68.3 ± 8
	Larson et al 2000	18	72.1 ± 23.8
	Van Sande et al 1976	13	66.2 ± 17.5
Citrulline	Larson et al 2000	18	0.26 ± 0.19
	Van Sande et al 1976	13	0.06 ± 0.02
Serine	Garseth et al 2001	25	2.49 ± 0.5
GABA	Abbott et al 1982	15	0.027 ± 0.012
	Altemus et al 2004	22	0.005 ± 0.0005
	Berretini et al 1982	41	0.018 ± 0.005
	Mertens et al 2000	5	0.021 ± 0.01
Glycine	Frye et al 2006	16	2.0 ± 1.07
	Garseth et al 2001	25	0.77 ± 0.17
	Larson et al 2000	18	0.77 ± 0.38
	Mertens et al 2000	5	0.19 ± 0.04
	Van Sande et al 1976	13	0.64 ± 0.14
Isoleucine	Garseth et al 2001	25	0.62 ± 0.21
	Van Sande et al 1976	13	0.66 ± 0.12
Leucine	Garseth et al 2001	25	1.52 ± 0.45
	Van Sande et al 1976	13	1.52 ± 0.31
Valine	Garseth et al 2001	25	1.67 ± 0.06
	Van Sande et al 1976	13	1.67 ± 0.47
Tyrosine	Garseth et al 2001	25	1.9 ± 0.71
	Van Sande et al 1976	13	1.43 ± 0.42

Appendix 8 Healthy normal control CSF cytokine concentrations from human studies

Substrate	Study	Number of control participants	Mean or median pg mL^{-1} ± SD	Range
IL-1β	Alexander et al 2005	16	0.02 ± 0.01	
	Backonja et al 2008	8	0.85 ± 0.28	
	Levine et al 1999	10	0.14 ± 0.7	
	Tarkowski et al 1999	59	0.2 ± 0.5	
IL-6	Alexander et al 2005	16	1.32 ± 0.53	
	Backonja et al 2008	8	0.67 ±1.1	
	Levine et al 1999	10	2.36 ± 1.3	
	Maier et al 2005	113	6	1 to 34
	Stoeck et al 2006	111	1.6	0.1 to 18
	Tarkowski et al 1999	59	2 ± 7	
IL-8	Backonja et al 2008	8	10.44 ± 8.17	
	Kotani et al 2004	50	18	17 to 24
	Maier et al 2005	113	6	1 to 23
	Stoeck et al 2006	111	12.6	4.2 to 19.9
IL-10	Backonja et al 2008	8	4.97 ± 1.1	
	Maier et al 2005	113	0.9	0 to 39
IL-12	Backonja et al 2008	8	1.68 ± 0.39	
	Stahel et al 1998	15	0.57 ±0.37	
TNFα	Alexander et al 2005	16	0.11 ± 0.04	
	Backonja et al 2008	8	< 0.1 ± 0.28	
	Levine et al 1999	10	0.47 ±0.62	

Appendix 9 Box plots showing outliers in control and OA groups for amino acids




Glutamine micromol/L
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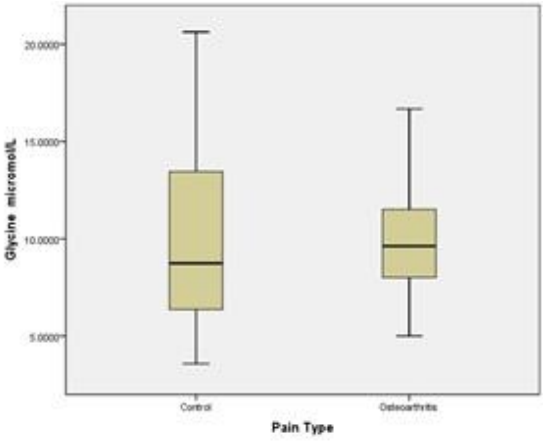
600.0000
400.0000
200.0000
0.0000
157
54
43
21
Control
Osteoarthritis
Pain Type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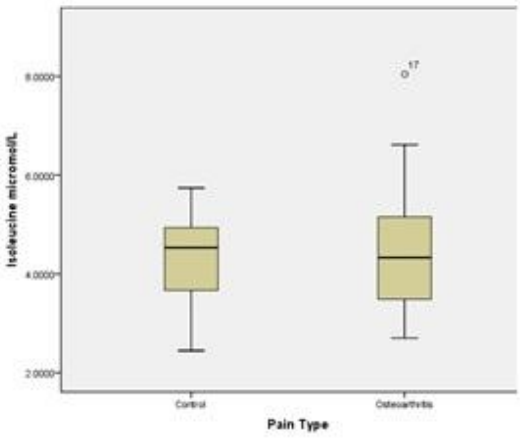
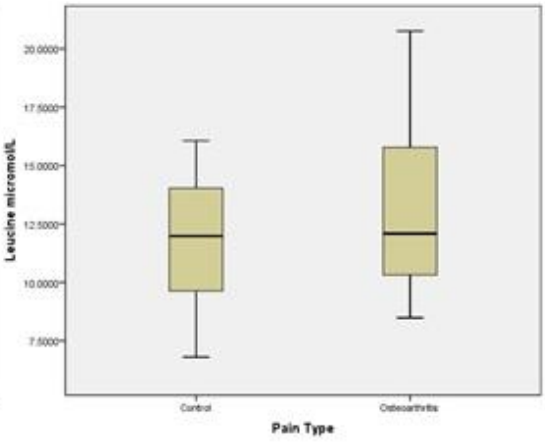
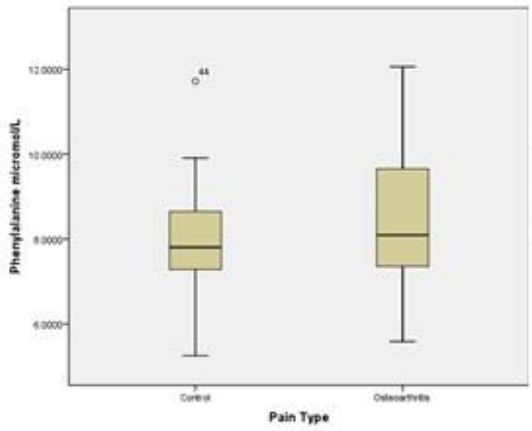
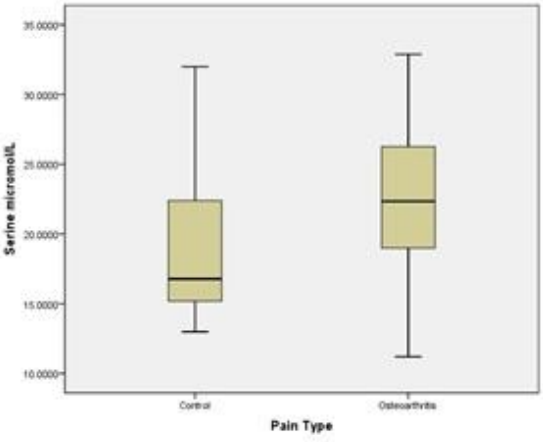
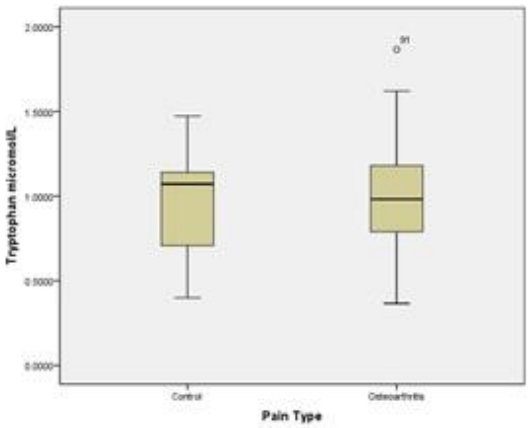
15.0000
10.0000
5.0000
Control
Osteoarthritis
Pain Type

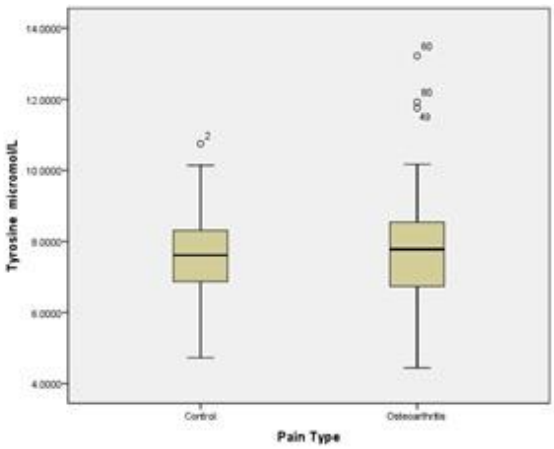

Isoleucine micromol/L
8.0000
6.0000
4.0000
2.0000
17
Control
Osteoarthritis
Pain Type

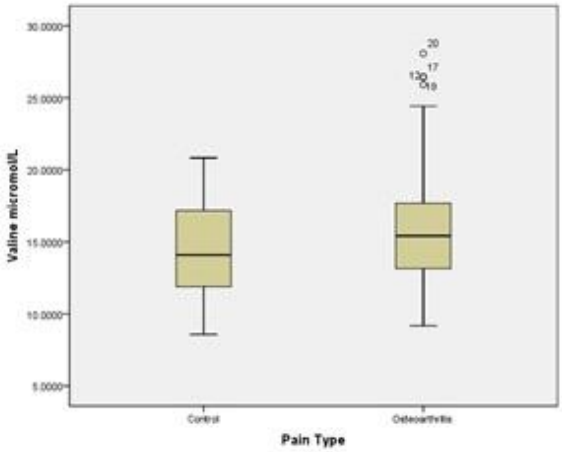

Leucine micromol/L
20.0000
17.5000
15.0000
12.5000
10.0000
7.5000
Control
Osteoarthritis
Pain Type


Phenylalanine micromol/L
12.0000
10.0000
8.0000
6.0000
44
Control
Osteoarthritis
Pain Type


Serine micromol/L
35.0000
30.0000
25.0000
20.0000
15.0000
10.0000
Control
Osteoarthritis
Pain Type



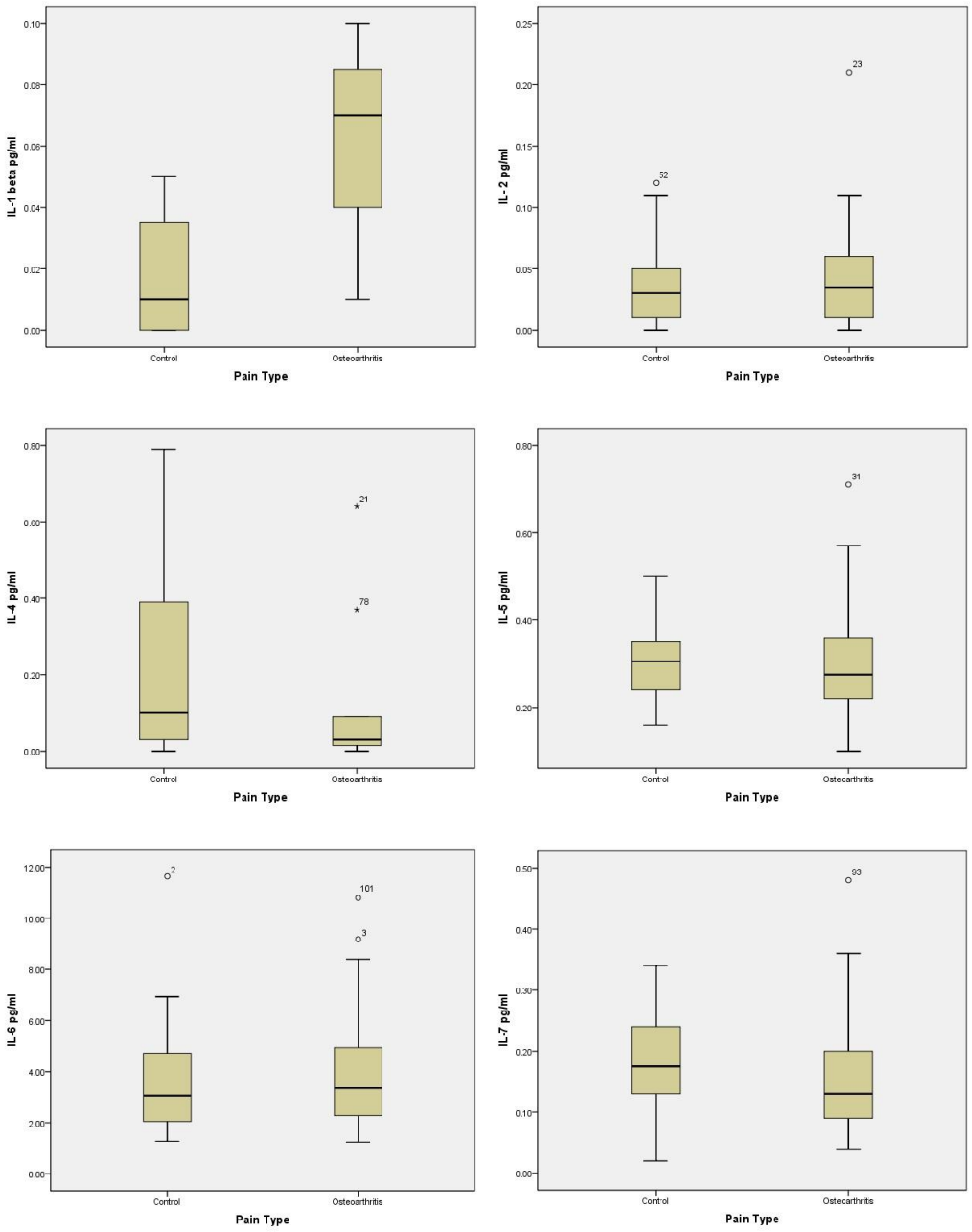


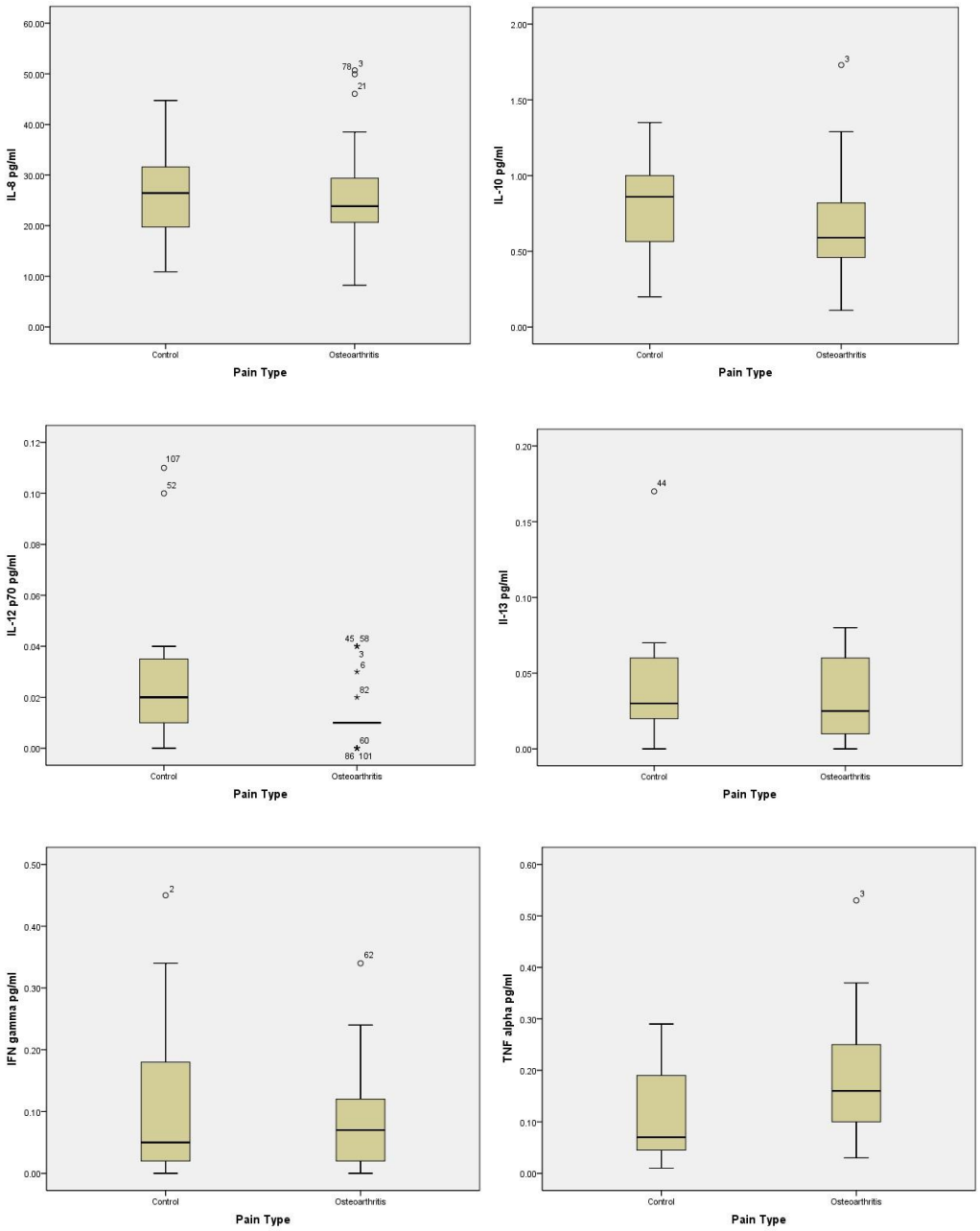


Outliers

	Control	OA
Arginine	75,	55
Aspartate	75, 25, 105	60,45
Citrulline	74	48
GABA		54,42
Glutamate	107	12*, 48*, 56, 59, 60
Glutamine	107	54, 63, 43, 40, 31, 21, 12, 11
Isoleucine		17, 59
Lycine		38, 12, 22, 32
Phenylalanine	44	
Tryptophan		91
Tyrosine	2	60, 80, 49
Valine		20, 17, 12, 19

Appendix 10 Boxplots showing outliers of cytokines in control and OA groups.

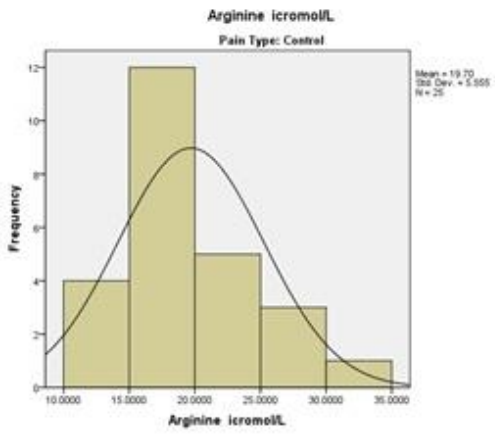


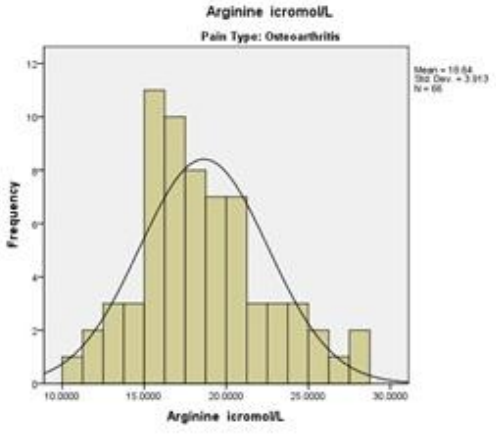

IL-8 pg/ml
IL-10 pg/ml
IL-12 p70 pg/ml
Il-13 pg/ml
IFN gamma pg/ml
TNF alpha pg/ml
Control
Osteoarthritis
Pain Type

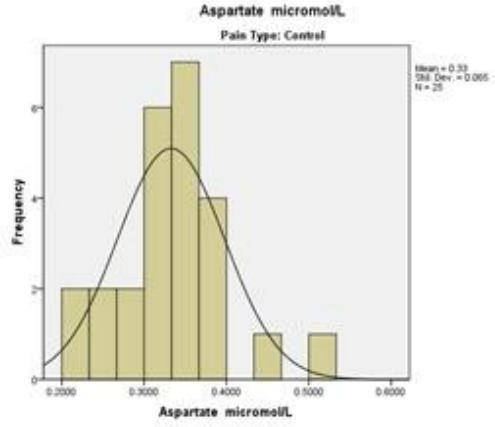
Outliers for cytokine assay

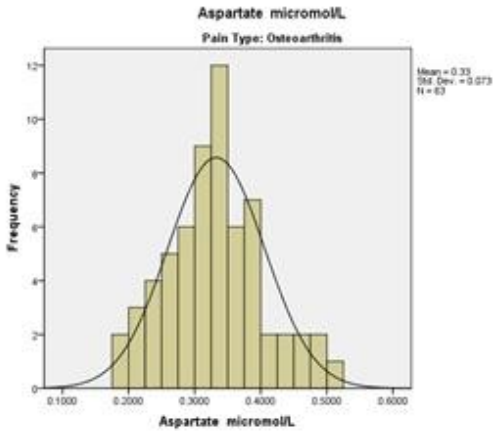
	Control	OA
IL2	52	23
IL4		78*, 21*
IL5		31
IL6	2	3, 101
IL8		3
IL12	107, 52	101*, 86, 60, 82*, 6*, 3*, 43*, 58*
IL13	44	
IFNg	2	62
TNFa		3

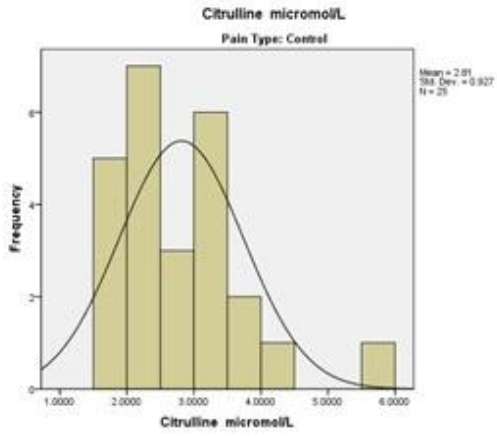
Appendix 11 Histograms and normal curves for amino acids and in the control and OA groups

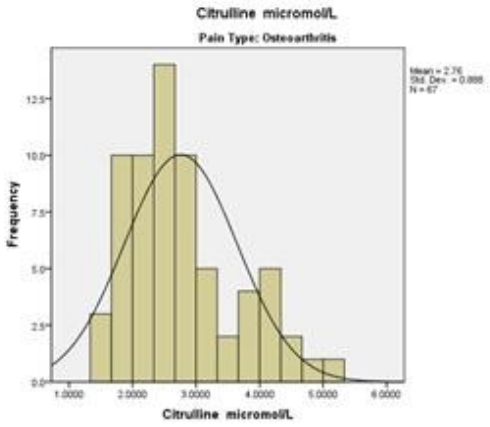


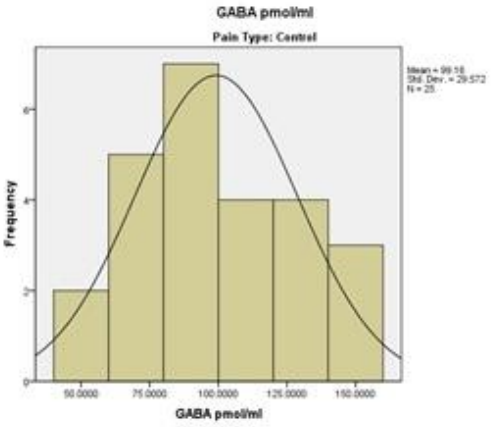


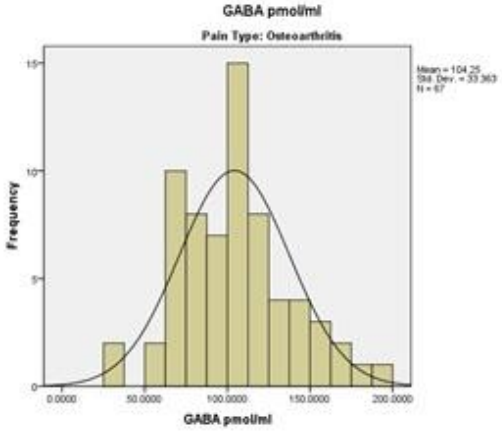


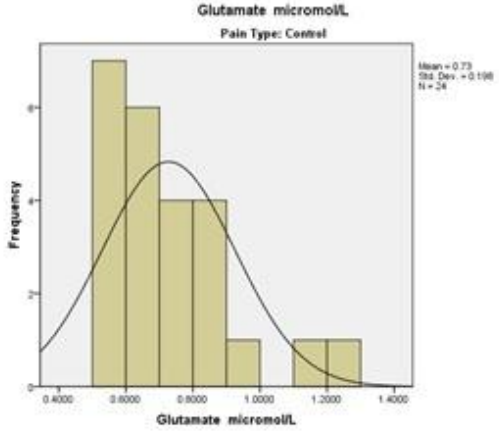


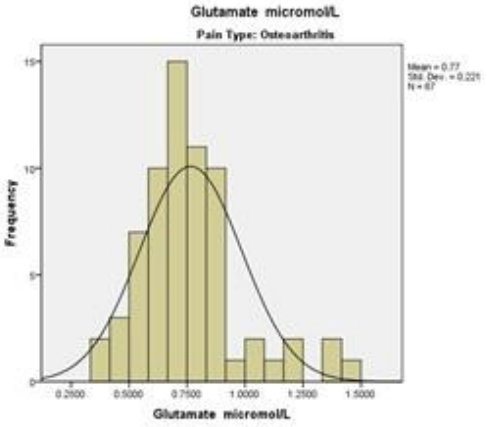


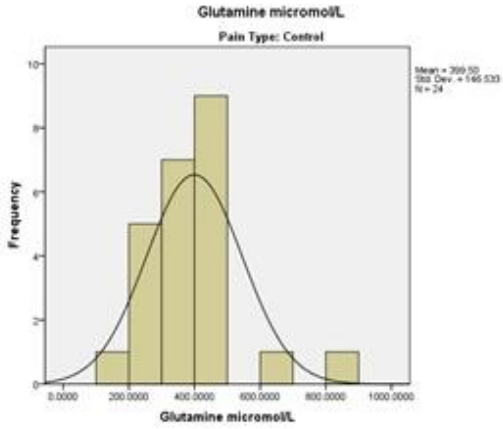


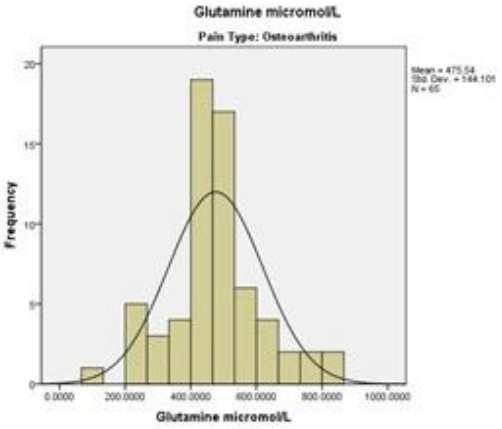

GABA pmol/ml
Pain Type: Control
Mean = 99.16
Std. Dev. = 29.572
N = 25
Frequency
GABA pmol/ml

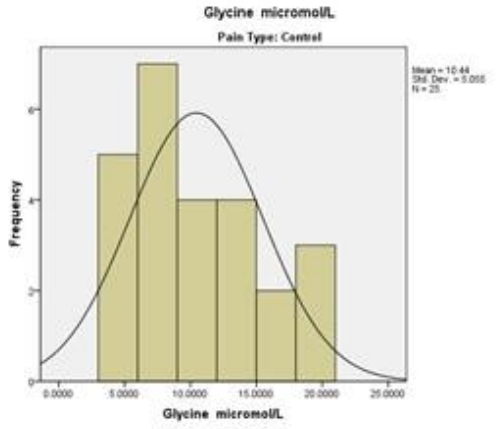

GABA pmol/ml
Pain Type: Osteoarthritis
Mean = 104.25
Std. Dev. = 33.363
N = 67
Frequency
GABA pmol/ml

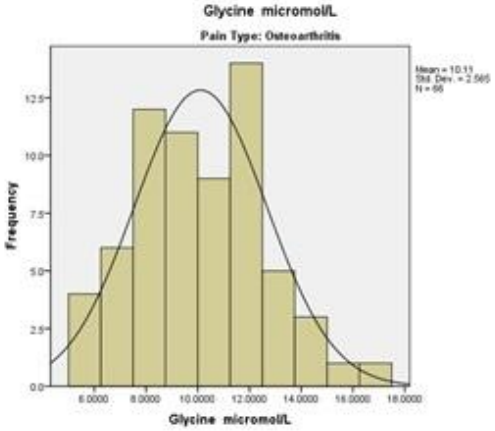

Glutamate micromol/L
Pain Type: Control
Mean = 0.73
Std. Dev. = 0.198
N = 24
Frequency
Glutamate micromol/L

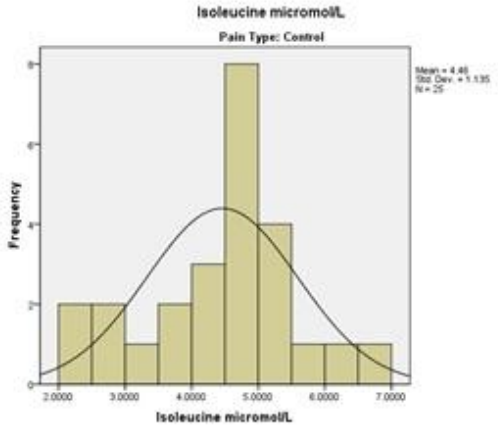

Glutamate micromol/L
Pain Type: Osteoarthritis
Mean = 0.77
Std. Dev. = 0.221
N = 67
Frequency
Glutamate micromol/L

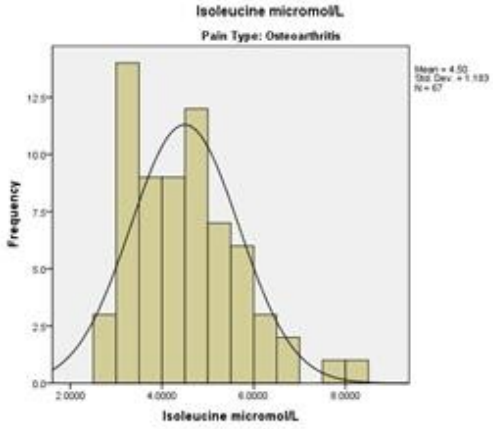

Glutamine micromol/L
Pain Type: Control
Mean = 399.50
Std. Dev. = 146.533
N = 24
Frequency
Glutamine micromol/L

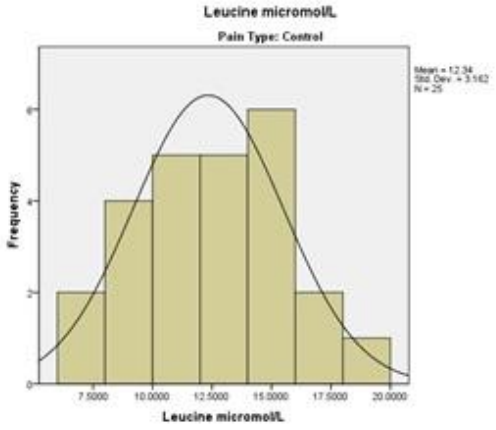

Glutamine micromol/L
Pain Type: Osteoarthritis
Mean = 475.54
Std. Dev. = 144.101
N = 65
Frequency
Glutamine micromol/L

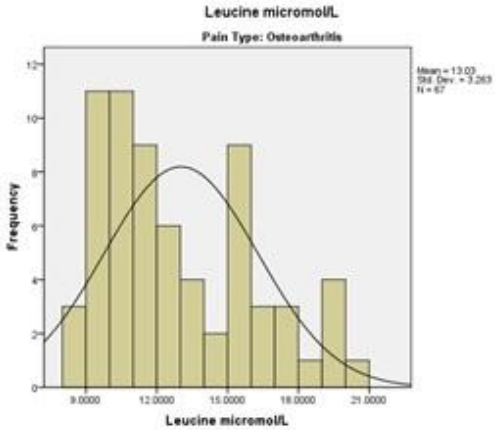

Glycine micromol/L
Pain Type: Control
Mean = 10.44
Std. Dev. = 5.055
N = 25
Frequency
Glycine micromol/L

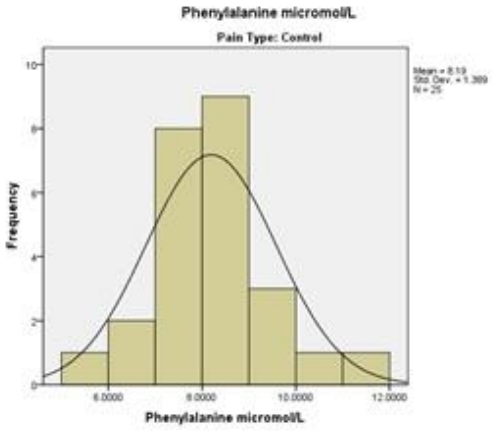

Glycine micromol/L
Pain Type: Osteoarthritis
Mean = 10.11
Std. Dev. = 2.565
N = 66
Frequency
Glycine micromol/L

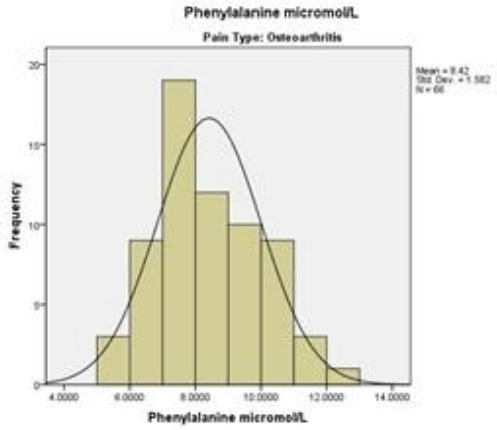

Isoleucine micromol/L
Pain Type: Control
Mean = 4.46
Std. Dev. = 1.135
N = 25
Frequency
Isoleucine micromol/L

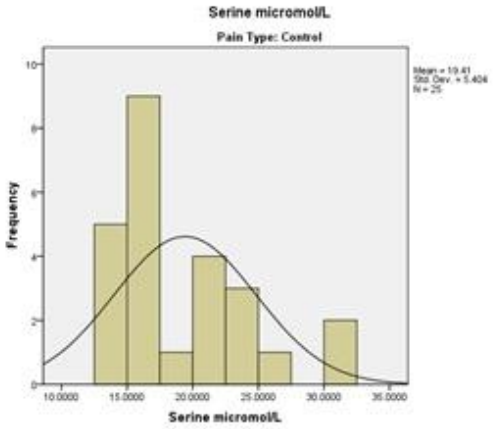

Isoleucine micromol/L
Pain Type: Osteoarthritis
Mean = 4.50
Std. Dev. = 1.183
N = 67
Frequency
Isoleucine micromol/L

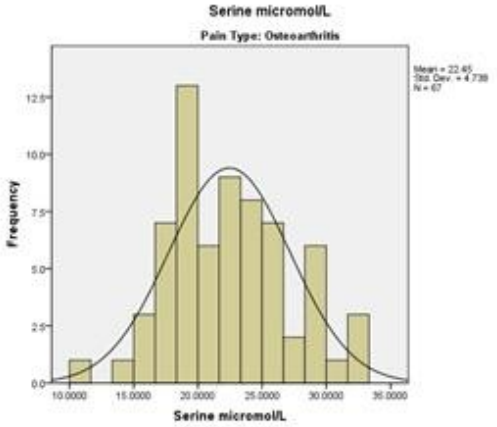

Leucine micromol/L
Pain Type: Control
Mean = 12.34
Std. Dev. = 3.162
N = 25
Frequency
Leucine micromol/L

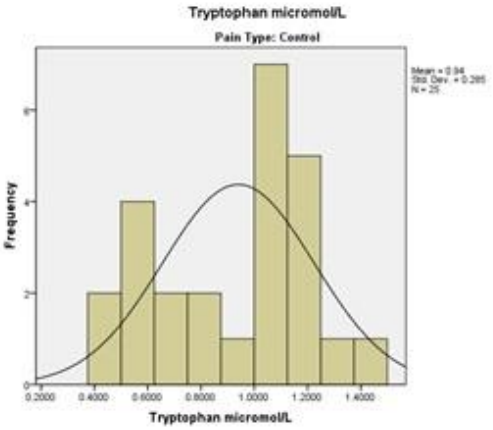

Leucine micromol/L
Pain Type: Osteoarthritis
Mean = 13.03
Std. Dev. = 3.263
N = 67
Frequency
Leucine micromol/L

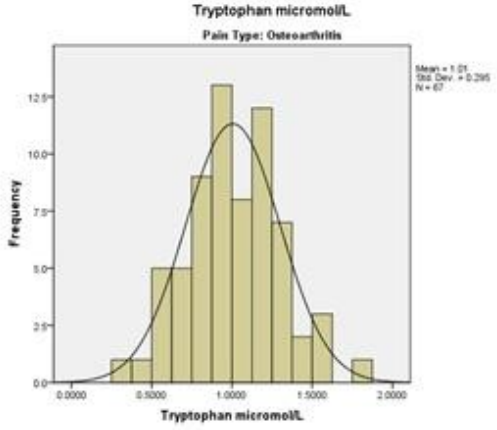

Phenylalanine micromol/L
Pain Type: Control
Mean = 8.19
Std. Dev. = 1.389
N = 25
Frequency
Phenylalanine micromol/L

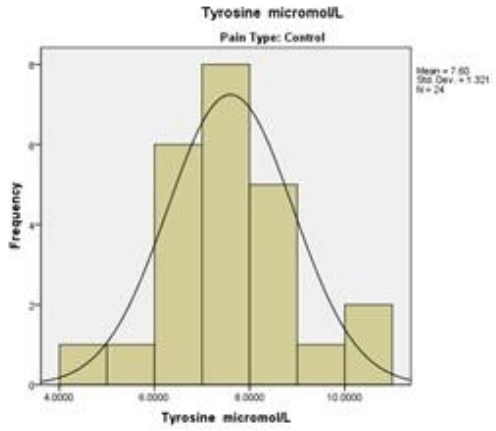

Phenylalanine micromol/L
Pain Type: Osteoarthritis
Mean = 8.42
Std. Dev. = 1.582
N = 66
Frequency
Phenylalanine micromol/L

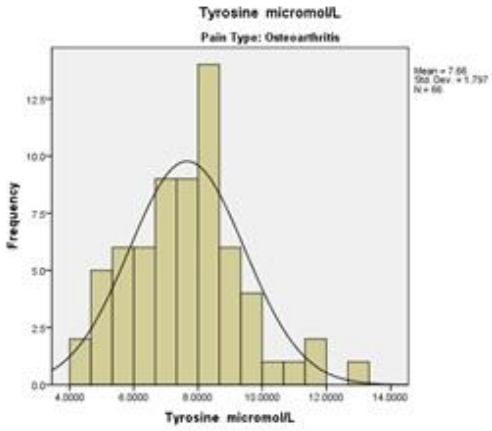

Serine micromol/L
Pain Type: Control
Mean = 19.41
Std. Dev. = 5.404
N = 25
Frequency
Serine micromol/L

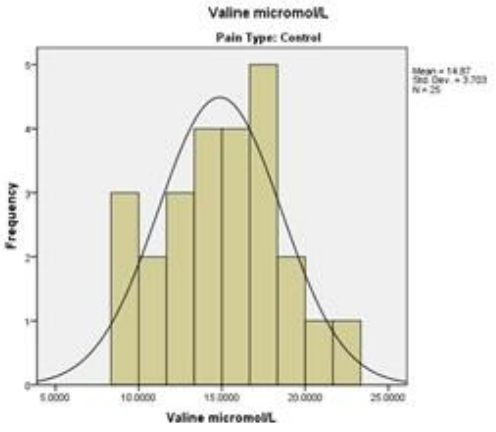

Serine micromol/L
Pain Type: Osteoarthritis
Mean = 22.45
Std. Dev. = 4.739
N = 67
Frequency
Serine micromol/L

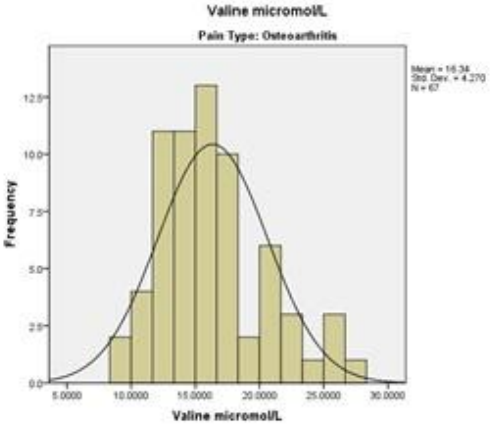

Tryptophan micromol/L
Pain Type: Control
Mean = 0.94
Std. Dev. = 0.295
N = 25
Frequency
Tryptophan micromol/L


Tryptophan micromol/L
Pain Type: Osteoarthritis
Mean = 1.01
Std. Dev. = 0.295
N = 67
Frequency
Tryptophan micromol/L


Tyrosine micromol/L
Pain Type: Control
Mean = 7.60
Std. Dev. = 1.321
N = 24
Frequency
Tyrosine micromol/L


Tyrosine micromol/L
Pain Type: Osteoarthritis
Mean = 7.66
Std. Dev. = 1.797
N = 66
Frequency
Tyrosine micromol/L

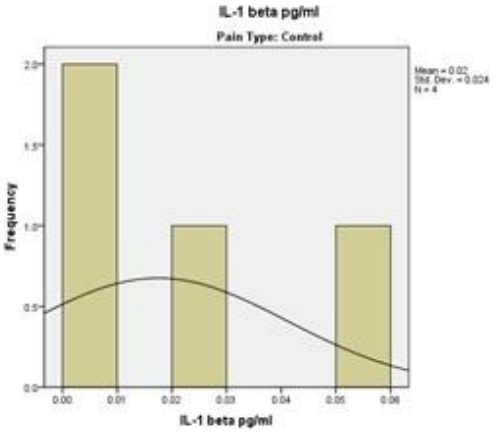

Valine micromol/L
Pain Type: Control
Mean = 14.87
Std. Dev. = 3.703
N = 25
Frequency
Valine micromol/L

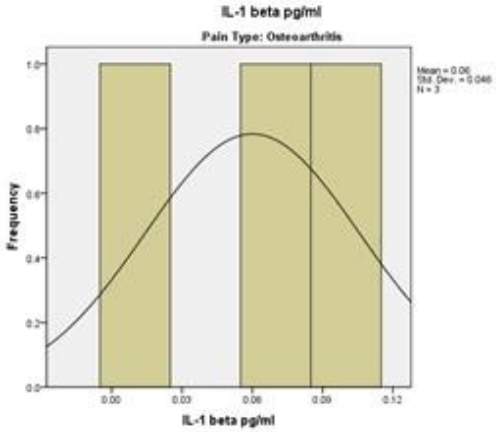

Valine micromol/L
Pain Type: Osteoarthritis
Mean = 16.34
Std. Dev. = 4.270
N = 67
Frequency
Valine micromol/L

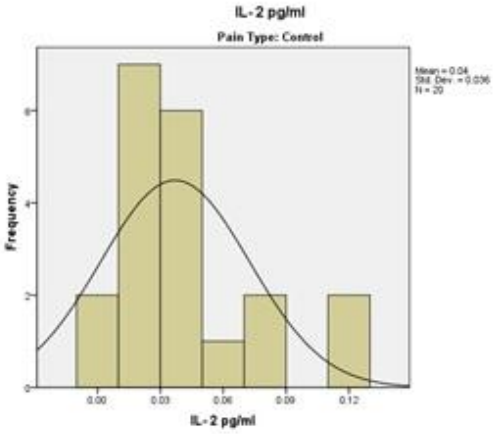
Appendix 12: Normality testing (Kolmorgorov-Smirnov) amino acid data OA and control groups

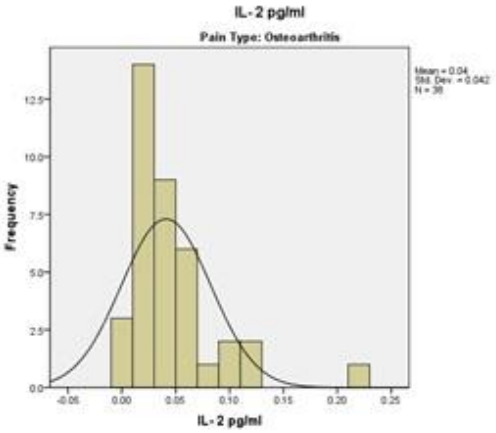
	Control			OA		
	Statistic	df	Sig.	Statistic	df	Sig.
Glutamate	0.177	20	0.101	0.108	59	0.084
Aspartate	0.121	21	0.200	0.076	56	0.200
Arginine	0.182	21	0.067	0.108	58	0.092
Citrulline	0.134	21	0.200	0.123	59	0.026[a]
Serine	0.220	21	0.009[a]	0.118	59	0.041[a]
Glutamine	0.174	20	0.113	0.149	58	0.003[a]
GABA	0.111	21	0.200	0.093	59	0.200
Glycine	0.144	21	0.200	0.065	58	0.200
Tryptophan	0.168	21	0.124	0.086	59	0.200
Leucine	0.107	21	0.200	0.132	59	0.013[a]
Isoleucine	0.104	21	0.200	0.080	59	0.200
Phenylalanine	0.139	21	0.200	0.087	58	0.200
Tyrosine	0.196	20	0.200	0.081	58	0.200
Valine	0.085	21	0.200	0.098	59	0.200
IL- 2	0.247	16	0.010[a]	0.193	36	0.002[a]
IL-6	0.163	19	0.200	0.128	48	0.047[a]
IL-7	0.133	18	0.200	0.146	45	0.017[a]
IL-8	0.133	19	0.200	0.106	49	0.200
IL-12	0.310	10	0.007[a]	0.362	21	<0.001[a]
IFNγ	0.304	11	0.005[a]	0.183	26	0.025[a]
TNFα	0.229	19	0.010[a]	0.123	49	0.063
IL-4	0.252	11	0.050[a]	0.366	10	<0.001[a]
IL-5	0.118	18	0.200	0.157	48	0.005[a]
IL-10	0.151	19	0.200	0.130	49	0.039[a]
IL-13	0.228	9	0.196	0.191	14	0.177

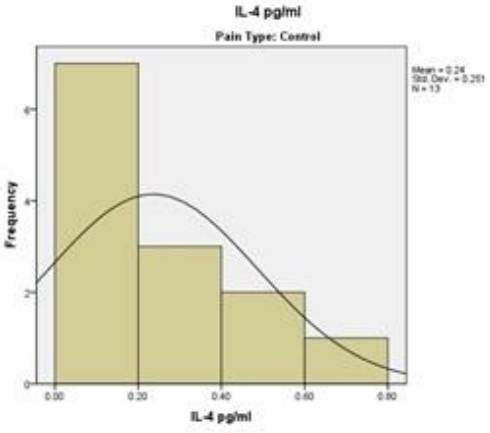
Appendix 13 Histograms of cytokines in control and OA groups

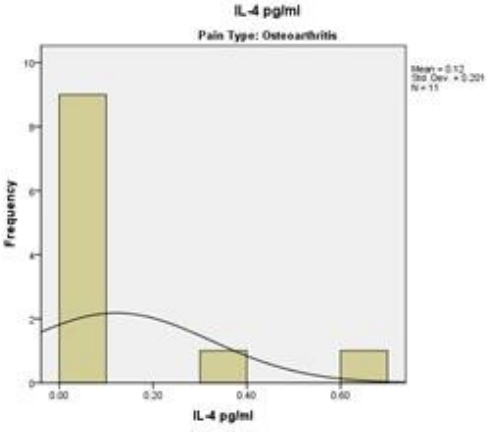


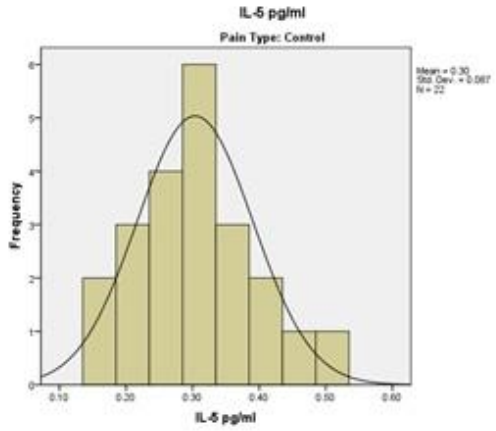


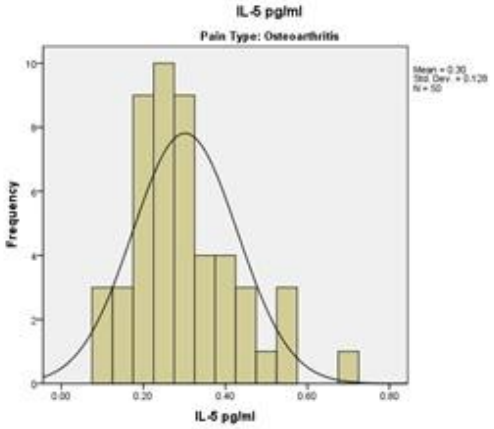


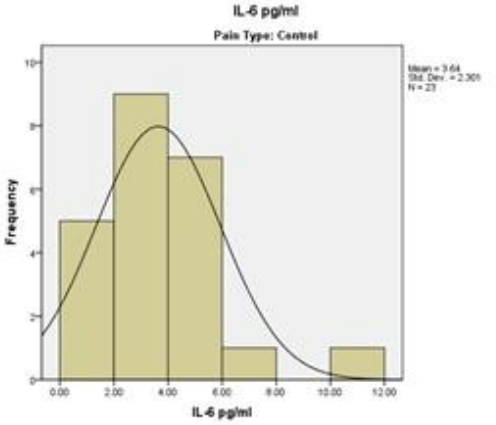


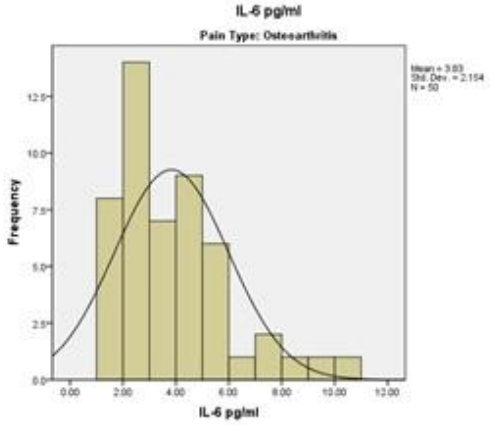


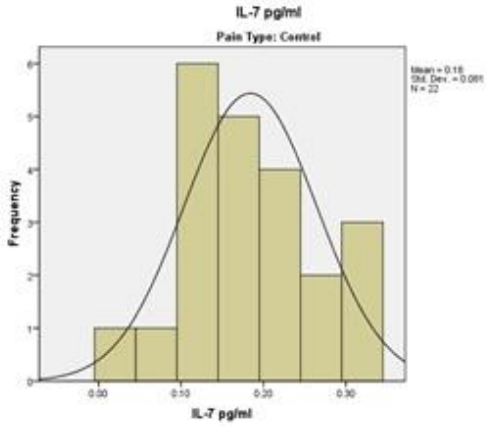


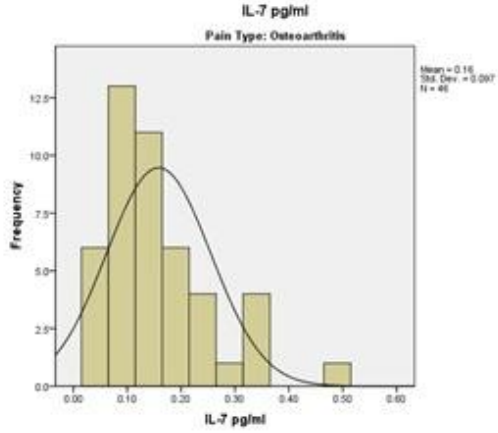

IL-5 pg/ml
Pain Type: Control
Frequency
IL-5 pg/ml

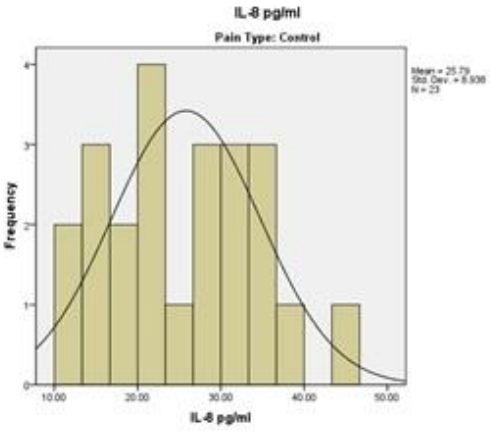

IL-5 pg/ml
Pain Type: Osteoarthritis
Frequency
IL-5 pg/ml

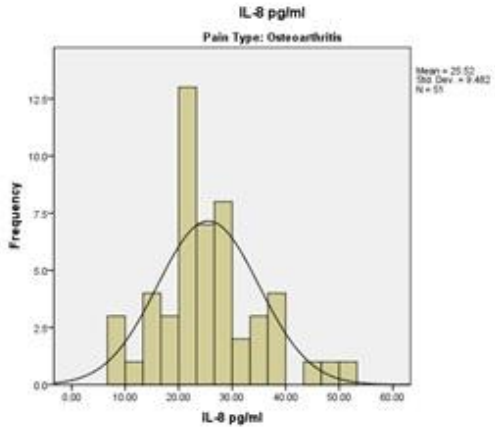

IL-6 pg/ml
Pain Type: Control
Frequency
IL-6 pg/ml

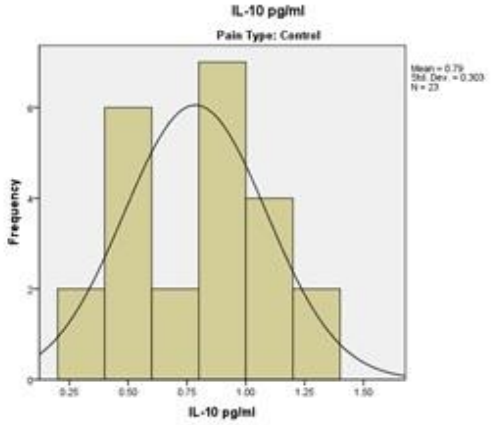

IL-6 pg/ml
Pain Type: Osteoarthritis
Frequency
IL-6 pg/ml

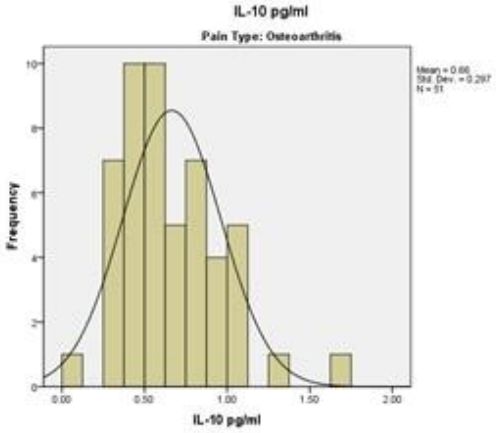

IL-7 pg/ml
Pain Type: Control
Frequency
IL-7 pg/ml

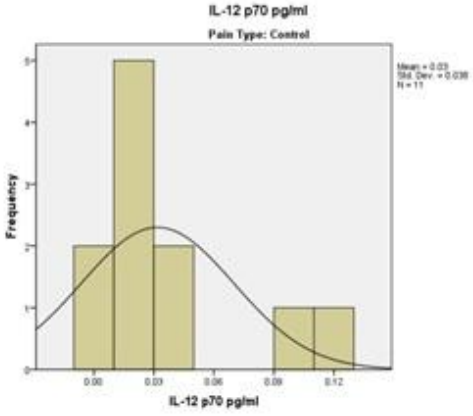

IL-7 pg/ml
Pain Type: Osteoarthritis
Frequency
IL-7 pg/ml

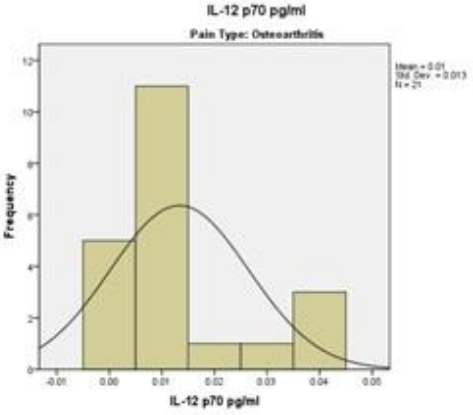

IL-8 pg/ml
Pain Type: Control
Frequency
IL-8 pg/ml

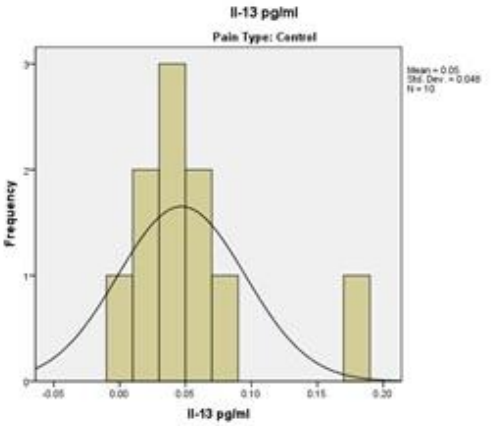

IL-8 pg/ml
Pain Type: Osteoarthritis
Frequency
IL-8 pg/ml

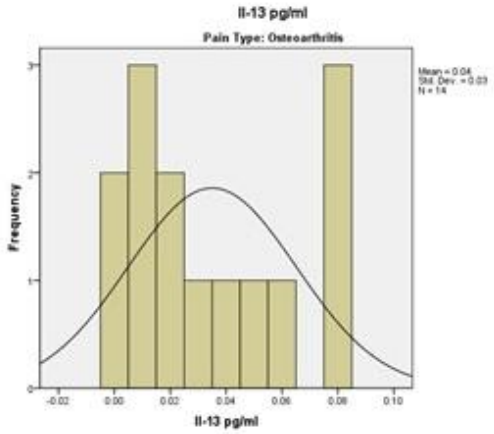

IL-10 pg/ml
Pain Type: Control
Frequency
IL-10 pg/ml

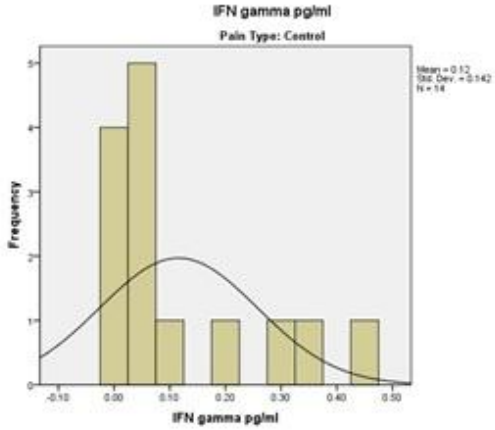

IL-10 pg/ml
Pain Type: Osteoarthritis
Frequency
IL-10 pg/ml

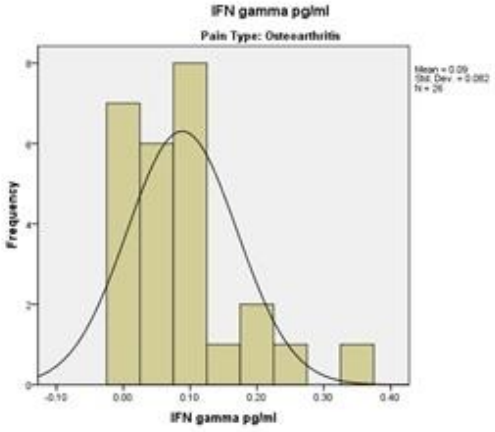

IL-12 p70 pg/ml
Pain Type: Control
Frequency
IL-12 p70 pg/ml

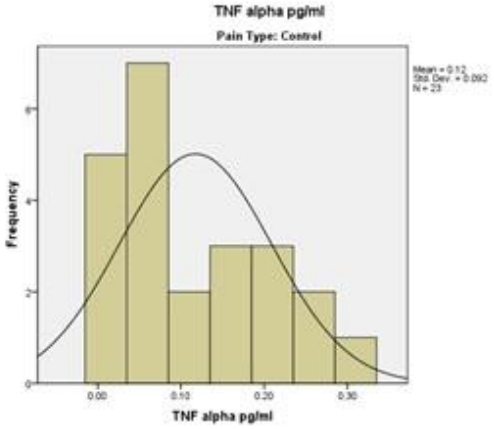

IL-12 p70 pg/ml
Pain Type: Osteoarthritis
Frequency
IL-12 p70 pg/ml

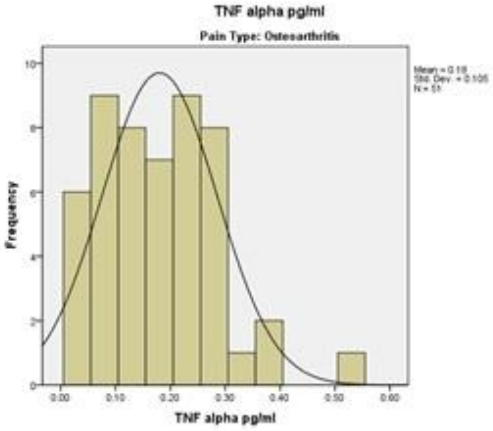

Il-13 pg/ml
Pain Type: Control
Frequency
Il-13 pg/ml


Il-13 pg/ml
Pain Type: Osteoarthritis
Frequency
Il-13 pg/ml


IFN gamma pg/ml
Pain Type: Control
Frequency
IFN gamma pg/ml


IFN gamma pg/ml
Pain Type: Osteoarthritis
Frequency
IFN gamma pg/ml


TNF alpha pg/ml
Pain Type: Control
Frequency
TNF alpha pg/ml


TNF alpha pg/ml
Pain Type: Osteoarthritis
Frequency
TNF alpha pg/ml

Appendix 14 Normality testing (Kolmogorov-Smirnov) cytokines OA and control groups

	0PAR			PAR		
	Statistic	n	p	Statistic	n	p
Glutamate	0.138	24	0.200	0.132	35	0.128
Aspartate	0.082	24	0.200	0.106	32	0.200
Arginine	0.143	23	0.200	0.092	35	0.200
Citrulline	0.127	24	0.200	0.150	35	0.046[a]
Serine	0.184	24	0.035[a]	0.079	35	0.200
Glutamine	0.206	23	0.012[a]	0.128	35	0.160
GABA	0.130	24	0.200	0.093	35	0.200
Glycine	0.090	23	0.200	0.083	35	0.200
Tryptophan	0.102	24	0.200	0.125	35	0.186
Leucine	0.142	24	0.200	0.152	24	0.039[a]
Isoleucine	0.108	24	0.200	0.108	24	0.200
Tyrosine	0.127	23	0.200	0.081	35	0.200
Phenylalanine	0.108	23	0.200	0.107	35	0.200
Valine	0.142	24	0.200	0.142	35	0.056
IL- 2	0.172	14	0.200	0.258	22	0.001[a]
IL-6	0.169	20	0.137	0.152	28	0.096
IL-7	0.183	18	0.113	0.155	27	0.093
IL-8	0.129	20	0.200	0.124	29	0.200
IL-12	0.353	12	<0.001[a]	0.353	9	0.021[a]
IFNγ	0.292	13	0.003[a]	0.232	13	0.054
TNFα	0.102	20	0.200	0.151	29	0.089
IL-4[b]	0.151	4		0.377	6	0.008[a]
IL-5	0.221	20	0.012[a]	0.145	28	0.140
IL-10	0.233	20	0.006[a]	0.083	29	0.200
IL-13	0.257	7	0.179	0.236	7	0.200