The effect of Regional Anaesthesia and Genetic Factors on the development of Chronic Pain following Total Knee Arthroplasty

A thesis submitted in fulfilment of the requirements for
the award of Doctorate in Philosophy

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Abstract

Background: Chronic Post Surgical Pain (CPSP) is common after Total Knee Arthroplasty (TKA). Factors, including demographic, genetic and possibly anaesthetic techniques, that may modify the risk of developing CPSP are still being investigated.

Aim and Objectives: The aims of this study were to show how anaesthetic techniques may influence CPSP and to evaluate potential polymorphisms in six genes that may also affect CPSP. Furthermore, this research studied the genomic variation of these genes in a sample of the local population. The genes investigated were *COMT*, *GCH1*, *SCN9A*, *KCNS1*, *OPRM1* and *OPRK1*.

Methods: Patients scheduled for a TKA were enrolled. Baseline characteristics were obtained, with a blood sample collected for genotyping. Patients were randomized to a spinal anaesthetic alone or to a general anaesthetic with femoral nerve block. Genotyping was performed using TaqMan™ SNP Genotyping assays. Patients were followed up at three and at six months with a telephone questionnaire that included a WOMAC® and S-LANSS score. The primary outcome was the WOMAC® score at six months. Secondary outcomes were the acute postoperative pain scores, the WOMAC® Pain score, the S-LANSS score and the incidence of chronic post-surgical pain (CPSP) at six months.

Results: 199 patients participated in the study. Patients who received a spinal anaesthetic had better function (WOMAC®: GA: 16.9 vs SP: 14.4, p-value 0.015) and less pain (WOMAC® pain: GA: 3.04 vs SP: 2.69, p-value 0.02) at three months, but not at six months. Overall, 11% of patients had chronic post-surgical pain (CPSP), with

Group GA having a higher incidence of (CPSP) at 6 months (OR 4.07, 95CI: 1.33 – 14.59, p-value 0.019). Neuropathic pain was strongly associated with CPSP.

Genotyping revealed that most SNPs had a frequency distribution similar to that found in European samples, except for rs998259 and rs3783641 (*GCH1*) and for rs495491 and rs533586 (OPRM1). Preoperative pain scores were lower in patients who carried the minor allele of rs2075572(*OPRM1*) (9 vs 11, p-value: <0.001), rs609148 (*OPRM1*) (9 vs 10, p-value: 0.028) and rs734784 (*KCNS1*) (9 vs 11, p-value: 0.046). Patients being homozygous for rs495491 (*OPRM1*) reported lower pain scores at rest (0 vs 2, p-value: 0.05).

On multivariate analysis, patients homozygous for rs4633 (*COMT*) had lower WOMAC® pain scores at six months (Estimate -1.78, 95CI: -2.98 – -0.58, p-value: 0.004). Patients who carried the rs2075572 (*OPRM1*) had higher pain scores at three months (Estimate: +7.30, 95CI:2.64 – 11.96, p-value: 0.002). Patients who had two copies of rs734784 (*KCNS1*) had lower WOMAC® scores throughout the study period (Estimate-3.94, 95CI: -6.97 – -0.91, p-value: 0.011).

Conclusion: Spinal anaesthesia appears to reduce CPSP when compared to general anaesthesia with a femoral block. Genetic polymorphisms may also play a role in the development of CPSP.

Publications and Presentations

Sciberras SC, Vella AP, Vella B, Spiteri J, Mizzi C, Borg-Xuereb K, LaFerla G, Grech G, Sammut F. A randomized, controlled trial on the effect of anesthesia on chronic pain after total knee arthroplasty*. Pain Manag*. 2022 Mar 30. doi: 10.2217/pmt-2021-0081.

Sciberras SC, Vella AP, Vella B, Spiteri J, Mizzi C, Borg-Xuereb K, LaFerla G, Grech G. The role of Genetic Factors in developing Acute and Chronic Pain following a Total Knee Arthroplasty. *17th World Congress of Anaesthesiologists, Prague, Czech Republic*. September 2021

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Sciberras SC, Vella B, Vella AP, Spiteri J, Mizzi C, Borg-Xuereb K, LaFerla G, Grech G. OP4.115 The influence of anaesthesia and genetic factors on the outcomes following total knee arthroplasty. *10th Malta Medical School Conference.* November 2018. https://www.um.edu.mt/__data/assets/pdf_file/0004/380497/Maltamedicalconference2018.pdf

In submission

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Abbreviations

AMPA	Alpha-amino-3-hydroxy-5-Methyl-4-isoxa-zoleProprionic Acid
BH4	tetrahydrobiopterin
cAMP	Cyclic Adenosine MonoPhosphate
COMT	Catechol-O-MethylTransferase
COX	CycloOXygenase enzyme
CPSP	Chronic Post-Surgical Pain
DN4	Douleur Neuropathique en 4 questions
DNA	Deoxyribose Nucleic Acid
DRG	Dorsal Root Ganglion
GPCR	G-protein Coupled Receptors
IASP	International Association for the Study of Pain
IPBSN	InfraPatellar Branch of the Saphenous Nerve
KA	Kainate
KOOS	Knee injury and Osteoarthritis Outcome Score
MAC	Minimum Alveolar Concentration
mGluR	Metabotropic Glutamate Receptors
MOR	M – Opiate receptor
NMDA	N-methyl-D-aspartate
NNT	Number needed to treat

NPQ	Neuropathic Pain Questionnaire
NRS	Numerical Rating Score
NSAIDS	Non-steroidal Anti-Inflammatory Drugs
PAG	PeriAqueductal Grey area
PCA	Patient Controlled Analgesia
PCR	Polymerase Chain Reaction
RVM	Rostral Ventromedial Medulla
QALY	Quality-Adjusted Life Year
S-LANSS	Self-reported Leeds Assessment of Neuropathic Symptoms and Signs
SNP	Single Nucleotide Polymorphism
TH4	Tetrahydrobiopterin
TIVA	Total Intravenous Anaesthesia
TKA	Total Knee Arthroplasty
TRP	Transient Response Protein
TRPA1	Transient Receptor Potential cation channel subfamily A member 1
TRPV1	Transient Receptor Potential cation channel subfamily V member 1
WHO	World Health Organization
WOMAC®	Western Ontario and McMaster Universities Osteoarthritis Index
VAS	Visual Analogue Scale
VGSC	Voltage Gated Sodium Channels

Chapter 1

Introduction

1.1 Introduction

All surgical procedures are associated with a considerable amount of pain in the acute post-operative period. For years, attempts were made to alleviate pain during and after surgery, until Morton demonstrated the effects of ether during the first public demonstration of an anaesthetic in October, 1846 (Boott, 1847).

Despite such attempts, a considerable number of patients still suffer from acute postoperative pain. Sommer showed that in a sample of more than 1,400 patients undergoing major surgery in a European country, 30% - 55% of patients suffered from severe acute post-operative pain (Sommer et al., 2008).

Such pain will negatively impact postoperative recovery. Furthermore, some patients with acute post-operative pain may further proceed to develop chronic pain at later stages.

1.2 Pain

Pain is defined by International Association for the Study of Pain (IASP) as 'an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage' (Baranowski et al., 2011).

1.2.1 The physiological role of Pain

Pain serves an important physiological role. It has a protective function as it prevents injury and also allows the body to guard injured areas against further damage (Grahek, 2007). Pain also forces us to rest injured tissue in order to promote healing.

The influence of these protective mechanisms can be clearly seen on those patients who have an insensitivity towards pain, either in the acquired or congenital forms. These patients end up with severe injuries due to repeated painless trauma (van Ness Dearborn, 1932; Melzack et al., 1996; Peddareddygari et al., 2014; Strotman et al., 2016).

Whilst pain in general is protective, prolonged or abnormally severe pain may cause restrictions in a way that affects the quality of life of the person. Examples of such syndromes include hyperalgesia (increased sensitivity to pain) and chronic pain (when the duration of pain exceeds the normal healing time for the injury). In the postoperative phase, patients who have severe pain will have a prolonged recovery and outcomes may be poorer in those patients with severe pain (Gan, 2017).

Besides having a direct influence on the body, pain also negatively effects the mental wellbeing of the person. In a questionnaire to nearly 10,000 patients in Iceland,

Björnsdóttir (2014) showed that patients with chronic pain showed a higher incidence of stress, depressive traits, sleep disruption and reduced quality of life. In a similar study involving more than 29,000 Spanish patients, the authors showed that those with chronic pain were five times more likely to suffer a worse health status and four times more likely to complain of depressive symptoms (Fernández-de-las-Peñas et al., 2011).

1.2.2 Classification of Pain

Pain may exist due to different causes and different mechanisms. There may be different therapeutic options for specific types of pain. For instance, acute pain may be relieved by anti-inflammatory drugs such as NSAIDS but neuropathic pain may be more amenable to atypical analgesics such as antiepileptic agents. Hence, classifying pain may offer help in the management of pain in individual patients.

A simple means of describing pain is by time, with pain being described as either acute or chronic depending on the duration of the pain. Acute pain is the result of tissue damage that settles once this damage resolves. On the other hand, chronic pain is defined as lasting for more than three months, and which persists after a normal healing time even though the injury itself has healed (Treede et al., 2015).

Another method for classifying pain was that adopted by Woolf (1998). This involves a method that describes the actual mechanism which incites the pain stimulus, such as an inflammatory process, a neuropathic involvement of the nervous system itself, or a mixture of both, as shown in Table 1-1.

Category	Cause	Symptoms	Examples
Physiologic	Brief exposure to a noxious stimulus	Rapid, yet brief pain perception	Touching a pin or hot object
Nociceptive / Inflammatory	Somatic or visceral tissue injury with mediators impacting on intact nervous tissue	Moderate to severe pain, described as crushing or stabbing; usually worsens after the first 24 h	Surgical pain, traumatic pain, sickle cell crisis
Neuropathic	Damage or dysfunction of peripheral nerves or CNS	Severe lancinating, burning, or electrical shock-like pain	Neuropathy, chronic regional pain syndrome, post-herpetic, neuralgia
Mixed	Combined somatic and nervous tissue injury	Combinations of symptoms; soft tissue pain plus radicular pain	Low back pain, back surgery pain

Table 1-1: Differences between the physiologic, nociceptive/inflammatory, neuropathic pain

One-dimensional classifications are usually arbitrary, and a lot of overlap exists between different categories. In view of this, the International Association for the Study of Pain has devised a multi-dimensional taxonomy, which integrates location of the pain, characteristics, patients' perception and possible aetiology (Baranowski et al., 2011). This has been integrated into the new International Classification of Disease, ICD-11, as shown in Table 1-2 (Treede et al., 2015).

Chronic pain (persistent or recurrent pain lasting longer than 3 months)	
1. Chronic primary pain	1.1. Widespread chronic primary pain (including fibromyalgia syndrome) 1.x. Other chronic primary pain 1.2. Localized chronic primary pain (including nonspecific back pain, chronic pelvic pain) 1.z. Chronic primary pain not otherwise specified
2. Chronic cancer pain	2.1. Chronic pain due to cancer and metastases 2.2. Chronic chemotherapy-induced pain (primary parent: chronic neuropathic pain) 2.3. Chronic pain due to cancer surgery (primary parent: chronic postsurgical and posttraumatic pain) 2.4. Chronic pain due to radiotherapy 2.x. Other chronic pain related to cancer 2.z. Chronic cancer pain not otherwise specified
3. Chronic postsurgical and posttraumatic pain	3.1. Chronic postsurgical pain 3.2. Chronic posttraumatic pain 3.x. Other chronic postsurgical and posttraumatic pain 3.z. Chronic postsurgical and posttraumatic pain not otherwise specified
4. Chronic neuropathic pain	4.1. Peripheral neuropathic pain 4.2. Central neuropathic pain 4.x. Other neuropathic pain 4.z. Neuropathic pain not otherwise specified
5. Chronic headache and orofacial pain	5.1. Chronic primary headaches 5.2. Chronic secondary headaches 5.3. Chronic orofacial pains 5.z. Headache and orofacial pain not otherwise specified

6. Chronic visceral pain	6.1. Chronic visceral pain from persistent inflammation 6.2. Chronic visceral pain from vascular mechanisms 6.3. Chronic visceral pain from obstruction/distension 6.4. Chronic visceral pain from traction/compression 6.5. Chronic visceral pain from combined mechanisms 6.6. Chronic visceral pain referred from other locations 6.7. Chronic visceral pain from cancer (primary parent: chronic cancer pain) 6.8. Functional or unexplained chronic visceral pain (primary parent: chronic primary pain) 6.x. Other chronic visceral pain 6.z. Chronic visceral pain not otherwise specified
7. Chronic musculoskeletal pain	7.1. Chronic musculoskeletal pain from persistent inflammation 7.2. Chronic musculoskeletal pain from structural osteoarticular changes 7.3. Chronic musculoskeletal pain due to disease of the nervous system (All neuropathic pain will be classified under 4. Chronic neuropathic pain. Here, other chronic musculoskeletal pain originating from diseases of the nervous system, eg, spastic pain will be listed.) 7.4. Chronic nonspecific musculoskeletal pain (primary parent: chronic primary pain) 7.x. Other chronic musculoskeletal pain syndromes 7.z. Chronic musculoskeletal pain not otherwise specified

Table 1-2: IASP classification for Chronic Pain, ICD-11. Reproduced from Treede RD, Rief W, Barke A, et al. A classification of chronic pain for ICD-11. Pain. 2015;156(6):1003-7.

1.2.3 Pain Pathway

The physiology of pain is quite complex. A series of receptors are stimulated at the site of pain to generate an action potential, which is relayed through peripheral nerves to the dorsal root ganglion. These nerves synapse in the spinal cord, and pain thence travels through specific pathways to the cortical areas of the brain.

Such nociceptive impulses may be modulated by either excitatory or inhibitory pathways that are present in the central nervous system. This will affect the ultimate perception of a painful stimulus.

An overview of the pain pathway is shown in Figure 1-1.

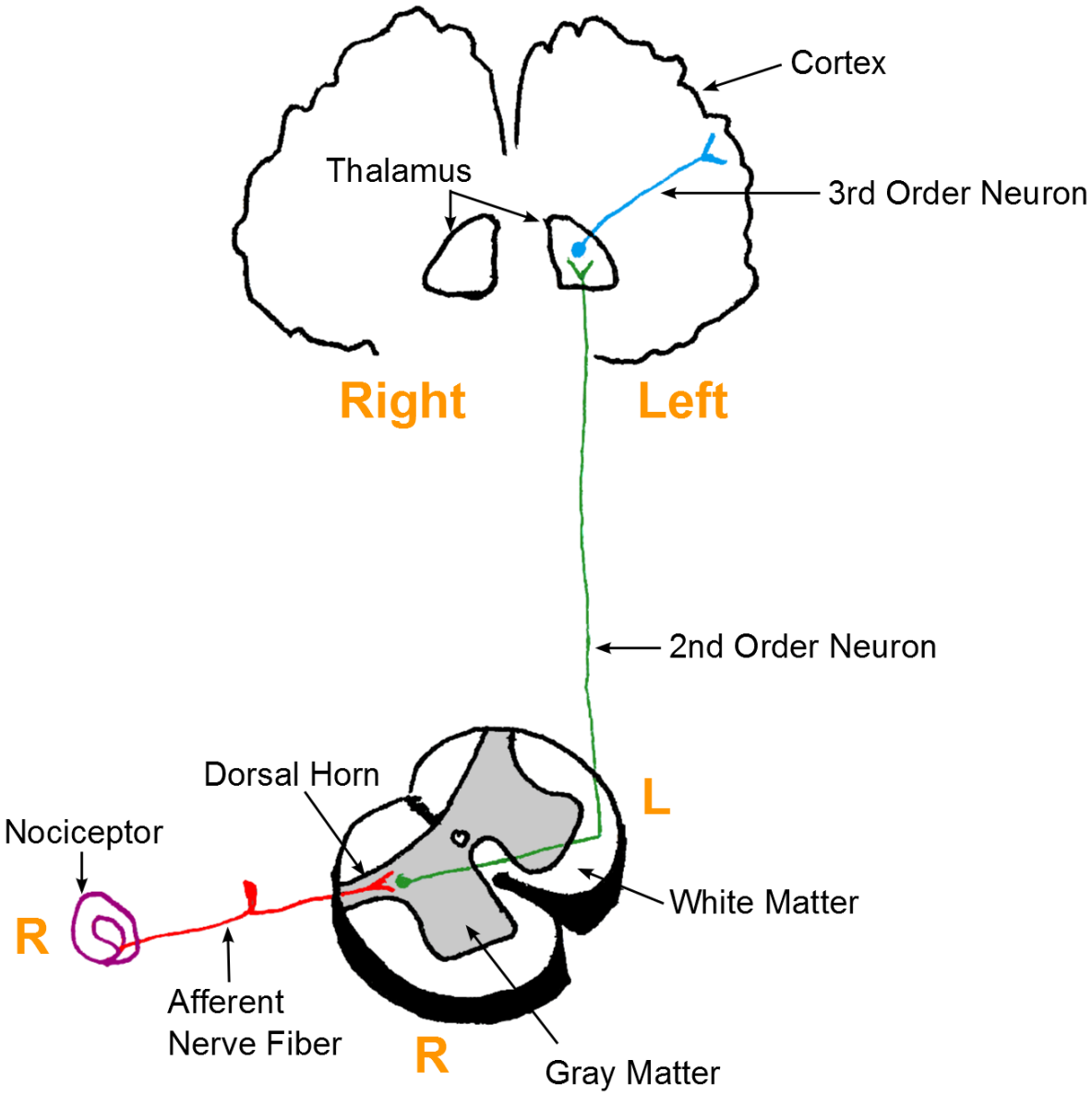

Figure 1-1: Simplified version of the Pain Pathway. B Guebeli, reproduced from https://commons.wikimedia.org/wiki/File:Afferents.png, licensed by Creative Commons 4.0

1.2.3.1 Nociceptors

The pain pathway starts with the nociceptor, which is the neuron that is responsible for sensing the painful stimulus. It is the nociceptor that initiates the action potential that will travel to the higher centres of the central nervous system.

Nociceptors are present in practically all tissues, including superficially in the skin epidermis and more deeply in muscular and bony structures. There are various types of receptors which respond to a variety of stimuli including pressure, extremes of temperature, actual tissue damage or chemical stimuli arising from such damage. Some nociceptors will respond to a specific stimulus, whereas others may be polymodal. There are also silent nociceptors that are generally quiescent, but fire impulses only when inflammation is present (Schmidt et al., 1995). Nociceptors generate an action potential when stimulated in an all-or-nothing manner (Dubin et al., 2010). This means that there is a threshold below which a stimulus will not incite an impulse.

Like other somatosensory neurons, nociceptors are pseudounipolar neurons (Basbaum et al., 2009). Such neurons have a cell body with only one process that branches into two. One branch will be synapsing with higher order spinal neurons, and the other becomes the peripheral axon that ends in the sensory ending (Figure 1-2). The terminal ends are free endings that branch into the tissue, rather than specialised corpuscles like the Ruffini corpuscle (Kruger et al., 1981, 2003).

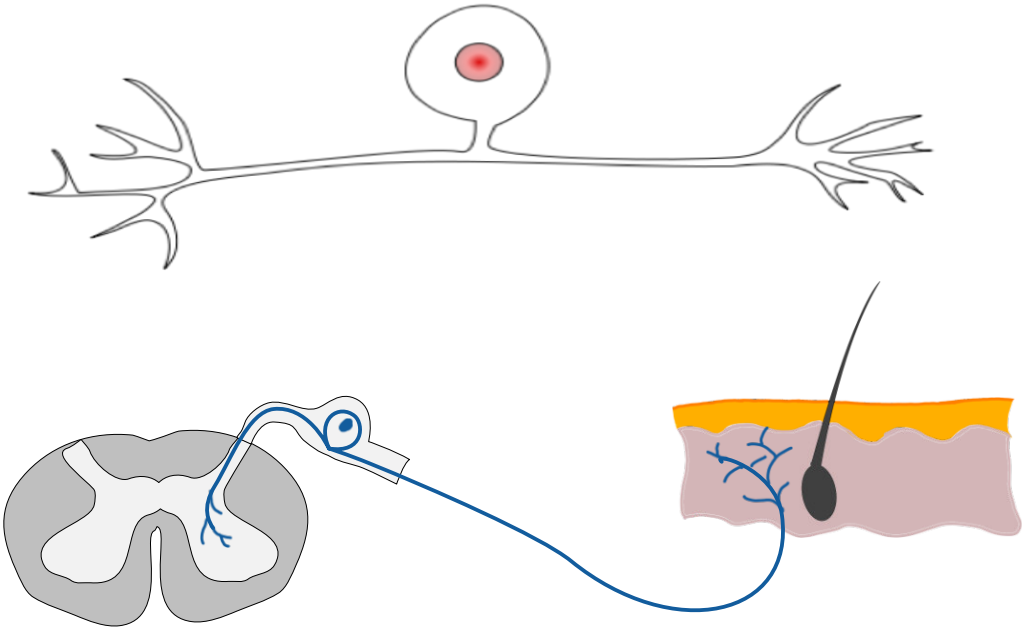

Figure 1-2: The structure of a nociceptor as a pseudounipolar neuron. At the periphery, it terminates as a number of free nerve endings, whereas proximally, the nociceptor would synapse with a variable number of higher order spinal neurons.

Neurons typically have a resting membrane potential of -70 mV, which is determined by the relative concentrations of the cations and anions present intraneuronally and in the surrounding extracellular fluid (Siegel et al., 2014). This is described by the Goldman-Hodgkin-Katz Equation, which is an expansion of the Nernst equation:

$$V_m = \frac{RT}{F} \ln\left(\frac{p_K[K^+]_o + p_{Na}[Na^+]_o + p_{Cl}[Cl^-]_i}{p_K[K^+]_i + p_{Na}[Na^+]_i + p_{Cl}[Cl^-]_o} \right)$$

where V_m is the membrane potential, R is the universal gas constant (8.314 $J.K^{-1}.mol^{-1}$), T is the temperature measured in Kelvin (K = °C + 273.15), F is the Faraday's constant (96485 $C.mol^{-1}$), pK is the membrane permeability for K^+, pNa is the relative membrane

permeability for Na^+, pCl is the relative membrane permeability for Cl^-, $[K^+]o$ is the concentration of K^+ in the extracellular fluid, $[K^+]i$ is the concentration of K^+ in the intracellular fluid, $[Na^+]o$ is the concentration of Na^+ in the extracellular fluid, $[Na^+]i$ is the concentration of Na^+ in the intracellular fluid, $[Cl^-]o$ is the concentration of Cl^- in the extracellular fluid, and $[Cl^-]i$ is the concentration of Cl^- in the intracellular fluid.

Regional changes in this membrane potential may lead to an action potential. Such changes occur when there is a new flux of ions in or out of the neuron, as would happen when ion channels open in response to a stimulus. The action potential then propagates through the axon or dendrites of the neuron in a unidirectional fashion.

The axons of the neurons may be of two types: C fibres or A-delta fibres. The C fibres are unmyelinated and have a small diameter. Because of this, the conduction velocity of such fibres is slow, at 2m/s. Such neurons are associated with the dull ache, which is diffuse in nature. C-fibres are polymodal, and may respond to different stimuli.

The A fibres have the largest diameter and are the most myelinated of the nerve fibres. Myelination improves nerve conduction velocity by saltatory action across the nodes of Ranvier. These fibres are classified into four main groups, according to size and speed of conduction (Table 1-3).

Type	Diameter (micron)	Conduction velocity (ms^{-1})	Function
A-alpha	10-20	60-120	Motor
A-beta	5-10	40-70	Touch/pressure
A-theta	3-6	15-30	Proprioception
A-delta	2-5	10-30	Pain, temperature

Table 1-3: Classification of A nerve fibres. Reproduced from Principles of physiology for the anaesthetist, by I Power and K Kam, Page 45, CRC Press, 2000.

Nociception involves A-delta fibres, which are of moderate diameter. These fibres are associated with nerve conduction speeds of up to 20m/s. They are stimulated by mechanical stimuli or temperature, and are responsible for the sharp, acute pain sensations (Fu et al., 2011).

1.2.3.2 Transduction

The actual mechanism on how receptors on the nociceptors are activated is still unclear. A number of proteins are involved, with some of these acting through secondary mechanisms, whilst other proteins are ionic channels for potassium, sodium and calcium.

All these mechanisms ultimately lead to a local depolarization, through the influx of sodium or calcium ions, or efflux of potassium ions. This makes the membrane potential more positive. The magnitude of these depolarizations is small, in the order of 0.1–10 mV, and do not last more than 100ms (Kandel et al., 2012). Furthermore,

they do not travel far, at most 1 – 2 mm from the position from where they are generated. For propagation of the signal through the axon, an action potential needs to be generated.

Isolated local depolarizations are not enough to start an action potential. However, their effect is summative, so that when a number of local depolarizations occur together, an action potential is triggered. The threshold for this to happen varies between nerve fibres, but is typically between -50 to -55 mV. This threshold may also vary due to modulation from ionic channels, that may be stimulated to make the membrane either more resistant or more sensitive to these local depolarizations.

Because of this threshold at which an action potential is initiated, neurons are activated in an all-or-nothing effect. The action potential amplitude will be the same for different grades of stimuli, but larger stimuli will cause a number of action potentials in succession.

1.2.3.3 Action Potential Generation

An action potential is a depolarization of the neuron cell membrane that is propagated through the neuron (Kandel et al., 2012). It is dependant on the influx of sodium ions into the cell through Voltage Gated Sodium Channels (VGSC). These transmembrane channels exist in three different states: closed, open and inactive.

In the closed state, the VGSC are impermeable to sodium, so that there is no disruption of the resting membrane potential. Once there is a local depolarization of sufficient magnitude, the VGSC open and allow the passage of sodium ions into the

neuron. This rapid influx of positive ions is responsible for the upstroke of the action potential, and the depolarization of the membrane to around +40 mV.

Soon after the open state, the VSGC becomes inactive, so that the channel itself is blocked and will not respond to further depolarizations until it enters its resting closed state.

This inactivation function is crucial for the transmission of the signal along the neuron. By having an inactive state, the VGSC cannot be kept open. Furthermore, the portion of the cell membrane that has just been depolarized will be refractory to any stimulus for a short duration of time: this prevents the action potential from spreading in a retrograde fashion. It also reduces hyperexcitability.

As will be discussed later, different conditions may affect the action potential. Inhibiting the action of the VGSC, such as with local anaesthetics, will effectively block the pain pathway: this forms the basis of regional anaesthesia. Furthermore, mutations of the VGSC will also cause altered sensations to pain.

1.2.3.4 Transmission

The action potential, once generated, will continue to propagate along the axon of the neuron. These neurons are known as the Dorsal Root Ganglion neurons, since their cell body is found in the dorsal root ganglion. The axons themselves end in the spinal cord and synapse with second-order neurons.

At the synapse, the action potential from the nociceptor needs to be transmitted to the post-synaptic neuron. This involves the release of a neurotransmitter, a small

molecule produced in the pre-synaptic neuron. The neurotransmitter will diffuse across the synaptic cleft to bind to receptors on the membrane of the post-synaptic membrane (Figure 1-3).

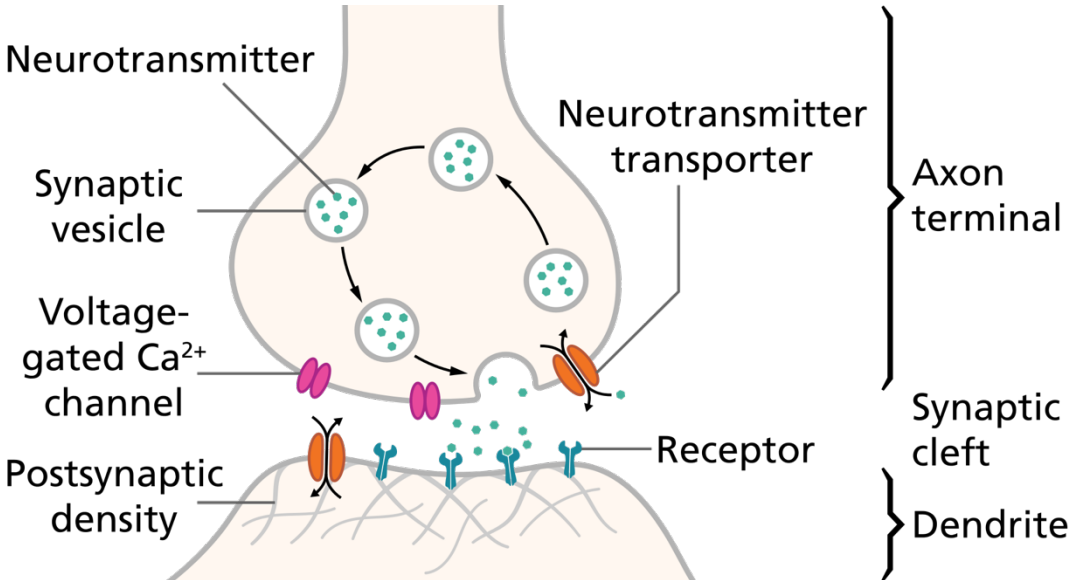

Figure 1-3: Release of neurotransmitter from presynaptic neurone, by Thomas Splettstoesser. Accessed from https://commons.wikimedia.org/w/index.php?curid=4134908. Reproduced under Creative Commons Licence.

There are two main types of such receptors. There are ligand-activated ionic channels which allow passage of ions into the cell to cause depolarization or hyperpolarization when activated. There are also metabotropic receptors, which trigger an intracellular secondary messenger mechanism that will then affect ionic channels, as described later.

The pain impulse passes from the peripheral nociceptors into the spinal cord. It will travel through the thalamus to reach the cortex. This is where localization of pain takes place (McMahon et al., 2013).

1.2.3.4.1 Spinal Cord

The nociceptors group together and with other nerve fibres to form the peripheral nerves. These travel to the spinal cord, and all sensory nerve fibres will pass through the dorsal horn of the spinal cord. The nerves then pass via the spinothalamic and spinoreticular tracts to reach the higher centres of the central nervous system (Figure 1-4).

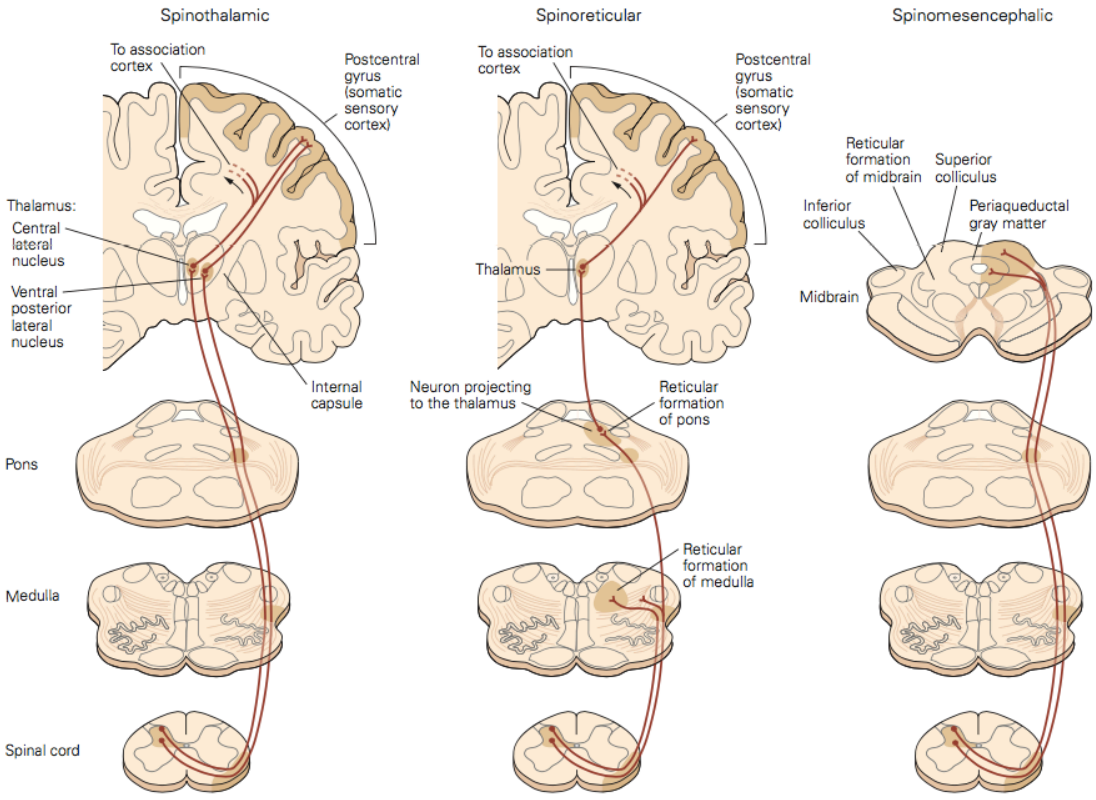

Figure 1-4: Ascending pathways through the spinal column. Reproduced from Principles of Neural Science, Kandel et al, 2012, Chapter 24, Page 544. Published by McGraw-Hill Education. Reproduced with permission.

The spinal cord is able to modulate nociception through descending pathways that inhibit transmission of nociception. These descending pathways are the site of action of opioid analgesics.

1.2.3.4.2 Cerebral Hemispheres

The ultimate destination of the pain pathway is the cerebrum, since this is where localization of the site of pain will occur. There are two main areas in the somatosensory cortex that seem important for pain localization. These are the primary somatosensory cortex (S1) and secondary somatosensory cortex (S2) regions.

The primary somatosensory cortex is located just behind the central sulcus, in the postcentral gyrus. It is the part of the cerebral cortex that is responsible for the localization of any sensation, including pain. This means that this region is more involved in the sharp pain, rather than in the feeling of dull non-localized pain. Neurones from the spinothalamic tract, through the ventral posterolateral nucleus in the thalamus, directly synapse in the S1 region, as shown by anatomical studies (Gingold et al., 1991) and by functional imaging (Apkarian et al., 2005). Pain studies also show that S1 is directly involved in the quantification of pain, with the activity as shown during magnetoencephalography of the S1 area being directly proportional to the pain scores as reported by subjects (Timmermann et al., 2001).

The secondary somatosensory cortex (S2) lies adjacent to S1 in the upper lip of the lateral sulcus. Contrary to S1, the S2 region has bilateral receptive fields – this means that a stimulus on one side of the body will affect both the S2 regions on either side of the brain (Chen et al., 2008). The secondary somatosensory cortex seems to be involved in higher processing of nociception (Treede et al., 2007).

The cingulate cortex is an area situated in the front of the corpus callosum. It is composed of the anterior and posterior sections. The anterior cingulate cortex

receives nociceptive input from the thalamus, particularly from the mediodorsal and parafascicular nuclei (Vogt et al., 1979). It is mainly involved in the emotional aspect of pain (Koyama et al., 2001). In fact, destruction of the area by surgery in rats prevented emotional responses typically shown after painful stimuli, such as paw lifting, licking and flinching (Johansen et al., 2001).

The insular cortex lies deep in the Sylvian fissure, and is involved in awareness, emotional states and regulation of body homeostasis, besides others (Uddin et al., 2017). It receives inputs from the thalamus (Ab Aziz et al., 2006), and direct stimulation of the area results in painful sensations (Ostrowsky et al., 2002).

The role of the cerebrum in the pain pathway cannot be underestimated. The cerebrum acts as a final stage in modulating pain, by increasing or decreasing the sensitivity to a painful stimulus. Hence, the same stimulus may be contrived as painful in an individual under particular circumstances, such as stress or sickness, or non-painful in other circumstances. Patients with chronic back pain showed a decreased activation of the cingulate cortex, prefrontal cortex and nucleus accumbens (Konno et al., 2018).

The psychological status of the patient may influence the level of pain after the procedure, even in acute post-operative pain, such as following a total knee arthroplasty. For instance, in a review of 1,500 patients across six studies, Sorel et al found that patients who had worse scores in mental health scores and who had symptoms of anxiety and/or depression, also showed poorer outcomes after a knee arthroplasty with regards to pain (Sorel et al., 2019).

The cerebrum is the site of action of centrally-acting analgesics, such as paracetamol (Graham et al., 2005).

1.2.3.4.3 Peri-Aqueductal Grey Area and Rostral Ventromedial Medulla

The periaqueductal grey (PAG) area is situated in the midbrain, in the area surrounding the cerebral aqueduct (Ottestad et al., 2013). It is the primary centre that controls the descending pathways that modulate transmission of pain through the spinal cord. Reynolds (1969) showed the potential of performing surgery in rodents solely under analgesia produced by stimulation of the PAG, whilst Richardson and Akil (1977) demonstrated the powerful analgesic effects of PAG stimulation in humans.

The Rostral Ventromedial Medulla (RVM) is found in the midline of the medulla, and sends neurones to the dorsal horn spinal cord neurones. Besides inhibitory fibres, there are also excitatory fibres, which produce descending facilitation of pain (Heinricher et al., 2010).

Both the PAG and the RVM are sites of action for endogenous and exogenous opioids, such as morphine (Fields et al., 1983; Vanegas et al., 2010).

1.2.4 Ion Channels involved in Nociception

A nerve impulse is the result of the interaction between the concentration of the different ions in the neuron. It is not surprising that there are specific ion channels that are involved in signal transduction and propagation of a nerve impulse. Furthermore, pain pathway has unique ion channels that are not present in other sensations.

Mutations of these channels will directly affect the response to pain. It is also possible to modulate the pain response by influencing or modifying the action of these ion channels.

1.2.4.1 Transient Response Protein Calcium Channels

One of the most important proteins in activation of the nociceptor is the Transient Response Protein (TRP) superfamily (Hwang et al., 2007). It is also one of the most studied ion channels involved in sensory neurons, given that these proteins are strongly conserved throughout evolution, from simple nematodes to humans (Harteneck et al., 2000).

The TRP channels regulate passage of calcium ions through the cell membrane, with seven different subfamilies (Moiseenkova-Bell et al., 2009; Li et al., 2011).

TRP channels are activated by a variety of stimuli, with different TRP channels responding to different triggers. For instance, TRPV1 is sensitive to heat, acid environment and pressure. TRPA1 is activated with temperature changes, and TRPC4, 5 are involved in the development of neuropathic pain (Hwang et al., 2007; Jardín et al., 2017).

TRPV1 is one of the TRP channels being most extensively studied. Cloned in 1997, it is the only TRP channel to be activated by capsaicin, which is present in hot chili peppers, and other vanilloids. It is temperature sensitive, and is responsible for noxious heat nociception- it is activated at a temperature of 43^{o}C (Caterina et al., 1997; Tominaga et al., 1998). Besides terminal endings of nociceptors, it is also found in neurons in the

dorsal root ganglion where it may modulate pain stimuli as they pass through the spinal cord. It is also widely distributed in the brain.

Another important member of the TRP family is the TRPA1 channel, which is the only member of the TRP-Ankyrin family present in mammals (Garrison et al., 2011). This channel is responsible for the nociception of cold noxious stimuli and inflammatory damage. It is also stimulated by a variety of compounds, both endogenous and exogenous. For instance, bradykinin, an inflammatory mediator that is produced after tissue damage, is a strong stimulant of TRPA1 (Bandell et al., 2004). The expression of TRPA1 channels in the neurons of the dorsal root ganglion and trigeminal ganglion appears to be coupled to that of the TRPV1 channels.

Both TRPV1 and TRPA1 have been implicated in pathological conditions that influence the perception of pain, such as migraines, inflammatory disorders, cancer and neuropathic pain (Ghilardi et al., 2005; Benemei et al., 2014; Malek et al., 2015).

It has been shown that both volatile and intravenous anaesthetic agents are capable of sensitizing both TRPV1 and TRPA1 receptors (Cornett et al., 2008). This means that a general anaesthetic has the potential of enhancing peripheral nociception, especially in the context of a surgical settings. Even morphine, a strong opioid used in the postoperative period to control pain, has been shown to elicit activity of the TRPV1 and TRPA1 receptors (Forster et al., 2009). This has only been shown in animal research not in a clinical setting, but it is plausible that by avoiding a general anaesthetic and reducing morphine requirements, a neuroaxial anaesthetic might improve outcomes, at least with regards to pain control.

1.2.4.2 Potassium Channels

The TRP family is the most common cation channel being investigated, but there are also other channels that influence pain. Potassium channels comprise the most commonly distributed channel in neurons, with around 78 genes being responsible for four different categories of potassium channels (Ocaña et al., 2004).

These potassium channels are not directly involved in signal transduction. Rather, they are important in regulating the membrane resting potential by facilitating or inhibiting action potential generation (Tsantoulas et al., 2014). Thus the activity of the potassium channels affects the threshold of firing of the neuron.

Voltage-gated potassium (K_v) channels are the most common of the potassium channels, and respond to differences in the membrane potential. Each channel pore is made up of a tetramer of four alpha subunits, with 12 families of these subunits being described. Furthermore, such families each contain a number of variants (Gutman et al., 2005). The K_v channels are responsible for the repolarization of cells, and the different types are reflected in different kinetics of pore-opening (Johnston et al., 2010).

Another potassium channel of note is the TREK-1, coded by the *KCNK2* gene on chromosome 1. It is present abundantly in brain tissue and in the heart (Fink et al., 1996). This channel is sensitive to heat, mechanical stimuli and a number of chemicals, including phospholipids and arachnidoic acid (Alloui et al., 2006). It is also affected by volatile anaesthetic agents, and might explain how such general anaesthetics reduce

the transmission of painful stimuli during surgery (Lazdunski et al., 1999; Patel et al., 2001).

TREK-1 has been shown to open when exposed to various volatile anaesthetic agents. This would have significant effects on membrane polarization and hence modulate neuronal response to nociception.

1.2.4.3 Voltage-Gated Sodium Channels

Once an ionic channel is activated due to a painful stimulus, there is a local depolarization around such channels. These ion channels cannot initiate an action potential directly. This is done via other ionic channels, namely the Voltage Gated Sodium Channels (VGSC).

The VSGC are a group of sodium channels with a common structure of α subunits, associated with smaller β subunits. The α subunits form the ionic channel, and the tetrameric unit is functional even in the absence of the β subunits. In fact, the classification of the different types of VSGC relies on the different types of α subunits, of which there are nine known variants: Nav 1.1 to Nav 1.9. Each of these variants is encoded by a separate gene, denoted as *SCN1A* to *SCN11A* (Catterall et al., 2005).

For every α subunit, there are two β subunits. The type of subunit depends on the channel type and location (Isom, 2001). The β subunits are not essential for function of the VSGC, but serve to modulate its function depending on the need.

The VGSC allows sodium influx into the cell when the cell membrane potential rises to a threshold, which is typically -50mV, from the resting membrane potential of -70mV.

This is sensed by the S4 segment of each homologous domain of the α subunit. The channel passes through three phases: voltage-gated active (or open) phase, an inactive phase, and a resting (or closed) phase.

In the closed phase, the central pore is blocked to sodium passage. Upon depolarization, there is a conformational change that opens the central pore to allow the passage of sodium. This is responsible for the fast upstroke of the action potential. At the peak of the action potential, there is another conformational change. This time, the intracellular loop that binds domains III and IV acts as a hinge, and closes the ionic channel. This stops the depolarization, and allows repolarization to occur (Yu et al., 2003).

With regards to signal amplification at the receptor site, the most important VGSC seem to be Nav1.7, Nav1.8 and to a lesser extent, Nav1.9 (Fang et al., 2002; Djouhri et al., 2003; Gold et al., 2008).

Nav1.8 is the main ionic channel in the nociceptor terminal endings, and in fact it is mainly expressed in nociceptors. It is encoded by the *SCN10A* gene, and is one of the VGSC that is resistant to tetrodotoxin (TTX). Similar to Nav1.9, Nav1.8 has a higher voltage gating threshold, of around -30mV (Elliott et al., 1993). This means that a bigger stimulus is needed before the action potential can be generated. Furthermore, Nav1.8 recovers faster from inactivation (Kwong et al., 2005), and this means that it can sustain more activity than the other VGSC.

Nav1.7 differs from Nav1.8 in a number of ways. It is sensitive to TTX, and recovery from inactivation is slower than with other VSGC, so neurons that preferentially

express Nav1.7 will not be able to fire at high frequencies. It seems that Nav1.7 has a role in determining the resting potential of the terminal endings, and this would mean that it would be involved in setting the threshold for the initiation of the action potential (Cummins et al., 1998). Hence, when activated, it would make the neuron more excitable, or amplify the potentials generated during signal transduction.

After being generated, the propagation of the action potential is dependant on another VGSC, Nav1.6 (Caldwell et al., 2000). This VGSC is present in both neuron fibres responsible for nociception, and in those neurone not involved in the pain pathway. It is present abundantly even in the central neural system, which means that it is not possibly clinically to target this specific VGSC for pain relief. Local anaesthetics target Nav1.6, and this allows the use of peripheral nerve blocks and wound infiltrations.

1.2.5 Secondary Messenger Pathways involved in Nociception

In contrast to ionotropic receptors, metabotropic receptors will activate a cascade of events which will ultimately influence ionic channels in an indirect manner. This influence may facilitate or inhibit the activity of the channel,. These cascades are known as secondary messenger pathways (Siegelbaum et al., 2012).

There are two main types of metabotropic receptors: the G-protein coupled receptors (GPCR) and the receptor tyrosine kinases.

1.2.6 Neurotransmitters involved in Nociception

Once the action potential reaches the end of the neurone, it has to be initiated in the following neurone across the synaptic membrane. This is the role of neurotransmitters, substances that are secreted by the pre-synaptic neuron and which activate the corresponding receptors on the post-synaptic membrane (Figure 1-5).

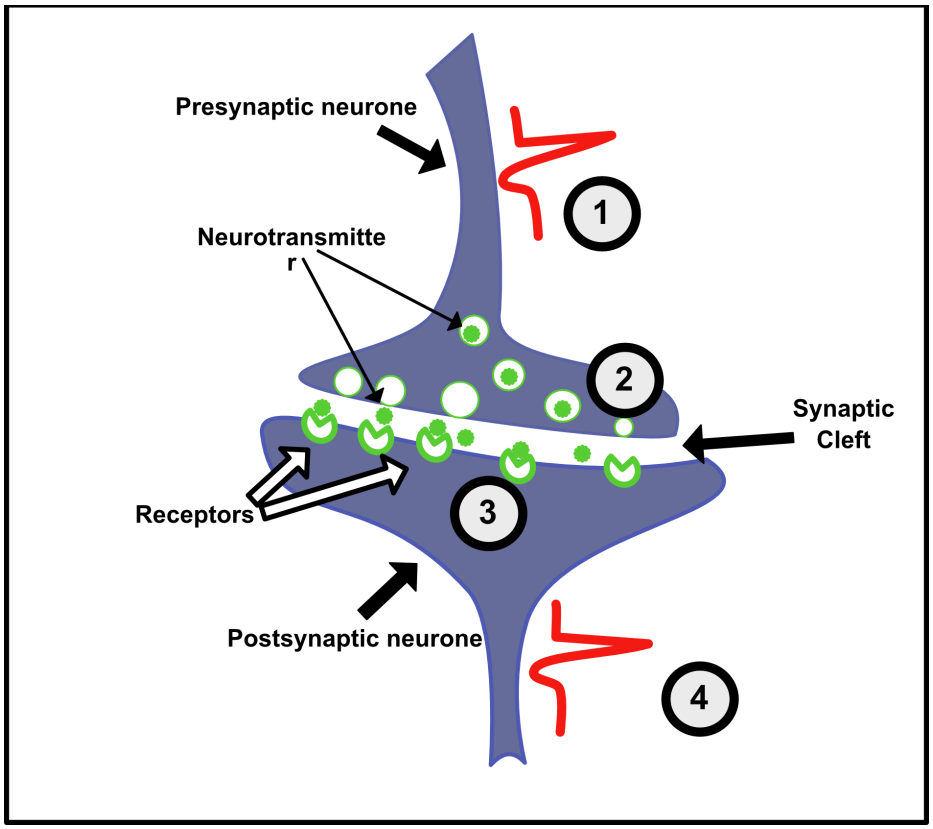

Figure 1-5: The role of neurotransmitters at the synaptic junction. Neurotransmitters are chemicals produced in the neurone, and stored in vesicles at the endplate of the presynaptic junction. Once an action potential arrives at the synapse (1), these are released from the vesicles into the synaptic cleft (2), and the neurotransmitters then bind to the receptors on the post-synaptic neurone (3). An action potential in this neurone is hence generated (4) and allows transmission of pain through the various neurones in the pain pathway.

There are various neurotransmitters involved in nociception, some of which will be described below.

1.2.6.1 Glutamate

Glutamate, an amino-acid, is recognised as being one of the most important excitatory neurotransmitters in the pain pathway. It may act directly on specific ion channels present on the post-synaptic membrane, or on metabotropic receptors that activate intracellular second messenger pathways (Kandel et al., 2012).

There are three ligand-activated ion channels that respond to glutamate: AMPA, kainite and NMDA receptors. These are named after synthetic agonists specific to each channel, namely alpha-amino-3-hydroxy-5-methyl-4-isoxa-zoleproprionic acid (AMPA) receptors; kainate (KA) receptors, and N-methyl-D-aspartate (NMDA) receptors. All three receptors are excitatory: activation causes an action potential to be generated. The AMPA and kainite receptors are usually grouped together as the non-NMDA receptors, as a result of their lack of response to APV (2-amino-5-phosphonovaleric acid), while both are blocked by CNQX (6-cyano-7-nitroquinoxaline-2,3-dione).

1.2.6.1.1 NMDA Receptor

The NMDA receptor is particular in a number of ways (Gonda, 2012). It is one of the receptors that requires the binding of two neurotransmitters simultaneously, glutamate and glycine. The NMDA receptor is both ligand- and voltage-gated: it will only respond to the presence of glutamate when the cell membrane is already depolarized. At rest, a magnesium ion will block calcium ions from flowing through the channel, even if glutamate binds to NMDA receptor in this state (Mayer et al., 1984). The magnesium ion is only displaced when the cell membrane becomes positive, allowing glutamate to activate the NMDA receptor in the presence of glycine. Thus,

NMDA receptors do not start a depolarization but serve to augment and sustain a larger depolarization. For this reason, NMDA receptors are implicated in modulating long-term changes in the pain pathway (Petrenko et al., 2003).

Once activated, the NMDA allows the influx of calcium into the cell, and this leads to activation of a series of intracellular pathways.

NMDA receptors are thought to be involved in excitatory pathways. They are also implicated in excitotoxicity, whereby prolonged activation of the NMDA receptor leads to toxic levels of intracellular calcium that lead to cell death (Zhou et al., 2013). On the other hand, NMDA receptors are necessary for the survival of neurons, with NMDA antagonists causing a decrease in the number of neurones present (Hetman et al., 2006).

Activation of the NMDA receptors in the spinal cord is considered to be of the main pathways for initiation of chronic pain (Woolf et al., 1991). Under normal conditions, magnesium ions block the NMDA receptor to glutamate. However, when intense, sustained release of glutamate and other neuropeptides occurs, sufficient depolarization occurs that overcomes the magnesium block – this would occur during significant tissue damage, such as in surgery. This is the first step towards central sensitization, whereby innocuous stimuli will elicit a painful response.

General anaesthesia is known to affect the NMDA receptor (Petrenko et al., 2014). Ketamine is a well-known intravenous anaesthetic agent that blocks the NMDA receptor. It is also known for its strong analgesic effect. Other anaesthetic agents such

as halothane, isoflurane and nitrous oxide also have an effect, albeit less pronounced, on the NMDA receptor (Yamakura et al., 1993; Hollmann et al., 2001).

Hence, there is a possibility that volatile anaesthetics, such as sevoflurane, might have an effect on the occurrence of post-operative chronic pain.

1.2.6.2 Endogenous opioids

In 1973, Snyder and Pert characterised the opiate receptor in an attempt to elucidate the effect of morphine and heroin on the human brain (Pert et al., 1973). In fact, the discovery of this receptor occurred even before the endogenous ligand had been known about. This led to the discovery by a number of independent investigators of naturally occurring peptides that act as agonists to the opioid receptor in 1975 (Hughes et al., 1975; Simantov et al., 1976). These were named endorphins.

Endorphins are small peptides that are secreted namely by neurones in the central and peripheral nervous system (Sprouse-Blum et al., 2010). These peptides are one of several peptides that act on opioid receptors. Besides endorphin, the other opioid peptides are encephalin and dynorphin. Each opioid peptide has an affinity for a subtype of opioid receptor, and hence each has differing effects.

1.2.6.2.1 The opioid receptor

The opioid receptor is a G-protein coupled receptor, and is linked to inhibitory G-proteins (McDonald et al., 2005). Activation of the receptor leads to a reduced cAMP level in the cell. There is also a hyperpolarization of the cell membrane due to an increased influx of potassium or a decreased efflux of calcium. This makes the neurone

less excitable with a decrease in transmission of nociceptive impulses. There are four types of opioid receptors, classified as MOR, KOR, DOR and NOR depending on their preferential binding to the opioid peptides. Each receptor is coded for by a different gene.

1.2.6.2.2 The MOP receptor

The MOP receptor, previously known as the mu-receptor, is present in the central nervous system, especially in the periaqueductal grey matter (PAG). As described earlier, this area in the midbrain is part of the descending inhibitory control pathway, and is involved in the inhibition of a nociceptive impulse transmitted to the thalamus. It is also present in the spinal cord, in the dorsal horn. It is activated by β-endorphin, and most of the clinically relevant opiates, such as morphine, diamorphine and fentanyl (Dickenson et al., 2013). It appears that the MOP is responsible for the analgesic effects and also for side effects, including respiratory depression, constipation and euphoria. Knockout mice that do not express MOP receptors show decreased latencies for some types of nociception, and a reduced response to morphine when compared to wild-type mice (Sora et al., 1997). It would hence seem that the MOP is responsible for the analgesic effects of morphine when given intravenously (Loh et al., 1998).

1.2.6.2.3 The KOP receptor

The KOP receptor is also widely distributed throughout the central nervous system including the dorsal horn of the spinal cord (Lemos et al., 2011). It is activated by dynorphin, and also by morphine. Although the MOP is responsible for morphine-

induced analgesia when this is administered systemically, the KOP seems to be responsible for the spinal effects of morphine (Yamada et al., 2006). The KOP also mediates some opposing effects to the MOP, such as dysphoria: this is attributed to the different distribution of the various types of opioid receptors.

1.2.6.3 Catecholamines

The roles of catecholamines, namely dopamine, noradrenaline and adrenaline, on pain modulation has been known since the early 20th century, when Weber (1904) applied adrenaline to the spinal cord of the cat. Noradrenaline and adrenaline are the main neurotransmitters in the sympathetic nervous system, but catecholamines are also involved in pain modulation both in the brain and spinal cord.

Catecholamines act on adrenergic receptors, of which there are two types: the alpha receptors, and the beta receptors, both of which are G-protein Coupled Receptors. There are two types of alpha receptors, classified as α1 and α2 receptors, whilst there are three beta receptors, β1, β2 and β3.

Ali *et al* showed that noradrenaline administered peripherally in inflamed tissue aggravated pain, whereas alpha-adrenergic antagonists relieved the pain (Ali et al., 2000). It is also known that α2 receptors are present in the dorsal spinal cord (Shi et al., 1999), namely in descending neurons from noradrenergic nuclei in the brainstem (Jones, 1991). Through the α2 receptors, intrathecal administration of catecholamines reduces nociception (Takano et al., 1992).

Furthermore, catecholamines may modulate pain perception at a cerebral level. Dopamine is influential in setting the emotional state of a person, and this may alter

the reaction to a painful condition such as surgery. In fact, dopamine efflux from the nucleus accumbens in the brain is higher when analgesics are administered (Xie et al., 2014). Furthermore, there is evidence suggesting that there is a central role for dopamine in modulation pain perception and analgesia (Jarcho et al., 2012). Patients who suffer from disorders know to be related to dopaminergic release, such as schizophrenia, Parkinsonism and anxiety states, also suffer from an altered sensitivity to pain.

There are several pharmacological means of altering the noradrenergic system. The most direct way is through agonists and antagonists of the $\alpha 2$ receptors. Clonidine and dexmedetomidine are two agonists of the $\alpha 2$ receptors, and both are well-known to be good analgesics (Bekhit et al., 2015). It is also possible to increase levels of catecholamines by promoting secretion, by amphetamines, for instance. Finally, yet another method of producing antinociception is by reducing breakdown of catecholamines so that their action persists for longer.

Degradation of catecholamines occurs by Catechol-O-MethylTransferase (COMT), and this occurs at the synaptic level. Variations of the COMT enzyme have been demonstrated to effect pain perception. Lower COMT activity is associated with increased catecholamine levels, which causes hyperalgesia through stimulation of β2-receptors (Khasar et al., 1999).

1.3 Acute Postoperative Pain

In 1968, Merskey proposed that acute pain is an unpleasant emotion triggered by any noxious stimulus which results in damage or potential damage to tissue (Merskey, 1968). This was reconfirmed by the International Association of Study of Pain in 1979 (Baranowski et al., 2011).

This implies that pain is a biological mechanism to avoid further injuries. Acute pain is self-limiting and will last only until the receptors are activated. Once the stimulus is over, the acute pain resolves. This is a distinguishing feature from chronic pain.

Surgical procedures are expected to result in pain: Apfelbaum (2003) demonstrated that 80% of postoperative patients suffered a degree of pain, from mild to severe. Unfortunately, despite the amount of literature and various methods of pain control, there is a large proportion of postoperative patients who experience severe pain (Apfelbaum et al., 2003; Gerbershagen et al., 2013; Gan et al., 2014).

1.3.1 Physiological effects of Postoperative Pain

Pain serves an important function in the body (Leknes et al., 2014). Without pain, we would not be able to defend ourselves from potentially harmful situations. However, pain limits a person's activities and reduces quality of life. Inadequate analgesia may lead to reduced mobility in patients after hip surgery (Morrison et al., 2003). Higher levels of pain were associated with longer hospital stays, less chance of being ambulated within 3 days of surgery, and lower locomotion scores at six months.

Besides the sensory component, pain has an influence on the endocrine system (Tennant, 2013). It activates the hypothalamic–pituitary–adrenal axis, the thyroid and the gonadal system. A summary of these effects may be seen in Figure 1-6.

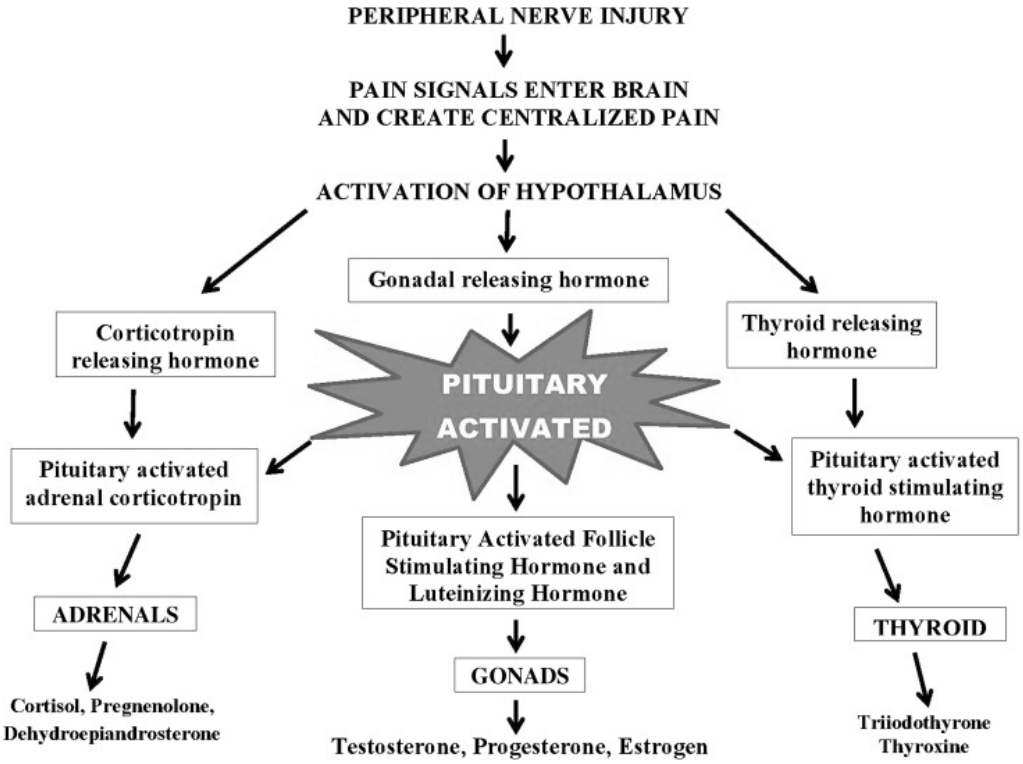

Figure 1-6: Endocrine pathways stimulated when pain is felt. The Hypothalamus is responsible for the widespread activity in the various glands. Reproduced from Tennant F, 2013, under Creative Commons CC BY license.

Given the large number of effects that all these hormones have on the body, there is a large influence of acute pain on various target organs. This includes the stress response, which is a neuroendocrine response, which includes an increased heart rate, increased blood pressure, and catabolism (Kehlet, 1989). It may affect the gastrointestinal system, to produce post-operative ileus (Gan, 2017). It also includes an immulogical response, since pain is known to be immunosuppressive (Page, 2005).

Pain has a psychological effect on the person. In a study in patients after ambulatory surgery, sleep has been shown to be disturbed in patients with high pain scores after surgery, with nearly half of the patients reported inability to sleep (Pavlin et al., 2004). Poor pain control is also associated with poorer psychological well being.

If the acute pain persists, it may also develop into chronic pain, and it may be an important factor in the development of the latter type of pain. In fact, severe acute pain is one of the risk factors for chronic pain in a variety of procedures (Kehlet et al., 2006; Wang et al., 2016).

1.3.2 Assessment of Pain

Assessment of the severity of pain is crucial, both in clinical practice and during the conduct of research. However, pain is subjective, which makes it intrinsically difficult to measure directly. Researchers have developed various scores that may be used in order to establish the severity of pain for an individual. These have been validated for different scenarios and populations.

1.3.2.1 Category Scales

The easiest scoring system is through descriptive terms, such as “mild”, “discomforting”, “distressing”, “horrible”, and “excruciating”, as used by Melzack (1971). A variation of such scales may use pictorial representation of facial expressions depicting varying degrees of pain, as shown in Figure 1-7.

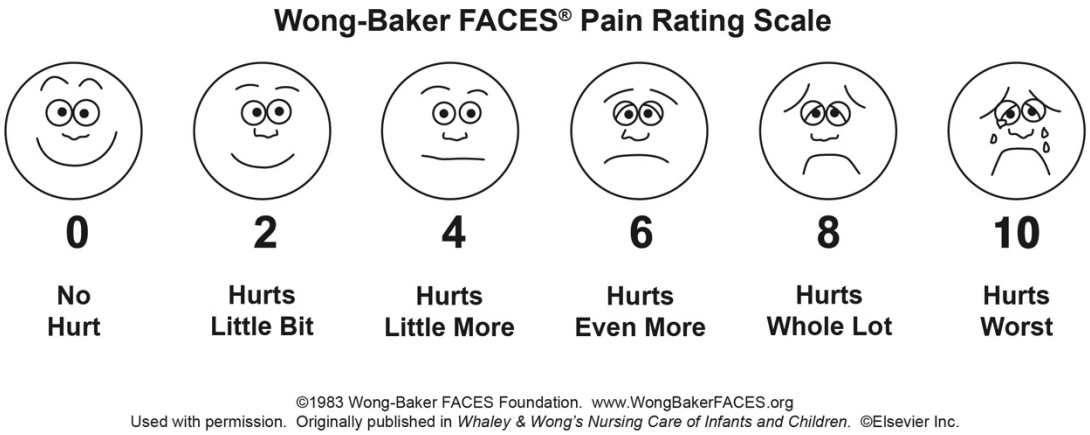

Figure 1-7: Wong Baker Pain Scale. Reproduced with permission from wongbakerfaces.org

Such systems are easy to use in persons who are not able to appropriately scale their pain, such as in children or the elderly. However, categorical scales or pictorial scales do not always show good agreement between different scales (Miró et al., 2016). This may be due to the way in which the tool is presented: Chambers et al showed that children tend to score a higher pain score when the lower end of the scale depicted smiling faces (Chambers et al., 1999).

Furthermore, since the scale is not continuous, statistical analysis is limited to non-parametric tests.

1.3.2.2 The Numerical Rating Scale

The Numerical Rating Scale (NRS) is a simple but effective of measuring pain, including postoperatively (Breivik et al., 2008). It involves asking the patient to rate their pain on a scale from 0 (no pain) to a maximum of 10 (worst pain). Although quite simple, the NRS has shown good correlation with other scores (Jensen et al., 1986). Since it may also be recorded verbally, it is useful for telephone interviews.

1.3.2.3 The Visual Analogue Scale

The Visual Analogue Scale (VAS) uses a graduated horizontal line, usually 100mm to 150mm, with one anchor labelled as “no pain”, and the other anchor labelled as “worst pain”, as shown in Figure 1-8 (Richard Chapman et al., 2001). The patient then marks a point on this line that would correspond to the pain felt at the time.

Figure 1-8: Visual Analogue Scale for pain. Patient marks on the line their perceived pain intensity, which is then measured from the left margin in millimetres.

A VAS score of more than 30mm would correspond to a pain that is usually described as moderate, and a score of more than 70mm would be described as considerable (Bodian et al., 2001).

The VAS score correlates well with the NRS score, and is also easy to use. However, it may be considered more tedious to implement, as the interviewer needs to measure the difference in millimetres of the marked line from the origin of the line. Since it requires paper and pen, it cannot be used over the phone (Hawker et al., 2011).

1.3.2.4 Morphine Consumption

Morphine consumption may be used as a surrogate to assess pain postoperatively – after all, a patient who is suffering from severe pain is more likely to need higher opiate consumption. However, patients who are receiving opiates would report lower pain scores, and this might complicate the interpretation of any study involving pain relief (Dai et al., 2013). Hence a composite parameter involving both pain scores and morphine consumption would seem ideal.

1.3.3 Pathophysiology of Postoperative Pain

Although similar in nature to non-operative pain, the mechanisms that underlie pain following a surgical incision may be different than other forms of nociception (Pogatzki-Zahn et al., 2017). Banik showed that in incised mouse plantar skin, there was an increased spontaneous activity of C-fibres, and increased heat sensitivity (Banik et al., 2008). Moreover, a skin incision on its own is enough to induce heat and mechanical hyperalgesia. Xu et al (2009) performed studies on rats involving a skin incision, a skin incision and deep tissue injury and sham surgery without any incision. Rats who had a skin incision showed behavioural signs of mechanical hyperalgesia, even without deep tissue damage. Guarding behaviour was only observed in those rats who had deep tissue damage.

Comparable results have been obtained in human studies. Kawamata et al (2002) performed incisions in human subjects, and recorded the intensity and duration of the pain thus produced. Pain was maximal during the incision itself, but after 30 minutes, the pain subsided. Despite the reduction in pain, there was still a reduced threshold for

mechanical stimulation of the area around the incision which resulted in secondary hyperalgesia. This was evident as early as 15 minutes after the incision, and lasted for up to 2 days.

In another study, Dorr et al (2007) looked at patients undergoing hip arthroplasty, half of which had the procedure done in a minimally invasive approach. In this study, 231 patients were split into two groups, one with a small skin incision (10cm) and another with a longer skin incision (20cm). The surgery performed in the small incision group was also more conservative, with less cutting of muscle and joint capsule. At the end of the procedure, the skin incision in the minimally invasive group was extended to 20cm, so that both groups had a similar skin incision. The patients who had the surgery done in a minimally invasive approach had better outcomes, and could be discharged significantly earlier. It was postulated that these patients had less injury to the deeper structures, and that this was responsible for the better outcomes.

Such studies indicate that any surgery will result in a degree of pain, and that this intensity of pain is related to the extent of tissue damage during surgery.

Besides the morbidity caused by acute pain, another danger is the risk of such pain persisting for more than would be expected and become chronic. in a meta-analysis of studies investigating the effect of acute pain on the incidence of chronic pain, Mei et al (2015) found that patients with severe acute postoperative pain had more than three times the risk of developing chronic pain. In a study by Brandsborg (2012) using data from 765 patients, severe acute postoperative pain was one of the main risk factors for chronic pain. Thomazeau et al investigated potential risk factors for the development of chronic postsurgical pain (CPSP) after total knee replacement. In this study of

around 100 patients, the incidence of CPSP was 28.8%, and the main perioperative factor associated with CPSP was the severity of acute pain after surgery (Thomazeau et al., 2016).

It is still not clear however, if reducing acute nociceptive pain after surgery allows for less development of chronic pain, or if there are other factors involved.

1.3.4 Pharmacological treatment for Postoperative Nociceptive Pain

Any drug that influences the pain pathway will be able to induce analgesia. However, the benefits gained would need to outweigh the risks. Hence, it is appropriate to start with simple analgesics and then proceed to stronger forms of analgesia if required.

1.3.4.1 Paracetamol

First used clinically in 1893 by von Mering (1893), paracetamol only gained popularity in the 1970's, especially for the treatment of pain and fever. Paracetamol is similar to NSAIDs: it inhibits the cyclooxygenase enzyme (COX) that is responsible for the production of prostaglandins (Flower et al., 1972). This explains the antipyretic effect of paracetamol. However, there are some peculiarities that do would not completely explain the analgesic effect of paracetamol in terms of its COX inhibition (Graham et al., 2005). Paracetamol is not a strong anti-inflammatory, and seems to exert its effect mainly in the brain itself. Högestätt et al (2005) demonstrated that paracetamol is metabolised to AM404 in the brain, which is a potent inhibitor of the TRPV1. AM404

also acts on cannabinoid receptors in the brain, and antagonism of the CB1 receptor inhibits the analgesic properties of paracetamol (Ottani et al., 2006).

Paracetamol is an effective form of analgesia following surgery. Toms et al (2008) showed that when given alone, paracetamol provided relief in half of the patients, for at least four hours. This resulted in a Number-Needed-to-Treat (NNT) of 3.6, with minimal side effects. In separate studies, Cakan and Shimia also showed an effect of paracetamol on patients after surgery, but without any difference in the consumption of morphine (Cakan et al., 2008; Shimia et al., 2014).

When compared to other forms of analgesia such as NSAIDs, paracetamol appears to be somewhat weaker (Hyllested et al., 2002). Its role in major surgery appears to be as an opioid-sparing agent.

1.3.4.2 NSAIDS

Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) are a diverse group of compounds that do not act like steroids or opiates. The first use of NSAIDs includes a description by Hippocrates, who used powdered willow bark to treat fever and pain. This contains salicylic acid, which was the first prototype of a NSAID. In 1897, Hoffmann synthesized acetylsalicylate, which was marketed in 1899 by Bayer as Aspirin (Sneader, 2000).

The main action of NSAIDs is the inhibition of cyclo-oxygenase (COX), which is an enzyme involved in prostaglandin synthesis (Cashman, 1996). The cyclo-oxygenase pathway is responsible for the production of cyclic prostaglandins such as PGD2, PGE2, PGH2 from arachnidoic acid. These prostaglandins are involved in the sensitization of terminal nerve endings. Since Prostaglandin E2 is the most predominant eicosanoid in

inflammatory conditions, the inhibition of COX with NSAIDs has a strong effect on inflammation and pain.

There are three forms of COX enzyme. COX-1 is expressed in non-inflammatory cells, such as platelets and gastric cells. On the other hand, COX-2 is found in inflammatory cells, including in the central nervous system. It is mainly COX-2 that is responsible for the analgesics properties of NSAIDs. COX-3 is a splice variant of COX-1, and is the site of action of paracetamol. The various NSAIDs may be classified as COX-1 specific, COX-2 specific or non-specific, depending on their specificity to the different COX enzymes.

The side effect profile of NSAIDs may be explained by their action on the COX enzyme. COX-1 is involved in a number of normal physiological functions in the body, including renal function, gastric mucosa protection, pulmonary airway regulation and platelet function. Hence, it is not surprising that COX-1 specific NSAIDs or non-specific NSAIDS will cause more peptic ulcer disease, or bleeding tendencies. COX-2 is also involved in some physiological functions such as renal function, but is more associated with analgesia and cardiac function. NSAIDs that are more COX-2 specific will have less bleeding but an increased risk of cardiac disease (Brogan et al., 2013).

Diclofenac is a NSAID of the phenylacetic acid class, and is one of the most commonly used NSAIDs worldwide (McGettigan et al., 2013). Like other NSAIDs, diclofenac shows anti-inflammatory, analgesic, and antipyretic properties (Altman et al., 2015). It is generally considered to be a non-specific COX inhibitor, however, laboratory studies show that it has more COX-2 inhibitory action than COX-1, and may be comparable to celecoxib, a typical COX-2 inhibitor (Grosser et al., 2006).

For post-operative pain relief, diclofenac appears to have a stronger effect than paracetamol. Jhuma Biswas et al (2014) showed that when administered to patients undergoing major gynaecological procedures, diclofenac was superior to paracetamol. Furthermore, the combination of diclofenac and paracetamol together was not superior to diclofenac. The NNT for diclofenac is around 2 (Collins et al., 2011). Hovorka et al (1993) showed that diclofenac administered peri-operatively reduced opioid analgesic requirements after gynaecological laparoscopic day-care. It also has an opioid-sparing effect in major surgery, with a magnitude of around 50% (Fayaz et al., 2004). This reduction in opioid use was also noted by Silvanto: following knee arthroplasties, patients receiving diclofenac had a reduced oxydocone use when compared to placebo (Silvanto et al., 2002).

1.3.4.3 Opiates

Opiates offer very good analgesia, at the expense of more side effects. This was known even as early as 3000BC, when opium poppy seeds were used for pain relief (Brownstein, 1993). Morphine, a naturally occurring opium alkaloid, was isolated in 1805, and is still the commonest opiate used in clinical practice.

Nowadays, there are a number of classes of opiates. Morphine is a natural alkaloid opiate, as is codeine. From these natural opiates, semi-synthetic opiates may be produced, such as diamorphine, dihydrocodeine and oxycodone. Completely synthetic opiates are also available: the most common class is the phenylpiperidines, such as fentanyl, alfentanil, sufentanil and remifentanil (Blakemore et al., 2002).

Opiates are available in a number of preparations and may be given in any mode possible: orally, intramuscularly, subcutaneously, intravenously, and also dermally or intranasally. This increases flexibility in offering an analgesic regimen that is tailored to the individual patient.

The main action of opiates is analgesia, but they also cause sedation and euphoria. Side effects include respiratory depression, nausea and vomiting, constipation, urinary retention and pruritus.

Such analgesics act upon the opioid receptors, which have been previously described (section 1.2.6.2.1 above). The main receptor involved in analgesia is the MOP. There is a high density of such receptors in the peri-aqueductal grey area (PAG) and rostral ventral medulla (section 1.2.3.4.3 above) (Fields et al., 1983; Vanegas et al., 2010), and also in the substantia gelatinosa of the spinal cord. Activation of these receptors causes activation of descending inhibitory pathways that then reduces the intensity of a nociceptive impulse from the peripheries to the brain.

1.3.4.4 Patient Controlled Analgesia (PCA)

Patient controlled analgesia allows a patient to control the administration of a bolus of analgesic without nursing intervention (Garimella et al., 2013). The analgesic most commonly used would be morphine administered intravenously with a syringe pump.

The analgesic efficiency of PCA methods over non-PCA forms of analgesia is debatable. Older studies had demonstrated better patient satisfaction with PCA, although the total dose of morphine given would have been the same (Ballantyne et al., 1993). However, newer evidence does not support a big difference in outcomes. A Cochrane

review in 2015 showed only a modest improvement in pain scores, with VAS score being around 10 points lower (out of 100) with a PCA (Mcnicol et al., 2015).

1.4 Chronic PostSurgical Pain

Once pain persists for three to six months after surgery, then the patient may be suffering from chronic postsurgical pain (CPSP) (Werner et al., 2014). The new ICD-11 classification requires the following criteria for a diagnosis (Schug et al., 2019):

1) Pain persisting at three months after surgery (some authors recommend six months)
2) The pain is a continuation of acute postoperateive pain or may develop after an asymptomatic period
3) Other causes for such pain (for example, infection, malignancy) should be excluded
4) The pain should be localised to the surgical area or to a referred area

Furthermore, CPSP is classified into subdiagnoses, dependant on the surgical procedure leading to such chronic pain. One of the seven listed procedures is in fact arthroplasty.

1.4.1 Incidence of CPSP

The overall incidence of CPSP is around 40 - 45% at six months (Sansone et al., 2015; Fletcher et al., 2015), with 15% of patients reporting pain even 2 years after surgery (Simanski et al., 2014). The incidence varies with the type of surgery, with amputations being the more likely to cause CPSP, as shown in Table 1-4 (Schug et al., 2017).

Type of surgery	Incidence of all CPSP	Incidence of severe CPSP (>5/10 of 10/10)	% neuropathic pain in CPSP
Abdominal surgery (bowel and colorectal)	17%-21%	Not reported	Not reported
Amputation	30%-85%	5%-10%	80%
Caesarean delivery	6%-55%	5%-10%	50%
Cholecystectomy	3%-50%	Not reported	Not reported
Craniotomy	7%-30%	25%	Not reported
Dental surgery	5%-13%	Not reported	Not reported
Hip arthroplasty	27%	6%	1%-2%
Inguinal herniotomy	5%-63%	2%-4%	80%
Knee arthroplasty	13%-44%	15%	6%
Melanoma resection	9%	Not reported	Not reported
Mastectomy	11%-57%	5%-10%	65%
Sternotomy	7%-17%	Not reported	Not reported
Thoracotomy	5%-65%	10%	45%
Vasectomy	0%-37%	Not reported	Not reported

Table 1-4: Incidence of chronic postsurgical pain depending on surgical procedure. Adapted from (Schug et al., 2017)

A number of factors that might influence the development of CPSP have been studied (Burke et al., 2009; Katz et al., 2009; Bruce et al., 2011). A younger age seems to predispose towards a higher incidence of CPSP after some types of surgery (Smith et al., 1999; Gjeilo et al., 2010). Female gender also seems to be associated with CPSP (Kalkman et al., 2003).

Different studies agree on one specific risk factor: preoperative pain. Across different studies, preoperative pain scores are associated with CPSP at six and twelve months (Liem et al., 2003; Lewis et al., 2015; Thomazeau et al., 2016; Rice et al., 2018).

Another important factor appears to be the intensity of acute pain during the early postoperative period (Hanley et al., 2007; Burke et al., 2009; Brandsborg, 2012; Mei et al., 2015; Rice et al., 2018). In fact, in some studies, it was the only factor that could predict CPSP (Katz et al., 1996).

It is not surprising that preoperative pain and acute postoperative pain might be implicated in the development of CPSP. Various reasons have been postulated, including physiological responses to such pain (see below). Psychosocial influence, such as anxiety and pain catastrophizing, has also been implicated in CPSP. Pain catastrophizing is defined as the tendency to magnify the threat from pain stimulus, to feel helpless in the context of pain and to ruminate about the pain experience (Sullivan, 2009).

Genetics also seem to play a role in CPSP. If a patient is already genetically predisposed to have a lower threshold for pain, CPSP will be more likely. It would also explain the relationship between pre-operative, acute post-operative and CPSP.

Several genes have been implicated in the development of CPSP. Examples of such genes has already been discussed, but these include *COMT*, *OPRM1*, *KCNS1*, *GCH1*, *OPRK1* and *SCN9A*.

1.4.2 Neuropathic Pain

Along tissues, nerves may also be damaged and injured. Intra-operative nerve damage may be due to nerve stretching, ischaemia, compression or direct cutting, and leads to a dysfunction of the sensory axons (Woolf, 2004; Devor, 2013). This results in axonal damage that ranges from disruption of axoplasmic transport to complete transection of the axon, or demyelination of the neurone.

Nerve injury may increase excitability of the neurones due to upregulation of voltage gated sodium channels (England et al., 1996) and down regulation of potassium channels in such neuromas (England et al., 1998). Ectopic activity of the neurone will occur, with spontaneous activity of the damaged nerve. This leads to symptoms of hyperaesthesia, such as feelings of numbness, tingling sensations, and paraesthesia.

In instances where the nerve is severely damaged, the proximal end seals off. Small ends start to sprout in an attempt to restore nerve connections. When this is not possible, these sprouts form a tangled knot of connections, known as a neuroma (Nikolajsen et al., 2010). A neuroma is an unorganised growth of nerve fibres which may create and transmit impulses which the nervous system may interpret as pain.

The association between nerve injury and CPSP is well established. A classic example is in limb amputations where the sciatic nerve has to be cut. In fact, limb amputation is the surgery that is most associated with CPSP.

In cases of nerve injury, neuronal sensitization and ectopic discharge is not limited only to the peripheral site of injury. The Dorsal Root Ganglion (DRG) and other uninjured nerves also undergo changes, with a concomitant increase in the number of voltage

gated sodium channels (Wu et al., 2001; Amir et al., 2005). This perpetuates the generation of neuropathic pain.

As neuropathic pain does not present in the same manner as nociceptive pain, it may be more difficult to diagnose, especially in the acute postoperative period (Searle et al., 2012). Treede et al (2008) offered a new definition of neuropathic pain, and a means of assessing the probability of a patient having neuropathic pain. This is shown in Figure 1-9.

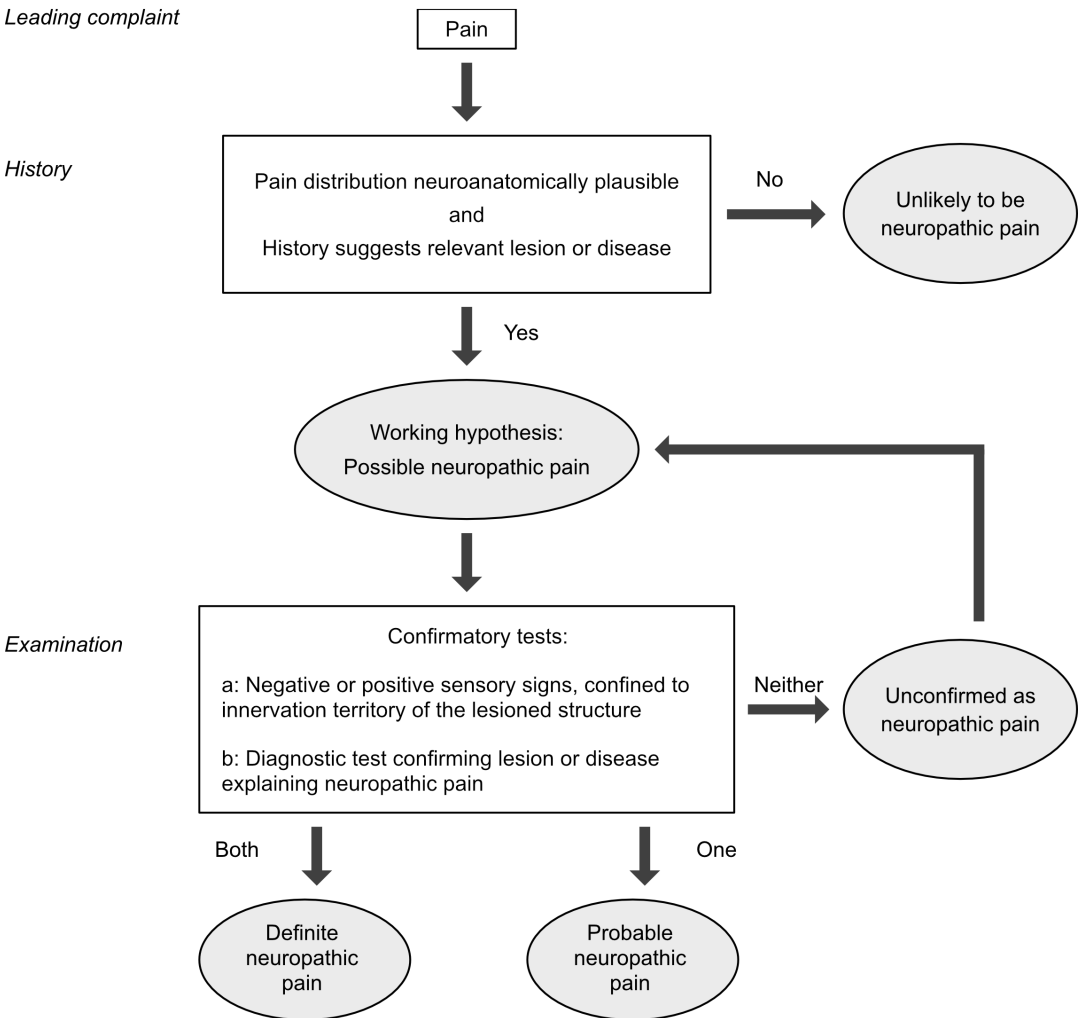

Figure 1-9: Flow chart for assessing probability of neuropathic pain. Adapted from Treede et al., 2008

1.4.2.1 Screening tools for Neuropathic Pain

Over the past few years, a number of tools have been used to help the researcher in screening for neuropathic pain (Bennett et al., 2007). The most commonly used of these tools include the Leeds Assessment of Neuropathic Symptoms and Signs (LANSS) (Bennett, 2001), the Neuropathic Pain Questionnaire (NPQ) (Krause et al., 2003), and the Douleur Neuropathique en 4 questions (DN4) (Bouhassira et al., 2005).

The LANSS questionnaire is the earliest of these questionnaires, and has been validated in a number of settings (Bennett et al., 2007). It is made up of two parts: a patient questionnaire with five questions and a simple clinical examination. It also exists in a self-reported version, the S-LANSS, where the patients check themselves for pain on light or deep touch (Bennett et al., 2005) (Appendix D).

Although the S-LANSS version has not yet been validated in Maltese, it has been translated and validated into several other languages, including Spanish, Greek and Arabic (Batistaki et al., 2016; Garoushi et al., 2017; López-de-Uralde-Villanueva et al., 2018). It has been used in post-operative or non-operative neuropathic pain, including cancer-related pain. It is important to note that a high score on the S-LANSS questionnaire does not imply a definitive diagnosis of neuropathic, but rather makes such a diagnosis highly probable.

The S-LANSS has been used to evaluate neuropathic pain after a knee arthroplasty by Fitzsimmons et al (2018). More than a third of the patients included in this study had a score suggestive of neuropathic pain at baseline. After surgery, nearly 25% of patients had neuropathic pain, with 11% of patients developing new onset neuropathic pain at

six months. Furthermore, in a study by Razmjou et al (2015), patients who had scored high on the S-LANSS had worse outcomes, such as more stiffness, depression and pain.

1.4.2.2 Incidence of Postoperative Neuropathic Pain

The incidence of postoperative neuropathic pain varies between different types of surgery (Table 1-5) from 7% to 70% (Haroutiunian et al., 2013; Tiippana et al., 2016). Fuzier reported that in a follow-up of over 2,000 orthopaedic patients, up to 43% of patients suffering from chronic pain have a strong neuropathic component (Fuzier et al., 2015).

Surgery	Chronic Postoperative Pain	Neuropathic pain
Thoracic surgery	37%	66%
Breast Surgery	41%	68%
Groin Hernia Repair	12%	31%
Hip or knee arthroplasty	27%	6%

Table 1-5: The incidence of chronic postoperative pain in various types of surgeries, together with the proportion of patients with neuropathic component for such pain. Adapted from Haroutiunian et al, 2013

1.4.3 Pathophysiology of CPSP

At first, it would seem logical to assume that CPSP is a result of acute postoperative pain that lingers on. However, tissue injury at three months should be minimal and would not be enough to explain the occurrence of CPSP. Still, it is important to note

that chronic post-operative pain evolves from acute pain, and that there is a transitional process involved (Katz et al., 2009). To paraphrase: “All chronic pain was once acute, but not all acute pain becomes chronic.” (Shipton, 2011)

Acute pain does not only elicit response in the brain but also affects the pain pathway itself (Scholz, 2014). Peripheral sensitization occurs when there is a prolonged inflammation at the site of injury. Chemical mediators such as Substance P, bradykinin, prostaglandins and cytokines, are released during prolonged inflammation. These cause an upregulation of voltage-gates sodium channel and a decrease in activation threshold (Basbaum et al., 2009).

At the level of the spinal cord, dorsal root ganglions also become more excitable. This is known as central sensitization, and this process involves NMDA glutamate receptors. It is an example of neuronal plasticity, and may be temporary or permanent. Usually, stimuli from uninjured tissues causes a small activation of such DRG neurons, but in the presence of prolonged, intense stimuli, NMDA receptors become activated and amplify the response (Bennett, 2000). Furthermore, NMDA receptor activation also increases synaptic activity, so that central sensitization results in a wider area of tissue that becomes more sensitive to lower degrees of pain.

Central sensitization also occurs in the brain itself, and this will cause diminished inhibition of the pain pathway.

1.4.4 Impact of CPSP

Besides pain, CPSP also negatively impacts patients' lives. In a study of 110 patients, Kinney et al (2012) observed reduced physical functioning and vitality in those patients with higher pain scores at three months after a thoracotomy. This is consistent with other studies which show how CPSP interferes with daily activities (Montes et al., 2015; Veal et al., 2015) and sleep (VanDenKerkhof et al., 2012).

CPSP could lead to an increased use of analgesics that may have potential adverse effects, especially if opioids are required to control pain.

Finally, chronic pain carries a cost to the society. There is an economic burden of pain-related costs, including treatment, hospitalizations and lost productivity that is difficult to quantify. Surgery accounted for 22.5% of all patients seen in 10 chronic pain clinics across North Britain (Crombie et al., 1998). A previous paper, published in 1996 by Labat, estimated that each patient suffering from chronic pain may cost as much as $1 million (Cousins et al., 2000).

1.4.5 Reducing the risk of CPSP

There are a number of non-modifiable factors that predispose to CPSP, such as age, gender, genetics, type of surgery. There are also numerous treatments that have been investigated in preventing the development of CPSP (Katz et al., 2009; Gan, 2017).

Modified surgical techniques that aim to reduce tissue and nerve injury have been attempted in order to reduce CPSP. For instance, laparoscopic procedures may be

beneficial, but the effect of laparoscopic surgery on CPSP is debatable (McCormack et al., 2003; Brandsborg, 2012).

Since inflammation is a strong influence for peripheral sensitization, anti-inflammatory drugs such as NSAIDS might be of use. In fact, NSAIDS are a mainstay of a multimodal approach for treatment of acute pain. Unfortunately, current evidence does not show a reduction of CPSP when NSAIDS are used peri-operatively (Clarke et al., 2015).

Acute postoperative pain is associated with CPSP. It seems intuitive that controlling such pain would reduce CPSP, but there are not a lot of good quality studies to assess such a hypothesis. In a small study involving 65 patients, Karanikolas et al (2011) did show that optimization of pain relief after an amputation reduced pain at six months, when compared to a more conventional approach.

Other drugs are being used in order to reduce neuropathic pain and CPSP. These include NMDA antagonists such as ketamine, anticonvulsants such as pregabalin and gabapentin, and antidepressant drugs. The evidence is however not convincing on any specific agent (Clarke et al., 2015).

Finally, locoregional and neuraxial anaesthesia, such as peripheral nerve blocks and epidural analgesia, have been studied (Thapa et al., 2018). By preventing intense stimulation of the DRG in the spinal cord, central sensitization may be reduced. There is considerable evidence to support the use of such techniques in reducing chronic pain. A Cochrane review shows the benefit of thoracic epidural analgesia for thoracotomy and breast surgery (Andreae et al., 2013).

It is plausible that no single intervention will be as useful as a combination of treatments. Furthermore, with so many factors involved, such as different phenotypes, it might be necessary to tailor treatment to the individual patient. For this reason, a pre-emptive approach may be more useful in an earlier detection of CPSP, as described by Katz et el (2015) in the Toronto Pain Service Clinic. It is their recommendation that all patients are followed up by the pain clinic, until it is clear that the patients are not suffering from CPSP.

1.5 Genetics of Pain

As discussed earlier, nociception involves various receptors encoded by different DNA sequences. Changes in such genes could play a significant role in nociception by altering the function of receptors and other proteins involved in nociception (James, 2013).

Mutations in a gene may involve three main different mechanisms: base substitution, insertion or deletion (Durland et al., 2022). Single nucleotide changes, or polymorphisms (SNPs) are more frequent than changes involves a series of bases.

In this research project, we shall be focussing on three main pathways that could be affected by different genotypes:

1. Ionic channels involved in the Initialization and transmission of nociceptive impulse
2. Modulation of pain pathway involving cathecolamines
3. Pharmacogenetic response to analgesics

1.5.1 Genetic Variations in Ionic Channels

In this research project, we shall be exploring two ionic channels: the sodium voltage-gated channel (section 1.2.4.3) and the potassium voltage-gated channel (section 1.2.4.2).

1.5.1.1 SCN9A

The nine different voltage gated sodium channels (VGSC) alpha subunits are encoded for by nine genes spread over four chromosomes (Catterall et al., 2005). In particular, one type of VGSC alpha subunit, Nav 1.7, which is implicated in channelopathy-associated insensitivity to pain, is encoded by *SCN9A.*

The *SCN9A* gene is found on chromosome 2 (2q24.3), and is 113.5-kbases long, with 26 exons. A series of mutations have been referenced. Loss of function mutations reduce sensitivity to pain (Shields et al., 2018). In fact, a number of Nav1.7 inhibitors have been looked into as possible analgesics (McKerrall et al., 2018).

The following mutations will be referenced in this study: rs6746030, rs7595255, rs12622743, rs11898284 and rs74449889 (**Error! Reference source not found.**Table 1-6). These are Single Nucleotide Polymorphisms (SNP), which means that there is only one nucleotide change between the wild type and the mutated variant.

Single Nucleotide Polymorphism	Position (GRCh38)	Type	Global Frequency of Minor Allele
rs6746030	Chr 2:166242648	Intron Variant: G to A	11%
rs7595255	Chr 2:166226468	Intron: C to T	11%
rs11898284	Chr 2:166325017	Intron: A to G	16%
rs74449889	Chr 2:166304225	Intron: A to C, G	5%

Table 1-6: The SNP's investigated for SCN9A. Data obtained from (The 1000 Genomes Project, 2015) *G: Guanine, A: Adenosine, C: Cytosine, T: Thymine,*

Estacion et al (2009) demonstrated that the change from the G allele to the A allele at rs6746030 results in a structurally different Nav1.7, which is more excitable. Indeed, rs6746030 has been implicated in higher pain scores in patients with lumbar disc herniation (Kurzawski et al., 2018). In a study of 27 different SNP's of the *SCN9A* gene, rs6746030 was the most influential in over 1,200 patients investigated, including in postoperative pain (Reimann et al., 2010). Specifically in a postoperative setting, Duan et al investigated the role of rs6746030 in the prediction of post-operative pain following gynaecological laparoscopic surgery. The presence of the minor allele of the SNP resulted in a higher Numerical Rating Score (Duan et al., 2016).

The other SNP's investigated have been less researched, and have been chosen on the basis of their potential, since they are intronic variants. rs7595255 was associated with pain in a cohort of 578 patients with osteoarthritis (Reimann et al., 2010). Unfortunately, in other studies, this SNP could not be evaluated due to technical reasons (Greenbaum et al., 2012; Duan et al., 2016). rs11898284 has been shown to be associated with increased heat pain sensitivity (Duan et al., 2015). As for rs74449889, this is one of the SNP's linked with neuropathic pain, especially in diabetic patients (Li et al., 2015).

1.5.1.2 KCNS1

Potassium voltage-gated channels do not participate directly in signal transduction but are important in modulating the resting membrane potential. In this way, such channels either facilitate or inhibit an action potential from being generated (Tsantoulas et al., 2014). Kcns1 is a Kv9.1 channel subunit, which is electrically silent on

its own, but modulates channel properties when combined with other potassium channels (Costigan et al., 2010; Bocksteins, 2016).

Found on chromosome 20 (20q13.12), *KCNS1* is a small gene with around 11,000 base pairs (Cunningham et al., 2022). There are five exons that when transcripted produce Kcns1, one of the many potassium voltage-gated channel proteins (Deloukas et al., 2001). It is expressed mainly in neuronal tissue, with much less activity elsewhere (Fagerberg et al., 2014).

Experimental data shows that mice that lack *KCNS1* suffer from a slight increase in acute pain under normal circumstances, but had an exaggerated response after nerve injury (Tsantoulas et al., 2018). Costigan et al (2010) also looked into neighbouring genes and found that nearly 80% of these were involved in membrane signalling, with nearly half of these associated with nociception. They conclude that *KCNS1* is central to many pathways that are integral to pain perception.

In this study, two mutations of the *KCNS1* gene will be investigated: these will be the single nucleotide polymorphisms rs4499491 and rs734784 (Table 1-7). The focus of this study centres on this particular gene as this has been shown to be associated with increased nociceptive excitability, especially after neuronal injury. Experimental data shows that mice that lack this channel suffer from a slight increase in acute pain under normal circumstances, but had an exaggerated response after nerve injury (Tsantoulas et al., 2018). In humans, changes in *KCNS1* has been associated with HIV-associated sensory neuropathy (Hendry et al., 2013). Furthermore, relevant to this study, changes in *KCNS1* are linked to neuropathic pain even in humans, in a variety of chronic pain

states including post-operative persistent pain (Langford et al., 2014; Tsantoulas et al., 2018).

Single Nucleotide Polymorphism	Position (GRCh38)	Type	Global Frequency of Minor Allele
rs734784	Chr 20: 45094986	Missense: T to C	41%
rs4499491	Chr 20: 45092778	3' UTR variant: C to A, G	47%

Table 1-7: The SNP's investigated for KCNS1. Data obtained from The 1000 Genomes Project, 2015 G: Guanine, A: Adenosine, C: Cytosine, T: Thymine

1.5.2 Modulation of Pain Pathways involving Cathecolamines

As described earlier (section 1.2.6.3), cathecolamines are integral to the modulation of nociception. Levels of noradrenaline, adrenaline and dopamine modulate the transmission of nociceptive impulses through the spinal cord (Takano et al., 1992), and affect the perception of pain in the brain (Jarcho et al., 2012).

1.5.2.1 GCH1

Synthesis of cathecolamines starts by uptake of tyrosine (Fernstrom et al., 2007). This is converted to dopamine by tyrosine hydroxylase, a process that requires tetrahydrobiopterin (TH4). This cofactor is produced by GTP cyclohydrolase 1, which is encoded by the *GCH1* gene.

In human volunteers, subjects who carried polymorphisms of *GCH1* had less pain when a topical high concentration of capsaicin was applied to their skin (Campbell et al.,

2009). In this small study, *GCH1* was shown to be responsible for 35% of the inter-individual response to pain.

Tegeder et al (2006) were the first to describe a pain-protective haplotype made up of 15 polymorphisms in the *GCH1* gene. In a study of 523 patients attending a tertiary care outpatient pain centre, homozygous carriers of this haplotype spent less time on specialized pain therapy (Doehring et al., 2009), although the effect was small. This might be due to the small number of patients who had this haplotype of 15 specific SNPs: only around 14% of patients carried this haplotype, with only 10 subjects being homozygous carriers. Lötsch et al (2007) later reduced this haplotype to three main polymorphisms, including rs3783641. Their work showed that two SNPs predicted the pain-protective haplotype with nearly 100% sensitivity. These SNPs were rs8007267 and rs3783641. We also note that the presence of rs3783641 without rs8007267 occurs infrequently (1.4%), as shown in Table 1-8**Error! Reference source not found.**. We hypothesize that rs3783641 should account for most of the variability in the influence of this haplotype, especially since the linkage disequilibrium between these two polymorphisms is very high (D' = 1.000) (Cunningham et al., 2022).

SNP	change	Haplotypes				
rs8007267*	G > A	G	G	***A***	G	G
rs2878172	T > C	T	T	***C***	***C***	***C***
rs2183080	G > C	G	G	G	***C***	G
rs3783641*	A > T	A	A	***T***	A	A
rs7147286	C > T	C	C	***T***	***T***	C
rs998259	G > A	G	***A***	G	G	G
rs8004445	C > A	C	C	C	***A***	C
rs12147422	A > G	A	A	A	***G***	A
rs7492600	C > A	C	C	C	***A***	C
rs9671371	G > A	G	G	***A***	G	***A***
rs8007201	T > C	T	T	***C***	T	***C***
rs4411417	A > G	A	A	***G***	A	A
rs752688	G > A	G	G	***A***	G	G
rs7142517	G > T	G	***T***	G	***T***	G
rs10483639*	C > G	C	C	***G***	C	C
		31.5%	19.8%	**14.6%**	9.7%	7.6%

*Table 1-8: Pain-protective haplotype of GCH1, as per Tegeder et al. Bold and italics: minor alleles; Light grey shading: SNPs investigated in our study; Dark grey shading: pain-protective haplotype; *SNPs investigated by Lötsch et al.*

In this study, we explore the effect of two SNPs in the *GCH1* gene, as shown in Table 1-9.

Single Nucleotide Polymorphism	Position (GRCh38)	Type	Global Frequency of Minor Allele
rs3783641	Chr 14: 54893421	Intron: T to A	23%
rs998259	Chr 14: 54888313	Intron: C to T	8%

Table 1-9: The SNP's investigated for the GCH1 gene. Data obtained from The 1000 Genomes Project, 2015 G: Guanine, A: Adenosine, C: Cytosine, T: Thymine

Besides its role in nociception, rs998259 is also implicated in atrial fibrillation (Huang et al., 2017) and mood (Hu et al., 2016).

1.5.2.2 COMT

The *COMT* gene on chromosome 22 codes for the enzyme Catechol-O-MethylTransferase (COMT). This enzyme metabolises catecholamine neurotransmitters (dopamine, epinephrine and norepinephrine), by adding a methyl group (Boussetta et al., 2019). COMT itself has been extensively studied as a possible therapeutic target, most notably in Parkinsonism.

The human *COMT* gene was first described by Tenhunen et al (1994). It contains six exons, spanning over around 27,000 base pairs. Two promoters control the transcription of the gene into two different mRNA: MB-COMT and S-COMT. The former is found predominantly in brain neurones, whereas the latter is found more in other tissues such as the liver, kidney and blood.

Over 8,000 single point mutations in the *COMT* gene are currently known. The SNP's that will be investigated in this study are shown in Table 1-10.

Single Nucleotide Polymorphism	Position (GRCh38)	Type	Global Frequency of Minor Allele
rs4680	Chr 22:19963748	Intron: G to A	37%
rs4633	Chr 22:19962712	Intron: C to T	37%
rs4818	Chr 22:19963684	Synonymous variant: C to G	30%

Table 1-10: The SNP's investigated for the COMT gene. Data obtained from The 1000 Genomes Project, 2015 G: Guanine, A: Adenosine, C: Cytosine, T: Thymine

rs4680 causes a structural change in the COMT enzyme, which lowers enzymatic activity. Hence, patients with the A variant will be able to metabolize catecholamines at a slower rate. The two variants are co-dominant, so heterozygous individuals will have an intermediate activity level (Lachman et al., 1996).

Similar to rs4680, rs4633 affects COMT enzyme activity, although polymorphism at this site is not associated with structural changes of the enzyme itself. The T allele is associated with lower COMT activity, and the C allele with the higher COMT activity.

rs4818 is not associated with any structural changes, but polymorphism at this allele is associated with even more variation of the COMT enzyme when compared to rs4680. Patients who are homozygous for the G variant will have increased enzymatic activity. Heterozygous individuals will have intermediate activity, and homozygous individuals with the C variant will have the least enzymatic activity (Barbosa et al., 2012).

The most commonly investigated of such mutations is the rs4680 mutation, also known as the Val 158 Met polymorphism. It has been implicated in more severe low back pain (Jacobsen et al., 2012), in patients with multiple sclerosis (Fernández-de-las-

Peñas et al., 2013), and also in predicting the opioid consumption after surgery (Candiotti et al., 2014). In the case of total knee replacements, Thomazeau et al (2016) found that the rs4680 mutation was more frequent (83%) in patients reporting chronic postsurgical pain, compared with 64% in the other patients. This conferred an odds risk ratio of 3.42 upon multivariate analysis.

Furthermore, studies have shown that certain haplotypes are more commonly associated with chronic pain syndromes. Haplotypes are combinations of different variations: in the case of the *COMT* gene, the most common haplotype describes the combination between rs6269, rs4633, rs4818 and rs4680 (Table 1-11). For instance, Zhang et al showed that patients with the haplotype ACCG had a higher fentanyl consumption than in patients with the haplotypes GCGG or ATCA (Zhang et al., 2015). This effect was not seen when individual SNP's were analysed.

rs6269	rs4633	rs4818	rs4680	COMT activity	Pain	Frequency
G	C	G	G	High	Least Pain	36.8%
A	T	C	A	Intermediate	Intermediate	54.6%
G	C	C	G	Low	Most Pain	7.0%
A	C	C	A	Unknown	Unknown	1.7%

Table 1-11: Various Haplotypes of the COMT gene, with relative COMT activity. Adapted from (Roten et al., 2011).

1.5.3 Pharmacogenetic response to analgesics

1.5.3.1 OPRM1

The MOP receptor is coded by the *OPRM1* gene, found on 6q25.2. A number of variations exist that may influence nociception. The allelic mutations that will be investigated are shown in Table 1-12**Error! Reference source not found.**.

Single Nucleotide Polymorphism	Position	Type	Global Frequency of Minor Allele
rs1799971	6:154039662	Intron: A to G	22%
rs2075572	6:154090869	Intron: G to C	16%
rs495491	6:154061407	Intron: A to G	29%
rs533586	6:154092539	Intron: C to T	25%
rs609148	6:154109880	Intron: G to A	15%
rs563649	6:154086832	UTR variant: C to T	11%

Table 1-12: The SNP's investigated for OPRM1. Data obtained from The 1000 Genomes Project, 2015 G: Guanine, A: Adenosine, C: Cytosine, T: Thymine

The main Single Nucleotide Polymorphism (SNP) of the *OPRM1* that has been investigated is rs1799971. The presence of a homozygous mutation in patients undergoing an elective caesarean section increased the morphine consumption, but did not have an effect on the development of chronic pain (Wang et al., 2019). This was also reproduced in a number of patients undergoing TKA (Chou et al., 2006). On

the other hand, patients who were homozygous for the wild type showed a worse outcome when an opioid was given epidurally (Song et al., 2013).

1.5.3.2 OPRK1

The gene that encodes for the KOP receptor is the *OPRK1*, which is present on 8q11.23 (Table 1-13**Error! Reference source not found.**).

Single Nucleotide Polymorphism	Position	Type	Global Frequency of Minor Allele
rs6985606	8:53248556	Intron: T to C	31%

Table 1-13: The SNP's investigated for the OPRK1 gene. Data obtained from The 1000 Genomes Project, 2015 G: Guanine, A: Adenosine, C: Cytosine, T: Thymine

rs6985606 has been shown to be a risk factor for pre-operative pain in a study of women with breast cancer who underwent breast surgery (Aouizerat et al., 2015). So far, there is no research on variations of *OPRK1* in orthopaedic surgery.

1.5.4 Genotyping

Determining the genotype may be done in a number of ways. The actual method used depends on a number of factors: sample to be used, volume, purity of sample and of course application of genotyping. KWOk P

1.5.4.1 DNA sequencing

First introduced by Padmanabhan (1974), and later refined by Sanger (1977), DNA sequencing involves a process known as 'chain termination method' or Sanger sequencing. It is possible to perform sequencing both manually or in an automated fashion.

There are three basic steps in Sanger sequencing (Dey, 2018; Furutani et al., 2022). The sample DNA is cleaved and denatured, in order to expose the DNA strands. Standard nucleotides are added together with a low dose of modified nucleotides (dideoxyribonucleotides). The former are involved in ampliflication of the sample DNA, whereas the latter do not allow this amplification to continue. This terminates the chain. The addition of modified nucleotides is random, and results in numerous copies of the sample DNA but with various lengths terminated by the 5'-modified nucleotides. Furthermore, the modified nucleotides are also labelled, either using a radionucleotide or a fluorescent label specific to each nucleotide.

The various chains are then separated using gel electrophoresis. This will sort the chains depending on the length of the chain.

Finally, the sequence of the DNA nucleotides may be read by exciting the fluorescent dye using a laser. Since each nucleotide would have been labelled with a specific dye, it would be possible to identify which nucleotide is present at a particular locus.

Sanger sequencing is a proven technology, and indeed has been used in the Human Genome Project (Waterston et al., 2002). It is very efficient, but it requires more

sample volume than other newer sequencing methods. Furthermore, it is slower than other sequencing methods. It is also possible to sequence multiple loci at one go.

1.5.4.2 TaqMan® Assays

Another option is realtime polymerase chain reaction (PCR) , also known as quantitative PCR. This amplifies DNA by using primers, which are short sequences of DNA that bind to a template. DNA polymerase then adds nucleotides to the primer to extend the strand, depending on the sequence of nucleotides of the source DNA (Bell, 1989).

For this research, TaqMan™ probes (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA) were used. These are presented in assays consisting of sequence-specific forward and reverse primers with two TaqMan® minor groove-binding (MGB) probes. These primers are labelled with a FAM™ or VIC™ reporter dye at the 5'end and a non-fluorescent quencher at the 3'end of each one .

The FAM™ and VIC™ reporter dyes are fluorescent at different wavelengths, at 517nm and 551nm respectively, with the FAM™ reporter dye being in the green wavelengths, and the VIC™ reporter dye being in the yellow wavelengths. The presence of the quencher in close proximity to the reporter reduces the luminescence of the dye: this results in luminescence occurring only when the dye and the quencher are separated. This will occur principally when the probe is attached to the expected DNA sequence, and then cleaved by the *Taq* polymerase during amplicon extension step.

Taq polymerase is a thermostable DNA polymerase first isolated from *Thermus aquaticus* by Chien (Chien et al., 1976). It is resistant to the high annealing

temperatures necessary to separate DNA strands, and hence it is not necessary to add DNA polymerase to each cycle. This facilitates automation of the PCR sequencing.

At the end of each PCR cycle, the sample is illuminated with light at wavelengths corresponding to the dye used. If the sequence being investigated is present in the sample DNA, then the primer would have attached to that sequence. *Taq* polymerase would then extend the chain, and in this process separate the dye from the quencher. This would allow flouresence of the dye that has been cleaved to be captured by a detection sensor, and quantified at each cycle.

By quantifying the threshold at which the fluorescent intensity is above a control, the cycle threshold, Ct, is calculated. This is then used to show the presence or absence of a SNP (**Error! Reference source not found.**).

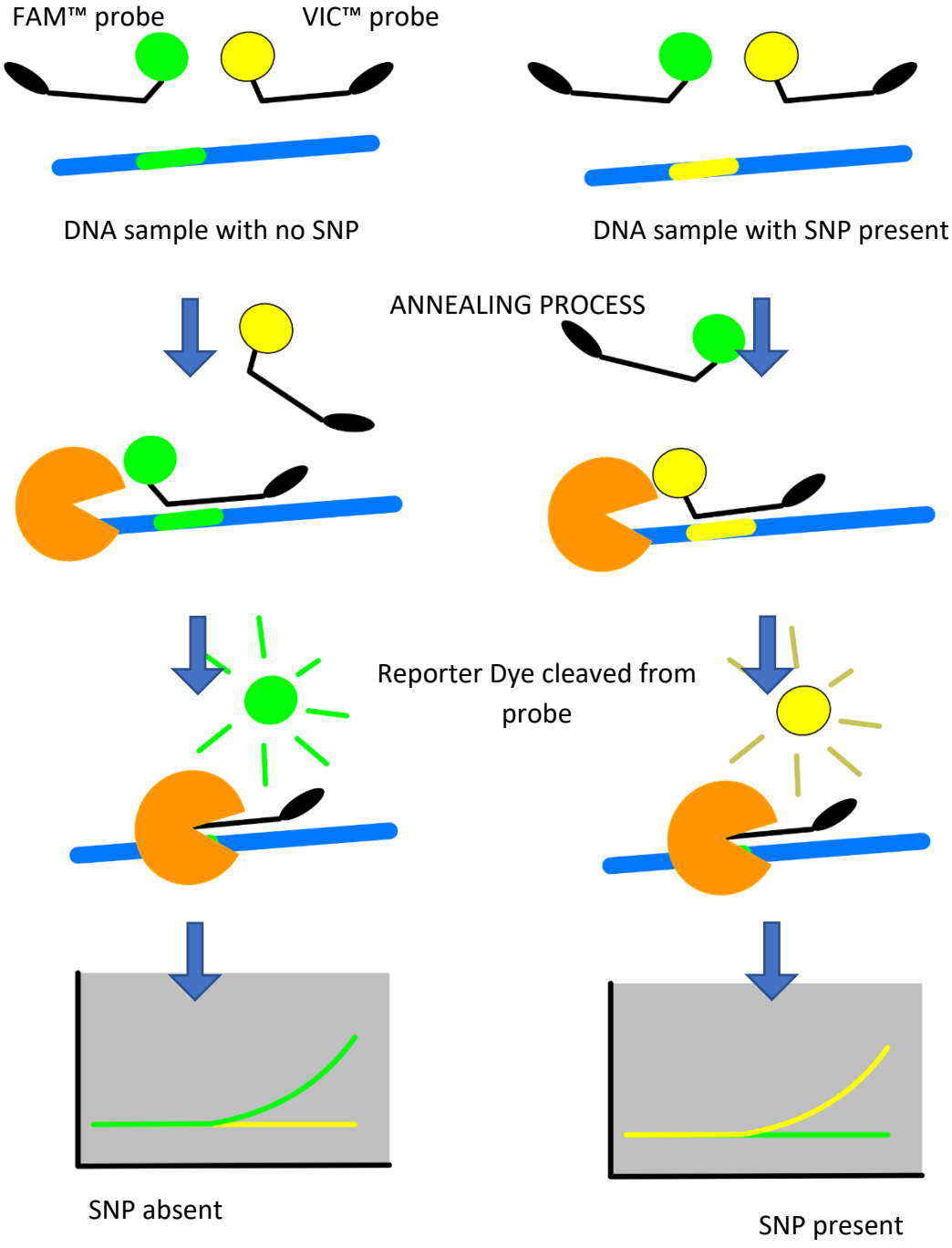

Figure 1-10: Detection of Single Nucleotide Polymorphism using realtime PCR.

Taqman™ genotyping has a number of advantages over Sanger sequencing. First of all, there is no need for gel substrates to analyse the reactions. This requires further manipulation with a possible risk of contamination. It is also much faster: a comparison done by Zhang et al showed that Taqman™ genotyping took around two hours wheareas Sanger sequencing took over 12 hours. The authors also note that TaqMan™ genotyping does not require expensive equipment or a high level of expertise.

There are few issues with TaqMan™ genotyping. *Taq* polymerase has a lower replication fidelity, with an error rate of around 1 nucleotide for each 9,000 nucleotides polymerized (Tindall et al., 1988). Also, the primers are allele specific, and hence can only be used for a particular sequence. For this reason, it is necessary to use at least two primers for each SNP in order to genotype a patient – one primer will attach to the 'normal' sequence of DNA, the other primer will bind to the mutated sequence.

Given the practility of TaqMan™ genotyping over other techniques, this was chosen to be the genotyping method for this research project.

1.6 Osteoarthritis of the Knee Joint

Osteoarthritis is a degenerative disease of the joint, most commonly associated with age. The WHO describes osteoarthritis as a 'a long-term chronic disease characterized by the deterioration of cartilage in joints which results in bones rubbing together and creating stiffness, pain, and impaired movement.' (World Health Organization, 2013) Osteoarthritis of the knee is a common disorder, which has been estimated to effect nearly 27 million people in the United States (Lawrence et al., 2008), and it is the most common indication for performing a total knee replacement (Van Manen et al., 2012).

1.6.1 Pathophysiology

Osteoarthritis is a disease of the chondrocyte, the cell type that produces cartilage. Cartilage is made up of proteoglycan molecules that form an elastic fibrous tissue. In osteoarthritis, the ability of this proteoglycan matrix to retain water is reduced, which makes the cartilage less elastic and more brittle and prone to damage.

1.6.2 Definition

A clear and standard definition of osteoarthritis is surprisingly lacking (Kraus et al., 2015). This is because osteoarthritis may be defined clinically, pathologically or radiologically. Since radiological findings are more objective, this has been used to define and grade osteoarthritis (Zhang et al., 2010). The Kellgren-Lawrence grading system (K/L) is the most commonly used scheme (Ball et al., 1963). It has five scoring grades, from 0 to 4, with osteoarthritis being defined as the presence of a definite osteophyte (Grade≥2) (Table 1-14).

Grade	Radiological Findings
grade 0	no radiographic features of OA are present
grade 1	doubtful joint space narrowing (JSN) and possible osteophytic lipping
grade 2	definite osteophytes and possible JSN on anteroposterior weight-bearing radiograph
grade 3	multiple osteophytes, definite JSN, sclerosis, possible bony deformity
grade 4	large osteophytes, marked JSN, severe sclerosis and definite bony deformity

Table 1-14: Kellgren-Lawrence grading system for Osteoarthritis, adapted from Kellgren et al., 1957*.*

However, not all patients who have radiological evidence of osteoarthritis will be symptomatic. Various studies have shown that pain as experienced by patients does not correlate with the radiological grading (Hannan et al., 2000; Bedson et al., 2008; Finan et al., 2013).

1.6.3 Incidence of Osteoathritis of the Knee

The incidence of osteoarthritis, defined by radiological evidence, has been estimated to be nearly 20% - 30% in adults older than 45 years in the Framingham study (Felson et al., 1987) and in the Johnston County Osteoarthritis Project (Jordan et al., 2007).

Symptomatic osteoarthritis occurs when there is pain, stiffness or swelling in a joint that already shows radiological signs of osteoarthritis. The incidence of symptomatic osteoarthritis of the knee was 16% in the Johnston County study (Jordan et al., 2007).

1.6.4 Scoring systems

It is important to select the appropriate patient and the timing for surgery, since a knee arthroplasty is an invasive procedure and is classified as major surgery (Baker et al., 2009). Before surgery, a non-invasive management approach should be considered first.

However, delaying surgery will have an impact on the patient's activity, even with an aggressive non-surgical approach. Skou et al (2015) demonstrated that there is a significant improvement in pain, quality of life and use of analgesics following TKA, as compared to an intensive non-surgical treatment and patient support, when measured at 12 weeks after the procedure.

A number of scoring systems have been devised to aid the clinician to select the appropriate patients and accurately gauge the timing of such intervention.

1.6.4.1 WOMAC® score

The Western Ontario and McMaster Universities Osteoarthritis Index was developed in 1988 in Canada, by Bellamy et al (1988). It is a questionnaire that aims to assess pain, stiffness and functional deficit in patients with osteoarthritis of the knee.

It consists of three sections, with a total of twenty-four questions, which may be answered on either a 5-point Likert scale, or a 100mm Visual Analogue Scale (VAS). Data may either be collected by an investigator, or by the patient himself. The score will then range from 0 to 96, with a lower score being associated with better outcome (Collins et al., 2011).

Since its introduction, the WOMAC® has been validated in a number of different settings. It has been translated into a number of languages and presented in both paper and electronic formats (Bellamy et al., 2011). It has been evaluated for validity, repeatability and internal consistency, with good results (Thumboo et al., 2001; McConnell et al., 2001). It has been used to assess patients after non-surgical and surgical treatment (American College of Rheumatology, 2015).

The score has some value in assisting the clinician in predicting the need for a total knee replacement. In a study of 1400 patients with established osteoarthritis of the knee, Faschingbauer (2017) found that the the likelihood of needing surgical treatment was 1.91 times higher if the WOMAC® score was greater than 24. Similarly, Hawker et al (2001) determined that a WOMAC® score greater than 39 should be necessary before surgery is considered.

The WOMAC® score is accurate in discriminating between treatment success and failure after TKA, with an AUC of 0.83 (Giesinger et al., 2015). In fact, Walker et al (2018) classified a postoperative 1-year WOMAC® score into four outcome groups: < 30 as excellent, 30 - 45 as good, 46 - 60 as fair and > 60 as poor.

The limitations of the score are few. The score is licensed against a fee, which makes it difficult to use in clinical practice. It requires around 10 minutes per patient to perform the questionnaire. Also, there are different variations in reporting the score, such as in the use of Lickert scales or VAS, and these make interpretation of research using the WOMAC® somewhat more difficult (Woolacott et al., 2012).

1.6.4.2 Oxford Knee Score

Described originally by Dawson et al (1998), the Oxford Knee Score is a screening test that is filled in by the patient, without input from a physician. This may better reflect the patients' experience, and hence be more objective (Jenny et al., 2012).

Like the WOMAC® score, the Oxford Knee Score has been extensively validated in a variety of clinical scenarios. Besides assessing surgery, it has also been used for non-operative treatment of osteoarthritis of the knee (Harris et al., 2013).

1.6.4.3 Knee injury and Osteoarthritis Outcome Score (KOOS)

The Knee injury and Osteoarthritis Outcome Score (KOOS) is an extension of the WOMAC® score designed by Roos et al (1998). It expands the WOMAC® to make it relevant for a younger age group and for knee injuries. It also aims to include short-term consequences of knee injuries, unlike the WOMAC, which is focused more on the long-term disabilities (Roos et al., 2003).

The KOOS has 42 questions, grouped into five categories: pain, symptoms, activities of daily living, sport and recreation function, and knee-related quality of life. Scores are transformed to a 0–100 scale, with a lower score representing extreme knee problems.

It has been translated into several languages, and has been validated in a number of settings.

The main disadvantage of the score is that it is time-consuming. It does not require a licence to use, and is freely available in a number of formats (Peer et al., 2013).

1.6.5 Treatment of Osteoarthritis of the Knee

Unless the symptoms of knee osteoarthritis are very mild, patients would be expecting some form of treatment. The most common complaint is pain, but stiffness is also a common ailment. Although there are no disease modifying treatments as for osteoarthritis, it is still possible to control both pain and stiffness using non-pharmacological and pharmacological treatments.

1.6.5.1 Surgical Treatment

In cases where daily function is hindered, or in cases of severe pain, analgesics may not be enough. Hence, surgery in the form of either a tibial osteotomy, unicondylar knee replacement, or a total knee replacement may be indicated.

There are yet no universally agreed criteria that must be met before a total knee arthroplasty is considered. Escobar et al (2003) used a Rand approach to develop criteria that would help the clinician decide when a patient with osteoarthritis of the knee would benefit most from surgery. This is shown in Table 1-15, and is based namely on radiological features, and mobilization of the patient.

Age	Radiology
<55 years 55 to 65 years > 65 years	Slight (Ahlbäck grade I) Moderate (Ahlbäck grades II and III) Severe (Ahlbäck grades IV and V)
Localization	**Knee Joint Mobility and Stability**
Unicompartmental tibiofemoral Unicompartmental plus patellofemoral Tricompartmental	Preserved mobility and stable joint (a minimum range of movement from 0° to 90° and absence of medial or lateral gapping of more than 5 mm. in the extended knee.)
	Limited mobility and/or unstable joint (a range of movement of less than 0° to 90° and/or medial or lateral gapping of more than 5 mm. in the extended knee.)
Symptomatology	
Slight	Sporadic pain, (e.g., when climbing stairs, daily activities typically carried out) nonsteroidal anti- inflammatory (NSAID) drugs for pain control).
Moderate	Occasional pain (e.g., when walking on level surfaces, some limitation of daily activities, NSAIDs to relieve pain.
Intense	Pain almost continuous (e.g. pain when walking short distances or standing for less than 30 minutes, limited daily activities, frequent use of NSAIDs, may require crutch or cane)
Severe	Pain at rest, daily activities always significantly limited, frequent use of analgesics- narcotics/NSAIDs, frequent use of walking aids.

Table 1-15: Criteria used to develop RAND-based appropriateness algorithm for total knee arthroplasty. Reproduced from Escobar et al., 2003

Other criteria have also been developed, such as the WOMAC® score, with a cutoff score of 39 being necessary before joint replacement is to be considered (Hawker et al., 2001).

However, when such criteria are applied to current practices, their validity is not always maintained. Ghomrawi (2014) evaluated the two above scores retrospectively in a sample of 500 cases, and found that using the Escobar criteria, only 80% of patients were deemed to have been appropriately operated. Using the Hawker criteria, nearly a third of replacements would have been inappropriate. Riddle et al (2014) showed that using the criteria developed by Escobar nearly 15 years earlier, only 44% of patients had received joint replacement surgery appropriately. This strong difference between recommendations and actual practice may be due to a number of reasons. The technology involved in the prosthesis has improved dramatically since the work by Escobar. The failure rate for the implants is low, and the risks involved in joint replacement have been reduced because of improved surgical techniques. Hence, with an increased benefit-to-risk ratio, the indications for joint replacement become less restrictive (Katz, 2014).

1.7 Total Knee Replacement

In 2016, a total of 668 patients underwent a knee replacement in Mater Dei Hospital, Malta [Annual Operation Statistics@Theatres 2016]. It is performed in patients who are suffering from severe pain in the knee due to arthritic changes, which could be due to osteoarthritis or to inflammatory diseases (Van Manen et al., 2012). Such pain must be interfering with normal function.

The patient presenting for a total knee replacement typically presents in the sixth or seventh decade of life, although it is becoming increasingly more common to perform such a procedure both in younger and in older populations (Ruiz et al., 2013; Lizaur-Utrilla et al., 2017). In either case, being restricted by pain will have a significant impact on the lifestyle of the person, and this may have to be borne out by his caregivers. In fact, it has been demonstrated that a total knee replacement may offer an increase in the quality-adjusted life year (QALY) per patient, with an additional 3.4 QALYs in the younger population, and an additional 1.8 QALYs per patient in the elder patient (Ruiz et al., 2013).

1.7.1 Surgical procedure

The surgical procedure for a total knee replacement is standard, although there are variations depending on the instrumentation used (Stern, 2001; Mihalko, 2013; Sanna et al., 2013).

The procedure is carried out under general or regional anaesthesia. An incision is made in the knee, with the commonest approach being through a midline incision or through

a medial parapatellar approach. The femur and tibia are then cut, and the prosthesis is then put in place.

1.7.2 Pain Following TKR

Pain in the acute postoperative period is common following TKA and tends to peak at 48 hours after surgery (Frassanito et al., 2010). Wylde et al (2011) found that 58% of such patients reported moderate to severe pain, as defined by a VAS pain score more than 40. Similar results were obtained locally by Sciberras (2011), by Zammit (2012) and by Santucci (2016).

1.7.2.1 Predictors for the severity of Post-Operative Pain

Patients vary in their response to pain. A number of studies have tried to elucidate which factors might predict the severity of pain after a total knee replacement. Liu et al identified younger age, female gender, increased BMI, increased severity of preoperative pain at the surgical site, prior surgery at the surgical site, preoperative use of opioids, anti-depressants and anti-convulsants, and use of general anaesthesia as risk factors for acute moderate to severe pain (Liu et al., 2012).

Genetic factors may also play a role in the development of pain following surgery. For instance, patients with a variant *OPRM1* gene who were undergoing an elective caesarean section showed increased morphine consumption (Wang et al., 2019). Likewise, Chou (2006) demonstrated the same effect in another study involving patients undergoing TKA. Postoperative pain also correlated with changes in the

activity of voltage-gated sodium channels following mutations in the *SCN9A* gene (Reimann et al., 2010; Duan et al., 2016).

1.7.2.2 Analgesia

Since pain itself may impact recovery in a negative way, as discussed in Section 1.3.1, it is important to treat such pain as best as possible.

Opiates are still the standard choice to treat severe pain that usually follows TKA. The mean average morphine consumption has been shown to be range from 6.6mg to 18mg in the first 24 hours after surgery (Seet et al., 2006; Sciberras et al., 2011). Opiates are also associated with a number of side-effects, most notably nausea and vomiting. This may be as high as 35 – 40% in patients after a TKA (Singelyn et al., 1998; Seet et al., 2006). Hence, finding a balance for opioid use after TKA would be useful.

As discussed earlier, nociception involves central, spinal and peripheral mechanisms (Section 1.2.3), a multimodal approach that targets different points in the pain pathway is better suited (Li et al., 2019). Wall et al (1988) were the first to describe multimodal analgesia for postoperative patients. This aims to use multiple concurrent mechanisms to treat pain. These mechanisms may be non-pharmaceutical or pharmaceutical, such as drugs listed in Section 1.3.4. Such an approach reduces the total dose of the individual drug regimes so that side-effects are limited. For instance, Huang et al (2008) compared the use of celecoxib and morphine PCA to morphine PCA only after TKA. Patients receiving both drugs had lower resting VAS pain scores and better rehabilitation. Furthermore, such patients needed 40% less opioid and had a reduction in post-operative nausea and vomiting.

NSAIDS and paracetamol may be administered preoperatively. Jianda et al (2016) administered celecoxib 30 – 60 minutes before TKA. Following this intervention, pain scores, morphine consumption and inflammatory markers were all decreased when compared to the control group.

The continued use of NSAIDs peri-operatively is effective in controlling post-TKA pain. Local data by Sciberras et al (2011) show that the use of diclofenac was associated with a decrease in pain on movement by 25%, and this was again confirmed locally by Santucci et al (2016). George et al (2017) demonstrated that adding diclofenac to a multimodal analgesic plan resulted in less opioid use, a lower length of stay in hospital, and importantly a lower pain intensity after 24 hours.

For a total knee arthroplasty, a number of peripheral nerve blocks may be used. The local anaesthetic used will act by blocking the voltage-gated sodium channels, and stops the pain from being propagated any further. Nerve blocks commonly used include: the femoral nerve block, the sciatic nerve block, the adductor canal block or a local infiltration. The most commonly used peripheral nerve block for TKA is a femoral nerve block. Local anaesthesia is injected around the femoral nerve as it travels beneath the inguinal ligament using ultrasound guidance or a peripheral nerve stimulator. It is also possible to leave a catheter next to the nerve for a continuous infusion of local anaesthesia in order to prolong the effect.

The analgesic efficiency of a femoral nerve block has been well documented (Dixit et al., 2018). A Cochrane review which included 2710 patients in 45 trials showed the non-inferiority of a femoral nerve block compared to an epidural, with a better safety profile, and a superior analgesic effect than PCA morphine (Chan et al., 2014). Total

morphine consumption is reduced when patients receive a continuous femoral nerve block with a catheter inserted (Seet et al., 2006).

1.7.3 CPSP Pain after TKR

1.7.3.1 Incidence

The incidence of CPSP after TKA is between 44% to 53% of patients, with 15% to 19% reporting severe pain (Petersen et al., 2015). Hence, CPSP after TKA carries a considerable burden, especially since the procedure is performed when patients have been in significant pain.

1.7.3.2 Factors associated with CPSP after TKA

Age and gender have been shown to play a role in CPSP. However, in patients after a TKA, such factors do not show such an association. A review of thirty-two studies by Lewis et al (2015) showed that age played a minor role in CPSP after TKA. Similarly, female gender also does not seem to increase the risk of CPSP (Lewis et al., 2015; Rice et al., 2018).

Pain scores before a TKA are highly associated with CPSP at six and twelve months (Liem et al., 2003; Lewis et al., 2015; Thomazeau et al., 2016; Rice et al., 2018). In a study of 300 patients who underwent a TKA, Rice et al (2018) found an increase of 6% in CPSP for every 1 point change in preoperative WOMAC. It is not clear if attempts at reducing such pain before surgery, such as performing surgery earlier, would have an impact on CPSP.

Acute postoperative pain is also strongly linked to CPSP. Thomazeau et al (2016) studied 104 patients for six months and assessed their pain scores over four days. In 28% of these patients, the acute pain scores over the first four postoperative days were persistently high, with a NRS above 5. Such patients were four times more likely to develop CPSP. Poulakka et al (2010) demonstrated an even higher likelihood, with patients in severe acute postoperative pain after a TKA having a ten times higher risk of CPSP.

Although some studies show a role of pain catastrophizing in CPSP after TKA, others do not (Lewis et al., 2015; Høvik et al., 2016).

No data has been yet published investigating the occurrence of CPSP after TKA as a result of the type of anaesthesia administered during TKA, although a number of studies are planned (Rantasalo et al., 2018). Similarly, the effect of genetic factors has not been widely investigated.

Chapter 2

Aims of the Research

2.1 Collection of Epidemiological Data

There is currently no local epidemiological data on the incidence of any of the outcomes investigated. It is unknown how many patients develop persistent post-surgical pain locally, including after major orthopaedic surgery. Having such information would be helpful in allocating resources for the care of such patients.

The 1000 Genomes Project (The 1000 Genomes Project, 2015) is an international effort to sequence DNA from at least 1000 individuals from a variety of ethnic groups across the world. This does not include the Maltese population with its unique cultural background. There is an effort to reproduce the 1000 Genomes Project specifically for the Maltese population as part of the Malta Biobank, but as yet there is no local data on the frequency of the single nucleotide polymorphisms involved in pain perception.

Different populations will have different genotypes, and the Maltese population is no different. This is known as Population Genomics, defined by the National Human Genome Research Institute as:

> *Population genomics is the application of genomic technologies to understand populations of organisms. In humans, population genomics typically refers to applying technology in the quest to understand how genes contribute to our health and well-being.*

This focuses more on genome-wide effects to help understand the evolutionary history of a population (Black et al., 2001). Luikart (2003) describes a four-step pathway in a population-genomic approach.

Such information is also useful to predict the characteristics of a population (Colomer-Vilaplana et al., 2022). For instance, a common example quoted is the lack of lactase, which causes lactose intolerance. Lactase is coded by the *LCT* gene and is regulated by another gene, *MCM6*. People who can consume lactose-containing food throughout their lives have a mutation in *MCM6* that prevents the otherwise gradual decline in expression of *LCT*. Indeed, 70 – 100% of people of East Asian descent lack the *MCM6* mutation and hence suffer from adult-onset lactose intolerance.

Furthermore, it may be possible to predict pharmacological responses in populations, should the prevalent genotypes be known for that population.

This research project aims to provide such information, in order to guide future research in this area.

2.2 Influence of Anaesthesia on Acute Pain

It is known that the type of anaesthesia given to a patient will influence perioperative outcomes, not only in the first few hours but also in the days after surgery and potentially for a considerable period afterwards (Harsten et al., 2013).

There is little local data on this effect: the principal investigator of this project has been involved in a number of audits on the incidence of pain and its severity after TKA, in relationship to the various methods used by both orthopaedic surgeons and anaesthetists to control such pain.

With such knowledge, it might be possible to better guide anaesthetists on their choice of anaesthesia, and also on titration of analgesia given in patients.

It is hypothesized that the post-operative pain during physiotherapy on the first postoperative day, measured using a Numerical Rating Scale, is influenced by the choice of either a spinal anaesthetic or a general anaesthetic with a femoral block.

2.3 Influence of Anaesthesia on Chronic Pain

There is an increasing amount of research being devoted to the emerging role of how anaesthesia may influence unrelated outcomes months after the procedure has been performed (Belfer et al., 2014).

So far, there is considerable research on finding factors that may predict the development of chronic post-surgical pain, but such research has mainly concentrated on demographic factors, such as age, gender, severity of acute post-operative pain (Bellville et al., 1971; Rosseland et al., 2004; Aubrun et al., 2005; Rakel et al., 2012; van Dijk et al., 2021). There is little evidence on modifiable factors such as different anaesthetic techniques. It is still unknown if the type of anaesthesia during a surgical procedure or if the adequacy of pain relief in the immediate post-surgical period has a bearing on the incidence of chronic post-surgical pain.

This research project aims to investigate the role of anaesthesia in the development of chronic post-surgical pain. We hypothesize that patients who receive a spinal anaesthetic during a TKA might have a different WOMAC® score than patients who receive a general anaesthesia with a femoral nerve block.

2.4 Influence of Genetics on Acute Pain

The role of genetics in predicting outcomes in medicine is not new: cancer research has established that certain genotypes are associated with worse outcomes. For

instance, the *BRCA1* germline mutations are associated with different prognosis (van ’t Veer et al., 2002). However, only a few studies have sought to relate pain and its severity to the genotype of patients undergoing a total knee arthroplasty (Edwards, 2006; Foulkes et al., 2008).

In this research, we hypothesize that the severity of acute postoperative pain is dependant on various genotypes. Twenty single-nucleotide polymorphisms (SNPs) across six genes will be targeted Table 2-1.

Gene	SNP	Chromoscome locus (GChr38)	Mutation
COMT	rs4680	chr 22:19963748	Intron: G to A
	rs4633	chr 22:19962712	Intron: C to T
	rs4818	chr 22:19963684	Synonymous variant: C to G
	rs6269	chr 22:19962429	Intron: A to G
GCH1	rs3783641	chr 14:54893421	Intron: T to A
	rs998259	chr 14:54888313	Intron: C to T
SCN9A	rs6746030	chr 2:166242648	Intron: G to A
	rs7595255	chr 2:166226468	Intron: C to T
	rs11898284	chr 2:166325017	Intron: A to G
	rs7444988	chr 2:166304225	Intron: A to C, G
KCNS1	rs734784	chr 20:45094986	Missense: T to C
	rs4499491	chr 20:45092778	3’ UTR variant: C to A, G
OPRM1	rs1799971	chr 6:154039662	Intron: A to G
	rs2075572	chr 6:154090869	Intron: G to C
	rs495491	chr 6:154061407	Intron: A to G
	rs533586	chr 6:154092539	Intron: C to T
	rs609148	chr 6:154109880	Intron: G to A
	rs563649	chr 6:154086832	UTR variant: C to T
OPRK1	rs6985606	chr 8:53248556	Intron: T to C

Table 2-1: List of Single Nucleotide Polymorphisms (SNPs) investigated in this research

2.5 Influence of Genetics on Chronic Pain

It is known that genetic factors have an influence on the incidence of chronic post-surgical pain, but research is scant. No such research has been done on a Maltese population.

This research project aims to check if there are particular genotypes that might be more common in patients with chronic post-surgical pain. This would help to allocate more resources, such as monitoring, analgesics, to such patients.

Chapter 3

Methodology

3.1 Introduction

This research will be a randomized controlled non-blinded study with analysis of both clinical and genetic outcomes. The tools used during the study, such as questionnaires as the WOMAC® and S-LANSS, have been validated before. The analysis will be quantitative using validated methods already used in other research projects.

The trial was registered with the US National Library of Medicine (clinicaltrials.gov, NCT04206046).

3.2 Sample size

A literature review showed that the WOMAC® score before surgery ranges from 35 to 49, out of a total score of 96. Following surgery, this range decreases to 12.6 to 18.4, with a change of 21.5 to 30.6 from the pre-operative WOMAC® score (Kahn et al., 2013; Wylde et al., 2015).

It was calculated that for a difference of 10 units between the WOMAC® scores in the two groups, eighty patients per trial arm would be required for a power of 80%. To allow for patients lost to follow up and exclusions, a total of 200 patients were included in this research project.

3.3 Ethical Approval

Approval from the various orthopaedic consultant surgeons caring for the patients involved in the trial was obtained. After clearance from the Data Protection Unit at Mater Dei Hospital was sought, the University Research and Ethics Committee was requested to approve the study.

Ethics approval was obtained in May 2017, with the reference number 05/2017 (Appendix B)

3.4 Recruitment

All patients who were to receive a Total Knee Arthroplasty (TKA) under the care of the participating orthopaedic firms, were identified from the elective surgical lists. Initial screening for suitability of the patients was done by inspecting the preoperative assessment sheet. This is usually done at the Preoperative Assessment Clinic at Mater Dei Hospital some weeks before the surgery.

Patients were excluded if:

- Age was more than 75 years
- Second TKA during the study period
- Rheumatoid arthritis as cause for TKA
- Revision TKA
- Any contraindication to any of the study drugs, including paracetamol, codeine or diclofenac
- Any contraindication for spinal anaesthesia
- Pre-existing evidence of chronic pain syndromes, such as fibromyalgia

Suitability was then confirmed by interviewing the patients before enrolment was considered.

Recruitment starting in April 2017, and lasted for a little over a year, until May 2018.

3.5 Consent

Consent was obtained from each patient after the nature of the study was explained to the patient. Possible participants were also informed of any requirements that would be additional to normal clinical practice, such as venipuncture, post-operative visits, and telephone questionnaires.

Patients who agreed to participate were given the opportunity to ask any questions Subsequently, written informed consent was obtained (Appendix A). Patients were also informed that they had the option to withdraw from the trial at any stage. In such cases, data from such patients would not be used in the analysis. The signed consent forms were kept until the research project ended.

3.6 Randomization

Randomization was performed using a minimization method as described by Pocock and Simon (1975). Normal randomization may lead to imbalances between the control and the intervention group, especially in smaller groups. Minimization calculates the imbalances before the patient is allocated to either group, and the allocation which leads to the least imbalance is then chosen. This was done in order to optimize matching between the two study groups, given the relatively small size of subjects in each arm.

A web-based application was used for such randomization. This was prepared and written by the principal investigator in PHP 5.6 and MySQL 8.0 (Appendix C). A secure platform was chosen with access limited only to the investigators. The randomization allocated patients to either a spinal anaesthetic (Group SP) or a general anaesthetic with a femoral block (Group GA) depending on five stratifications:

- Age
- Gender
- BMI
- Surgical Firm
- Pre-operative WOMAC®

For each stratification factor, the number of patients previously assigned in either group was calculated. The difference between these totals is the imbalance score, and the imbalance score of each factor was added together to give the total imbalance score.

The patient was then assigned to the group with the smallest marginal total in order to reduce the total imbalance score. This method has been previously described (Taves, 1974; Scott et al., 2002).

In cases where the difference was the same, the randomization was then done by chance, with a 70% chance of being allocated to the spinal group. This was done in anticipation of a greater amount of crossover from the spinal group to the general anaesthesia group.

3.7 Treatment Arms

In all cases, subjects followed a standard analgesic protocol that had been established by the researcher in prior studies.

All subjects received Paracetamol 1g and Diclofenac 50mg orally preoperatively upon being prepared for transfer to theatre. Furthermore, all enrolled patients were prescribed post-operative analgesia. This consisted of:

Morphine	as Patient Controlled Analgesia (PCA), 1mg boluses and a lockout time of 5 minutes
Paracetamol	1g orally every six hours
Codeine	30mg orally every eight hours
Diclofenac	50mg orally every eight hours
Ondansetron	4mg – 8mg intravenously when needed

The following day, the Morphine PCA was changed to oral Morphine (OroMorph®). The first 10mg dose was given in the morning, and repeated every four hours if required.

These were prescribed on the prescription chart by the investigators in order to minimise errors.

3.7.1 General Anaesthesia (GA) group

Subjects randomized to the GA group would receive a standard general anaesthetic regime as per current practices. This would include:

Induction	Propofol titrated to effect Fentanyl, at 1 – 2 μg / kg If necessary, Muscle relaxant of choice
Maintenance	Sevoflurane, adjusted to maintain depth of anaesthesia
Analgesia	Morphine or Pethidine as required Femoral nerve block
Femoral Nerve Block	Ultrasound-Guided Femoral nerve, with Bupivicaine dose set by anaesthetist

Any long-acting opiate analgesics that were administered during the procedure were included in the total morphine consumption. Shorter acting drugs, principally Fentanyl, was not included in this total, since these would not have had an impact on pain relief for more than thirty minutes.

3.7.2 Spinal Anaesthesia (SP) group

Subjects that were randomized to the SP group would receive an intrathecal injection of 0.5% Heavy Bupivacaine at a dose set by the anaesthetist, and Diamorphine, at a recommended dose of 300μg.

The intrathecal injection was performed by the attending anaesthetist, as per current recommendations.

3.7.3 Cross-over between groups

In cases where the caring anaesthetist felt that the randomization was not appropriate on clinical grounds or whenever a spinal anaesthetic could not be administered, then cross-over to the other group was allowed.

Given that it had been decided to perform statistical analysis on a per-protocol basis, patients who crossed over to the other group were then included in that group, and not excluded from the trial.

3.8 Blinding

Due to the nature of the treatment, it was not possible to blind either the patient or the clinician to the treatment. Neither was it possible to blind the investigators collecting the data in the peri-operative phase of the research project, since it was necessary to identify any patients who would cross over to the other group.

However, care was taken to limit the exposure of the treatment provided when collecting data at later stages of the study. For instance, during the telephone interviews the investigators only had access to patient name, the date of surgical procedure and the telephone number. This was done in order to avoid bias during the conduct of the telephone questionnaires.

3.9 Data Collection

Investigators collected data during enrolment, on the day following surgery, and at three months and at six months after surgery.

This was collected on a web platform on a secure server used specifically for the project. This was programmed by the principal investigator using a MySQL database and a PHP backend, and optimized for use on a mobile phone. The application was designed to allow limited exposure of data collected to the investigators, both for Data Protection and for blinding purposes.

Upon registration of the subject into the system, a unique four-digit reference number was randomly generated and assigned to each entry. This was used to anonymize blood samples that were taken and to label the genetic samples.

3.9.1 Baseline

Once enrolled, demographic data was collected. This included:

- Name, Surname, ID Numbers (for identification purposes only)
- Age
- Body Mass Index
- ASA
- Telephone number(s) of the patient
- Date of surgical procedure
- Surgeon responsible for the procedure

- Baseline questionnaires were used to assess pain, function, disability and incidence of neuropathic pain. These were the:
    - Western Ontario and McMaster Universities Osteoarthitis Index (WOMAC®) - score for pain, function and disability[1]
    - Self-reported Leeds Assessment of Neuropathic Symptoms and Signs (S-LANSS) – score for assessment of neuropathic pain

The WOMAC® and S-LANSS questionnaires were obtained in English, and then translated into Maltese. This was initially done by the investigator, but then checked by external translators not associated with the study. Validation was performed with healthy volunteers and with a small group of patients prior to the start of the study. The actual version used during interviews depended on patient preference, but the English version was used as reference when any clarification was required.

The 5-point Likert scale was used for the WOMAC® questionnaire, for a score total of 96.

3.9.2 Early Postoperative Period

On the morning following the surgery, an investigator visited the patient. The anaesthetic that had been administered during the procedure was confirmed. An analysis-by-protocol had been agreed *a priori*, so changes in the anaesthesia from the randomization were marked as crossover cases.

[1] Licensed from Prof N Bellamy, Australia

The investigator then collected data on:

- The current intensity of pain at rest, before mobilisation, using a Numerical Rating Scale from 0 – 10 (0: no pain, 10: worst pain)
- The intensity of pain during physiotherapy, using a Numerical Rating Scale from 0 – 10 (0: no pain, 10: worst pain)
- Morphine consumption, including intra-operative and PCA consumption, for the first 24 hours
- The intensity of any nausea, on a scale of 0 to 4 (0: no nausea, 1: mild, 3: moderate, 4: severe)
- Any vomiting episodes, and if any antiemetics were given

If other long-acting opiates besides morphine were used, namely pethidine, then these were converted to morphine equivalents. Every 25mg of intravenous pethidine were considered equivalent to 10mg of intravenous morphine. Oral morphine was converted to intravenous equivalents assuming a 50% bioavailability (Back, 2001).

3.9.3 Assessment at 3-months

Three months after surgery, all subjects were interviewed by phone. The investigator collected the following data:

- The intensity of pain at rest, using a Numerical Rating Scale from 0 – 10 (0: no pain, 10: worst pain)
- The intensity of pain on exercise, using a Numerical Rating Scale from 0 – 10 (0: no pain, 10: worst pain)

- The use of any analgesic during the previous two weeks
- S-LANSS
- WOMAC®

3.9.4 Assessment at 6-months

Six months after surgery, all subjects were interviewed by phone. The investigator collected the following data:

- The intensity of pain at rest, using a Numerical Rating Scale from 0 – 10 (0: no pain, 10: worst pain)
- The intensity of pain on exercise, using a Numerical Rating Scale from 0 – 10 (0: no pain, 10: worst pain)
- The use of any analgesic during the previous two weeks
- S-LANSS
- WOMAC®

3.10 Genetic Sampling

3.10.1 Blood sampling

A sample of blood was taken during anaesthesia to avoid patient discomfort. A total of 3mL was collected in an EDTA sample bottle and labelled with a unique four-digit number to ensure blinding. This sample was stored in a temperature-monitored refrigerator at 4^{o}C to 8^{o}C, then transported to the Biomedical Sciences Building at the University of Malta to be stored in the cold rooms at 4^{o}C. Storage was limited to a few days at most.

3.10.2 Preparation, DNA extraction

The blood samples were allowed to warm to room temperature, then were properly mixed before DNA extraction commenced. Each was then given a sequence number, so as to further blind the investigator.

For this study, a rapid DNA extraction kit was chosen, the Qiagen® DNeasy Blood & Tissue Kits (Qiagen GmbH, Dusseldorf, Germany).

This kit utilises proprietary reagents to lyse cells, purify and extract DNA from a sample of blood. A silica-based filter is used to adsorb the DNA until the final stages of the process. The yield from such a process is slightly less than a salting-out method, but at a fraction of time (Maurya et al., 2013). It also needs a much smaller blood sample, in the order of 100μL compared to 1mL for the salting out method.

The process used followed the recommended procedures by the company, with some minor modifications to improve the concentration of the final sample:

1. 80 μL of Phosphate-buffered Saline, 20 μL of Proteinase K were mixed with 120 μL of blood in a 1.5mL microcentrifuge tube, for a total volume of 220 μL. 200 μL of Buffer AL solution was added, and the solution was then mixed by vortexing for 5 – 10 seconds.
2. This mixture was then incubated at 56°C for 11 minutes. 200 μL of pure ethanol was added to the mixture, which was then mixed thoroughly by gentle shaking.
3. This mixture was transferred to the DNeasy mini spin column, and centrifuged at 6000 x g for 1 minute 15 seconds. The resulting flow-through was discarded, and the spin column placed in another 2 mL microcentrifuge tube.
4. 500 μL of Buffer AW1 solution was placed in the spin column and centrifuged at 6000 x g for 1 minute 15 seconds. The flow-through was discarded.
5. The process was repeated with 500 μL of Buffer AW2 solution, and centrifuged at 20,000 x g for 3 minutes 15 seconds.
6. To remove as much ethanol as possible, the spin column was then centrifuged with no solution at 20,000 x g for another 1 minute 15 seconds.
7. A volume of 30 - 50 μl of Buffer AE was used to elute the DNA. This volume was pipetted into the spin column, and left to stand for 5 minutes.
8. The spin column was centrifuged at 6000 x g for 1 minute 15 seconds, and the eluate collected in a 1.5 mL microcentrifuge tube suitably labelled.

The sample was then checked for DNA concentration and purity using a Nanodrop™ 2000 spectrophotometer (Thermo Fisher Scientific, US). This uses UV light to measure

absorbance of a sample, and depending on the ratio of absorbance at 230 nm, 260 nm and 280 nm wavelengths, it measures the concentration of DNA.

1. A sample of AE buffer was used to calibrate the machine, and this was repeated to confirm that the system was not biased.
2. 1.2 μL of the sample was placed on the measuring tray, and the DNA concentration and purity was assessed.
3. A cut-off concentration of 20 ng/μL was taken to consider the sample as being adequate for realtime PCR analysis. A 260 nm / 280 nm ratio, which is a measure of DNA purity, was also measured, and a minimum of 1.7 was considered adequate. If these criteria were not met, then another attempt was made at obtaining an adequate sample.

Aliquots containing 30 μL of sample DNA solution at a concentration of 25 μg were prepared for each sample. This was done to standardize each sample for use with the realtime PCR. These were stored at –20^{o}C until further use. This was limited to a few months until genotyping was performed.

Blood that was not used was stored at -20^{o}C in the cold storage rooms in the Biomedical Sciences Building, University of Malta.

3.10.3 DNA analysis

Genetic analysis to determine the presence of the investigated SNPs was performed using quantitative polymerase chain reaction (qPCR), also known as realtime PCR. During a qPCR, a sequence of a sample DNA is amplified (amplicon) using specific

primers, and this reaction is performed repeatedly. After each cycle, the number of amplicons generated after each duplication is measured with the use of a fluorescent dye attached to the primer.

The procedure used is described below:

1. The aliquots containing DNA were thawed.
2. A master mix with TaqMan™ Universal Master Mix II, with UNG (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, US), and the TaqMan™ predesigned SNP Genotyping Assay was prepared.
3. The required amount was pipetted to 0.1 mL strip tubes, which were individually labelled.
4. One µL of DNA sample was added to the mixtures, with one strip tube designated as the Negative Template Control. This did not contain any DNA sample, rather, 1µL of distilled water was added.
5. qPCR was then performed using the Rotor-Gene Q (Qiagen GmbH, Dusseldorf, Germany). The sequence used was recommended by the probe assay manufacturer (Figure 3-1).

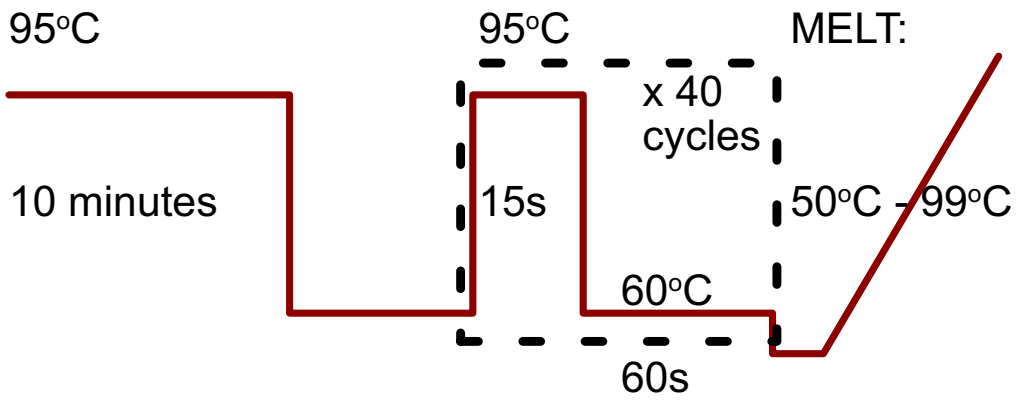

Figure 3-1: qPCR protocol used on the Rotor-Gene Q for the detection of the various SNP's

The Rotor-gene Analysis Software, version 2.4.1 (Qiagen GmbH, Dusseldorf, Germany) was used for the analysis the results of each realtime PCR run. The threshold was set manually for each channel, depending on the Negative Template Control (Figure 3-2).

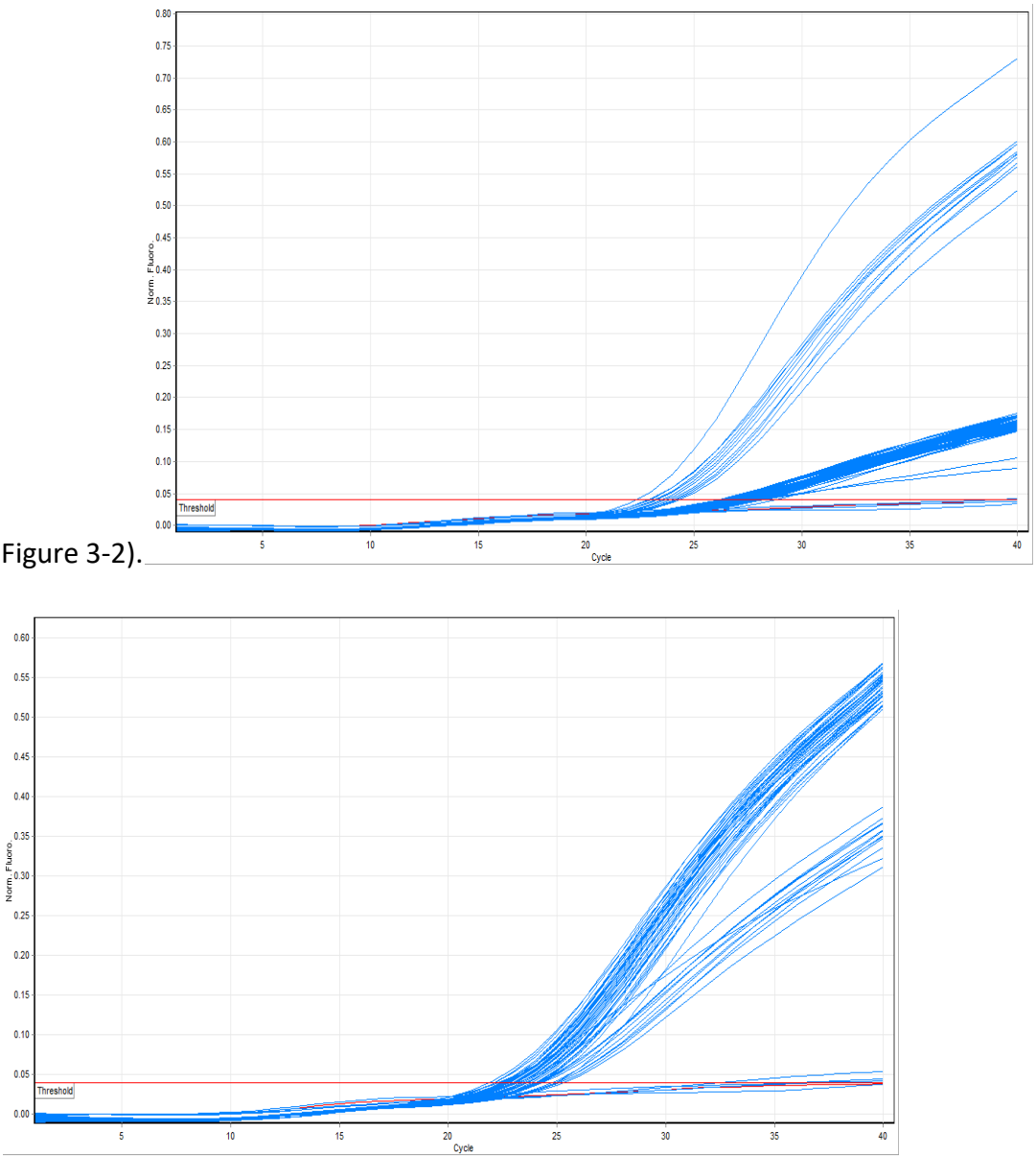

Figure 3-2: An example of the output from the Rotor-Gene Q. The blue lines indicate the flourescence from each sample, per channel, whilst the red line is the negative template control. The horizontal red line is the threshold set manually.

6. A Scatter plot analysis was then used to identify the genotype of each sample (Figure 3-3).

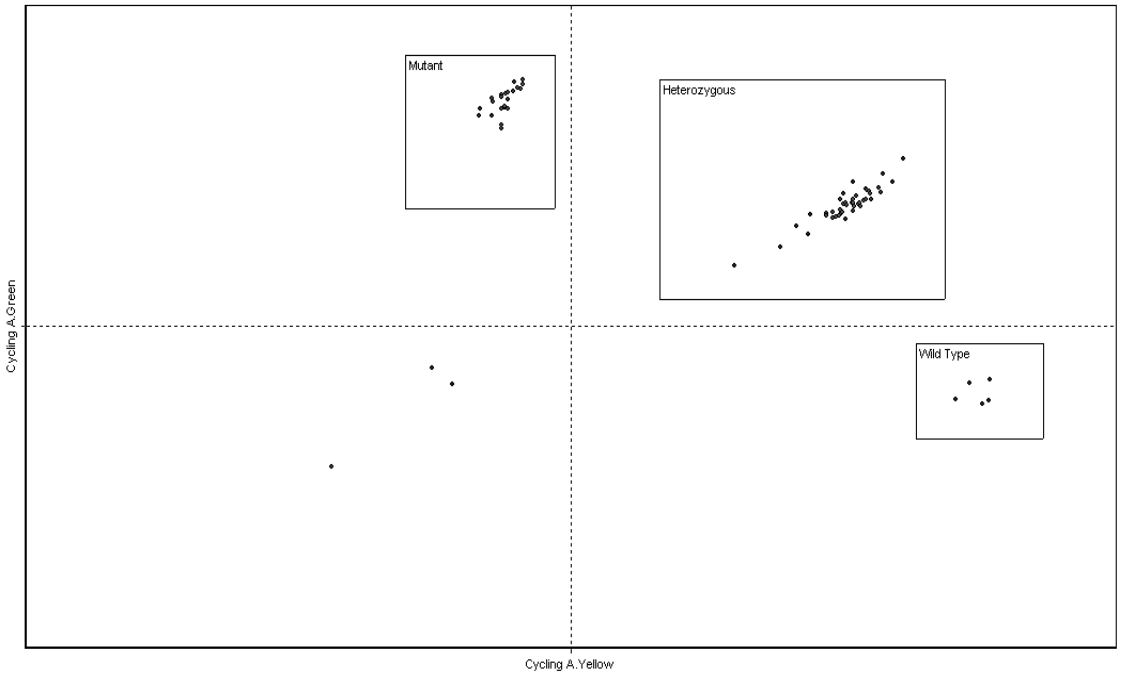

Figure 3-3: An example of a scatter plot output from the Rotor-Gene Q. Clusters of samples can be noted, this depends on the flourescence detected in each channel. Homozygous samples would be clustered to the top-left and bottom-right quadrants, whereas heterozygous samples would be clustered in between.

7. The data was then exported to a comma separated spreadsheet for further analysis.

The Rotor-Gene Q allows for 72 samples to be run at the same time. Hence a maximum of 71 samples and one negative template control could be analysed during one run.

Details regarding the specific assays are described in Table 3-6 to Table 3-6. Each probe assay contains two probes, one with the FAM™ reporter dye and one with the VIC™ reporter dye.

COMT	Probe Details	Preparation
rs4633 Chr 22: 19962712	CCAAGGAGCAGCGC ATCCTGAACCA**[C/T]**GTGCTGCAGC ATGCGGAGCCCGGGA Ancestral allele: C VIC: C FAM: T	1 µL DNA (25 ng) 12.5 µL Universal Master Mix 0.625 µL SNP Genotyping Assay C___2538747_20 0.625 µL TE Buffer 10.25µL distilled water Total: 25 µL
rs4680 Chr 22: 19963748	CCAGCGGATGGTGGATTTC GCTGGC**[A/G]**TGAAGGACAAGGTG TGCATGCCTGA Ancestral allele: G VIC: A FAM: G	1 µL DNA (25 ng) 12.5 µL Universal Master Mix 0.625 µL SNP Genotyping Assay C__25746809_50 0.625 µL TE Buffer 10.25µL distilled water Total: 25 µL
rs4818 Chr 22: 19963684	GCCTGCTGTCACCAGGGG CGAGGCT**[C/G]**ATCACCATCGAGAT CAACCCCGACT Ancestral allele: C VIC: C FAM: G	1 µL DNA (25 ng) 12.5 µL Universal Master Mix 0.625 µL SNP Genotyping Assay C___2538750_10 0.625 µL TE Buffer 10.25µL distilled water Total: 25 µL

Table 3-1: Details of the probes to genotype the COMT gene, with details of sample preparation for the qPCR as determined after optimization. Chromosomal locus as determined on GRCh38. Assay numbers as provided by Thermo Fisher Scientific

GCH1	Probe Details	Preparation
rs3783641 Chr 14: 54893421	ATTACAGTCCTCATATAGAAAT CAC[**A / T**]GGCAAATGAGT CAGGTGGGGAATGC Ancestral Allele: T VIC: A FAM: T	1 µL DNA (25 ng) 10 µL Universal Master Mix 0.5 µL SNP Genotyping Assay C__25800745_10 0.5 µL TE Buffer 8.2 µL distilled water Total: 20.2 µL
rs998259 Chr 14: 54888313	AGCTGCTGGAAGTCAACAGAG TGAG[**C / T**]GATGACAATTCT GACAGGCCCACCC Ancestral Allele: C VIC: C FAM: T	1 µL DNA (25 ng) 10 µL Universal Master Mix 0.5 µL SNP Genotyping Assay C___7593515_10 0.5 µL TE Buffer 8.2 µL distilled water Total: 20.2 µL

Table 3-2: Details of the probes to genotype the GCH1 gene, with details of sample preparation for the qPCR as determined after optimization. Chromosomal locus as determined on GRCh38. Assay numbers as provided by Thermo Fisher Scientific

SCN9A	Probe Details	Preparation
rs6746030 Chr 2: 166242648	TTAACTTGGCAGCATGAG AACCTCC**[A/G]**TACACAACCTGACA AGAAAGACAT Ancestral allele: G VIC: A FAM: G	1 µL DNA (25 ng) 10 µL Universal Master Mix 0.5 µL SNP Genotyping Assay C__29330435_10 0.5 µL TE Buffer 8.2 µL distilled water Total: 20.2 µL
rs7595255 Chr 2: 166226468	AAAAATAAATGAAGTTCT AATAAAA**[C/T]**TAATCATGAATTGAT ATCAAATTAA Ancestral allele: C VIC: C FAM: T	1 µL DNA (25 ng) 12.5 µL Universal Master Mix 0.625 µL SNP Genotyping Assay C__29330446_10 0.625 µL TE Buffer 10.25µL distilled water Total: 25 µL
rs11898284 Chr 2: 166325017	TTTTGTTAATTGTGACAA ATGCGCC**[A/G]**TACTAATATAAACT GTTAATAACAG Ancestral allele: A VIC: A FAM: G	1 µL DNA (25 ng) 12.5 µL Universal Master Mix 0.625 µL SNP Genotyping Assay C____422328_10 0.625 µL TE Buffer 10.25µL distilled water Total: 25 µL
rs74449889 Chr 2: 166304225	TATGAGTGGCCTAATGC TTCACACC**[A/G]**ATTACTTCTTACCT GGGATTACAGA Ancestral allele: A VIC: A FAM: G	1 µL DNA (25 ng) 12.5 µL Universal Master Mix 0.625 µL SNP Genotyping Assay C__99847737_10 0.625 µL TE Buffer 10.25µL distilled water Total: 25 µL

Table 3-3: Details of the probes to genotype the SCN9A gene, with details of sample preparation for the qPCR as determined after optimization. Chromosomal locus as determined on GRCh38. Assay numbers as provided by Thermo Fisher Scientific

KCNS1	Probe Details	Preparation
rs734784 Chr 20: 45094986	AGAGATGCCTCCGACA CCCCATCAA **[T / C]** GCTGCTCA GCAAGTCCTCAAACTCT Ancestral allele: T VIC: T FAM: C	1 µL DNA (25 ng) 10 µL Universal Master Mix 0.5 µL SNP Genotyping Assay C___2457087_10 0.5 µL TE Buffer 8.2 µL distilled water Total: 20.2 µL
rs4499491 Chr 20: 45092778	ATTCTCTCTGCTTGGAGTACTCCCC **[C / A]** CTGGAACCTCA TTTGCTTAATTAGC Ancestral allele: C VIC: C FAM: A	1 µL DNA (25 ng) 10 µL Universal Master Mix 0.5 µL SNP Genotyping Assay C___2457091_10 0.5 µL TE Buffer 8.2 µL distilled water Total: 20.2 µL

Table 3-4: Details of the probes to genotype the KCNS1 gene, with details of sample preparation for the qPCR as determined after optimization. Chromosomal locus as determined on GRCh38. Assay numbers as provided by Thermo Fisher Scientific

OPRM1	Probe Details	Preparation
rs1799971 Chr 6: 154039662	GGTCAACTTGTCCCACTTAG ATGGC**[A / G]** ACCTGTCCGAC CCATGCGGTCCGAA Ancestral allele: A VIC: A FAM: G	1 µL DNA (25 ng) 12.5 µL Universal Master Mix 0.625 µL SNP Genotyping Assay C___8950074_10 0.625 µL TE Buffer 10.25µL distilled water Total: 25 µL
rs2075572 Chr 6: 154090869	GTTAGCTCTGGTCAAGGCT AAAAAT**[C / G]** AATGA GCAAAATGGCAGTATTAACA Ancestral allele: G VIC: C FAM: G	1 µL DNA (25 ng) 12.5 µL Universal Master Mix 0.625 µL SNP Genotyping Assay C___1691815_10 0.625 µL TE Buffer 10.25µL distilled water Total: 25 µL
rs495491 Chr 6: 154061407	TTGTCACCAGACTTAGGA GAGATAT**[A / G]**TCTC ACTGTAGAACCAGTGCCTATC Ancestral allele: A VIC: A FAM: G	1 µL DNA (25 ng) 12.5 µL Universal Master Mix 0.625 µL SNP Genotyping Assay C____809956_10 0.625 µL TE Buffer 10.25µL distilled water Total: 25 µL
rs533586 Chr 6: 154092539	TCAGGACTGTGAGG ACAGATGGCTC**[C / T**] GGAGA AATGAATAGCAAGTCAAATG Ancestral allele: C VIC: C FAM: T	1 µL DNA (25 ng) 10 µL Universal Master Mix 0.5 µL SNP Genotyping Assay C___1691813_10 0.5 µL TE Buffer 8.2 µL distilled water Total: 20.2 µL

rs609148 Chr 6: 154109880	TTCTAAGCCAAAGTTCA GTTCTCCA [**G / A**] TTCATCT GAGCTCAGGCCCAGTTTT Ancestral allele: G VIC: G FAM: A	1 µL DNA (25 ng) 10 µL Universal Master Mix 0.5 µL SNP Genotyping Assay C___3073591_10 0.5 µL TE Buffer 8.2 µL distilled water Total: 20.2 µL
rs563649 Chr 6: 154086832	TTAGATCATGCAGGTCT ATAACCAA [**C / T**] GGTGAATC TAGCAAAAGTTATTTTC Ancestral allele: C VIC: C FAM: T	1 µL DNA (25 ng) 12.5 µL Universal Master Mix 0.625 µL SNP Genotyping Assay C____809947_10 0.625 µL TE Buffer 10.25µL distilled water Total: 25 µL

Table 3-5: Details of the probes to genotype the OPRM1 gene, with details of sample preparation for the qPCR as determined after optimization. Chromosomal locus as determined on GRCh38. Assay numbers as provided by Thermo Fisher Scientific

OPRK1	**Probe Details**	**Preparation**
rs6985606 Chr 8: 53248556	AACCCACTTCATGCCACCCTCTCTC [**T / C**] GATCTTCAGTCT CTTCATTTCCTAA Ancestral Allele: T VIC: T FAM: C	1 µL DNA (25 ng) 10 µL Universal Master Mix 0.5 µL SNP Genotyping Assay C___2898341_20 0.5 µL TE Buffer 8.2 µL distilled water Total: 20.2 µL

Table 3-6: Details of the probes to genotype the OPRK1 gene, with details of sample preparation for the qPCR as determined after optimization. Chromosomal locus as determined on GRCh38. Assay numbers as provided by Thermo Fisher Scientific

Where necessary, agarose gel electrophoresis was performed on the sample obtained after the realtime PCR, in order to check for the possibility of primer dimers. This was suspected when the scatter plot showed a wide spread of samples in the bottom-left quadrant, or a spread along the heterozygous cluster (Figure 3-4).

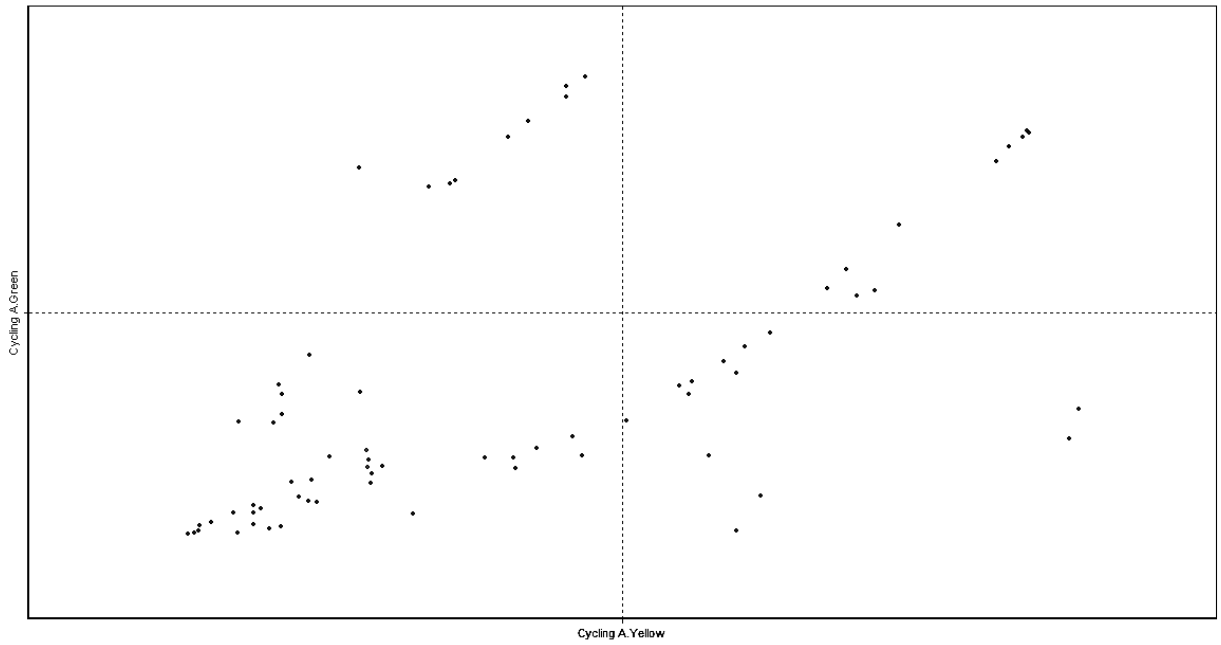

Figure 3-4: Scatterplot with spread of samples indicating the possibility of primer dimers

Primer dimers are an artefact generated during PCR, whereby the primers attach to themselves during the annealing process. This reduces the efficiency of the reactions.

Using a TaqMan® system, it is not possible to use a melting curve analysis to detect primer dimers, since the TaqMan® probes are hydrolysis probes. Hence, it was necessary to use agarose gel electrophoresis in order to check for primer dimers.

This was done as follows:

- Tris base, acetic acid and EDTA mixture (TAE) was prepared by dissolving 242g of Tris base in 500 mL of distilled water, 57.1 mL of glacial acetic acid, and 100 mL of 0.5M EDTA. This resulting in a 50X concentrated TAE mixture, which was then diluted to 1X TAE solution. The final solution had a pH of 8.3.
- The agarose gel to be used was prepared by mixing 1g of agarose with 100mL of 1X TAE buffer, in a conical flask, and stirred.
- The mixture was then heated in a microwave, at a power of 400W, for 4 minutes, or until the agarose was completely dissolved in the buffer.
- Ethidium bromide was added, at a volume of 10 μL to every 100 mL of agarose. This fluoresces under UV light, when attached to DNA, and was used to visualize the DNA after electrophoresis.
- The solution was slowly poured into a cast, making sure to avoid any bubble formation. If such bubbles occurred, these were removed with a pipette tip. The gel was checked to make sure that it was as level as possible.
- A comb was inserted into the solution, to make wells in the agar to accommodate the samples.
- The setup was left to cool for 20 – 30 minutes, and then the cast and agarose gel were them placed in the electrophoresis equipment. The whole setup was covered with TAE buffer solution, and the comb was then removed.
- The gel was adjusted to be parallel to the walls of the electrophoresis equipment.
- A volume of 2 μL of sample to be investigated was pipetted into the wells, together with a dye and precipitant (ReddyMix™ Master Mix). One well was loaded with a 100 kbase ladder, which served to identify the size of the molecules at the end of the procedure.

- The electrodes were then attached, and the setup was run at 95 V, for 45 minutes.
- The gel was then removed from the electrophoresis equipment, and placed on an ultraviolet transilluminator so that the resulting bands could be analysed.

Bands that were occurring at the lowest marker (100 kbases) were assumed to be due to primer dimers, and if these were present, the qPCR run was optimised by a reduction in reagents.

3.11 Outcome Measures

The primary outcomes for the study were:

- the Numerical Rating Score during physiotherapy in the early phase
- the WOMAC® score at three and six months
- the WOMAC® Pain subscore at three and six months

Secondary outcomes were also considered

- Acute pain, as measured by the Numerical Pain Scales at rest
- Total morphine consumption over 24 hours
- the S-LANSS at three and six months (Appendix D)
- The incidence of chronic post-surgical pain (CPSP) at three and at six months, defined as the number of patients with a high WOMAC® pain subscore (equal or higher than 5 out of a total of 20)

3.12 Statistical analysis

Given the strong possibility of having a considerable amount of cross-over, an analysis by protocol, rather than by intention to treat, was considered. In order to reduce bias, a further analysis by intention to treat was performed, to validate the primary results.

The questions of the WOMAC® score can be scored on a 5-point (0 – 4) Likert scale, or a Visual Analogue Scale (0 – 100mm). The 5-point Likert scale was used, and the values reported in this research are not transformed. Hence, the values for WOMAC® score are out of a maximum of 96 points, and the values for WOMAC® Pain subscore are out of a maximum of 25 points.

Statistical analysis was performed with R (version 3.5.1), using R Studio (Version 1.1.442). A p-value of 0.05 was taken as significant. Univariate analysis was performed initially, using parametric or non-parametric tests where appropriate. The data was first checked for normality and skewness using visual methods, and other tests such as Shapiro's test of normality. When appropriate, t-tests, Mann-Whitney U tests, Kruskal-Wallis test and chi-squared tests were used for univariate analysis. Linear regression, polynomial linear regression and logistic regression (as part of generalized linear models) were used for multivariate analysis when indicated. For such analysis, any factors found to have a p value of 0.25 or less during univariate analysis were used as predictive variables in such models, unless otherwise stated (Zhang, 2016). A stepdown modelling was performed, with the predictive variables with least significance being dropped during each iteration (Calcagno et al., 2010).

Statistical analysis using the WOMAC® score was performed using non-parametric tests. Kersten (2010) had shown that the score exhibits good ordinal scaling tests, and also the ability to be transformed into an interval scale. This meant that is was not necessary to treat the WOMAC® score as a categorical variable, and that the WOMAC® score could be interpreted as an ordinal variable. Hence, it was possible to compare median scores between two samples.

Since the WOMAC® scores and the WOMAC® Pain subscores were collected repeatedly over six months, a linear mixed model was used to compare these scores between the two groups using the packages *lme4* (version 1.1-28) (Bates et al., 2015) and *lmerTest* (version 3.1-3) (Kuznetsova et al., 2017). The WOMAC® or WOMAC® Pain scores were used as the outcome, with an interaction of anaesthesia and postoperative time as fixed factors, and the patient reference as a random effect. This was then checked for normality of residuals, normality of random effects, and heteroscedasticity. The relevance of the final model was tested with a likelihood ratio test between the final model and a model containing only the intercept and random effect.

The same approach was performed for each SNP investigated, both with a dominant and with a recessive analysis. In such analysis, we found significant heteroscedasticity, so robust estimation of standard errors and covariances was performed using the packages *sandwich* (version 3.0-1) (Zeileis et al., 2020).

With regards to the population genetics, the sample was first checked for Hardy-Weinberg equilibrium (Mayo, 2008). Deviations from such equilibrium might indicate selection bias, errors in genotyping or population stratification (Namipashaki et al., 2015). Testing for Hardy-Weinberg equilibrium involves a Chi-squared test: if the

resulting p-value is low, then the distribution of the genotypes in the population are not in Hardy-Weinberg equilibrium.

The genotype distribution of each SNP was then compared to that reported globally and also to that reported in Europe. Such figures were obtained from the 1000 Genome Project (The 1000 Genomes Project, 2015).

When multiple SNP's were tested for a particular gene, then the linkage disequilibrium was checked. This is done to check if the presence of an allele is dependent on another allele, and may happen for a number of reasons, for instance, due to the close proximity of the two alleles (Slatkin, 2008). If two alleles are linked physically, then the effect of either cannot be elucidated independently from the other.

Haplotyping was performed using the *haplo.stats* package in R (Sinnwell et al., 2020). This estimates the haplotypes of each patient and provides the probability for this estimation. Only those samples that had a probability of being correct greater than 0.9 were analysed.

Chapter 4

Results

4.1 Demographics

Out of a total 298 patients who were screened during the study period, with 212 patients meeting the eligibility criteria. Two patients refused participation, so 210 subjects were enrolled and included in the study. Eleven patients were excluded from the study after enrolment, leaving 199 candidates for analysis (Figure 4-1).

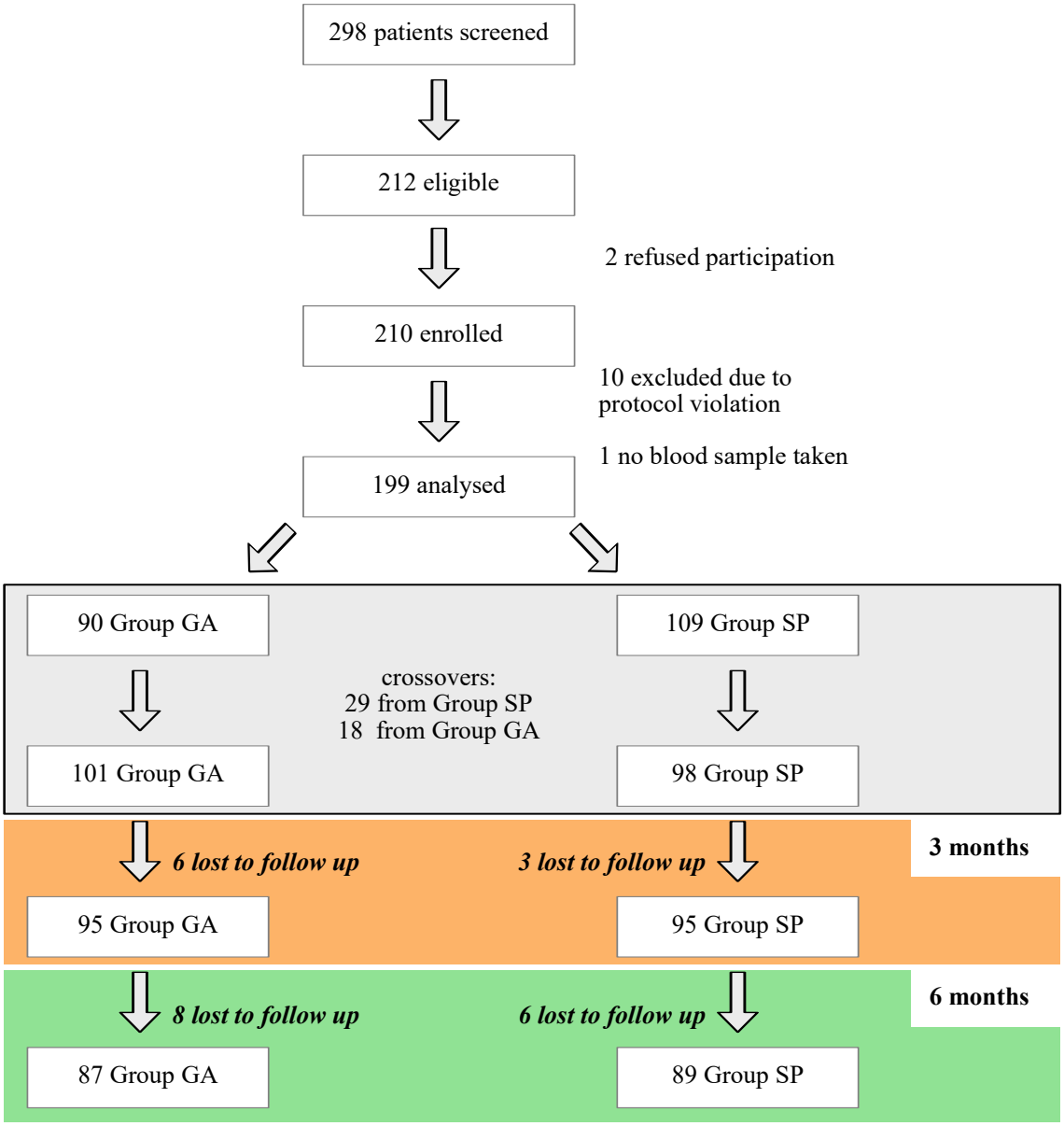

Figure 4-1: CONSORT Flow chart. Group GA: Patients received general anaesthetic and femoral block, Group SP: patients received spinal anaesthetic

Table 4-1 shows the reasons for exclusion, the most common being a protocol violation with no NSAIDs being prescribed for various reasons. Blood sampling was not done in one patient, and it was decided not to include this patient in any of the analysis.

Patient	Randomization	Reasons for Exclusion
1	GA	no block due to ST depressions during surgery
2	Spinal	No NSAIDs used
3	Spinal	No NSAIDs used
4	GA	No NSAIDs used
5	GA	Rheumatoid Arthritis
6	Spinal	Myelodysplasia, cannot take diclofenac
7	Spinal	Operation cancelled
8	GA	Refused block, and refused NSAIDS
9	GA	No NSAIDS due to creatinine and ischaemic heart disease
10	Spinal	received GA and intrathecal opiates
11	Spinal	Blood sample not taken

Table 4-1: Reasons for exclusion from the study

There were 47 patients who crossed over to the other group. More crossovers (n=29 vs n=18) were from Group SP to Group GA. This was expected and anticipated, given that there is an inherit failure rate in performing spinal anaesthesia.

The demographics of the population studied are listed in Table 4-2.

	All	Group GA	Group SP	p-value
n	199	101	98	
Surgical Firm **A** **B** **C** **D** **E** **F**	 17 19 40 34 33 57	 6 11 20 19 16 29	 11 8 20 15 17 28	0.79
Age (years)	66.2 ± 5.85	66.1 ± 6.27	66.3 ± 5.4	0.92
Sex (female)	128 (64%)	66 (65%)	62 (63%)	0.77
BMI	34.3 ± 6.97	34.3 ± 7.61	34.1 ± 6.00	0.89
ASA I – II	114 (57%)	58 (57%)	56 (57%)	0.30
S-LANSS (High)	25 (12%)	14 (14%)	11 (11%)	0.67
WOMAC® **(median)**	41 [IQR: 31 – 53]	39 [IQR: 29 – 52]	45 [IQR: 31 – 53]	0.23
WOMAC®-Pain **(median)**	10 [IQR: 7 – 12]	9 [IQR: 7 – 12]	10 [IQR: 8 – 12]	0.06

Table 4-2: Demographics of the study population.

There were no significant differences between the two groups. A non-significant difference was present with patients in the spinal group reporting a higher WOMAC® score.

The demographics for the study group is similar to those reported in other centres: a mean age of around 66 years, a female-male ratio of 2:1, and an ASA predominately

being I – II (Souza et al., 2016). A WOMAC® score of 42 would be consistent with recommendations of when to perform surgery (Faschingbauer et al., 2017).

Patients assigned to the spinal group had a higher median baseline WOMAC® Pain subscore, although this was not statistically significant.

4.1.1 Genetic Analysis

4.1.1.1 COMT gene

The frequency of the various single nucleotide polymorphisms in the Catechol-O-Methyltransferase gene and their relative distributions, are shown in Table 4-3.

SNP	Homozygous Major	Heterozygous	Homozygous Minor	Study MAF	Global MAF	p-value
rs4633	C|C 50 (26%)	C|T 101 (52%)	T|T 43 (22%)	48%	37%	***<0.001***
rs4680	G|G 49 (25%)	G|A 100 (44%)	A|A 44 (23%)	49%	37%	***<0.001***
rs4818	C|C 81 (42%)	C|G 84 (44%)	G|G 26 (14%)	37%	30%	0.06

Table 4-3: Distribution of the genotypes and Minor Allele Frequency (MAF) in the COMT gene for the various Single Nucleotide Polymorphisms (SNP) investigated in this study. p-value quoted is derived by Chi² test for difference between local and global MAF. (The 1000 Genomes Project, 2015)

All three variants were in Hardy-Weinberg equilibrium, as shown in Table 4-4.

Gene	SNP	Chi2	p-value
COMT	rs4633	0.239	0.63
	rs4680	0.166	0.68
	rs4818	0.198	0.65

Table 4-4: Hardy-Weinberg Equilibrium statistics for COMT variants investigated in the study.

The genotype distribution in the population studied is similar to that reported in Europe, but significantly different from the global distribution (Figure 4-2).

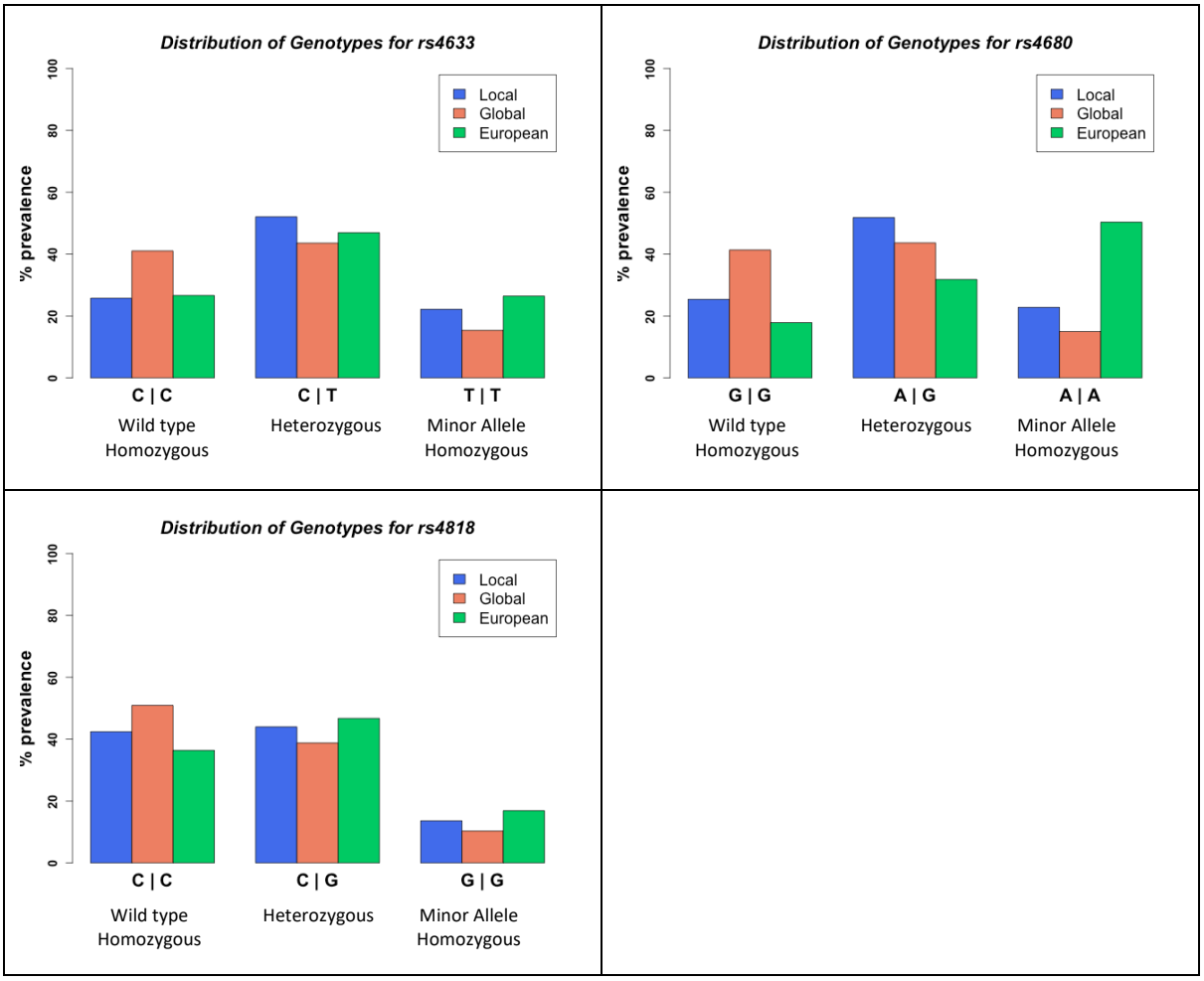

Figure 4-2: Comparison of the genotypes in different Single Nucleotide Polymorphism for the COMT gene, between study, global and European frequencies. (The 1000 Genomes Project, 2015)

A total of 192 (99%) out of 194 patients had at least one copy of a polymorphism of the *COMT* gene. The presence of more than one SNP in the same patient is shown in Table 4-5. Most patients who had the rs4633 variant also had the rs4680 variant (139 out of 187 patients). This is consistent with data from the 1000 Genome Project where these two variants show a high degree of coinheritance, especially in Europe (The 1000 Genomes Project, 2015). The linkage equilibrium factors are shown in Figure 4-3, as calculated using the LD function of the genetics package (version 1.3.8.1.3) in R.

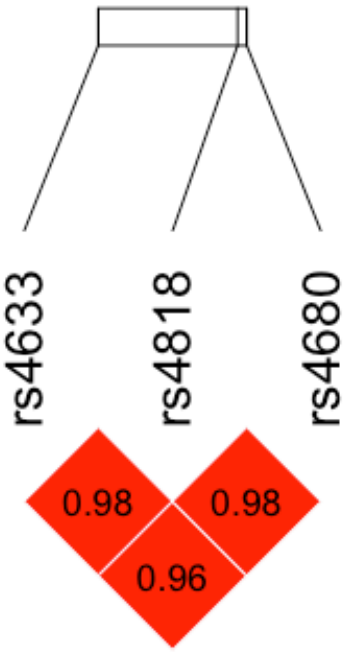

Figure 4-3: LD plot for COMT, showing coefficient D'. There is strong linkage dysequilibrium between all three variants, with all coefficients being close to 1.

Haplotype estimation was successful in 194 patients. It was not possible to estimate haplotypes in 5 patients with the required 90% certainty.

The most common haplotype was the TCA haplotype, as shown in Table 4-5. There was no difference between Group SP and Group GA in the incidence of the most common haplotype (TCA).

rs4633	rs4818	rs4680	Haplotype Frequency
T	C	A	46.9%
C	G	G	35.4%
C	C	G	15.2%
C	C	A	1.1%
T	C	G	0.8%
C	G	A	0.3%
T	G	G	0.3%
T	G	A	0.0%

Table 4-5: Frequency of multiple single nucleotide polymorphisms of the COMT gene in the same patient (haplotypes)

Th most common diplotype, which is the combination of the two possible haplotypes in a patient, was the TCA-CGG combination. This was present in 67 patients (34.5%). The TCA-TCA diplotype was the second most frequent, in 42 patients (21.5%).

There was no effect of carrying the variants of the *COMT* gene on the baseline WOMAC®, WOMAC® Pain score and the S-LANSS score, as shown in Table 4-6, Table 4-7 and Table 4-8.

SNP	Baseline WOMAC® Score		Wilcoxon W score	p-value
	Minor Allele Carrier	*NonCarrier*		
rs4633	40	48	3269.5	0.44
rs4680	40	45	3778	0.33
rs4818	42	42	4575.5	0.66

Table 4-6: Effect of the COMT variants on the baseline WOMAC® scores. Wilcoxon-Mann-Whitney test.

SNP	Baseline WOMAC® Pain subscore		Wilcoxon W score	p-value
	Minor Allele Carrier	*Non Carrier*		
rs4633	9	10	3246	0.40
rs4680	9	10	3768	0.35
rs4818	10	10	4527	0.76

Table 4-7: Effect of the COMT variants on the baseline WOMAC® Pain subscores. Wilcoxon-Mann-Whitney test.

SNP	S-LANSS score > 11		OR	CI	p-value
	Minor Allele Carrier	*Non Carrier*			
rs4633	9.7% (14 / 144)	16.3% (8 / 49)	0.55	0.20 – 1.64	0.21
rs4680	9.0% (13 / 144)	16.7% (8 / 48)	0.50	0.18 – 1.49	0.18
rs4818	11.9% (13 / 109)	6.2% (5 / 81)	2.1	0.65 – 7.68	0.21

Table 4-8: Effect of the COMT variants on the Baseline S-LANSS scores. OR: odd's ratio of high S-LANSS score. Fisher's exact test.

There was no difference in the incidence of carriers of either of the three variants in the spinal or general anaesthesia groups.

4.1.1.2 SCN9A gene

The frequency of the various single nucleotide polymorphisms in the *SCN9A* gene and their relative distributions are shown in Table 4-9.

SNP	Homozygous Major	Heterozygous	Homozygous Minor	Local MAF	Global MAF	p-value
rs6746030	G | G 131 (69%)	G | A 49 (26%)	A | A 11 (6%)	19%	11%	***<0.001***
rs7595255	C | C 134 (68%)	C | T 53 (27%)	T | T 11 (6%)	19%	11%	***<0.001***
rs11898284	A | A 151 (77%)	A | G 40 (20%)	G | G 6 (3%)	13%	16%	0.11
rs74449889	A | A 140 (100%)	A | G 0 (0%)	G | G 0 (0%)	0%	5%	***<0.001***

Table 4-9: Distribution of the genotypes and Minor Allele Frequency (MAF) in the SCN9A gene for the various Single Nucleotide Polymorphisms (SNP) investigated in this study. p-value quoted is derived by Chi² test for difference between local and global MAF . (The 1000 Genomes Project, 2015)

Given that all patients were homozygous ancestral for rs74449889, this particular variant was not included in any further analysis.

All three variants were in Hardy-Weinberg equilibrium, as shown in Table 4-10.

Gene	**SNP**	**Chi^2**	**p-value**
SCN9A	rs6746030	3.62	0.06
	rs7595255	2.59	0.11
	rs11898284	1.75	0.19

Table 4-10: Hardy-Weinberg Equilibrium statistics for SCN9A variants investigated in the study.

The distribution of the genotypes in the investigated variations, as compared to global and European frequencies, is shown in Figure 4-4. The local population shows a similar distribution to the European distribution but it is significantly different from the global distribution.

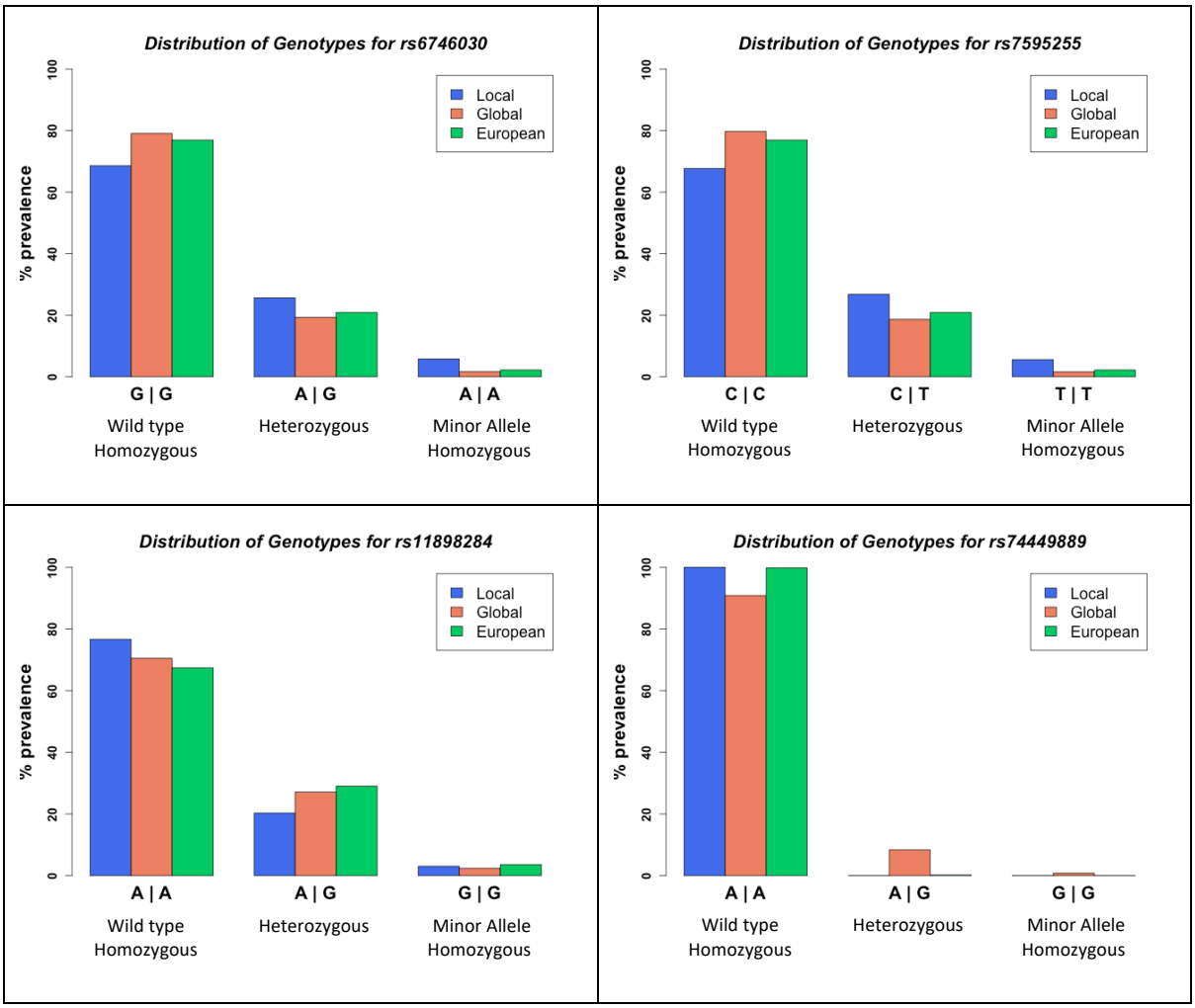

Figure 4-4: Comparison of the genotypes in different Single Nucleotide Polymorphism for the SCN9A gene, between local, global and European frequencies. (The 1000 Genomes Project, 2015)

A total of 101 (52%) out of 196 patients had at least one allele which was a variant of the *SCN9A* gene. All patients who had the rs7595255 variant also had the rs6746030. This is consistent with co-inheritance as reported in the the 1000 Genome Project, where these two variants show a high degree of coinheritance (The 1000 Genomes Project, 2015). The linkage equilibrium factors are shown in Figure 4-5, as calculated using the LD function of the genetics package (version 1.3.8.1.3) in R.

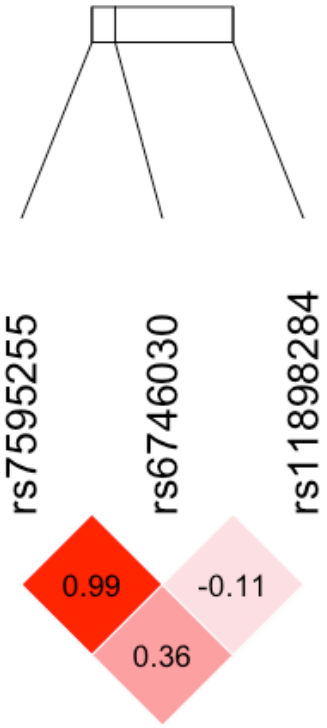

Figure 4-5: LD plot for SCN9A, showing coefficient D'. There is strong linkage dysequilibrium between rs755255 and rs6746030, with a coefficient close to 1.

The most common haplotype was the G in the rs6746030, C in the rs7595255 and A in rs11898284 (GCA), as shown in Table 4-11. There was no difference between Group SP and Group GA in the incidence of the most common haplotype (GCA).

rs6746030	rs7595255	rs11898284	Haplotype Frequency
G	C	A	65.9%
A	T	A	16.4%
G	C	G	10.6%
Others			7.1%

Table 4-11 Frequency of multiple single nucleotide polymorphisms of the SCN9A gene in the same patient (haplotypes)

There was no effect of carrying the variants of the *SCN9A* gene on the baseline WOMAC®, WOMAC® Pain score and the S-LANSS score, as shown in Table 4-12, Table 4-13 and Table 4-14.

SNP	Baseline WOMAC® score		Wilcoxon W score	p-value
	Minor Allele Carrier	*Non Carrier*		
rs6746030	44.5	40.0	4252	0.32
rs7595255	44	40	3984	0.47
rs11898284	43	42	3080	0.27

Table 4-12: Effect of the SCN9A variants on the baseline WOMAC® scores. Wilcoxon-Mann-Whitney test

SNP	Baseline WOMAC® Pain subscore		Wilcoxon W score	p-value
	Minor Allele Carrier	*Non Carrier*		
rs6746030	10	9	4264	0.30
rs7595255	10	9	3937	0.39
rs11898284	10	9	2925	0.12

Table 4-13: Effect of the SCN9A variants on the baseline WOMAC® Pain subscores. Wilcoxon-Mann-Whitney test

SNP	S-LANSS score > 11		OR	CI	p-value
	Minor Allele Carrier	*Non Carrier*			
rs6746030	6.7% (4 / 60)	13.1% (17 / 130)	0.47	0.11 – 1.55	0.22
rs7595255	6.3% (4 / 64)	13.5% (18 / 133)	0.43	0.10 – 1.38	0.15
rs11898284	17.3% (8 / 46)	9.3% (14 / 150)	2.0	0.69 – 5.68	0.18

Table 4-14: Effect of the SCN9A variants on the Baseline S-LANSS scores. Fisher's exact test.

The distribution of the *SCN9A* variants between the spinal and the general anaesthesia groups is shown in Table 4-15. There is a significant preponderance of patients who had the variant allele rs7595255 in Group GA, and a slightly non-significant difference in the distribution of rs6746030. This might have acted as a confounding effect when analysing outcomes on the basis of the type of anaesthesia.

SNP	Group GA	Group SP	OR	CI	p-value
rs6746030	36 / 95 (38%)	24 / 95 (25%)	0.56	0.28 – 1.08	0.08
rs7595255	40 / 100 (40%)	24 / 97 (25%)	0.49	0.25 – 0.94	***0.02***
rs11898284	22 / 100 (22%)	24 / 96 (24%)	0.55	0.58 – 2.42	0.73

Table 4-15: Frequencies of the variants of the SCN9A gene, across the two groups. Fisher's exact Test.

4.1.1.3 OPRM1 gene

The frequency of the various single nucleotide polymorphisms in the *OPRM1* gene and their relative distributions are shown in Table 4-16.

SNP	Homozygous Major	Heterozygous	Homozygous Minor	Local MAF	Global MAF	p-value
rs1799971	A | A 140 (73%)	A | G 45 (23%)	G | G 7 (4%)	15%	22%	***0.01***
rs2075572	C | C 56 (29%)	G | C 100 (52%)	G | G 38 (20%)	45%	39%	***<0.001***
rs495491	A | A 81 (44%)	A | G 84 (45%)	G | G 21 (11%)	34%	29%	***0.015***
rs533586	T | T 70 (37%)	T | C 90 (48%)	C | C 29 (15%)	39%	25%	***<0.001***
rs609148	G | G 104 (53%)	G | A 74 (38%)	A | A 15 (8%)	27%	15%	***<0.001***
rs563649	C |C 152 (83%)	C | T 31 (17%)	T | T 1 (<1%)	9%	11%	0.43

Table 4-16: Distribution of the genotypes and Minor Allele Frequency (MAF) in the OPRM1 gene for the various Single Nucleotide Polymorphisms (SNP) investigated in this study. p-value quoted is derived by Chi² test for difference between local and global MAF. (The 1000 Genomes Project, 2015)

All six variants were in Hardy-Weinberg equilibrium, as shown in Table 4-17.

Gene	SNP	Chi^2	p-value
OPRM1	rs1799971	1.28	0.26
	rs2075572	0.20	0.65
	rs495491	0.0019	0.96
	rs533586	0.013	0.91
	rs609148	0.04	0.83
	rs563649	0.0073	0.93

Table 4-17: Hardy-Weinberg Equilibrium statistics for OPRM1 variants investigated in the study.

The distribution of the genotypes in the investigated variations as compared to global and European frequencies is shown in Figure 4-6. In all but one variation, rs563649, the local population show a significantly different from the global distribution. All variations except two, rs495491 and rs533586, show a similar distribution as to a European population.

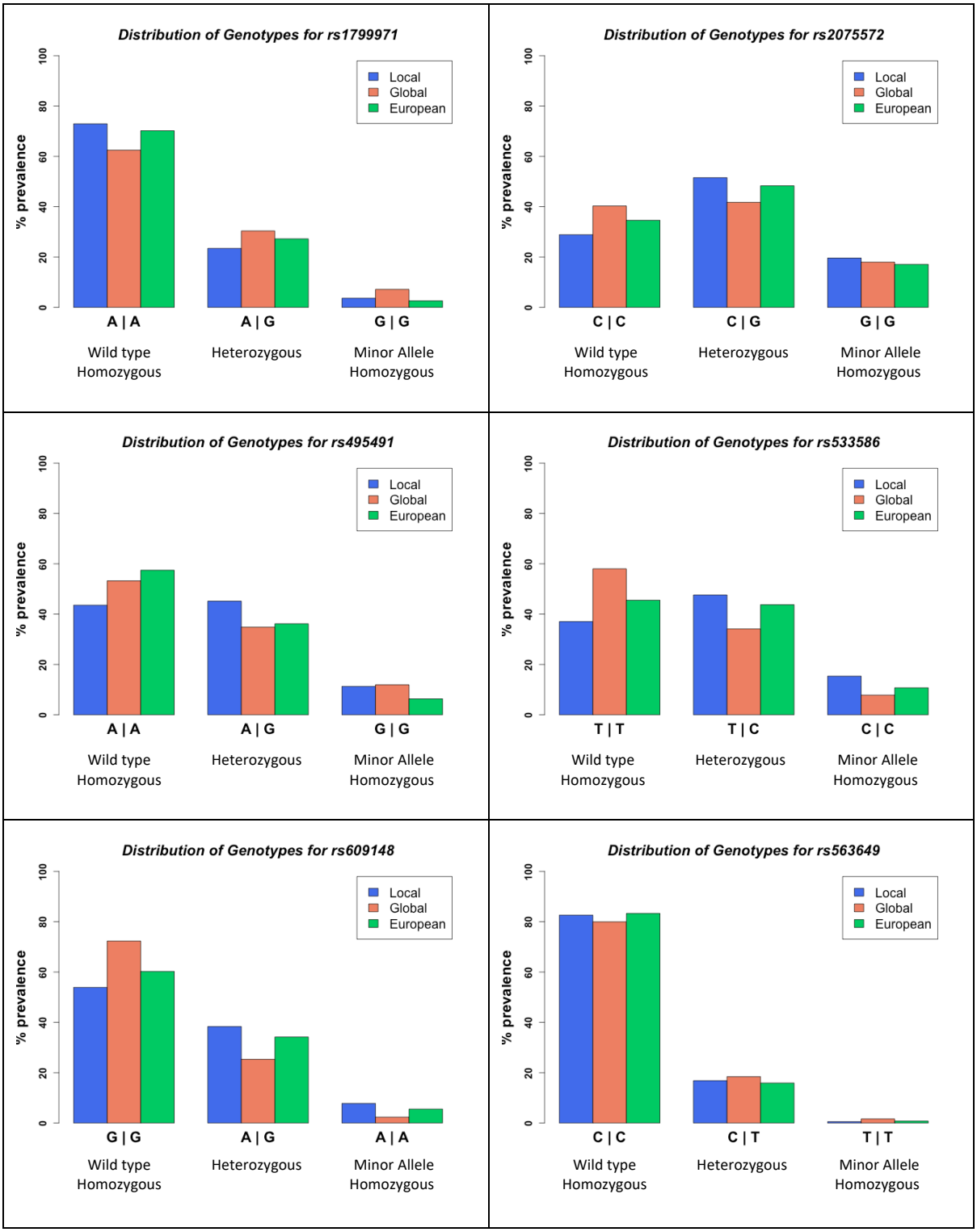

Figure 4-6: Comparison of the genotypes in different Single Nucleotide Polymorphism for the OPRM1 gene, between local, global and European frequencies. (The 1000 Genomes Project, 2015)

Only 19 (10%) patients did not have at least one of the variants investigated, with an average of 3.9 variants per patient in those patients who had at least one SNP.

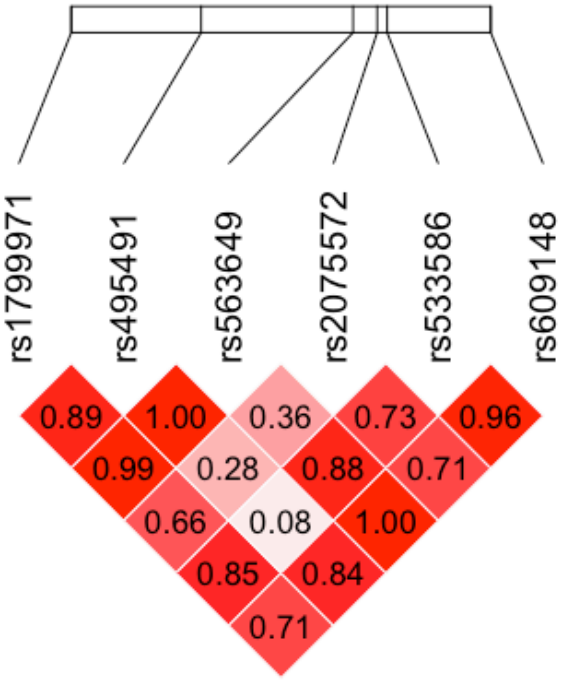

Figure 4-7: LD plot for OPRM1, showing coefficient D'. There is strong linkage dysequilibrium between rs1799971 / rs563649, between rs563649 / rs609148 and between rs533586 / rs609148, with a coefficient close to 1.

There was a decrease in the baseline WOMAC® score and in the baseline WOMAC® Pain subscore in patients who were carrying the rs2075572 variant (minor allele C) of the *OPRM1* gene, as shown in Table 4-18 and Table 4-19. Patients who had the minor allele A of rs609148 had lower pain scores.

SNP	**Baseline WOMAC® Score**		**Wilcoxon W score**	**p-value**
	Minor Allele Carrier	*NonCarrier*		
rs1799971	41.5	42.0	3480	0.69
rs2075572	39.5	49	3049	***0.02***
rs495491	39	45.5	3620	0.11
rs533586	40	44.5	3717	0.25
rs609148	40	43.5	4023	0.15
rs563649	37.5	43	2032.5	0.16

Table 4-18: Effect of the OPRM1 variants on the baseline WOMAC® score. Wilcoxon-Mann-Whitney test.

SNP	Baseline WOMAC® Pain subscore		Wilcoxon W score	p-value
	Minor Allele Carrier	Non Carrier		
rs1799971	9	10	3629	0.97
rs2075572	9	11	2632	***<0.001***
rs495491	9	10	3714	0.18
rs533586	9	10	3548	0.11
rs609148	9	10	3737.5	***0.028***
rs563649	9.5	10	2333.5	0.76

Table 4-19: Effect of the OPRM1 variants on the baseline WOMAC® Pain subscore. Wilcoxon-Mann-Whitney test.

There was a decrease in the incidence of patients reporting a high baseline S-LANSS score in patients with the rs1799971 variant, as shown in Table 4-20. Other variants did not show any significant effect.

SNP	S-LANSS score > 11		OR	CI	p-value
	Minor Allele Carrier	*Non Carrier*			
rs1799971	2% (1 / 52)	14% 20 / 139 (14%)	0.12	0.003–0.77	***0.017***
rs2075572	11% (15 / 138)	12% (7 / 56)	0.85	0.30 – 2.63	0.80
rs495491	11% (12 / 105)	11% (9 / 80)	1.02	0.34 – 2.90	1
rs533586	11% 14 / 118 (11%)	10% 7 / 70 (10%)	1.21	0.43 – 3.74	0.82
rs609148	11% 10 / 88 (11%)	11% 11 / 104 (11%)	1.08	0.39 - 2.98	1
rs563649	13% 4 / 32 (13%)	10% 15 / 151 (10%)	1.29	0.29 - 4.48	0.75

Table 4-20: Effect of the OPRM1 variants on the Baseline S-LANSS scores. Fisher's exact test.

The distribution of the OPRM1 variants between the spinal and the general anaesthesia groups is shown in Table 4-21. There is no difference between the incidence of any of the variants in both groups.

SNP	Group GA	Group SP	OR	CI	p-value
rs1799971 (G)	23 / 95 (24%)	29 / 96 (24%)	1.35	0.68 – 2.71	0.42
rs2075572 (G)	73 / 97 (75%)	65 / 97 (67%)	0.67	0.34 – 1.31	0.27
rs495491 (G)	54 / 92 (59%)	51 / 93 (55%)	0.86	0.45 – 1.59	0.66
rs533586 (C)	56 / 93 (60%)	62 / 95 (65%)	0.81	0.43 - 1.51	0.55
rs609148 (A)	48 / 97 (49%)	40 / 95 (42%)	1.34	0.74 - 2.48	0.32
rs563649 (T)	18 / 93 (19%)	14 / 90 (16%)	1.30	0.56 - 3.05	0.56

Table 4-21: Frequencies of the variants of the OPRM1 gene, across the two groups. Fisher's exact test.

4.1.1.4 GCH1 gene

The frequency of the various single nucleotide polymorphisms in the *GCH1* gene and their relative distributions are shown in Table 4-22.

SNP	Homozygous Major	Heterozygous	Homozygous Minor	Local MAF	Global MAF	p-value
rs998259	C | C 88 (51%)	C | T 64 (37%)	T | T 19 (11%)	30%	8%	***< 0.001***
rs3783641	T | T 144 (76%)	T | A 41 (22%)	A | A 4 (2%)	13%	23%	***< 0.001***

Table 4-22: Distribution of the genotypes and Minor Allele Frequency (MAF) in the GCH1 gene for the various Single Nucleotide Polymorphisms (SNP) investigated in this study. p-value quoted is derived by Chi² test for difference between local and global MAF. (The 1000 Genomes Project, 2015)

Both variants were in Hardy-Weinberg equilibrium, as shown in Table 4-23.

Gene	SNP	Chi^2	p-value
GCH1	rs998259	0.0647	0.80
	rs3783641	1.54	0.21

Table 4-23: Hardy-Weinberg Equilibrium statistics for GCH1 variants investigated in the study

The local distribution of both rs998259 and rs3783641 were significantly different from the global distribution ($p < 0.0001$) and from the European distribution ($p = 0.005$ in both cases), as shown in Figure 4-8.

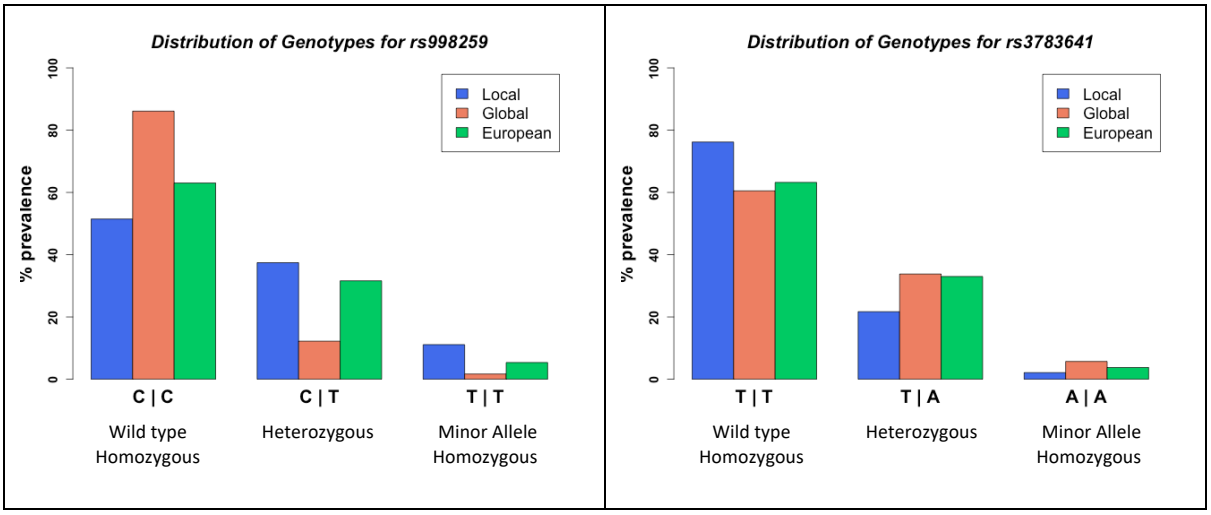

Figure 4-8: Comparison of the genotypes in different Single Nucleotide Polymorphism for the GCH1 gene, between local, global and European frequencies. (The 1000 Genomes Project, 2015)

A total of 117 (66%) out of 176 patients had at least one allele which was a variant of the *GCH1* gene, with 11 patients (7%) who had both variants. There is a strong correlation between the two SNP's, with a coefficient D' for linkage dysequilibrium of 0.999. This is consistent with data from the 1000 Genome Project, where these two variants show a high degree of coinheritance, especially in Europe (The 1000 Genomes Project, 2015).

The most common haplotype was the combination of the wild types of both SNP's, as shown in Table 4-24.

rs998259	**rs3783641**	**Haplotype Frequency**
C	T	57%
T	T	29%
C	A	13%
T	A	<0.01%

Table 4-24: Frequency of multiple single nucleotide polymorphisms of the GCH1 gene in the same patient (haplotypes).

There was no effect of carrying the variants of the GCH1 gene on the baseline WOMAC®, WOMAC® Pain score and S-LANSS score, as shown in Table 4-25, Table 4-26, and Table 4-27.

SNP	Baseline WOMAC® score		Wilcoxon W score	p-value
	Minor Allele Carrier	*Non Carrier*		
rs998259	47	40	3993	0.23
rs3783641	40	43	3431	0.50

Table 4-25: Effect of the GCH1 variants on the Baseline WOMAC® score. Wilcoxon-Mann-Whitney test.

SNP	Baseline WOMAC® Pain subscore		Wilcoxon W score	p-value
	Minor Allele Carrier	*Non Carrier*		
rs998259	10	9	4056	0.16
rs3783641	9	10	3341	0.70

Table 4-26: Effect of the the GCH1 variants on the baseline WOMAC® Pain subscore. Wilcoxon-Mann-Whitney test.

SNP	S-LANSS score > 11		OR	CI	p-value
	Minor Allele Carrier	*Non Carrier*			
rs998259	12% (10 / 83)	8.0% (7 / 87)	1.56	0.51 – 5.10	0.45
rs3783641	9.0% (5 / 45)	10.5% (15 / 143)	1.07	0.28 – 3.33	1.0

Table 4-27: Effect of the GCH1 variants on the Baseline S-LANSS scores. Fisher's exact test.

There was no difference in the incidence of carriers of either of the two variants in the spinal or general anaesthesia groups, as shown in Table.

SNP	Group SP	Group GA	p-value
rs998259	49%	48%	0.88
rs3783641	22%	26%	0.82

Table 4-28: Frequency of patients having at least one copy of the variant SNP being investigated, per study arm.

4.1.1.5 KCNS1 gene

The frequency of the various single nucleotide polymorphisms in the *KCNS1* gene, and their relative distributions, are shown in Table 4-29.

SNP	Homozygous Major	Heterozygous	Homozygous Minor	Local MAF	Global MAF	p-value
rs4499491	C | C 76 (40%)	C | A 99 (52%)	A | A 16 (8%)	34%	47%	***0.001***
rs734784	T | T 54 (30%)	T | C 85 (46%)	C | C 44 (24%)	47%	41%	***0.049***

Table 4-29: Distribution of the genotypes and Minor Allele Frequency (MAF) in the KCNS1 gene for the various Single Nucleotide Polymorphisms (SNP) investigated in this study. p-value quoted is derived by Chi² test for difference between local and global MAF . (The 1000 Genomes Project, 2015)

The rs4499491 variant was not in Hardy-Weinberg equilibrium, as shown in Table 4-30. This might indicate some form of bias.

Gene	SNP	Chi^2	p-value
KCNS1	rs4499491	4.06	***0.04***
	rs734784	0.661	0.42

Table 4-30: Hardy-Weinberg Equilibrium statistics for KCNS1 variants investigated in the study

The local distribution of both rs4499491and rs734784 were significantly different from the global distribution ($p < 0.001$ and $p=0.049$ respectively), but were similar to the European distribution ($p=0.14$ and $p=0.95$ respectively), as shown in Figure 4-9.

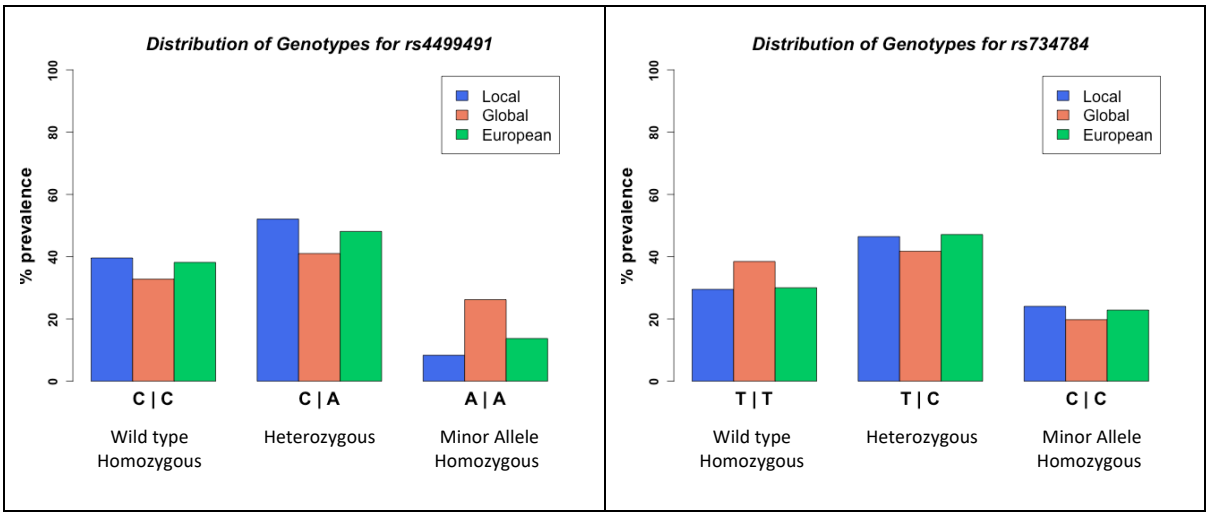

Figure 4-9: Comparison of the genotypes in different Single Nucleotide Polymorphism for the KCNS1 gene, between local, global and European frequencies. (The 1000 Genomes Project, 2015)

A total of 154 (82%) out of 184 patients had at least one allele which was a variant of the *KCNS1* gene. There were 54 patients (34%) who had both variants.

The two SNP's do not seem to be co-inherited, with a linkage coefficient D' of 0.402. Comparison with data from the 1000 Genome Project is not possible, as little information exists about any European population (The 1000 Genomes Project, 2015).

rs4499491	rs734784	Haplotype Frequency
C	T	42%
A	T	24%
A	C	23%
C	C	11%

Table 4-31: Frequency of multiple single nucleotide polymorphisms of the KCNS1 gene in the same patient (haplotypes)

Patients with the rs734784 variant of the *KCNS1* gene had lower scores on the baseline WOMAC® score and WOMAC® Pain scores as shown in Table 4-32, Table 4-33 and Table 4-34. There were no other statistically significant effects of rs449941 on the baseline scores.

SNP	**Baseline WOMAC® score**		**Wilcoxon W score**	**p-value**
	Minor Allele Carrier	*Non Carrier*		
rs4499491	40	43	4004	0.32
rs734784	42	47	2810	***0.047***

Table 4-32: Effect of the KCNS1 variants on the baseline WOMAC® score. Wilcoxon-Mann-Whitney test.

SNP	**Baseline WOMAC® Pain subscore**		**Wilcoxon W score**	**p-value**
	Minor Allele Carrier	*Non Carrier*		
rs4499491	10	9	4094.5	0.46
rs734784	9	11	2809	***0.046***

Table 4-33: Effect of the KCNS1 variants on the baseline WOMAC® Pain subscore. Wilcoxon-Mann-Whitney test.

SNP	S-LANSS score > 11		OR	CI	p-value
	Minor Allele Carrier	*Non Carrier*			
rs4499491	7.8% (9 / 115)	15.6% (12 / 76)	0.45	0.29 – 4.30	0.10
rs734784	8.0% (10 / 128)	15.0% (8 / 54)	0.49	0.16 – 1.52	0.17

Table 4-34: Effect of the KCNS1 variants on the Baseline S-LANSS scores. Fisher's exact test.

Patients in Group SP were more likely to have the rs4499491 variant, as shown in Table 4-35. There was no difference in the incidence of carriers of rs3783641 in the spinal or general anaesthesia groups.

SNP	Group SP	Group GA	p-value
rs4499491	68%	53%	***0.04***
rs3783641	71%	70%	0.87

Table 4-35: Frequency of patients having at least one copy of the variant SNP of the KCNS1 being investigated per study arm.

4.1.1.6 OPRK1 gene

The frequency of rs6985606, a single nucleotide polymorphism in the K-opiate receptor-1 (*OPRK1*) gene, and its relative distribution is shown in Table 4-36.

SNP	Homozygous Major	Heterozygous	Homozygous Minor	Local MAF	Global MAF	p-value
rs6985606	T|T 51 (29%)	T|C 87 (51%)	C|C 33 (19%)	43%	31%	***<0.001***

Table 4-36: Distribution of the genotypes and Minor Allele Frequency (MAF) in the OPRK1 gene for the Single Nucleotide Polymorphisms (SNP) rs6985606 investigated in this study. p-value quoted is derived by Chi² test for difference between local and global MAF. (The 1000 Genomes Project, 2015)

The SNP was in Hardy-Weinberg equilibrium, as shown in Table 4-37.

Gene	SNP	Chi^2	p-value
OPRK1	rs6985606	0.0702	0.79

Table 4-37: Hardy-Weinberg Equilibrium statistics for OPRK1 variant investigated in the study

When comparing these frequencies with global and European distributions, there is a similarity in the frequencies found locally and those reported in Europe ($p = 0.32$). The local differences were significantly different from the global distribution ($p < 0.001$).

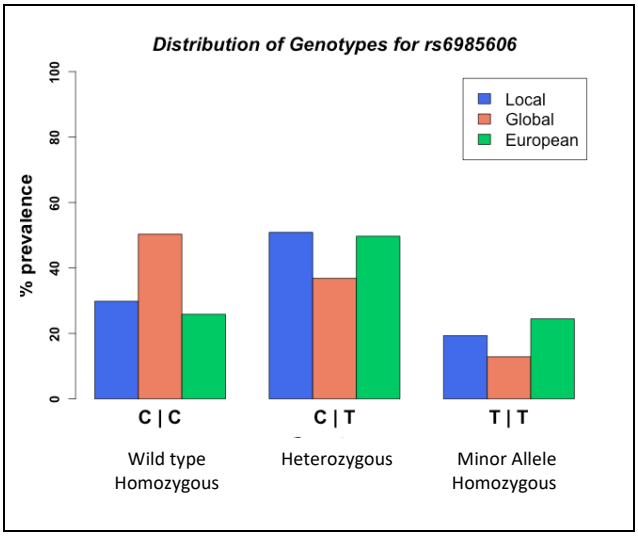

Figure 4-10: Comparison of the genotypes in different Single Nucleotide Polymorphism for the OPRK1 gene, between local, global and European frequencies. (The 1000 Genomes Project, 2015)

There was no effect of carrying the variant rs6985606 of the *OPRK1* gene on the baseline WOMAC®, WOMAC® Pain score and the S-LANSS score, as shown in Table 4-38, Table 4-39 and Table 4-40.

SNP	Baseline WOMAC® score		Wilcoxon W score	p-value
	Minor Allele Carrier	*Non Carrier*		
rs6985606	41.5	42.0	3058	0.84

Table 4-38: Effect of the rs6985606 variant of the OPRK1 gene on the Baseline WOMAC® score. Wilcoxon-Mann-Whitney test.

SNP	Baseline WOMAC® Pain subscore		Wilcoxon W score	p-value
	Minor Allele Carrier	*Non Carrier*		
rs6985606	10	9	3475	0.10

Table 4-39: Effect of the rs6985606 variant of the OPRK1 gene on the baseline WOMAC® Pain subscore. Wilcoxon-Mann-Whitney test.

SNP	S-LANSS score > 11		OR	CI	p-value
	Minor Allele Carrier	Non Carrier			
rs6985606	11.7% (14 / 120)	8.7% (4/ 50)	1.52	0.44 – 6.66	0.59

Table 4-40: Effect of the rs6985606 variant of the OPRK1 gene on the Baseline S-LANSS scores. Fisher's exact test.

Carriers of the variant were more likely to receive a spinal anaesthetic, but this difference was not statistically significant (Group SP: 54% vs Group GA: 42%, p = 0.18).

4.1.1.7 Summary

In summary, the following points are noted:

- In most variants investigated, the distribution between genotypes was similar to a European distribution.
- Some variants exhibited co-inheritance: this would make analysis of some variants redundant.
- Reduced baseline WOMAC® scores were found in patients with either the minor allele (G) of rs2075572 in the *OPRM1* gene, or the minor allele (C) of rs734784 variant of the *KCNS1* gene.
- A decreased baseline WOMAC® Pain subscore was found in patients carrying the minor allele (G) of rs2075572 in the *OPRM1* gene.
- The minor allele (G) of the rs1799971 variant of the *OPRM1* gene was associated with a higher baseline S-LANSS score.

4.2 Early Postoperative Pain

4.2.1 Descriptive Statistics

The average pain scores in the early postoperative period for the patients in the study are shown in Table 4-41.

	Median	**No pain**	**1 – 3**	**4 – 7**	**8 - 10**
Numerical Rating Score, at rest	2 [IQR: 0 – 5]	72 (36.1%)	52 (26.1%)	58 (29.2%)	17 (8.5%)
Numerical Rating Score, during physiotherapy	5 [IQR: 3 – 7]	20 (10.3%)	46 (23.1%)	85 (43.1%)	47 (23.6%)
Morphine Consumption over 24 hours (mg)	11.6 (SD 9.18)				

Table 4-41: Median scores for pain at rest and during physiotherapy, and mean morphine consumption over 24 hours

On the morning of the first day postoperatively, severe pain at rest was not common, with only 17 patients (8.5%) reporting a pain score equal to or higher than 8. In fact, a high proportion of patients (36.1%) reported no pain at all. This is reflected in Figure 4-11.

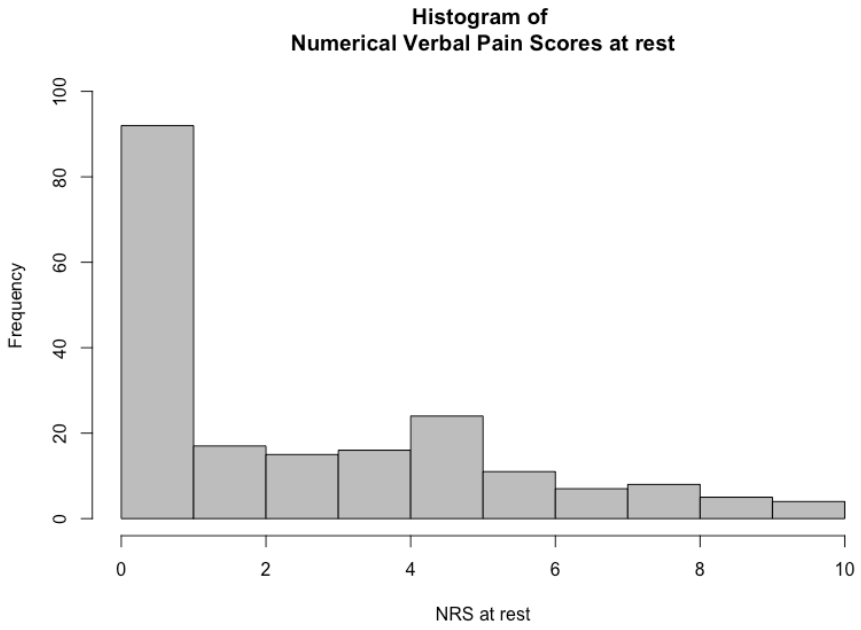

Figure 4-11: Distribution of Numerical Rating Scores (NRS) for pain at rest

Total morphine consumption up to this point was also low, with an average of only 11.6 mg: this included long-acting opiates given during the procedure and in the early stages of recovery. In fact, the majority of patients used less than 10 mg of morphine over 24 hours (Figure 4-12).

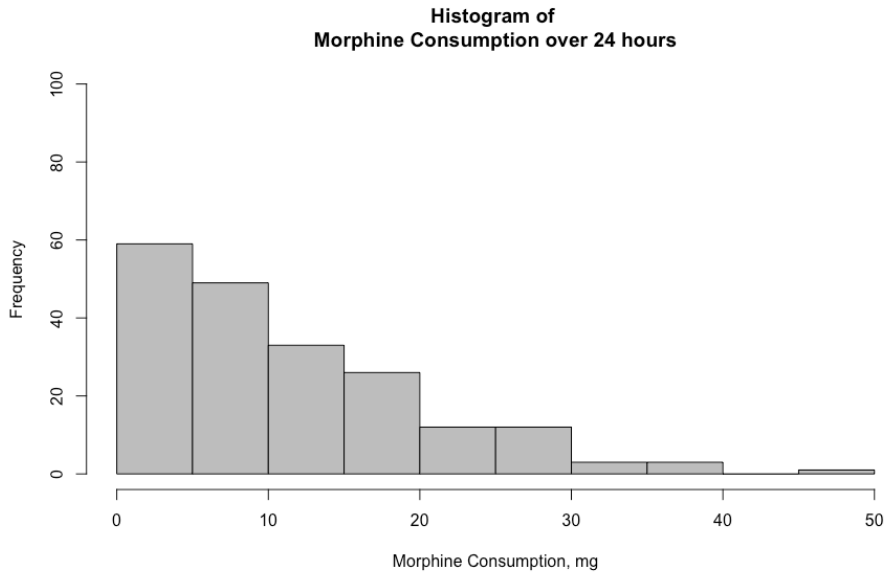

Figure 4-12: Distribution of morphine consumption over 24 hours

There was no relationship between the total amount of morphine consumption and the pain score at rest. Hence, it is not possible to assume that the more morphine a patient used, the better the pain scores (Figure 4-13). This might be due to the effect of the intrathecal diamorphine, whose action lasts for around 10 hours.

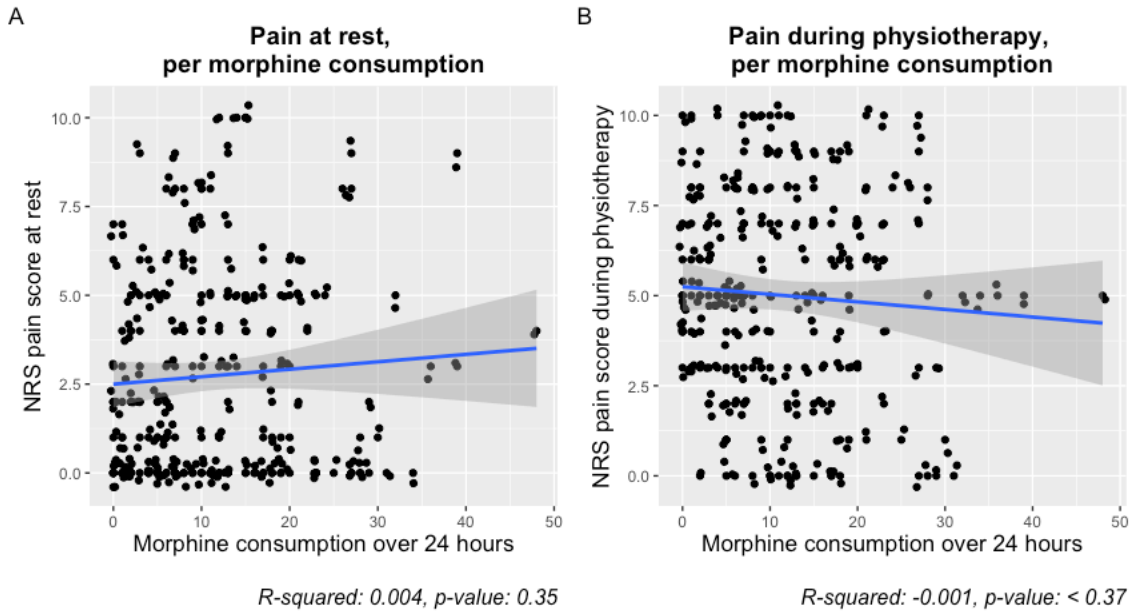

Figure 4-13: A) Pain scores at rest vs Morphine consumption. B) A) Pain scores at rest vs Morphine consumption. No relationship exists in either graph, with a flat linear regression line in blue, and an R^2 being very close to zero. This would mean that both Pain at rest and Pain during physiotherapy are not related to morphine consumption.

Pain on physiotherapy was more severe, which was expected, with a median score of 5 (Figure 4-14). Although some patients fared well, reporting low levels of pain, 46 patients (23.6%) has a pain score of 8 or greater. This was despite the use of a multimodal analgesic plan.

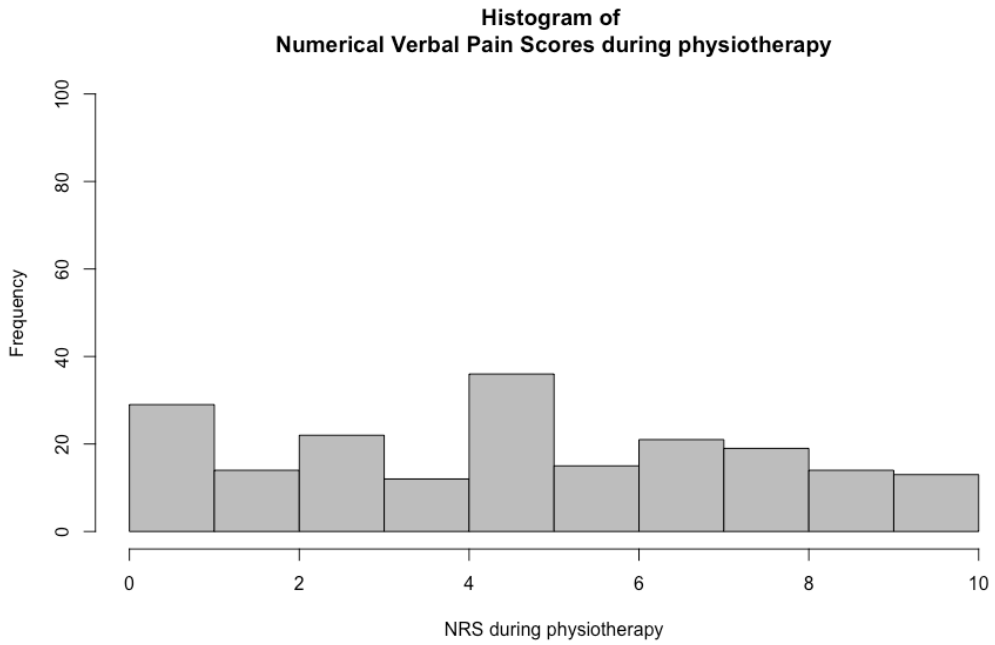

Figure 4-14: Distribution of pain scores during physiotherapy

4.2.2 Factors influencing Early Postoperative Pain

4.2.2.1 Demographic Factors

On univariate analysis, age, sex, BMI, ASA, surgical firm did not show any influence on pain at rest or during physiotherapy.

There was also no effect of S-LANSS on pain during physiotherapy, but the baseline WOMAC® score was associated with increased pain score by 2% for every point, albeit at a slightly non-statistical significance (estimate: 1.02, 95%CI: 0.99 to 1.05, p = 0.06). The pain score component of the WOMAC® score did not have any influence on the postoperative pain.

Ordinal regression analysis of the effect of various demographic factors on pain at rest and during physiotherapy was performed. The results are being shown in Table 4-42 and Table 4-43. Age was significantly associated with a decreased pain at rest: for an increase by one year, patients had a 6% probability of having less pain.

	Coefficient	**Odds Ratio**	**CI**	**p-value**
Age	-0.058	0.94	0.90 – 0.98	***0.014***
Male	-0.208	0.84	0.48 – 1.47	0.55
BMI	-0.018	0.99	0.94 – 1.02	0.52
Low S-LANSS	0.506	1.42	0.83 – 244	0.19
Baseline WOMAC® pain	-0.039	0.97	0.89 – 1.05	0.49
Residual Deviance: 786.498 AIC: 816.498			Pulkstenis-Robinson test: 0.31 Lipsitz test: 0.90 Hosmer and Lemeshow test: 0.36	

Table 4-42: Effect of demographic data on pain scores at rest. Polynomial Ordinal Regression, odds ratio describe probability

Male patients and higher BMI were associated with less probability of being in pain during physiotherapy, with males having 50% less chance of being in pain.

	Coefficient	Odds Ratio	CI	p-value
Age	-0.03	0.97	0.92 – 1.01	0.15
Male	-0.63	0.53	0.31 – 0.92	***0.025***
BMI	-0.039	0.96	0.92 – 1.00	***0.057***
Low S-LANSS	0.10	1.10	0.63– 1.93	0.72
Baseline WOMAC® pain	-0.061	1.06	0.98 – 1.15	0.12
Residual Deviance: 851.6026 AIC: 881.6026			Pulkstenis-Robinson test: 0.17 Lipsitz test: 0.90 Hosmer and Lemeshow test: 0.18	

Table 4-43: Effect of demographic data on pain scores during physiotherapy. Polynomial Ordinal Regression.

4.2.2.2 Influence of Anaesthesia

The influence of the type of anaesthetic on early postoperative pain following a total knee replacement is shown in Table 4-44.

	Group GA	Group SP	p-value
Median Numerical Rating Score, at rest	1 [IQR: 0.0 – 4.3]	2 [IQR: 0.0 – 5.0]	0.14
Number of patients with severe pain (NRS between 8 – 10)	8 (8%)	9 (9%)	0.80
Median Numerical Rating Score, during physiotherapy	5 [IQR: 2.0 – 7.0]	5 [IQR: 4.0 – 8.0]	***0.0008***
Number of patients with severe pain (NRS between 8 – 10)	17 (18%)	29 (30%)	0.06
Morphine Consumption over 24 hours (mg)	15 [IQR: 10.0 – 21.0]	5 [IQR: 2.0 – 9.0]	***< 0.001***

Table 4-44: Pain scores and morphine consumption, categorized by type of anaesthesia. NRS: Numerical Rating Score. Wilcoxon-Mann-Whitney test, Chi^2 test.

There is no difference between the two groups at rest, but during physiotherapy, a higher proportion of patients in the spinal group reported severe pain. On the other hand, morphine consumption over 24 hours was much lower in the spinal group.

This might be explained by the fact that physiotherapy usually occurred hours after the last morphine administration, so patients in the spinal might have been at a disadvantage. It has also been documented that neuroaxial anaesthesia might cause rebound pain, but this would have been reflected also in the pain at rest. An effect of the femoral nerve block, done only in the GA group, cannot be excluded.

Other confounding variables could include genetic factors: it has already been described that there was an increased frequency of the rs7595255 variant, and possibly also the rs6746030 variant of the *SCN9A* gene in the patients in Group GA.

A multivariate analysis was performed for pain during physiotherapy. All demographic data and the anaesthetic type were used as dependant variables, and a step-down approach was used (Table 4-45).

Pain during physiotherapy ~ male + anaesthesia				
	Estimate	**Std. Error**	**t-value**	**p-value**
Intercept	4.68	0.322	14.566	
male	-0.944	0.419	-2.25	***0.025***
Spinal anaesthesia	1.53	0.404	3.794	***0.0002***
Null deviance: 1663.8 on 193 degrees of freedom Residual deviance: 1513.9 on 191 degrees of freedom AIC: 957.13 Number of Fisher Scoring iterations: 2				

Table 4-45: Linear regression model, stepdown analysis. Only gender and anaesthesia were retained in the final model, since these retained statistical significance.

As shown in Table 4-45, the two main predictors for pain during physiotherapy were gender and anaesthesia. Males had less pain during physiotherapy (mean NRS score 3.7 vs 4.6, mean difference -0.994, 95%CI -1.77 to -0.12, p = 0.025). Patients who had a spinal anaesthetic had a higher pain score (mean NRS score 6.1 vs 4.6, mean difference 1.53, 95%CI: 0.74 to 2.33, p < 0.01), even after taking into account the consumption of morphine during the first 24 hours.

4.2.2.3 Influence of Genetic Factors

The effect of the various polymorphisms in the *COMT* gene on the outcomes of this research is shown in Table 4-46. No significant effect of these polymorphisms could be found on either of the outcomes.

	Wild Type	Heterozygous	Recessive	p-value
rs4633				
n	49	101	43	
NRS (rest)	2 [IQR: 0.0 – 5.3]	2 [IQR: 0.0 – 5.0]	3 [IQR: 0.0 – 5.0]	0.48
severe (8 – 10)	3 (6%)	7 (7%)	5(12%)	0.56
NRS (physiotherapy)	5 [IQR: 2.0 – 7.0]	5 [IQR: 3.0 – 7.0]	5 [IQR: 3.0 – 8.0]	0.44
severe (8 – 10)	8 (17%)	21 (21%)	14(33%)	0.18
Morphine over 24 hours (mg)	10 [IQR: 5.5– 17.0]	11 [IQR: 5.0 – 18.0]	7 [IQR: 2.0 – 15.5]	0.30
rs4680				
n	48	100	44	
NRS (rest)	2 [IQR: 0.0 – 5.0]	2 [IQR: 0.0 – 5.0]	3 [IQR: 0.8 – 5.0]	0.41
severe (8 – 10)	4 (8%)	7(7%)	5 (12%)	0.65
NRS (physiotherapy)	5 [IQR: 2.0 – 7.0]	5 [IQR: 3.0 – 7.0]	5 [IQR: 3.0 – 8.0]	0.59
severe (8 – 10)	9 (20%)	22 (22%)	13 (30%)	0.48
Morphine over 24 hours (mg)	9 [IQR: 5.3 – 16.8]	10 [IQR: 5.0 – 18.0]	7 [IQR: 2.8 – 17.0]	0.47
rs4818				
n	81	84	25	
NRS (rest)	2 [IQR: 0.0 – 5.0]	2 [IQR: 0.0 – 5.0]	2.5 [IQR: 0.0 – 4.5]	0.88
severe (8 – 10)	8 (9%)	5 (6%)	2 (8%)	0.74
NRS (physiotherapy)	5 [IQR: 3.0 – 8.0]	5 [IQR: 3.0 – 7.0]	5 [IQR: 2.0 – 6.5]	0.57
severe (8 – 10)	22 (27%)	19(23%)	3 (13%)	0.37
Morphine over 24 hours (mg)	9 [IQR: 4.0 – 18.0]	10 [IQR: 5.0 – 16.0]	9 [IQR: 3.0 – 15.0]	0.82

Table 4-46: Effect of the COMT variations on pain scores and morphine consumption grouped by genotype of the variant allele. Wilcoxon-Mann-Whitney test for comparison of medians, Fisher's exact test for incidences. NRS: Numerical Rating Score

The effect of the various polymorphisms in the *SCN9A* gene on the outcomes of this research is shown in Table 4-47.

	Wild	Heterozygous	Recessive	p-value
rs6746030				
n	130	49	11	
NRS (rest)	2 [IQR: 0.0 – 5.0]	2 [IQR: 0.0 – 5.0]	3.5 [IQR: 1.3 – 5.8]	0.38
severe (8 – 10)	10 (8%)	4 (8%)	2 (20%)	0.31
NRS (physiotherapy)	5 [IQR: 3.0 – 7.0]	5 [IQR: 3.0 – 8.0]	4.5 [IQR: 2.3 – 7.8]	0.58
severe (8 – 10)	16 (22%)	13 (27%)	3 (30%)	0.72
Morphine over 24 hours (mg)	8 [IQR: 4.0– 15.8]	13 [IQR: 7.0– 19.0]	6.5 [IQR: 3.0– 17.2]	0.06
rs7595255				
n	133	53	11	
NRS (rest)	2 [IQR: 0 – 5]	2 [IQR: 0 – 5]	2 [IQR: 0.3 – 5]	0.75
severe (8 – 10)	10 (8%)	5 (9%)	1 (10%)	0.73
NRS (physiotherapy)	5 [IQR: 3.0 – 7.0]	6 [IQR: 3.0 – 8.0]	3.5 [IQR: 2.0 – 6.5]	0.16
severe (8 – 10)	28 (22%)	15 (28%)	2 (20%)	0.61
Morphine over 24 hours (mg)	8 [IQR: 4.0 – 16.0]	13 [IQR: 7.0 – 19.2]	9 [IQR: 3.0 – 17.2]	***0.025***
rs11898284				
n	150	40	6	
NRS (rest)	1 [IQR: 0.0 – 5.0]	3 [IQR: 0.8 – 4.3]	1.5 [IQR: 0.3 – 2.0]	0.38
severe (8 – 10)	12 (8%)	4 (10%)	0 (0%)	0.85
NRS (physiotherapy)	5 [IQR: 3.0 – 7.0]	5 .5 [IQR: 3.0 – 7.8]	5 [IQR: 3.5 – 5.8]	0.74
severe (8 – 10)	33 (22%)	10 (26%)	1 (17%)	0.69
Morphine over 24 hours (mg)	10 [IQR: 5.0 – 17.2]	9.5 [IQR: 4.8 – 17.0]	15.5 [IQR: 7.5 – 19.0]	0.77

Table 4-47: Effect of the SCN9A variations on pain scores and morphine consumption, grouped by genotype of the variant allele. Wilcoxon-Mann-Whitney test for comparison of medians, Fisher's exact test for incidences.

Overall, there was no significant effect of these polymorphisms on most of the outcomes, except for total morphine consumption over 24 hours. However, it has already been established that there were more carriers of the rs7595255 and possibly of the rs6746030 variants in the patients who received a general anaesthestic. Thus this might be considered a confounding factor.

To check for this, a linear regression model was used, with morphine consumption as the dependant variable, and presence of rs7595255 and anaesthesia type as the dependant variables:

Morphine consumption ~ rs7595255 + anaesthesia

Although the adjusted R^2 was only 0.26, there was a strong association with anaesthesia type rather than presence of rs7595255 variant (Table 4-48).

Morphine consumption ~ rs7595255 + anaesthesia				
	Estimate	**Std. Error**	**t-value**	**p-value**
Intercept	15.574	0.942	16.528	
rs7595255 TC	1.535	1.332	1.152	0.25
rs7595255 TT	0.528	2.612	0.202	0.84
Spinal anaesthesia	-9.174	1.169	-7.849	***<0.001***
Residual standard error: 7.962 on 191 degrees of freedom				
Multiple R-squared: 0.2683R			Adjusted R-squared: 0.2568	
F-statistic: 23.34 on 3 and 191 DF, p-value*: <0.001*				

Table 4-48: Linear Regression model for Morphine consumption, using rs7595255 and anaesthesia type as independent variables

The effect of the various polymorphisms in the *OPRM1* gene on the outcomes of this research is shown in Table 4-49. Patients who were homozygous for the rs495491 had lower pain scores at rest, but this effect was not seen in pain scores during physiotherapy.

Furthermore, there was only one patient who was homozygous for rs563649, and this patient required no morphine at all.

	Wild Type	Heterozygous	Recessive	p-value
rs1799971				
n	139	45	7	
NRS (rest)	2 [IQR: 0.0 – 5.0]	1 [IQR: 0.0 – 5.0]	3 [IQR: 0.0 – 4.0]	0.89
severe (8 – 10)	12 (9%)	45 (11%)	0 (0%)	0.78
NRS (physiotherapy)	5 [IQR: 3.0 – 7.0]	5 [IQR: 2.0 – 8.0]	7 [IQR: 5.5 – 8.0]	0.42
severe (8 – 10)	30 (22%)	12 (27%)	3 (43%)	0.63
Morphine over 24 hours (mg)	9.0 [IQR: 5.0– 17.0]	10.0 [IQR: 5.0 – 15.0]	19.0 [IQR: 8.0 – 22.0]	0.25
rs2075572				
n	56	100	38	
NRS (rest)	3 [IQR: 0.0 – 6.0]	2 [IQR: 0.0 – 5.0]	1 [IQR: 0.0 – 5.0]	0.49
severe (8 – 10)	6 (11%)	8 (8%)	3 (8%)	0.89
NRS (physiotherapy)	5 .5 [IQR: 3.0 – 7.0]	5 [IQR: 3.0 – 7.2]	5 [IQR: 3.0 – 8.0]	0.50
severe (8 – 10)	12 (21%)	24 (24%)	10 (26%)	0.66
Morphine over 24 hours (mg)	10.5 [IQR: 5.8 – 17.0]	9 [IQR: 5.0 – 15.0]	13 [IQR: 5.0 – 21.0]	0.32
rs495491				
n	80	84	21	
NRS (rest)	2 [IQR: 0.0 – 5.0]	2 [IQR: 0.0 – 5.0]	0 [IQR: 0.0 – 2.2]	***0.05***
severe (8 – 10)	3 (4%)	12 (14%)	1 (5%)	***0.05***
NRS (physiotherapy)	5 [IQR: 3.0 – 8.0]	5 [IQR: 3.0 – 7.0]	5 [IQR: 2.0 – 7.2]	0.99
severe (8 – 10)	22 (25%)	19 (23%)	5 (24%)	0.37
Morphine over 24 hours (mg)	10 [IQR: 5.0 – 17.0]	10 [IQR: 4.5 – 17.5]	8.5 [IQR: 3.0 – 23.5]	0.98

rs533586				
n	70	89	29	
NRS (rest)	2 [IQR: 0.0 – 5.0]	2 [IQR: 0.0 – 5.0]	3 [IQR: 0.0 – 5.0]	0.69
severe (8 – 10)	4 (6%)	8 (9%)	3 (10%)	0.67
NRS (physiotherapy)	5 [IQR: 3.0 – 8.0]	5 [IQR: 3.0 – 7.0]	5 [IQR: 3.0 – 7.0]	0.80
severe (8 – 10)	16 (23%)	19 (21%)	7 (24%)	0.82
Morphine over 24 hours (mg)	10 [IQR: 5.0 – 15.0]	9 [IQR: 4.0 – 18.0]	10 [IQR: 4.0 – 18.0]	0.85
rs609148				
n	104	73	15	
NRS (rest)	2 [IQR: 0.0 – 5.0]	2 [IQR: 0.0 – 5.0]	2 [IQR: 0.0 – 5.0]	0.92
severe (8 – 10)	8 (8%)	7 (10%)	1 (7%)	0.92
NRS (physiotherapy)	5 [IQR: 3.0 – 8.0]	5 [IQR: 3.0 – 7.0]	5 [IQR: 2.5 – 8.0]	0.46
severe (8 – 10)	27 (26%)	13 (18%)	5 (33%)	0.50
Morphine over 24 hours (mg)	10.5 [IQR: 5.0 – 17.0]	8 [IQR: 4.0 – 17.0]	10 [IQR: 4.5 – 19.0]	0.45
rs563649				
n	151	31	1	
NRS (rest)	2 [IQR: 0.0 – 5.0]	2.5 [IQR: 0.2 – 5.0]	0 [IQR: 0.0 – 4.5]	0.35
severe (8 – 10)	13 (9%)	3 (10%)	0 (0%)	0.76
NRS (physiotherapy)	5 [IQR: 3.0 – 7.0]	5 [IQR: 3.2 – 8.0]	5 [IQR: 5.0 – 5.0]	0.53
severe (8 – 10)	34 (23%)	10 (32%)	0 (0%)	0.56
Morphine over 24 hours (mg)	10 [IQR: 5.0 – 18.0]	6 [IQR: 3.0 – 14.0]	0 [IQR: 0.0 – 0.0]	0.05

Table 4-49: Effect of the OPRM1 variations on pain scores and morphine consumption, grouped by genotype of the variant allele. Wilcoxon-Mann-Whitney test for comparison of medians, Fisher's exact test for incidences.

The effect of the various polymorphisms in the *GCH1* gene on the outcomes of this research is shown in Table 4-50. No significant effect of these polymorphisms could be found on either of the outcomes.

	Wild Type	Heterozygous	Recessive	p-value
rs998259				
n	87	64	19	
NRS (rest)	2 [IQR: 0.0 – 5.0]	2.5 [IQR: 0.0 – 5.0]	0 [IQR: 0.0 – 4.0]	0.23
severe (8 – 10)	11 (13%)	3 (5%)	1(15%)	0.22
NRS (physiotherapy)	5 [IQR: 3.0 – 7.0]	5.5 [IQR: 3.0 – 7.0]	5 [IQR: 2.0 – 7.0]	0.44
severe (8 – 10)	20 (26%)	15 (24%)	4 (21%)	1.00
Morphine over 24 hours (mg)	10 [IQR: 6.0– 18.8]	9.5 [IQR: 3.0 – 17.0]	9 [IQR: 6.5 – 17.5]	0.41
rs3783641				
n	143	41	4	
NRS (rest)	2 [IQR: 0.0 – 5.0]	2.5 [IQR: 0.0 – 5.2]	2.0 [IQR: 0.8 – 3.0]	0.63
severe (8 – 10)	13 (9%)	4 (10%)	0 (0%)	0.84
NRS (physiotherapy)	5 [IQR: 2.0 – 7.0]	5 [IQR: 3.0 – 7.0]	5.5 [IQR: 3.0 – 8.0]	0.65
severe (8 – 10)	39 (28%)	7 (18%)	0 (0%)	0.47
Morphine over 24 hours (mg)	10 [IQR: 4.2 – 17.0]	8.5 [IQR: 5.5 – 14.0]	10.5 [IQR: 8.2 – 11.8]	0.97

Table 4-50: Effect of the GCH1 variations on pain scores and morphine consumption grouped by genotype of the variant allele. Wilcoxon-Mann-Whitney test for comparison of medians, Fisher's exact test for incidences.

The effect of the various polymorphisms in the *KCNS1* gene on the outcomes of this research is shown in Table 4-51. No significant effect of these polymorphisms could be found on either of the outcomes.

	Wild Type	Heterozygous	Recessive	p-value
rs734784				
n	54	85	43	
NRS (rest)	1.5 [IQR: 0.0 – 4.8]	1.5 [IQR: 0.0 – 5.0]	2.0 [IQR: 0.0 – 5.0]	0.44
severe (8 – 10)	5 (9%)	3 (4%)	6 (14%)	0.10
NRS (physiotherapy)	6.0 [IQR: 3.0 – 7.0]	5.0 [IQR: 2.0 – 7.5]	5.0 [IQR: 3.0 – 7.0]	0.28
severe (8 – 10)	12 (22%)	21 (25%)	10 (23%)	0.69
Morphine over 24 hours (mg)	10 [IQR: 6.0– 19.0]	9.0 [IQR: 5.0 – 15.0]	7.0 [IQR: 3.5 – 14.5]	0.39
rs4499491				
n	76	99	16	
NRS (rest)	1.0 [IQR: 0.0 – 4.5]	2.0 [IQR: 0.0 – 5.0]	5.0 [IQR: 0.0 – 6.0]	0.26
severe (8 – 10)	5 (7%)	8 (8%)	3 (19%)	0.24
NRS (physiotherapy)	5 [IQR: 3.0 – 7.0]	5 [IQR: 3.0 – 8.0]	6.0 [IQR: 4.8 – 8.0]	0.19
severe (8 – 10)	12 (16%)	26 (26%)	6 (38%)	0.26
Morphine over 24 hours (mg)	11 [IQR: 5.2 – 18.8]	8 [IQR: 5.0 – 15.0]	7 [IQR: 2.8 – 13.0]	0.11

Table 4-51: Effect of the KCNS1 variations on pain scores and morphine consumption grouped by genotype of the variant allele. Wilcoxon-Mann-Whitney test for comparison of medians, Fisher's exact test for incidences.

With respect to rs6985606, for the *OPRK1* gene, the outcome of this research is shown in Table 4-52.

	Wild	Heterozygous	Recessive	p-value
rs6985606				
n	50	87	33	
NRS (rest)	2 [IQR: 0.0 – 5.0]	1 [IQR: 0.0 – 4.0]	3.0 [IQR: 0.0 – 5.0]	0.09
severe (8 – 10)	4 (8%)	4 (5%)	4 (12%)	0.34
NRS (physiotherapy)	5 [IQR: 3.0 – 8.0]	5 [IQR: 3.0 – 8.0]	5 [IQR: 3.8 – 7.0]	0.57
severe (8 – 10)	14 (28%)	18 (21%)	7 (21%)	0.66
Morphine over 24 hours (mg)	10 [IQR: 5.2– 16.8]	9 [IQR: 4.0– 17.0]	7 [IQR: 3.0– 12.0]	0.47

Table 4-52: Effect of the rs6985606 variation of OPRK1 on pain scores and morphine consumption, in carriers and non-carriers of the variant allele. Wilcoxon-Mann-Whitney test for comparison of medians, Fisher's exact test for incidences.

No significant effect could be noted. Since the KOP receptor is implicated in the spinal effects of opiates, the relationship between rs6985606 and a spinal anaesthetic was checked. Still there were no significant effects noted. This might be due to the fact that these pain scores would have been collected 24 hours after surgery, when the effect of the intrathecal opiate would have worn off.

4.2.2.4 Summary

In summary, the following was noted:

- On ordinal regression, age, male gender and higher BMI were associated with less acute pain.
- Patients who had a spinal anaesthetic were more likely to be in pain the following day but consumed much less morphine over 24 hours.
- Patients who were homozygous recessive for rs495491 variant of the *OPRM1* gene were more likely to have lower pain scores at rest.

4.3 Postoperative Pain at 3 and 6 months

4.3.1 Descriptive Statistics

At three months follow-up, 9 patients could not be contacted, with 4 of these completely lost to follow up. 23 patients were lost to follow-up at six months. In total, data from 175 patients was used in the final analysis.

4.3.1.1 Progression of WOMAC® Score, WOMAC® Pain Subscore

The median WOMAC® scores and WOMAC® Pain subscores decreased significantly throughout the study period, as shown in Figure 4-15. The baseline WOMAC® score was 41. At three months, this dropped by 27 points to 13. At six months, the WOMAC® score was 10, dropping by 28 points.

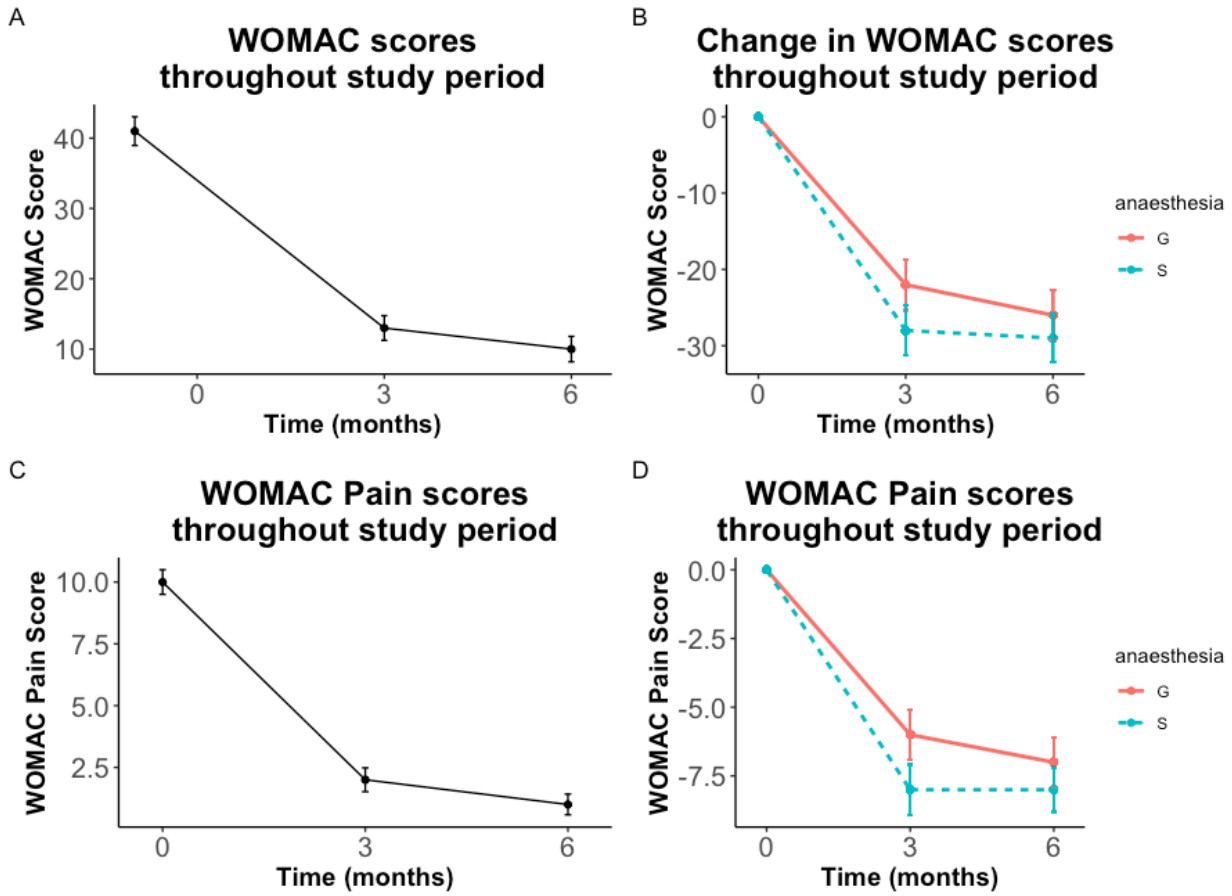

Figure 4-15: A) Median WOMAC® score reported by patients before surgery, at three months and at six months after surgery. B) Median change from baseline across the two study groups C) Median WOMAC® Pain scores before surgery, at three months and at six months after surgery. D) Median change from baseline across the two study groups. Error bars denote 95% confidence intervals

Most of the improvement occurred before three months, with a small further improvement within the next three months. A total of 162 patients (96%) had improvement in their WOMAC® score, but 7 patients actually were found to be worse off six months after surgery.

The improvement in WOMAC® score over 6 months was more than expected during the design of the trial.

Still, it is worrying to see that the incidence of Chronic Post-Surgical Pain at 6 months is 11%, when defined as a WOMAC-Pain score of 5 or greater out of a maximum of 20. In fact, only 62 patients (37.5%) reported no pain at all.

4.3.1.2 Progression of S-LANSS Score

The incidence of neuropathic pain, as assessed by a high S-LANSS, increased from a baseline of 12.8% to 23.4% at six months (Figure 4-16).

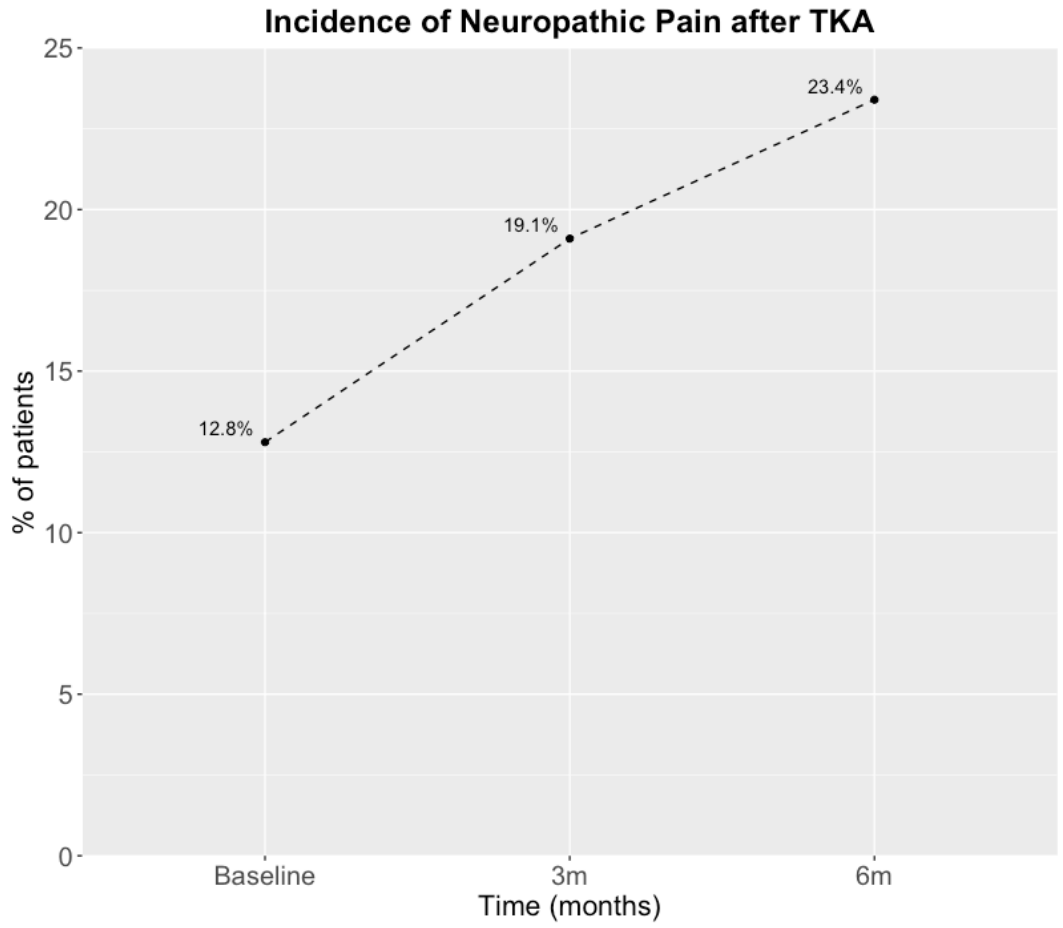

Figure 4-16: Incidence of Neuropathic pain in the first six months after TKA, as assessed by high S-LANSS score.

The most common symptom from the S-LANSS questionnaire at six months was paraesthesia, followed by abnormal touch sensation (Table 4-53).

	Baseline		3 months		6 months	
Question	Total (n = 196)	High S-LANSS (n=25)	Total (n = 199)	High S-LANSS (n = 37)	Total (n = 187)	High S-LANSS (n = 41)
Paraesthesia	45 (23%)	17 (68%)	97 (49%)	32 (86%)	95 (51%)	36 (88%)
Colour changes	43 (22%)	21 (84%)	23 (12%)	13 (35%)	15 (8%)	14 (34%)
Sensitive to touch	31 (16%)	14 (56%)	55 (28%)	25 (68%)	60 (32%)	32 (78%)
Sudden pain	88 (45%)	19 (76%)	52 (26%)	26 (70%)	41 (22%)	21 (51%)
Temperature changes	93 (47%)	22 (88%)	77 (39%)	27 (73%)	38 (20%)	24 (59%)
Pain on rubbing	27 (14%)	16 (64%)	47 (24%)	32 (86%)	48 (26%)	36 (88%)
Pain on pressing	33 (17%)	14 (56%)	27 (14%)	17 (46%)	25 (25%)	19 (46%)

Table 4-53: Frequency of symptoms as per S-LANSS questionnaire, at baseline, at three months and at six months

At six months, neuropathic pain resolved in 8.6%, remained the same in 4,6%, but increased in 18.9% (Figure 4-17).

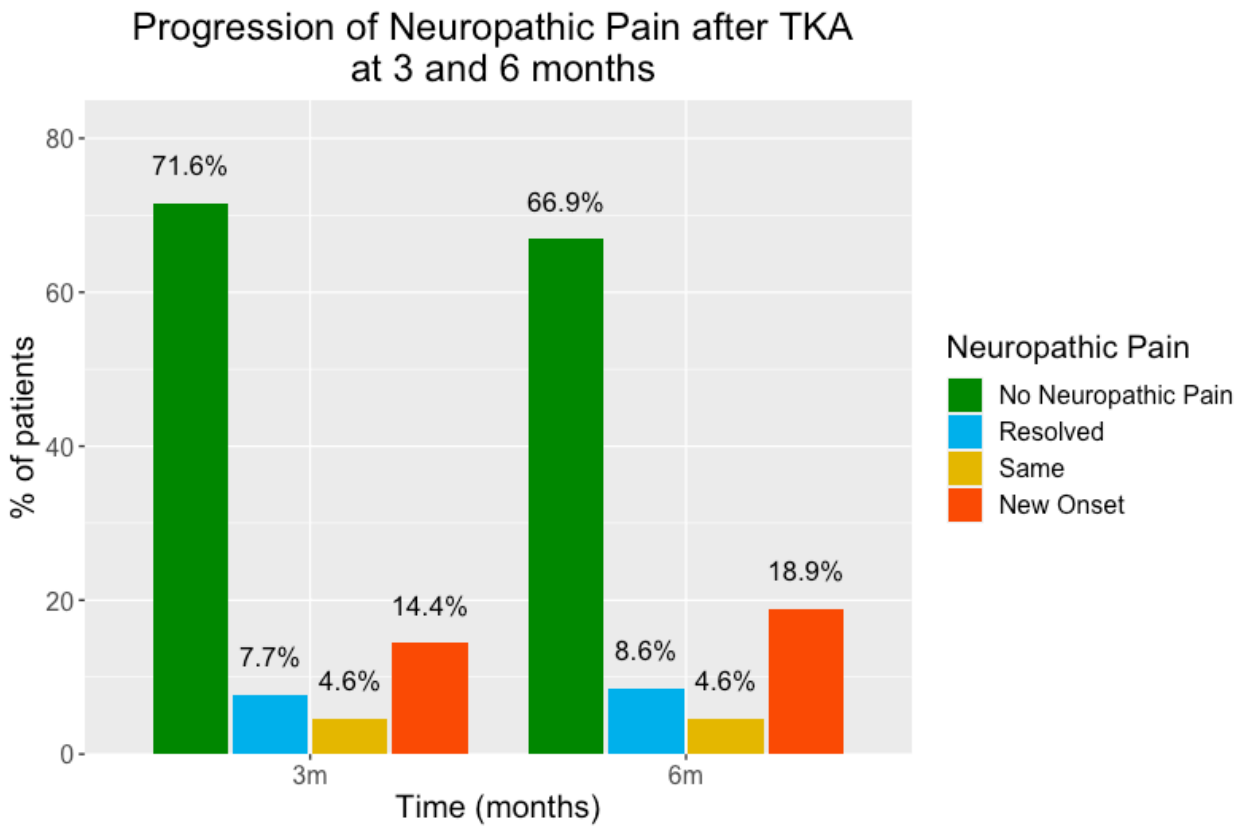

Figure 4-17: Progression of neuropathic pain in the first six months after TKA.

Furthermore, patients who developed CPSP were 10 times more likely to suffer from neuropathic pain (32% vs 4%, OR 9.8, 95%CI 3.2 – 34.3, $p < 0.0001$). In fact, throughout the study period, median WOMAC® pain scores were twice as high in patients who reported a high S-LANSS score as shown in Figure 4-18.

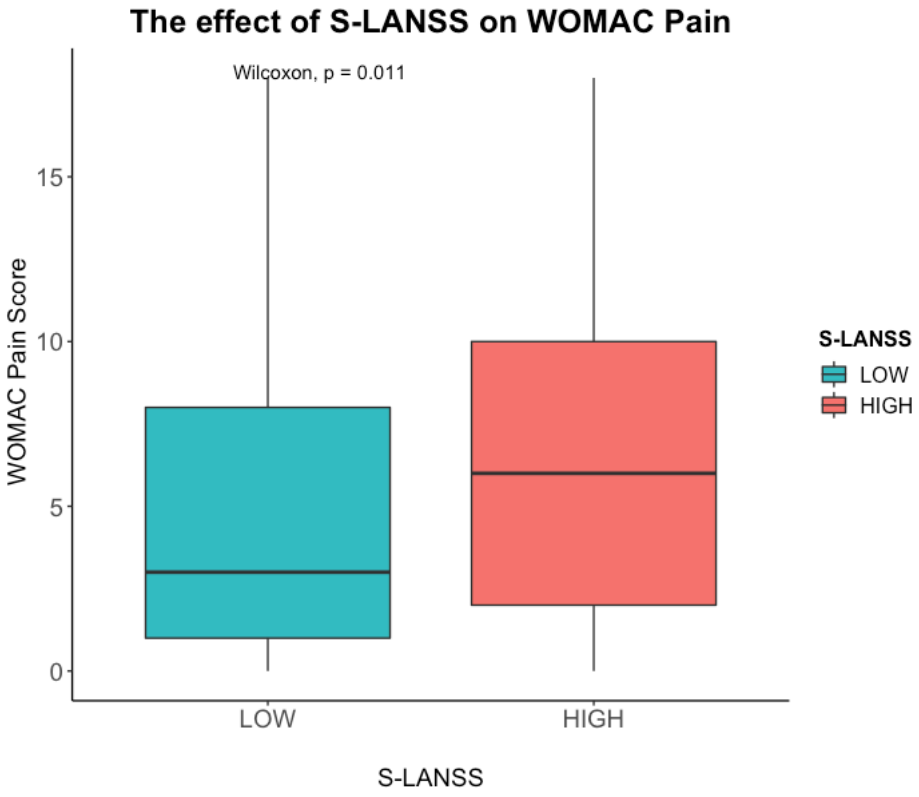

Figure 4-18: Effect of S-LANSS score on WOMAC® pain scores

4.3.2 Factors influencing CPSP at six months

4.3.2.1 Demographic Factors

A linear mixed-effect analysis of demographical data was done for WOMAC® and WOMAC® Pain scores, since these were repeated observations. Patient reference and surgical firm were used as random effects.

The surgical firm caring for the patient during the study period showed no influence on the outcomes.

The effect of gender, BMI and age on WOMAC® and WOMAC® Pain scores throughout the study period are shown in Table 4-54 and Table 4-55.

	Estimate	**95% Conf Interval**	**p-value**
Age	-0.24	-0.56 – 0.07	0.132
Male Gender	-7.38	-11.13 – -3.63	**<0.001**
BMI	0.48	0.22 – 0.75	**<0.001**

Table 4-54: Effect of Age, Gender and BMI on WOMAC® Scores. (Linear Mixed Effects Model)

	Estimate	**95% Conf Interval**	**p-value**
Age	-0.13	-0.21 – -0.05	***0.002***
Male Gender	-1.11	-2.06 – -0.16	***0.023***
BMI	0.094	0.029 - 0.159	***0.005***

Table 4-55: Effect of Age, Gender and BMI on WOMAC® Pain Scores. (Linear Mixed Effects Model)

Male patients reported lower WOMAC® score but WOMAC® pain scores were only lower at baseline, shown in Figure 4-19.

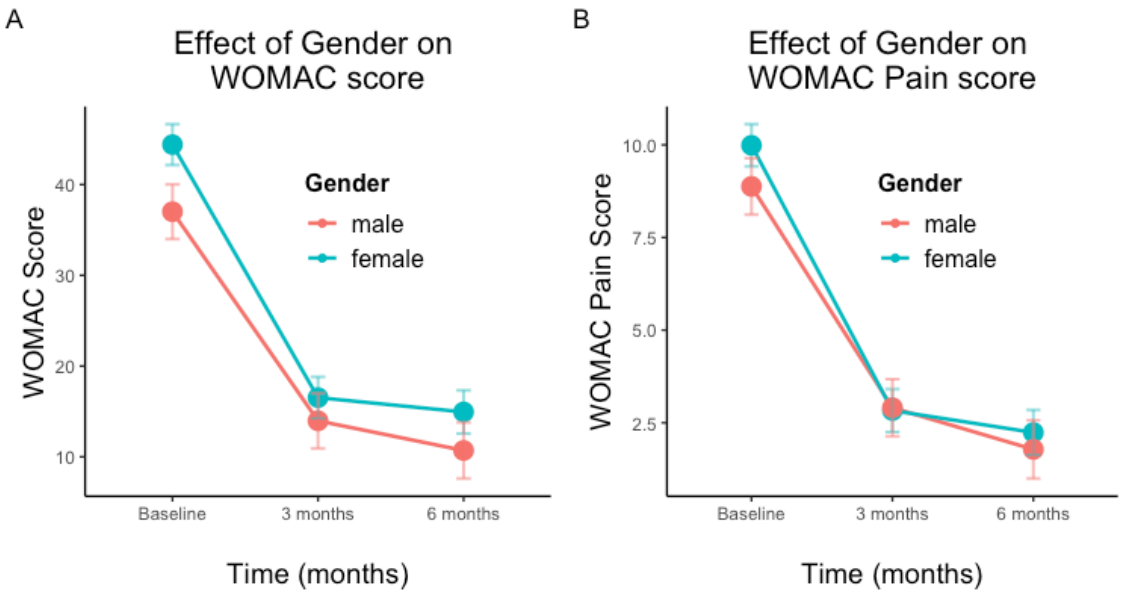

Figure 4-19: Effect of gender on A) WOMAC®, B) WOMAC® Pain scores, throughout the study.

Age had no effect on WOMAC® scores, and a small effect on the baseline WOMAC® pain score (Figure 4-20).

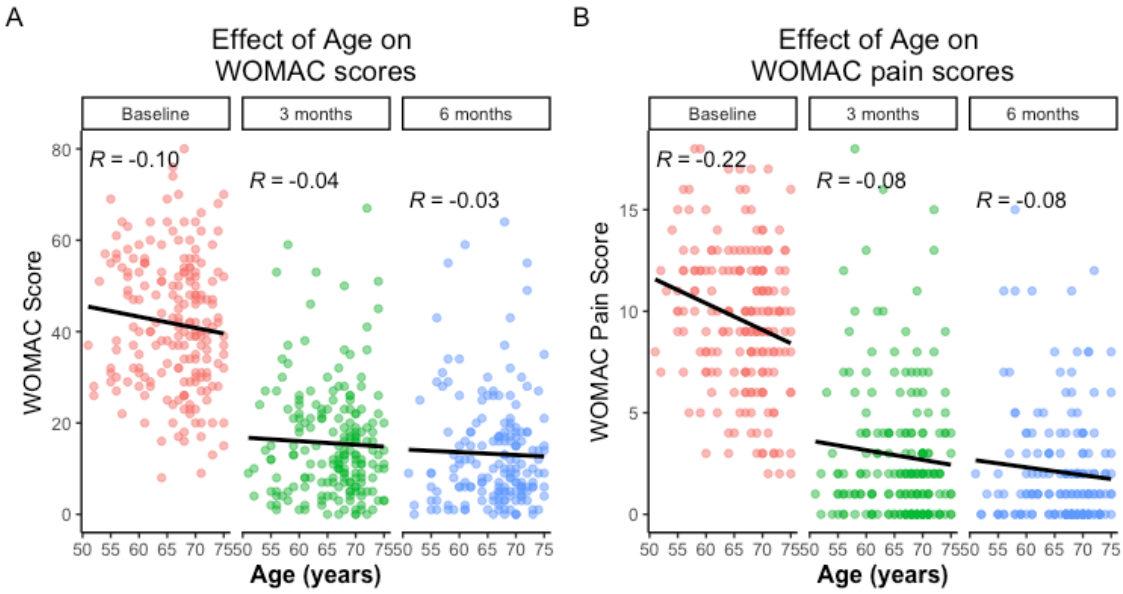

Figure 4-20: Effect of age on A) WOMAC®, B) WOMAC® Pain scores, grouped per interval of postoperative time.

Obese patients reported worse WOMAC® and WOMAC® pain scores before surgery (Figure 4-21). Before surgery, an increase of 10 kg/m² in BMI would result in an increase of nearly five units on the WOMAC® score, and nearly 1 unit on the WOMAC® pain score.

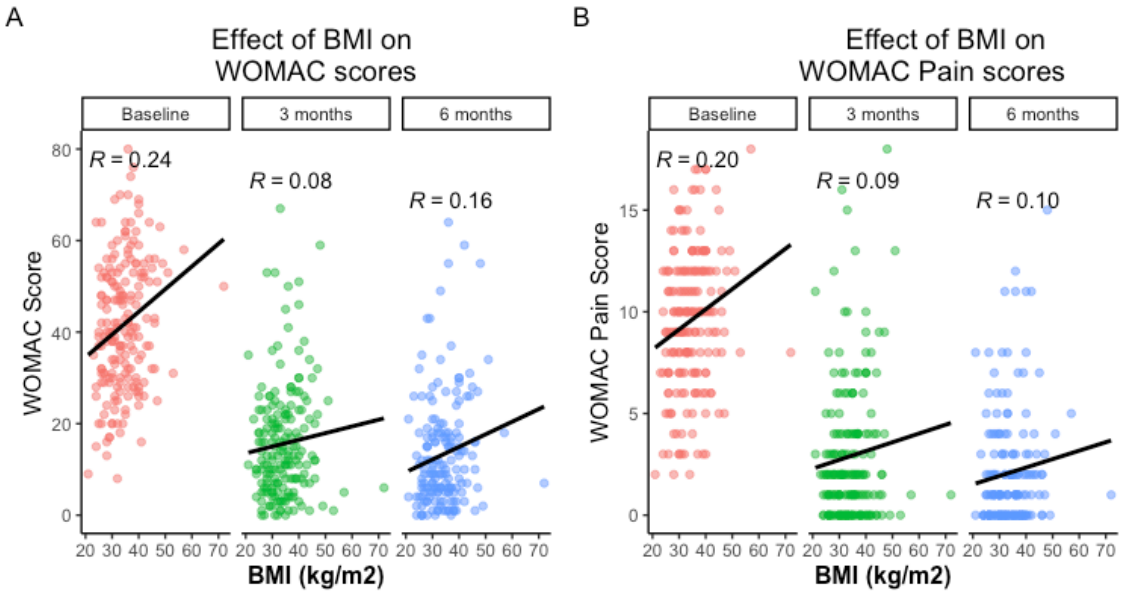

Figure 4-21: Effect of BMI on A) WOMAC®, B) WOMAC® Pain scores, grouped per interval of postoperative time.

Baseline pain scores had a significant effect on the level of pain at six months. Patients who had a higher baseline WOMAC® Pain subscore tended to have a higher incidence of postoperative pain at six months. This was highly statistically significant ($p = 0.002$, $R^2 = 0.046$), but had a poor predictive value (Figure 4-22A).

Pain at six months was also dependant on pain at three months (Figure 4-22B). Patients who were in pain at three months were more likely to remain in pain at six months. The intensity of this pain was also dependant on the pain scores at three months (estimate: 0.55, 95%CI: 0.46 - 0.65, p: <0.001, R^2: 0.42). In fact, pain at three months was the most significant factor throughout the study.

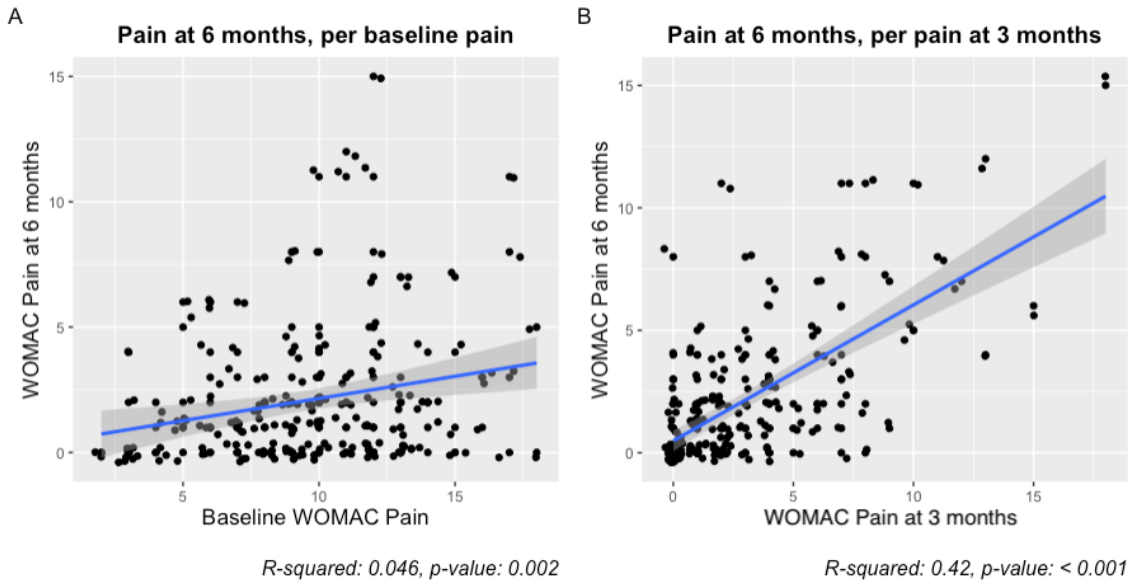

Figure 4-22: Scatterplots, with fitted lines, for pain at six months.Pain at six months, against baseline pain score. B) Pain at six months against pain at three months.

Patients with a high preoperative S-LANSS score reported a higher median WOMAC® Pain score at six months, but this was not statistically significant (high S-LANSS: 2 vs low S-LANSS: 1, Mann-Whitney test, p = 0.29). However, patients with a high S-LANSS score at three months and at six months were at a risk of higher WOMAC® pain scores at six months. This is shown in Figure 4-23.

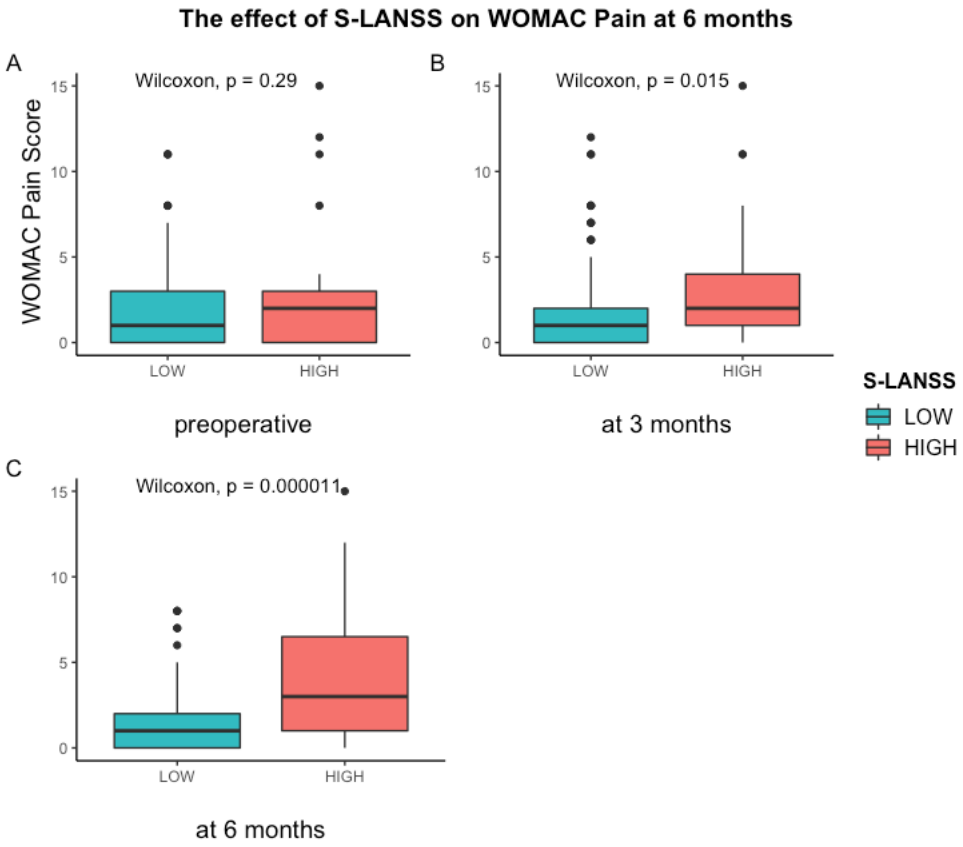

Figure 4-23: Effect of S-LANSS at various intervals on WOMAC® Pain scores at six months. A) Preoperative S-LANSS B) S-LANSS at 3 months C) S-LANSS at six months.

4.3.2.2 The effect of Acute Postoperative Pain on CPSP

Since the pain scores at rest were low throughout both study groups, no further analysis was made.

With regards to the effect of pain during physiotherapy, there was no statistical effect on any of the outcomes investigated at three and at six months.

4.3.2.3 Influence of Anaesthesia

The effect of anaesthesia on pain scores at three and at six months after total knee replacement is shown in Table 4-56. Univariate analysis showed no significant differences between the two groups in most parameters.

	Characteristic	GA	Spinal	p-value[2]
3 months	WOMAC®	14 [IQR: 7.0 – 22.3]	12 [IQR: 6.0- 21]	0.31
	WOMAC® Pain	2 [IQR: .8 – 4.0]	2 [IQR: 0.0 – 4.0]	0.81
	Chronic Pain n (%)	19 (21%)	19 (20%)	-
	S-LANSS ≥ 12	20 (22%)	17 (18%)	0.59
6 months	WOMAC®	8 [IQR: 4.0 - 17.0]	11 [IQR: 6.0 – 18.0]	0.23
	WOMAC® Pain	1 [IQR: 0.0 – 2.0]	2 [IQR: 0.0 – 3.0]	0.06
	Chronic Pain n (%)	13 (16%)	12 (13%)	0.67
	S-LANSS ≥ 12	21 (25%)	20 (22%)	0.72

[1]median [IQR] for continuous variables; n / N (%) categorical variables

[2]Fisher's exact test; Wilcoxon rank sum test

Table 4-56: Differences in pain scores, WOMAC® scores and S-LANSS at three and six months, in the two groups. Chronic Pain is defined as patients with a WOMAC® Pain subscore greater than five.

Since the study involved repeated measures of observation for WOMAC® and WOMAC® Pain scores, further analysis was done using a linear mixed-effect model. This was done using WOMAC® as the outcome, an interaction of anaesthesia and postoperative time as fixed factors, and the patient reference as a random effect. The results are shown in Table 4-57 and Table 4-58.

Linear mixed model for WOMAC® score vs Type of Anaesthesia **WOMAC® ~ Anaesthesia + Time + Anaesthesia:Time + (1|subject id)**					
	Estimate	**95% Conf Interval**		**t value**	**p-value***
Intercept	42.93	40.3	45.5	32.8	
3 months	-28.51	-31.22	-25.80	-20.60	***< 0.001***
6 months	-28.57	-31.34	-26.80	-20.91	***< 0.001***
GA at baseline	-2.37	-6.00	1.26	-1.28	0.20
GA at 3 months	4.83	0.96	8.70	2.45	***0.015***
GA at 6 months	2.46	-1.52	6.44	1.21	0.23
	parameters	AIC	logLik	Chisq	Df
Reference model	3	4822	-2408		
Current model	8	4344	-2164	488	5
				p-value	< 0.001

**p values calculated using Satterthwaite d.f.*

Table 4-57: Differences in WOMAC® scores at three and six months, in the two groups. Linear Mixed-model.

Linear mixed model for WOMAC® Pain subscore vs Type of Anaesthesia WOMAC® Pain ~ Anaesthesia + Time + Anaesthesia:Time + (1|subject id)					
	Estimate	**95% Conf Interval**		**t value**	**p-value***
Intercept	10.06	9.42	10.69	30.82	
3 months	-7.36	-8.12	-6.61	-19.08	***< 0.001***
6 months	-9.95	-8.72	-7.18	-20.18	***< 0.001***
GA at baseline	-0.93	-1.84	-0.031	-2.03	***0.043***
GA at 3 months	1.28	0.21	2.36	2.33	***0.020***
GA at 6 months	0.87	-0.24	1.98	1.54	0.12
	parameters	AIC	logLik	Chisq	Df
Reference model	3	3304	-1649		
Current model	8	2853	-1418	262	5
				p-value	< 0.001

**p values calculated using Satterthwaite d.f.*

Table 4-58: Differences in WOMAC® Pain subscores at three and six months, in the two groups. Linear Mixed-model analysis.

Patients who had had a general anaesthetic reported higher (worse) WOMAC® and WOMAC® pain scores at three months, but not at six months (Figure 4-24).

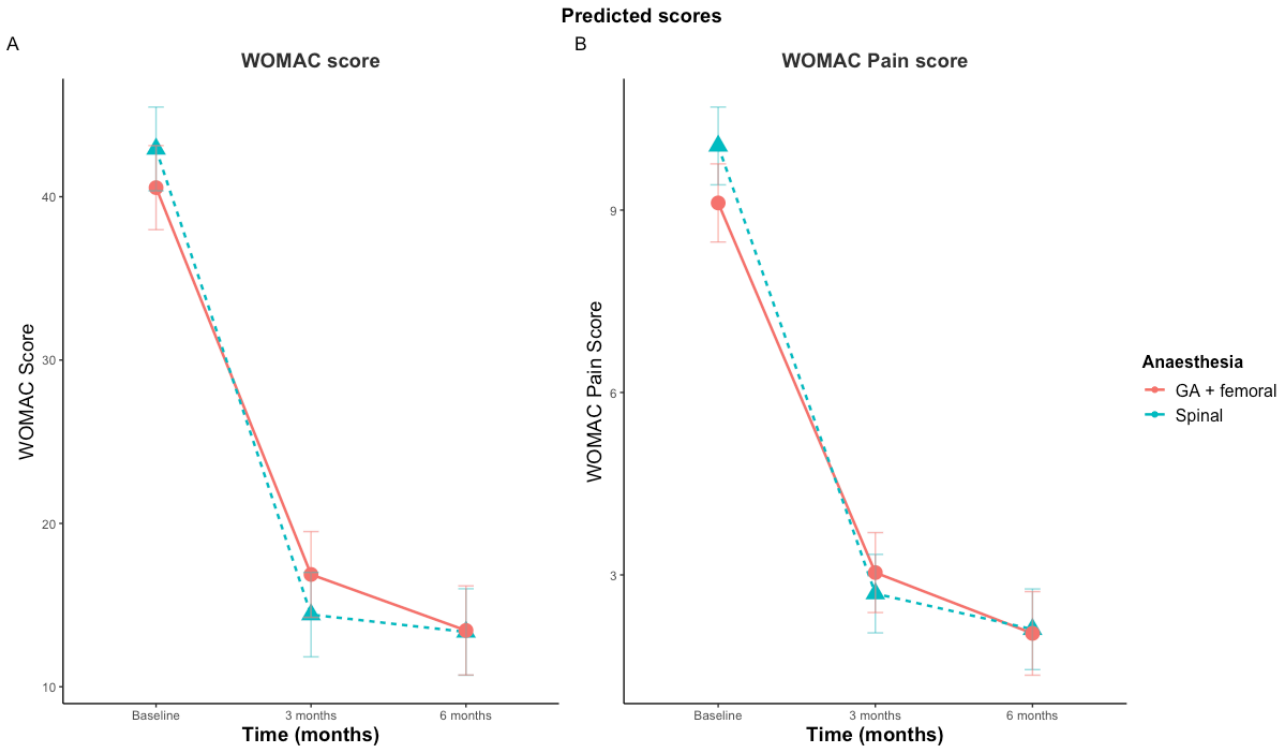

Figure 4-24: Predicted A) WOMAC® scores, B) WOMAC® Pain scores according to linear mixed effect model.

4.3.2.4 Multivariate Analysis of the Effect of Anaesthesia

A multivariate analysis using a generalized linear model was performed to assess which factors were most likely to be associated with a higher incidence of chronic postoperative surgical pain (Table 4-59). This was done in a step down manner.

All parameters were first checked for significance. Age, gender and BMI had a p-value greater than 0.5, and were excluded from further analysis. The other parameters were checked for collinearity. As anticipated from univariate analysis, there was a strong correlation between morphine consumption and choice of anaesthetic, so morphine consumption was removed from the model.

Using Chi-squared tests, the most non-significant factors were dropped, and the Akaike information criterion (AIC) was checked at each stage. The model with the lowest AIC was then used.

Seven outliers were removed from the model. These were chosen on the basis of Cook's distance being greater than four times the standard deviation, and also on the Bonferroni outlier test.

Binomial logistic regression model for incidence of CPSP at six months				
	Estimate	**OR**	**95% Conf Interval OR**	**p-value**
Initial model				
Intercept	-6.03			
Baseline WOMAC® Pain	0.15	1.16	0.96 – 1.43	0.13
Baseline S-LANSS	-0.17	0.85	0.21 – 2.48	0.78
General Anaesthesia	1.04	2.83	0.72 – 12.57	0.15
Morphine consumption	0.03	1.03	0.97 – 1.09	0.32
Pain at rest	0.21	1.23	0.99 – 1.54	0.06
Pain during physiotherapy	-0.02	0.97	0.76 – 1.21	0.76
Age	-0.02	0.98	0.89 – 1.07	0.61
Male gender	0.27	1.32	0.39 – 4.30	0.64
BMI	-0.03	0.97	0.88 – 1.06	0.55
Null Deviance: 103.172			Residual deviance: 90.142	
AIC: 110.14				
Hoslem test: p = 0.62				
Final model				
Intercept	-3.78			
Baseline WOMAC® Pain	0.15	1.17	1.00 – 1.38	0.065
General Anaesthesia	1.40	4.07	1.33 – 14.59	***0.019***
Postoperative Pain at rest	0.21	1.23	1.02 – 1.48	***0.026***
Null Deviance: 109.24			Residual deviance: 97.54	
AIC: 105.54				
Hoslem test: p = 0.39			Analysis of Deviance: 97.54 vs 109.23, p=***0.008***	

Table 4-59: Logistic regression model for incidence of chronic pain at six months, as defined by WOMAC® Pain subscore greater than 5.

Hence, the main three factors that could predict the incidence of CPSP at six months were:

- Baseline pain, with an increase in the odds ratio of 17% for every unit on the WOMAC® Pain subscore
- Pain at rest 24 hours after surgery, with an increase in the odds of 23% for every unit in the NRS score
- Type of anaesthesia, with patients administered general anaesthesia being 4 times more likely to develop CPSP at six months.

It should be noted that the spinal group had a higher baseline median WOMAC® Pain subscore when compared to the GA group. This might explain the lack of statistical significance on univariate analysis, as the two factors had opposing effects.

4.3.2.5 Influence of Genetic Factors on WOMAC®, WOMAC® Pain

4.3.2.5.1 COMT

rs4633

Univariate analysis of rs4633 using a dominant model is summarized in Table 4-60. This assumes that the minor allele T would have a dominant effect over the major allele C.

		rs4633 minor allele T as dominant allele		
	Characteristic	**CT | TT [1]**	**CC[1]**	**p-value[2]**
3 months	n	138	46	
	WOMAC®	12 [5 - 20.75]	16.5 [9.25 - 23]	0.042
	WOMAC® Pain	2.00 [0.00 - 4.00]	2.50 [1.00 - 4.75]	0.10
	% Chronic Pain	26 / 138 (19%)	12 / 46 (22%)	0.30
	S-LANSS ≥ 12	27 / 140 (20%)	10 / 47 (22%)	-
6 months	n	126	43	
	WOMAC®	9 [4 - 19]	12 [6 - 19]	0.14
	WOMAC® Pain	1.0 [0.00 - 2.8]	1.0 [1.00 - 3.25]	0.09
	% Chronic Pain	17 / 127 (13%)	7 / 44 (16%)	0.62
	S-LANSS ≥ 12	26 / 127 (20%)	13 / 44 (30%)	0.30

[1]median [IQR] for continuous variables; n / N (%) categorical variables

[2]Fisher's exact test; Wilcoxon rank sum test

Table 4-60: Pain scores at three and six months between patients with and without the T allele (minor allele) of rs4633. Dominant analysis: assuming that rs4633 has a dominant effect.

Repeated measures analysis, using a linear mixed model, did not show any difference between the two groups, as shown in Table 4-61. Due to a high presence of heteroscedasticity, a robust analysis of standard errors and covariance was performed.

rs4633	WOMAC			WOMAC Pain		
Predictors	**Estimates**	**CI**	**p**	**Estimates**	**CI**	**p**
CT | TT	-1.44	-6.33 – 3.45	0.56	-0.40	-1.63 – 0.84	0.53
CT | TT at 3 m	-1.30	-7.55 – 4.94	0.68	-0.40	-2.09 – 1.29	0.64
CT | TT at 6 m	-0.99	-6.35 – 4.38	0.72	0.08	-1.25 – 1.41	0.90
			Random Effects			
σ^2	94.09			7.37		
ICC	0.44			0.30		
Marginal R^2 / Conditional R^2	0.502 / 0.720			0.526 / 0.667		

Table 4-61: Linear Mixed Model analysis for rs4633 as dominant allele. CI: confidence intervals, calculated using robust analysis of standard errors.

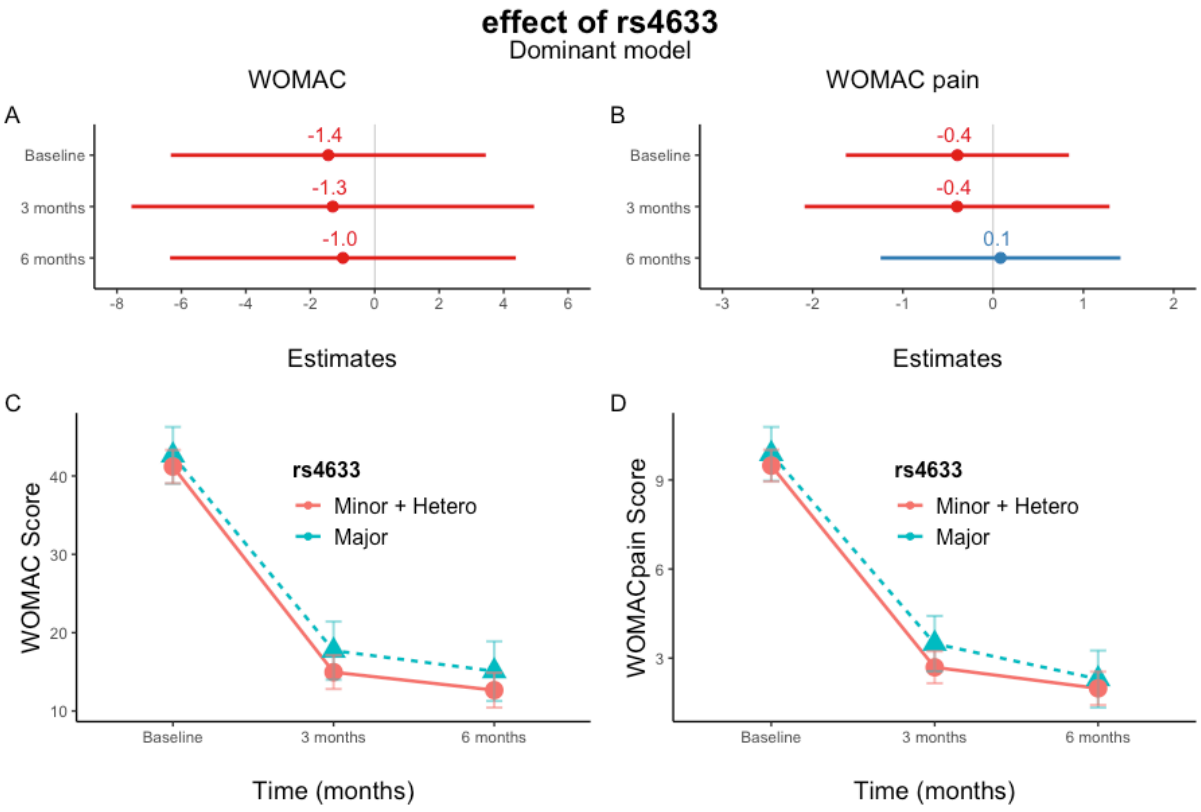

Figure 4-25: Analysis of rs4633 using dominant model. A, B) Forest plot of estimates for WOMAC®, WOMAC® pain with robust confidence intervals C, D) predicted values for WOMAC®, WOMAC® pain as per above model

Using a recessive model, where only homozygous carriers of the minor allele would show a clinical effect, there was evidence of a possible interaction between rs4633 and WOMAC® Pain scores. Patients who had at least one copy of rs4633 had more pain than those without.

		rs4633 minor allele T as recessive allele		
	Characteristic	**CC | CT** [1]	**TT**[1]	**p-value**[2]
3 months	n	143	41	
	WOMAC®	13 [7 - 22]	12 [5 - 21]	0.41
	WOMAC® Pain	2 [1 - 4]	1 [0 - 3]	0.24
	% Chronic Pain	31 (22%)	7 (17%)	0.66
	S-LANSS ≥ 12	28 (20%)	9 (22%)	0.66
6 months	n	135	34	
	WOMAC®	10 [5.5 - 18]	7.5 [2.25 - 15.75]	0.076
	WOMAC® Pain	1 [0 - 3]	0 [0 - 2]	***0.028***
	% Chronic Pain	22 (16%)	2 (6%)	0.17
	S-LANSS ≥ 12	33 (24%)	6 (18%)	0.50

[1]median [IQR] for continuous variables; n / N (%) categorical variables

[2]Fisher's exact test; Wilcoxon rank sum test

Table 4-62: Pain scores at three and six months between patients with and without the C allele (major allele) of rs4633. Recessive analysis: assuming rs4633 has a recessive effect.

A robust linear mixed model did show a significance of rs4633 on WOMAC® Pain. However, on comparing this model to a base model without rs4633 with a likelihood

ratio test, there was no difference. This would mean that the model including rs4633 is no better than that without rs4633.

rs4633	WOMAC			WOMAC Pain		
Predictors	**Estimates**	**CI**	**p**	**Estimates**	**CI**	**p**
TT	1.26	-3.24 – 5.76	0.58	0.51	-0.60 – 1.62	0.37
TT at 3 m	-2.61	-8.09 – 2.86	0.35	-0.99	-2.51 – 0.53	0.20
TT at 6 m	-4.17	-8.88 – 0.55	0.08	-1.48	-2.73 – -0.23	***0.02***
Random Effects						
σ^2	93.43			7.28		
ICC	0.44			0.31		
Marginal R^2 / Conditional R^2	0.501 / 0.722			0.526 / 0.671		

Table 4-63: Linear Mixed Model analysis for rs4633 using recessive model. CI: confidence intervals, calculated using robust analysis of standard errors.

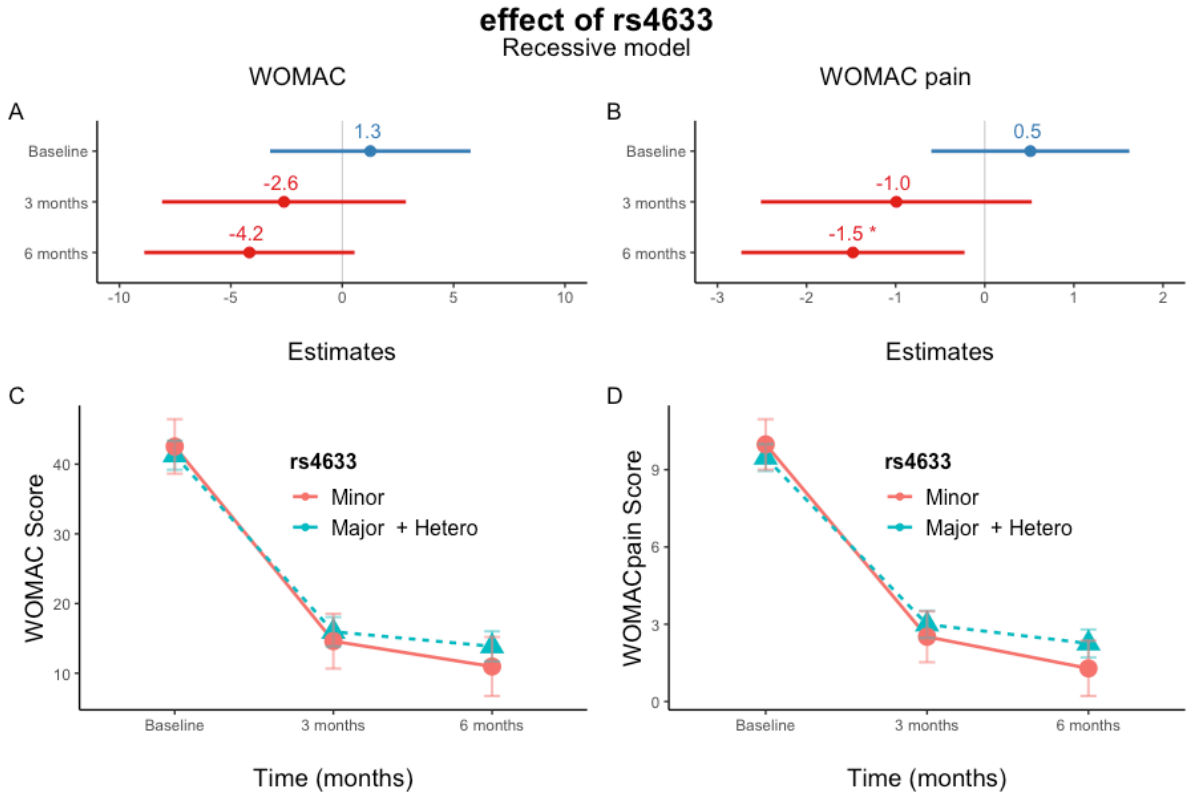

Figure 4-26: Analysis of rs4633 using recessive model. A, B) Forest plot of estimates for WOMAC®, WOMAC® pain with robust confidence intervals C, D) predicted values for WOMAC®, WOMAC® pain as per above model

rs4680

Univariate analysis of rs4680 using a dominant model is summarized in Table 4-64. This assumes that the minor allele A would have a dominant effect over the major allele G. There was no difference between carriers of rs4680 and non-carriers in any of the outcomes.

		rs4680 minor allele A as dominant allele		
	Characteristic	**GA | AA[1]**	**GG[1]**	**p-value[2]**
3 months	n	137	44	
	WOMAC®	13 [5 - 21]	14 [9.75 - 23]	0.09
	WOMAC® Pain	2 [0 - 4]	2 [1 - 4]	0.11
	% Chronic Pain	27 (20%)	10 (23%)	0.67
	S-LANSS ≥ 12	27 (20%)	7 (16%)	0.52
6 months	n	125	41	
	WOMAC®	9 [4 - 17]	12 [6 - 18]	0.16
	WOMAC® Pain	1 [0 - 3]	2 [1 - 3]	0.10
	% Chronic Pain	18 (14%)	5 (12%)	1
	S-LANSS ≥ 12	27 (22%)	11 (27%)	0.53

[1]median [IQR] for continuous variables; n / N (%) categorical variables

[2]Fisher's exact test; Wilcoxon rank sum test

Table 4-64: Pain scores at three and six months between patients with and without the A allele (minor allele) of rs4680. Dominant analysis: assuming rs4680 has a dominant effect.

Repeated measures analysis, confirmed that there were no difference between the two groups, as shown in Table 4-65 and Figure 4-27.

rs4680	WOMAC			WOMAC Pain		
Predictors	**Estimates**	**CI**	**p**	**Estimates**	**CI**	**p**
GA | AA	-1.99	-6.97 – 2.98	0.43	-0.52	-1.79 – 0.75	0.42
GA | AA at 3 m	-0.25	-6.57 – 6.07	0.94	-0.18	-1.91 – 1.55	0.84
GA | AA at 6 m	-0.07	-5.30 – 5.16	0.98	0.43	-0.86 – 1.73	0.51
Random Effects						
σ^2	92.81			7.35		
ICC	0.45			0.30		
Marginal R^2 / Conditional R^2	0.498/ 0.725			0.523 / 0.667		

Table 4-65: Linear Mixed Model analysis for rs4680 using dominant model. CI: confidence intervals, calculated using robust analysis of standard errors.

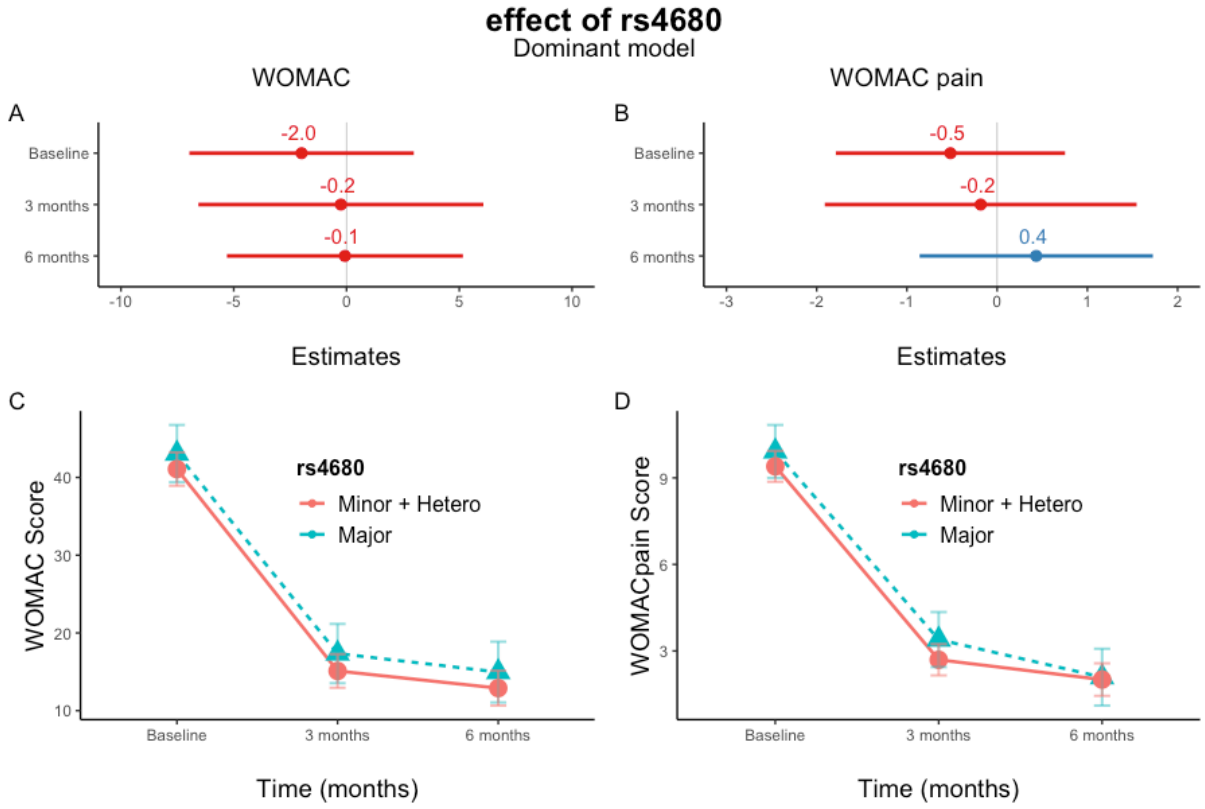

Figure 4-27: Analysis of rs4680 using dominant approach. A, B) Forest plot of estimates for WOMAC®, WOMAC® pain with robust confidence intervals C, D) predicted values for WOMAC®, WOMAC® pain as per above model

Similarly, there was no difference between patients were homozygote carriers for rs4680 and non-homozygotes (recessive approach), as shown in Table 4-66. A linear mixed model confirmed the lack of influence, as shown in Table 4-67 and Figure 4-28.

		rs4680 minor allele A as recessive allele		
	Characteristic	**GG | GA [1]**	**AA[1]**	**p-value[2]**
3 months	n	140	41	
	WOMAC®	13 [7 - 21.25]	12 [5 - 22]	0.70
	WOMAC® Pain	2 [1 - 4]	1 [0 - 4]	0.23
	% Chronic Pain	29 (21%)	8 (20%)	1
	S-LANSS ≥ 12	25 (18%)	9 (22%)	0.51
6 months	n	132	34	
	WOMAC®	10 [5 - 18]	8.5 [2.25 - 16]	0.18
	WOMAC® Pain	1 [0 - 3]	0.5 [0 - 2]	0.066
	% Chronic Pain	20 (15%)	3 (9%)	0.42
	S-LANSS ≥ 12	29 (22%)	9 (26%)	0.65

[1]median [IQR] for continuous variables; n / N (%) categorical variables

[2]Fisher's exact test; Wilcoxon rank sum test

Table 4-66: Pain scores at three and six months between patients with and without the G allele (major allele) of rs4680. Recessive analysis: assuming rs4680 has a recessive effect.

rs4680	WOMAC			WOMAC Pain		
Predictors	**Estimates**	**CI**	**p**	**Estimates**	**CI**	**p**
AA	0.29	-4.16 – 4.73	0.90	0.18	-0.93 – 1.29	0.75
AA at 3 m	-0.83	-6.20 – 4.53	0.76	-0.60	-2.12 – 0.92	0.44
AA at 6 m	-2.21	-6.90 – 2.48	0.36	-0.95	-2.21 – 0.31	0.14
Random Effects						
σ^2	92.63			7.33		
ICC	0.46			0.31		
Marginal R^2 / Conditional R^2	0.496/ 0.726			0.523 / 0.668		

Table 4-67: Linear Mixed Model analysis for rs4680 using recessive model. CI: confidence intervals, calculated using robust analysis of standard errors.

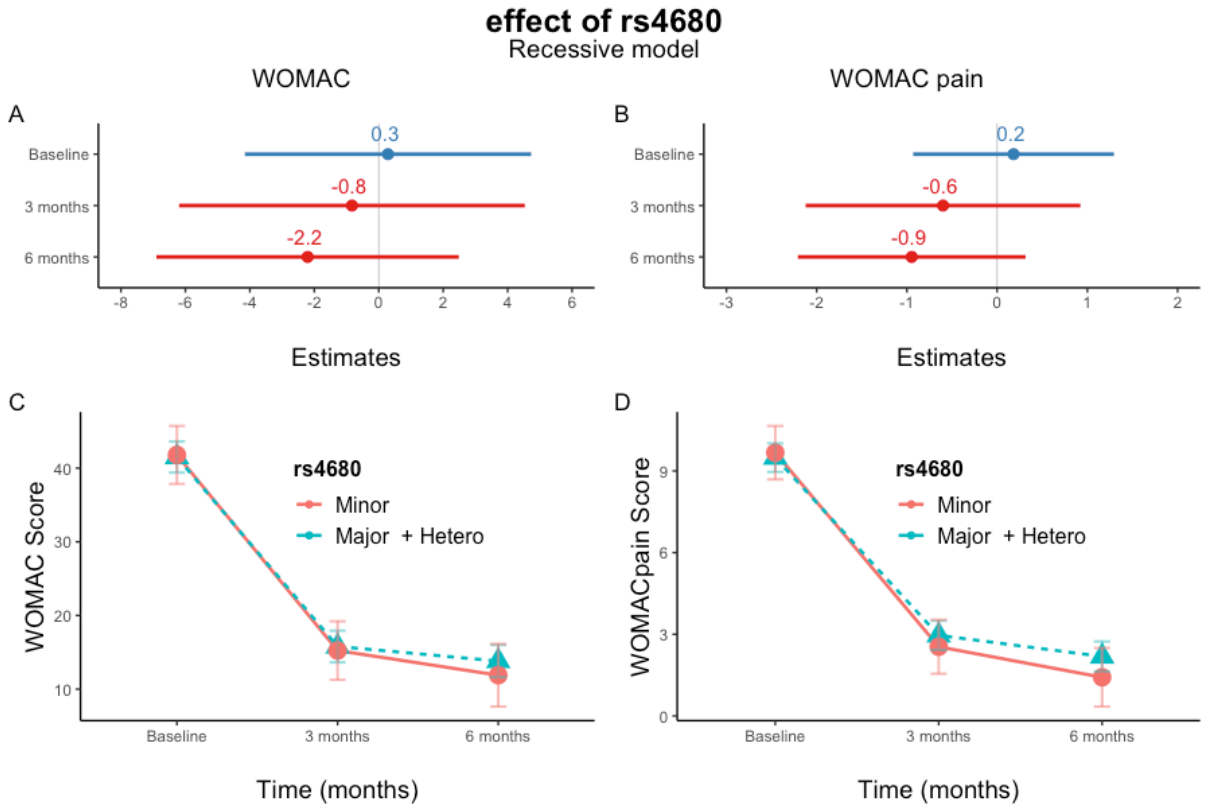

Figure 4-28: Analysis of rs4680 using recessive approach. A, B) Forest plot of estimates for WOMAC®, WOMAC® pain with robust confidence intervals C, D) predicted values for WOMAC®, WOMAC® pain as per above model

rs4818

On univariate analysis, non-carriers of rs4818 appeared to have a better outcome for WOMAC® and WOMAC® Pain scores at six months, as shown in Table 4-68.

However, this was not confirmed on linear mixed model analysis.

		rs4818 minor allele G as dominant allele		
	Characteristic	**CG | GG [1]**	**CC[1]**	**p-value[2]**
3 months	n	102	78	
	WOMAC®	14 [8 - 22]	12 [5 - 21]	0.14
	WOMAC® Pain	2 [1 - 4]	1 [0 - 4]	0.19
	% Chronic Pain	21 (21%)	15 (19%)	0.85
	S-LANSS ≥ 12	18 (18%)	14 (18%)	1
6 months	n	97	69	
	WOMAC®	12 [6 - 18]	6 [3 - 16]	***0.01***
	WOMAC® Pain	2 [0 - 4]	1 [0 - 2]	***0.004***
	% Chronic Pain	16 (16%)	6 (9%)	0.17
	S-LANSS ≥ 12	23 (24%)	15 (22%)	0.71

[1]median [IQR] for continuous variables; n / N (%) categorical variables

[2]Fisher's exact test; Wilcoxon rank sum test

Table 4-68: Pain scores at three and six months between patients with and without the G allele (minor allele) of rs4818. Dominant analysis: assuming rs4818 has a dominant effect.

rs4818	WOMAC			WOMAC Pain		
Predictors	**Estimates**	**CI**	**p**	**Estimates**	**CI**	**p**
CG | GG	-0.25	-4.45 – 3.95	0.91	-0.09	-1.11 – 0.93	0.86
CG | GG at 3 m	1.78	-2.95 – 6.51	0.46	0.25	-1.08 – 1.58	0.71
CG | GG at 6 m	3.41	-0.99 – 7.82	0.129	0.78	-0.38 – 1.94	0.19
Random Effects						
σ^2	92.98			7.39		
ICC	0.45			0.29		
Marginal R^2 / Conditional R^2	0.500/ 0.726			0.531 / 0.669		

Table 4-69: Linear Mixed Model analysis for rs4818 using dominant model. CI: confidence intervals, calculated using robust analysis of standard errors.

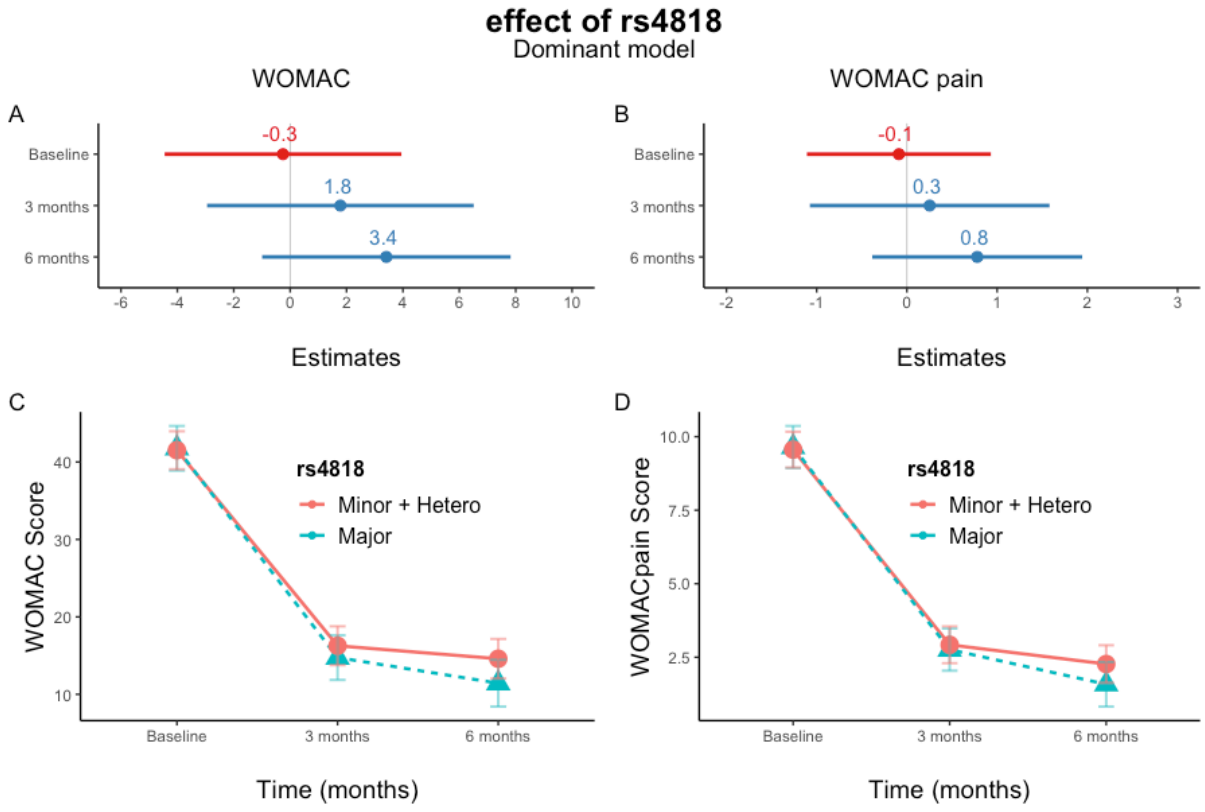

Figure 4-29: Analysis of rs4818 using dominant approach. A, B) Forest plot of estimates for WOMAC®, WOMAC® pain with robust confidence intervals C, D) predicted values for WOMAC®, WOMAC® pain as per above model

A recessive approach, with the major allele C being dominant over the minor allele, showed no difference in outcomes in any of the measures investigated.

		rs4818 minor allele G as recessive allele		
	Characteristic	**CC | CG [1]**	**GG[1]**	**p-value[2]**
3 months	n	155	25	
	WOMAC®	13 [6.5 - 21]	14 [8 - 22]	0.62
	WOMAC® Pain	2 [0 - 4]	2 [0 - 4]	0.81
	% Chronic Pain	31 (20%)	5 (20%)	1
	S-LANSS ≥ 12	28 (18%)	4 (16%)	1
6 months	n	142	24	
	WOMAC®	9 [5 - 17.75]	11.5 [6 - 18.5]	0.37
	WOMAC® Pain	1 [0 - 2]	1.5 [1 - 3.25]	0.10
	% Chronic Pain	19 (13%)	3 (12%)	1
	S-LANSS ≥ 12	32 (23%)	6 (25%)	0.80

[1]median [IQR] for continuous variables; n / N (%) categorical variables

[2]Fisher's exact test; Wilcoxon rank sum test

Table 4-70: Pain scores at three and six months between patients with and without the C allele (major allele) of rs4818. Recessive analysis: assuming rs4818 has a recessive effect.

rs4818	WOMAC			WOMAC Pain		
Predictors	**Estimates**	**CI**	**p**	**Estimates**	**CI**	**p**
GG	-2.47	-8.85 – 3.91	0.45	-0.18	-1.83 – 1.47	0.83
GG at 3 months	2.64	-4.96 – 10.23	0.50	0.54	-1.43 – 2.50	0.59
GG at 6 months	3.47	-2.74 – 9.69	0.27	0.39	-1.00 – 1.78	0.58
Random Effects						
σ^2	93.32			7.42		
ICC	0.45			0.29		
Marginal R^2 / Conditional R^2	0.498/ 0.725			0.529 / 0.667		

Table 4-71: Linear Mixed Model analysis for rs4818 using recessive model. CI: confidence intervals, calculated using robust analysis of standard errors.

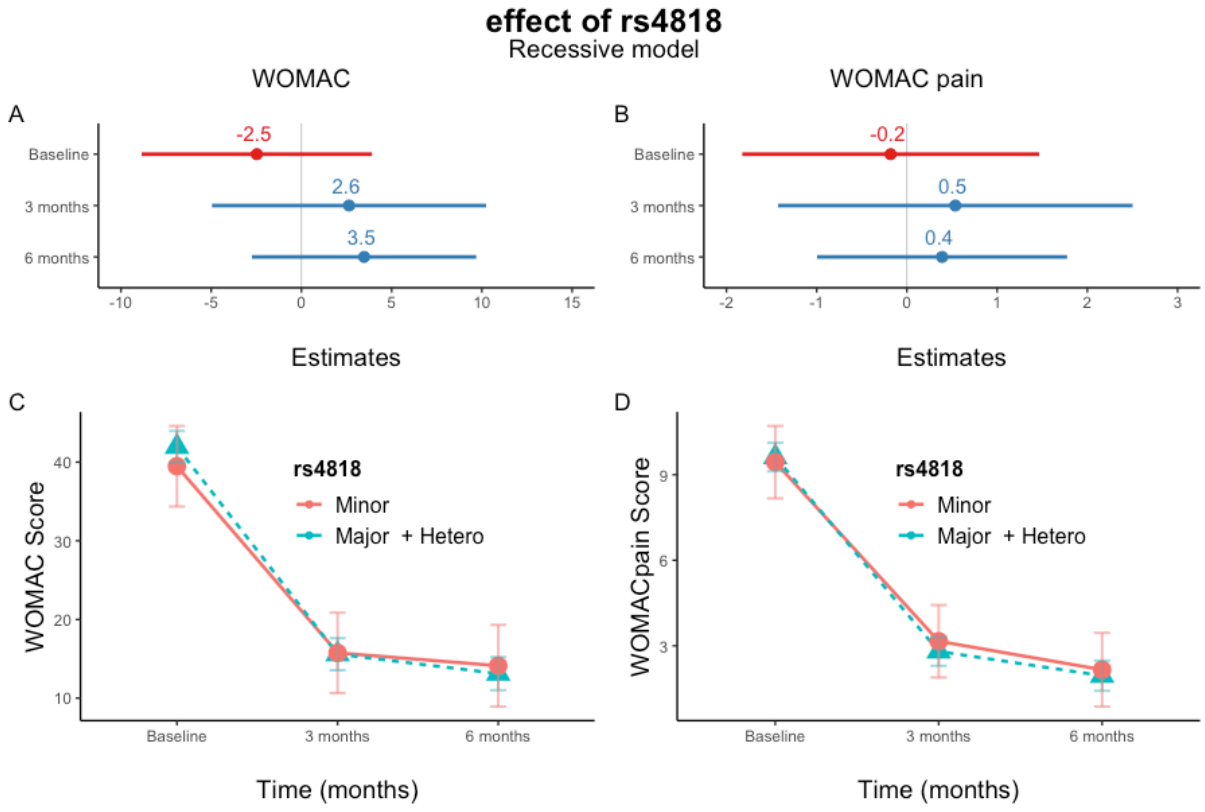

Figure 4-30: Analysis of rs4818 using recessive approach. A, B) Forest plot of estimates for WOMAC®, WOMAC® pain with robust confidence intervals C, D) predicted values for WOMAC®, WOMAC® pain as per above model

Overall, none of the polymorphisms investigated in the *COMT* gene showed any influence on WOMAC® and WOMAC® Pain scores.

Haplotype analysis was performed comparing patients who were homozygous at rs4680, rs4818 and rs4633. The two most frequent haplotypes were chosen, TCA (61 patients, 48%) and CGG (33 patients, 35%). The effect on the WOMAC® Score, WOMAC® Pain subscore, change in WOMAC® Pain score and incidence of chronic pain at 6 months is being shown in Figure 4-31.

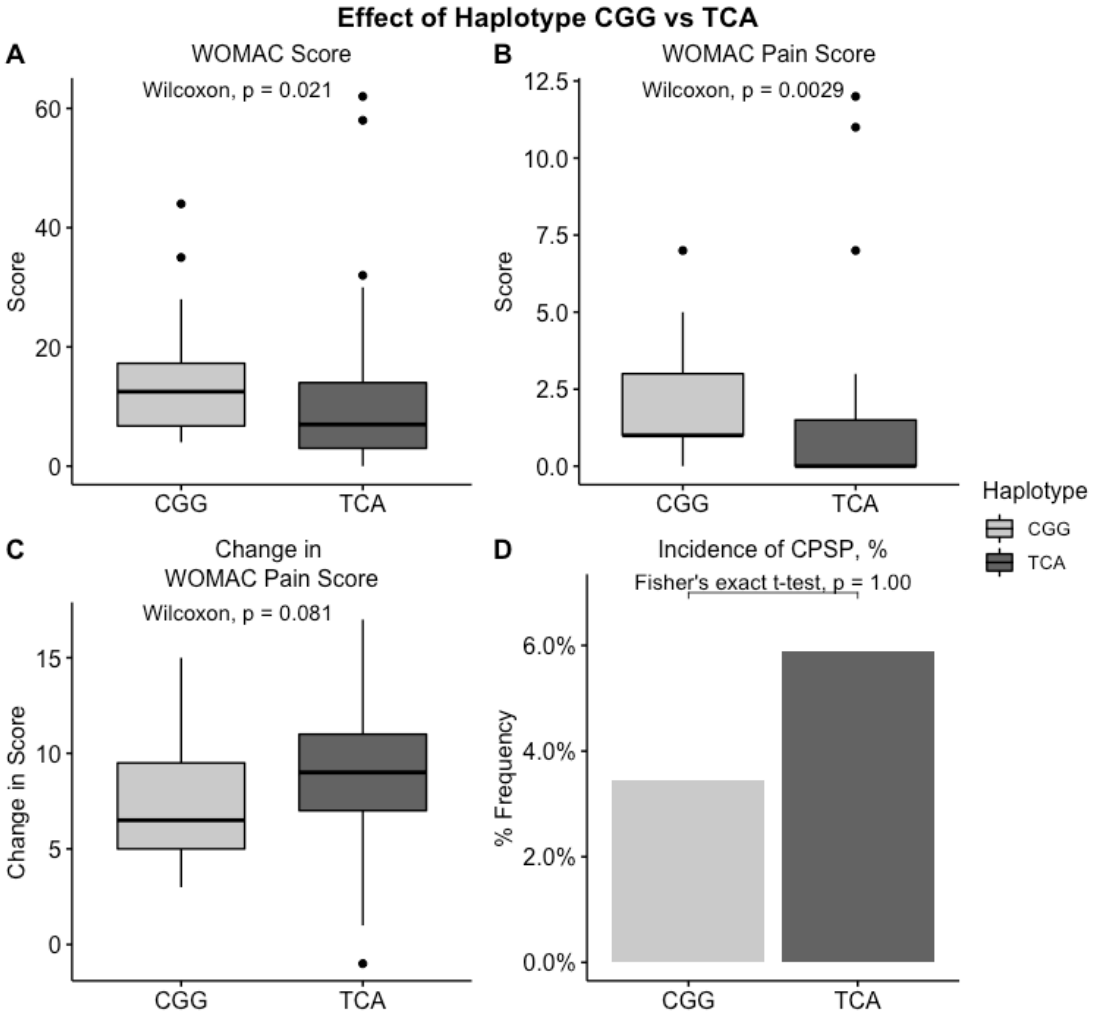

Figure 4-31: Effect of the two most common haplotypes of COMT, Haplotype CGG and Haplotype TCA, on various indices at six months

There is beneficial effect in having the TCA haplotype. Patients with such a combination of SNP's had better functionality, less pain, a bigger decrease in pain after six months.

4.3.2.5.2 SCN9A

rs6746030

The effects of rs6746030 using either a dominant or a recessive approach are summarized in Table 4-72 to Table 4-75, and in Figure 4-32 to Figure 4-33.

No significant effect could be seen. It must be noted that only 11 patients were homozygous for rs6746030, so the recessive approach was not expected to be statistically significant unless a large effect would be present.

		rs6746030 minor allele A as dominant allele		
	Characteristic	**GA | AA [1]**	**GG[1]**	**p-value[2]**
3 months	n	56	123	
	WOMAC®	11.5 [4.75 - 21]	13 [8 - 22]	0.14
	WOMAC® Pain	2 [0.75 - 3]	2 [0 - 4]	0.57
	% Chronic Pain	9 (16%)	28 (23%)	0.32
	S-LANSS ≥ 12	9 (16%)	26 (21%)	0.55
6 months	n	56	116	
	WOMAC®	9 [3.75 - 18]	10 [6 - 18]	0.59
	WOMAC® Pain	1 [0 - 2.25]	1 [0 - 3]	0.68
	% Chronic Pain	7 (15%)	16 (14%)	1
	S-LANSS ≥ 12	10 (21%)	30 (26%)	0.69

[1]median [IQR] for continuous variables; n / N (%) categorical variables

[2]Fisher's exact test; Wilcoxon rank sum test

Table 4-72: Pain scores at three and six months between patients with and without the A allele (minor allele) of rs6746030. Dominant analysis: assuming rs6746030 has a dominant effect.

rs6746030	WOMAC			WOMAC Pain		
Predictors	Estimates	CI	p	Estimates	CI	p
GA | AA	2.49	-2.17 – 7.14	0.30	0.48	-0.60 – 1.57	0.380
GA | AA at 3 m	-4.23	-9.62 – 1.15	0.12	-0.78	-2.21 – 0.66	0.289
GA | AA at 6 m	-2.59	-7.50 – 2.31	0.30	-0.78	-1.99 – 0.44	0.212
Random Effects						
σ^2	88.83			7.18		
ICC	0.47			0.31		
Marginal R^2 / Conditional R^2	0.500 / 0.736			0.526 / 0.674		

Table 4-73: Linear Mixed Model analysis for rs6746030 using dominant model. CI: confidence intervals, calculated using robust analysis of standard errors.

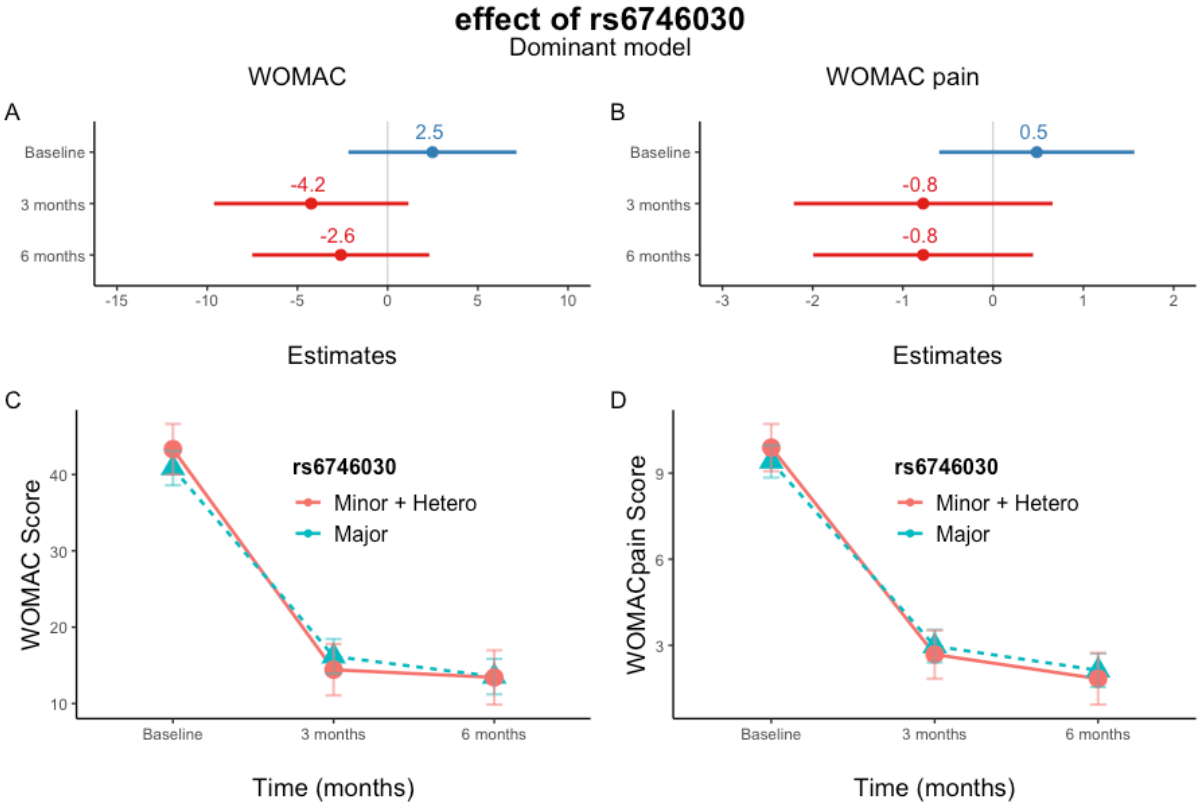

Figure 4-32: Analysis of rs6746030 using dominant model. A, B) Forest plot of estimates for WOMAC®, WOMAC® pain with robust confidence intervals C, D) predicted values for WOMAC®, WOMAC® pain as per above model

		rs6746030 minor allele A as recessive allele		
	Characteristic	**GG | GA [1]**	**AA[1]**	**p-value[2]**
3 months	n	168	11	
	WOMAC®	13 [7 - 22]	6 [3.5 - 13.5]	0.11
	WOMAC® Pain	2 [0 - 4]	2 [0.5 - 3.5]	0.99
	% Chronic Pain	35 (21%)	2 (18%)	1
	S-LANSS ≥ 12	33 (20%)	2 (18%)	1
6 months	n	153	11	
	WOMAC®	10 [5 - 18]	11 [6 - 18]	0.69
	WOMAC® Pain	1 [0 - 3]	2 [1 - 4.5]	0.18
	% Chronic Pain	20 (13%)	3 (27%)	0.19
	S-LANSS ≥ 12	39 (25%)	1 (9%)	0.30

[1]median [IQR] for continuous variables; n / N (%) categorical variables

[2]Fisher's exact test; Wilcoxon rank sum test

Table 4-74: Pain scores at three and six months between patients with and without the G allele (major allele) of rs4633. Recessive analysis: assuming rs6746030 has a recessive effect.

rs6746030	WOMAC			WOMAC Pain		
Predictors	**Estimates**	**CI**	**p**	**Estimates**	**CI**	**p**
AA	-0.96	-9.21 – 7.29	0.82	1.34	-1.20 – 3.88	0.30
AA at 3 m	-1.36	-15.60 – 12.87	0.85	-0.44	-4.80 – 3.92	0.84
AA at 6 m	2.95	-8.50 – 14.40	0.61	-0.52	-3.43 – 2.40	0.73
Random Effects						
σ^2	89.34			7.21		
ICC	0.47			0.31		
Marginal R^2 / Conditional R^2	0.498 / 0.734			0.527 / 0.673		

Table 4-75: Linear Mixed Model analysis for rs6746030 using recessive model. CI: confidence intervals, calculated using robust analysis of standard errors.

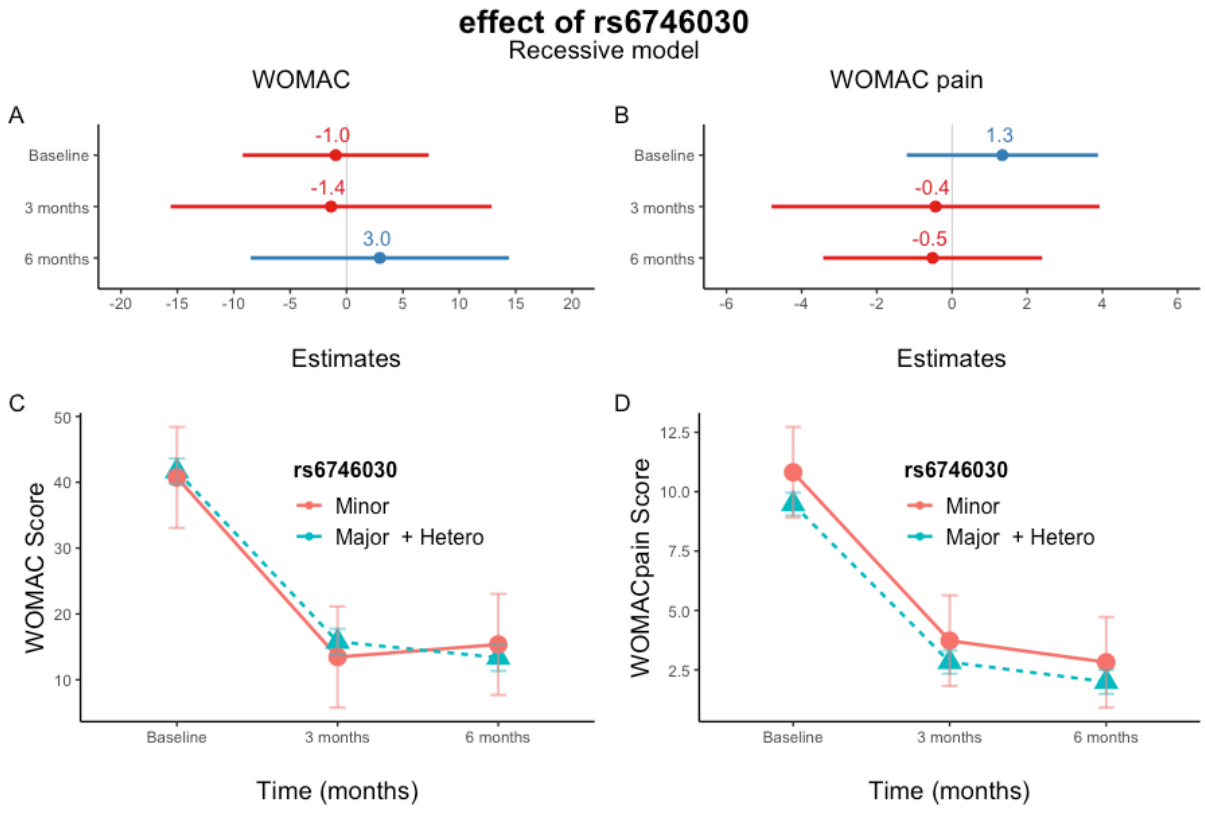

Figure 4-33: Analysis of rs6746030 using recessive model. A, B) Forest plot of estimates for WOMAC®, WOMAC® pain with robust confidence intervals C, D) predicted values for WOMAC®, WOMAC® pain as per above model

rs7595255

There seems to be no effect of rs7595255 on any of the outcomes measured, both when a dominant or a recessive approach were used. This is summarized in Table 4-76 to Table 4-79 and in Figure 4-34 to Figure 4-35.

The increased WOMAC® Pain score for homozygous carriers of rs7595255 at baseline, as shown in Table 4-79, may be explained by the increased number of patients in Group GA who carried the polymorphism (40% of patients, compared to 25% of patients in Group SP). Furthermore, the total number of such patients was limited to 11 patients only.

		rs7595255 minor allele T as dominant allele		
	Characteristic	**CT | TT [1]**	**CC[1]**	**p-value[2]**
3 months	n	60	126	
	WOMAC®	1.5 [4.75 - 21]	13 [8 - 21.75]	0.20
	WOMAC® Pain	2 [0.75 - 3]	2 [0 - 4]	0.62
	% Chronic Pain	10 (17%)	27 (21%)	0.56
	S-LANSS ≥ 12	10 (17%)	27 (21%)	0.56
6 months	n	52	119	
	WOMAC®	9 [3.75 - 18]	10 [5 - 17.5]	0.61
	WOMAC® Pain	1 [0 - 2.25]	1 [0 - 3]	0.77
	% Chronic Pain	8 (15%)	15 (13%)	0.63
	S-LANSS ≥ 12	11 (21%)	29 (24%)	0.84

[1]median [IQR] for continuous variables; n / N (%) categorical variables

[2]Fisher's exact test; Wilcoxon rank sum test

Table 4-76: Pain scores at three and six months between patients with and without the T allele (minor allele) of rs7595255. Dominant analysis: assuming rs7595255 has a dominant effect.

rs7595255	WOMAC			WOMAC Pain		
Predictors	**Estimates**	**CI**	**p**	**Estimates**	**CI**	**p**
CT | TT	1.70	-2.82 – 6.22	0.46	0.36	-0.70 – 1.41	0.50
CT | TT at 3 m	-3.10	-8.38 – 2.17	0.25	-0.59	-1.97 – 0.80	0.41
CT | TT at 6 m	-1.74	-6.63 – 3.16	0.49	-0.48	-1.71 – 0.75	0.44
Random Effects						
σ^2	94.25			7.36		
ICC	0.44			0.29		
Marginal R^2 / Conditional R^2	0.505 / 0.721			0.529 / 0.666		

Table 4-77: Linear Mixed Model analysis for rs7595255 using dominant model. CI: confidence intervals, calculated using robust analysis of standard errors.

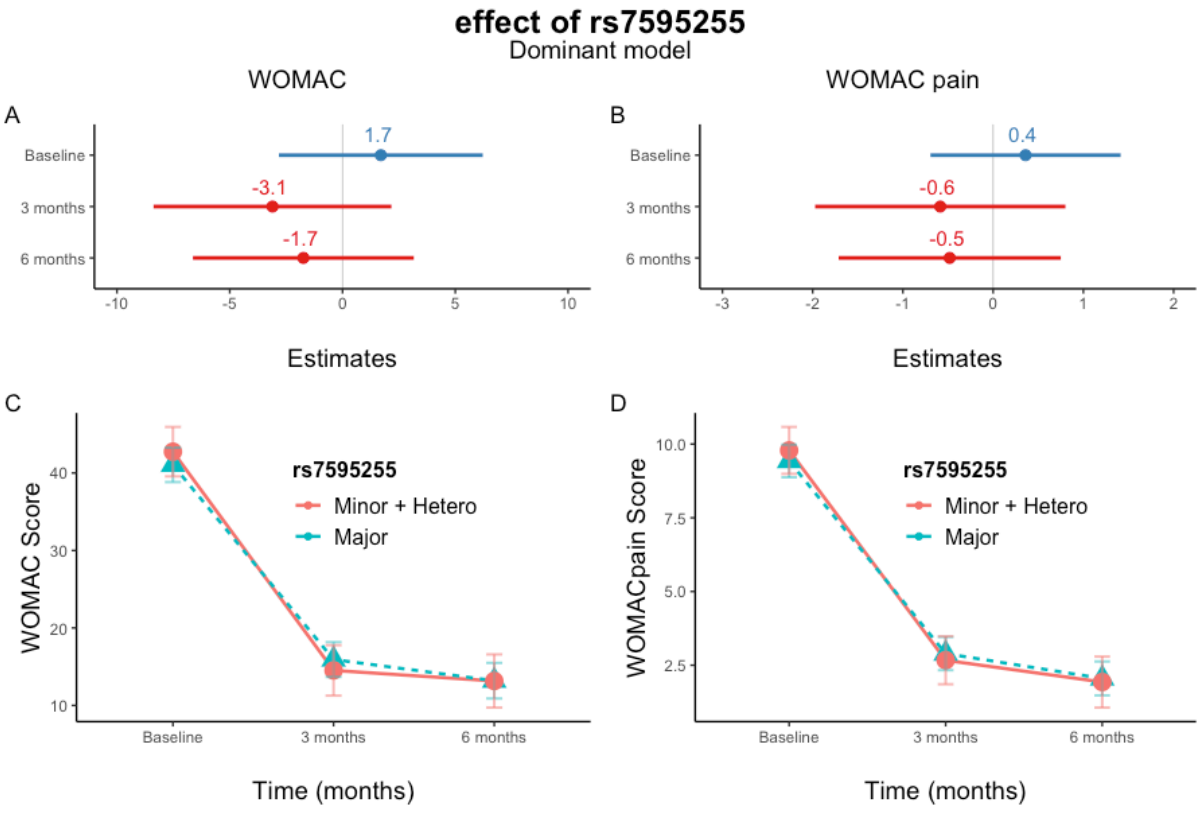

Figure 4-34: Analysis of rs7595255 using dominant model. A, B) Forest plot of estimates for WOMAC®, WOMAC® pain with robust confidence intervals C, D) predicted values for WOMAC®, WOMAC® pain as per above model

		rs7595255 minor allele T as recessive allele		
	Characteristic	**CC | CT [1]**	**TT[1]**	**p-value[2]**
3 months	n	175	11	
	WOMAC®	13 [7 - 21.5]	6 [3.5 - 13.5]	0.12
	WOMAC® Pain	2 [0 - 4]	2 [1 - 3.5]	0.77
	% Chronic Pain	35 (20%)	2 (18%)	1
	S-LANSS ≥ 12	36 (21%)	1 (9%)	0.69
6 months	n	160	11	
	WOMAC®	9 [5 - 17.25]	11 [6 - 18]	0.58
	WOMAC® Pain	1 [0 - 3]	2 [1 - 4.5]	0.16
	% Chronic Pain	20 (12%)	3 (27%)	0.17
	S-LANSS ≥ 12	39 (24%)	1 (9%)	0.46

[1]median [IQR] for continuous variables; n / N (%) categorical variables

[2]Fisher's exact test; Wilcoxon rank sum test

Table 4-78: Pain scores at three and six months between patients with and without the C allele (major allele) of rs7595255. Recessive analysis: assuming rs7595255 has a recessive effect.

rs7595255	WOMAC			WOMAC Pain		
Predictors	**Estimates**	**CI**	**p**	**Estimates**	**CI**	**p**
TT	2.34	-4.08 – 8.76	0.47	2.32	0.39 – 4.25	***0.029***
TT at 3 m	-4.39	-18.30 – 9.52	0.54	-1.26	-5.55 – 3.04	0.57
TT at 6 m	0.08	-11.62 – 11.79	0.99	-1.47	-4.21 – 1.28	0.30
Random Effects						
σ^2	94.25			7.34		
ICC	0.44			0.28		
Marginal R^2 / Conditional R^2	0.504 / 0.721			0.534 / 0.667		

Table 4-79: Linear Mixed Model analysis for rs7595255 using recessive model. CI: confidence intervals, calculated using robust analysis of standard errors.

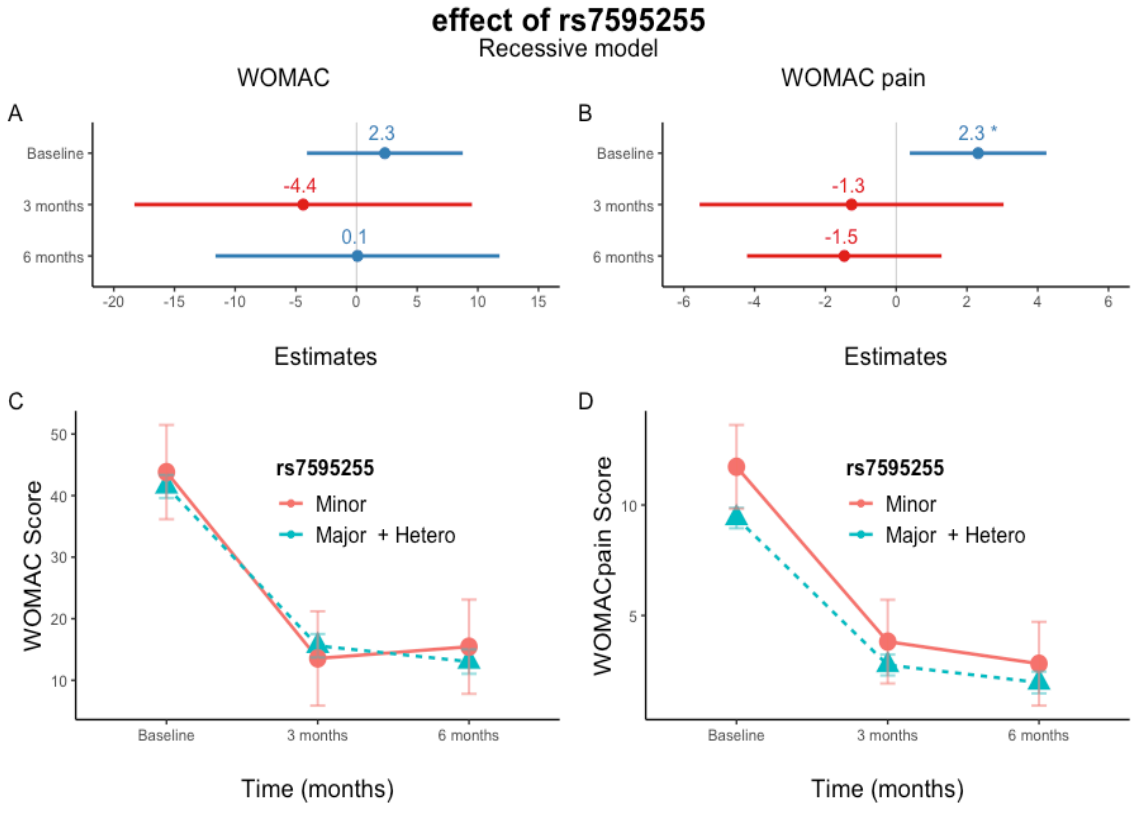

Figure 4-35: Analysis of rs7595255 using recessive model. A, B) Forest plot of estimates for WOMAC®, WOMAC® pain with robust confidence intervals C, D) predicted values for WOMAC®, WOMAC® pain as per above model

rs11898284

The effects of rs11898284 are summarized in Table 4-92 to Table 4-83, and in Figure 4-36 to Figure 4-37.

Univariate analysis of rs11898284 revealed WOMAC® Pain was significantly different at 3 months using a dominant approach, and significantly different at 6 months using a recessive approach. However, such results were not confirmed on a linear mixed model analysis. However, with only six patients being homozygous for rs11898284, any interpretation must be done cautiously.

		rs11898284 minor allele G as dominant allele		
	Characteristic	**GA | GG [1]**	**AA[1]**	**p-value[2]**
3 months	n	44	141	
	WOMAC®	13.5 [7 - 24]	13 [6 - 21]	0.28
	WOMAC® Pain	3 [1 - 5.25]	2 [0 - 4]	***0.047***
	% Chronic Pain	13 (30%)	25 (18%)	0.13
	S-LANSS ≥ 12	10 (23%)	27 (19%)	0.52
6 months	n	43	127	
	WOMAC®	12 [6 - 19]	9 [5 - 17]	0.20
	WOMAC® Pain	1 [0 - 4]	1 [0 - 3]	0.63
	% Chronic Pain	7 (16%)	17 (13%)	0.62
	S-LANSS ≥ 12	13 (30%)	28 (22%)	0.31

[1]median [IQR] for continuous variables; n / N (%) categorical variables

[2]Fisher's exact test; Wilcoxon rank sum test

Table 4-80: Pain scores at three and six months between patients with and without the G allele (minor allele) of rs11898284. Dominant analysis: assuming rs11898284 has a dominant effect.

rs11898284	WOMAC			WOMAC Pain		
Predictors	**Estimates**	**CI**	**p**	**Estimates**	**CI**	**p**
GA | GG	1.91	-3.30 – 7.12	0.47	0.74	-0.51 – 1.99	0.24
GA | GG at 3 m	0.53	-4.80 – 5.86	0.85	0.27	-1.32 – 1.86	0.74
GA | GG at 6 m	0.96	-4.11 – 6.04	0.71	-0.08	-1.52 – 1.36	0.91
Random Effects						
σ^2	93.36			7.32		
ICC	0.44			0.29		
Marginal R^2 / Conditional R^2	0.505 / 0.723			0.531 / 0.668		

Table 4-81: Linear Mixed Model analysis for rs11898284 using dominant model. CI: confidence intervals, calculated using robust analysis of standard errors.

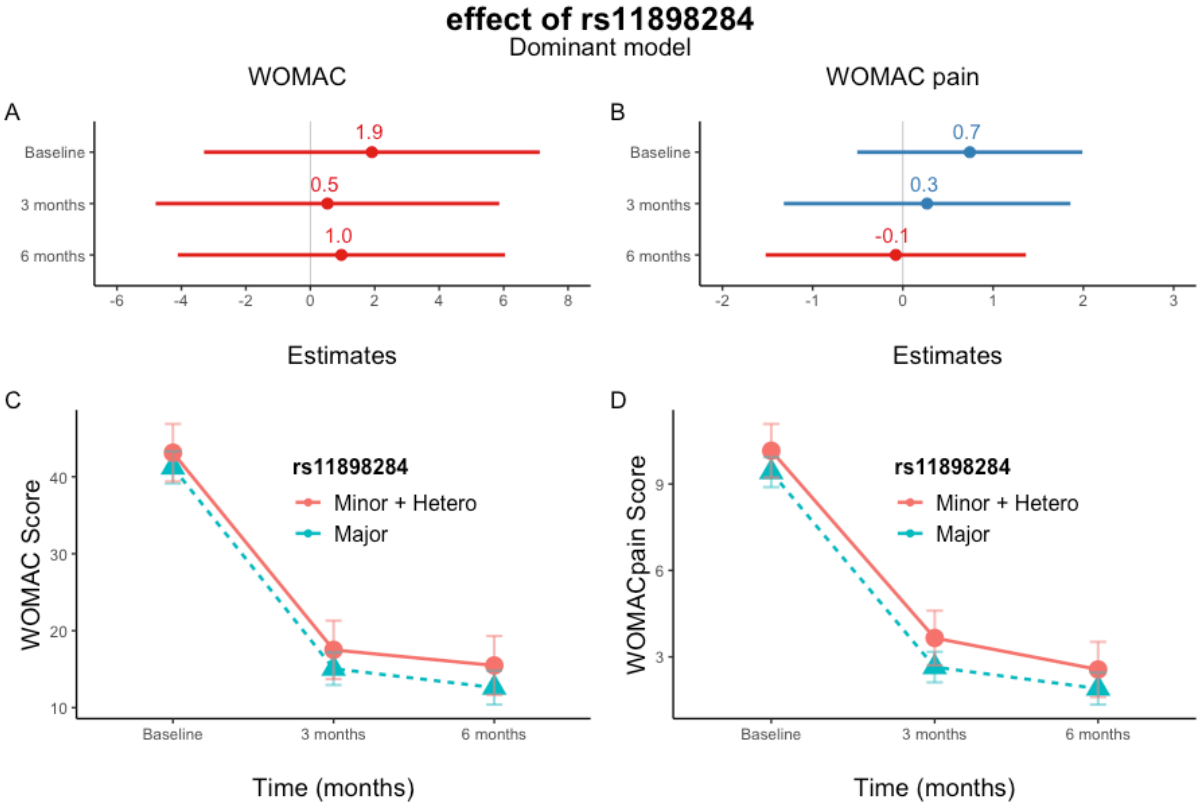

Figure 4-36: Analysis of rs11898284 using dominant model. A, B) Forest plot of estimates for WOMAC®, WOMAC® pain with robust confidence intervals C, D) predicted values for WOMAC®, WOMAC® pain as per above model

		rs11898284 minor allele G as recessive allele		
	Characteristic	**AA | AG [1]**	**GG[1]**	**p-value[2]**
3 months	n	179	6	
	WOMAC®	13 [7 - 21.5]	8.5 [6.25 - 20.5]	0.70
	WOMAC® Pain	2 [0 - 4]	1 [1 - 3.25]	0.76
	% Chronic Pain	37 (21%)	1 (17%)	1
	S-LANSS ≥ 12	36 (20%)	1 (17%)	1
6 months	n	164	6	
	WOMAC®	9.5 [5 - 18]	7 [1.75 - 13.75]	0.38
	WOMAC® Pain	1 [0 - 3]	0 [0 - 0]	***0.019***
	% Chronic Pain	24 (15%)	0 (0%)	0.60
	S-LANSS ≥ 12	40 (24%)	1 (17%)	1

[1]median [IQR] for continuous variables; n / N (%) categorical variables

[2]Fisher's exact test; Wilcoxon rank sum test

Table 4-82: Pain scores at three and six months between patients with and without the G allele (major allele) of rs11898284. Recessive analysis: assuming rs11898284 has a recessive effect.

rs11898284	WOMAC			WOMAC Pain		
Predictors	**Estimates**	**CI**	**p**	**Estimates**	**CI**	**p**
GG	2.75	-14.56 – 20.06	0.76	-0.61	-4.91 – 3.70	0.78
GG at 3 m	-4.96	-23.00 – 13.08	0.59	0.04	-4.50 – 4.58	0.99
GG at 6 m	-6.18	-23.72 – 11.35	0.49	-1.36	-5.76 – 3.05	0.55
			Random Effects			
σ^2	92.99			7.30		
ICC	0.45			0.30		
Marginal R^2 / Conditional R^2	0.502 / 0.724			0.528 / 0.669		

Table 4-83: Linear Mixed Model analysis for rs11898284 using recessive model. CI: confidence intervals, calculated using robust analysis of standard errors.

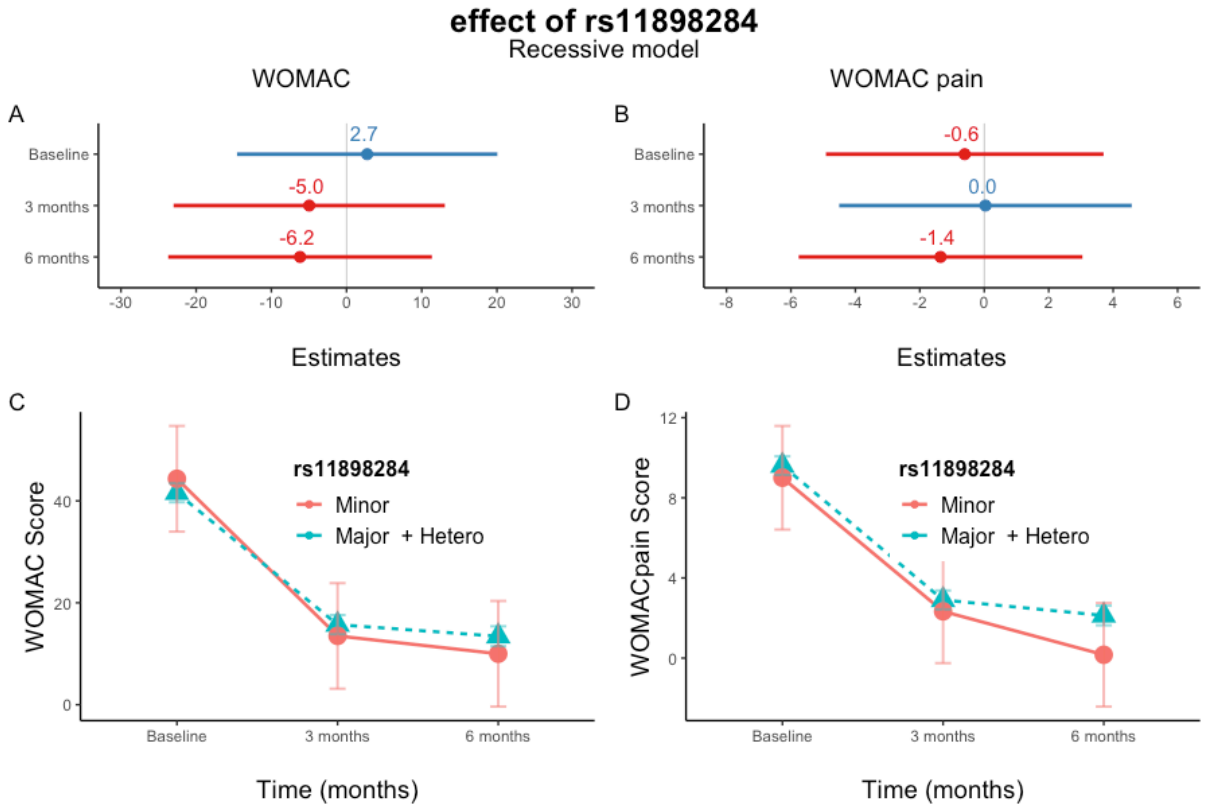

Figure 4-37: Analysis of rs11898284 using recessive model. A, B) Forest plot of estimates for WOMAC®, WOMAC® pain with robust confidence intervals C, D) predicted values for WOMAC®, WOMAC® pain as per above model.

4.3.2.5.3 OPRM1

rs1799971

The presence of at least one allele of rs1799971 (dominant approach) did not have any effect on outcomes, as summarized in Table 4-84 to Table 4-87 and in Figure 4-38 to Figure 4-39.

		rs1799971 minor allele G as dominant allele		
	Characteristic	**AG | GG [1]**	**AA[1]**	**p-value[2]**
3 months	n	50	131	
	WOMAC®	11 [6 – 20]	14 [8 – 23]	0.15
	WOMAC® Pain	2.00 [0.00 – 3.00]	2.00 [0.00 – 4.00]	0.46
	% Chronic Pain	7.0 [4.0 – 10.0]	7.0 [4.0 – 10.0]	0.80
	S-LANSS ≥ 12	8 / 51 (16%)	28 / 134 (21%)	0.53
6 months	n	45	120	
	WOMAC®	10 [4 - 18]	10 [6 - 20]	0.54
	WOMAC® Pain	1.00 [0.00 - 2.00]	1.00 [0.00 - 3.00]	0.60
	% Chronic Pain	3 / 47 (6.4%)	15 / 121 (12%)	0.40
	S-LANSS ≥ 12	9 / 47 (19%)	29 / 121 (24%)	0.55

[1]median [IQR] for continuous variables; n / N (%) categorical variables

[2]Fisher's exact test; Wilcoxon rank sum test

Table 4-84: Pain scores at three and six months between patients with and without the G allele (minor allele) of rs1799971. Dominant analysis: assuming rs1799971 has a dominant effect.

rs1799971	WOMAC			WOMAC Pain		
Predictors	Estimates	CI	p	Estimates	CI	p
GA | AA	-1.31	-6.00 – 3.38	0.58	0.48	-0.60 – 1.57	0.380
GA | AA at 3 m	-1.38	-6.57 – 3.81	0.602	-0.78	-2.21 – 0.66	0.289
GA | AA at 6 m	-1.36	-5.97 – 3.24	0.561	-0.78	-1.99 – 0.44	0.212
Random Effects						
σ^2	89.85			7.18		
ICC	0.46			0.31		
Marginal R^2 / Conditional R^2	0.512 / 0.734			0.526 / 0.674		

Table 4-85: Linear Mixed Model analysis for rs1799971 using dominant model. CI: confidence intervals, calculated using robust analysis of standard errors.

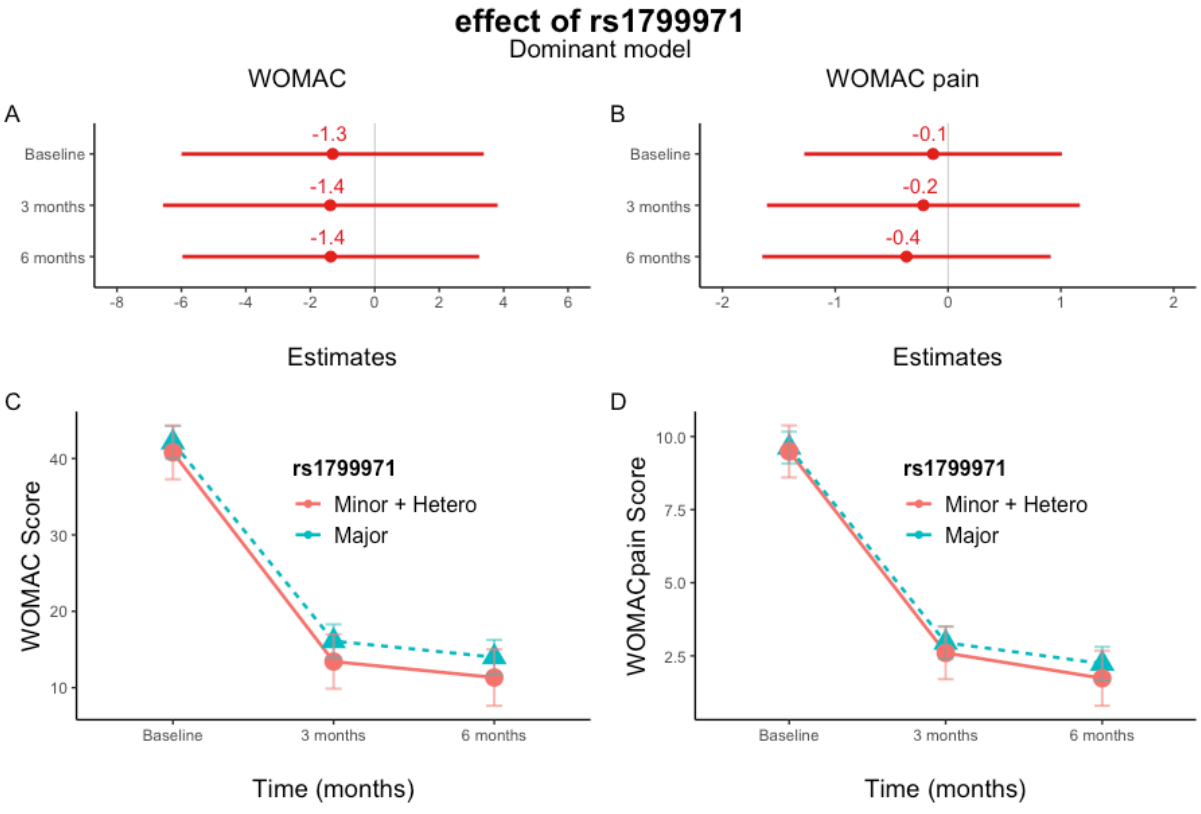

Figure 4-38: Analysis of rs1799971 using dominant model. A, B) Forest plot of estimates for WOMAC®, WOMAC® pain with robust confidence intervals C, D) predicted values for WOMAC®, WOMAC® pain as per above model

A recessive approach showed that rs1799971 might have an effect on WOMAC® scores and WOMAC® Pain scores, as shown in Table 4-86 to Table 4-89, and Figure 4-39. This was more evident on a linear mixed model analysis, where patients homozygous for the minor allele G were more likely to have lower WOMAC® and WOMAC® Pain scores.

Although the estimates for WOMAC® scores were borderline significant, the likelihood-ratio test comparing the fitted model with a simpler model that excluded rs1799971. Similarly, the estimates for the WOMAC® Pain scores were highly significant, but the model was not better than a more parsimonious model.

Both results could be explained by the low number of patients who were homozygous for rs1799971.

		rs1799971 minor allele G as recessive allele		
	Characteristic	**AA | AG [1]**	**GG[1]**	**p-value[2]**
3 months	n	175	6	
	WOMAC®	14 [8 – 23]	10 [5 – 16]	0.28
	WOMAC® Pain	2.00 [0.00 – 4.00]	1.00 [1.00 – 3.00]	0.91
	% Chronic Pain	32 / 176 (18%)	1 / 7 (14%)	>0.99
	S-LANSS ≥ 12	36 / 178 (20%)	0 / 7 (0%)	0.35
6 months	n	159	6	
	WOMAC®	11 [5 - 19]	10 [4 - 11]	0.29
	WOMAC® Pain	1.00 [0.00 - 3.00]	0.00 [0.00 - 2.00]	0.41
	% Chronic Pain	18 / 161 (11%)	0 / 7 (0%)	>0.99
	S-LANSS ≥ 12	35 / 161 (22%)	3 / 7 (43%)	0.19

[1]median [IQR] for continuous variables; n / N (%) categorical variables

[2]Fisher's exact test; Wilcoxon rank sum test

Table 4-86: Pain scores at three and six months between patients with and without the G allele (major allele) of rs1799971. Recessive analysis: assuming rs1799971has a recessive effect.

rs1799971	WOMAC*			WOMAC Pain		
Predictors	**Estimates**	**CI**	**p**	**Estimates**	**CI**	**p**
GG	8.17	-2.22 – 18.56	0.12	2.32	0.11 – 4.53	***0.040***
GG at 3 m	-13.22	-28.13 – 1.69	0.08	-2.17	-5.81 – 1.46	0.241
GG at 6 m	-13.83	-28.30 – 0.63	0.06	-3.29	-6.16 – -0.42	***0.025***
			Random Effects			
σ^2	87.81			7.03		
ICC	0.47			0.33		
Marginal R^2 / Conditional R^2	0.512 / 0.740			0.527 / 0.682		
	*LRT p-value : ***0.046***			LRT p-value: 0.64		

Table 4-87: Linear Mixed Model analysis for rs1799971 using recessive model. CI: confidence intervals, calculated using robust analysis of standard errors.

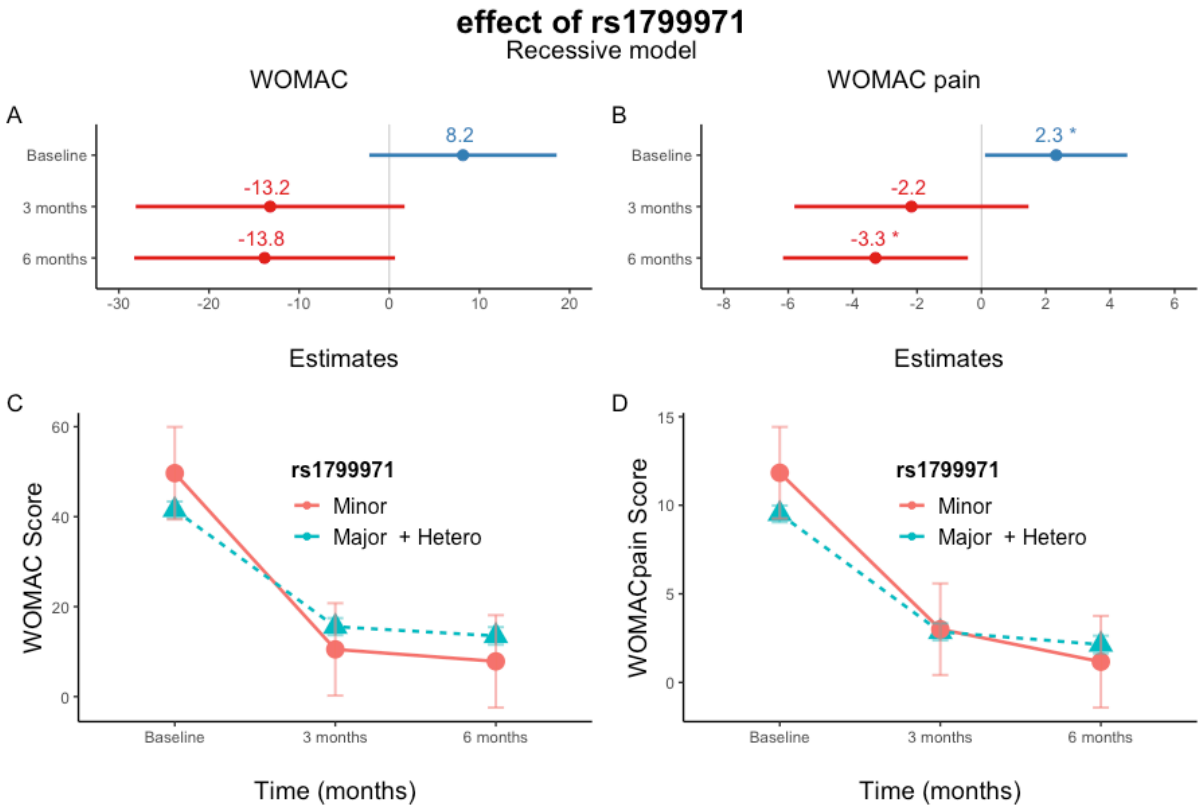

Figure 4-39: Analysis of rs1799971 using recessive model. A, B) Forest plot of estimates for WOMAC®, WOMAC® pain with robust confidence intervals C, D) predicted values for WOMAC®, WOMAC® pain as per above model

rs2075572

The effect of rs2075572 on the measured outcomes is shown in Table 4-88 to Table 4-91, and Figure 4-40, and Figure 4-41.

Linear mixed effects analysis with a dominant approach showed that patients who carried the G allele of rs2075572 had lower WOMAC® and WOMAC® pain scores.

The recessive approach showed no influence of homozygous carriage of rs2075572. This is not unexpected, given that the dominant approach showed significant effects.

		rs2075572 minor allele G as dominant allele		
	Characteristic	**CG | GG [1]**	**CC[1]**	**p-value[2]**
3 months	n	130	53	
	WOMAC®	14 [8 – 24]	12 [5 – 21]	0.22
	WOMAC® Pain	2.00 [0.00 – 4.00]	2.00 [1.00 – 4.00]	0.89
	% Chronic Pain	24 / 131 (18%)	9 / 54 (17%)	>0.99
	S-LANSS ≥ 12	27 / 133 (20%)	11 / 54 (20%)	>0.99
6 months	n	120	48	
	WOMAC®	11 [5 - 19]	10 [7 - 20]	0.50
	WOMAC® Pain	1.00 [0.00 - 3.00]	1.00 [0.00 - 4.00]	0.76
	% Chronic Pain	13 / 122 (11%)	6 / 49 (12%)	0.79
	S-LANSS ≥ 12	29 / 122 (24%)	11 / 49 (22%)	>0.99

[1]median [IQR] for continuous variables; n / N (%) categorical variables

[2]Fisher's exact test; Wilcoxon rank sum test

Table 4-88: Pain scores at three and six months between patients with and without the G allele (minor allele) of rs2075572. Dominant analysis: assuming rs2075572 has a dominant effect.

rs2075572	WOMAC*			WOMAC Pain*		
Predictors	**Estimates**	**CI**	**p**	**Estimates**	**CI**	**p**
CG | GG	-5.37	-9.71 – -1.03	***0.015***	-1.74	-2.75 – -0.72	***0.001***
CG | GG at 3 m	7.43	2.82 – 12.04	***0.002***	1.80	0.44 – 3.16	***0.010***
CG | GG at 6 m	3.92	-0.84 – 8.67	0.106	1.43	0.19 – 2.67	***0.024***
			Random Effects			
σ^2	89.60			7.01		
ICC	0.46			0.31		
Marginal R^2 / Conditional R^2	0.513 / 0.738			0.542 / 0.686		
	*LRT p-value : ***0.0058***			*LRT p-value: ***0.0049***		

Table 4-89: Linear Mixed Model analysis for rs2075572 using dominant model. CI: confidence intervals, calculated using robust analysis of standard errors.

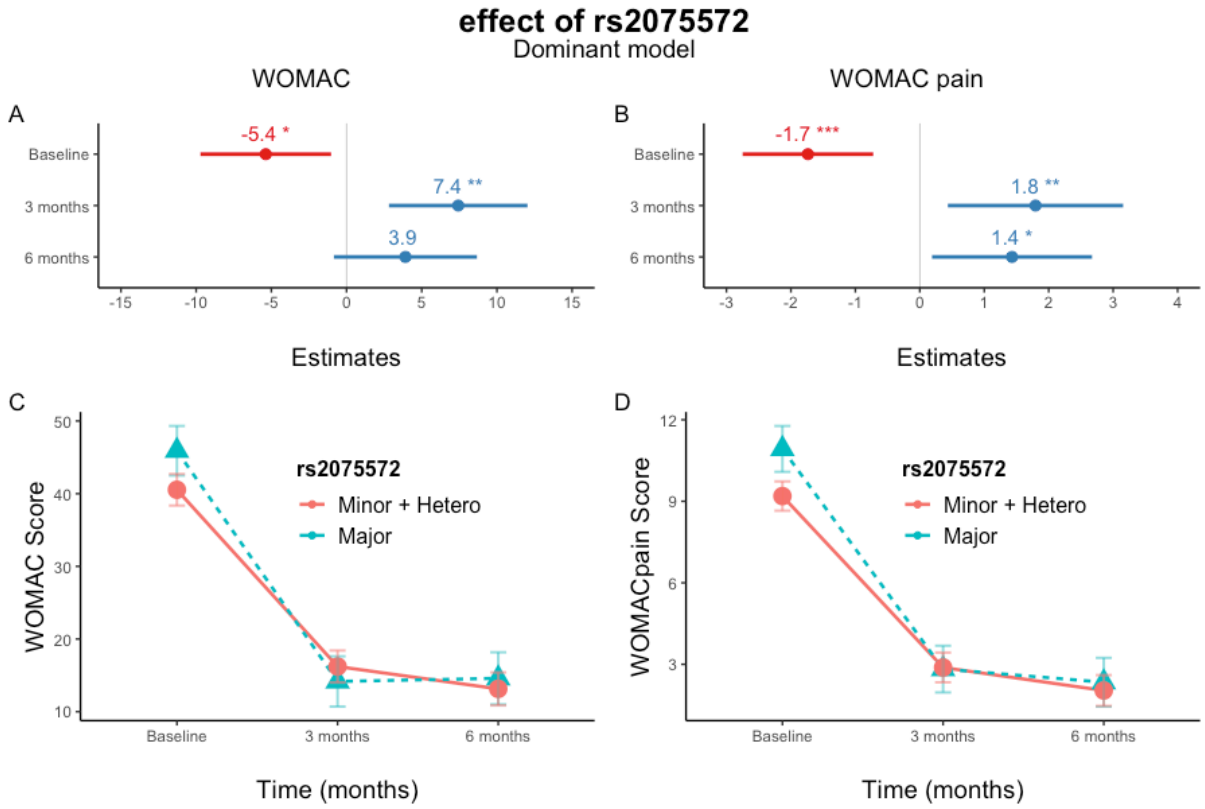

Figure 4-40: Analysis of rs2075572 using dominant model. A, B) Forest plot of estimates for WOMAC®, WOMAC® pain with robust confidence intervals C, D) predicted values for WOMAC®, WOMAC® pain as per above model

		rs2075572 minor allele G as recessive allele		
	Characteristic	**CC | CG [1]**	**GG[1]**	**p-value[2]**
3 months	n	147	36	
	WOMAC®	14 [8 – 23]	12 [5 – 22]	0.29
	WOMAC® Pain	2.00 [1.00 – 4.00]	1.00 [0.00 – 4.00]	0.23
	% Chronic Pain	27 / 149 (18%)	6 / 36 (17%)	>0.99
	S-LANSS ≥ 12	29 / 151 (19%)	9 / 36 (25%)	0.49
6 months	n	137	36	
	WOMAC®	10 [6 – 19]	12 [4 – 18]	0.60
	WOMAC® Pain	1.00 [0.00 – 3.00]	1.50 [0.00 – 3.00]	0.37
	% Chronic Pain	16 / 139 (12%)	3 / 32 (9.4%)	>0.99
	S-LANSS ≥ 12	30 / 139 (22%)	10 / 32 (31%)	0.25

[1]median [IQR] for continuous variables; n / N (%) categorical variables

[2]Fisher's exact test; Wilcoxon rank sum test

Table 4-90: Pain scores at three and six months between patients with and without the C allele (major allele) of rs2075572. Recessive analysis: assuming rs2075572 has a recessive effect.

rs2075572	WOMAC*			WOMAC Pain		
Predictors	**Estimates**	**CI**	**p**	**Estimates**	**CI**	**p**
GG	0.31	-4.81 – 5.44	0.90	0.35	-0.90 – 1.60	0.58
GG at 3 m	-3.10	-8.85 – 2.65	0.29	-0.90	-2.45 – 0.65	0.26
GG at 6 m	-2.18	-6.95 – 2.59	0.37	-0.33	-1.72 – 1.07	0.66
Random Effects						
σ^2	92.17			7.17		
ICC	0.45			0.31		
Marginal R^2 / Conditional R^2	0.507 / 0.730			0.533 / 0.679		

Table 4-91: Linear Mixed Model analysis for rs2075572 using recessive model. CI: confidence intervals, calculated using robust analysis of standard errors.

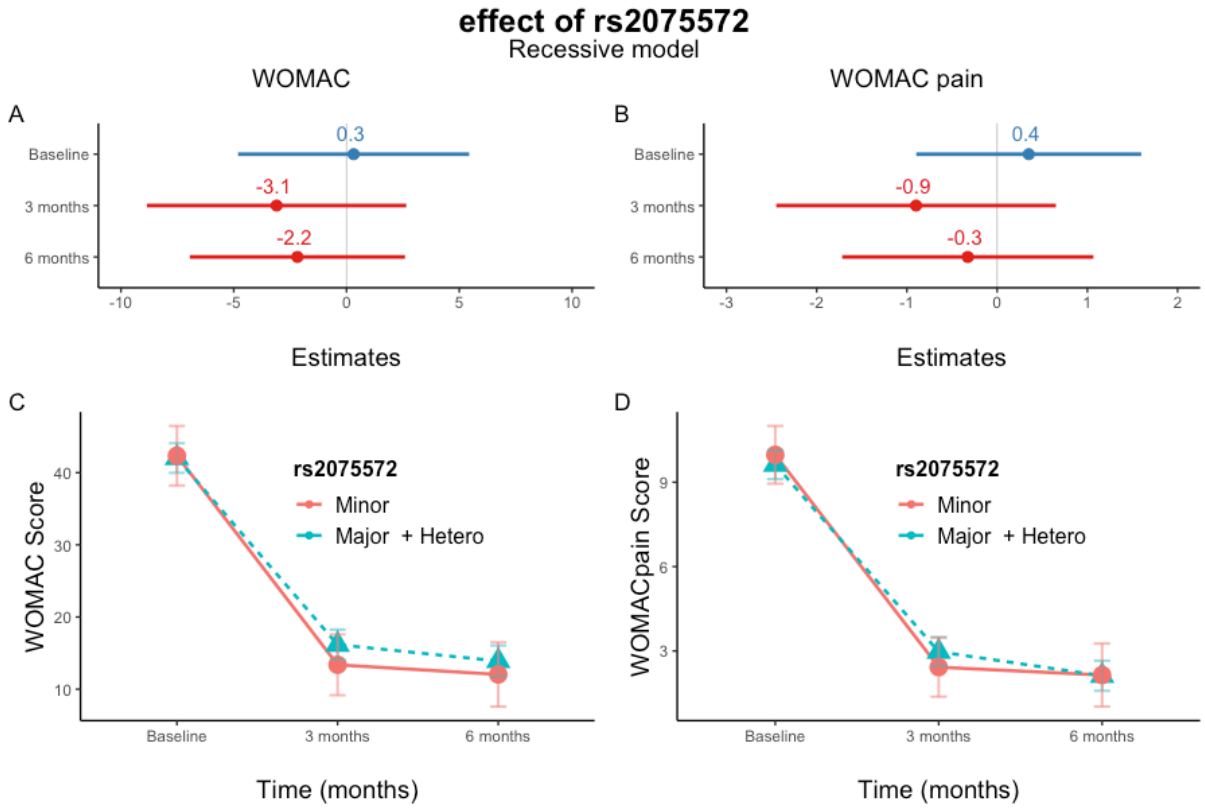

Figure 4-41: Analysis of rs2075572 using recessive model. A, B) Forest plot of estimates for WOMAC®, WOMAC® pain with robust confidence intervals C, D) predicted values for WOMAC®, WOMAC® pain as per above model

rs495491

There was no influence of rs495491 on any of the investigated outcomes, both using a dominant and a recessive approach. This is shown in Table 4-92 to Table 4-95, and in Figure 4-42 to Figure 4-43.

		rs495491 minor allele G as dominant allele		
	Characteristic	**AG | GG** [1]	**AA** [1]	**p-value** [2]
3 months	n	102	72	
	WOMAC®	12 [6 – 23]	15 [9 – 25]	0.27
	WOMAC® Pain	2.0 [0.0 – 4.0]	2.0 [0.0 – 4.0]	0.95
	% Chronic Pain	21 / 102 (21%)	13 / 74 (18%)	0.70
	S-LANSS ≥ 12	22 / 103 (21%)	14 / 75 (19%)	0.71
6 months	n	93	67	
	WOMAC®	10 [6 – 20]	10 [6 – 18]	0.99
	WOMAC® Pain	1.00 [0.00 – 3.00]	1.00 [0.00 – 2.25]	0.76
	% Chronic Pain	11 / 95 (12%)	7 / 68 (10%)	>0.99
	S-LANSS ≥ 12	19 / 95 (20%)	20 / 68 (29%)	0.19

[1]median [IQR] for continuous variables; n / N (%) categorical variables

[2]Fisher's exact test; Wilcoxon rank sum test

Table 4-92: Pain scores at three and six months between patients with and without the G allele (minor allele) of rs495491. Dominant analysis: assuming rs495491has a dominant effect.

rs495491	WOMAC			WOMAC Pain		
Predictors	**Estimates**	**CI**	**p**	**Estimates**	**CI**	**p**
AG | GG	-3.19	-7.40 – 1.03	0.13	-0.63	-1.64 – 0.37	0.22
AG | GG at 3 m	1.69	-3.07 – 6.44	0.49	0.72	-0.61 – 2.04	0.29
AG | GG at 6 m	2.09	-2.44 – 6.61	0.37	0.60	-0.58 – 1.78	0.32
			Random Effects			
σ^2	96.51			7.44		
ICC	0.44			0.31		
Marginal R^2 / Conditional R^2	0.500 / 0.722			0.519 / 0.667		

Table 4-93: Linear Mixed Model analysis for rs495491 using dominant model. CI: confidence intervals, calculated using robust analysis of standard errors.

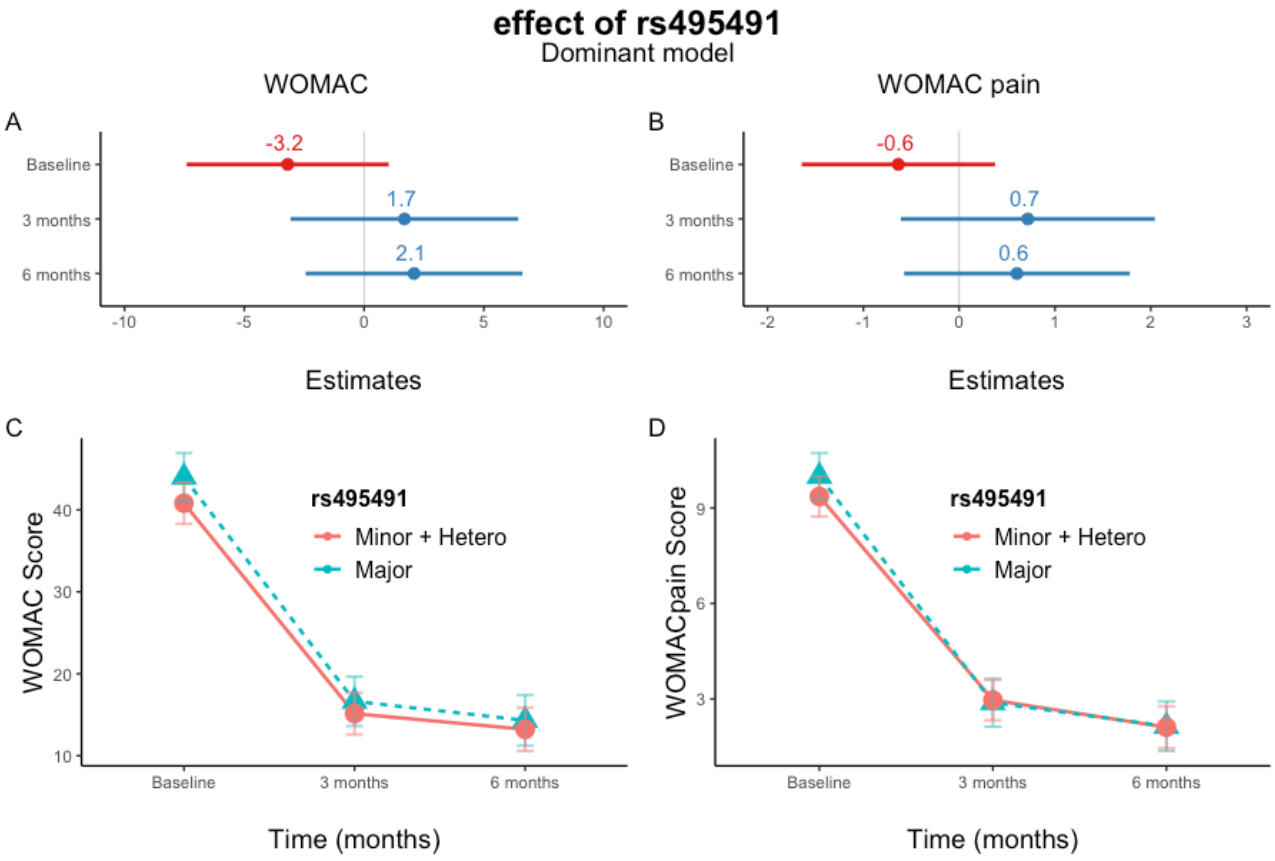

Figure 4-42: Analysis of rs495491 using dominant model. A, B) Forest plot of estimates for WOMAC®, WOMAC® pain with robust confidence intervals C, D) predicted values for WOMAC®, WOMAC® pain as per above model

		rs495491 minor allele G as recessive allele		
	Characteristic	**AA | AG [1]**	**GG[1]**	**p-value[2]**
3 months	n	153	21	
	WOMAC®	13 [8 – 22]	14 [4 – 29]	0.89
	WOMAC® Pain	2.0 [0.0 – 4.0]	2.0 [1.0 – 4.0]	0.73
	% Chronic Pain	29 / 155 (19%)	5 / 21 (24%)	0.56
	S-LANSS ≥ 12	32 / 157 (20%)	4 / 21 (19%)	>0.99
6 months	n	140	20	
	WOMAC®	10 [5 – 18]	14 [7 – 20]	0.57
	WOMAC® Pain	1.00 [0.00 – 3.00]	2.00 [0.00 – 3.00]	0.39
	% Chronic Pain	15 / 143 (10%)	3 / 20 (15%)	0.47
	S-LANSS ≥ 12	36 / 143 (25%)	3 / 20 (15%)	0.41

[1]median [IQR] for continuous variables; n / N (%) categorical variables

[2]Fisher's exact test; Wilcoxon rank sum test

Table 4-94: Pain scores at three and six months between patients with and without the A allele (major allele) of rs495491. Recessive analysis: assuming rs495491 has a recessive effect.

rs495491	WOMAC			WOMAC Pain		
Predictors	**Estimates**	**CI**	**p**	**Estimates**	**CI**	**p**
GG	3.02	-4.15 – 10.20	0.41	0.15	-1.99 – 2.29	0.89
GG at 3 m	-3.16	-10.96 – 4.65	0.43	-0.07	-2.55 – 2.40	0.95
GG at 6 m	-3.64	-9.23 – 1.95	0.20	0.07	-1.78 – 1.92	0.94
Random Effects						
σ^2	96.20			7.47		
ICC	0.45			0.31		
Marginal R^2 / Conditional R^2	0.496 / 0.723			0.517 / 0.666		

Table 4-95: Linear Mixed Model analysis for rs495491 using recessive model. CI: confidence intervals, calculated using robust analysis of standard errors.

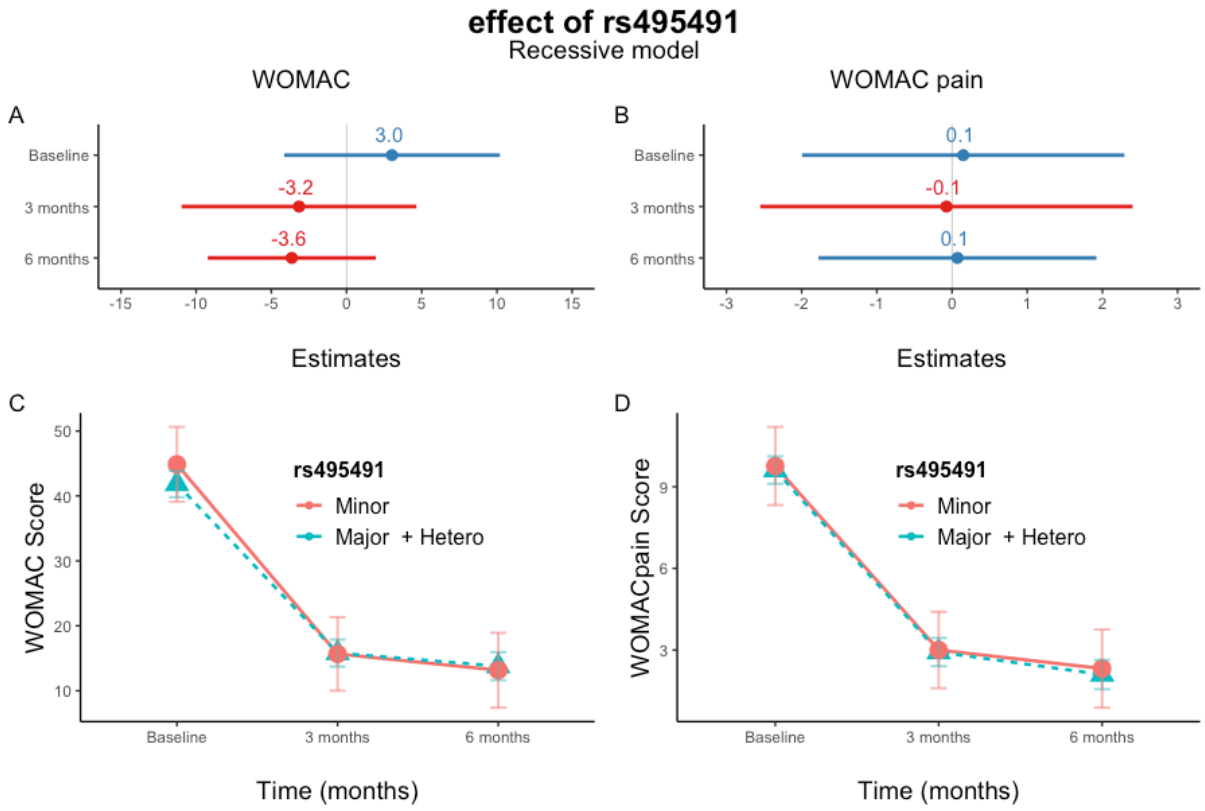

Figure 4-43: Analysis of rs495491 using recessive model. A, B) Forest plot of estimates for WOMAC®, WOMAC® pain with robust confidence intervals C, D) predicted values for WOMAC®, WOMAC® pain as per above model

rs533586

There was no influence of rs495491 on any of the investigated outcomes, both using a dominant and a recessive approach. This is shown in Table 4-96 to Table 4-99, and in Figure 4-44 to Figure 4-45.

		rs533586 minor allele C as dominant allele		
	Characteristic	**TC | CC [1]**	**TT[1]**	**p-value[2]**
3 months	n	113	65	
	WOMAC®	14 [8 – 24]	12 [7 – 22]	0.50
	WOMAC® Pain	2.0 [0.0 – 4.0]	2.0 [1.0 – 3.0]	0.84
	% Chronic Pain	22 / 114 (19%)	11 / 66 (17%)	0.70
	S-LANSS ≥ 12	23 / 115 (20%)	12 / 67 (18%)	0.85
6 months	n	102	61	
	WOMAC®	10 [4 – 19]	11 [7 – 20]	0.24
	WOMAC® Pain	1.00 [0.00 – 3.00]	2.00 [0.00 – 3.50]	0.61
	% Chronic Pain	10 / 103 (9.7%)	7 / 63 (11%)	0.80
	S-LANSS ≥ 12	25 / 103 (24%)	13 / 63 (21%)	0.70

[1]median [IQR] for continuous variables; n / N (%) categorical variables

[2]Fisher's exact test; Wilcoxon rank sum test

Table 4-96: Pain scores at three and six months between patients with and without the C allele (minor allele) of rs533586. Dominant analysis: assuming rs533586 has a dominant effect.

rs533586	WOMAC*			WOMAC Pain		
Predictors	**Estimates**	**CI**	**p**	**Estimates**	**CI**	**p**
TT	-2.52	-6.87 – 1.82	0.25	-0.74	-1.82 – 0.34	0.18
TT at 3 m	3.85	-1.04 – 8.74	0.12	0.84	-0.53 – 2.22	0.23
TT at 6 m	0.92	-3.78 – 5.62	0.70	0.36	-0.85 – 1.58	0.56
Random Effects						
σ^2	93.59			7.43		
ICC	0.45			0.30		
Marginal R^2 / Conditional R^2	0.506 / 0.727			0.527 / 0.667		

Table 4-97: Linear Mixed Model analysis for rs533586 using dominant model. CI: confidence intervals, calculated using robust analysis of standard errors.

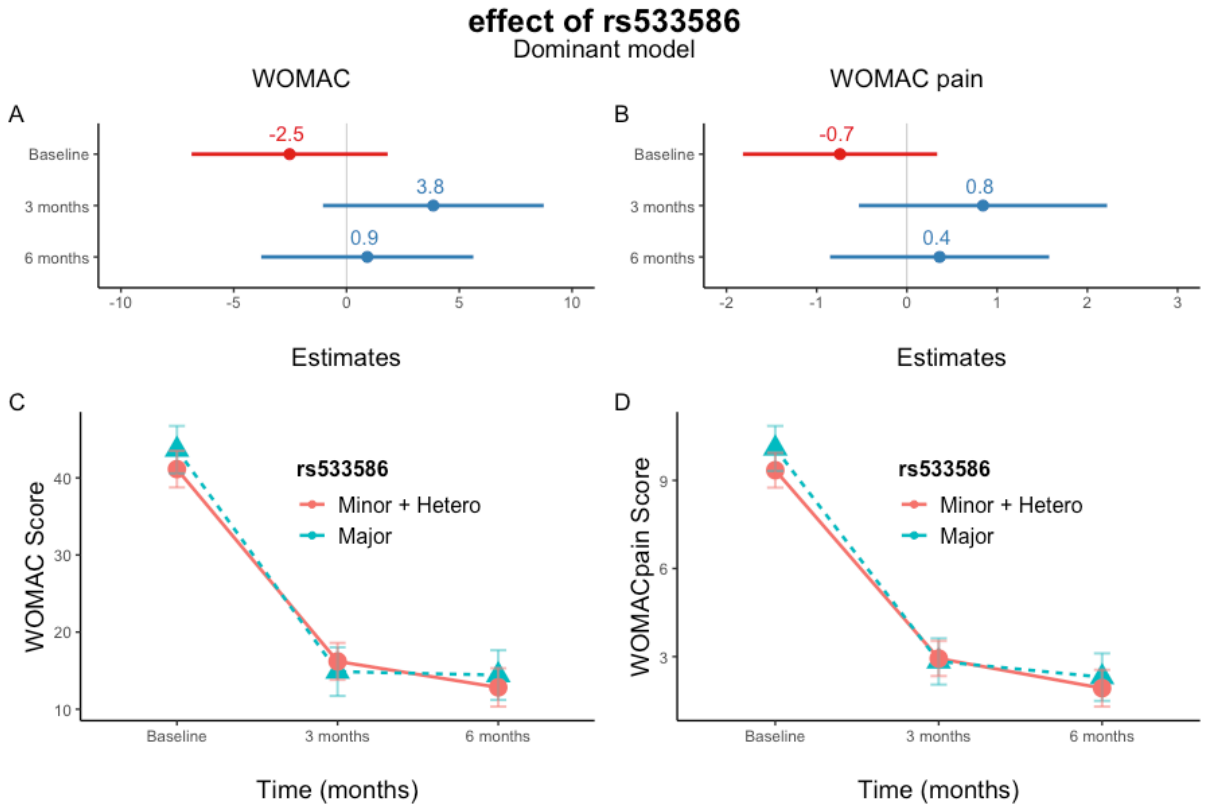

Figure 4-44: Analysis of rs533586 using dominant model. A, B) Forest plot of estimates for WOMAC®, WOMAC® pain with robust confidence intervals C, D) predicted values for WOMAC®, WOMAC® pain as per above model

		rs533586 minor allele C as recessive allele		
	Characteristic	**TT | TC [1]**	**CC[1]**	**p-value[2]**
3 months	n	150	28	
	WOMAC®	13 [8 – 22]	13 [4 – 25]	0.79
	WOMAC® Pain	2.0 [1.0 – 4.0]	2.5 [0.0 – 4.2]	0.98
	% Chronic Pain	27 / 152 (18%)	6 / 28 (21%)	0.60
	S-LANSS ≥ 12	28 / 154 (18%)	7 / 28 (25%)	0.44
6 months	n	139	24	
	WOMAC®	10 [6 – 19]	12 [3 – 23]	0.68
	WOMAC® Pain	1.00 [0.00 – 3.00]	1.00 [0.00 – 3.00]	0.68
	% Chronic Pain	14 / 142 (9.9%)	3 / 24 (12%)	0.72
	S-LANSS ≥ 12	33 / 142 (23%)	5 / 24 (21%)	>0.99

[1]median [IQR] for continuous variables; n / N (%) categorical variables

[2]Fisher's exact test; Wilcoxon rank sum test

Table 4-98: Pain scores at three and six months between patients with and without the T allele (major allele) of rs533586. Recessive analysis: assuming rs533586 has a recessive effect.

rs533586	WOMAC*			WOMAC Pain		
Predictors	**Estimates**	**CI**	**p**	**Estimates**	**CI**	**p**
TT	0.16	-6.18 – 6.49	0.96	0.41	-1.03 – 1.84	0.58
TT at 3 m	0.34	-7.08 – 7.76	0.93	0.10	-2.02 – 2.22	0.93
TT at 6 m	0.19	-6.07 – 6.46	0.95	-0.07	-1.95 – 1.81	0.94
Random Effects						
σ^2	94.48			7.47		
ICC	0.45			0.29		
Marginal R^2 / Conditional R^2	0.503 / 0.725			0.525 / 0.665		

Table 4-99: Linear Mixed Model analysis for rs533586 using recessive model. CI: confidence intervals, calculated using robust analysis of standard errors.

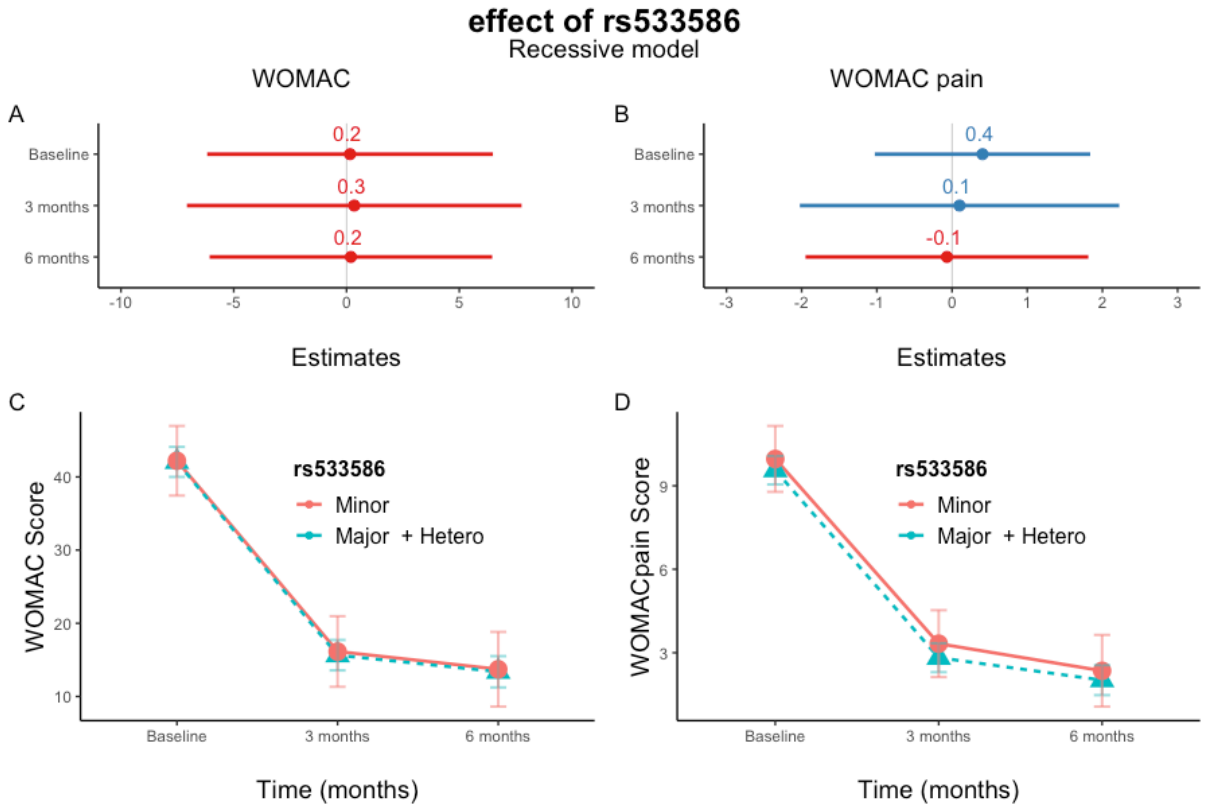

Figure 4-45: Analysis of rs533586 using recessive model. A, B) Forest plot of estimates for WOMAC®, WOMAC® pain with robust confidence intervals C, D) predicted values for WOMAC®, WOMAC® pain as per above model

rs609148

Patients with the minor allele A of rs609148 showed lower baseline WOMAC® pain scores. There was no other difference in any of the other investigated outcomes, both using a dominant and a recessive approach. This is shown in Table 4-96 to Table 4-103, and in Figure 4-46 to Figure 4-47.

		rs609148 minor allele A as dominant allele		
	Characteristic	**GA | AA** [1]	**GG**[1]	**p-value**[2]
3 months	n	83	98	
	WOMAC®	14 [7 – 22]	13 [8 – 22]	0.90
	WOMAC® Pain	2.00 [0.00 – 4.00]	2.00 [1.00 – 3.00]	0.68
	% Chronic Pain	16 / 84 (19%)	16 / 99 (16%)	0.70
	S-LANSS ≥ 12	15 / 85 (18%)	20 / 100 (20%)	0.71
6 months	n	76	90	
	WOMAC®	10 [4 – 22]	10 [6 – 18]	0.57
	WOMAC® Pain	1.00 [0.00 – 3.00]	1.00 [0.00 – 3.00]	0.56
	% Chronic Pain	9 / 77 (12%)	8 / 92 (8.7%)	0.61
	S-LANSS ≥ 12	21 / 77 (27%)	17 / 92 (18%)	0.20

[1]median [IQR] for continuous variables; n / N (%) categorical variables

[2]Fisher's exact test; Wilcoxon rank sum test

Table 4-100: Pain scores at three and six months between patients with and without the A allele (minor allele) of rs609148. Dominant analysis: assuming rs609148 has a dominant effect.

rs609148	WOMAC			WOMAC Pain*		
Predictors	**Estimates**	**CI**	**p**	**Estimates**	**CI**	**p**
GA | AA	-3.07	-7.20 – 1.06	0.14	-1.08	-2.08 – -0.08	***0.034***
GA | AA at 3 m	3.86	-0.80 – 8.52	0.11	1.15	-0.17 – 2.46	0.088
GA | AA at 6 m	3.64	-0.66 – 7.94	0.10	1.24	0.09 – 2.38	***0.035***
Random Effects						
σ^2	92.91			7.24		
ICC	0.45			0.30		
Marginal R^2 / Conditional R^2	0.505 / 0.730			0.535 / 0.675		
				*LRT p-value: 0.08		

Table 4-101: Linear Mixed Model analysis for rs609148 using dominant model. CI: confidence intervals, calculated using robust analysis of standard errors.

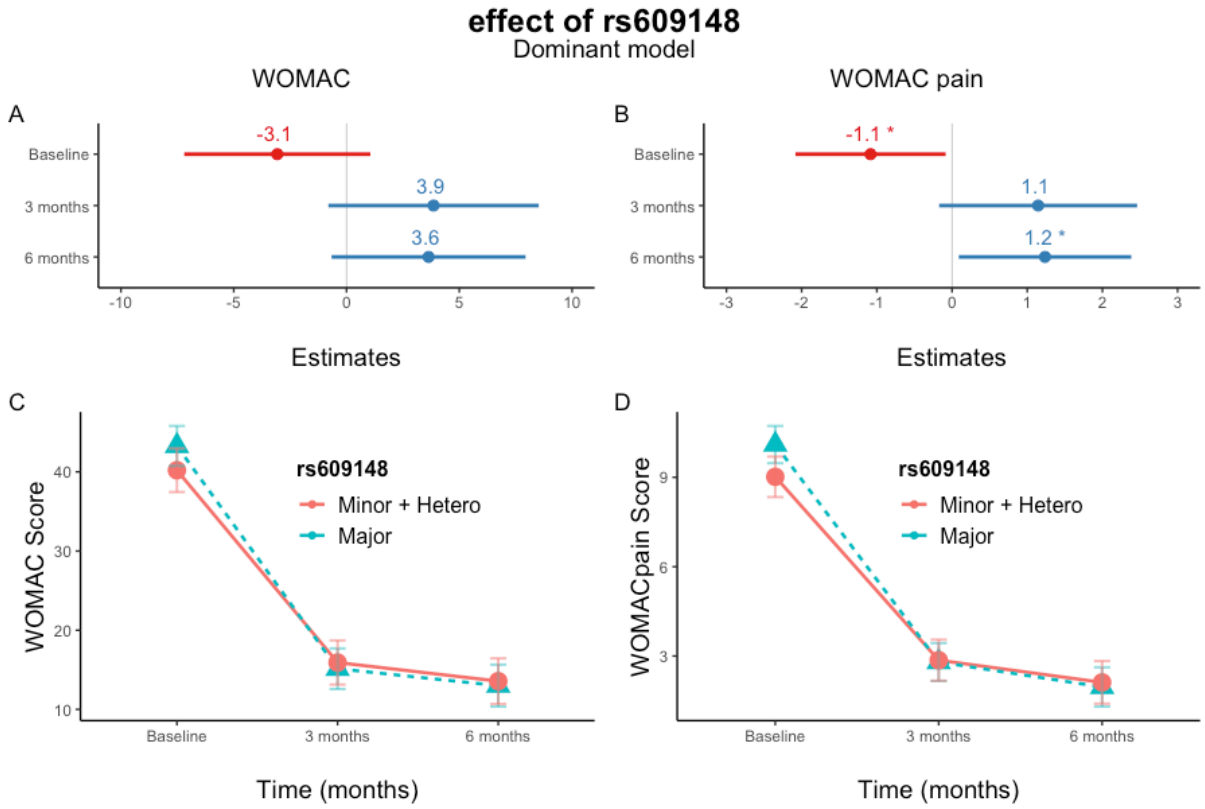

Figure 4-46: Analysis of rs609148 using dominant model. A, B) Forest plot of estimates for WOMAC®, WOMAC® pain with robust confidence intervals C, D) predicted values for WOMAC®, WOMAC® pain as per above model

		rs609148 minor allele A as recessive allele		
	Characteristic	**GG | GA [1]**	**AA[1]**	**p-value[2]**
3 months	n	168	13	
	WOMAC®	13 [8 – 23]	13 [4 – 22]	0.69
	WOMAC® Pain	2.00 [1.00 – 4.00]	0.00 [0.00 – 4.00]	0.50
	% Chronic Pain	30 / 170 (18%)	2 / 13 (15%)	>0.99
	S-LANSS ≥ 12	31 / 172 (18%)	4 / 13 (31%)	0.27
6 months	n	156	10	
	WOMAC®	10 [6 – 19]	6 [2 – 12]	0.090
	WOMAC® Pain	1.00 [0.00 – 3.00]	0.50 [0.00 – 1.00]	0.27
	% Chronic Pain	17 / 159 (11%)	0 / 10 (0%)	0.60
	S-LANSS ≥ 12	38 / 159 (24%)	0 / 10 (0%)	0.12

[1]median [IQR] for continuous variables; n / N (%) categorical variables

[2]Fisher's exact test; Wilcoxon rank sum test

Table 4-102: Pain scores at three and six months between patients with and without the G allele (major allele) of rs609148. Recessive analysis: assuming rs609148 has a recessive effect.

rs609148	WOMAC*			WOMAC Pain		
Predictors	**Estimates**	**CI**	**p**	**Estimates**	**CI**	**p**
TT	0.43	-7.22 – 8.09	0.91	1.01	-0.56 – 2.57	0.21
TT at 3 m	-0.59	-11.16 – 9.97	0.91	-1.22	-3.93 – 1.50	0.38
TT at 6 m	-4.37	-11.68 – 2.94	0.24	-1.87	-4.11 – 0.36	0.10
Random Effects						
σ^2	94.11			7.31		
ICC	0.45			0.30		
Marginal R^2 / Conditional R^2	0.504 / 0.726			0.532 / 0.672		

Table 4-103: Linear Mixed Model analysis for rs609148 using recessive model. CI: confidence intervals, calculated using robust analysis of standard errors.

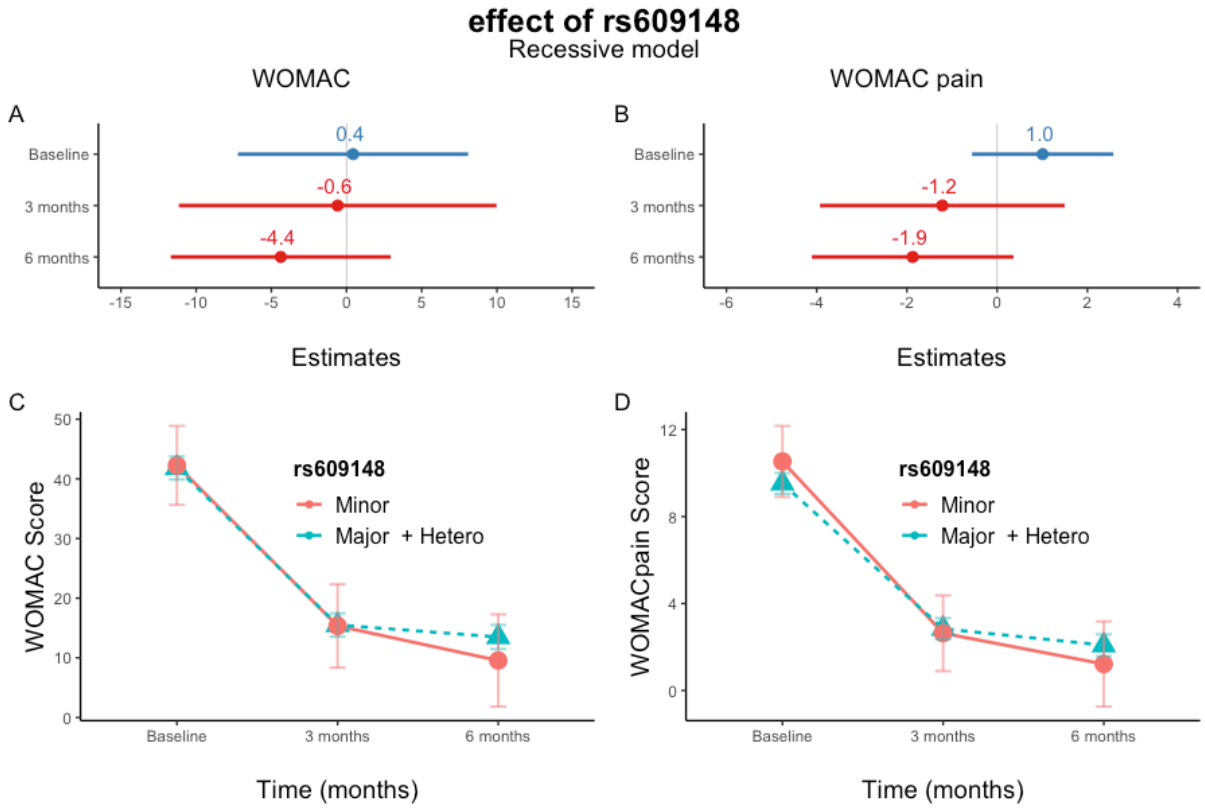

Figure 4-47: Analysis of rs609148 using recessive model. A, B) Forest plot of estimates for WOMAC®, WOMAC® pain with robust confidence intervals C, D) predicted values for WOMAC®, WOMAC® pain as per above model

rs563649

There was no influence of rs563649 on any of the investigated outcomes using a dominant approach. This is shown in Table 4-96 to Table 4-97, and in Figure 4-44.

		rs563649 minor allele T as dominant allele		
	Characteristic	**CT | TT [1]**	**CC[1]**	**p-value[2]**
3 months	n	31	143	
	WOMAC®	13 [7 – 22]	14 [8 – 24]	0.76
	WOMAC® Pain	2.0 [1.0 – 4.0]	2.0 [0.0 – 4.0]	0.63
	% Chronic Pain	6 / 31 (19%)	27 / 145 (19%)	>0.99
	S-LANSS ≥ 12	8 / 32 (25%)	27 / 146 (18%)	0.46
6 months	n	28	131	
	WOMAC®	15 [7 – 20]	10 [5 – 19]	0.13
	WOMAC® Pain	2.00 [0.25 – 4.00]	1.00 [0.00 – 3.00]	0.07
	% Chronic Pain	5 / 30 (17%)	12 / 132 (9.1%)	0.32
	S-LANSS ≥ 12	10 / 30 (33%)	25 / 132 (19%)	0.09

[1]median [IQR] for continuous variables; n / N (%) categorical variables

[2]Fisher's exact test; Wilcoxon rank sum test

Table 4-104: Pain scores at three and six months between patients with and without the T allele (minor allele) of rs563649. Dominant analysis: assuming rs563649 has a dominant effect.

rs563649	WOMAC			WOMAC Pain		
Predictors	**Estimates**	**CI**	**p**	**Estimates**	**CI**	**p**
GA | AA	-3.20	-8.82 – 2.41	0.26	-0.31	-1.67 – 1.06	0.66
GA | AA at 3 m	3.02	-3.54 – 9.59	0.37	0.67	-1.25 – 2.59	0.50
GA | AA at 6 m	5.32	-0.64 – 11.28	0.08	1.35	-0.26 – 2.97	0.10
			Random Effects			
σ^2	93.90			7.45		
ICC	0.46			0.30		
Marginal R^2 / Conditional R^2	0.495 / 0.726			0.526 / 0.666		

Table 4-105: Linear Mixed Model analysis for rs563649 using dominant model. CI: confidence intervals, calculated using robust analysis of standard errors.

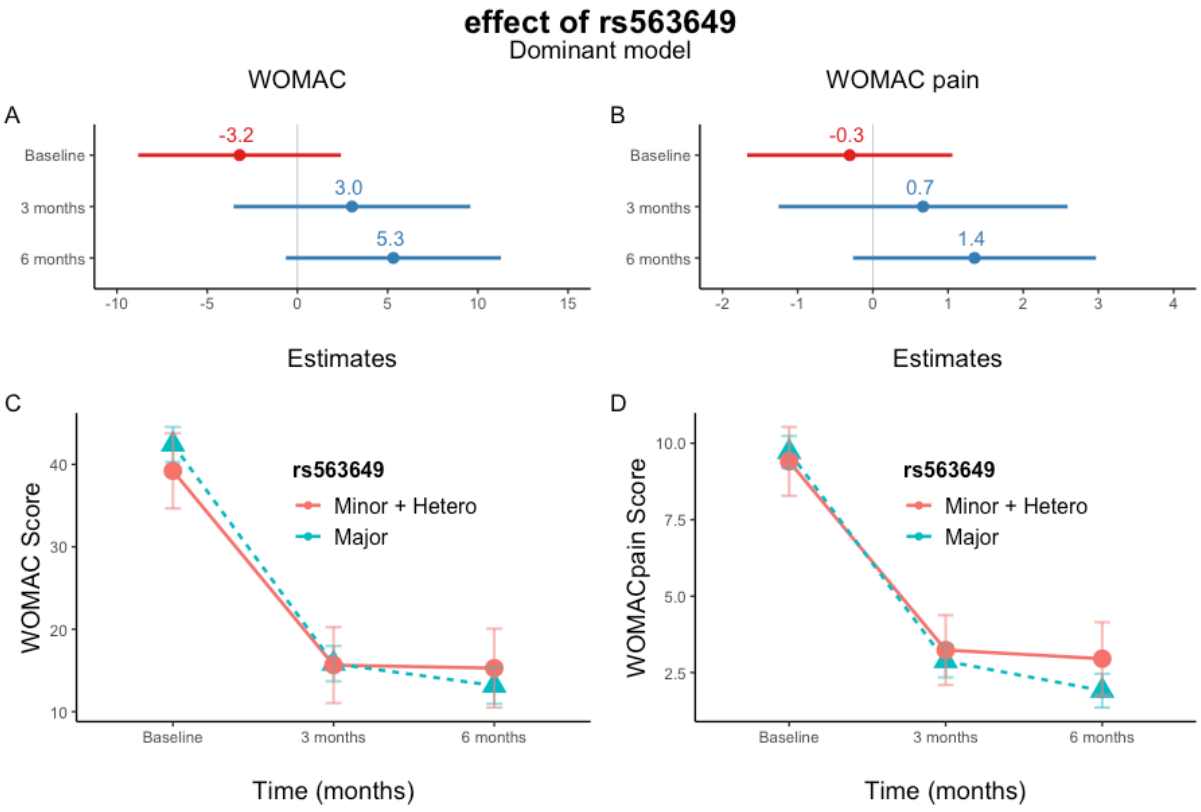

Figure 4-48: Analysis of rs6746030 using dominant model. A, B) Forest plot of estimates for WOMAC®, WOMAC® pain with robust confidence intervals C, D) predicted values for WOMAC®, WOMAC® pain as per above model

Only one patient was homozygous for the rs563649 variant. Hence a recessive approach was not attempted.

4.3.2.5.4 GCH1

rs998259

The role of rs998259 in the *GCH1* gene on the development of pain at three and six months is shown in Table 4-106 to Table 4-109, and Figure 4-49, Figure 4-50. This investigated if the minor allele T had a dominant effect over the major allele C.

There was no effect of this polymorphism on any of the outcomes, both on univariate analysis and using a linear mixed effect model with robust estimations of standard errors.

		rs998259 minor allele T as dominant allele		
	Characteristic	**CT | TT [1]**	**CC[1]**	**p-value[2]**
3 months	n	78	84	
	WOMAC®	14 [6 - 22]	11 [7 - 21]	0.38
	WOMAC® Pain	2 [0.25 - 4]	2 [0 - 3]	0.18
	% Chronic Pain	19 (24%)	11 (13%)	0.07
	S-LANSS ≥ 12	19 (24%)	12 (14%)	0.16
6 months	n	69	76	
	WOMAC®	9 [5 - 19]	10.5 [6 - 18]	0.78
	WOMAC® Pain	1 [0 - 3]	1 [0 - 3]	0.70
	% Chronic Pain	10 (14%)	11 (14%)	1
	S-LANSS ≥ 12	20 (29%)	13 (17%)	0.11

[1]median [IQR] for continuous variables; n / N (%) categorical variables

[2]Fisher's exact test; Wilcoxon rank sum test

Table 4-106: Pain scores at three and six months between patients with and without the T allele (minor allele) of rs998259. Dominant analysis: assuming that rs998259 has a dominant effect.

rs998259	WOMAC			WOMAC Pain		
Predictors	**Estimates**	**CI**	**p**	**Estimates**	**CI**	**p**
CT | TT	2.40	-2.01 – 6.81	0.29	0.70	-0.39 – 1.79	0.21
CT | TT at 3 m	-0.08	-5.16 – 5.00	0.98	0.34	-1.06 – 1.73	0.64
CT | TT at 6 m	-0.53	-5.37 – 4.32	0.83	-0.60	-1.84 – 0.65	0.35
Random Effects						
σ^2	96.77			7.25		
ICC	0.43			0.27		
Marginal R^2 / Conditional R^2	0.513 / 0.720			0.550 / 0.672		

Table 4-107: Linear Mixed Model analysis for rs998259 using dominant model. CI: confidence intervals, calculated using robust analysis of standard errors.

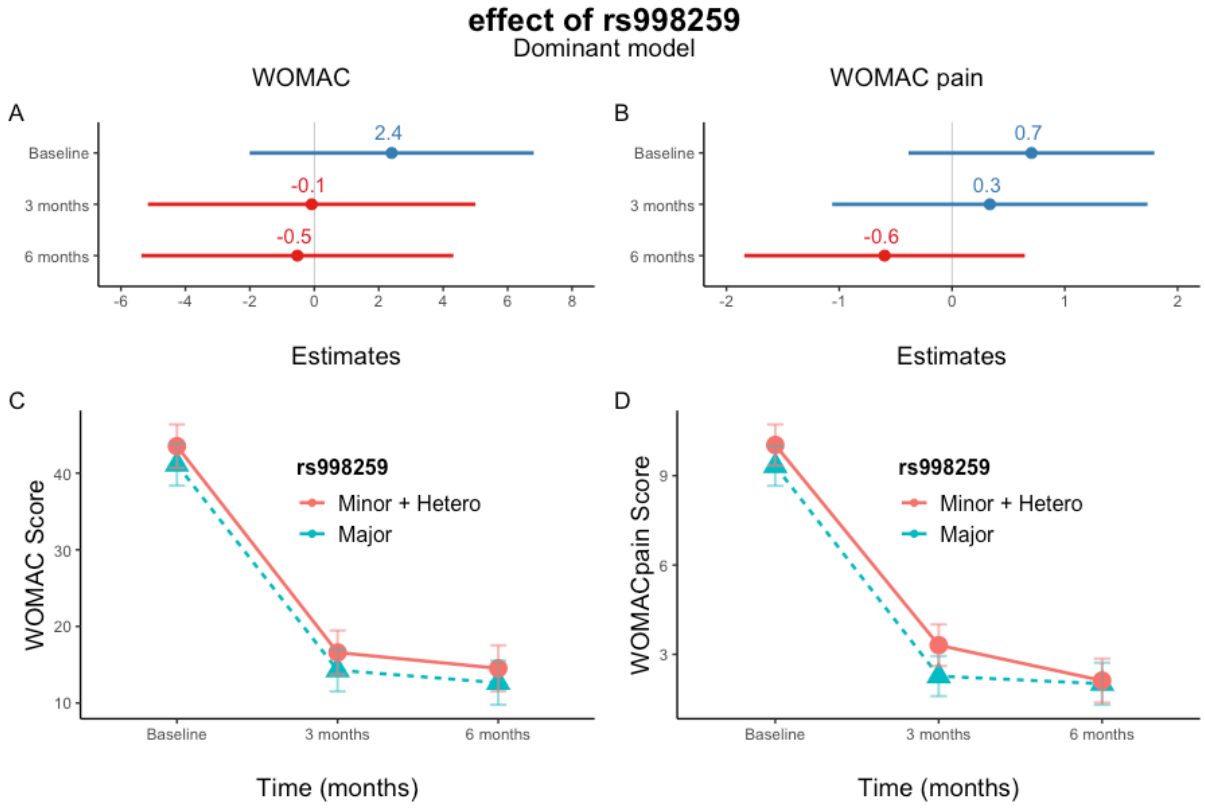

Figure 4-49: Analysis of rs998259 using dominant model. A, B) Forest plot of estimates for WOMAC®, WOMAC® pain with robust confidence intervals C, D) predicted values for WOMAC®, WOMAC® pain as per above model

Using a recessive model, where only homozygous carriers of the minor allele would show a clinical effect, there was no evidence of a possible interaction between rs998259 and outcomes.

		rs998259 minor allele T as recessive allele		
	Characteristic	**CC | CT [1]**	**TT[1]**	**p-value[2]**
3 months	n	145	17	
	WOMAC®	12 [7 - 22]	11 [5 - 19]	0.63
	WOMAC® Pain	2 [0 - 4]	2 [0 - 6]	0.71
	% Chronic Pain	25 (17%)	5 (29%)	0.32
	S-LANSS ≥ 12	28 (19%)	3 (18%)	1
6 months	n	129	16	
	WOMAC®	9 [5 - 18]	13.5 [6 - 16.25]	0.66
	WOMAC® Pain	1 [0 - 3]	1.5 [0.75 - 4.25]	0.28
	% Chronic Pain	17 (13%)	4 (25%)	0.25
	S-LANSS ≥ 12	28 (22%)	5 (31%)	0.54

[1]median [IQR] for continuous variables; n / N (%) categorical variables

[2]Fisher's exact test; Wilcoxon rank sum test

Table 4-108: Pain scores at three and six months between patients with and without the C allele (major allele) of rs998259. Recessive analysis: assuming rs998259 has a recessive effect.

rs998259	WOMAC			WOMAC Pain		
Predictors	**Estimates**	**CI**	**p**	**Estimates**	**CI**	**p**
TT	2.73	-4.66 – 10.13	0.47	0.31	-1.63 – 2.25	0.75
TT at 3 m	-2.59	-14.54 – 9.36	0.67	0.34	-2.82 – 3.49	0.83
TT at 6 m	-0.19	-10.61 – 10.22	0.97	0.10	-2.48 – 2.67	0.94
Random Effects						
σ^2	96.67			7.30		
ICC	0.43			0.27		
Marginal R^2 / Conditional R^2	0.511 / 0.721			0.545 / 0.669		

Table 4-109: Linear Mixed Model analysis for rs998259 using recessive model. CI: confidence intervals, calculated using robust analysis of standard errors.

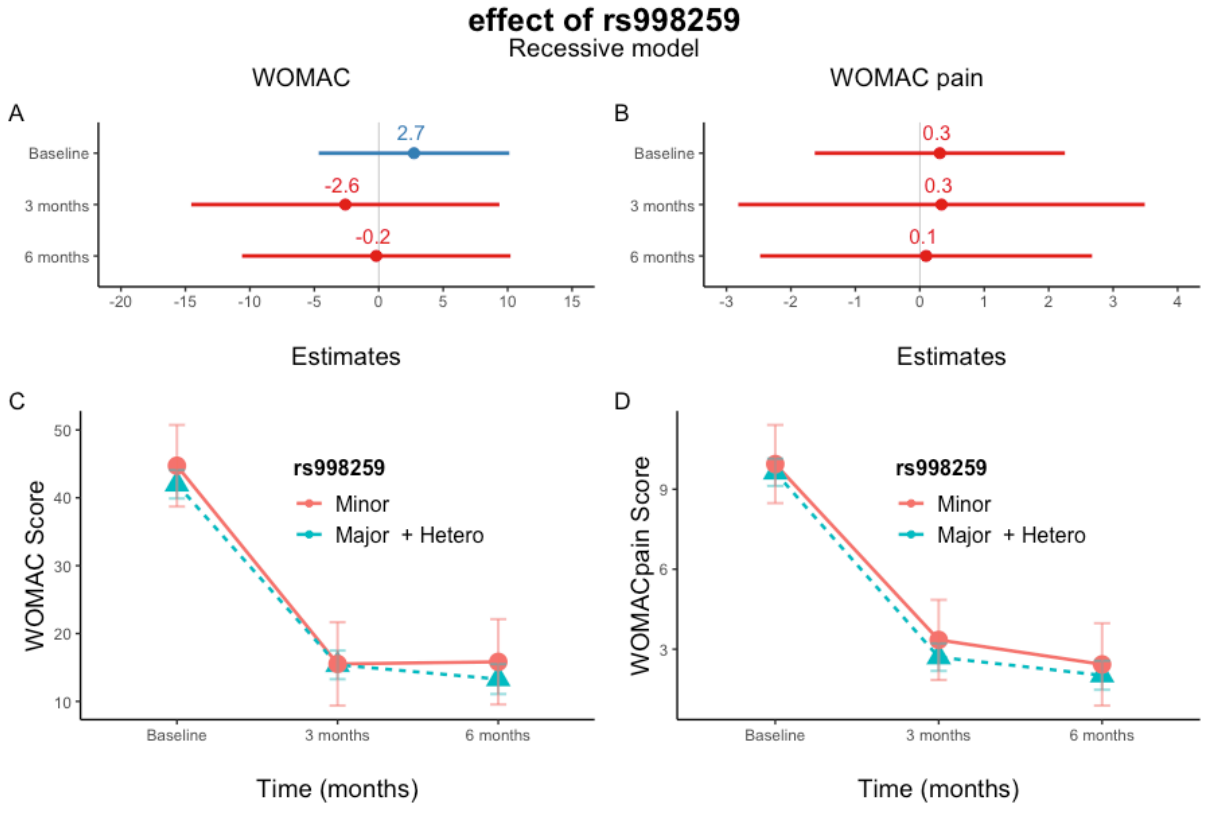

Figure 4-50: Analysis of rs998259 using recessive model. A, B) Forest plot of estimates for WOMAC®, WOMAC® pain with robust confidence intervals C, D) predicted values for WOMAC®, WOMAC® pain as per above model

rs378364

The role of rs378364 in the *GCH1* gene on the development of pain at three and six months is shown in Table 4-110 to Table 4-111, and in Figure 4-51.

Analysis using a dominant showed no effect of this polymorphism on any of the outcomes, both on univariate analysis and using a linear mixed effect model with robust estimations of standard errors.

It should be noted that the incidence of rs378364 was low, so only 4 subjects were homozygous for this polymorphism. Hence analysis using recessive approach was not performed.

		rs378364 minor allele T as dominant allele		
	Characteristic	**TA | TT [1]**	**AA[1]**	**p-value[2]**
3 months	n	43	135	
	WOMAC®	12 [5 - 19]	13 [7 - 21.5]	0.54
	WOMAC® Pain	1 [1 - 3.5]	2 [0 - 4]	0.76
	% Chronic Pain	9 (21%)	25 (19%)	0.82
	S-LANSS ≥ 12	8 (19%)	26 (19%)	1
6 months	n	40	123	
	WOMAC®	9 [4.75 - 18]	10 [5 - 18]	0.78
	WOMAC® Pain	1 [0 - 3]	1 [0 - 3]	0.71
	% Chronic Pain	8 (20%)	15 (12%)	0.29
	S-LANSS ≥ 12	12 (30%)	26 (21%)	0.29

[1]median [IQR] for continuous variables; n / N (%) categorical variables

[2]Fisher's exact test; Wilcoxon rank sum test

Table 4-110: Pain scores at three and six months between patients with and without the A allele (minor allele) of rs378364. Dominant analysis: assuming that rs378364 has a dominant effect.

rs3783641	WOMAC			WOMAC Pain		
Predictors	**Estimates**	**CI**	**p**	**Estimates**	**CI**	**p**
TA | TT	-1.91	-6.98 – 3.17	0.46	-0.08	-1.34 – 1.17	0.89
TA | TT at 3 m	1.09	-4.16 – 6.34	0.68	0.20	-1.27 – 1.68	0.79
TA | TT at 6 m	0.30	-4.36 – 4.96	0.90	0.42	-0.99 – 1.84	0.56
Random Effects						
σ^2	95.01			7.38		
ICC	0.44			0.30		
Marginal R^2 / Conditional R^2	0.500 / 0.721			0.528 / 0.670		

Table 4-111: Linear Mixed Model analysis for rs378364 using dominant model. CI: confidence intervals, calculated using robust analysis of standard errors.

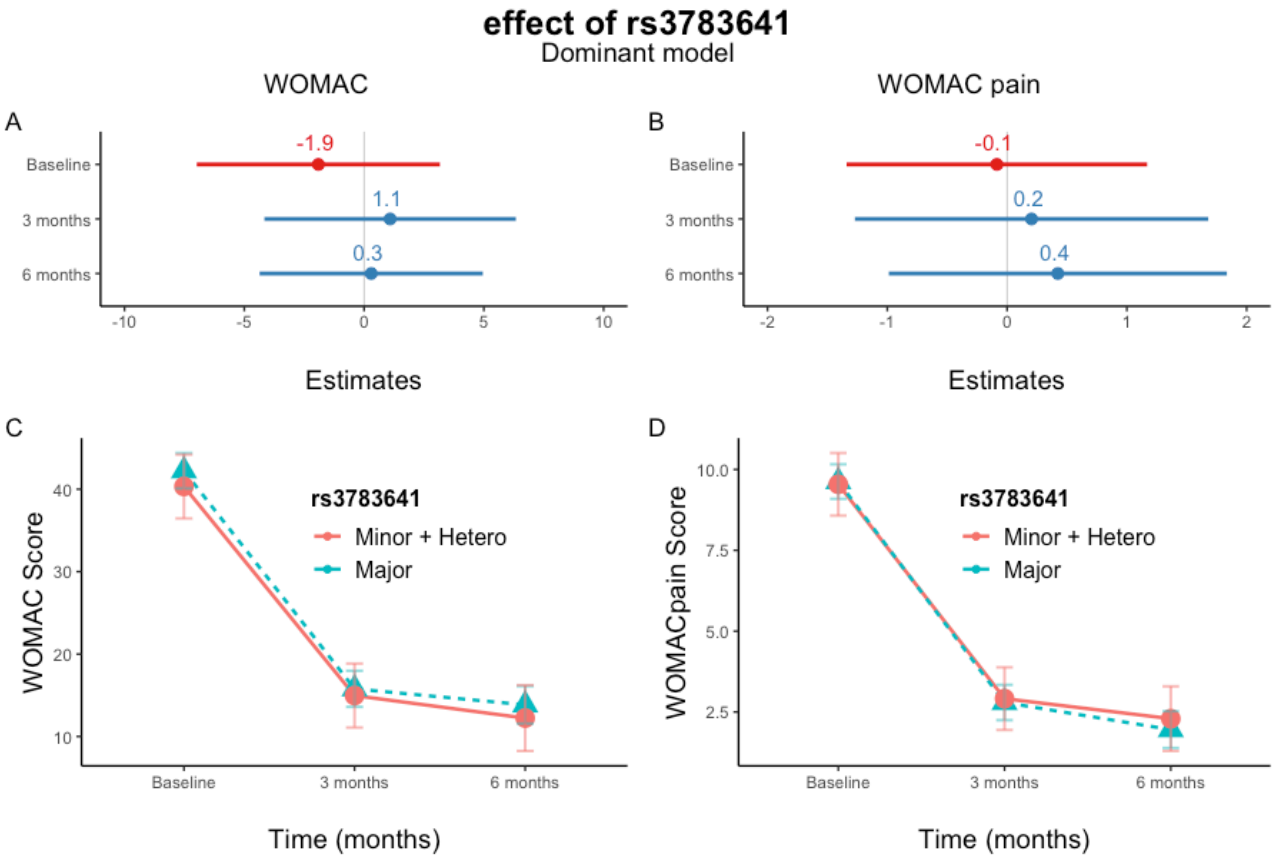

Figure 4-51: Analysis of rs378364 using dominant model. A, B) Forest plot of estimates for WOMAC®, WOMAC® pain with robust confidence intervals C, D) predicted values for WOMAC®, WOMAC® pain as per above model

4.3.2.5.5 KCNS1

rs4499491

The role of rs4499491 in the *KCNS1* gene on the development of pain at three and six months is shown in Table 4-112 to Table 4-115, and Figure 4-52 to Figure 4-53.

Analysis using a dominant or a recessive approach showed no effect of this polymorphism on any of the outcomes, both on univariate analysis and using a linear mixed effect model with robust estimations of standard errors.

		rs4499491 minor allele A as dominant allele		
	Characteristic	**CA | AA [1]**	**CC[1]**	**p-value[2]**
3 months	n	109	71	
	WOMAC®	13 [8 - 21]	12 [5 - 21]	0.41
	WOMAC® Pain	2 [1 - 4]	2 [0 - 4]	0.45
	% Chronic Pain	21 (19%)	10 (14%)	0.42
	S-LANSS ≥ 12	25 (22%)	10 (14%)	0.18
6 months	n	100	66	
	WOMAC®	10.5 [5 - 18]	9 [5 - 17]	0.55
	WOMAC® Pain	1 [0 - 3]	1 [0 - 2]	0.37
	% Chronic Pain	14 (14%)	8 (12%)	0.82
	S-LANSS ≥ 12	23 (23%)	15 (23%)	1

[1]median [IQR] for continuous variables; n / N (%) categorical variables

[2]Fisher's exact test; Wilcoxon rank sum test

Table 4-112: Pain scores at three and six months between patients with and without the A allele (minor allele) of rs4499491. Dominant analysis: assuming rs4499491 has a dominant effect.

rs4499491	WOMAC			WOMAC Pain		
Predictors	**Estimates**	**CI**	**p**	**Estimates**	**CI**	**p**
CA | AA	-1.58	-5.72 – 2.55	0.45	-0.47	-1.49 – 0.55	0.367
CA | AA at 3 m	1.91	-3.07 – 6.90	0.45	0.67	-0.67 – 2.02	0.326
CA | AA at 6 m	2.07	-2.53 – 6.66	0.38	0.81	-0.32 – 1.95	0.161
Random Effects						
σ^2	92.35			7.24		
ICC	0.46			0.30		
Marginal R^2 / Conditional R^2	0.497 / 0.728			0.529 / 0.671		

Table 4-113: Linear Mixed Model analysis for rs4499491 using dominant model. CI: confidence intervals, calculated using robust analysis of standard errors.

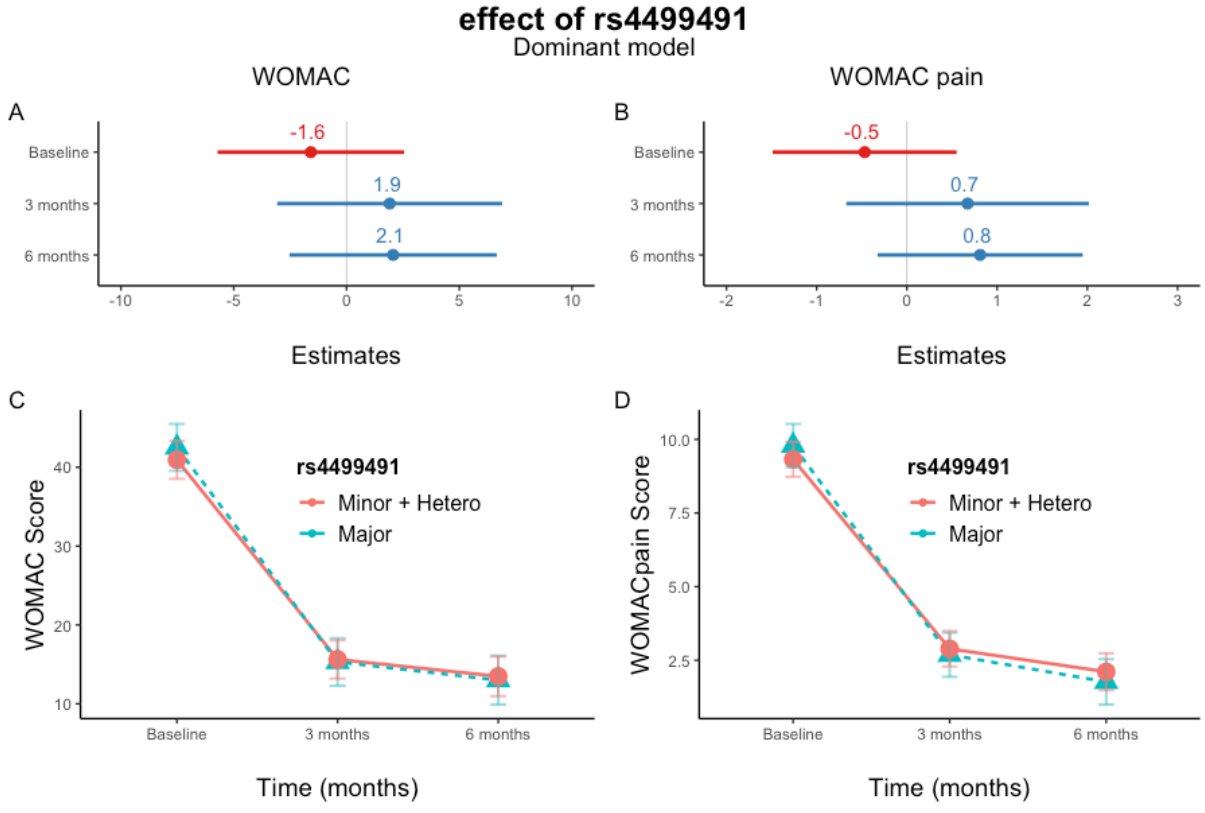

Figure 4-52: Analysis of rs4499491 using dominant model. A, B) Forest plot of estimates for WOMAC®, WOMAC® pain with robust confidence intervals C, D) predicted values for WOMAC®, WOMAC® pain as per above model

		rs4499491 minor allele A as recessive allele		
	Characteristic	**CC | CA [1]**	**AA[1]**	**p-value[2]**
3 months	n	166	14	
	WOMAC®	13 [7 - 21]	9.5 [4 - 17.75]	0.20
	WOMAC® Pain	2 [1 - 4]	1 [0 - 3.75]	0.34
	% Chronic Pain	29 (17%)	2 (14%)	1
	S-LANSS ≥ 12	33 (19%)	2 (13%)	0.74
6 months	n	152	14	
	WOMAC®	9 [5 - 17]	15 [4.25 - 22.5]	0.62
	WOMAC® Pain	1 [0 - 2.25]	1.5 [0 - 4]	0.60
	% Chronic Pain	14 (9%)	3 (21%)	0.16
	S-LANSS ≥ 12	35 (23%)	3 (21%)	1

[1]median [IQR] for continuous variables; n / N (%) categorical variables

[2]Fisher's exact test; Wilcoxon rank sum test

Table 4-114: Pain scores at three and six months between patients with and without the C allele (major allele) of rs4499491. Recessive analysis: assuming rs4499491 has a recessive effect.

rs4499491	WOMAC			WOMAC Pain		
Predictors	**Estimates**	**CI**	**p**	**Estimates**	**CI**	**p**
AA	-4.24	-12.09 – 3.62	0.29	-1.24	-3.07 – 0.59	0.18
AA at 3 m	0.67	-5.61 – 6.96	0.83	0.97	-1.34 – 3.28	0.41
AA at 6 m	7.18	-0.79 – 15.15	0.08	1.93	-0.20 – 4.07	0.08
Random Effects						
σ^2	91.26			7.20		
ICC	0.47			0.30		
Marginal R^2 / Conditional R^2	0.498 / 0.732			0.530 / 0.673		

Table 4-115: Linear Mixed Model analysis for rs4499491 using recessive model. CI: confidence intervals, calculated using robust analysis of standard errors.

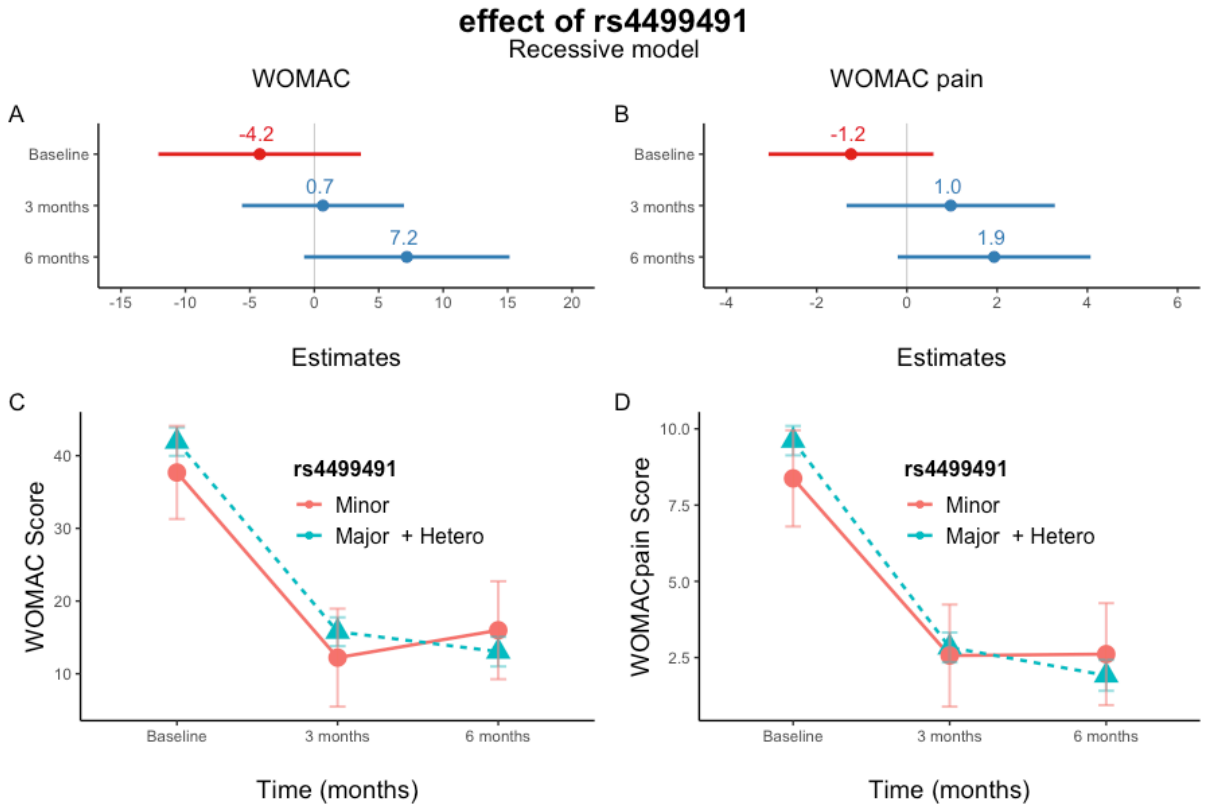

Figure 4-53: Analysis of r rs4499491 using recessive model. A, B) Forest plot of estimates for WOMAC®, WOMAC® pain with robust confidence intervals C, D) predicted values for WOMAC®, WOMAC® pain as per above model

rs734784

The role of rs734784 in the *KCNS1* gene on the development of pain at three and six months is shown Table 4-116 to Table 4-119, and in Figure 4-54, Figure 4-55.

Analysis using a dominant or a recessive approach showed no effect of this polymorphism on any of the outcomes, both on univariate analysis and using a linear mixed effect model with robust estimations of standard errors.

		rs734784 minor allele C as dominant allele		
	Characteristic	**TC | CC [1]**	**TT[1]**	**p-value[2]**
3 months	n	119	52	
	WOMAC®	13 [6.5 - 21]	13.5 [8 - 26]	0.30
	WOMAC® Pain	2 [0 - 4]	2 [1 - 6]	0.38
	% Chronic Pain	18 (15%)	14 (27%)	0.09
	S-LANSS ≥ 12	18 (15%)	14 (27%)	0.15
6 months	n	112	47	
	WOMAC®	9 [5 - 17.25]	12 [5 - 22.5]	0.22
	WOMAC® Pain	1 [0 - 3]	1 [0 - 3]	0.89
	% Chronic Pain	10 (9%)	7 (15%)	0.27
	S-LANSS ≥ 12	22 (19%)	16 (33%)	0.07

[1]median [IQR] for continuous variables; n / N (%) categorical variables

[2]Fisher's exact test; Wilcoxon rank sum test

Table 4-116: Pain scores at three and six months between patients with and without the C allele (minor allele) of rs734784. Dominant analysis: assuming rs734784 has a dominant effect.

rs734784	WOMAC			WOMAC Pain		
Predictors	**Estimates**	**CI**	**p**	**Estimates**	**CI**	**p**
TC | CC	-4.47	-9.31 – 0.37	0.07	-0.96	-2.19 – 0.27	0.13
TC | CC at 3 m	1.48	-3.84 – 6.80	0.59	0.43	-0.98 – 1.84	0.55
TC | CC at 6 m	1.41	-3.55 – 6.37	0.58	0.74	-0.56 – 2.04	0.27
Random Effects						
σ^2	86.40			6.91		
ICC	0.44			0.33		
Marginal R^2 / Conditional R^2	0.520 / 0.743			0.530 / 0.684		

Table 4-117: Linear Mixed Model analysis for rs734784 using dominant model. CI: confidence intervals, calculated using robust analysis of standard errors.

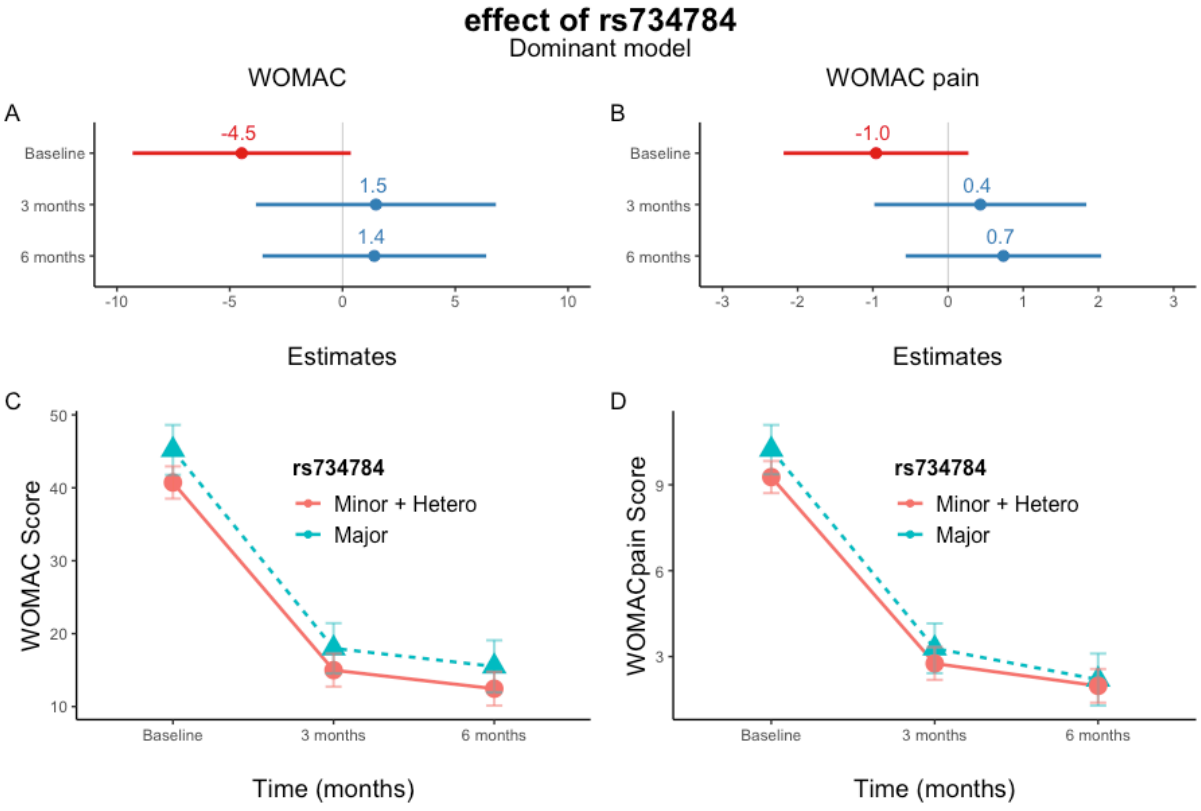

Figure 4-54: Analysis of rs734784 using dominant model. A, B) Forest plot of estimates for WOMAC®, WOMAC® pain with robust confidence intervals C, D) predicted values for WOMAC®, WOMAC® pain as per above model

		rs734784 minor allele C as recessive allele		
	Characteristic	**TT | TC [1]**	**CC[1]**	**p-value[2]**
3 months	n	131	40	
	WOMAC®	13 [7.5 - 23]	14 [5 - 21]	0.46
	WOMAC® Pain	2 [0.5 - 4]	2 [0 - 3.25]	0.34
	% Chronic Pain	27 (21%)	6 (15%)	0.35
	S-LANSS ≥ 12	27 (21%)	8 (20%)	1
6 months	n	122	37	
	WOMAC®	11 [5 - 18]	9 [3 - 14]	0.068
	WOMAC® Pain	1 [0 - 3]	1 [0 - 2]	0.27
	% Chronic Pain	16 (13%)	1 (3%)	0.12
	S-LANSS ≥ 12	33 (27%)	5 (14%)	0.12

[1]median [IQR] for continuous variables; n / N (%) categorical variables

[2]Fisher's exact test; Wilcoxon rank sum test

Table 4-118: Pain scores at three and six months between patients with and without the T allele (major allele) of rs734784. Recessive analysis: assuming rs734784 has a recessive effect.

rs734784	WOMAC			WOMAC Pain		
Predictors	**Estimates**	**CI**	**p**	**Estimates**	**CI**	**p**
CC	-3.99	-8.41 – 0.42	0.08	-0.62	-1.88 – 0.65	0.34
CC at 3 m	1.02	-3.99 – 6.03	0.69	-0.16	-1.72 – 1.39	0.84
CC at 6 m	-0.71	-5.18 – 3.77	0.76	-0.18	-1.59 – 1.22	0.80
Random Effects						
σ^2	86.46			6.94		
ICC	0.46			0.32		
Marginal R^2 / Conditional R^2	0.520 / 0.743			0.531 / 0.682		

Table 4-119: Linear Mixed Model analysis for rs734784 using recessive model. CI: confidence intervals, calculated using robust analysis of standard errors.

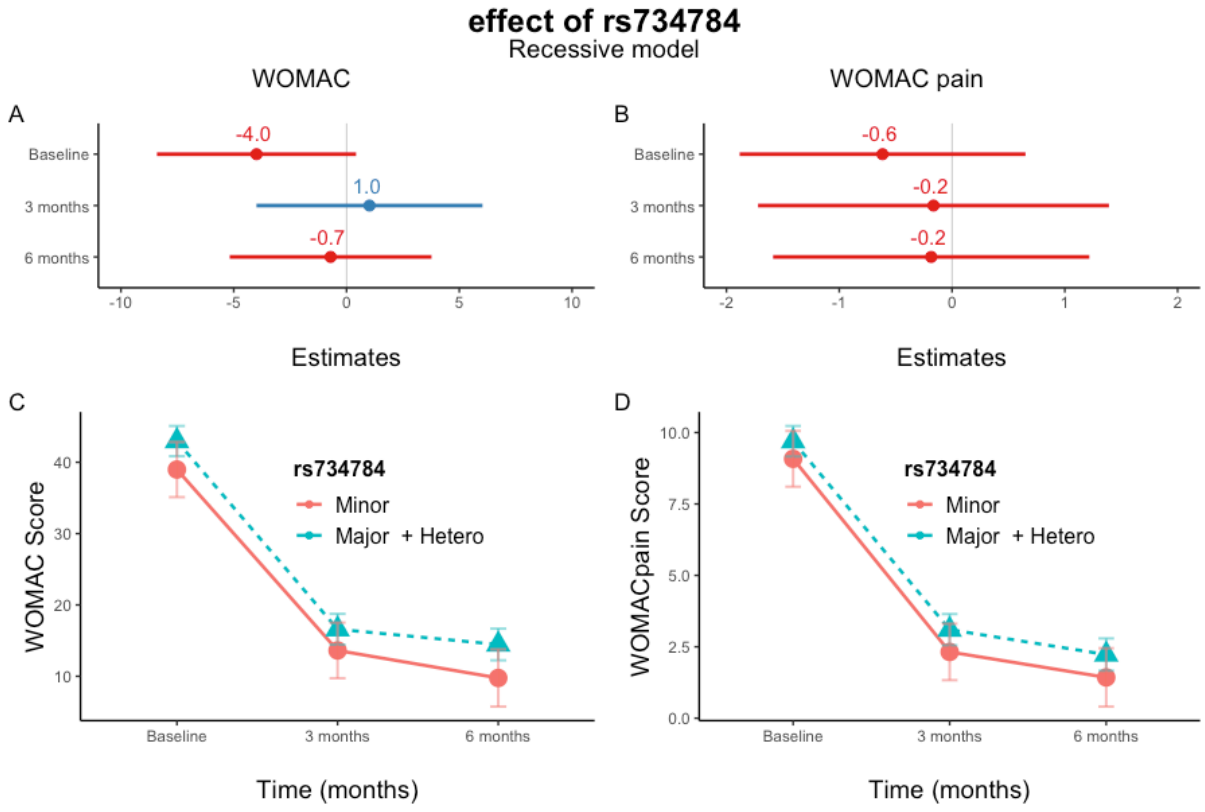

Figure 4-55: Analysis of rs734784 using recessive model. A, B) Forest plot of estimates for WOMAC®, WOMAC® pain with robust confidence intervals C, D) predicted values for WOMAC®, WOMAC® pain as per above model.

4.3.2.5.6 OPRK1

rs6985606

The impact of rs6985606 in the *OPRK1* gene on the development of pain at three and six months is shown in Table 4-120 to Table 4-123, and in Figure 4-56 to Figure 4-57.

Analysis using a dominant or a recessive approach showed no effect of this polymorphism on any of the outcomes, both on univariate analysis and using a linear mixed effect model with robust estimations of standard errors.

		rs6985606 minor allele C as dominant allele		
	Characteristic	**TC | CC [1]**	**TT[1]**	**p-value[2]**
3 months	n	114	46	
	WOMAC®	14 [7 - 23]	13 [5 - 18]	0.25
	WOMAC® Pain	2 [0 - 4]	2 [0.25 - 4]	0.62
	% Chronic Pain	26 (23%)	7 (15%)	0.38
	S-LANSS ≥ 12	22 (19%)	11 (24%)	0.68
6 months	n	104	44	
	WOMAC®	10 [4 - 18]	8 [5 - 15.25]	0.53
	WOMAC® Pain	1 [0 - 3]	1 [0 - 2]	0.72
	% Chronic Pain	17 (16%)	3 (7%)	0.19
	S-LANSS ≥ 12	24 (23%)	10 (23%)	1

[1]median [IQR] for continuous variables; n / N (%) categorical variables

[2]Fisher's exact test; Wilcoxon rank sum test

Table 4-120: Pain scores at three and six months between patients with and without the C allele (minor allele) of rs6985606. Dominant analysis: assuming rs6985606 has a dominant effect.

rs6985606	WOMAC			WOMAC Pain		
Predictors	**Estimates**	**CI**	**p**	**Estimates**	**CI**	**p**
TC | CC	0.26	-3.91 – 4.43	0.90	0.90	-0.21 – 2.01	0.11
TC | CC at 3 m	2.60	-2.28 – 7.47	0.30	-0.36	-1.74 – 1.02	0.60
TC | CC at 6 m	2.12	-2.34 – 6.58	0.35	-0.27	-1.48 – 0.93	0.66
			Random Effects			
σ^2	81.24			6.83		
ICC	0.50			0.33		
Marginal R^2 / Conditional R^2	0.519 / 0.758			0.540 / 0.693		

Table 4-121: Linear Mixed Model analysis for rs6985606 using dominant model. CI: confidence intervals, calculated using robust analysis of standard errors.

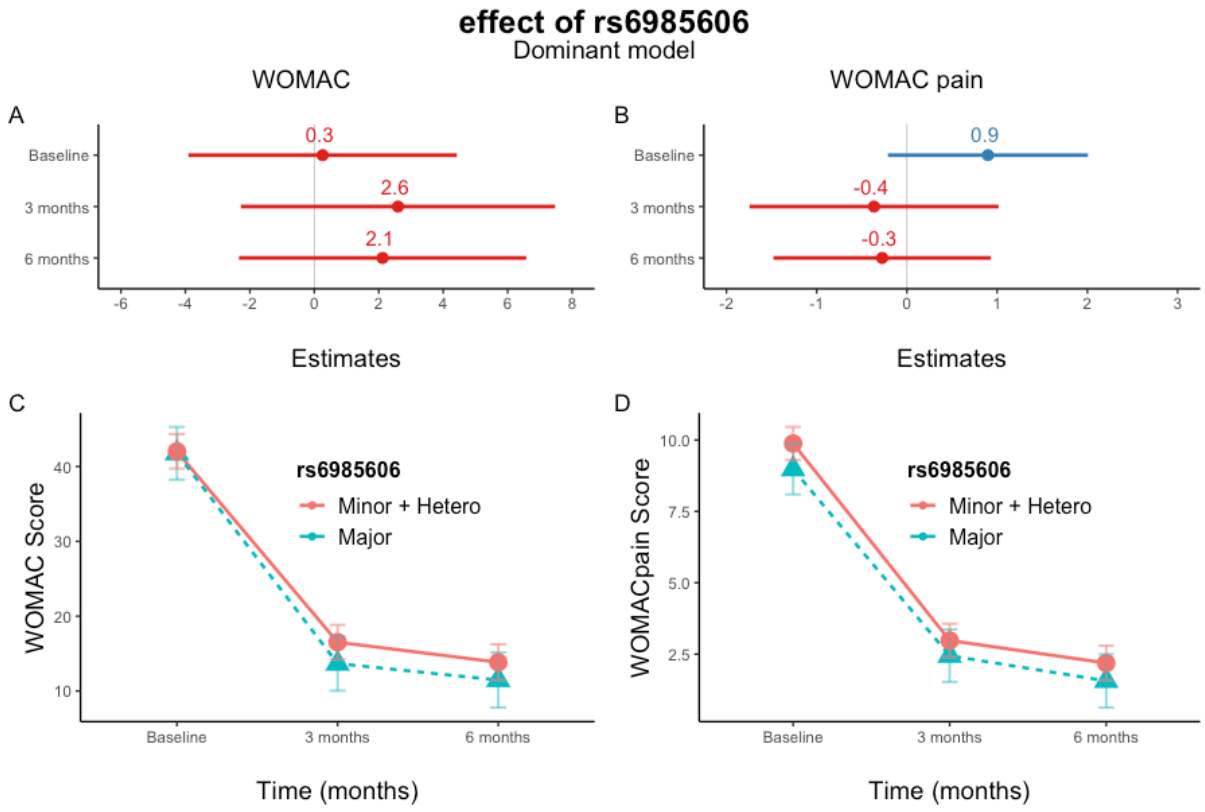

Figure 4-56: Analysis of rs6985606 using dominant model. A, B) Forest plot of estimates for WOMAC®, WOMAC® pain with robust confidence intervals C, D) predicted values for WOMAC®, WOMAC® pain as per above model.

		rs6985606 minor allele C as recessive allele		
	Characteristic	**TT | TC [1]**	**CC[1]**	**p-value[2]**
3 months	n	127	33	
	WOMAC®	13 [7 - 21]	15 [6 - 23]	0.70
	WOMAC® Pain	2 [0 - 4]	2 [1 - 4]	0.67
	% Chronic Pain	25 (20%)	8 (24%)	0.63
	S-LANSS ≥ 12	27 (21%)	6 (18%)	1
6 months	n	118	30	
	WOMAC®	8.5 [5 - 17]	10 [6.25 - 18]	0.31
	WOMAC® Pain	1 [0 - 2]	1 [0 - 4]	0.23
	% Chronic Pain	15 (13%)	5 (17%)	0.56
	S-LANSS ≥ 12	29 (25%)	5 (17%)	0.47

[1]median [IQR] for continuous variables; n / N (%) categorical variables

[2]Fisher's exact test; Wilcoxon rank sum test

Table 4-122: Pain scores at three and six months between patients with and without the T allele (major allele) of rs6985606. Recessive analysis: assuming rs6985606 has a recessive effect.

rs6985606	WOMAC			WOMAC Pain		
Predictors	**Estimates**	**CI**	**p**	**Estimates**	**CI**	**p**
CC	0.55	-4.49 – 5.60	0.83	0.22	-1.05 – 1.49	0.73
CC at 3 m	0.43	-5.08 – 5.94	0.88	0.46	-1.38 – 2.30	0.62
CC at 6 m	2.28	-3.28 – 7.83	0.421	0.40	-1.19 – 1.98	0.62
Random Effects						
σ^2	81.47			6.83		
ICC	0.50			0.34		
Marginal R^2 / Conditional R^2	0.518 / 0.757			0.537 / 0.693		

Table 4-123: Linear Mixed Model analysis for rs6985606 using recessive model. CI: confidence intervals, calculated using robust analysis of standard errors.

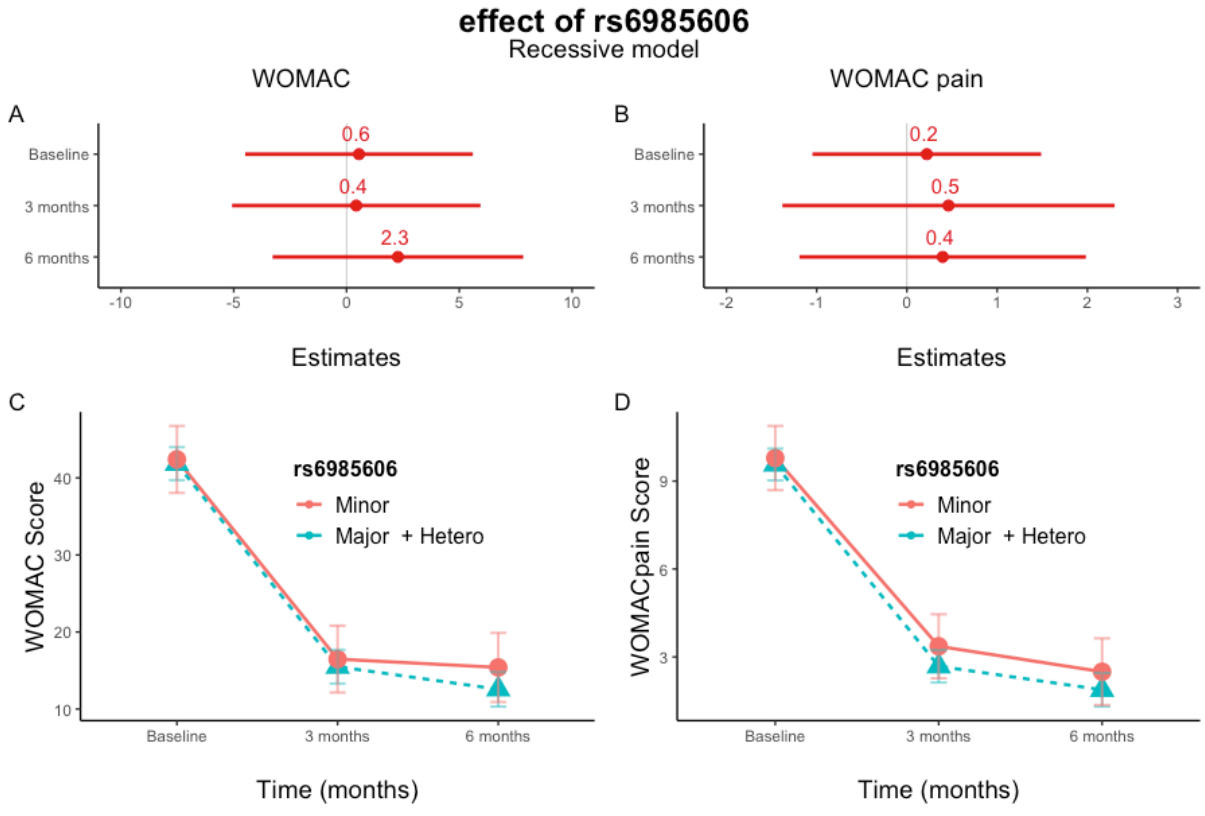

Figure 4-57: Analysis of rs6985606 using recessive model. A, B) Forest plot of estimates for WOMAC®, WOMAC® pain with robust confidence intervals C, D) predicted values for WOMAC®, WOMAC® pain as per above model.

4.3.2.5.7 Step-Down Modelling for WOMAC®, WOMAC®Pain

Zuur et al describe suggest that the best practice in fitting a mixed model is by a step-down approach (Zuur et al., 2009). All possible explanatory variables are first introduced in the model, and then remove each term. If this does not affect the model, then this term is removed. Indeed, this is the preferred method even by the authors of *lmerTest* package (Kuznetsova et al., 2017).

Hence, this approach was used to validate the above findings. For each polymorphism, four models were constructed:

- WOMAC® Score as outcome variable, using dominant approach
- WOMAC® Pain Score as outcome variable, using dominant approach
- WOMAC® Score as outcome variable, using recessive approach
- WOMAC® Pain Score as outcome variable, using recessive approach

Most of the final fitted models did not include the polymorphism investigated, so only the significant models are being presented here.

rs4633

Using a recessive approach, WOMAC® pain was influenced by the presence or absence of the major allele C. This confirms the previous findings that patients homozygous for the C allele of the rs4633 polymorphism have a reduced WOMAC® Pain score at six months.

The final model also included age and BMI as explanatory variables. The initial model and final model are shown in Table 4-124. Figure 4-58 shows the estimates for each

variable with its respective robust confidence interval, and the predicted effect of rs4633 on WOMAC® pain.

rs4633	**Initial Model**				**Final Model**		
Predictors	**Estimates**	**CI**	**p**	**Eliminated**	**Estimates**	**CI**	**p**
TT	0.19	-0.90 – 1.28	0.73		0.27	-0.84 – 1.38	0.64
TT at 3 m	-1.00	-2.55 – 0.55	0.21		-1.01	-2.56 – 0.54	0.20
TT at 6 m	-1.79	-2.99 – -0.58	***0.004***		-1.78	-2.98 – -0.58	***0.004***
Age	-0.06	-0.12 – -0.01	***0.032***		-0.06	-0.12 – 0.00	0.052
BMI	0.04	-0.01 – 0.10	0.13		0.05	-0.01 – 0.11	0.107
Pain at rest	-0.03	-0.17 – 0.11	0.70	*1*			
Pain while Physio	0.04	-0.10 – 0.18	0.61	*2*			
Anaesthesia (GA)	-0.19	-0.90 – 0.52	0.60	*3*			
Sex (M)	-0.41	-1.11 – 0.29	0.25	*4*			
S-LANSS at Baseline	-0.85	-2.12 – 0.42	0.19	*5*			
Random Effects							
σ^2	6.95				6.95		
ICC	0.27				0.29		
Marginal R^2 / Conditional R^2	0.561 / 0.681				0.554 / 0.682		

Final model

WOMAC® pain ~ rs4633 + Interval + Age + BMI + rs4633:interval + (1 | Subject)

Table 4-124: Step down model fitting for Linear Mixed Model analysis for rs4633 as recessive allele. CI: confidence intervals, calculated using robust analysis of standard errors.

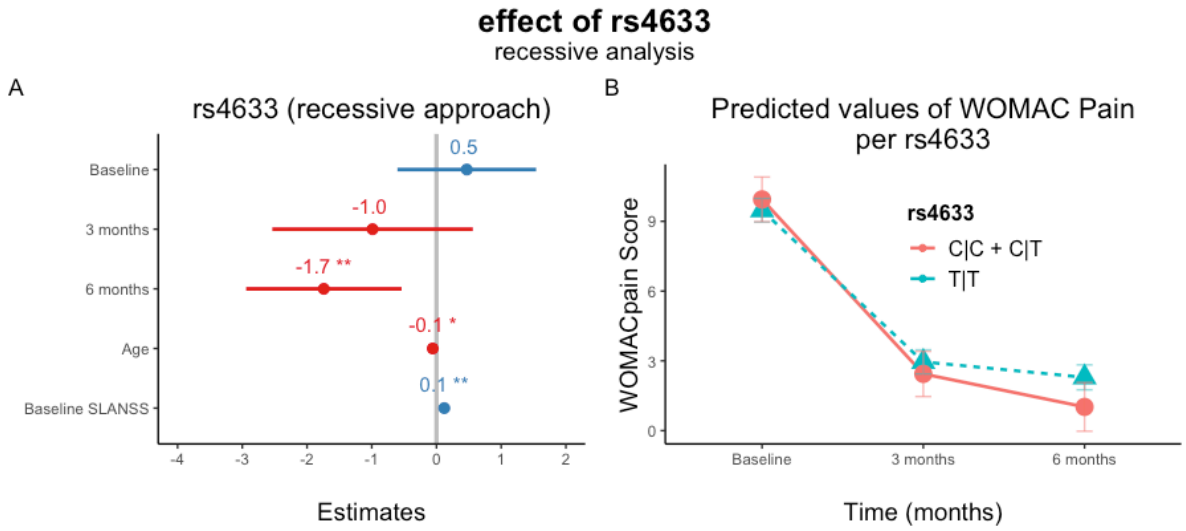

Figure 4-58: rs4633: A) Forest plot of estimates for WOMAC® pain with robust confidence intervals B) predicted values for WOMAC® pain as per above model.

rs2075572

Using a dominant approach, the WOMAC® score was influenced by the presence of the minor allele G. This confirms the previous findings that patients carrying the G allele of the rs2075572 polymorphism have increased WOMAC® scores at six months.

The final model also included sex and BMI as explanatory variables. The initial model and final model are shown in Table 4-125. Figure 4-59 shows the estimates for each variable with its respective robust confidence interval, and the predicted effect of rs2075572 on WOMAC® scores.

rs2075572	Initial Model				Final Model		
Predictors	**Estimates**	**CI**	**p**	**Eliminated**	**Estimates**	**CI**	**p**
CG | GG	-4.48	-8.69 – -0.26	0.038		-4.85	-9.07 – -0.64	***0.024***
CG | GG at 3 m	7.33	2.67 – 11.99	***0.002***		7.30	2.64 – 11.96	***0.002***
CG | GG at 6 m	3.74	-1.05 – 8.54	0.13		3.69	-1.11 – 8.50	***0.13***
Sex	-3.25	-6.05 – -0.45	0.023		-3.72	-6.64 – -0.80	***0.013***
BMI	0.25	0.03 – 0.48	***0.029***		0.25	0.02 – 0.48	***0.030***
Age	0.00	-0.26 – 0.26	-	*1*			
Anaesthesia (GA)	0.65	-2.64 – 3.94	0.70	*2*			
Pain at rest	0.16	-0.48 – 0.80	0.61	*3*			
S-LANSS at Baseline	-2.98	-7.78 – 1.82	0.22	*4*			
Pain while Physio	0.40	-0.24 – 1.03	0.22	*5*			
Random Effects							
σ^2	88.53				6.93		
ICC	0.43				0.26		
Marginal R^2 / Conditional R^2	0.549 / 0.743				0.568 / 0.682		

Final model

WOMAC® ~ rs2075572 + Interval + Sex + BMI + rs2075572:interval + (1 | Subject)

Table 4-125: Step down model fitting for Linear Mixed Model analysis for rs2075572 as dominant allele. CI: confidence intervals, calculated using robust analysis of standard errors.

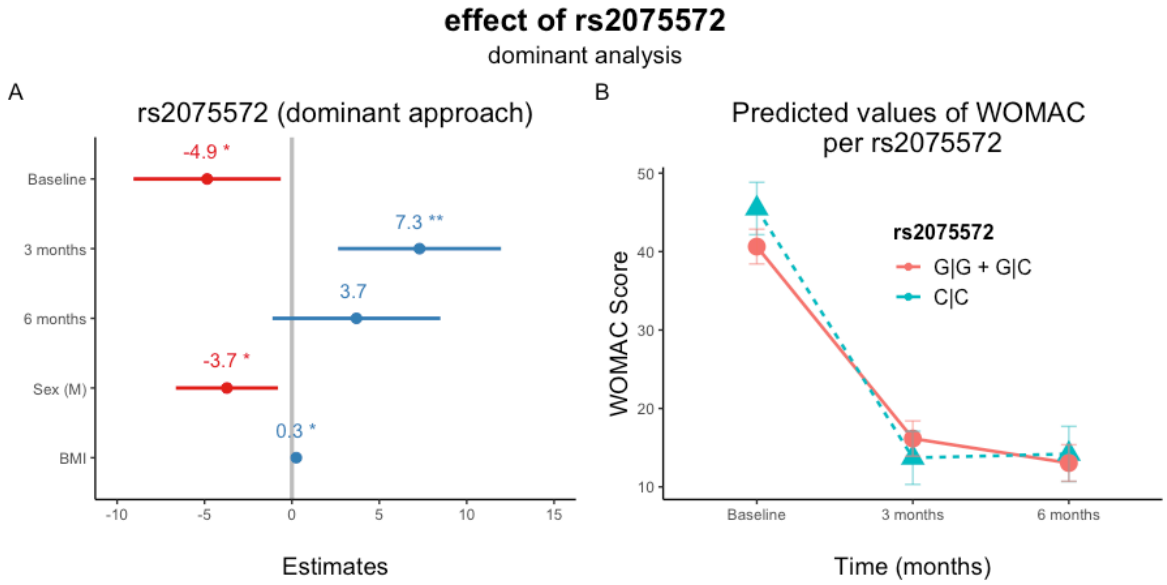

Figure 4-59: rs2075572: A) Forest plot of estimates for WOMAC® with robust confidence intervals B) predicted values for WOMAC® as per above model

The baseline WOMAC® score was lower in patients carrying the G allele of rs2075572, with the score being around 5 points less. However, at three months after surgery, the WOMAC® score in such patients was higher by around 2 points. There was no difference at six months.

The WOMAC® Pain score was also influenced by the presence of the minor allele G. This confirms the previous findings that patients carrying the G allele of the rs2075572 polymorphism have decreased WOMAC® Pain score at baseline.

The final model also included age as explanatory variables. The initial model and final model are shown in Table 4-126. Figure 4-60 shows the estimates for each variable with its respective robust confidence interval, and the predicted effect of rs2075572 on WOMAC® pain.

rs2075572 was associated with less pain before surgery by nearly two points. This effect was cancelled at three and at six months.

rs2075572	Initial Model				Final Model		
Predictors	**Estimates**	**CI**	**p**	**Eliminated**	**Estimates**	**CI**	**p**
CG | GG	-1.66	-2.66 – -0.66	***0.001***		-1.78	-2.78 – -0.79	***<0.001***
CG | GG at 3 m	1.78	0.43 – 3.13	***0.010***		1.77	0.41 – 3.12	***0.011***
CG | GG at 6 m	1.44	0.21 – 2.68	***0.022***		1.44	0.19 – 2.68	***0.024***
Age	-0.05	-0.11 – 0.01	0.10		-0.06	-0.12 – 0.00	0.060
Pain at rest	-0.00	-0.14 – 0.13	0.98	*1*			
Anaesthesia (GA)	-0.07	-0.80 – 0.67	0.86	*2*			
Pain while Physio	0.02	-0.12 – 0.16	0.77	*3*			
Sex (M)	-0.24	-0.92 – 0.44	0.48	*4*			
BMI	0.04	-0.02 – 0.10	0.17	*5*			
S-LANSS at Baseline	-0.79	-2.06 – 0.49	0.23	*6*			
Random Effects							
σ^2	6.69				6.69		
ICC	0.29				0.31		
Marginal R^2 / Conditional R^2	0.570 / 0.696				0.562 / 0.696		

Final model

WOMAC® ~ rs2075572 + Interval + Age + rs2075572:interval + (1 | Subject)

Table 4-126: Step down model fitting for Linear Mixed Model analysis for rs2075572 as dominant allele. CI: confidence intervals, calculated using robust analysis of standard errors.

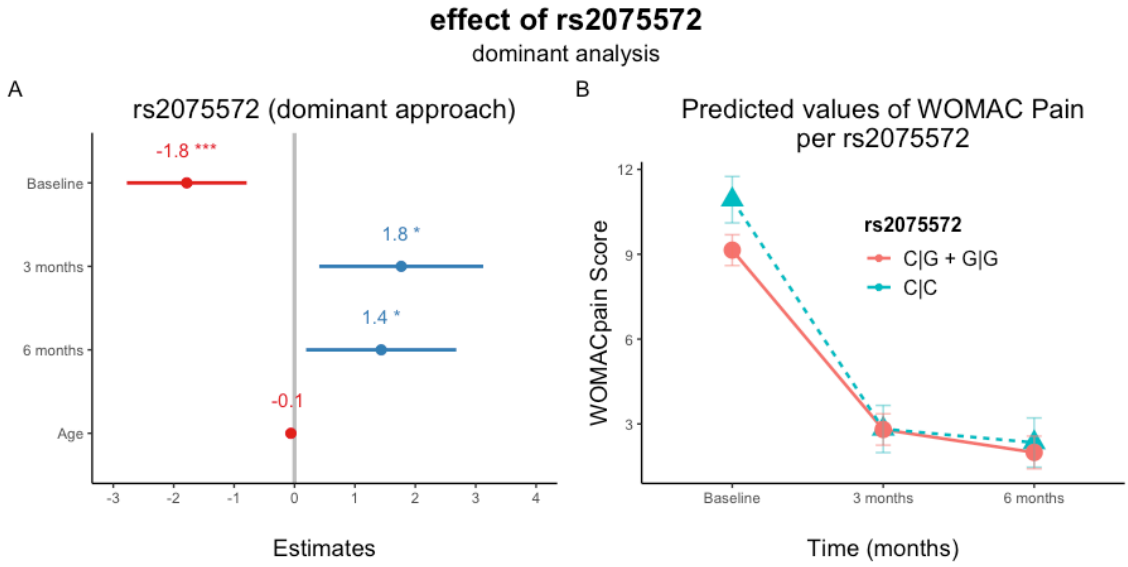

Figure 4-60: rs2075572: A) Forest plot of estimates for WOMAC® pain with robust confidence intervals B) predicted values for WOMAC® pain as per above model

rs734784

Using a recessive approach, the WOMAC® score was influenced by the presence of the major allele C. This confirms the previous findings that patients homozygous for the C allele of the rs734784 polymorphism have a reduced WOMAC® Pain score at all points investigated.

The final model also included sex and BMI as explanatory variables. The initial model and final model are shown in Table 4-127. Figure 4-61 shows the estimates for each variable with its respective robust confidence interval, and the predicted effect of rs734784 on WOMAC®.

rs734784	Initial Model				Final Model		
Predictors	**Estimates**	**CI**	**p**	**Eliminated**	**Estimates**	**CI**	**p**
CC	-3.87	-8.28 – 0.55	0.086		-3.94	-6.97 – -0.91	***0.011***
CC at 3 m	0.18	-4.78 – 5.14	0.94				
CC at 6 m	-0.92	-5.48 – 3.63	0.69				
Sex	-3.91	-6.71 – -1.11	***0.006***		-3.67	-6.55 – -0.79	***0.013***
BMI	0.26	0.03 – 0.50	***0.029***		0.31	0.08 – 0.55	***0.009***
Pain while Physio	0.36	-0.33 – 1.05	0.31		0.53	0.05 – 1.01	***0.029***
Anaesthesia (GA)	0.00	-3.31 – 3.32	-	*1*			
Age	-0.08	-0.35 – 0.19	0.55	*2*			
Pain at rest	0.32	-0.39 – 1.03	0.38	*3*			
S-LANSS at Baseline	-3.50	-8.49 – 1.49	0.17	*4*			
Random Effects							
σ^2	85.07				85.02		
ICC	0.41				0.42		
Marginal R^2 / Conditional R^2	0.571 / 0.748				0.563 / 0.748		

Final model

WOMAC® ~ rs734784 + Interval + Sex + BMI + Pain while Physio + (1 | Subject)

Table 4-127: Step down model fitting for Linear Mixed Model analysis for rs4633 as recessive allele. CI: confidence intervals, calculated using robust analysis of standard errors.

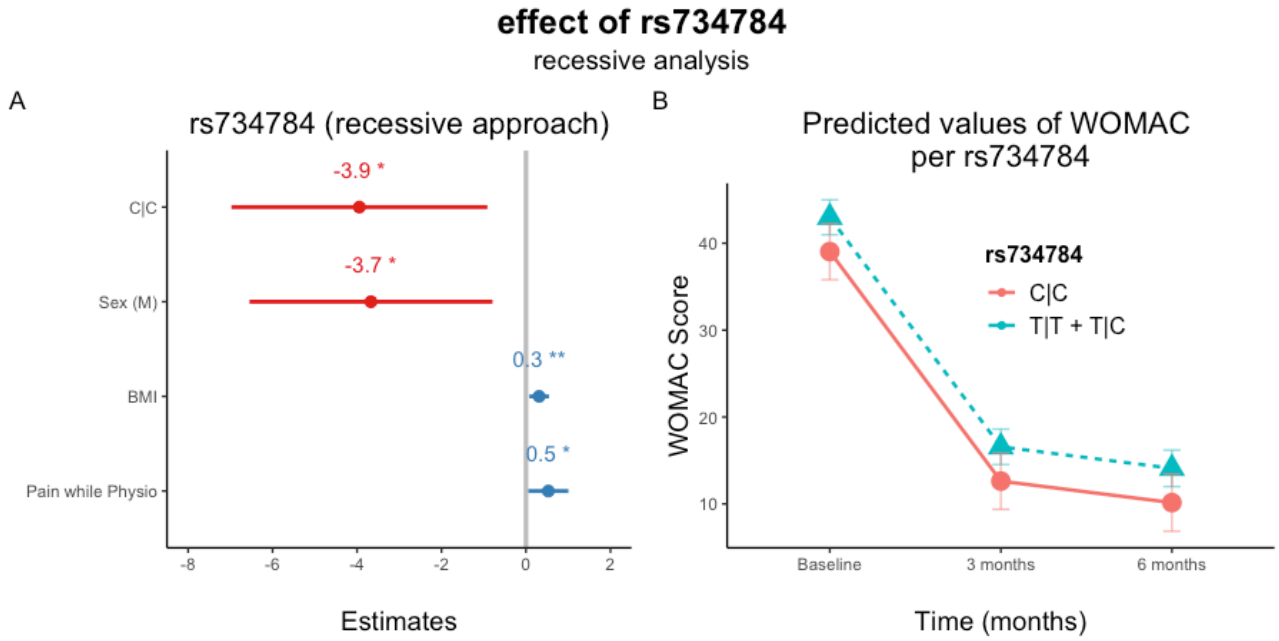

Figure 4-61: rs734784 A) Forest plot of estimates for WOMAC® pain with robust confidence intervals B) predicted values for WOMAC® pain as per above model

Throughout the study, rs734784 appears to be associated with better WOMAC®

scores, with a decrease of around 4 points.

4.3.2.6 Incidence of CPSP and Neuropathic Pain as per various factors

Table 4-128 shows the frequency of the various SNP's (dominant analysis) in patients who developed CPSP at six months, whereas Table 4-129 demonstrates the same for patients with a high S-LANSS score at six months.

		CPSP	No CPSP	p-value
n		25	148	
COMT	rs4633	17 (68%)	109 (74%)	0.62
	rs4680	18 (72%)	107 (72%)	1
	rs4818	16 (64%)	81 (55%)	0.17
SCN9A	rs6746030	7 (28%)	41 (28%)	1
	rs7595255	8 (32%)	44 (30%)	0.63
	rs11898284	7 (28%)	36 (24%)	0.62
OPRM1	rs1799971	5 (20%)	40 (27%)	0.62
	rs495491	13 (52%)	80 (54%)	0.66
	rs563649	6 (24%)	22 (15%)	0.25
	rs2075572	17 (68%)	103 (70%)	0.81
	rs533586	14 (56%)	88 (59%)	1
	rs609148	13 (52%)	63 (43%)	0.37
GCH1	rs3783641	8 (32%)	32 (22%)	0.29
	rs998259	10 (40%)	59 (40%)	1
KCNS1	rs4499491	14 (56%)	86 (58%)	0.82
	rs734784	14 (56%)	98 (66%)	0.32
OPRK1	rs6985606	17 (68%)	87 (59%)	0.19
Spinal Anaesthesia		12 (48%)	78 (53%)	0.67
Baseline WOMAC® Score		50.5 [35.25 - 56.5]	40 [30 - 51]	0.10
Baseline WOMAC® Pain Score		11.5 [9 - 12.25]	9 [7 - 12]	***0.046***

Table 4-128: Frequency counts of all SNP's investigated, Spinal Anaesthesia and median baseline scores for WOMAC® and WOMAC® Pain, in patients with and without CPSP at 6 months after TKA

		Low S-LANSS	High S-LANSS	p-value
n		134	41	
COMT	rs4633	100 (75%)	26 (63%)	0.30
	rs4680	99 (74%)	27 (66%)	0.53
	rs4818	74 (55%)	23 (56%)	0.71
SCN9A	rs6746030	38 (28%)	10 (24%)	0.69
	rs7595255	41 (31%)	11 (27%)	0.84
	rs11898284	31 (23%)	13 (32%)	0.31
OPRM1	rs1799971	38 (28%)	9 (22%)	0.54
	rs495491	76 (57%)	19 (46%)	0.19
	rs563649	20 (15%)	10 (24%)	0.14
	rs2075572	92 (69%)	29 (71%)	1
	rs533586	77 (57%)	25 (61%)	0.70
	rs609148	55 (41%)	21 (51%)	0.20
GCH1	rs3783641	29 (22%)	12 (29%)	0.29
	rs998259	50 (37%)	20 (49%)	0.11
KCNS1	rs4499491	78 (58%)	23 (56%)	1
	rs734784	91 (68%)	22 (54%)	0.07
OPRK1	rs6985606	81 (60%)	24 (59%)	1
Spinal Anaesthesia		70 (52%)	20 (49%)	0.72
Baseline WOMAC® Score		40 [29 - 51]	46.5 [32 - 54]	0.07
Baseline WOMAC® Pain Score		9 [7 - 12]	10.5 [9 - 12.25]	***0.023***

Table 4-129: Frequency counts of all SNP's investigated, Spinal Anaesthesia and median baseline scores for WOMAC® and WOMAC® Pain, in patients with low or high S-LANSS at 6 months after TKA

4.3.2.7 Multivariate Analysis of the Effect of Various SNP's

A multivariate analysis using a generalized linear model was used to assess which SNP's were most likely to be associated with a higher incidence of chronic postoperative surgical pain (Table 4-130).

The analysis was performed according to a Dominant approach. Homozygous non-variants (A/A) were compared to heterozygous and homozygous variants (A/B, B/B). This approach would show if the variant allele (B) would have a dominant effect (Zhao et al., 2016).

The initial model included all SNP's investigated, together with baseline WOMAC® Pain scores and anaesthesia type. Collinearity between the various SNP's was checked by using Spearman's correlation test, and by pairwise Chi-squared tests. There was a strong collinearity between the variants of each gene investigated. Hence, one SNP was chosen for each gene and used in the initial model.

A step-down technique was used. Using Chi-squared tests, the most non-significant factors were dropped, and the Akaike information criterion (AIC) was checked at each stage. This was repeated until the lowest AIC was found. This model was then considered as the final model.

Three outliers were removed from the model. These were chosen on the basis of Pearson residuals being less than 3, and also on the Bonferroni outlier test as provided by the outlier function in the *car* package (version 3.0-10) of R.

The initial model and the derived model are shown in Table 4-130. It can be seen that three SNP's had a significant clinical effect:

- the rs4633 variant of *COMT* was associated with an odds ratio of 18.7 for CPSP
- the rs563649 variant of *OPRM1* was associated with an odds ratio of 59.3 for CPSP
- the rs734784 variant of *KCNS1* was associated with an odds ratio of 0.08 for CPSP

This would mean that rs4633 and rs563649 would increase the chances of CPSP, whereas rs734784 would have a protective effect.

Binomial logistic regression model for incidence of CPSP at six months					
	Estimate	**OR**	**95% Conf Interval OR**		**p-value**
Initial model					
Intercept	-6.83				
Baseline WOMAC® Pain	0.19	1.21	0.99	1.49	0.07
General Anaesthesia	1.28	3.58	0.92	13.99	0.07
COMT – rs4633	2.13	8.42	0.82	86.48	0.07
SCN9A – rs6746030	-0.60	0.55	0.12	2.45	0.43
OPRM1 – rs563649	1.43	4.20	0.93	18.95	0.06
GCH1 – rs998259	-0.08	0.92	0.25	3.40	0.90
KCNS1 – rs734784	-0.87	0.42	0.09	1.86	0.25
OPRK1 – rs6985606	0.95	2.57	0.47	13.99	0.27
Null Deviance: 80.91			Residual deviance: 65.69		
		AIC: 83.69			
Final model					
Intercept	-10.60				
Baseline WOMAC® Pain	0.36	1.44	1.01	2.04	***0.04***
General Anaesthesia	2.14	8.52	1.02	70.90	***0.05***
COMT – rs4633	2.93	18.69	0.55	638.96	0.10
OPRM1 – rs563649	4.08	59.3	3.07	11143.9	***0.01***
KCNS1 – rs734784	-2.52	0.08	0.01	0.92	***0.04***
Null Deviance: 51.84			Residual deviance: 30.53		
		AIC: 42.53			
Hoslem test: p = 0.56			Analysis of Deviance: 51.8 vs 30.5, p=0.0007		

Table 4-130: Logistic regression model for incidence of chronic pain at six months, as defined by WOMAC® Pain subscore greater than 5.

Using the same methods listed above, an attempt was made to prepare a model using a Recessive approach. In this approach, the homozygous variant is compared to the heterozygous and homozygous non-variants. However, it was not possible to obtain meaningful statistical results, possibly due to the small number of homozygous variants in some of the populations.

4.3.2.8 Summary

4.3.2.8.1 Type of Anaesthesia

CPSP, as defined by a WOMAC® Pain Subscore greater than 5, was more frequent in patients who

- had a higher WOMAC® Pain subscore preoperatively
- had a higher WOMAC® Pain subscore at three months
- pain at rest 24 hours after surgery
- had a general anaesthetic

4.3.2.8.2 Genetic Factors

WOMAC® score was reduced in patients:

- with the G allele of the rs2075572 polymorphism in the *OPRM1* gene at baseline, but not at 3 or 6 months (Table 4-88, Table 4-89)
- homozygous for the C allele of the rs734784 polymorphism of the KCNS1 gene throughout all of the study period, when adjusted for sex and BMI

The WOMAC® Pain subscore was lower in patients:

- with the G allele of the rs2075572 polymorphism in the *OPRM1* gene at baseline, but not at 3 or 6 months (Table 4-88, Table 4-89)

CPSP may be:

- higher in patient with the rs4633 variant of the *COMT* gene
- higher in patients with the rs563649 variant of the *OPRM1* gene
- lower in patients with the rs734784 variant of the *KCNS1* gene

Chapter 5

Discussion

5.1 Introduction

This is the first study locally to investigate the incidence and risk factors for the development of chronic pain after surgery, specifically after TKA. Furthermore, it is also the first study to describe the prevalence of a variety of polymorphisms in the *COMT*, *SCN9A*, *OPRM1*, *GCH1*, *KCNS1* and *OPRK1* genes.

There is a lack of studies on Maltese genotypes, although the Malta Genome Project aims to give a better insight into such genotypes. Studies by Capelli et al (2006) demonstrated that the variations shown in the Y chromosome in the Maltese population resemble closely that of Sicilians and Calabrians.

In this study, we have assumed that the genetics of the population sampled would be representative of the general local population. It is possible that this may not be true. For instance, it is possible that some of the genes investigated might be implicated in the pathogenesis of osteoarthritis of the knee. This would have introduced a form of selection bias. However, we have compared our samples to a more generalised dataset obtained from the 1000 Genomes Project to mitigate this effect.

The results of our research will help focus more interest in these areas.

5.2 Demographics

5.2.1 Acute Postoperative Pain following a TKA

It is to be expected that such major surgery would be associated with a considerable amount of pain, especially in the first few days. There are a number of treatment options available for analgesia, which should help patients to feel no or mild pain postoperatively.

Total knee arthroplasty is considered major surgery, even though it is routine. There is significant soft tissue and bone trauma, and this will inevitably result in considerable post-operative pain.

Local research by Sciberras (2011), by Zammit (2012) and by Santucci (2016) highlighted that pain following TKA may be severe and a cause of considerable morbidity. Santucci et al repeated work done by Sciberras and coworkers after a period of five years. Despite the time interval, the latter study showed minimal improvements in outcomes: 67% of patients in a local cohort had more than mild pain even at rest, and 25% of patients rated their pain as being severe.

Other studies also corroborate such findings. Kornilov et al (2016) assessed a hundred consecutive patients after TKA and found an average pain score of 5.5 out of 10. In another study by Essex et al (2018), one-third of patients rated their pain as severe before trial treatment. In a large meta-analysis of 113 studies in which different analgesic protocols were compared, patients in the control groups had a pain score at rest of 80mm on a Visual Analog Scale (Karlsen et al., 2017). Such high pain scores also

affect initial postoperative outcomes, as shown by Robinson et al (2014). Median pain scores for patients were above five on a numerical rating score, and this caused more than a third of patients to delay their discharge.

In the present cohort of patients, there was a significant improvement in the pain scores at rest on the first day, when compared to the other local data by Sciberras (2011) and by Santucci (2016). Only 8.5% of our patients rated their pain as severe, with a median score of 2 on a numerical rating score. This can be attributed to the controlled manner by which analgesics were prescribed. In fact, in the unpublished work by Zammit, which also provided a strict analgesic regimen, the pain scores at rest were similar to the ones found in this study.

On the other hand, there was not much of an improvement in controlling pain during physiotherapy, with the median score of 5 being slightly better than a median score of 6 found by Sciberras in 2011. One may conclude that the analgesic protocol, which included regular paracetamol, diclofenac and codeine with morphine given only on request, was not enough to improve pain scores during the first session of physiotherapy. Indeed, other studies included a regular opioid for the first 48 hours and this might have led to better pain control during the initiation of physiotherapy (Harsten et al., 2013). The reasons for not administering opiates to post-operative patients locally are unclear and could be more of a cultural nature.

Ordinal regression in our cohort showed that older patients were 6% more likely to have less pain at rest. Male patients were twice more likely to have reduced pain scores during physiotherapy. This is consistent with evidence from other studies, where increased body mass index, female gender and younger age have been linked to

increased postoperative pain and morphine consumption (Rakel et al., 2012; Abrecht et al., 2019).

The other demographic factors which were studied in our study did not show any association with postoperative acute pain.

The relationship between gender and post-operative pain has been studied extensively. Several studies have shown female patients to be at risk of post-operative pain (Rosseland et al., 2004; Aubrun et al., 2005; Hussain et al., 2013), but most of these studies looked into the early post-operative period. For instance, Hussain et al (Hussain et al., 2013)reported increased pain scores and opioid consumption in 60 females undergoing laparoscopic cholecystectomy, compared to a group of 60 males. Such increased pain was present only in the immediate post-operative periods (less than two hours) and was not continued in the late postoperative phase (more than 24 hours). Furthermore, this effect is not consistent throughout the literature, and there is variation between different types of surgeries (Pereira et al., 2015).

Age is also known to affect pain scores after surgery. The PAIN OUT study examined the incidence and intensity of pain after surgery in over 11,500 patients over eight years (van Dijk et al., 2021). Postoperative pain was less in more elderly patients. Our results are in agreement with such evidence. The reasons for such an effect are unclear: it might be subjective, with older patients feeling less pain in general (Halaszynski, 2013). It could also be due to an increased response to analgesics. Bellville et al (1971) studied the effect of opioids in over 700 patients after surgery and found that age was the most important factor determining efficacy of analgesics administered.

Pre-operative knee pain did not impact the level of pain scores at rest or on physiotherapy in this study. Other studies have demonstrated a strong effect of pre-operative pain on acute post-operative pain after TKA (Gramke et al., 2009; Rakel et al., 2012). Most of these studies include a Pain Catastrophizing Scale or equivalent, to assess the individual psychological response toward pain. Unfortunately, this research did not investigate this response, since the main aim of the study was a comparison of the type of anaesthesia. However, this study used the baseline pain scores as a reference for future scores. This should have reduced the influence of internal bias on the results of the study.

5.2.2 Chronic PostSurgical Pain following TKA

Patients rate the success of any surgical procedure depending on many factors (Ware et al., 1983; Choi et al., 2016). One of the most important of these is pain, both in the acute phase, but more especially in the longer term. In fact, Howells et al found a strong correlation between patient satisfaction and WOMAC® Pain scores after TKA (Howells et al., 2016). Similarly, in more than 500 patients after TKA, Bryan et al (2018) suggest that the difference in WOMAC® pain after 6 months is a significant factor in predicting patient satisfaction.

CPSP is not uncommon and the incidence varying different surgical procedures. For instance, thoracotomy, amputations and inguinal hernia repair are well known to be associated with CPSP (Fletcher et al., 2015). Orthopaedic procedures are also associated with a high incidence of CPSP. In patients who have had a TKA, the

incidence of mild CPSP seems to range from 29 to 75% of the patients undergoing surgery (Wylde et al., 2015; Thomazeau et al., 2016).

The study was powered to assess a difference in mean WOMAC® scores between each group. We used the WOMAC® pain subscore to assess pain. This is a widely used score, as shown by Woolacott et al (2012), who reported that such score was used as an outcome in 45% of studies in osteoarthritis of the knee.

The WOMAC® pain score assesses five aspects of daily activities: walking, stair climbing, sitting, lying down, and standing. The stem of the scale asks the patient to focus on the extent of pain in the involved knee during these activities. The extent of pain experienced during each of these activities is reported on a 5-point Likert scale ranging from “none” to “extreme.” The sum of these is the total WOMAC pain score, which ranges from 0 to 20, with lower scores meaning less pain.

The WOMAC Pain score is part of the WOMAC score, which includes pain, function and stiffness, to give a range of 0 – 96, with lower scores meaning better outcomes.

Throughout this study, the WOMAC® score decreased by 28 points (SD +/- 15.64), from a median score of 41 at baseline to a median score of 10 at six months. Similarly, the WOMAC® Pain subscore decreased from 10 points to 1 point with a median change of 8 points (SD +/-4). These results are slightly better than those reported elsewhere. Allyson Jones et al (2003) reported a preoperative score of 11 points and a postoperative score of 4 points at six months. Similar findings were reported by Bryan et al (2018), with a preoperative score of 10.1, falling to 3.4 at six months and 2.9 at 12 months. Liebensteiner et al (2019) reported a preoperative WOMAC® Pain score of 10

points and a postoperative median score of 1.2 points after one year. Compared to these studies, we found lower WOMAC® pain scores. This would have made it more difficult to detect any differences between groups.

In our cohort of patients, WOMAC® scores and WOMAC® pain scores improved mostly within the first three months after surgery. Lenguerrand et al (2016) had similar results in a study of 84 patients having a knee arthroplasty. This is not surprising, as this may be consistent with the time taken for a scar to mature (Guo et al., 2010).

The incidence of chronic post-surgical pain was used as a secondary outcome. Being a dichotomous variable, this would have required the use of a much larger population, so multivariate analysis using logistic regression was performed to reduce a Type II error.

Chronic post-surgical pain (CPSP) was defined as a WOMAC® Pain subscore greater than five at six months after the procedure. This would be equivalent to mild pain when compared to the numerical rating scale. Using this definition, the incidence of chronic post-surgical pain was 11% in the study population.

A stricter definition, such as no pain at all, would have resulted in an incidence of 62.5%. This may be compared to a study by Thomazeau et al (2016), where 28.8% of patients reported some degree of pain after TKA, whereas Wylde et al (2015) had found that 75% of patients reported some degree of pain after six months.

Several studies have shown that certain demographic factors might influence the development of chronic pain (Kim et al., 2018). These include age, gender, body mass index, educational level, and psychosocial factors.

In our study, age, gender and BMI did have an effect on pain outcomes. Male patients reported lower scores for WOMAC® throughout the study period. WOMAC® pain was only lower for males at baseline. Parsley et al (2010) suggest that females might fare worse than males after TKA, possibly because women requiring a TKA present later in life. This difference could also be due to differences between males and females, with the current prosthesis being anatomically more suitable for male patients (Kim et al., 2015). However, more recent studies show no effect of gender on functional outcomes (Nassif et al., 2015).

Some studies have shown that younger age is correlated with worse pain scores (Singh et al., 2008; Chodór et al., 2016; Townsend et al., 2018). No clear mechanism has been postulated, but it could be the result of a combination of factors. Pain sensitivity could be less in the elderly (Yezierski, 2012), although a meta-analysis by El Tumi (2017) found different and conflicting results across several studies. Pain sensitivity could be impaired due to other conditions, such as diabetes or peripheral neuropathies, that may be more frequent in the elderly. It is also possible that younger patients presenting for surgery suffer from more severe and aggressive forms of arthritis. In our study, age had a small effect on pain scores. The main correlation was found to be at baseline, with the difference between age groups being less at three and at six months. This could mean that younger patients might benefit more from TKA in terms of pain and function since the change in scores was larger.

This study is in agreement with several other studies that show that a higher BMI leads to higher pain scores (Singh et al., 2010; Merle-Vincent et al., 2011). In 264 patients who underwent a TKA with a follow-up of two years, Merle-Vincent et al report that

patient satisfaction is much less in patients with a BMI greater than 27 kg/m². Another study by Singh et al using the Mayo Clinical Total Joint Registry, with over 7,000 patients, demonstrated that limitations in function were related to an increase in BMI.

The role of postoperative acute pain in the development of chronic pain is being extensively investigated. Increased pain seems to predispose to an increased incidence of chronic pain (Thomazeau et al., 2016; Kim et al., 2018; Buvanendran et al., 2019). In our study, there was no strong relationship between acute pain and the development of chronic pain. This might be explained by the single measurement taken 24 hours postoperatively, which might not have been sensitive enough to capture the pain profile of the patients appropriately. Ideally, patients might have been questioned about their pain even after their discharge from the hospital.

Preoperative WOMAC® Score and Pain subscore were associated with pain at six months. Higher pain scores at baseline and at three months were most significant in predicting pain at six months. This might be partly due to the subjectivity of the patients. It is not surprising that patients who report higher pain scores initially would also report higher pain scores later on, as pain scores are a subjective measure. It could also be due to other factors that might predispose a patient to feel more pain, such as psychiatric or genetic associations. There is also evidence that osteoarthritis itself may increase sensitization of the pain pathways by virtue of the persistent pain (Wylde et al., 2013; Fitzsimmons et al., 2018)

However, it must be noted that 22% of patients had an increase in pain after the third month: in fact, 14 of 44 patients who reported no pain at three months later reported some pain at six months. Since the telephone questionnaires were done by the same

researcher, inter-researcher variability was not responsible. Most of these cases had modest increases (median increase of 2), but two patients reported an increase in the WOMAC® Pain subscore of 8 and 9 respectively.

The progression of neuropathic pain throughout the study period highlights the need to look specifically for such pain. Indeed, this increases from 11% at baseline to 23.4% at six months. Fitzsimmons et al (2018) studied 99 patients after TKA using the S-LANSS questionnaire and found a suspected neuropathic pain in 35.5% of patients before surgery. This decreased to 23.6% of patients at six months.

5.2.2.1 Neuropathic Pain

Neuropathic pain is widely accepted as being caused by lesions in the somatosensory system (Colloca et al., 2017). Despite being clinically different from nociceptive pain, it is difficult to diagnose neuropathic pain or to distinguish it from nociceptive pain. For this reason, various screening tools have been designed with the most commonly used being the Leeds Assessment of Neuropathic Symptoms and Signs (LANSS) (Bennett, 2001), the Neuropathic Pain Questionnaire (NPQ) (Krause et al., 2003), and the Douleur Neuropathique en 4 questions (DN4) (Bouhassira et al., 2005).

We assessed the risk of neuropathic pain using the S-LANSS since this is a validated and commonly used questionnaire (Bennett et al., 2005). The S-LANSS is a modification of the original LANSS questionnaire, designed to be easier for self-reporting. It has been used to evaluate neuropathic pain after a knee arthroplasty by numerous authors (Fitzsimmons et al., 2018; Bryan et al., 2018).

Neuropathic pain after TKA is not uncommon. Osteoarthritis itself may be a cause of neural sensitization that may predispose to neuropathic pain (Rienstra et al., 2021). Haroutiunian (2013) report that 6% of patients had symptoms of neuropathic pain after a hip or knee arthroplasty. Bryan et al (2018) described the progression of the S-LANSS score throughout one year, with mean scores that decreased from 7.0 to 5.7 in a year. Fitzsimmons et al (2018) found a high S-LANSS in 36% preoperatively, which decreased to 24% at six months.

We were surprised to note that the number of patients who had high scores on the S-LANSS questionnaire nearly doubled in the six months after surgery. Nearly 50% of patients reported paraesthesia around the operated knee: this rose to 88% in patients who reported a high S-LANSS. Similarly, a high proportion of patients had allodynia (painful response to light touch) and pain when rubbing the affected area.

Furthermore, a large number of patients, nearly one in five, reported new-onset neuropathic pain.

Paraesthesia after TKA may be due to iatrogenic injury to the infrapatellar branch of the saphenous nerve. Yang et al (2022) investigated this condition in 59 patients who reported possible infrapatellar saphenous neuralgia after knee arthroscopy. Neuralgia to such injury was not uncommon and occurred in 55% - 84% of patients after TKA. The mean time between surgery and patient symptoms was 6.4 months, which is consistent with our data of an increase in patients reporting a high S-LANSS score at six months.

Studies have shown that patients with high S-LANSS scores had worse outcomes, such as more stiffness, depression and pain (Razmjou et al., 2015; Rienstra et al., 2021). Other studies have demonstrated that patients with neuropathic pain also had higher overall pain scores when compared to patients with nociceptive pain (Hasegawa et al., 2021; Şahin et al., 2021).

Our study showed that CPSP was linked to a high S-LANSS at any stage of the study. A high preoperative S-LANSS was associated with higher WOMAC® pain scores, although this was not significant statistically. This effect has already been noted in studies by numerous authors (Warner et al., 2017; Kurien et al., 2018). Moreover, a high S-LANSS at three months was strongly linked to an increased WOMAC® pain score at three months. Such patients might have suffered injury to nerves such as the saphenous nerve, and it might be useful to investigate further these patients using simple tests such as an ultrasound (Batistaki et al., 2019; Yang et al., 2022).

Patients with a high S-LANSS score at six months were more likely to have had a higher preoperative WOMAC® Pain score. Additionally, patients with rs734784 (*KCNS1*) showed a decreased incidence of neuropathic pain, although this effect did not reach statistical significance ($p=0.07$). This gene is thought to be influential in the development of neuropathic pain (Tsantoulas et al., 2018).

We did not account for medical conditions that might contribute to neuropathic pain, such as diabetes mellitus (Colloca et al., 2017). However, by obtaining the S-LANSS pre-operatively, we hope to have compensated for this lack of information. This allowed us to establish if the patients would already have suffered from some form of neuropathic pain due to other causes other than surgery.

Another limitation is that we did not physically examine patients with a high S-LANSS to confirm a potential diagnosis of neuropathic pain.

5.3 Choice of Anaesthesia

Total knee arthroplasty lends itself both to a general anaesthetic and to a neuraxial approach (Turnbull et al., 2017). Furthermore, there exist many peripheral nerve blocks that help with anaesthesia and/or postoperative analgesia.

There is an ongoing debate on whether a general or a spinal anaesthetic is better for TKA. A general anaesthetic offers multiple advantages: it is technically less demanding, carries little risk of failure, and may be better tolerated by patients. Indeed, a general anaesthetic is possible in any individual patient, and it should always be considered if a spinal anaesthetic is not possible or has failed (Schwenk et al., 2020). Advances in anaesthesia mean that newer techniques such as total intravenous anaesthesia are associated with less postoperative nausea and vomiting and a better recovery profile.

On the other hand, regional anaesthesia offers some advantages also and is considered by some as being the default choice (Schwenk et al., 2020). A large retrospective study of more than 14,000 patients found lower incidences of short-term complications (Pugely et al., 2013). Blood loss was less with spinal anaesthesia, as was post-operative nausea and vomiting. In another large retrospective study of more than 200,000 patients who had surgery for TKA or hip arthroplasty, 30-day mortality was nearly half in patients who had a spinal anaesthetic compared to a general anaesthetic (Perlas et al., 2016). Indeed, a meta-analysis done by Memtsoudis et al (2013) analysing 94 studies showed a decrease in mortality, pulmonary complications, acute renal failure, infections and deep vein thrombosis. The only complication that was higher in patients who had received spinal anaesthesia was urinary retention.

Our choice of including a femoral nerve block in the general anaesthesia means that we cannot directly compare a regional to a general anaesthetic technique. However, a femoral nerve block is considered a standard of care if a spinal anaesthetic is not done (Rodriguez-Patarroyo et al., 2021). Other peripheral nerve blocks do exist, such as an adductor canal nerve block, IPACK nerve block, and sciatic nerve blocks, but the femoral nerve block remains an easy nerve block that is reproducible by different operators. Furthermore, the current use of other blocks in our local practice is limited.

5.3.1 The influence of Anaesthesia on Acute pain

Our study demonstrated that a spinal anaesthetic does not seem to improve pain outcomes on the first postoperative day. At this point, the effects of both a spinal anaesthetic and a general anaesthetic should have worn off. For instance, Gramke et al (2009) showed that in day-care patients, a regional anaesthetic technique only affected pain scores on the day of surgery, but had no effect on other postoperative days.

We expected that pain scores would be similar in both groups, but in fact a higher proportion of patients in the spinal group reported severe pain during physiotherapy.

There may be several explanations for this. First of all, it may be that a general anaesthetic might have an analgesic effect that lingers on for more than 24 hours. General anaesthetic agents are known to provide intraoperative analgesia (Ryu et al., 2017), possibly through their action on TREK-1 potassium channels, which have been associated with nociception (Pavel et al., 2020; Mathie et al., 2021). Tomi et al (1993) however showed that such pain relief does not occur with very low concentrations (0.2

MAC) of volatile anaesthetic agents. Hence, it is unlikely that a general anaesthetic would influence pain on the first post-operative day.

Morphine consumption over 24 hours was much lower in the spinal group. Physiotherapy usually occurred hours after the last morphine administration, which would place patients in the spinal group at a disadvantage. To mitigate this effect in this research, the analgesia protocol called for a dose of oral morphine at 6 am for all patients, but there was still a very marked difference in the morphine consumption between the two groups. Also, one of the metabolites of morphine is morphine-6-glucuronide, which has strong analgesic properties and is long-lasting (Christrup, 1997; van Dorp et al., 2006). In fact, morphine-6-glucuronide is being investigated as a potential analgesic therapy in the postoperative setting (Dahan et al., 2008).

The patients who received general anaesthesia also received a femoral nerve block, which might have provided extended analgesia in some patients. Various authors have observed low VAS scores (2.5cm) at 24 hours during physiotherapy in patients with a femoral block after TKA (Affas et al., 2011; Tan et al., 2018). Sciberras (2011) had shown that a femoral nerve block was useful in reducing pain at rest, but not during physiotherapy. However, this was before the use of ultrasound-guided nerve blocks, which have been shown to improve the success rate and duration of analgesia (Abrahams et al., 2009). Indeed, although the presence of a femoral nerve block might have influenced the results, it was felt that it would have been unethical not to offer such a standard of care to patients receiving a general anaesthetic.

It is also possible that patients who receive a spinal anaesthetic might suffer from rebound pain after the spinal anaesthetic wears off (Muñoz-Leyva et al., 2020). In a

review of literature about the possibility of rebound pain in ambulatory surgery, Lavand'homme describes patients who have an increased level of pain after a regional anaesthetic wears off (Williams et al., 2007; Galos et al., 2016; Lavand'homme, 2018). This has not been extensively described for patients receiving a spinal anaesthetic. In a study of 120 patients, Harsten et al (2013) compared spinal anaesthesia with bupivacaine alone was compared to a total intravenous general anaesthesia and found that patients receiving a spinal anaesthetic had higher pain scores after six hours. However, there were differences in the analgesic protocol between the two groups and this might have affected the outcomes. Similar findings were found by Erdogan et al (2018) in a study of 100 patients for inguinal hernia repair. Patients who had a spinal anaesthetic were more comfortable for over six hours after surgery when compared to patients who had had a general anaesthetic but then had slightly worse pain scores at 24 hours.

It is not clear why rebound pain would occur after regional anaesthesia. This phenomenon appears to occur more frequently when regional anaesthesia is used as the sole anaesthetic, rather than as a form of postoperative analgesia (Muñoz-Leyva et al., 2020). Furthermore, rebound pain is usually severe but temporary: it is transient and should not be confused with CPSP (Sunderland et al., 2016). There might be upregulated sensitivity after a nerve block since animal studies do show heat hyperalgesia after a nerve block (Kolarczyk et al., 2011). However, this has yet to be shown in humans. It is also possible that there could be peripheral sensitization, even though regional anaesthesia would reduce central sensitization. Such mechanisms

however are still unclear, so it would seem that rebound pain does not represent an exaggerated physiological response to pain (Muñoz-Leyva et al., 2020).

Rebound pain could be attributed to the fact that patients with a spinal anaesthetic were practically pain-free initially. This might skew their perception of the intensity of pain later during their stay.

Finally, other factors might explain the increased pain during physiotherapy for patients who had received a spinal anaesthetic. Genetic factors could not be catered for during randomization, and the frequency of the rs7595255 variant, and possibly also the rs6746030 variant, of the *SCN9A* gene was higher in the patients who had received a general anaesthetic.

5.3.2 The influence of Anaesthesia on CPSP

This single-centre, nonblinded randomized study has shown that patients who receive an SP instead of a GA might be at a lower risk of CPSP at 6 months. To our knowledge, this is the first randomized controlled study to have shown such an effect.

Indeed, although the patients in Group GA had better baseline scores for WOMAC® and WOMAC® Pain, such patients reported worse outcomes at three months. This effect did not extend to six months.

On logistic regression, a spinal anaesthetic was associated with a significant decrease in the incidence of chronic pain (WOMAC® Pain subscore less than 5). On univariate analysis, there was a decrease of 4% in the incidence of CPSP, but this was not statistically significant. This was not surprising, as power analysis shows that at least

600 patients would be needed for statistical significance to be obtained. Furthermore, the baseline WOMAC® Pain subscore was higher in the patients who received a spinal anaesthetic, and this variable was associated with an opposing influence.

There are only a few other studies that have investigated the influence of different anaesthetic types on chronic postoperative pain.

The use of peri-operative opioids is associated with opioid-induced hyperalgesia (Lee et al., 2011). This is thought to be due to various mechanisms, including neuroplastic changes in the pain pathway secondary to NMDA receptor activation. In cardiac surgery, the use of remifentanil infusions for CABG has been associated with chronic sternotomy pain after one year (van Gulik et al., 2012). Even in studies that have disputed such findings, such as the study by de Hoogd et al (2018), analgesic use in the first three postoperative months was more common in patients who had received a remifentanil infusion intraoperatively compared to those receiving standard doses of fentanyl.

Patients who received a spinal anaesthetic also received a propofol infusion for sedation. The use of intravenous anaesthesia using propofol has been compared to volatile anaesthetic agents such as Sevoflurane and Desflurane. There are only a few studies that investigate this effect. Song et al (2012) randomized 366 patients undergoing a thoracotomy to either total intravenous anaesthesia or to inhalation anaesthesia. Patients in the total intravenous group had nearly half the incidence of CPSP at six months. Similarly, in 80 women who had undergone a hysterectomy, total intravenous anaesthesia with propofol had less frequent CPSP (Ogurlu et al., 2014). The mechanisms responsible for the reduced CPSP with propofol are unclear. It may be

that volatile anaesthetics might be implicated in the initiation of CPSP or that propofol might be protective. Indeed, propofol is known to inhibit subtypes of the NMDA receptor (Orser et al., 1995). Propofol also increases anti-oxidant activity (Hans et al., 1997), which might have led to reduced inflammation. It would be interesting to compare total intravenous anaesthesia, inhalational anaesthesia and spinal anaesthesia.

Brandsborg et al (2007) investigated risk factors for chronic pain occurring after twelve months in women who had undergone a hysterectomy. Over one thousand Danish women were involved in the study. Using multivariate analysis, a spinal anaesthetic was found to be protective, with less than half of the incidence of chronic pain when compared to women who received a general anaesthetic. In another cohort of over two hundred women, spinal anaesthesia seemed to reduce chronic pain following a caesarean section (Nikolajsen et al., 2004). This was also the case in another retrospective study of 220 patients by Nardi et al (2013), with an odds ratio of 0.15 for the development of chronic pain after a caesarean performed under spinal anaesthesia when compared to a general anaesthetic. Epidural anaesthesia also reduced chronic pain by 80% after open abdominal surgery (Bouman et al., 2014).

On the other hand, other studies have not shown such an effect. Erdogan et al (2018) investigated 100 patients, with half receiving a spinal anaesthetic, and the other half a general anaesthetic, for inguinal hernia repair. There was no difference between the two groups for chronic pain at three months. However, this study was small and furthermore, the outcome chosen was specific to neuropathic pain which is only one component of chronic pain. In a study of over 700 patients after TKA, regional

anaesthesia combined with a general anaesthetic did not influence chronic pain outcomes (Yao et al., 2019). However, this study investigated a single-shot femoral nerve block, which is unlikely to affect long-term outcomes (Bugada et al., 2017). Another study compared the effects of anaesthesia and peripheral nerve blocks in over 500 patients, with no effect on chronic post-surgical pain (Bugada et al., 2017). However, this study was focused more on the use of regional anaesthesia as analgesia, and did not report the use of such treatment in patients receiving a general or a spinal anaesthetic. Only the study by Erdogan et al was a randomized controlled trial.

The results of the current study would indicate that the use of a spinal anaesthetic would be protective towards CPSP, at least for the first three months, and possibly even six months.

Several reasons could explain this effect. It is unlikely that a spinal anaesthetic would last for more than 24 hours, even using long-lasting local anaesthetics such as bupivacaine and intrathecal diamorphine or morphine.

The reduced opioid consumption in the first 24 hours associated with a spinal anaesthetic might lead to less opioid-induced hyperalgesia, as described above. The use of propofol sedation could have influenced the frequency of CPSP as well.

It is also possible that a spinal anaesthetic might dampen the stress response that occurs during and after surgery.

Eroğlu et al (2016) showed that there was no difference in inflammatory markers in patients who had a TKA under a spinal anaesthetic or a general anaesthetic. However, these markers were sampled at baseline, just after the procedure and after 24 hours. It

would be possible for such a stress response to manifest itself before 24 hours. Indeed, El-Radaideh et al (2019) showed how in patients who were randomized to either a spinal or general anaesthetic, the blood glucose increased more in patients with a general anaesthetic when measured 30 minutes after surgery. Davis et al (1987) compared cortisol levels in spinal or general anaesthesia in patients undergoing a hip arthroplasty and found that general anaesthesia was associated with a three-fold increase in cortisol levels. Such findings were also corroborated in a prospective controlled study involving 75 patients by Milosavljevic et al (2014), who showed that cortisol levels were higher in patients who received a general anaesthetic. Even more comprehensively, studies done by Brandt et al (1978) have shown how epidural anaesthesia reduced catabolism after surgery, as assessed by a cumulative 5-day nitrogen balance.

The stress response of surgery is well known to cause transcriptional changes in the dorsal spinal cord, although the exact nature of such transcriptional changes remains incompletely defined. In a rat model, Raithel et al (2018) used a plantar incision to cause pain. On the fifth day, the lumbar spine was examined histologically for evidence of gene expression and found induced expression of 70 genes in the dorsal spinal cord. This was despite successful analgesia with Resiniferatoxin, which causes prolonged TRPV1 channel opening. This active role of the spinal cord in self-modulating its function has been demonstrated in other studies (Iadarola et al., 1988; Obara et al., 2009; Liu et al., 2011).

Such changes in the spinal cord lead to a phenomenon known as central sensitization (Woolf, 2011). This manifests as pain hypersensitivity. Central sensitization involves

spinal cord and central nervous system changes in synaptic activity that lead to the facilitation of nociceptive impulses. Ultimately, this would lead to CPSP.

One of the earliest genes to be expressed following injury is c-*fos*, and this has been used as a marker of stress (Svendsen et al., 2001; Stenberg et al., 2005). Various studies have shown that different anaesthetic agents and different types of anaesthesia influence c-*fos* expression. Ether, nitrous oxide and ketamine are associated with a dose-dependent increase in c-*fos* expression (Ma et al., 2002; Shehab et al., 2002) Propofol, a commonly used anaesthetic agent, was shown to induce c-*fos* expression in some studies (Yin et al., 2011), but inhibit in others (Nagata et al., 1998). Halothane did not affect c-*fos* expression, but isoflurane diminished the rise in expression (Liu et al., 2011).

The use of lidocaine infiltration during surgery in rats appears to reduce c-*fos* expression in the dorsal horn of the spinal cord but not in higher centres of the brain, such as the paraventricular nucleus (Stenberg et al., 2005). In a similar study, rats injected with formalin showed an intense c-*fos* expression, which was reduced when lidocaine was either administered together with the formalin injection or soon after (Tokunaga et al., 1995). Kfoury et al (2020) even performed a parietal nerve block in rats, using bupivacaine. They demonstrated that the use of such nerve block reduced c-*fos* expression in response to carrageenan injection.

Even intrathecal opiates seem to influence genetic expression in the spinal cord (Crosby et al., 1994). Rats injected with intrathecal morphine showed a marked attenuation in the normal increase in preproenkephalin expression following injury.

The authors of such research conclude that morphine blocks noxious stimuli to neurons to prevent this increase in gene expression.

There is no human data to support the inhibition of gene expression in the spinal cord with a spinal anaesthetic. This would involve obtaining histological samples and would be considered unethical. Still, it would seem natural to conclude that the above mechanisms would apply to humans as well.

Hence, it may be postulated that the reduction in CPSP seen in this study with spinal anaesthesia may arise from the reduced stress response that occurs during surgery. This might lead to an attenuated expression of genes that are involved in central sensitization.

5.4 Genetic Analysis

Genetic factors are expected to have a wider influence on the development of CPSP after TKA. Like modifiable factors such as anaesthesia, these can affect the progression of pain throughout the postoperative period. Furthermore, genetic factors may also play a role in overall pain sensitivity that could alter the baseline pain of a patient.

A variety of gene loci have been implicated in the development of CPSP (Buskila, 2007). We have chosen six different genes for this study. *OPRM1* and *OPRK1* are both genes responsible for opioid receptors, and it is expected that changes in these receptors would lead to altered pain perception and an altered response to opioids used in the treatment of pain. *COMT* and *GCH1* are genes that encode for enzymes responsible for the metabolism of adrenergic neurotransmitters such as dopamine, norepinephrine and epinephrine. The genes *SCN9A* and *KCNS1* code for sodium and potassium ion channels present on nociceptors, and hence would be important in the transmission of pain impulses along neurones.

5.4.1 *COMT* gene

The *COMT* gene on chromosome 22 codes for the enzyme Catechol-O-MethylTransferase (COMT). This enzyme metabolises catecholamine neurotransmitters (dopamine, epinephrine and norepinephrine), by adding a methyl group (Boussetta et al., 2019). COMT itself has been extensively studied as a possible therapeutic target, most notably in Parkinsonism.

The human *COMT* gene was first described by Tenhunen et al (1994). It contains six exons, spanning over around 27,000 base pairs. Two promoters control the transcription of the gene into two different mRNA: MB-COMT and S-COMT. The former is found predominantly in brain neurones, whereas the latter is found more in other tissues such as the liver, kidney and blood.

Polymorphism of the *COMT* gene is known to affect enzyme activity. For instance, a well-studied mutation is the Val158Met, also known as rs4680, which results from a substitution of a G base to A in exon 4. The A allele of the rs4680 causes a structural change that lowers enzymatic activity so that patients metabolize catecholamines at a slower rate. Heterozygous individuals should have an intermediate activity level (Lachman et al., 1996). Similarly, rs4633 affects COMT enzyme activity, although polymorphism at this site is not associated with structural changes of the enzyme itself. The T allele is associated with lower COMT activity and the C allele with the higher COMT activity. rs4818 is not associated with any structural changes, but polymorphism at this allele is associated with even more variation in the activity of the COMT enzyme when compared to rs4680. Patients who are homozygous for the G variant should have increased enzymatic activity. Heterozygous individuals will have

intermediate activity, and homozygous individuals with the C variant should have the least enzymatic activity (Barbosa et al., 2012).

5.4.1.1 Epidemiology

Polymorphisms in the *COMT* gene appear to be common. The allele frequencies in our patients for rs4680, rs4818 and rs4633 were among the highest in the polymorphisms studied in this research. Up to half of the patients investigated in this study had at least one variant in the *COMT* gene.

This is relatively high when compared to the global prevalence of such variations, but it is similar to the incidence in other European populations (Maria et al., 2012; The 1000 Genomes Project, 2015; Machoy-Mokrzyńska et al., 2019). Even throughout Europe itself, the quoted frequencies for rs4633 range from 44% to 54%, for rs4680 from 41% to 55% and for rs4818 from 27% to 48%.

The high incidence of a minor allele implies that there would be significant clinical implications for such polymorphisms in clinical practice. It could be that *COMT* polymorphisms predispose to higher rates of osteoarthritis of the knee. This is unlikely, given that the incidence of the minor alleles in our study is comparable to that of other cohorts that involved normal populations. There could also be unrelated beneficial effects that were not explored in this study. A comparable example would be the high incidence of thalassaemia trait in Mediterranean populations, which confers protection against malaria (Hedrick, 2011).

In our study, there was evidence of a coinheritance between rs4633 and rs4680. This was also found in various other studies, with a coefficient of linkage disequilibrium D

of 1.0 (strongly coinherited) (The 1000 Genomes Project, 2015). There was still coinheritance between rs4633 and rs4818, but to a lesser degree. This would mean that future studies might focus more on either rs4633 or rs4680, since the effects of such variations may be explained by either SNP.

The high level of linkage disequilibrium between these polymorphisms is due to their physical closeness on the chromosome. It also indicates that these three polymorphisms would form part of a haploblock (Wall et al., 2003), which would mean that it would be best to analyse the effects of the haplotypes rather than just the single variation. This is also true for the *COMT* gene, with the variations in rs6269, rs4633, rs4818 and rs4680 being described most commonly in the literature (Dai et al., 2010; Zhang et al., 2015; Machoy-Mokrzyńska et al., 2019). Diatchenko et al (2005, 2006) showed that these four polymorphisms gave rise to 7 different haplotypes with a frequency of more than 0.5%. Furthermore, three haplotypes (GCGG, ATCA, and ACCG) were present in more than 95% of patients.

Unfortunately, due to technical reasons, genotyping for rs6269 was not successful, despite repeated attempts. Hence, the full haplotype for our patients could not be established. Even so, rs6269 is known to have a high linkage disequilibrium with rs4818 (Cunningham et al., 2022), so it is rare to find a patient who has only one of either polymorphism. For this reason, we still proceeded with haplotyping patients for the three polymorphisms that we could successfully genotype for.

The most common haplotype in our patients was the TCA haplotype, where patients had both rs4633 and rs4680. Such patients would have COMT with average enzyme activity and average pain sensitivity. The second most common haplotype was the

CGG, with patients having only the rs4818 polymorphism. The haplotype distribution in our study is similar to that described in other studies (Diatchenko et al., 2005; Roten et al., 2011; Zhang et al., 2015; Machoy-Mokrzyńska et al., 2019).

Given the relatively high frequency of polymorphisms in a general population, it is feasible to investigate the effects of *COMT* polymorphisms in future research. *COMT* variations are implicated in a variety of disorders besides pain. It has been studied in schizophrenia (Ma et al., 2021), ADHD (Kang et al., 2020; Liu et al., 2021), dementia (Hayek et al., 2021) and pre-eclampsia (Sljivancanin Jakovljevic et al., 2020) amongst others. It is also implicated in the different pharmacological responses to opioids and quetiapine (Zubiaur et al., 2021).

5.4.1.2 The influence of COMT *on Acute Postoperative Pain*

Lower COMT activity is associated with increased catecholamine levels, which may cause hyperalgesia through stimulation of β2-receptors (Khasar et al., 1999). Indeed, various studies have shown that polymorphisms in *COMT* genes are associated with altered pain perception. In a study of 149 children undergoing adenotonsillectomy, Sadhasivam et al (2014) found that acute pain was associated with rs6269, rs4633, rs4818 and rs4680. In the postoperative phase after cardiac surgery, patients who carried the rs4680 G>A variant had higher pain scores (Ahlers et al., 2013). Similarly, de Gregori et al (2013) studied *COMT* polymorphisms in 109 patients who underwent major abdominal and urological surgery and found that rs6269, rs4633, rs4818 and especially rs4680 are associated with higher consumption of morphine postoperatively.

On the other hand, some studies have refuted such associations. Kim et al (2006) studied 221 patients who underwent major oral surgery, and only found a weak contribution of *COMT* variations toward postoperative pain. In another study, COMT was only associated with morphine consumption in Chinese subjects, but not in patients of other ethnicities (Somogyi et al., 2016). Hu et al (2018) could not find any difference in opioid consumption in the first 48 hours postoperatively in patients with or without COMT polymorphism, in a meta-analysis of 10 studies with over 800 patients.

The present study does not support the association between *COMT* variations (rs4633, rs4818 and rs4680) and pain scores on the first day after surgery. The reasons for this lack of association compared to other studies might be numerous. For instance, the rs6269 variant was not checked for at this stage, as there were technical difficulties with genotyping this sequence with the TaqMan® assay.

Our study might have lacked the power to assess differences that might be attributable to *COMT* variations. Even so, there was no appreciable difference in the median scores in our cohort, which means that any potential effect present would be clinically small. A compounding problem was that the pain scores after surgery were unexpectedly quite low, so a much larger number of patients would have been required to detect differences.

The above studies quoted also concentrated on the first 24 hours after surgery, whereas in this study, the pain scores were assessed after 24 hours.

Morphine consumption was an end-point in a number of these studies. In our study, assessing this for *COMT* polymorphisms would be difficult since patients in Group SP did not use much morphine postoperatively. These patients accounted for half of the cohort. It would be useful to repeat such a study in different types of surgery, but excluding patients who receive neuroaxial anaesthesia.

5.4.1.3 The influence of COMT *on CPSP*

The noradrenergic system may potentially affect nociception at the peripheral, spinal and supraspinal levels (Andersen et al., 2009). In normal healthy tissue, norepinephrine has little effect. However, after injury, levels of norepinephrine may correlate with either hyperalgesia or analgesia, depending on an interplay of different receptors and neuronal pathways. Furthermore, noradrenergic neurotransmitters such as dopamine also affect the brain itself. For instance, dopamine D-1 receptors are pronociceptive, whereas stimulation of D-2 receptors appears to be effective against tonic pain (Wood, 2008).

It is no surprise that mutations in the *COMT* gene would lead to altered pain perceptions. Individuals who are homozygous for rs4680 report higher pain ratings. Zubieta et al (2003) demonstrated reduced activation of the opioid receptor in the brain by using PET scans and an opioid selective radiotracer. Fibromyalgia, a classic example of chronic pain syndromes, appears to be associated with rs4680 in various studies (Lee et al., 2015).

With regards to CPSP, the evidence for *COMT* is still somewhat inconclusive. Wang et al (2018) did not find a relationship between CPSP and the genotype of women who

had undergone a caesarean section, but the number of patients with CPSP was admittedly small. On the other hand, in patients after TKA, Thomazeau et al (2016) found a borderline significance between the rs4680 A allele and chronic pain, with an odds ratio of 3.2, but the authors comment that the study was most likely underpowered to find significant differences. Rut et al (2014) demonstrated a protective association of the minor allele of rs4633 (T) in patients one year after a lumbar discectomy. However, the same study showed that the G allele of rs4680 was associated with a better outcome, not the minor A allele as in this study or the study by Thomazeau. It is could be that COMT variations may have a different effect on different types of surgeries.

In this study, rs4633 appears to reduce the median WOMAC® Pain score at six months. This is similar to the study by Rut et al (2014) in lumbar spinal surgery, homozygous patients for the T allele of rs4633 had lower WOMAC® and WOMAC® pain scores, but only six months after surgery. Also, the G allele of rs4818 seems to increase WOMAC® and WOMAC® pain scores at six months, but this finding was not observed on linear mixed model analysis. Linear mixed models are more sensitive to outlier data and to missing data, which could explain some of the lack of consistency between the univariate analysis and the linear mixed models.

COMT polymorphisms are increasingly being researched as a haplotype, using rs6269, rs4633, rs4818 and rs4680 respectively as a haploblock. Diatchenko et al (2005) were the first to observe that these four polymorphisms produced seven haplotypes that had a frequency of more than 0.5%. The most common three haplotypes account for over 95% of all haplotypes: these are the GCGG, ATCA and ACCG haplotypes. Patients

with the GCGG haplotype possess the rs4818 mutation only, and these patients would have the highest COMT activity. Hence GCGG is classically defined as the Low Pain Sensitivity (LPS) haplotype. Conversely, ACCG is associated with the lowest COMT activity and is defined as the High Pain Sensitivity (HPS) haplotype. Finally, the ATCA haplotype confers intermediate COMT activity and is defined as the Average Pain Sensitivity (SPS) haplotype (Zhang et al., 2015).

Unfortunately, due to technical reasons, genotyping for rs6269 was not successful, despite repeated attempts. Hence, the full haplotype for our patients could not be established. On the other hand, rs6269 is known to have a high linkage disequilibrium with rs4818, so it is rare that a patient would not have both polymorphisms. Hence, we still proceeded with haplotyping patients for the three polymorphisms that we could successfully genotype for.

Contrary to the observations by Diatchenko (2005), our study found that the TCA haplotype was linked to lower pain scores. This could also be due to a number of reasons, as discussed below.

Diatchenko investigated haplotype blocks in *COMT* polymorphism in four loci, whereas we could only work genotype three loci. This should not have had any major impact on our results, since rs6269 had a strong linkage disequilibrium with rs4818. It is highly unlikely that patients with the TCA haplotype in our study would have not been classified as ATCA in Diatchenko's work: for example, the incidence for the GCGG haplotype was quoted to be 36.5%, whereas that of the ACGG was only 1%. Diatchenko compared combinations of haplotypes, whereas we only selected diplotypes with the same haplotype. Even when we used the method used by

Diatchenko, there was no relationship between pain scores and haplotype combination.

A major difference between our work and that of Diatchenko is in the cohort of patients. Both studies are comparable in number, but the patients in Diatchenko's study suffered from temporomandibular joint disorder, whereas our patients suffered from osteoarthritis of the knee. COMT activity might influence pain depending on the condition being investigated.

In the study by Rut et al (2014), data from 176 orthopaedic patients was collected for a year after lumbar spinal surgery. Contrary to Diatchenko's findings, but similar to our study, rs4633 showed a protective effect. Another study of 69 patients after lumbar spinal surgery, this time by Dai et al (2010), also found that patients with the T allele for rs4633 had better functional outcomes after twelve months. Furthermore, the ATCA haplotype was associated with better outcomes, like in our study. On the other hand, Machoy-Mokryńska et al (2019) observed higher levels of pain with the TCA haplotype.

Finally, the work by Diatchenko might not have included factors that might further influence pain, including other possible polymorphisms. Rakvåg et al (2008) investigated morphine efficiency in nearly 200 cancer patients, using 11 single nucleotide polymorphisms including rs4680, rs4818 and rs6269. This study included polymorphisms in region 1 and other promoter regions of the *COMT* gene. The authors note that direct comparison between different studies is difficult to make and that the individual polymorphism would not be the sole reason for the effect of COMT polymorphism on pain.

One limitation of our study is that we did not correlate genetic polymorphism with enzymatic activity. This has been done by Dharaniprasad et al (2020), in 216 patients after cardiac surgery. rs4680 was associated with a 14-fold lower activity in COMT activity. Indeed, patients with this polymorphism all developed CPSP.

5.4.1.4 Summary

Our study has shown that rs4633 possibly reduces WOMAC® pain scores at six months. Similarly, rs4818 appears to increase WOMAC® and WOMAC® pain scores at six months. We have found that contrary to other studies, but similar to a few others, the TCA haplotype is protective against CPSP.

5.4.2 *SCN9A* gene

The *SCN9A* gene is responsible for the coding of the alpha-subunit of the voltage-gated sodium channel, $Na_v1.7$. It is present on the short arm of chromosome 2 and spans nearly 217,000 base pairs. There are 29 exons in the gene, as characterized by Raymond et al. This work also showed how *SCN9A*, like other genes responsible for voltage-gated sodium channels, exhibit alternative splicing of some of these exons. This mechanism allows for even more variability in the resulting protein structure. Indeed, exon 5A of *SCN9A* is preferentially expressed in the peripheral nerves and central nervous system, whereas exon 5A was transcripted only in dorsal root ganglion neurones (Raymond et al., 2004).

SCN9A polymorphism is responsible for structural differences in Nav1.7, which may lead to differences in channel activity. Reimann et al (2010) investigated the functional effects of rs6746030, which is a mutation in exon 18 involving a substitution of an amino acid at position 1150. Although peak currents and time of activation or fast inactivation were not different, slow inactivation was shorter in subjects with the minor allele A of rs6746030. Slow inactivation regulates the firing frequency of neurons, so this could explain how this mutation predisposes to a greater sensitivity to pain.

Polymorphisms in this gene are implicated in erythromelalgia and similar neuropathic pain syndromes (Hisama et al., 1993), congenital insensitivity to pain (Drissi et al., 2020) and possibly epilepsy (Wallace et al., 1998; Lossin et al., 2002; Zhang et al., 2020), schizophrenia (Chen et al., 2021). *SCN9A* is also associated with Paroxysmal Extreme Pain Disorder, which is characterized by skin flushing and episodes of severe

pain (Fertleman et al., 2006). Zhong et al (2017) also related propofol sensitivity to rs6746030, with carriers of the minor allele requiring lower propofol plasma concentrations for the same effect.

The variations investigated in this study were all related to nociception, with rs6746030 being the variant most quoted in literature so far.

5.4.2.1 Epidemiology

Around half of the subjects had at least one variant of the *SCN9A* in our study, with around one-fifth of patients carrying rs7595255 or rs6746030. This prevalence of 19% is much higher than the global prevalence of 11%. Interestingly, although the European population has a prevalence of around 13%, the Maltese incidence is most similar to a British population (The 1000 Genomes Project, 2015).

There was a strong coinheritance between rs7595255 and rs6746030, which has also been demonstrated in other studies (Reimann et al., 2010; The 1000 Genomes Project, 2015). rs11898284 showed much less linkage with either of the other polymorphisms. This is not surprising, given that rs11898284 is not located as close to either 7595255 or rs6746030, and is separated by around 82 base pairs.

It is important to note that in the case of rs74449889, only the homozygous wild type (AA) was found in the population studied. This particular variant is more common in America and Eastern Asia, but otherwise absent in African and European countries (The 1000 Genomes Project, 2015). This difference in incidence might explain some heterogenicity between different studies performed in different locations. It also

means that future research using this particular polymorphism would be difficult to conduct locally.

5.4.2.2 The influence of SCN9A *on Acute Postoperative Pain*

SCN9A polymorphism is responsible for structural differences in Nav1.7, which may lead to differences in activity. Indeed, rs6746030 codes for a more excitable sodium channel (Estacion et al., 2009), and has been associated with higher pain scores in patients with back pain (Kurzawski et al., 2018).

We have not found evidence of a difference in pain scores with any of the polymorphisms investigated. We did find an association between rs7595255 and to a lesser extent rs6746030, with more morphine consumption on univariate analysis. However, such two variants were more present in patients receiving a general anaesthetic, and multivariate analysis did not confirm the association between acute postoperative pain and the investigated SNPs.

In a large study of 421 patients undergoing laparoscopic gynaecological surgery, Duan et al (2016) showed that *SCN9A* polymorphism is associated with higher pain scores. In another study of 200 patients who underwent pancreatectomy, the presence of the 3312T allele was associated with less pain after surgery (Duan et al., 2013). Yeo et al (2020) also found that rs16851799 and rs6754031 were associated with differences in postoperative VAS scores in more than 1,000 women undergoing elective total hysterectomy.

These studies did include rs6746030, but the other polymorphisms in this study have not been widely investigated. Only one other study investigated rs7595255 and

rs11898284, and this study was performed on healthy young women (Duan et al., 2015).

5.4.2.3 The influence of SCN9A *on CPSP*

Voltage-gated sodium channels are important in the generation and transmission of an action potential. Nav1.7 is involved in the initiation of an action potential and hence is important in setting the sensitivity for nociceptive signals to be transmitted (Cummins et al., 1998). Although mutations in Nav1.7 are known to be important for specific diseases, there is a lack of data on the effect of *SCN9A* polymorphism on the development of chronic pain, including postoperative pain.

The main variant of the *SCN9A* gene that showed an influence on any of the outcomes investigated was rs11898284. Carriers of the minor allele showed worse outcomes in most of the outcomes, with pain at rest at three and at six months, and WOMAC® Pain subscore at three months being significant. This was not confirmed on repeated measures analysis, but this could be the result of outlier or inconsistent data.

The lack of significance for the other outcomes would probably stem from the reduced frequency of the allele, as this was present in only 23% of the patients. There were only 11 patients who were homozygous for the minor allele of rs11898284, so interpretation of recessive analysis needs to be done cautiously. This was also the case for the other two polymorphisms.

The lack of statistical significance with wide ranges of confidence could be due to a lack of power. Since we did not know the local minor allele frequencies, it was not possible to calculate accurately the number of subjects required to refute the null

hypothesis. For this reason, we cannot categorically state that *SCN9A* polymorphism is not associated with chronic pain, but that the effect in clinical practice is likely to be limited to a small number of patients. Ideally, this should be researched with a larger number of patients.

5.4.2.4 Summary

Our study does not show that rs6746030 or rs7595255 influenced any outcomes of the study in the acute or chronic postoperative period.

There is some evidence that rs11898284 may be linked to worse outcomes, but the low frequency of the minor allele makes interpretation of such results difficult.

5.4.3 *OPRM1* gene

The *OPRM1* gene resides on the long arm of chromosome 6, and it is about 230,000 base pairs long over 18 exons (Shabalina et al., 2009). It is responsible for the MOP receptor which was previously known as the μ-opioid receptor.

The MOP receptor is a G-coupled protein receptor that binds to endomorphins and endorphins (Crist et al., 2014). Activation of the receptor leads to reduced cAMP intracellularly which causes a hyperpolarization of the cell membrane (McDonald et al., 2005). The MOP receptor is present namely in the central nervous system, especially in the periaqueductal grey zone. This is involved in descending inhibitory pathways that act on second-order neurons in the spinal cord to reduce nociception and hence induce analgesia.

Given the large size of the gene, it is not surprising that there are 3,324 documented polymorphisms of the *OPRM1* gene. Only 1,395 of these variants have a minor allele frequency greater than 1% (Spampinato, 2015; The 1000 Genomes Project, 2015).

The most commonly investigated variant is rs1799971, a mutation in exon 1 of *OPRM1*. The change of residue 40 from asparagine to aspartic acid creates a novel CpG-methylation site that prevents the upregulation of *OPRM1* (Crist et al., 2014). This change results in a three-fold increase in the binding of β-endorphin compared to the wild-type receptor (Bond et al., 1998). One would expect that this would mean that subjects with rs1799971 would have an augmented response to opioids, but in fact, the opposite seems to be true. Lötsch et al (2002) demonstrated that the pupils

constricted less in patients with the G allele and that this response was related to the number of G alleles.

rs1799971, also known as the A118G mutation, is frequently found in Asian populations (40% - 60%), less so in European populations (around 15%) and very infrequently in populations of African American descent (4%) (Levran et al., 2021). It has been linked to a poor response to opiates in several studies, both in cancer pain and postoperatively. It has also been linked to alcoholism.

Other polymorphisms also show a strong association with pain sensitivity, although more work needs to be done to confirm such findings. Shabalina et al (2009) investigated 30 candidate SNPs over *OPRM1*, focussing on polymorphisms in exons and promoter genes. With nearly 200 Caucasian subjects, the authors showed that rs563649 and the rs2075572 - rs533586 haplotype were associated with pain sensitivity. Furthermore, they showed that morphine produced less analgesia in subjects with at least one copy of rs563649, although statistical significance was not reached.

5.4.3.1 Epidemiology

In this research, the variants of *OPRM1* locally show a significantly different distribution from the global distribution. The Maltese population seems to have a lower incidence of the G allele of rs1799971, but higher incidences of the other variants. This would mean that studies performed locally involving this polymorphism might be underpowered for an analysis of a recessive model.

The most common variant in this study was rs2075572, which is associated with smoking initiation and possibly dependence (Zhang et al., 2006) The high incidence of this polymorphism locally makes it feasible to consider rs2075572 as a potential target for future studies in such a topic.

We found a very low incidence of patients who were homozygous for the minor alleles of rs1799971, rs609148 and rs563649.

Overall, a normal *OPRM1* gene with no variants was present in only 10% of patients. This makes *OPRM1* an attractive target for further research.

5.4.3.2 The influence of OPRM1 *on Acute Postoperative Pain*

The *OPRM1* gene encodes for the MOP opioid receptor, and hence it is not surprising that it has been the attention of several studies investigating postoperative pain and opioid consumption. However, such studies have yielded different conclusions.

In a study of nearly 600 women undergoing a caesarean section, Sia et al (2008) showed that pain scores and morphine consumption were higher with each copy of G allele. Similar results were obtained by Tan et al (2009), again in post-caesarean section women, and in hysterectomy patients (Sia et al., 2013). Bartosova et al (2022) compared pain scores and opioid consumption in 104 patients after inguinal hernioplasty with variants of rs1799971 and found that the G allele increased pain and opioid consumption. In the context of total knee arthroplasties, Chou et al (2006) had shown that again, the G allele conferred a poorer response to morphine after surgery.

In the present study, patients with the G allele tended to show higher pain scores and opioid consumption, but this was not statistically significant. This did not change when a dominant or recessive approach was used to analyse the genetic effect. At first, this would seem to contradict current findings, but it must be noted that most of the studies on rs1799971 were conducted on Asian patients.

A meta-analysis by Hwang et al (2014) showed that in over 4,600 patients across 18 studies, patients who had the G allele of rs1799971 had an increased need for opioids. However, this was more evident in Asian patients and in patients that had abdominal surgery. The authors note that the effect of rs1799971 was not as strong in Caucasian patients.

Thomazeau et al (2016) investigated the effect of rs1799971 on pain in 109 patients and found no there was no statistically significant effect. In their discussion, the authors mention that the incidence of rs1799971 in Caucasian populations is much smaller than in Asian populations, and that this might account for the lack of findings. In their study, only 2 patients were homozygous for the G allele: in our study, we had 7 such patients. In both studies, these patients exhibited higher pain scores and higher morphine consumption.

Hence, it cannot be excluded that there is an effect of rs1799971 on postoperative pain after TKA. However, given the low incidence of this allele, it is unlikely to play a major role in post-operative care, except in individualized protocols.

From all the other polymorphisms of *OPRM1* investigated in this study, only rs495491 showed an effect on pain scores at rest. Patients homozygous for the minor allele G

would have less pain. No data could be found in the literature related rs495491 to acute postoperative pain, so a comparison cannot be made. It would seem promising to investigate this polymorphism further to confirm these results.

5.4.3.3 The influence of OPRM1 *on CPSP*

Our study has shown that some polymorphisms in *OPRM1* are related to the development of CPSP after TKA. Patients who had the G allele of rs2075572 had lower WOMAC® scores before surgery, but not at three or six months. Similarly, patients who had the G allele of rs2075572 or the A allele of rs609148 had lower WOMAC® pain scores at baseline, but not at three or six months.

This would imply that patients with these polymorphisms might not benefit as much from surgery as patients without such polymorphisms. This result could also be due to a lack of power in the study to find better outcomes at later stages of the study.

There is also a possible interaction between rs563649 and WOMAC® pain scores, with patients who had the minor T allele appearing to be at a higher risk of CPSP. However, this did not reach statistical significance, possibly due to a lack of power.

Shabalina et al (2009) describe rs563649, rs2075572 and rs533586 as forming a potential haploblock. We did not find any relationship between our outcomes and haplotype combinations in our study. This could be the result of the different frequencies of the haplotypes that were found in their and in our study. For instance, in their study, the most common haplotype was the C-G-T haplotype, present in 61% of subjects. This was present in only 0.6% of patients in our study. It is also possible that our results would be different because of the different nature of the two studies.

Our study was a clinical study with rather elderly patients presenting for a TKA, whereas the study by Shabalina and co-workers involved healthy female volunteers characterizing their sensitivity to various noxious stimuli.

Contrary to some other studies, we did not find a strong association between CPSP and rs1799971, the most studied SNP in *OPRM1*. As discussed above, this SNP is more frequent in people of Asian descent. This was reflected in the low frequency of this polymorphism in patients enrolled in our study.

Similar to our study, there was a lack of association of polymorphisms in *OPRM1* and CPSP in other studies (Montes et al., 2015).

5.4.3.4 Summary

We have found that patients with rs2075572 had lower WOMAC® scores before surgery, and patients with rs2075572 or rs609148 had lower WOMAC® pain scores at baseline. We found a possible link between rs563649 and increased pain at 6 months after TKA, although this was not statistically significant.

5.4.4 *GCH1* gene

Found on chromosome 14 and measuring around 60,800 base pairs, *GCH1* encodes for GTP cyclohydrolase 1. This enzyme is involved in the production of the tetrahydrobiopterin (BH4), which is a cofactor for the neurotransmitters serotonin, dopamine, norepinephrine and epinephrine (Hahn et al., 2001; Zheng et al., 2019). Although not directly related to nociception, these neurotransmitters are still important in the modulation of pain control (Latremoliere et al., 2011).

In rats, BH4 levels have been associated with pain, specifically neuropathic pain. Tegeder et al (2006) demonstrated how axonal injury increased the upregulation of *GCH1* and consequently levels of BH4 in primary sensory neurons. Inhibiting the increase in BH4 levels alleviated pain, whereas administering BH4 intrathecally exacerbated the pain.

5.4.4.1 Epidemiology

The two polymorphisms investigated were quite commonly found in the local population: the minor allele of rs998259 was present in 30% of the samples, and the minor allele for rs3783641 was present in 13% of samples. These incidences were significantly different from both the global and the European incidence. The frequency of the minor allele of rs998259 is the highest found in any population studied (Cunningham et al., 2022). We cannot exclude that this could be due to some form of bias, although this is unlikely.

Both SNP's were strongly co-inherited. Indeed, Kim et al (2010) showed that there is a high linkage disequilibrium throughout the *GCH1* gene region, with the possibility of

only one haplotype being present. For their study, rs998259 was used as a marker for the pain-protective haplotype.

5.4.4.2 The influence of GCH1 *on Acute Postoperative Pain*

In this study, there was no evidence that rs998259 or rs3783641 influence the severity of acute postoperative pain after TKA. It must be pointed out that only a few patients were homozygous for the minor allele of either polymorphism.

The only study that assessed the influence of *GCH1* on acute postoperative pain was done by Lee et al (2011). Hundred patients who underwent teeth extraction under general anaesthesia were followed up for seven days with daily pain diaries. A telephone questionnaire was also used to assess pain at three months. Patients who had the wild type of allele for three separate polymorphisms of *GCH1* as described by Lötsch et al (2007), including rs3783641, had a shorter duration of pain. Our study only investigated acute postoperative pain on the first postoperative day, so we cannot easily compare our findings with this study.

Dabo et al (2010) postulate that *GCH1* polymorphism may be more related to neuropathic rather than nociceptive pain. In their study on nearly 700 women who were in labour, *GCH1* polymorphisms did not seem to affect pain during labour. They attributed this lack of effect to the fact that pain during labour is more nociceptive than neuropathic. We did not assess acute neuropathic pain after TKA, so we are not able to confirm such a hypothesis.

5.4.4.3 The influence of GCH1 *on CPSP*

Our study did not find any association between either rs3783641 or rs998259 on any outcomes at three or six months.

Tegeder et al (2006) were the first to show an effect of a pain-protective haplotype on pain scores 12 months after a lumbar discectomy. 162 patients were enrolled, with successful follow-up in 147 subjects. An additive effect of the haplotype was found: patients with no copy of the haplotype fared worse, patients homozygous for the haplotype were all better, and the heterozygous patients had an intermediate response. The authors themselves note that rs3783641 and rs8007267 would have contributed most to this effect. Furthermore, as in our study, there were only four patients that were homozygous for the pain-protective haplotype. We performed only a dominant approach, as with an additive or recessive approach the results were inconclusive.

Kim et al (2010) also showed a protective effect of rs998259 and the above-mentioned haplotype in 69 patients after surgical treatment of lumbar disc degeneration. These patients were followed up for 12 months. Functional scores improved more in patients with the minor allele of rs998259.

Similar findings to our study were shown in a study of 345 patients after elective hysterectomy (Hoofwijk et al., 2019). The presence of rs3783641 actually increased the odds of CPSP at 3 and at 12 months, although this was not statistically significant. We demonstrated the same effect in our study.

Multiple studies were either inconclusive or showed no effect of GCH1 on CPSP (Hickey et al., 2011; Belfer et al., 2015; Montes et al., 2015). A meta-analysis of studies involving rs3783641 concludes that any associations demonstrated so far are probably spurious (Chidambaran et al., 2020).

Although the minor allele was not infrequent in our sample, the low number of homozygous carriers of either SNP might have diluted a possible effect. However, our number of patients was actually larger than most of the other studies so far.

5.4.4.4 Summary

We did not find any evidence that either rs998259 or rs3783641 influence pain in the acute or chronic phase.

5.4.5 *KCNS1* gene

Found on chromosome 20, *KCNS1* is a small gene with around 11,000 base pairs (Cunningham et al., 2022). There are five exons that when transcripted produce Kcns1, one of the many potassium voltage-gated channel proteins (Deloukas et al., 2001). It is expressed mainly in neuronal tissue, with much less activity elsewhere (Fagerberg et al., 2014).

Potassium voltage-gated channels do not participate directly in signal transduction but are important in modulating the resting membrane potential. In this way, such channels either facilitate or inhibit an action potential from being generated (Tsantoulas et al., 2014). Kcns1 is a Kv9.1 channel subunit, which is electrically silent on its own, but modulates channel properties when combined with other potassium channels (Costigan et al., 2010; Bocksteins, 2016). Costigan et al (2010) also looked into neighbouring genes and found that nearly 80% of these were involved in membrane signalling, with nearly half of these associated with nociception. They conclude that Kcns1 is central to many pathways that are integral to pain perception.

Experimental data shows that mice that lack *KCNS1* suffer from a slight increase in acute pain under normal circumstances, but had an exaggerated response after nerve injury (Tsantoulas et al., 2018).

The most common polymorphism in *KCNS1* studied so far is rs734784, which is found in exon 5. This missense SNP is common in the general population (around 40 – 45%) and leads to one isoleucine amino acid being changed to a valine residue. rs734784

has been associated with increased pain in volunteers and in patients with sciatica (Costigan et al., 2010).

No functional data on rs4499491 could be found, but it does occur in a highly conserved region within *KCNS1* (Langford et al., 2014).

5.4.5.1 Epidemiology

Both rs4499491 and rs734784 are commonly found in the population studied, with 82% of patients carrying at least one copy of the variant. There is no strong co-inheritance between these two polymorphisms, which would mean that their effects may be investigated separately.

In the current population studied, rs4499491 was not in Hardy-Weinberg equilibrium. This would indicate some form of bias in the proportion of homozygotes and heterozygotes for this polymorphism. Such bias can exist if there are mutations, selection, non-random mating, genetic drift or gene flow. Given the circumstances of this research, it is probable that some form of selection occurred. For instance, since the frequency of the homozygous minor allele is much lower than found in a general population, such polymorphism might protect against osteoarthritis.

5.4.5.2 The influence of KCNS1 *on Acute Postoperative Pain*

There was no effect of either rs734784 or rs4499491 on postoperative acute pain in this study. We cannot comment on rs4499491, since this was not in Hardy-Weinberg equilibrium in our study, and this might have introduced bias.

Langford et al (2014) reported that *KCNS1* was the only potassium channel gene, out of four investigated, that was associated with breast pain before cancer surgery. The authors postulate that this pain would be due to altered neuronal excitability and that potassium channels would be a major influence on such excitability. Indeed, they found that individuals homozygous for the minor allele A of rs4499491 had a three-fold increase in reporting preoperative breast pain.

On the other hand, in another cohort of patients presenting with sciatica and who underwent a discectomy, rs734784 accounted for greater pain pre-operatively, but not after surgery (Costigan et al., 2010).

Other studies on *KCNS1* have focused on CPSP, rather than acute pain.

5.4.5.3 The influence of KCNS1 *on CPSP*

Research in *KCNS1* is relatively new, with only three studies identified before ours. Indeed, a recent meta-analysis only included two studies on the topic (Chidambaran et al., 2020).

Costigan et al (2010) looked into the pain of 151 patients a year after lumbar discectomy and found an association of greater pain with rs734784. They quote that this SNP accounted for around 5% of the variance in pain scores in these patients. The same authors also demonstrated that rs734784 was more frequent in patients who had suffered from chronic phantom pain after an amputation.

In a study of 345 women who underwent an elective hysterectomy, Hoofwijk et al (2019) found no correlation between polymorphisms of *KCNS1*, including rs734784,

and CPSP at 3 and at 12 months. Similarly, in 300 patients post-mastectomy, Langford et al (2015) did not find a difference in patients with or without this SNP. Costigan et al (2010) also did not find an association between pain at 12 months following surgery and rs734783.

We found that patients homozygous for the C allele of rs734784 had significantly less WOMAC® scores throughout the study period. Clinically, this translated to a WOMAC® score of nearly 4 points less. The WOMAC® pain scores did not reach statistical significance, but there was a similar trend. This was also seen in the percentage of patients with CPSP at six months and in the S-LANSS scores. The incidence of rs734783 in patients with possible neuropathic pain at six months was also much lower, nearly reaching significance (p=0.07).

Given such findings, we would conclude that rs734784 might have a protective benefit, seen only in homozygous patients. This is the opposite of the findings in the work done by Costigan et al. Our studies were of comparable size, and both were orthopaedic procedures although on different sites of the body. We found an association only on a recessive model, whereas Costigan et al used only an additive model.

There might be other interactions that we did not explore that could have led to our findings. For example, Kcns1 channels do not have any effect on their own but exert their influence on other potassium channels. These findings could be the result of other mutations not investigated in either study. This highlights the difficulties in genetic studies.

5.4.5.4 Summary

We found that patients who were homozygous for rs734784 had lower WOMAC® scores, and tended to have lower incidences of CPSP and evidence of neuropathic pain.

5.4.6 *OPRK1* gene

The human gene *OPRK1* has been characterized only in 2004, and it is the gene responsible for the KOP opioid receptor (Yuferov et al., 2004). It is 26,000 base pairs long on chromosome 8, spread over 4 exons.

The KOP receptor mediates analgesia without causing respiratory depression (Pathan et al., 2012). Indeed, although all opioids act on MOP receptors, some opioids such as morphine and oxycodone exhibit some activity also on KOP receptors.

The primary ligand to KOP is dynorphin, which induces analgesia. The KOP receptor is widely distributed in the central nervous system, including in the spinal cord and brainstem (Cahill et al., 2014). Dynorphin is emerging as an important factor in the development of chronic pain (Podvin et al., 2016). The pain appears to induce an increase in dynorphin levels in the spinal cord, as shown by Wagner et al (1993)in a neuropathic pain model in rats. This increase in dynorphin occurred 21 days after injury and was observed bilaterally in the spinal cord. It is not clear if such a consequence further augments chronic pain, or if this is protective (Caudle et al., 2000). Dynorphin injected intrathecally induces analgesia, but it has only been tested in animal models – unfortunately, it is associated with paralysis of the hind limbs when used in this manner. Caudle et al postulate that dynorphin may act to reduce pain in the initial phases of injury: this effect has also been seen in knockout mice who had the KOP receptors deleted (Schepers et al., 2008). Such mice exhibited increased hyperalgesia after injury.

rs6985606 has been linked with opioid and alcohol dependence (Zhang et al., 2008; Karpyak et al., 2013), and also with the severity of HIV infection and response to treatment (Proudnikov et al., 2013).

5.4.6.1 Epidemiology

rs6985606 was commonly found in the study population, with a local minor allele frequency of 43%. Like most other polymorphisms investigated in this study, this is similar to the frequency found in European populations.

Such a high frequency of this polymorphism means that it is a good candidate for future studies since it is common enough to allow for the comparison of different genotypes.

5.4.6.2 The influence of OPRK1 *on Acute Postoperative Pain*

Nielson et al (2016) showed how gene polymorphisms at *OPRK1* may affect pain sensitivity, with carriers of variant *OPRK1* having a higher pain tolerance. This was not confirmed in other studies, such as by Huang et al (2008) in 72 females or by Olesen et al (2018) in nearly a hundred patients with osteoarthritis of the hip.

Mutations in *OPRK1* might also affect pain by influencing sensitivity to opioid analgesics, such as morphine or oxycodone. However, Olesen et al (2015) and Nielsen et al (2016) found no such effect in eight polymorphisms of *OPRK1.* However, Ho et al (Ho et al., 2020) did observe that rs720764 increased the pain threshold in response to butorphanol, which is a specific KOR agonist. rs6985606 was not one of the polymorphisms investigated in either of these studies.

In our study, rs6985606 did not have any influence on any of the pain scores in the acute setting.

5.4.6.3 The influence of COMT *on CPSP*

We hypothesized that rs6985606 might have an influence on chronic pain, based on a potential interaction with opioid use. We chose this SNP because it was the most common polymorphism at the *OPRK1* gene that could be analysed using a validated TaqMan® SNP genotype assay – other polymorphisms had a minor allele frequency of less than 10%. Furthermore, it shows a high linkage disequilibrium with a large number of other polymorphisms of *OPRK1* (Cunningham et al., 2022). This means that it is highly likely that other polymorphisms would be present if the patient would be a carrier for rs6985606.

Literature on *OPRK1* polymorphisms and pain development is still scarce, and less is known on rs6985606. Most of such literature reflects research on opioid dependence (Crist et al., 2018) and on the analgesic response to opioids.

Our study does not support our hypothesis, and there was no influence of rs6985606 on any of the outcomes for CPSP. There might be no effect of rs6985606 on KOR receptors. rs6985606 is an intronic mutation, which means that the change in DNA code occurs in a part of the gene which is not expressed in the final protein (Sherry et al., 2001). Still, since rs6985606 is highly linked with other polymorphisms including exon mutations such as rs702764 (Cunningham et al., 2022), it is likely that *OPRK1* mutations in general do not affect CPSP.

Our study only investigated the presence of a mutation. Due to the nature of the study, we could not assess the expression of mRNA that could vary during chronic pain. Wawrzczak-Bargieła et al (2020) observed increases in mRNA expression of both *PDYN* which expresses dynorphin, and *OPRK1* in mice in a neuropathic pain model. However, if such increased mRNA expression had occurred in our patients, we should have been able to see a larger effect, rather than no effect at all.

Furthermore, high levels of dynorphin cause a down-regulation of opioid receptors, including KOP receptors (Podvin et al., 2016). It may have been more appropriate for us to target *PDYN*, the gene for dynorphin expression, rather than *OPRK1*. However, using *OPRK1* in our study allowed us to check for any differences in opioid use. For instance, Kringel et al (2017) explored the use of a number of biomarkers that could be used to identify patients requiring high doses of opioids. Nine potential SNP's in the *OPRK1* gene were flagged for future research.

5.4.6.4 Summary

We did not find any evidence that rs6985606 influences pain in the acute or chronic phase.

5.5 Summary

5.5.1 Demographics

Our study showed that:

- On the first postoperative day, older patients tend to report lower pain scores at rest than younger patients.
- Similarly, male patients reported lower pain scores during physiotherapy.
- Female gender, younger age and increasing BMI were associated with higher WOMAC® pain scores throughout the study period.
- Neuropathic pain seems to be an important factor in the development of CPSP.

5.5.2 Influence of Anaesthesia

Our study showed that

- Patients who have received spinal anaesthesia have much lower consumption of morphine, but show increased pain scores during physiotherapy on the first day after the surgery.
- A spinal anaesthetic seems to reduce WOMAC® and WOMAC® pain scores
- The incidence of CPSP appears to be reduced with a spinal anaesthetic compared to general anaesthesia and a femoral block.

5.5.3 Influence of Genetics

Most of the polymorphisms included were frequently encountered in the study population. The genetic frequencies of these mutations are very similar to those found in a European population, except for rs998259 and rs3783641 (*GCH1*) and for rs495491 and rs533586 (OPRM1). This is consistent with previous studies that show that Maltese genomics is strongly related to Sicilian and mainland Italy (Capelli et al., 2006; Caruana, 2012; Lazaridis et al., 2014).

Importantly, some of the polymorphisms investigated exhibited high levels of co-inheritance. It would be possible to choose one of such polymorphisms rather than a genotype for all the co-inherited mutations. Of note, no patient in the study had the G allele of rs74449889. Future research might wish to avoid this particular polymorphism unless specifically indicated.

We found an association between preoperative pain scores and polymorphism in *OPRM1* (rs2075572, rs609148 and rs495491) and *KCNS1* (rs734784).

The only gene to influence acute postoperative pain was *OPRM1* (rs495491, possibly rs1799971).

An interaction between *COMT* (rs4633, rs4818), *SCN9A* (rs11898284), *OPRM1* (rs2075572), *KCNS1* (rs734784) and outcomes was seen at six months.

5.6 Limitations

The study was conducted in a single centre which may limit generalisability to other health-care settings. Indeed, Bellomo et al (2009) advice against using single-centre studies for preparing practice guidelines due to the risk of selective bias. Furthermore, single centre studies tend to be smaller and have limited resources (Meinert et al., 1986). However, TKA is a well-standardized technique across different centres, with minimal variations. Hence, we believe that our results should be applicable to other centres.

The main focus of this study was on CPSP, rather than acute post-operative pain. One of the major limitations of the study would be the limited data collection in the early postoperative phase, for instance few hours after the procedure. Other authors have already investigated acute postoperative pain, even locally (Sciberras et al., 2011; Zammit et al., 2012; Santucci et al., 2016). In this study, the main aim was to assess acute pain as a potential influence on CPSP.

Another limitation was a lack of standardization in anaesthetic care. For instance, the drugs and doses used for the femoral nerve block were not defined in the trial protocol. This was done to ensure that the study would better reflect clinical practice. Still, most femoral nerve blocks were done in a comparable manner, using 0.5% plain bupivacaine.

The use of a femoral block in Group GA but not in Group SP could be considered a limitation in that our study compares general anaesthesia with a femoral block to spinal anaesthesia. This would mean that this study is not a study comparing spinal

anaesthesia to general anaesthesia. Furthermore, all patients in the spinal group received intravenous infusion of propofol using a Target-Controlled Infusion (TCI) syringe pump. This might have influenced results.

Our data on pain outcomes in the later stages of the study were conducted by telephone questionnaires. This means that there is an element of subjectivity, although the use of validated scores like the WOMAC® and the S-LANSS should have mitigated this limitation. Ideally, it would have been best if patients identified to suffer from CPSP were to have been examined in hospital. However, most patients were elderly. Most of such patients had already been discharged from medical care, and we feared that most would not have accepted an examination at hospital. For example, Montes et al (2015), who did examine and performed such examinations in person, lost nearly 24% of patients to follow-up, compared to our study where we lost only 11% of patients.

The initial assessments done pre-operatively in person were done by a number of data collectors. This might have introduced an element of inter-individual variation. However, both the WOMAC® and the S-LANSS have been extensively validated and show limited variation between observers. Furthermore, every observer was trained in the use of such questionnaires.

The telephone questionnaires were performed by only two observers, in order to minimise inter-observer variation. Although there could be internal bias, we tried to minimiise this by blinding each step. Indeed, at the time of the telephone questionnaires, the observers did not have access to previous scores or to the allocation of the intervention.

Most of the upregulation responses noted in animal studies were noted hours and days after injury, since this involved sacrificing the animal to obtain histological specimens of the spinal cord. It is possible that the duration of our study reserved for acute pain was not long enough to allow for an increase in genetic transcription. On the other hand, other studies that collected data did show differences in the early postoperative phase, possibly due to altered medication sensitivity and effect. We did not assess for such interactions.

The biggest limitation of the genetic analysis was the lack of power to detect the influence of some of the polymorphisms investigated. These occurred in low frequency in the population, and this would make detecting any changes between groups more difficult. Montes et al (2015) calculated that 500 cases and 500 controls were required to detect an odds ratio greater than 1.5 in a simple allelic test. If the SNP might have a recessive role, that is, a patient would need to be homozygous for the polymorphism, then it is possible that such differences might not be clinically relevant on a large scale. Latremoliere et al (2011) point out that for a minor allele with a frequency of 1%, 400 patients would need to be included to obtain just 4 homozygous carriers of the allele.

Furthermore, the design of the study might not have helped to explore genetic factors. The use of a randomized control trial for exploring the association between the type of anaesthesia and CPSP means that this could be a confounding factor. Indeed, spinal anaesthesia was found to be protective. Although we tried to control for the effect of anaesthesia using a linear mixed model analysis for each SNP, splitting the group into four combinations (anaesthesia vs genotype) reduces the sensitivity of any statistical method. We also tried to explore a possible interaction of genotype and type of

anaesthesia. We did this to check if any genotype would benefit from a particular form of anaesthesia. Unfortunately, this was even more complicated and would require a large number of patients.

5.7 Conclusion

This is the first study to investigate the role of anaesthesia in the development of chronic pain after TKA. Furthermore, it is the first study to offer genotyping information of several polymorphisms in the local population.

We used a standardized analgesic protocol for our patients. This was well tolerated, and showed better pain relief than conventional local practice. There is still room for improvement, since nearly 9% of patients rated their pain in the first 24 hours as being severe.

The patients in our cohort had good outcomes after TKA. WOMAC® scores were lower than in other comparable studies. The incidence of CPSP at 6 months was 11% when defined as a WOMAC® pain score greater than 5, but a significant number of pains had more pain than before surgery. Indeed, the number of patients who reported a high S-LANSS was higher at six months than at three months.

We have shown that a spinal anaesthetic may reduce the intensity of CPSP at three months and may reduce the incidence of CPSP at six months. We hypothesize that this may be due to a reduced stress response during surgery amongst other possible causes.

With regards to genetic factors, our findings show that the Maltese population is very similar to a European population with regards to most SNP frequencies. However, rs998259 and rs3783641 (*GCH1*) and for rs495491 and rs533586 (OPRM1) were more frequent in our cohort.

A number of genetic loci show promise in predicting CPSP after TKA. However, our study lacked the power to confidently detect differences in outcomes. Of note, polymorphism in *OPRM1* seems to influence pain preoperatively, peri-operatively and in the longer term. *COMT*, *SCN9A* and *KCNS1* also merit further research in this subject.

Chapter 6

Further Work

Although comprehensive, our study still leaves some unanswered questions, such as confounding effects like propofol used for sedation in patients with spinal anaesthesia. Furthermore, the full significance of neuropathic pain and CPSP was not investigated.

It would be useful to validate these results with a multi-centre trial. This would help to enroll more patients and give more significance to the applicability of this research. Such a multi-centre study may involve societies like the European Society of Anaesthesia and Intensive Care and equivalents, who might offer the necessary funding to perform larger trials.

We would propose extending this research by comparing other forms of anaesthesia. It would be interesting to compare total intravenous anaesthesia (TIVA) to a spinal anaesthesia. This would explore the role of propofol infusions intra-operatively on CPSP. This should be another randomized controlled trial, with at least two treatment arms. TIVA is not routine for TKA, although Harsten et al (2013) did compare TIVA with propofol and remifentanil to a spinal anaesthesia. This study looked at short term outcomes after TKA.

The increase in neuropathic pain throughout the study period merits further investigation. We did not explore non-genetic factors that might explain the occurrence of neuropathic pain, such as diabetes mellitus. Indeed, literature on diabetes and CPSP is very scarce. Only one retrospective observational study describes how diabetes seems to increase the risk of CPSP after thoracotomy (Wang et al., 2012). Ideally, such research should include HbA1c levels to better define the presence of diabetes, but such research should be relatively easy to perform.

More difficult to perform would be investigating the role of injury of the infrapatellar branch of the saphenous nerve (IPBSN). Hopton et al describe (2004) how 60% of 113 patients after a TKA had lateral skin numbness. Our study showed that half of our patients had medial skin numbness, an area supplied by the IPBSN. Clendeden et al (2015) showed resolution of pain in 9 out of 16 patients after infiltration with local anaesthesia around the IPBSN. It would be feasible to ask patients who show signs of CPSP to undergo a simple ultrasound scan, and if positive for such injury, to have said infiltration.

The research project only included patients who had undergone a TKA which could limit the applicability of this study. Therefore it would be worth repeating the study in other forms of surgical procedures with high risk of CPSP, such as inguinal hernia repair, caesarean sections. There are issues however. Inguinal hernias are being done laparoscopically more frequently, which precludes a spinal anaesthetic. Similary, caesarean sections are routinely done under spinal anaesthesia, except in very urgent and emergent cases.

One of the main limitations of the study was a lack of power for certain genotypes. Increasing the number of patients would of course help, although one might argue that certain genotypes are too infrequent to be of clinical use in predicting individual risk.

Clinically, this study could help in two ways. First of all, genotyping for *KCNS1*, *OPRM1* and *COMT* might be used to predict individual risk of CPSP. This has already been done to predict risk in other disorders (Wray et al., 2008). Of course, other factors also play

a role, and the ultimate phenotype cannot always be derived from a genotype . Still, such work could help allocate more resources to patients with certain genotypes.

Secondly, and most importantly, this research project highlights how common CPSP. Furthermore, it is possible to identify at risk patients even at 3 months after surgery. Hence, we propose that patients who present with a high WOMAC® pain subscore at three months should be referred to a Pain Clinic. This will allow better follow-up and management of such cases.

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APPENDIX A Consent Form

Consent For Participation in Clinical Trial

We are investigating the occurrence of Chronic Pain after a Total Knee Replacement. We would like to check:

- the number of patients who develop chronic pain 3 – 6 months after the operation
- if Chronic Pain is dependant on different types of anaesthesia
- if Chronic Pain is dependant on genetic factors

For this reason, the research will involve:

- Taking a blood sample from all patients to determine genetic profile
- Half of the patients will receive a SPINAL anaesthetic and half will receive a GENERAL anaesthetic
- We shall be checking pain scores within 48 hours of the operation, and after 3 – 6 months (by telephone)

Your data will be kept personal, and will not be divulged to third parties. The data and your genetic profile will be used only for the purposes of this research. Blood samples will be stored for 10 years, then destroyed once all research is complete. Furthermore, you have the right to withdraw your consent at any point: in such cases, we shall not be using any of your data.

Should you have any queries, before or after enrolment, you may contact the researchers as shown below.

Name:

I agree to the above, and am willing to participate in the study.

MAIN RESEARCHER
Dr Stephen Sciberras
Consultant Anaesthesia,
Mater Dei Hospital
7984 7658
(Ethics approval number:)

Kunsens ghal Ricerka

Nixtiequ nistudjaw kemm il-persuna jkollhom ugigh kroniku wara li jkollhom Total Knee Replacement. Nixtiequ naraw:

- In-numru ta' nies li ikollhom ugigh kroniku 3 – 6 xhur wara l-operazzjoni
- Jekk dan l-ugigh kroniku huwiex dependenti fuq it-tip ta' loppju li tkun hadt
- Jekk hemm fatturi genetici ghal dan l-ugigh

Minhabba f'hekk, nixtiequ:

- Niehdu ftit demm biex inkunu nistghu naraw fatturi genetici
- Nofs il-pazjenti se jkollhom loppju SPINAL, u n-nofs l-iehor se jkollhom loppju GENERALI
- Se naraw l-ammont ta' ugigh fl-ewwel 48 siegha wara l-operazzjoni, u wara 3 – 6 xhur (b'telefonata)

Id-dettalji tieghek se jinzammu private, u mhux se jinghataw lil terzi persuni. L-informazzjoni migbura, kif ukoll testijiet genetici se jintuzaw biss ghal dan l-istudju. Dawn se jithassru wara r-ricerka tkun lesta. Id-demm li jittiehed se jinzamm ghal 10 snin, imbaghad jintrema'. Tista' ukoll tiddecidi li ma' tkomplix tiehu sehem f'din ir-ricerka: f'dak il-kaz, l-informazzjoni migbura fuqek mhix se tintuza.

Jekk ghandek aktar mistoqsijiet, qabel jew wara li tibda tiehu sehem f'din ir-ricerka, tista' tikkuntatja lill-persuni indikati isfel.

Isem:

Naqbel ma' dak li ntqal lili, u nixtieq niehu sehem f'din ir-ricerka

RESEARCH SUPERVISOR
Prof G Laferla
Faculty of Medicine and Surgery

APPENDIX B Ethics Approval

L-UNIVERSITÀ TA' MALTA
Msida – Malta
Skola Medika
Sptar Mater Dei

UNIVERSITY OF MALTA
Msida – Malta
Medical School
Mater Dei Hospital

Ref No: **05/2017**

Thursday 4th May 2017

Dr Stephen Sciberras
Department of Anaesthesia
Mater Dei Hospital

Dear Dr Stephen Sciberras,

Please refer to your application submitted to the Research Ethics Committee in connection with your research entitled:

The effect of Regional Anaesthesia and Genetic Factors on the development of Chronic Pain following Knee Arthroplasty

The University Research Ethics Committee granted ethical approval for the above mentioned protocol.

Yours sincerely,

Dr. Mario Vassallo
Chairman
Research Ethics Committee

Email: umms@um.edu.mt • Web: http://www.um.edu.mt/hms

APPENDIX C Code used for randomization

```
<?php

include ("db_connect.php");

try {
    $conn = new PDO("mysql:host=$servername;dbname=$myDB",
$username, $password);
    // set the PDO error mode to exception
    $conn->setAttribute(PDO::ATTR_ERRMODE, PDO::ERRMODE_EXCEPTION);
    }
catch(PDOException $e)
    {
    echo "Connection failed: " . $e->getMessage();
    }

if ($_REQUEST['test'] == 'true') {echo "test:  <br>";}

// get this patient's details
$stmt = $conn->prepare("SELECT age, sex, bmi, surgeon, prewomac,
randomized FROM patients WHERE refnumber = (:refnumber)");
$stmt->bindParam(':refnumber', $refnumber);
$refnumber = $_REQUEST["refnumber"];
$stmt->execute();
$subject = $stmt->fetch(PDO::FETCH_ASSOC);

if ($subject['randomized'] != "N") {
   return;
 }

// Get characteristics of all those already randomized
// prepare sql and bind parameters
$stmt = $conn->prepare("SELECT * FROM patients WHERE consented ='Y'
AND randomized !='N' AND included ='Y'");
$stmt->execute();

// prepare sql
$stmt = $conn->prepare("SELECT anaesthesia FROM patients WHERE age
> (:agelower) AND age < (:agehigher) AND randomized != 'N' AND
included ='Y'");
$stmt->bindParam(':agelower', $agelower);
$stmt->bindParam(':agehigher', $agehigher);
switch (true) {
  case ( $subject["age"] < 65 ):
    $agelower = 0;
    $agehigher = 65;
    break;
  case ( $subject["age"] > 69 ):
    $agelower = 69;
    $agehigher = 80;
    break;
  default:
    $agelower = 64;
    $agehigher = 70;
    break;
}
```

```
$stmt->execute();
$age_results = $stmt->fetchAll(PDO::FETCH_ASSOC);
$age_counts = array("spinal"=>0, "general"=>0);
for ( $i=0; $i <= $stmt->rowCount();$i++)
{
  if ($age_results[$i]['anaesthesia'] == "S") {
$age_counts["spinal"]++; }
  if ($age_results[$i]['anaesthesia'] == "G") {
$age_counts["general"]++; }
}


$stmt = $conn->prepare("SELECT anaesthesia FROM patients WHERE bmi
> (:bmilower) AND bmi < (:bmihigher) AND randomized != 'N' AND
included ='Y'");
$stmt->bindParam(':bmilower', $bmilower);
$stmt->bindParam(':bmihigher', $bmihigher);
switch (true) {
  case ( $subject["bmi"] < 35 ):
    $bmilower = 0;
    $bmihigher = 35;
    break;
  default:
    $bmilower = 34;
    $bmihigher = 70;
    break;
}
$stmt->execute();
$bmi_results = $stmt->fetchAll(PDO::FETCH_ASSOC);
$bmi_counts = array("spinal"=>0, "general"=>0);
for ( $i=0; $i <= $stmt->rowCount();$i++)
{
  if ($bmi_results[$i]['anaesthesia'] == "S") {
$bmi_counts["spinal"]++; }
  if ($bmi_results[$i]['anaesthesia'] == "G") {
$bmi_counts["general"]++; }
}

$stmt = $conn->prepare("SELECT anaesthesia FROM patients WHERE sex
= (:gender) AND randomized != 'N' AND included ='Y'");
$stmt->bindParam(':gender', $gender);
$gender= $subject["sex"];
$stmt->execute();
$sex_results = $stmt->fetchAll(PDO::FETCH_ASSOC);
$sex_counts = array("spinal"=>0, "general"=>0);
for ( $i=0; $i <= $stmt->rowCount();$i++)
{
  if ($sex_results[$i]['anaesthesia'] == "S") {
$sex_counts["spinal"]++; }
  if ($sex_results[$i]['anaesthesia'] == "G") {
$sex_counts["general"]++; }
}

$stmt = $conn->prepare("SELECT anaesthesia FROM patients WHERE
surgeon = (:surgeon) AND randomized != 'N' AND included ='Y'");
$stmt->bindParam(':surgeon', $surgeon);
$surgeon = $subject["surgeon"];
$stmt->execute();
```

```
$surgeon_results = $stmt->fetchAll(PDO::FETCH_ASSOC);
$surgeon_counts = array("spinal"=>0, "general"=>0);
for ( $i=0; $i <= $stmt->rowCount();$i++)
{
  if ($surgeon_results[$i]['anaesthesia'] == "S") {
$surgeon_counts["spinal"]++; }
  if ($surgeon_results[$i]['anaesthesia'] == "G") {
$surgeon_counts["general"]++; }
}

$stmt = $conn->prepare("SELECT anaesthesia FROM patients WHERE
prewomac > (:prewomaclower) AND prewomac < (:prewomachigher) AND
randomized != 'N' AND included ='Y'");
$stmt->bindParam(':prewomaclower', $prewomaclower);
$stmt->bindParam(':prewomachigher', $prewomachigher);
switch (true) {
  case ( $subject["prewomac"] < 25 ):
    $prewomaclower = 0;
    $prewomachigher = 25;
    break;
  case (( $subject["prewomac"] > 26 ) AND ( $subject["prewomac"] <
51 )):
    $prewomaclower = 26;
    $prewomachigher = 50;
    break;
  case ( $subject["prewomac"] > 51 ):
    $prewomaclower = 51;
    $prewomachigher = 75;
    break;
  default:
    $prewomaclower = 76;
    $prewomachigher = 100;
    break;
}
$stmt->execute();
$prewomac_results = $stmt->fetchAll(PDO::FETCH_ASSOC);
$prewomac_counts = array("spinal"=>0, "general"=>0);
for ( $i=0; $i <= $stmt->rowCount();$i++)
{
  if ($prewomac_results[$i]['anaesthesia'] == "S") {
$prewomac_counts["spinal"]++; }
  if ($prewomac_results[$i]['anaesthesia'] == "G") {
$prewomac_counts["general"]++; }
}


//calculate total score for spinals
$spinals = $age_counts["spinal"] + $bmi_counts["spinal"] +
$sex_counts["spinal"] + $surgeon_counts["spinal"] +
$prewomac_counts["spinal"];
$general = $age_counts["general"] + $bmi_counts["general"] +
$sex_counts["general"] + $surgeon_counts["general"] +
$prewomac_counts["general"];

echo $_REQUEST['test'];
if ($_REQUEST['test'] == 'true')
{
```

```
  echo "spinals - age: " . $age_counts["spinal"] . ",  bmi: " .
$bmi_counts["spinal"] . " , sex: " . $sex_counts["spinal"] . "
surgeon: " . $surgeon_counts["spinal"] . ",  prewomac:  ".
$prewomac_counts["spinal"];
  echo "<br>general - age: " . $age_counts["general"] . ",  bmi: "
. $bmi_counts["general"] . " , sex: " . $sex_counts["general"] . "
surgeon: " . $surgeon_counts["general"] . ",  prewomac:  ".
$prewomac_counts["general"];
  echo "<br>spinals: ". $spinals . " general: ".$general;

  return false;
}


switch (true) {
  case ( $spinals > $general ):
    echo json_encode ( array('RNDM' => "General") );
    $stmt = $conn->prepare("UPDATE patients SET randomized = 'G'
WHERE refnumber = (:refnumber)");
    $stmt->bindParam(':refnumber', $refnumber);
    $refnumber = $_POST["refnumber"];
    $stmt->execute();
    return;
  case ( $spinals < $general):
    echo json_encode ( array('RNDM' => "Spinal") );
    $stmt = $conn->prepare("UPDATE patients SET randomized = 'S'
WHERE refnumber = (:refnumber)");
    $stmt->bindParam(':refnumber', $refnumber);
    $refnumber = $_POST["refnumber"];
    $stmt->execute();
    return;
  case ( $spinals == $general):
    // randomize to one group
    if ( (rand ( 0 , 1000 )) > 250 ){
      echo json_encode ( array('RNDM' => "Spinal") );
      $stmt = $conn->prepare("UPDATE patients SET randomized = 'S'
WHERE refnumber = (:refnumber)");
      $stmt->bindParam(':refnumber', $refnumber);
      $refnumber = $_POST["refnumber"];
      $stmt->execute();
    }  else {
      echo json_encode ( array('RNDM' => "General") );
      $stmt = $conn->prepare("UPDATE patients SET randomized = 'G'
WHERE refnumber = (:refnumber)");
      $stmt->bindParam(':refnumber', $refnumber);
      $refnumber = $_POST["refnumber"];
      $stmt->execute();
    }

    return;
}
?>
```

APPENDIX D S-LANSS questionnaire

In the area where you have pain, do you also have “pins and needles”, tingling or prickling sensations?

Fil-post li għandek l- uġigħ, thossha mtarxa, jew bhal tnemnim, jew qisu tordqodlok?

Yes (5)	No (0)
Iva	Le

Does the painful area change colour (perhaps look mottled or more red) when the pain is particularly bad?

Fil-post li għandek l- uġigħ, tinduna li jkollok tbiddil fil-kulur (aktar abjad jew ahmar) meta l-ugigħ ikun qawwi?

Yes (5)	No (0)
Iva	Le

Does your pain make the affected skin abnormally sensitive to touch? Getting unpleasant sensations or pain when lightly stroking the skin might describe this.

Thoss li fil-post li għandek l- uġigħ, il-ġilda hija aktar sensitiva?

Yes (3)	No (0)
Iva	Le

Does your pain come on suddenly and in bursts for no apparent reason when you are completely still? Words like “electric shocks”, jumping and bursting might describe this.

Ikollok drabi meta l-ugigħ jigi f’salt, mingħajr raguni, anke meta ma tkunx qegħda ticcaclaq? Affarjiet bhal: “xokkijiet”, “taqbez”, jew “tinfaqa’”.

Yes (2)	No (0)
Iva	Le

In the area where you have pain, does your skin feel unusually hot like a burning pain?

Fil-post li għandek l-uġigħ, thoss li l-ġilda tieghek tkun qisha taħraq bl-ugigħ?

Yes (1)	No (0)
Iva	Le

Gently rub the painful area with your index finger and then rub a non-painful area (for example, an area of skin further away or on the opposite side from the painful area). How does this rubbing feel in the painful area?

Bil-mod, ħokk il- ġilda tal-post fejn tħoss l-ugigħ, u post ieħor fejn ma jkollox ugigħ. Tħoss differenza bejn iż-żewg naħat?

Yes (3)	No (0)
Iva	Le

Gently press on the painful area with your finger tip and then gently press in the same way onto a non-painful area (the same non-painful area that you chose in the last question). How does this feel in the painful area?

Bil-mod, għafas il- ġilda tal-post fejn tħoss l-ugigħ, u post ieħor fejn ma jkollox ugigħ. Tħoss differenza bejn iż-żewg naħat?

A score of 12 or higher suggests pain is predominantly neuropathic in origin