Optimising opioids for cancer pain: Investigating the basis of inter-individual analgesic response and side-effect profile

by

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Declaration of Originality

I, Joanne Droney, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

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This work could not have been carried out without the support of a number of people and organisations and it is my pleasure to thank them now.

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Abstract

There is significant inter-individual variation in response to morphine in terms of analgesia and side-effects. In recent years there has been growing interest in the possibility that genetic factors may play a role in variability in morphine response.

The aims of this thesis were to develop a clinically relevant method of defining response to morphine, to investigate how multiple clinical and genetic factors may interact together to influence response to morphine and to explore constipation as a common side-effect of morphine.

Clinical and biological data were collected as part of two clinical trials: 1) A prospective observational study which included patients taking oral morphine for cancer pain (N=298) and 2) A prospective follow-up randomised controlled trial of oral morphine versus oral oxycodone for cancer pain (recruitment ongoing). Symptom complexes were examined using Principal Components Analysis. Genetic association testing was carried out using both the candidate gene approach (sequence-specific primers with polymerase chain reaction) and genome-wide assays. Multivariate regression analyses were used to explore gene-gene and gene-environment interactions. Preliminary testing of a constipation assessment tool was performed.

Analgesic response and central side-effects appear to be distinct components of morphine response. The genetic and clinical factors associated with these clinical outcomes and with daily morphine dose requirements are markedly different.
There is inter-individual variation in bowel function in cancer patients on oral morphine. Constipation is a common symptom and is generally poorly managed.

It is too early to be able to apply the results of genetic association studies of morphine response in cancer pain to clinical practice. Response to morphine is complex, both in terms of clinical confounders and pharmacogenetics. Challenges for future research include carrying out carefully designed studies of adequate power, standardising outcome measures of response to morphine and expansion of the genetic association testing.

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List of Abbreviations

AA	Amino acid
ADORA1	A1-adenosine receptor
ADRA2A	α_{2A} adrenergic receptor
ALOX12	arachidonate 12-lipoxygenase
ARRB2	B-arrestin 2 gene
Asn	Asparagine
Asp	Aspartic acid
bp	Base pair
CEU	HapMap population of Utah residents of northern and western European ancestry
CI	Confidence Interval
COMT	catechol-O-methyltransferase
CRF	Clinical research file
dH2O	Distilled water
DIP	deletion-insertion polymorphism
DNA	Deoxyribonucleic acid
dsDNA	Double stranded DNA
DOR	Delta opioid receptor
dATP	deoxyadenosine triphosphate
dCTP	deoxyguanosine triphosphate
dGTP	deoxycytidine triphosphate
dNTP	Deoxyribonucleotide triphosphate
dTTP	deoxythymidine triphosphate
DRG	Dorsal Root Ganglion
ds	Double stranded
EDTA	Ethylenediaminetetraacetic acid
GABA	*gamma*-Aminobutyric acid
GCH1	Guanosine Triphosphate Cyclohydrolase 1
GCP	Good Clinical Practice
GWAS	Genome Wide Association study
HWE	Hardy Weinberg Equilibrium
KOR	Kappa opioid receptor
LD	Linkage Disequilibrium
LDG	Laxative Dose Group
LOD score	logarithm (base 10) of odds score. An estimate of whether two loci are likely to lie near each other on a chromosome and thus likely to be inherited together. A LOD score of ≥3 suggests that the odds are 1000 to 1 in favour of genetic linkage i.e. a LOD score of ≥3 suggests that the two loci are close in terms of genetic distance.
mAF	Marker Allele Frequency
MAF	Minor Allele Frequency
MDA	Multiple Displacement Amplification
MDR1	Multidrug resistance 1 gene
Met	Methionine
M-3-G	morphine-3-glucuronide
M-6-G	morphine-6-glucuronide

MHRA	Medicines and Healthcare Products Regulatory Agency
MOR	Mu-opioid receptor
mRNA	Messenger RNA
NRS	Numerical rating scale
OPRM	Mu opioid receptor gene
OPRD	Delta opioid receptor gene
OPRK	Kappa opioid receptor gene
OR	Odds Ratio
PCA	Principal Components Analysis
PCR	Polymerase Chain Reaction
PR	Prolonged release
R&D	Research and Development Committee
REC	Research Ethics Committees
rpm	Revolutions per minute
sAF	Susceptibility allele frequency
SC	subcutaneous
SD	Standard Deviation
SEM	Standard error of the mean
SERT	Serotonin reuptake transporter
SLC6A4	Gene coding for the serotonin reuptake transporter
SNP	Single Nucleotide Polymorphism
SSP	Sequence specific primer
Ta	Annealing Temperature (During PCR)
Tm	Melting Temperature (During PCR)
TRPV1	Transient receptor potential cation channel, subfamily V / capsaicin receptor
UGT2B7	uridine diphosphate-glucuronosyltransferase 2B7
Val	Valine
VNTR	Variable Number Tandem Repeat
WHO	World Health Organisation
12(S)-HPETE	12(S)-hydroperoxy-5,8,10,14-eicosatetraenoic acid

Colour scheme for LD plots (Haploview version 4.0, Broad Institute, Cambridge, USA): Each box represents pairwise LD between markers. Numbers represent D' or R^2 x 100. No number represents D' value of 1. Pink-Red boxes represent D'≤1 and LOD≥2. White boxes represent D' <1 and LOD <2. Blue boxes represent areas where D' = 1 and LOD < 2.

1 Chapter 1: Background and General Introduction

1.1 Cancer pain and opioids: worldwide perspective

Most patients with cancer experience pain at some stage in their illness and many require opioid analgesia. For the past 20 years cancer pain has been managed according to The World Health Organisation (WHO) analgesic ladder. This three-step ladder recommends sequential increases in the strength of analgesia, starting with paracetamol (step 1), adding in a weak opioid (step 2) if the pain persists and finally progressing to a strong opioid (step 3) if pain is still not controlled . Adjuncts (e.g. non steroidal anti-inflammatories) are permissible at any stage along the ladder. This three-step analgesic ladder is under scrutiny currently with a proposal to exclude step 2, however the results of these trials are outstanding.

Morphine is the WHO first line strong opioid of choice for cancer pain [1;2]. This recommendation is based on availability, familiarity and cost rather then evidence of superior efficacy or tolerability. Despite this recommendation however, until relatively recently morphine was unavailable or not commonly used in many countries. In 1991 twenty countries accounted for 86% of morphine consumption [3]. In 2006 Europe and North America accounted for 89% of global morphine consumption with developing countries, which account for 80% of the world's population, only consuming 6% of worldwide morphine [4]. Reasons for this inequality in morphine use / availability include:

- Inadequate health care infrastructures or pain control as a low priority item in a country's national health agenda
- Lack of knowledge / skills in pain treatment. Even in the USA, a survey of 897 physicians, reluctance to prescribe opioids for cancer pain was cited by 61% of respondents [5].
- Legal and regulatory issues surrounding the use and availability of opioids[6].
- Fear of addiction and misuse or re-direction of opioids into the illegal drug trade [7].

In 1984 the WHO Cancer Pain Relief Programme was established in which a country's morphine consumption was considered to be an indicator of progress to improve cancer pain relief [1;3]. Between 1984 and 1992, global consumption of morphine increased by nearly 300%[3;8]. Since then morphine availability has increased worldwide and it has been introduced to many more countries.

Other alternative opioids have also become more widely available, although as with morphine, availability and consumption is not uniform worldwide e.g. 94% of oxycodone is consumed by USA, UK, Canada, Germany and Australia [4]. In a recent survey of 3030 cancer patients across Europe, morphine (oral or systemic) was the most commonly used strong opioid (50%), followed by fentanyl (14%) , oxycodone (4%), methadone (2%) and hydromorphine (1%) [9]. There are marked differences in the choice of first line strong opioid in different countries, eg in Belgium it is fentanyl while in Finland it is oxycodone [9]. International differences in choice of opioid and opioid consumption was also shown in a study comparing opioid consumption in Denmark, Finland, Germany, Iceland, The Netherlands, Norway and Sweden [10].

Some opioids differ in their mode of metabolism and therefore some are recommended as being safer in certain circumstances than others e.g. alfentanil in renal failure. There is little evidence however to suggest superiority of one opioid over another in terms of efficacy [2]. There have been very few randomized controlled trials comparing different opioids and those that have been carried out have tended to be small.

1.2 Inter-individual variation in response to morphine for cancer pain

Although there appears to be little difference between opioids on a population level, on an individual level, variation in response to opioids is a well recognised phenomenon. Patients taking morphine for cancer pain may be categorised clinically into "morphine responders" and "morphine non-responders" depending on their sensitivity to the analgesic effects of morphine, and their tolerance or intolerance of morphine-related side-effects:

- *"Morphine responders":*
    - Most patients who are prescribed morphine for cancer pain are "Morphine responders". These patients achieve good pain control without problematic side-effects. These patients are thus sensitive to the analgesic effects of morphine and are tolerant of (or resistant to) morphine-related side-effects.

- *"Morphine non-responders":*
  A significant proportion of patients, (up to 30%), do not have a satisfactory clinical outcome on morphine [11]. These "morphine non-responders" present in a number of different ways [12]:

    - Patients who achieve good analgesia but with intolerable side-effects. These patients appear to be sensitive to the analgesic actions of morphine but are intolerant of the side-effects.

    - Patients who do not achieve good analgesia because of dose-limiting side-effects. It is unclear whether these patients are in fact sensitive to the analgesic effects of morphine because their intolerance of the side-effects precludes upwards dose titration.

      It is not uncommon for morphine side-effects such as nausea, vomiting, dry mouth, nightmares, sedation, confusion, hallucinations and myoclonus to become apparent as the dose is increased. These toxicities may often be managed with other medications e.g. antiemetics / antipsychotics, but sometimes they persist or become intolerable and thus dose-limiting. In this

case current practice in our palliative care department is to switch these patients (morphine non-responders) from morphine to an alternative opioid.

- Patients who do not achieve good analgesia but do not experience side-effects either, despite escalating morphine doses. These patients are insensitive to the analgesic effects of morphine but are also tolerant of any potential side-effects.

There also appears to be considerable variation in the doses of morphine required to achieve pain control.

At present the clinical use of morphine and other opioids in cancer pain management involves careful titration of the dose of an initial opioid (usually morphine) according to response (analgesia and side-effects). As there is currently no way of prospectively predicting such response, in many instances patients experience significant pain and/or side-effects or delay in adequate symptom management.

1.2.1 Opioid analgesia and side-effects: Mechanism of action:

The pharmacokinetic and pharmacodynamic factors which may underpin inter-individual variation in response to morphine have yet to be elucidated. Based on current knowledge of opioid action however, it may be hypothesised that such variation in analgesic sensitivity and side-effect tolerance may be, in part, associated with:

- Morphine metabolism
- Site of u-opioid receptor activation
- The influence of second messengers

Morphine is metabolised in the liver to the metabolites, morphine-3-glucuronide (M3G) (55%) and morphine-6-glucuronide (M6G) (15%). M6G is a potent analgesic agent [13] but M3G, whilst being considered to be analgesically inactive, is thought to have neuroexcitatory properties [14]. M6G has been associated with drowsiness, nausea and vomiting, unconsciousness and respiratory depression. M3G has been proposed to play a role in morphine-related agitation, myoclonus, hyperalgesia and delirium [15].

To date, serum measurements of morphine and its metabolites have not demonstrated any reproducible association with analgesic sensitivity or development of opioid side-effects [16;17]. There is however, continued interest in this field. Variation in a number of factors might influence the formation and action of morphine and its metabolites in the central nervous system. These include:

- Expression and activity of UDP-glucuronosyltransferase (UGT) 2B7, the major enzyme involved in morphine metabolism.
- Morphine oral bioavailability. This varies from 35-75% [18]. Morphine uptake into the systemic circulation from the gastrointestinal tract and circulating levels of morphine and its metabolites in the central nervous system are influenced by P-glycoprotein levels and expression [19;20].
- Renal function. Morphine and its metabolites (including morphine-6-glucuronide) are eliminated via the kidneys [21;22]. Therefore in renal impairment, the effect of morphine is prolonged and more pronounced.

The location of opioid receptors throughout the central nervous system has been demonstrated by immunohistochemical and in-situ hybridization techniques. Neuroimaging advances such as fMRI has increased our knowledge of which areas of the central nervous system are involved in pain and its modulation [23]. Opioids produce analgesia and/or opioid-related side-effects through action at different areas in the central nervous system.

Opioids produce analgesia by acting at opioid receptors along the ascending pain pathways, which involves the dorsal horn, the brain stem, thalamus and cerebral cortex. Opioids also have an action on descending inhibitory pain pathways, which include the midbrain periaqueductal grey, the nucleus raphe magnus and the rostral ventral medulla[18].

Opioids produce analgesia though a reduction in presynaptic neurotransmitter release, neurone hyperpolarisation and a reduction in cellular excitability and thus a decrease in synaptic transmission. Opioids also inhibit GABAergic transmission [18;24].

Opioids cause nausea and vomiting through action on the chemoceptor trigger zone which is close to the area postrema in the floor of the fourth ventricle. This is outside the blood brain

barrier. They also have a direct action on the vomiting centre, which is located in the brainstem.

Persistent sedation and drowsiness due to opioids is problematic because it impairs quality of life and impinges on the patient's ability to function at home and in the workplace. Other common central side-effects include nausea, confusion and hallucinations. The central side-effects of drowsiness, confusion, hallucinations, nausea and myoclonus can occur independently of each other. There is a phenomenon known as opioid-induced neurotoxicity which may manifest as any or all of the following symptoms: hallucinations, cognitive dysfunction, severe sedation, myoclonus and opioid hyperalgesia.

Despite the fact that these effects of opioids are quite common, little is known about their precise pathogenesis [25]. Possible contributing factors include:

- The accumulation of opioid metabolites, especially M-3-G (although the evidence for this is conflicting)
- The anticholinergic effects of opioids
- An interaction between opioids and catecholamines in the central nervous systems
- Activation of NMDA receptors and the stimulation of excitatory second messengers e.g. glutamate [26;27].

The mechanism of opioid-induced constipation is discussed in section 1.6.2.

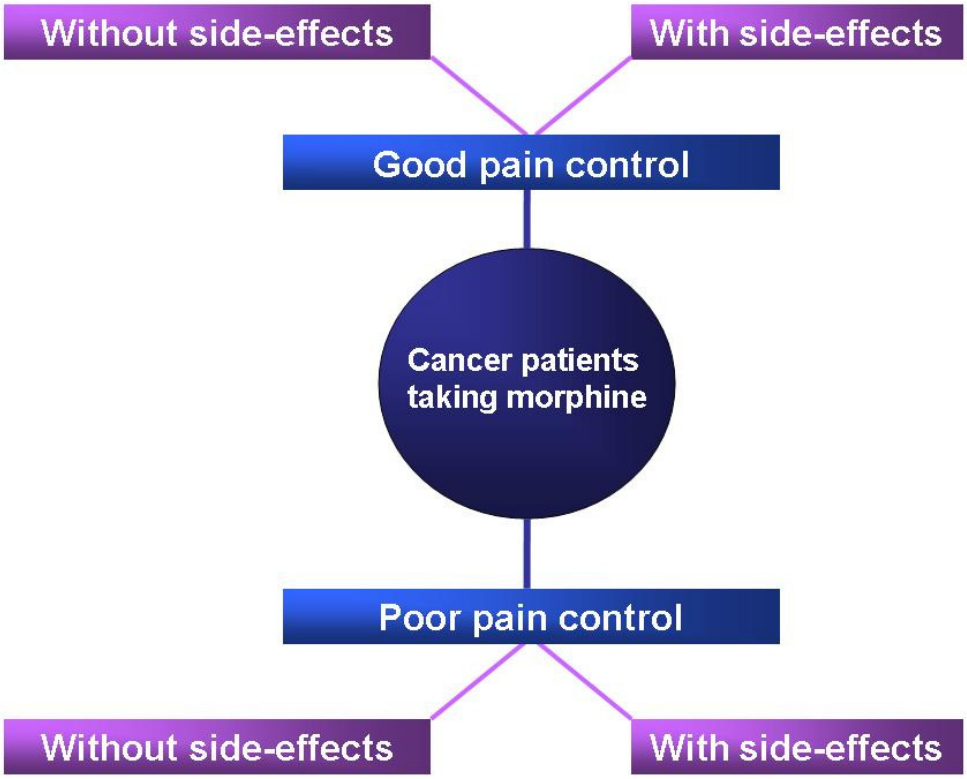

Figure 1.1 Inter-individual variation in response to morphine

Table 1.1Morphine side-effects

Common	**Less common**	**Rare***	**Possible**
Confusion	Prutitus	Respiratory depression	Suppression of immune system
Drowsiness	Sweating		Suppression of hypothalamic-pituitary axis
Hallucinations	Myoclonus		
Myoclonus	Allodynia		
Bad dreams	Hyperalsgesia		
Constipation	Delerium		
Nausea			
Dry mouth			

* These side-effects are rare in cancer patients if opioids are titrated carefully according to response

Recognition of this substantial inter-individual variation in response to morphine for cancer pain has resulted in the emergence of two clinical phenomena. Firstly "opioid switching" as a clinical manoeuvre to redress the balance between analgesia and side-effects has become widely accepted. Secondly there has been a surge of interest in the mechanisms (clinical and molecular) underlying inter-individual variation in morphine response with a number of clinical studies being carried out in this area.

1.2.2 Opioid switching

Morphine “non-responders” often benefit from changing to an alternative opioid in a practice known as “opioid switching”. The same principle applies for patients who do not respond well to any other opioid also. When used carefully the practice of opioid switching is a powerful therapeutic tool in the individualisation of cancer pain management.

The terms “opioid switching” and “opioid rotation” are often used interchangeably in the literature. For the purpose of this thesis opioid switching refers to the use of an alternative opioid for the purpose of improving analgesia and/or reducing intolerable side-effects. Opioid rotation is taken to refer to the use of an alternative opioid because of availability, patient or physician choice or a change in the route of administration of the opioid. In some studies patients undergoing opioid switching and opioid rotation are included together [28;29].

A Cochrane review in 2004 examined the evidence base for the effectiveness of opioid switching to improve drug tolerability. 52 studies were included in this review, including 23 case reports, 15 retrospective studies or audits and 14 prospective uncontrolled studies. All studies except one reported opioid switching as a beneficial clinical practice. The numbers of patients included in these studies tended to be small. At that time no randomized controlled trials or prospective controlled studies had been carried out in this area. Therefore the conclusion of the systematic review was that there was no substantial evidence to support the practice of opioid switching. The author called for further research in a number of areas: 1) to establish the true efficacy of opioid switching and 2) to determine which opioid should be used first line and second line [30].

A further systematic review in 2006 suggested that opioid switching is associated with improvement in response to opioids in at least 50% of patients [12]. A more detailed look at some of the larger studies suggests that careful opioid switching may in fact be associated with much higher response rates. Table 1.2 details the results of some of the largest studies in this area.

To date there has been only one prospective case-controlled study of opioid switching. This study was initially carried out at the Royal Marsden Hospital by Drs Joy Ross and Dag Rutter and upon this study much of this present thesis is based. In the original papers from this study 186 patients were included which comprised two cohorts. 74% (138/186) were morphine "responders", who had been on morphine for at least 4 weeks and who had good pain control with minimal side-effects. 26% (48/186) were morphine "non-responders" who had either poor pain control and / or intolerable side-effects despite adequate morphine dose-titration. Morphine non-responders were switched to oxycodone as the second-line strong opioid, resulting in an overall 87% good clinical outcome in terms of pain and side-effect profile [31].

After an opioid switch is undertaken, it usually takes a few days of dose titration for the maximum effect to become apparent. Time to stabilization on the second opioid may be slightly longer if the patient was switched because of both pain and side-effects [29].

The dose of the original opioid at which an opioid switch is required varies widely. In one study in which 54 patients were switched from morphine to methadone, the median daily morphine dose was 200mg (range 30-1000mg) [32]. In another study the morphine equivalent daily dose was 577 +/- 1535mg [33]. In the Royal Marsden study in which morphine non-responders were switched to oxycodone, the median dose of morphine at time of switching was 70mg (range 15-580mg) [31].

Similarly the duration of the initial opioid therapy is variable. In one study patients were on the initial opioid for a median of 44 days (range 20-240 days) before switching to an alternative opioid [32]. In another study the mean duration of the previous opioid therapy is reported as 25.8 weeks (180.6 days) (range 1-104 weeks) [34]. There appears to be two broad groups of patients which require opioid switching. The first group require switching shortly after initiation of the initial opioid, when the opioid dose is relatively low. The second group appear to become non-responsive to the initial opioid at either higher doses or after chronic opioid therapy. It has recently been proposed that these two groups of patients represent

different clinical phenomona [35]. The first group, i.e. those failing on the initial opioid shortly after opioid initiation, may represent the fact that not all drugs are efficacious in all patients. "Heterogeneity of treatment effects" is seen with most pharmaceutical medications and may be explained in part by individual pharmacokinetic or pharmacodynamic factors. The second group of patients however, i.e. those patients who experience pain or intolerable side-effects after chronic opioid therapy, may be the result of physical tolerance to the initial opioid.

Table 1.2 Studies assessing efficacy of opioid switching

Author	Type of study	Number of patients undergoing opioid switch	Successful outcome after opioid switching	Reason for switching	Comment
Mercadante 2009 *[29]	Prospective uncontrolled study	118	87%	Uncontrolled pain 15.2% Adverse effects 28.8% Uncontrolled pain and adverse effects 50.8%	More than 1 opioid switch required in 7 patients (6%)
Riley 2006 [31]	Prospective case-controlled study	47	87%	Morphine responders compared to morphine non responders: Confusion and drowsiness $p=5 \times 10^{-12}$ Pain relief $p=2.4 \times 10^{-6}$ Nightmares $p=5 \times 10^{-5}$ Nausea $p=7.3 \times 10^{-3}$	More than 1 opioid switch required in 4 patients (8.5%)
Wirz 2006[34]	Prospective uncontrolled study	50	64%	Uncontrolled pain 60% Sedation 4% Itch 2% Gastroimtestinal effects (nausea, constipation) 40%	
Mercadante 2001[36]	Prospective uncontrolled study	52	80%	Uncontrolled pain 20% Adverse effects 16% Uncontrolled pain and adverse effects 64%	
Kloke 2000*[28]	Retrospective uncontrolled study	103	65%	Uncontrolled pain 43% Adverse effects 20% Uncontrolled pain and adverse effects 15%	
De Stoutz 1995[33]	Retrospective uncontrolled study	80	73%	Uncontrolled pain 16% Cognitive failure 39% Hallucinations 24% Myoclonus 11% Nausea 9% Local irritation 1%	More than 1 opioid switch required in some patients

* studies included changing from one opioid to another in order to improve pain and side-effect profile (i.e. opioid switching) and also for other reasons (opioid rotation)

1.2.2.1 Opioid switching: what dose?

One of the most challenging aspects of switching from one opioid to another is deciding on the starting dose of the second opioid. Although equianalgesic dose ratios are available, these are merely guidelines to the relative potencies of different opioids. These ratios were derived largely from single dose studies and do not take into account individual patient factors which may contribute to effect, such as previous opioid therapy, opioid tolerance or underlying disease [37;38]. The reported equianalgesic dose ratios between opioids also may be quite variable depending on the study. The generally accepted equianalgesic dose ratio for oral morphine: oxycodone lies between 1:1 and 1:2 [37]. In one study the median dose ratio of morphine: oxycodone was 1.7 with a broad range from 0.25 – 12 [39]. To make matters even more complicated the equianalgesic dose ratios may change according to the initial opioid dose, as has been proposed when switching morphine to methadone [32].

One of the major factors contributing to the difficulties in determining the dose of the alternative opioid is the phenomenon of opioid tolerance. This is defined as a reduction in response to a drug after repeated administration. Tolerance to both opioid induced analgesia and side-effects may occur, although not necessarily in tandem together [40]. Incomplete cross tolerance to different opioids exists, which may be explained in part by differing pharmacodynamic and pharmacokinetic factors. There appears to be no defined relationship between the dose of the initial opioid at time of switching and the final stable alternative opioid dose after switching [12;29]. In some cases opioid switching facilitates upward dose titration to achieve good analgesia [41]. In others the final equivalent dose of opioid required was reduced [33]. These data demonstrate the importance of individualised dose titration when switching opioids [12]. Most authors recommend deciding on the dose of the alternative opioid in the context of a) the individual patient experience (ie whether switching because of uncontrolled pain or side-effects) and b) the type of opioid used. Conservative dose ratios [37] and an initial dose reduction of the equianalgesic dose by 25-50% [37;38] are recommended with subsequent careful individualised dose titration.

1.3 Clinical Factors influencing morphine response

Response to morphine is potentially influenced by a number of different clinical and environmental factors.

Many studies have demonstrated gender-related differences in pain sensitivity and analgesic response to opioids. A recent review suggests that women experience more pain post-operatively and that women are more sensitive to experimental pain [42]. The data pertaining to analgesic response however are quite mixed and often inconsistent. Animal studies have demonstrated that males are more sensitive to both morphine and alfentanil mediated analgesia [43].A prospective cohort study of a heterogeneous group of 423 women and 277 men in the post-operative period concluded that women had more severe pain and required larger doses of morphine than men [44]. A human healthy volunteer study however demonstrated that morphine has a greater potency and a slower speed of analgesic onset and offset in women rather than in men [45]. A further study found that post-operative kappa-opioid agonists were associated with greater analgesia in females than males [46]. Any potential gender-related differences may be species-related or may be due to differences in the study population or pain outcomes. It is not clear why gender-related differences occur. It is not thought to be related to differences in the pharmacokinetics of opioids [43;45]. Hormonal differences or neurobiological factors may play a role [42;47].

Age has been shown to be associated with variation in pain perception and analgesia in other studies. In most studies there was a negative correlation between age and pain sensitivity / analgesic response. Younger patients appear to have higher pain scores and greater morphine requirements post-operatively [48-50]. One retrospective study went so far as to suggest a formula for estimating post-operative morphine dose requirements based on the patient's age [51]. A recent systematic review however suggests that the association between age and pain / analgesia is somewhat conflicting and inconsistent [47]. Again, it is not entirely clear why such differences may occur. Animal studies have demonstrated differences in receptor levels of dopamine, serotonin and enkephaline with age [52]. Other suggested explanations are differences in

drug handling in terms of pharmacokinetics or age –related differences in the number or binding of opioid receptors [51].

In a retrospective study of cancer patients taking morphine, differences in patient age, white cell and platelet count were found when 100 "morphine responders" were compared to 70 "morphine non-responders". These factors only accounted for 6.9% of variability in the study population and there were a number of limitations to the study due to its retrospective nature [53]. In another retrospective analysis, 103 cancer patients who underwent an opioid change (due to inadequate pain control, adverse effects from the opioid or other reasons) were compared to 170 patients who responded well to their initial opioid. In this study there was no difference in terms of age, gender, tumour diagnosis, pain syndrome or co-analgesics, with the exception of corticosteroids [28].

In the original cohort of patients included in the Royal Marsden study of cancer patients on morphine carried out in our department, regression analysis was used to develop a model to predict "morphine responders" (n=138) versus "morphine non-responders" (n=47). Morphine non-response was found to be associated with white cell count, weight, tumours of the lower gastrointestinal tract, recent chemotherapy and concomitant use of beta blockers, proton pump inhibitors or 5HT3 antiemetics [31]. These findings have not been replicated in other studies. A further prospective study by a different group found no relationship between the need for an opioid switch (due to uncontrolled pain, adverse opioid effects or convenience) and age, gender, pain type, the use of adjuvant medications (including corticosteroids) or biochemical factors [29].

There are few data explaining why not all patients benefit from opioid switching. In a study by Mercadante et al, patients who had both poor pain control and adverse opioid effects were more likely to be unsuccessful in switching (p=0.004). No association was found with clinical factors (including age of patient, gender), type or dose of opioid, pain type, the use of adjuvant medications or renal impairment. The number of patients who did not respond to opioid switching in this study however was small (N=15/118), therefore it is difficult to extrapolate these data into meaningful clinical practice [29].

If a switch from one opioid to another is unsuccessful a number of factors should be taken into consideration:

- A further opioid switch may be indicated. More than 1 opioid switch is sometimes required to achieve an optimum balance between analgesia and side-effects [29;31;33].
- The pain may not be opioid responsive.
- Central delivery of opioids may be required (via the epidural or intrathecal route). Eight patients in Mercadante's study required intrathecal pain management [29]. In the Royal Marsden study, 5/48 patients did not have a successful outcome after opioid switching. One of these required an epidural and the authors suggest that for 3 others anaesthetic intervention would have been appropriate [31].
- Other factors may be contributing to the side-effects experienced e.g. renal impairment, concurrent medications or factors related to the underlying disease.

To date the only clinical factor which is consistently and reliably associated with altered morphine response is renal dysfunction (see section 1.2.1). However marked inter-individual variability in morphine response occurs even in patients with normal renal function.

At present, for the large majority of patients, no clinical factor has been identified which allows prospective prediction of which patient will respond best to which opioid. Therefore in recent years much attention has been focused on pharmacogenetics: the notion that an individual's genetic makeup may underpin variability in morphine response.

1.4 Pharmacogenetics of morphine

In many areas of medicine, pharmacogenetics has yielded some very exciting results. An individual's response to drugs such as irinotecan, abacavir and warfarin can now be accurately predicted through integration of genetic and clinical data. This facilitates prospective decision making regarding choice of the correct dose of the correct drug for a given patient and reduces subsequent side-effects. Thus in a number of different areas of medicine including oncology and haematology, pharmacogenetics has revolutionised drug prescribing.

Irinotecan in a chemotherapeutic agent used in the treatment of colorectal cancer. The active metabolite of irinotecan, SN-38, is associated with potentially serious side-effects such as diarrhoea and neutropenia. SN-38 is inactivated through glucuronidation by the enzyme uridine diphosphate glucuronosyl transferase (UGT1A1). Reduced activity of UGT1A1, which is seen in patients with specific variations in the gene coding for this enzyme, is associated with increased risk of serious side-effects. Prospective genotyping of patients who are prescribed irinotecan allows dose reduction (with a subsequent reduction in side-effects) in patients carrying these genetic variants [54;55].

Polymorphisms in two genes, VKORC1 and CYP2C9 account for up to 30% and 10% of variability in warfarin therapeutic dose requirements [56;57].

Similarly, side-effects with Abacavir, a nucleoside analogue reverse transcriptase inhibitor used in the treatment of HIV and AIDS, are predicted through genotyping for HLA-B*5701 [58].

Recently there has been a growing interest in the possibility that genetic factors might play a role in variability of opioid response. The hypothesis held by many researchers is that changes at a molecular level in an opioid's pharmacokinetic and pharmacodynamic pathways may be responsible for altered opioid response [59].

In terms of cancer pain, pharmacogentics may potentially allow prospective prediction of response to different opioids. Personalised prescribing involving choosing the correct dose of the correct opioid for the each individual patient would result in more rapid pain management, reduced side-effects and potentially a better quality of life.

1.4.1 Evidence to support genetic variation in response to opioids

There are a few well defined pain-related conditions in which a definitive causal gene has been identified. Mutations in SCN9A, which codes for a peripheral sodium channel, have been associated with either a complete inability to sense pain or, quite the opposite, an extreme pain disorder [60;61].

There are substantial animal data to support the hypothesis that opioid response may be under genetic control. Morphine acts primarily through the mu-opioid receptor (MOR). MOR-knockout mice (in which the MOR gene has been "turned off") are entirely refractory to the analgesic effects of morphine [62-64] and do not experience any side-effects from morphine [63;65]. Other genetically bred strains of mice exhibit differential response to pain perception and opioid analgesia. For example, the CXBK mouse is a genetically inbred strain which has decreased MOR expression [66]. These animals experience markedly reduced analgesia from morphine compared to other mice [67]. Current research summarised in the "Pain Genes database" suggests that there are over 300 genes, variation in which may play a role in differential pain perception and analgesia in animals [68].

A number of genetic association studies involving pain and response to opioids have been carried out in healthy volunteers and non-cancer patients. As detailed later in this chapter, these studies have often been influential in the design of research in cancer patients taking opioids for pain.

One of the best characterised pharmacogenetic phenomena involves codeine. Codeine, a weak opioid, is metabolised to morphine via the genetically heterogenous enzyme CYP2D6 (debrisoquine/spartenine hydroxylase) [69]. Up to 10% of Caucasians (and varying proportions of other populations) lack CYP2D6 activity. These are known as

"poor metabolisers" and thus experience little analgesia from codeine, as compared to "extensive metabolisers [70]. On the other hand, CYP2D6 gene duplication (where the individual has an extra copy of the region of DNA containing the gene) is associated with ultra-rapid metabolism of codeine to morphine, found in 3% of Caucasians [71]. There are case reports of fatal neonatal opioid toxicity in children who are breastfed by a codeine-ingesting CYP2D6 ultra-rapid metabolising mother [72;73]. Variation in at least 16 alleles has been shown to influence CYP2D6 activity [74]. Recent data suggest that CYP2D6 phenotype also plays a role in response to tramadol [75], with CYP2D6 gene duplication playing a role in development of respiratory depression [76]. Surprisingly, the impact of genetic variation in CYP2D6 on codeine response has not been extensively studied in cancer patients.

1.4.2 Genetic association studies: The genetic code

A number of genetic association studies have been carried out in cancer patients taking opioids. The aim of genetic association studies is to identify areas of genetic variation which are associated with an altered clinical outcome or phenotype. Genetic association studies are based on the central dogma of molecular biology i.e. the expression of genetic information stored in DNA involves transcription of this information into mRNA which subsequently specifies the synthesis of protein via the process of translation.

Most of the genetic information which is interpreted to give the final protein product is packaged in segments known as exons. The intervening intronic sequences do not contain information which is used in production of the protein product. Only a proportion of DNA encodes the amino acid sequence for a protein. The untranslated region (UTR) (which may be exonic and may be upstream to the gene (5'UTR) or downstream (3'UTR)) undergoes transcription but not translation. These UTRs are important for efficiency of translation. The promoter region is the DNA sequence which is located just upstream to the gene and is involved in initiation of transcription.

Differences in response to drugs may be brought about by genetic variation at a DNA, RNA or protein level. (Figure 1.2)

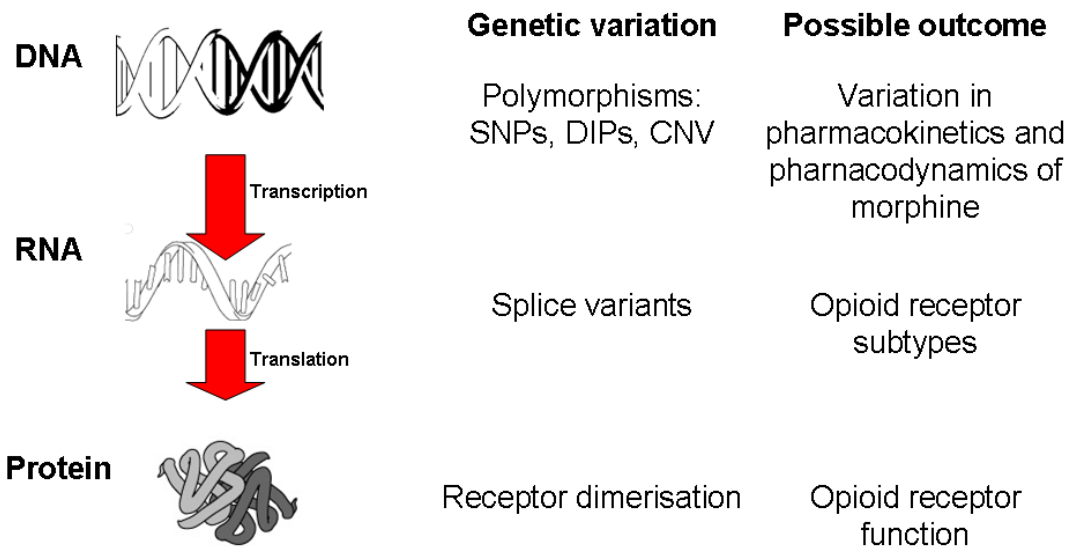

Figure 1.2 Genetic variation at DNA, RNA or protein level may influence response to morphine

1.4.3 DNA variation: Single Nucleotide Polymorphisms

To date all genetic association studies in cancer patients taking opioids have examined DNA variation.

DNA is made up of two opposing strands of nucleotides, with each nucleotide consisting of one of four bases (adenine (A), guanine (G), thymine (T) and cytosine (C)), a sugar and a phosphate bond. Each stretch of DNA constitutes a chromosome, which in turn consists of a number of genes. A gene is a stretch of DNA which carries the genetic information encoding a specific protein. The Human Genome project, which was completed in 2003, set out to map all of the genes in human DNA (approximately 30,000) and to sequence the 3 billion chemical base pairs that make up human DNA [77].

Polymorphisms are differences in the genetic structure which occur in greater than 1% of the population. Genetic variants which occur with a frequency of less than 1% are known as mutations. Single nucleotide polymorphisms (SNPs) are the most common type of genetic variant at a DNA level. A SNP represents a change in a single nucleotide at a particular position along the DNA strand. The different variants that a polymorphism may take are called alleles. In most cases there are two (but sometimes more) possible alternative alleles; the common (major) allele, which is carried by most people in the population, and the variant (minor) allele. Each SNP has a unique identifier, often known as an "rs number" (reference SNP number). SNPs are also often given names which are derived from their location in the gene and the amino acid change they are associated with. There are approximately 12 million known SNPs [78]. Genetic association studies search for a link between the clinical outcome measured (phenotype) and these genetic variants.

SNPs may influence clinical outcome through a change in the amino acid sequence produced by the gene, thus altering the structure or function of the enzyme / receptor / protein coded for by that gene. This is thought to occur in a number of ways:

1) The SNP may occur in a region of the gene (known as an exon) which is translated into protein.

2) SNPs may also have an indirect association with changes in the final protein product of the gene if, through a process known as linkage disequilibrium (non-random association), the SNP represents a marker for the true disease-susceptibility SNP. In this way SNPs occurring in non-coding (intronic) regions of the gene may *appear* to be associated with changes in clinical outcomes in genetic association studies.

3) The SNP may occur in an area of the gene which subsequently alters the process and products of transcription and translation.

As each individual has two versions of each chromosome, the genotype of an individual at a particular position is denoted by the combination of alleles at that position. In a situation where there are two possible allelic variants at a given position,

an individual's genotype is determined by whether he is homozygous for either the major or the minor allele (i.e. carry two copies of the same allele) or heterozygous (carrying one copy of the major allele and one of the minor).
Apart from SNPs there are a number of other genetic variants which may be associated with disease or clinical outcome, including deletion-insertion polymorphisms (DIPs), copy number variation (CNV) and VNTRs (variable nucleotide tandem repeat sequences) which alter chromosomal structure.

1.4.4 Genetic association studies: Candidate genes and Genome-Wide Association Studies

Until the recent emergence of genome-wide assays, candidate gene studies have traditionally been the standard for population-based (rather than family-based) genetic association studies. Genes are chosen for candidate gene association analysis because their end product (the protein they code for) is known to play a role in the outcome of interest, e.g. in opioid response. Candidate gene studies are therefore hypothesis driven, based on biologically plausible pathways. Candidate gene association studies have a number of advantages:

- In general, the population frequency of most SNPs tested is known; therefore it is relatively easy to carry out power calculations.
- The required sample size is often smaller than in genome-wide association studies (GWAS) because fewer tests are being carried out.
- As the number of SNPs / genes tested is limited, the resulting genetic data are generally of a manageable volume and may be analysed using standard statistics and commonly used desktop statistical programmes.
- Replication of candidate gene studies is not prohibitively expensive.

However, candidate gene association studies are not without their limitations. As these studies are hypothesis driven, the amount of data and especially the amount of new information generated is limited by the breath of current biological and genetic knowledge.

In recent years much attention has been focused on the concept of linkage disequilibrium (LD), the non-random association of genetic variants within a

population. It is now known that stretches of the genome are inherited together. Although the number of SNPs in any gene may be enormous, it is not necessary to analyse SNPs which are in tight LD with each other. Recent advances such as the International HapMap Project have involved extensive genotyping of common SNPs and have increased our understanding of the architecture of LD in different populations [79]. Such data have facilitated methods of choosing subsets of SNPs (tag SNPs) which provide information on most of the allelic variation in a particular region. These data underpin the practical application of GWAS. These data also support the notion that all genetic association studies are indirect studies in that the genetic variant shown to be associated with the clinical outcome in the study may actually be merely a marker for the true association variant [80].

GWAS allow the hypothesis-free simultaneous testing of multiple genetic variants across the whole genome. GWAS have recently become more feasible in terms of cost and statistical support. The most recent GWA platforms provide information on up to 1 million SNPs.

The first GWAS in pain was published in 2009 in a cohort of 221 patients undergoing oral surgery. 255,785 SNPs spanning the entire genome were simultaneously analysed in this study. The study outcomes included maximum post-operative pain, post-operative pain onset and time to analgesia post ketorolac administration [81]. To date all published genetic association studies involving cancer patients taking opioids for pain have been candidate gene studies. No GWAS has been yet published in this field.

1.4.5 Pharmacogenetics of response to morphine: published data

All published genetic association studies of variation in response to opioids for cancer pain involve analysis of SNPs. The genes involved have been chosen because their end product (the protein they code for) is known to play a role in opioid response. The opioid pharmacogenetic studies in cancer patients which have shown some positive results have primarily focused attention on three genes; OPRM (Mu opioid

receptor gene), COMT (catechol-O-methyltransferase gene) and MDR1 (Multidrug resistance 1 gene).

1.4.5.1 OPRM

OPRM is the gene which codes for the mu opioid receptor (MOR). This is a natural candidate gene as morphine and other opioids exert their effects primarily through this receptor. OPRM has been associated with variation in opioid response in non-cancer studies, including post-operative pain [82-84], labour pain [85] and in human volunteer studies [86-88].

Over 800 polymorphisms in OPRM have been described [89]. The most commonly studied SNP is *rs1799971*. This SNP is also known as A118G, the two alleles at this location being A and G. The frequency of the polymorphism (the G allele) varies depending on the population being studied, from 16% in Northern and Western Europeans to 46% in Japanese [90]. A118G results in an amino acid change from asparagine to aspartic acid. This polymorphism occurs in the region of the gene which codes for the extracellular amino-terminus of MOR, the part of the receptor involved in ligand binding.

Lotsch studied the association of A118G on the central effects of morphine and M6G in a single blind two-way crossover study healthy volunteers (N=12). Pupil constriction in response to IV administration of morphine or M6G was used as a measure of the central effects of the drug. Individuals carrying the G allele (GG or AG genotype) required larger doses of M6G to achieve 50% pupil constriction than subjects with AA genotype. In this study there was no association between morphine response and A118G [91]. Another study of 20 healthy volunteers demonstrated that higher doses of alfentanil were needed to achieve analgesia in subjects with GG compared to AA genotype, whereas the former appeared to be protected against respiratory depression [86].

In terms of cancer pain and response to morphine, A118G has been associated with variation in daily morphine dose requirements and also in overall analgesic response to morphine. In a single point observational study, Klepstad et al examined this SNP

in 99 cancer patients who were deemed to be well pain-controlled on morphine (i.e. their average pain score on a numerical rating scale (NRS) of 0-10 was 4 or less). Patients with the AA genotype (N=78) at this position required a lower dose of morphine (mean +/- standard deviation: 97 +/- 89 mg/day) compared to patients with GG genotype (N=4) (225 +/- 143mg/day), p=0.006 [92]. In a larger cohort of the same study, carriage of the G allele (genotype AG or GG) at this position was also associated with increased morphine dose requirements, p=0.012 [93].

In a study by Campa et al, 145 cancer patients who were being started on morphine for pain were titrated according to their individual needs and the patients were followed up prospectively for eight weeks. Pain scores were assessed using an 11 point NRS and a verbal rating scale. At the end of week 1, patients with the AA genotype (N=106) had a mean drop in pain scores from baseline of 3.73 +/- 1.72 (mean +/- SD) compared to 0.3 +/- 1.77 in those with GG genotype (N=10), p<0.001 [94].

The clinical relevance of OPRM A118G is difficult to interpret at this point. There are a number of inconsistencies in the results of studies in this area. A single time point observational study by Ross et al demonstrated no association between A118G and "morphine responders" versus "non-responders". In this study morphine responders had been on morphine for at least a month and had good pain control without intolerable side-effects. Morphine non-responders had inadequate analgesia despite morphine dose-escalation and / or intolerable side-effects. [95] A study of fentanyl requirements in patients in labour suggested that the A allele (and not the G allele as in other studies) is associated with higher fentanyl dose requirements (p=0.0091) [85]. A recent meta-analysis of eight clinical studies (cancer and non-cancer) concluded that at best there is only a weak association between this SNP and opioid dose requirements and that currently available data do not support personalised prescribing of opioids based on genotyping of this polymorphism [96]. In this meta-analysis, the study by Klepstad et al [92] was excluded in favour of the larger study by Reyes-Gibby et al [93]because of the overlap in the study population. The study by Campa et al was also excluded as the genotype frequencies were not in Hardy-Weinberg equilibrium (HWE) i.e. they deviated from expected frequencies (see chapter 2, section 2.4.2 for discussion about HWE).

A limited number of other SNPs across OPRM have also been studied in cancer patients on morphine. Klepstad et al examined three other OPRM SNPs (-172 G>T, IVS2+31 G>A and IVS2+691 G>C) but these were not found to associated with variation in the study endpoint, daily morphine dose requirements [92]. Ross et al examined six other OPRM SNPs in addition to A118G (-172 G>T, 5433 C>T, 32459 C>T, 50665 A>G, 51325 G>C and 80547 T>C). There was no difference in the frequencies of these SNPs between "morphine responders" versus "non-responders" [95].

There is also controversy about the functional relevance of the A118G polymorphism. One study demonstrated altered receptor binding affinity for β-endorphin, but not for commonly used MOR agonists such as morphine or fentanyl [97]. Another study however reported no difference in binding affinities for beta-endorphin between wild type receptor and the mu opioid receptor coded for by this variant but did find binding differences for other MOR agonists [98]. A further study found no differences in binding of either morphine or beta-endorphin [99]. One study reported reduced mRNA and receptor expression associated with the G allele [100]. Overall, it is unclear whether A118G truly plays a significant role in inter-individual variation morphine response.

1.4.5.2 COMT

The COMT gene codes for catechol-O-methyltransferase, an enzyme involved in the metabolism of catecholamines including adrenaline, noradrenaline and dopamine. These substances act as neurotransmitters in the brain. There is considerable evidence of interaction between the opioidergic and catecholamine systems [101-103]. Reduced COMT activity leads to elevated catecholamine levels which have been associated with increased experimental pain sensitivity [102;104].

The most commonly studied polymorphism in this gene is *rs4680*, also known as Val158Met. This SNP is associated with an amino acid change from valine (val) to methionine (met), which is associated with reduced enzyme activity [105]. One of the earliest genetic studies of this polymorphism genotyped 29 healthy volunteers and measured pain response to saline infused into the masseter muscle. The volume of saline necessary to reach and maintain a certain level of pain intensity was used as a

measurement of pain sensitivity. Patients with the *met-met* genotype had increased pain sensitivity, i.e. they experienced pain at lower volumes compared to those with the *val-val* genotype. Subjects homozygous for *met* also exhibited decreased regional brain opioid system activation [102].

Diatchenkos group subsequently identified haplotypes (combinations of SNPs, see section 2.4.3) which were associated with variability in pain sensitivity [106]. It has been proposed that the COMT haplotypes alter mRNA structure [107]. Interestingly the haplotype study did not find a significant association between pain sensitivity and Val158Met. Indeed, the haplotypes associated with "low pain sensitivity" and "high pain sensitivity" both contained the allele at this position which coded for the *val* variant and it was the haplotype representing the intermediate pain sensitivity which had the allele coding for the *met* variant [106]. In a later paper the same group examined the influence of COMT haplotypes and the Val158Met SNP on different types of painful stimuli and concluded that while the haplotypes were associated with differential resting pain sensitivity, the Val158Met SNP was associated with the rate of temporal summation of heat pain [103]. Like OPRM however, there are inconsistencies in the reported data. Kim et al did not find any strong association between Val158Met and experimental pain sensitivity (n=500) or post-operative oral pain [108;109]. In the latter study results of COMT haplotype analysis also gave conflicting results to previous data.

Intriguingly in two Norweigan studies (N=207, taken from the same study population) of response to morphine for cancer pain, patients with the *met-met* genotype required lower doses of morphine than those with *val-val*, (mean 24 hour dose 95mg *met-met* versus 155mg *val-val*)[93;110]. As the *met* allele is associated with reduced COMT activity, it is associated with increased dopamine concentrations with subsequent suppression of enkephalins [101]. This in turn is associated with compensatory upregulation of mu-opioid receptor expression [102;111]. The authors therefore propose that the opioid receptor upregulation might be associated with an increased effect from morphine in *met-met* individuals with subsequent lower morphine dose requirements [110]. The same group also analysed haplotypes across the entire COMT gene and found an association between a haplotype containing the A allele coding for *met* and morphine dose requirements, p=0.006. Other haplotypes however which also

contained the A allele at this position were not associated with variability in morphine dose [112].

The same Norweigan study population was used to examine the joint effects of COMT Val158Met and OPRM A118G on variability in morphine dose requirements for cancer pain. Consistent with the group's previous findings, they concluded that subjects with genotype OPRM A118G AA and COMT *met-met* required the lowest morphine dose, p=0.012 [93].

One study in cancer pain compared inter-individual variation in response to oral morphine in terms of development of central side-effects, showing an association between two intronic SNPS in COMT (*rs7290221* and *rs5746849*) but not Val158Met and an increase in confusion, drowsiness and hallucinations [113].
Overall, as for OPRM, the number of studies examining genetic variation in COMT in cancer pain is small and the data regarding genetic variation in COMT and opioid response are as yet incomplete.

1.4.5.3 MDR-1

MDR-1 (Multidrug resistance 1 gene), also known as ABCB1 (ATP-binding cassette B1), codes for p-glycoprotein which regulates the transport (efflux) of morphine from the brain into the blood across the blood-brain barrier [114]. Reduced p-glycoprotein levels / activity results in enhanced analgesia from systemically (but not centrally) administered morphine [115]. MDR1 knockout mice exhibit an increased ratio of brain: plasma morphine concentrations compared to normal mice [116]. In animal studies, pharmacological inhibition of p-glycoprotein increased the concentration of morphine in the brain, with improved analgesic effect [117;118].

Most genetic studies have involved three MDR-1 SNPs; C3435T (*rs1045642*), GT2677A (*rs2032582*) and C1236T (*rs1128503*). A study of 126 Korean patients having intravenous fentanyl found an association between MDR-1 SNPs C3435T and GT2677A and respiratory suppression (p=0.0056) [119]. Subjects homozygous for the variant of SNP C3435T (genotype TT) have a reduced level of p-glycoprotein expression [120].

SNPs in MDR-1 have been examined in two studies of cancer patients taking morphine. C3435T was found to be associated with variability in pain relief in Campa's study of patients starting morphine for cancer pain. Patients with genotype TT (N=38) at this position had greater pain relief than those with CC (N=49), p<0.001. In this study the joint effects of OPRM A118G and MDR-1 C3435T were also examined. Patients with genotype OPRM A118G AA and MDR-1 C3435T TT appeared to have better pain relief compared to other combinations. The interaction analysis however was not statistically significant [94]. GT2677A and C1236T, but not C3435T, were associated with a decreased level of central side-effects (drowsiness, confusion, hallucinations) in a study of 228 patients on morphine for cancer pain. In this study the protective alleles MDR-1 2677G and COMT -4873G were independently associated with fewer central side-effects on multivariate analysis [113].

There remains some controversy about the functional relevance of MDR-1 polymorphisms in opioid response [121-123].

1.4.5.4 Other Genes

The association between the response to morphine for cancer pain and a number of other genes has also been studied with mixed results.

One study has examined the influence of cytokine gene polymorphisms on analgesic response based on the idea that tumour-induced mediators such as cytokines may cause inflammation and modulate cancer pain. This study included 140 patients with lung cancer who were referred for pain management to the supportive care specialists. Subjects scored their pain on an 11-point NRS at baseline and 30 days after their first assessment. TNFα -308G>A was associated with variation in pain severity at 30 days, p=0.04. IL-6 -174G>C was associated with differences in morphine equivalent daily dose, p=0.004 [124].

A SNP in the gene coding for β-arrestin (*ARRB2*) was found to be associated with differences between morphine responders and morphine non-responders (p=0.013) [95]. β-arrestin is an intracellular protein which acts as inhibitor of receptor coupling and is a negative regulator of opioid receptor signalling. [125] B-arrestin2 knockout mice have enhanced morphine analgesia.[126] Two SNPs in stat6, a gene involved in MOR

transcription, were found in the same study to be weakly associated with morphine responders versus non-responders [127].

An obvious candidate gene in pharmacogenetic studies of opioids is UGT2B7 (uridine diphosphate-glucuronosyltransferase 2B7). The enzyme coded for by this gene metabolizes morphine to morphine-3- and morphine-6-glucuronide (M-3-G and M-6-G). A small study (N=12) of patients using morphine via a patient-controlled analgesic pump found an association between a SNP in the promoter of UGT2B7 and morphine, M-3-G and M-6-G concentrations [128]. Polymorphisms in this gene were not found to be associated with concentrations of morphine or its metabolites in two larger studies in cancer patients (N=70 and 239) [129;130]. Similarly UGT2B7 SNPs were not found to be associated with any difference between morphine responders and non-responders [95].

GCH1 (Guanosine Triphosphate Cyclohydrolase 1) is an enzyme involved in the production of BH4 (tetrahydrobiopterin), which in turn is associated with pain sensitivity. SNPs in GCH1 have been associated with reduced BH4 expression and subsequently reduced non-cancer pain [131]. A recent study of 251 cancer patients found an association between SNPs in GCHC1 and time between cancer diagnosis and opioid therapy initiation. The authors proposed that a longer time interval suggested protection from pain [132]. The clinical application of such data are uncertain.

There have been a number of other genes which have been analyzed in animal, human volunteer or non-cancer pain studies which may play a role in opioid responsiveness but which not to date been studied extensively in cancer pain. These include MC1R (melanocortin-1 receptor gene)[133] and CYP2D6 [134].

EPOS: multicentre European pharmacogenetics of opioids study

The largest pharmacogenetics of opioids study in cancer patients has just recently been published. Two thousand two hundred and ninety four cancer patients on a variety of opioids, from 17 centres in 11 European countries were included. This was a single time point observational study. One hundred and twelve SNPs in 25 genes were analysed and the study population was divided into a test set and a validation set. None of the SNPs tested were significant in the validation set and none were significant on subgroup analyses which included stratification according to opioid [135]. The genes tested were *OPRM (Mu opioid receptor), OPRD (Delta opioid receptor), OPRK (Kappa opioid receptor), ARRB2 (B-arrestin 2), GNAZ (guanine nucleotide binding protein), HINT1 (histidine triad nucleotide binding protein 1) , Stat6 (signal transducer and activator of transcription 6), ABCB1 (p-glycoprotein transporter/ Multidrug resistance 1), COMT (Catachol-O-methyl transferase), HRH1 (Histamine Receptor 1), ADRA2A (α_{2A} adrenergic receptor), MC1R (melanocortin 1 receptor), TACR1 (neurokinin 1 receptor), GCH1 (GTP cyclohydrolase 1), DRD2 (Dopamine receptor D2), DRD3 (Dopamine receptor D3), HTR3A (serotonin 5HT-3A receptor), HTR3B(serotonin 5HT-3B receptor), HTR2A(serotonin 5HT-2A receptor), HTR3C(serotonin 5HT-3C receptor), HTR3D(serotonin 5HT-3D receptor), HTR3E(serotonin 5HT-3E receptor), HTR1 (Histamine receptor 1)* and *CNR1(cannabinoid receptor 1).*

The current data supporting a practical role for genetic variation on response to opioids for cancer pain is limited. A recent study analyzing the prospective impact of the most commonly studied genetic variants in OPRM, COMT, MDR1 and CYP2D6 on opioid dose requirements, pain scores and side-effects in 352 patients attending an outpatient pain clinic concluded that, based on current knowledge, genotyping for current known genetic variants "barely merits the laboratory effort" [136]. Current data do not support incorporation of genetic assays into the clinical management of cancer pain [137]. None of the studies to date have identified any genetic variant which could be used to prospectively predict opioid response.

1.4.6 RNA variation: Splice Variants

A single stretch of DNA can result in a number of different protein products, mainly through RNA modification. The existence of multiple mu opioid receptor subtypes as a result of a process known as alternative splicing has been recently proposed. Splicing refers to a process of post-transcription modification in which intronic material is removed and exons are joined together to form messenger RNA (mRNA). mRNA is subsequently translated into the protein product, which in the case of OPRM is the mu-opioid receptor. Different splicing products with potentially different functions can be formed through different arrangements of the exons in the mRNA.

The concept of multiple receptor subtypes is not new, suggested by a) incomplete cross tolerance between various mu-opioid receptor agonists [138] and b) differential actions of mu-opioid receptor agonists and antagonists [139]. Sophisticated rodent studies using inbred strains and knockout strains have further strengthened this concept. Mu-opioid receptor knockout mice demonstrate a complete loss of all mu-opioid receptors from the brain, suggesting that if mu-opioid receptor subtypes exists, they must be derived from the same gene [64]. The CXBK mouse, as mentioned above (section 1.4.1), is poorly responsive to morphine. CXBK mice have reduced levels of OPRM1 mRNA, with subsequent reduced expression of mu-opioid receptors and less signal transduction [66]. CXBK mice however retain analgesic sensitivity to morphine-6-glucuronide (M-6-G), heroin and fentanyl, all mu-opioid receptor agonists, suggesting that M-6-G, heroin and fentanyl act via a different mu-opioid receptor than morphine [140].

Opioid receptors are G-protein coupled receptors. These receptors have an extracellular amino terminus and an intracellular carboxyl tail. (Figure 1.3) They span the cell membrane seven times resulting in three intracellular and three extracellular domains. The three opioid receptors (mu, delta and kappa) share approximately 60% structural homology [141], with the main differences being in the transmembrane regions and extracellular loops [142]. These extracellular loops are important for ligand binding. The first extracelluler loop has been shown to be critical for binding of the MOR-

specific peptide, DAMGO.[143] The extracellular amino chain is also important in ligand binding in MOR (mu-opioid receptor). The sixth transmembrane domain and the third extracellular loop of DOR (delta opioid receptor) is critical for DOR selectivity. [144] The 2nd and 3rd intracellular loops as well as the carboxyl tail are important in the interaction between the receptors and G-proteins [145]. Receptor signaling is markedly impaired in DOR lacking the 3rd intracellular domain [146].

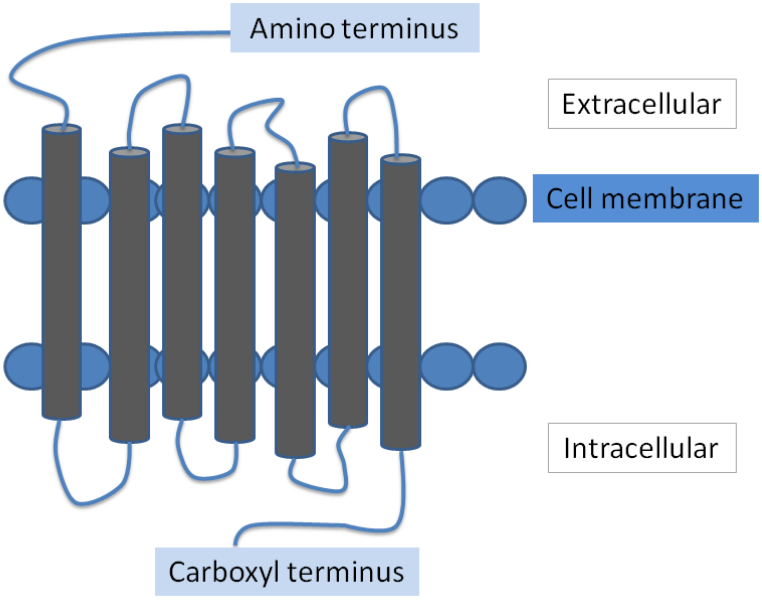

Figure 1.3 Structure of opioid receptor

The structure of the human mu-opioid receptor gene has been cloned [147]. OPRM was initially thought to be made up of 4 exons, although more recent work suggests that it is in fact much more complex [148]. Each exon is thought to be important for expression of different parts of the mu-opioid receptor. Exon 1 codes for the amino-terminal of the receptor and exons 1, 2 and 3 code for the transmembrane domains and intra/extracelluler loops [149]. Exon 4 codes for the distal portion of the carboxyl chain of the receptor which is important in coupling the receptor to intracellular signalling systems and events. Knockout animals and antisense probes have been used to examine the impact of blocking expression of parts of a gene of interest. Rodents without expression of exon 1 have significantly reduced morphine analgesia but retain sensitivity to M-6-G [150;151]. Conversely, rodents with loss of exon 2 expression exhibit decreased M-6-G and not morphine analgesia [150]. These data suggest that morphine

and M-6-G may act on different mu-opioid receptor subtypes, which may be brought about through variation at an RNA level. Even more intriguing is that although antisense blockade of exon 1 expression inhibited supraspinal morphine analgesia, it did not have any effect on spinal morphine analgesia, suggesting that perhaps different mu-opioid receptors are involved in both processes [152]. Antisense blockade of exon 4 inhibited all morphine effects [150;152]. Immunohistochemical labelling of mu-opioid receptor splice variants demonstrated differential distribution in the brain and spinal cord suggesting region specific processing of OPRM into different mu-opioid receptor subtypes [153].

Practical challenges of examining human brain RNA means that most research in this area has been carried out in vitro using cell lines expressing mu opioid receptors [154]. Ten human splice variants have been identified resulting from alternative splicing at exon 4 [154;155]. Since exons 1-3 remain conserved, all of these splice variants bind mu-opioid agonists, but there are differences in terms of function eg receptor internalisation and regional distribution [152]. One study examining human brain tissue resected at time of temporal lobectomy for epilepsy demonstrated the presence of mu-opioid receptor splice variants through reverse transcriptase polymerase chain reaction [156]. Alternative splicing of delta and kappa opioid receptors has also been demonstrated [157].

There are some human data supporting the role of splice variants in pain perception. A recent study by Diatchenko et al analysed patterns of genetic material between mice and humans. Although there are differences in the structure of mouse and human OPRM, this study demonstrated that a) most mouse OPRM exons (of which there are 20) also exist in humans and b) the architecture of the human OPRM is probably more complex than previously thought. A SNP in OPRM, *rs563649*, was associated with both altered pain response and differential translation of splice variants of mu opioid receptor [148]. To date no studies have been carried out examining the association between opioid receptor expression / splice variants and response to morphine in cancer patients in the clinical setting.

1.4.7 Protein variation: receptor dimerisation

Finally, even after the genetic code is translated into a protein product, modifications and variation in function may occur through the process of receptor dimerisation. Morphine acts primarily through the mu-opioid receptor. Animal studies have demonstrated that disruption of the mu-opioid receptor gene abolishes the analgesic effect of morphine despite the presence of functional delta and kappa opioid receptors [63]. However binding studies suggest that morphine does bind to delta and kappa receptors, albeit with less affinity than to mu-opioid receptors [24;158]. Although the role of delta and kappa opioid receptors in morphine response is not entirely clear, there is a suggestion that opioid receptors interact with each other and influence function [159-162]. For example delta-receptor knockout mice do not develop analgesic tolerance to morphine [163]. Opioid receptors can form homo and hetero-dimers, thus producing novel functional properties through altered ligand binding and differential intracellular receptor trafficking [164-166]. Opioid receptor dimerisation adds further to the complexity of inter-individual variation in opioid response. There have been no clinical studies examining the role of opioid receptor dimers on analgesic response to morphine for cancer pain.

1.5 Clinical phenotype in opioid genetic studies

One of the main challenges in interpreting genetic association studies in cancer and palliative care is that there is little consensus with respect to definition of outcome measures in pain and analgesia studies [167]. Most opioid genetic association studies have chosen one aspect of pain or side-effects to determine the primary outcome. This approach risks overlooking the complexity that defines morphine response. Some studies have examined the level of pain sensation before and after administering the drug [94;168]. Other studies have studied the dose of opioid required by individual patients [82;83;85;92;93;110;135]. In cancer studies the dose of morphine for pain control [92;93;110] and the change in numerical rating scores (NRS) for pain [94] have been used as measures of response to opioids. One study explored differences in central side-effects in cancer patients on morphine as an outcome measure [113] however most studies included side-effects as a secondary outcome. Another study has examined the

length of time between cancer diagnosis and initiation of opioid therapy as an outcome measure. [132]

The situation is made more complex by the fact that there is no standardised system for cancer pain assessment or indeed choice of pain intensity scale. [169;170] This lack of consensus and standardisation makes it difficult to compare results from different studies and may be one of the reasons underlying non-replication of genetic data in this field [171].

For genetic association studies to be useful and translational to clinical practice, the phenotype of response to a drug must be clinically meaningful.

Previous work in our department focused on the genetic and clinical associations with morphine responders and non-responders. [39;53;95] In the Royal Marsden study, patients were recruited into one of two cohorts according to patients' subjective experience and the observations of the experienced clinical team. "Morphine responders" were patients who had been on morphine for at least one month and who were experiencing adequate analgesia without problematic side-effects. "Morphine non-responders" were defined as those patients who failed to achieve adequate analgesia despite escalating morphine doses or who experienced subjectively intolerable side-effects. Morphine response / non-response was reported by the patient and assessed and determined by the clinical palliative care team. The phenotype of morphine responder versus non-responder was used in subsequent genetic association studies [95]. To date no clinical or genetic factors have been identified which are significant or robust enough to be used in clinical practice to prospectively predict morphine responders / non-responders.

Perhaps one of the reasons for this is that response to morphine must be considered a complex trait. Most complex human diseases are multi-factorial in nature. Pain is one of the most complex measurable traits, contributed to by a number of physical, genetic, environmental and psychological components [172]. Therefore response to morphine for cancer pain must be equally as complex.

Morphine non-response is broadly defined as inadequate pain control or intolerable side-effects. What constitutes morphine response / non-response in one individual may be very different in another individual. Morphine response is not a homogenously defined outcome; rather it is determined by a balance between a number of interacting predictor variables contributing to pain control and different side-effects (see fig 1.4). There are no data to suggest that pain and side-effects may not be modified by different clinical and genetic factors.

Two of the challenges in examining such a complex phenotype are 1) trait heterogeneity and 2) phenotypic variability [173]. In terms of response to morphine, trait heterogeneity occurs when the definition of response / non-response does not take into account the fact that it is actually made up of a number of distinct underlying traits, e.g. pain and different side-effects. A patient who is considered a morphine non-responder due to lack of analgesic efficacy does not have the same clinical phenotype as one which is a morphine responder because of intolerable drowsiness. Phenotypic variability occurs when there are differences in the time of onset of analgesia / side-effects and / or the severity of side-effects or degree of analgesia in morphine responders / non-responders. Trait heterogeneity and phenotype variability result in a heterogeneous model of response to morphine.

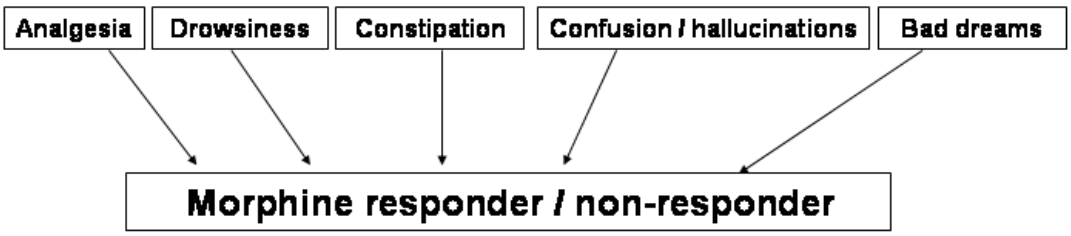

Figure 1.4 Trait heterogeneity in morphine response.
Morphine response is not a homogenously defined outcome
Adapted from [173]

1.6 Opioids for neuropathic pain

The role of opioids in the treatment of neuropathic pain in both cancer and non-cancer settings has been controversial. Evidence exists however supporting the analgesic efficacy of these agents for neuropathic pain.

A Cochrane review in 2009 examined the efficacy and tolerability of opioids for neuropathic pain of mixed aetiologies. The authors divided the studies according to study duration. Short term studies were defined as those in which subjects received a single opioid dose or an intravenous infusion and in which outcomes were measured for less than 24 hours. All other studies included in the review were considered to be "intermediate-term" studies. The median duration of these latter studies was 28 days (range 8-70 days). Only studies of pure opioid agonists were included, therefore studies of tramadol or buprenorphine were excluded. The short terms studies did not provide convincing evidence for the use of opioids in the management of neuropathic pain. The intermediate studies however did demonstrate a significant reduction in neuropathic pain intensity of 13 points (CI -16 to -9) on a 100 point VAS. The subjects in these studies had a variety of neuropathic pains including post herpetic neuralgia, diabetic neuropathy and phantom limb pain [174].

A review of RCTs between 2005 and 2010 calculated the NNT (number needed to treat) to achieve 50% pain relief for different neuropathic pain treatments. This review included RCTs which used chronic dosing and placebo but excluded studies of cancer neuropathic pain except for a number of defined post-surgical cases. The NNT for opioids in painful polyneuropathies (2.6 (CI 1.7-6) was less than that of gabapentin (6.4 (CI 4.3-12) but more than that for tricyclic antidepressants (2.1 (CI 1.9-2.6). The combined overall NNH (number needed to harm) were comparable between opioids (17.1 (CI 9.9-66)) and TCAs (15.9 (CI 11-26) but the NNH for gabapentin was much higher (32.5 (CI 18-222)) [175].

Most published guidelines on the management of neuropathic pain recommend the use of opioids as second or third line treatment, after other agents such as tricyclic antidepressants, pregabalin and duloxetine. This recommendation is based on the lack of reliable quality evidence for strong opioids in neuropathic pain and the concerns of

long term dependence and the risk of serious adverse effects or overdose [176;177]. The recent NICE guidelines suggest that strong opioids should only be started after review in a specialist clinic [176]. However in some select clinical circumstances, such as cancer related neuropathic pain, opioids may be recommended as first line therapy [178].

A review of 22 studies including 13,683 cancer patients demonstrated that neuropathic pain is a significant problem, with a prevalence among this population of 19-39% [179]. Despite the lack of reliable randomised controlled trials of opioids in neuropathic cancer pain, there are studies which promote the efficacy of these drugs in this setting. A study of 593 cancer patients demonstrated the efficacy of pain management according to the WHO analgesic ladder, the outcomes of which were not dependent on whether the pain was nociceptive (N=380), neuropathic (N=32) or mixed (N=181) [180]. It appears however that neuropathic cancer pain may need a slightly different approach with opioids, as compared to non-neuropathic cancer pain. A prospective study of 167 cancer patients who were commenced on opioid therapy demonstrated the analgesic efficacy of opioids for patients with "definite neuropathic pain", as classified by defined criteria. As compared to patients with "unlikely neuropathic cancer pain", patients with "definite neuropathic pain" required more aggressive opioid titration and management, including opioid switching and the management of opioid side-effects [181]. There is a lack of direct comparisons between opioids for cancer-related neuropathic pain.

Cancer related neuropathic pain is often treated with additional adjunct analgesic agents which are used alongside opioids because they target specific neuropathic pain mechanisms. Compared to non-cancer neuropathic pain however, the analgesic effect sizes of these adjuncts are generally smaller and there is a high incidence of side-effects [182].

1.7 Constipation on morphine

1.7.1 Constipation in cancer patients taking opioids

Constipation is a significant problem in cancer patients, occurring in 30 - 50% of this population [183-185]. Constipation in hospice patients is almost as prevalent as pain, (52% versus 62% respectively) [186]. Constipation has also been shown to be a cause of considerable distress in patients approaching end of life.[187] Although the pathogenesis of constipation is multi-factorial [188], opioid medication plays a key role [189]. The reported prevalence of constipation amongst all patients taking opioids ranges from 50 – 100% [189;190]. Constipation in palliative care patients on opioids also has financial implications [191].

Although there have been a number of papers examining the effects of morphine in cancer patients in terms of analgesia and side-effects [192;193], there have been few studies specifically focusing on the constipating effects of opioids in cancer patients. The numbers of patients included in these studies have tended to be small [194-196]. The current literature is conflicting in terms of the clinical relationship between opioids and constipation. The questions of whether there is a dose-response relationship and whether tolerance to constipation develops remain to be answered.

Constipation in cancer patients taking morphine was chosen for in-depth evaluation as part of this thesis because 1) there has been relatively little comprehensive study in this area to date and 2) there is recent interest in this topic with the advent of new opioid antagonists for the treatment of constipation.

1.7.2 Action of opioids on the gastrointestinal tract

Endogenous opioids are thought to play a role in regulating bowel function in healthy individuals who are not taking opioids [197]. Unlike opioid-analgesia, opioid-induced constipation is thought to be mediated predominately by peripheral rather than central mechanisms [198]:

- In vitro experiments have demonstrated inhibitory effect of opioids on isolated gut muscle preparations [199].
- The constipating effects of centrally administered morphine are abolished in animals in which the intestine has been rendered refractory to central influences through vagal resection. In the same animals however, subcutaneous morphine still produced anti-diarrhoeal action [200].
- Peripherally restricted opioid agonists such as loperamide which do not cross the blood-brain barrier also cause a reduction in gut transit [201].

MOR, KOR and DOR are found throughout the gastrointestinal tract and are involved in gastrointestinal function [202;203]. In humans MOR are found primarily in the submucosal and myenteric plexuses and also in cells of the lamina propria. KOR are confined to the myenteric plexus and are also found in mucle cells. DOR in humans are localised on both the myenteric and submucosal plexuses, in the muscle and in the mucosa [202]. Morphine is thought to act directly on these peripheral opioid receptors in the bowel.

Opioids cause constipation by increasing gut transit time and reducing gut luminal fluid content. Opioid receptor activation results in an overall reduction in neuronal excitability through an increase in membrane potassium permeability[204], an inhibition of voltage-gated calcium channels[205] and a reduction in cAMP production [206]. This causes a disruption in the integrated propulsive gut contractions and enhances non-propulsive contractions which may also lead to tonic spasm and abdominal cramping [207]. The stool remains in the gut for longer due to increased transit time resulting in more water re-absorption and thus the stool becomes hard and dry. Opioid receptor activation also has an indirect inhibitory effect on secretomotor neurones which results in less fluid in the gut lumen [208].

Opioid receptor activation results in changes in some of the key hormones, receptors and molecules involved in gastrointestinal function. Release of acetylcholine, an important excitatory neurotransmitter in the gastrointestinal tract, is reduced. Serotonin is released which acts through 5HT2 receptors to increase the release of noradrenaline. Noradrenaline plays a role in the inhibition of intestinal secretion [208].

1.7.3 Current management of constipation in patients taking opioids

Published studies suggest that constipation in cancer patients is generally poorly managed [209]. There may be a number of reasons related to definition, assessment and treatment. There is currently no gold standard for the management of constipation in cancer and palliative care patients because robust clinical trials in this area are lacking [210].

The management of constipation has traditionally been based around oral and rectal laxative use. There are a number of different agents available but to date there is no evidence that any one laxative is superior to another, mainly because there have been few studies in this area [210;211]. All laxatives, given in sufficient quantities, are capable of normalising bowel function in most constipated patients [194;212] Drug compliance is often an issue in cancer patients, not least because of poly-pharmacy. Most symptom control guidelines recommend prescribing concurrent laxatives for all patients taking opioids. In cancer patients the use of combination laxatives (a softening agent and a stimulant) is recommended in order to maximise efficacy and minimise side-effects [213]. It has been suggested that it is not possible to define accuarately any fixed opioid-laxative dose relationship because of inter-individual variation in terms of bowel function and response to medications [195]. It is also recommended that laxative treatment is titrated according to individual patient need and symptoms [196;214], rather like opioids are titrated to pain.

1.7.4 Opioid antagonists for opioid-induced constipation

In recent years there has been increased interest in the use of opioid receptor antagonists for the management of opioid induced constipation. Theoretically opioid receptor blockade by an agent which does not cross the blood brain barrier would inhibit peripherally mediated gastrointestinal side-effects without interruption of centrally-mediated opioid analgesia. The main three opioid receptor antagonists which have been studied for this purpose: naloxone, methylnaltrexone and alvimopan. Naloxone is a pan-opioid receptor antagonist with a high affinity for mu-opioid receptors. Systemically administered naloxone is used to reverse life-threatening

opioid toxicity and acts both peripherally and centrally. Oral naloxone however undergoes significant hepatic first pass glucuronidation to produce inactive metabolites [215], resulting in a very low (2%) oral bioavailability [216]. The role of oral naloxone as a peripheral opioid antagonist in cancer patients has been examined in a number of small early studies with good effect [217-219]. These studies however all demonstrated that the risk of precipitating pain or opioid withdrawal is substantial. Recently there has been increased interest in the use of fixed dose combination of prolonged release (PR) oral oxycodone / naloxone as a method to provide analgesia while preventing opioid-induced constipation. A multicentre prospective placebo-controlled randomized double-blind phase II trial of 202 patients with severe chronic pain concluded that a 2:1 oxycodone:naloxone ratio was optimal in terms of safety and efficacy. Only 2.5% of study participants in this study had a cancer diagnosis [220]. There was no significant difference in the mean pain intensities between treatment groups but there was an improvement in bowel function according to naloxone dose ($p<0.05$). Opioid withdrawal was not formally assessed in this study. Similar studies with 12 week follow-up periods comparing oxycodone / naloxone (2:1 ratio) PR with oxycodone PR alone demonstrated a significant improvement in bowel function in those taking the oxycodone/naloxone combination ($p<0.0001$) [221;222]. The maximum oxycodone dose (as part of the oxycodone/naloxone combination) in these studies was 80mg/day. Studies involving higher opioid doses with longer follow-up are warranted. Large studies of this combination of oxycodone / naloxone combination in a predominately cancer population have not yet been published.

Methylnaltrexone is a quaternary opioid antagonist which does not readily cross the blood brain barrier because of its reduced lipid solubility and increased polarity [223]. It is a non-selective opioid antagonists with preferential mu-opioid receptor binding [207]. Methylnaltrexone was originally studied in healthy volunteers who were given intravenous placebo, placebo + morphine or methylnaltrexone + morphine. Methylnaltrexone significantly prevented morphine-induced increase in oral-caecal transit time but did not affect morphine analgesia [224]. A randomized placebo-controlled trial was carried out involving 22 subjects who were enrolled on a methadone maintainance programme. All subjects who received methylnaltrexone (intravenous) had a laxation response without opioid withdrawal or significant side-effects [225].

A large randomized placebo-controlled phase III study using subcutaneous methylnaltrexone in 133 patients with advanced illness has been carried out [226]. Study subjects were administered 0.15mg/kg methylnaltrexone SC or placebo every second day for two weeks. The primary endpoints of this study were the proportion of subjects who had a rescue-free laxation (without the use of an enema or suppository) within four hours of the first dose and the proportion that had a rescue-free laxation within four hours after two or more of the first four doses. Subjects were also assessed for overall pain scores and opioid withdrawal. Forty eight percent of patients receiving methylnaltrexone had a rescue-free laxation within the first four hours after the first dose, compared to 15% who received placebo ($p<0.001$). Approximately 50% of patients who had a rescue-free laxation within four hours had a response within 30minutes, suggesting a rapid onset of action. There were no differences in pain scores between the methylnaltrexone and placebo groups and there was no evidence of significant opioid withdrawal. Only 59% (78/133) of study patients had a cancer diagnosis [226]. A similar study in 154 patients with advanced illness receiving a single dose of 0.15 or 0.3mg/kg SC methylnaltrexone or placebo demonstrated a laxation within 4 hours for 58-62% of patients, depending on the dose administered [227]. SC methylnaltrexone is licensed in the UK for palliative care patients as either 8 or 12mg depending on body weight [228]. Large studies evaluating the safety of methylnaltrexone are warranted [211].

Alvimopan is a large oral peripherally restricted opioid antagonist with high affinity for mu-opioid receptors [229]. It is metabolised in the gut [230]. Systemic absorption ranges from 0.03% in dogs to 6% in humans [231]. Studies in non-cancer and post-operative patients are encouraging [232-235]. A large study in patients with cancer pain has been carried out but the final results have not yet been published fully. Concern about cardiovascular side-effects have been raised [236-238].

Just as patients have a variable response to the constipating effects of opioid agonists such as morphine, there also appears to be inter-individual variation in response to opioid-antagonists. The study of 133 patients taking methylnaltrexone for opioid-induced constipation demonstrated a 48% response rate [226]. Although highly significant as compared to placebo, these results suggest that, as with opioid agonists, response to opioid antagonists may vary at an individual level. The authors theorised

that the lack of response in some patients may have been attributable to centrally-mediated constipation or to other non-opioid constipating factors [226].

Endogenous opioids are thought to play a role in the physiological regulation of gut function [239-241]. Studies with opioid receptor antagonists support this idea. Early studies on isolated pieces of human ileum demonstrated increased muscle contraction when bathed in methylnaltrexone, even in the absence of morphine [242]. Furthermore, in the absence of morphine, repeated intravenous administration of methylnaltrexone in healthy human volunteers demonstrated a significant reduction in gut transit time (as measured by the hydrogen breath test) [243]. Oral naloxone has been shown to improve gut function in patients with chronic idiopathic constipation, although the numbers of patients studied was small [244]. In vitro studies using guinea pig ileum demonstrated that alvimopan increases contractions and mechanical activity in morphine-naïve animals [245].

The development of opioid receptor antagonists is exciting; however a recent Cochrane review urges some caution [238]. These agents have not been directly compared against each other and they have not been compared to best current clinical practice with oral and rectal laxatives. In some of the larger studies, as constipation was a pre-requisite to entering the study, these individuals were clearly on an inadequate laxative regimen from the outset [226;246]. Most data suggest that these agents are more efficacious than placebo in improving opioid-induced bowel dysfunction. Long term safety data however is warranted. Animal studies suggest that chronic use of opioid-receptor antagonists may actually result in an increase in the number of opioid receptors, which in turn may lead to increased sensitivity to the constipating effect of opioid agonists and a resultant dependence on opioid antagonists [247]. It has also been suggested that the effects of opioid-antagonists may in fact be proportional to the degree of opioid agonist tolerance and that patients on higher doses of opioid agonists may in fact be more at risk of adverse effects from drugs such as naloxone [248].

1.7.5 Assessment of constipation in cancer and palliative care patients

Assessment of constipation has been reported to be poor in palliative care patients [249]. This may be for a number of reasons. Firstly there is no standardised definition of constipation in cancer patients, which makes objective assessment difficult. Secondly, constipation often means different things to different people [250], which makes interpretation of subjective assessment difficult. Finally, few constipation assessment tools have been specifically designed and validated for use in cancer patients and such tools are not in general use in UK hospitals.

Two dimensions of constipation definition in palliative care have been proposed: 1) measurable objective symptoms i.e. frequency of bowel movements and characteristics of defaecation and 2) the patient's subjective perception of constipation[251].

1.7.5.1 Constipation: subjective assessment

Constipation in cancer and palliative care patients differs from constipation in gastroenterology clinics in that in the former it should be treated as a symptom while in the latter it may be a disease process in its own right. The scale of distress caused by symptoms (including constipation) and the perception of severity of the symptom are largely subjective assessments [252]. In gastroenterology, subjective assessment of the presence of constipation does not correlate with the presence of constipation as defined by standardised criteria [253]. The same appears to be true in cancer and palliative care [254]. In cancer and palliative care patients therefore, the symptom of constipation from the patient's subjective perspective is as important as the more objective medical assessment.

1.7.5.2 Constipation: objective assessment

Unlike other symptoms in palliative care, with regards to constipation, there are some dimensions which may be objectively assessed [250]. Incorporating an objective measure into constipation assessment may be useful in order to facilitate rational pharmacological management.

On review of the literature, three key aspects of objective constipation assessment appear to be frequency of bowel movements, ease of defecation and stool consistency. There are many different sources which support the choice of these key features:

- Firstly, a suggested definition of constipation is the "passage of small, hard faeces infrequently and with difficulty" [251]. Data from studies of bowel function suggest that the key characteristics in defining constipation are faeces that are difficult to pass, infrequency of bowel movements compared to normal, smaller bowel movements compared to normal and hard stools [255].

- Secondly, in gastroenterology, the disorder of constipation may be classified into three categories: disorder of rectal evacuation, slow transit or a combination of both [256]. Although constipation in cancer and palliative care patients is often secondary to illness or medications rather than being a primary disorder, this classification is also useful for this group of patients in order to guide assessment of constipation as a symptom. Assessment of stool frequency, stool form and the need to strain provides information on both of these parameters. Increased gut transit time may be demonstrated by decreased stool frequency [256] and by assessment of stool form [257]. Difficulty in rectal evacuation may be assessed by stool consistency or the need to strain [256].

- Finally, as laxative therapies are either stool softeners or stool stimulants, detailed assessment of frequency, stool consistency and straining may provide adequate information to prompt rational laxative use.

1.7.5.3 Constipation assessment tools

Many different constipation assessment tools exist however there is no such tool which has been designed, validated and is commonly used in cancer and palliative care patients.

Although a heterogeneous group, cancer and palliative care patients have different needs and burdens compared to other patient populations. Many are undergoing rigorous chemotherapy, have multiple co-morbidities, and it is likely that constipation

is only one of many symptoms. Most existing tools are not suitable for this group of patients e.g. recall period may be too long (leading to error or bias) or the tool may take too long to complete (thus possibly decreasing the accuracy and completeness of the answers) [258]. Other constipation tools are not suitable for cancer and palliative care patients as they include questions which would be non-discriminatory in this population. For example abdominal distension / bloating may be due to disease / comorbidities and may not be solely related to the presence of constipation. A suitable constipation assessment tool for the cancer and palliative care population needs to be brief, easy to understand and non-invasive. Recall period must be brief enough to limit recall burden (taking into account the nature of cancer and palliative care patients), yet long enough to pick up sufficient occurrences of relatively infrequent bowel movement events [258].

A constipation symptom measurement scale is essential for the adequate management of constipation as it will provide the means for detecting symptoms, grading their severity and assessing effectiveness of management [259].

Table 1.3 summarises a number of the other available constipation assessment tools. Some of these are stand alone tools, others form part of general symptom assessment tools. As can be seen, few studies combine objective and subjective assessments of constipation.

Two constipation assessment tools were proposed for use in palliative care. The first was a bowel score which required patients to recall details about their bowel movements over the preceding three days. The score was made up of a composite of a) frequency and b) stool consistency[196]. The second was a bowel function score based on frequency (current compared to usual), stool consistency and ease of passing stool [260;261]. Unfortunately neither of these was tested in large prospective studies.

Mundipharma recently devised a novel constipation assessment tool, the Bowel Function Index (BFI) which is based on both subjective and objective assessment measures: ease of defaecation, sensation of incomplete bowel evacuation and subjective assessment of constipation. The patient scores each parameter on a numerical rating scale 0-100 and the BFI is the mean of the three scores. This tool was

validated against the Pac-Sym. This tool however does not include any assessment of frequency or stool consistency [262].

A Bowel Function Diary has recently been validated in a non-cancer population who were started on opioids. The results of this study suggest that assessing the number of spontaneous bowel movements (SBM, bowel movements without the use of laxatives etc) can differentiate between patients who are constipated or not on opioids [263]. The use of SBM per week however does not take into account the usual bowel habit of the study subject and the mean number of SBM per week is very varied. In the validation study (N=238), patients who reported that they were constipated had a mean SBM of 5.6 +/- 4.3 compared to 7.3 +/-3.6. A further limitation of this tool is that the assessment diary includes a number of items e.g. abdominal pain and lack of appetite which are non-discriminatory in cancer patients, therefore its use in a palliative care population is likely to be limited.

Table 1.3 Summary of available Constipation Assessment Tools

Note: this is not an exhaustive list Questionnaire	Subjective assessment	Objective assessment	Tested in cancer or palliative care patients	Scoring system	Comments
Clinical constipation assessment tools					
Bristol Stool Chart [264]	X	√	√	Descriptive scoring system (verbal and visual descriptives)	
Constipation Assessment Scale [265]	X	√	√	3 point VRS: no problem, some problem, severe problem.	2 minutes to complete
Constipation Scoring System (Cleveland Clinic Score)[266]	X	√	X	Structured questionnaire, each question scoring 0-2 or 0-4 marks	
PAC-SYM (Patient Assessment of Constipation Symptoms)[267]	X	√	X	5 point VRS (absent, mild, moderate, severe, very severe)	2 week recall period, 4 minutes to complete
KESS (Knowles-Eccersley-Scott Symptom Questionnaire)[268;269]	X	√	X	Structured questionnaire, each question scoring 0-3 or 0-4 marks.	5 minutes to complete
Rome Criteria [270-272]	X	√	X	Presence of certain criteria	Developed for diagnosis of functional constipation. Symptoms need to be present for at least 12 weeks in the preceding 12 months.
Bowel Function Index [273]	√	√	X	0-100 NRS	3-item questionnaire: ease of defaecation, incomplete bowel evacuation and subjective assessment
Bowel Function Diary[263]	X	√	X	Adjectival Likert scales	Validated in non-cancer patients on a variety of opioids for two weeks.

General symptom assessment tools incorporating constipation assessment *				
EORTC (European Organisation for Research and Treatment of Cancer QLQ-C30)[274-276]	√	X	√	4 point VRS: Not at all, a little, quite a bit, very much.
Rotterdam Symptom Checklist [277;278]	√	X	√	4 point VRS: Not at all, a little, quite a bit, very much.
Constipation assessment tools in palliative care research				
Bowel function scores [260]	X	√	√	Numerical score 0-2 or 0-3 for 3 items
Bowel score[196]	X	√	√	Numerical score for 2 items
* Note some general symptom assessment scales do not incorporate any question about constipation e.g. ESAS (the Edmonton Symptom Assessment System)				

1.8 Aims of the thesis:

The overall objective of this thesis was to test the following hypotheses:

1. Inter-individual variation in response to morphine may be clinically defined.
2. Inter-individual variation in response to morphine is associated with genetic variation.

It is hoped that the data presented in this thesis may contribute towards building a tool to allow prospective prediction of response to morphine for cancer pain.

My original aims for this thesis were:

- To identify genetic factors associated with inter-individual variation in response to morphine in patients with cancer pain using candidate gene assays.

- To explore the prevalence and management of constipation in cancer patients taking morphine.

Preliminary results and data analyses, together with developments in pharmacogenetics in terms of techniques and approaches, allowed the generation of new hypotheses resulting in the following additional aims:

- To develop a clinically relevant and robust method of defining "response" to morphine
- To explore how multiple clinical and genetic factors may interact together to influence response to morphine
- To carry out an exploratory genome wide association study to identify genetic factors associated with inter-individual variation in response to morphine in patients with cancer pain
- To examine the clinical assessment of constipation in cancer patients taking morphine
- To develop a constipation assessment tool

1.8.1 Clinical data presented in this thesis:

The clinical data were derived from two clinical trials:

- The *Prospective Morphine Study*: A prospective observational study of morphine responders and non-responders. Clinical and genetic data from a smaller number of patients (N=228) in this study have been previously published [31;95;113;279]. Data from the final completed study (N=298) are presented in this thesis.
- *A Prospective Randomized Controlled Trial of Morphine versus Oxycodone for Cancer Pain*. This study is ongoing and therefore the final clinical results are not available for this thesis. Interim results for the primary aims and objectives of this study form part of another PhD thesis [280]. Interim data pertaining to constipation assessment are presented in this thesis.

The clinical data were interrogated to:

- Explore the influence of clinical and environmental factors on inter-individual variation in response to morphine

- Develop a robust and meaningful definition (phenotype) of clinical response to morphine for cancer pain which would be used in further genetic association studies

- Constipation as a side-effect of opioids was chosen for in-depth analysis in terms of phenotype definition, prevalence, management and assessment.

1.8.2 Genetic data presented in this thesis

Genetic analysis was carried out on samples from the *Prospective Morphine Study*.

- Candidate genes were analysed on the whole study population.
- A genome-wide association study was carried out on a subset of the study population. This is the first GWA in patients taking morphine for cancer pain that we know of.

1.8.2.1 Candidate genes

The hypothesis tested in this study was that polymorphisms in genes coding for proteins and enzymes involved in biological pathways which interact with opioids and their receptors play a role in inter-individual variation in morphine response in cancer patients. A number of candidate genes were chosen for investigation based on known biological pathways involved in pain and morphine response and also on previously published animal studies in this area. Twelve genes were investigated in the palliative medicine department, comprising the Royal Marsden Hospital / Imperial College "Pain Plate". I optimised, ran and analysed the assays for "Pain Plate 1" which included the following genes: *OPRK (Kappa opioid receptor gene), OPRD (Delta opioid receptor gene), TRPV1 (capsaicin receptor gene), ADORA1 (A1-adenosine receptor gene), ADRA2A (α_{2A} adrenergic receptor gene)* and *ALOX12 (arachidonate 12-lipoxygenase gene).* "Pain Plate 2" included *BDNF (Brain derived neurotropic factor), CSEN (Calsenilin gene), GRIN1 (Glutamate receptor subunit 1), GRIN2A GRIN1 (Glutamate receptor subunit 2a), GCH1 (GTP cyclooxygenase 1) and MC1R (Melanocortin 1 receptor),* which were designed and tested on the study population by Dr Gretton and presented in her PhD thesis [280]. I also chose two other genes which had been run on a smaller sample of the study population to be included for more in-depth analysis, *OPRM (mu-opioid receptor gene) and ARRB2 (B-arrestin 2 gene)*, as well as *SLC6A4 (serotonin reuptake transporter gene).* For each of the genes presented in this thesis there is a biologically plausible association with pain perception and response to morphine, as detailed in relevant results chapters.

1.9 Thesis outline

Chapter 2: This chapter contains details of the two clinical trials (The *Prospective Morphine Study* and the *Prospective Randomized Controlled Trial of Morphine versus Oxycodone for Cancer Pain*) which were carried out to generate clinical and laboratory data for this thesis. The laboratory methods for genotyping are described including candidate gene and whole-genome testing. The statistical methodologies used to interrogate the data are presented.

Chapter 3: The clinical results of the *Prospective Morphine Study* are described. This chapter focuses on the results of data exploration in terms of phenotype definition. The clinical phenotypes to be used in the subsequent genetic association studies are proposed.

Chapter 4: This chapter describes the results of the candidate gene association study involving genes coding for the opioid receptors. This study was carried out using data from the *Prospective Morphine Study*.

Chapter 5: This chapter describes the results of the candidate gene association study involving other genes involved in pain and analgesia (*ADORA1, TRPV1, ADRA2A, ALOX12, ARRB2* and *SLC6A4*). This study was carried out using data from the *Prospective Morphine Study*.

Chapter 6: This chapter presents the results of the genome-wide association study which was carried out on a subset of patients from the *Prospective Morphine Study.*

Chapter 7: This chapter presents the results of a number of studies investigating constipation in cancer patients taking oral morphine. An exploration of the current management of constipation is presented using data from the *Prospective Morphine Study.* The results of a retrospective audit of documentation of constipation assessment which was carried out on a subset of the patients in the *Prospective Morphine Study* are detailed. A definition of a working phenotype of constipation in cancer patients on morphine is presented. The results of a genetic association analysis

are detailed. Finally the results pertaining to the development of a constipation assessment tool as part of the *Prospective Randomized Controlled Trial of Morphine versus Oxycodone for Cancer Pain* are reported.

Chapter 8: The findings of this thesis are discussed with particular emphasis on future challenges and opportunities for research in this area.

2 Chapter 2 Methods

The work presented in this thesis involved the integration of carefully collected clinical data with genetic data to explore inter-individual variation in response to morphine. This chapter contains detailed descriptions of the clinical studies and laboratory and analytical methods used.

2.1 Clinical studies

The clinical data were collected as part of two clinical studies:

- *The Prospective Morphine Study*
- *A Prospective Randomized Controlled Trial of Morphine versus Oxycodone for Cancer Pain*

I was involved in running both of these studies along with a fellow PhD student, Dr Sophy Gretton, and a number of research nurses. My involvement in both studies included:

- Study participant recruitment. This included screening, identification, consenting and follow up of patients as appropriate
- Biological specimen collection, storage (as appropriate) and analysis
- Maintenance of study specific databases and data entry
- Clinical trial governance. This included including liaison with research governance bodies; Research Ethics Committee (REC), Research and Development Committee (R&D) and the Medicines and Healthcare Products Regulatory Agency (MHRA).

Both studies were carried out in accordance with the principles of Good Clinical Practice (GCP) [281]. Material Transfer Agreements were set up for the transfer of material from the Royal Marsden Hospital to external organisations, in accordance with the Human Tissue Act 2004 [282].

2.1.1 Prospective Morphine Study

2.1.1.1 Study design

This study was originally set up in 2002 and some analysis of the first 228 study subjects has been published [31;95;279]. I was involved in the continued running of the trial until it was closed with a final total study population of 298.

The aim of this study was to investigate the effect of genetic variants on the clinical outcome of morphine treatment. This was an observational single time-point study. Adult patients (over 18 years of age) who were taking oral morphine for cancer pain were included. Patients with predominately neuropathic or incident pain, patients with renal impairment (plasma creatinine >1.5 upper limit of normal) and patients who were on subcutaneous, intramuscular or intravenous morphine or other regular opioids were not included in this study.

Approval for this study was given by the Research and Ethics Committee and all patients signed a written consent form before entering the study. The clinical palliative care team titrated morphine dose according to individual response. Patients were recruited into one of two cohorts according to patients' subjective experience and the observations of the experienced clinical team. Subjects who had a successful outcome on morphine were called "Morphine responders". These were patients who had been on morphine for at least one month and who were experiencing adequate analgesia without problematic side-effects. "Morphine non-responders" were defined clinically as those patients who did not have a satisfactory clinical outcome i.e. those who failed to achieve adequate analgesia despite escalating morphine doses and/or who experienced subjectively intolerable side-effects. Morphine non-responders underwent an opioid switch (as determined by the clinical team, but usually to oxycodone as the hospital's second line strong opioid [283]). Clinical data on morphine non-responders were collected before they underwent the opioid switch. This cohort was also followed up after one week to assess the efficacy of the switch.

2.1.1.2 Data collection

Pain severity was recorded using the modified Brief Pain Inventory (BPI) [284]. This pain scoring system has been used in other studies in this area [92;110]. Five dimensions of pain intensity were assessed; worst pain, least pain and average pain in the previous 24 hours, current pain and percentage pain relief from morphine. For each of these pain modalities, study subjects recorded pain severity on an 11-point numerical rating scale (NRS) of 0 – 10 (0 = no pain, 10 = worst pain imaginable).

Morphine side-effects (drowsiness, confusion and hallucinations, constipation, dry mouth, myoclonus, itch and nausea) were recorded using a four point Likert scale; grade 0 "not at all", grade 1 "a little", grade 2 "quite a bit", grade 3 "very much". This scale is similar to that used in the European Organisation for Research and Treatment of Cancer Quality of Life Questionnaire (EORTC-Q30) [276], and is also similar to those used in other studies of response to opioids [29;92;112]. Demographic data (including age, gender and ethnicity), cancer diagnosis, current morphine dose, length of time on morphine and concomitant medications were documented. For those patients who had died at time of analysis, the number of days between recruitment to the study and death were recorded as a measure of prognosis. Details of the study questionnaires and proformas are found in Appendix B.

2.1.1.3 Biological samples

Biological samples were collected at the time of recruitment to the study:

- Routine haematology (full blood count) and biochemistry (urea, creatinine, electrolytes, calcium, albumin, liver function tests): 3ml blood in EDTA and 4.5ml in Lithium Heparin tubes respectively. These were processed by the laboratories at the Royal Marsden Hospital.
- Pharmacogenetic analysis: 9mls blood in EDTA tubes for DNA extraction. These were processed and stored in Imperial College, as detailed in section 2.3.1.
- Immunology (lymphocyte subset analysis): 3ml blood in EDTA tube. This sample was processed and analysed in the immunology laboratories of Chelsea and Westminster Hospital within 24 hours of the blood being taken.

- Morphine metabolites: 9mls blood in Lithium Heparin tubes. These samples were centrifuged for 20minutes at 3000rpm within two hours of the blood being taken and the plasma stored at -40 degrees for subsequent analysis. (Metabolite analysis is presented as part of Dr Gretton's PhD thesis[280]).
- Urine was also collected for future proteomic and metabonomic analysis. This was centrifuged at 3000rpm for 30 minutes and the supernatant was stored at minus 80 degrees.

2.1.1.4 Limitations of the Prospective Morphine Study

The data in this study were collected from a single time point which does not reflect the dynamic nature of symptoms in cancer patients. Therefore, in order to more accurately capture the subjective nature of the symptoms necessitating an opioid switch, a prospective follow-up study design, such as in the *Prospective Randomized Controlled Trial of Morphine versus Oxycodone for Cancer Pain (*described in section 2.1.2), is required.

The decision to switch opioids was made empirically by the palliative care team. This was based on the patient's subjective experience and subjective threshold of sensitivity of opioid analgesia and tolerability of side-effects rather than being based on pre-determined standardised measures of pain or side-effect intensity. This is in keeping with guidelines on opioid switching which emphasise the need for individual assessment and flexibility in decision-making based on inter-individual variation [285].

A more standardised definition of when to consider opioid switching might result in a homogeneous cohort. Very little work has been carried out in this area. Most studies on opioid switching either do not define the switching criteria or define them according to the subjective patient experience. A number of studies by Mercadante et al have used standardised cut off point in terms of pain and side-effect intensity as criteria to define the need for an opioid switch. One prospective study defined patients who required an opioid switch in terms of opioid dose escalation and/or intensity of opioid side-effects. Patients with unacceptable side-effects were those with symptom intensity of ≥2 on a scale of 0-3, which corresponded to a verbal scale of 0 = not at all,

1 = slight, 2 = a lot and 3 = awful. Patients experiencing a lack of opioid analgesic sensitivity necessitating an opioid switch were defined as those who had a poor analgesic response despite having had their opioid dose doubled in one week. Poor analgesic response was not defined in terms of pain intensity. In that study, the opioid switch was considered successful when the pain intensity or symptom score was reduced by 33% within seven days. The authors stated however that although they tried to use objective and reproducible assessments, they did not adapt a very rigid switching protocol as this would preclude the flexibility which is necessary in obtaining a balance between analgesia and opioid side-effects in patients with challenging clinical conditions [29]. In another study the criteria for switching from morphine to methadone included uncontrolled pain (VAS >4) and/or moderate to severe opioid side-effects [36].

There has been no study on opioid switching which incorporates objective testing of symptoms as a definition of the need to initiate an opioid switch. For example, the clock drawing test has been used to determine opioid-related cognitive dysfunction [286]. There has been one case report of the benefits of opioid switching in a patient with an abnormal clock drawing test [287] but such objective assessments have not been used in opioid switching studies. As a measure of cognitive function the clock drawing test or other assessment may provide objective criteria for opioid switching and an objective measure of assessing the efficacy of the switch.

There are little data on the factors which influences a clinician's decision to initiate an opioid switch. The proportion of patients switched due to pain or adverse effects varies widely, depending on the study [12]. Although guidelines on opioid switching recommend assessment of demographics such as age, race, disease status and concomitant medications, there is scant evidence as to how these and other factors influence decision making. Furthermore there is undoubtedly an element of inter-clinician variation in terms of when and why opioid switches are initiated. Further research in this area would help define the clinical phenotype of morphine responder versus morphine non-responder.

2.1.2 A Prospective Randomized Controlled Trial of Morphine versus Oxycodone for Cancer Pain

This study was designed by Dr Sophy Gretton, Dr Julia Riley and Dr Joy Ross. The interim analysis of the primary outcome forms part of Dr Gretton's PhD[280]. Recruitment to this study is ongoing and therefore any further analysis of clinical outcomes is not possible at time of writing this thesis. A brief overview of the study design is detailed below. I was involved in running the study until I finished my work as a research fellow, at which time 109 study subjects had been recruited. I had a specific primary role in amending the protocol to comply with MHRA standards, submitting substantial amendments to the research governance agencies (including an amendment to include the constipation on opioids assessment tool), designing and maintaining the study specific database and maintaining the pharmacovigilance records for the study.

2.1.2.1 Study design

The primary aim of this study is to compare the response rates of morphine and oxycodone when these drugs are used as first line strong opioids in moderate to severe cancer pain.

The eligibility criteria for this study includes adult patients (over 18 years of age) with a diagnosis of cancer for whom pain is not controlled on Step 2 (weak opioid) analgesics (as defined by the 1996 WHO analgesic ladder) and who clinically require a strong opioid. Like in the *Prospective Morphine Study*, patients with predominately neuropathic or incident pain, patients with renal impairment (plasma creatinine >1.5 upper limit of normal), patients requiring parenteral opioids and patients with a clearly defined history of intolerance to morphine or oxycodone are excluded.

Approval for this study was granted by the Royal Marsden Hospital R&D department and the REC and the MHRA. Written consent is obtained from all study participants. Potential study participants are recruited from inpatient and outpatient settings at the Royal Marsden Hospital.

The study was designed and is conducted out in accordance with the principles of EU

Clinical Trials Directive (2001/20/EC), The Medicines for Human Use (Clinical Trials) Regulations (2004) and the Human Tissue Act (2004) [282;288;289].

This is a single centre, prospective, randomised controlled trial. On entry to the study, participants are randomised via a computer to receive either oral morphine or oral oxycodone. Study subjects are titrated on short-acting preparations of oral morphine or oral oxycodone as per Palliative Care clinical guidelines in the Trust [283] until pain control is achieved or intolerable side-effects are experienced. If the study subject is a non-responder to the initial opioid (i.e. he/she experiences inadequate analgesia despite escalating doses and/or intolerable side-effects), he/she is switched to the alternative opioid (morphine if the patient started on oxycodone and vice versa) and this is titrated to effect. Study subjects complete a daily opioid titration diary until they are stable on either the initial or second opioid. Once they are stable they are converted to the long acting preparation of the opioid if appropriate. Study subjects are followed up for a maximum period of 12 months or until they die or withdraw from the study.

2.1.2.2 Data collection

Clinical data and biological specimens are collected from study subjects at certain defined time points during their follow up as detailed in figure 2.1. At each time point clinical data are collected about pain character and pain severity (using the Modified Brief Pain Inventory, as for the Prospective Morphine Study). Neuropathic pain is screened for using the S-LANSS (self-report version of the Leeds Assessment of Neuropathic Symptoms and Signs pain scale) [290]. Side-effect scores are collected as 11 point numerical rating scales where 0 represents no symptom and 10 the worst symptom severity imaginable. Data about the opioid dose, schedule and breakthrough opioid are collected, as well as data about concomitant medications. Demographic data including ethnicity, gender, age, tumour diagnosis are also collected at baseline. The daily titration diaries include pain and toxicity scores as detailed above. At each time point the "Constipation on Opioids Assessment" is completed. Details of the study questionnaires and proformas are found in appendix B.

Biological samples are also collected at each time point. These are the same as are collected in the prospective Morphine Study (section 2.1.1.3), except that whole blood for DNA is only collected once, at time of recruitment.

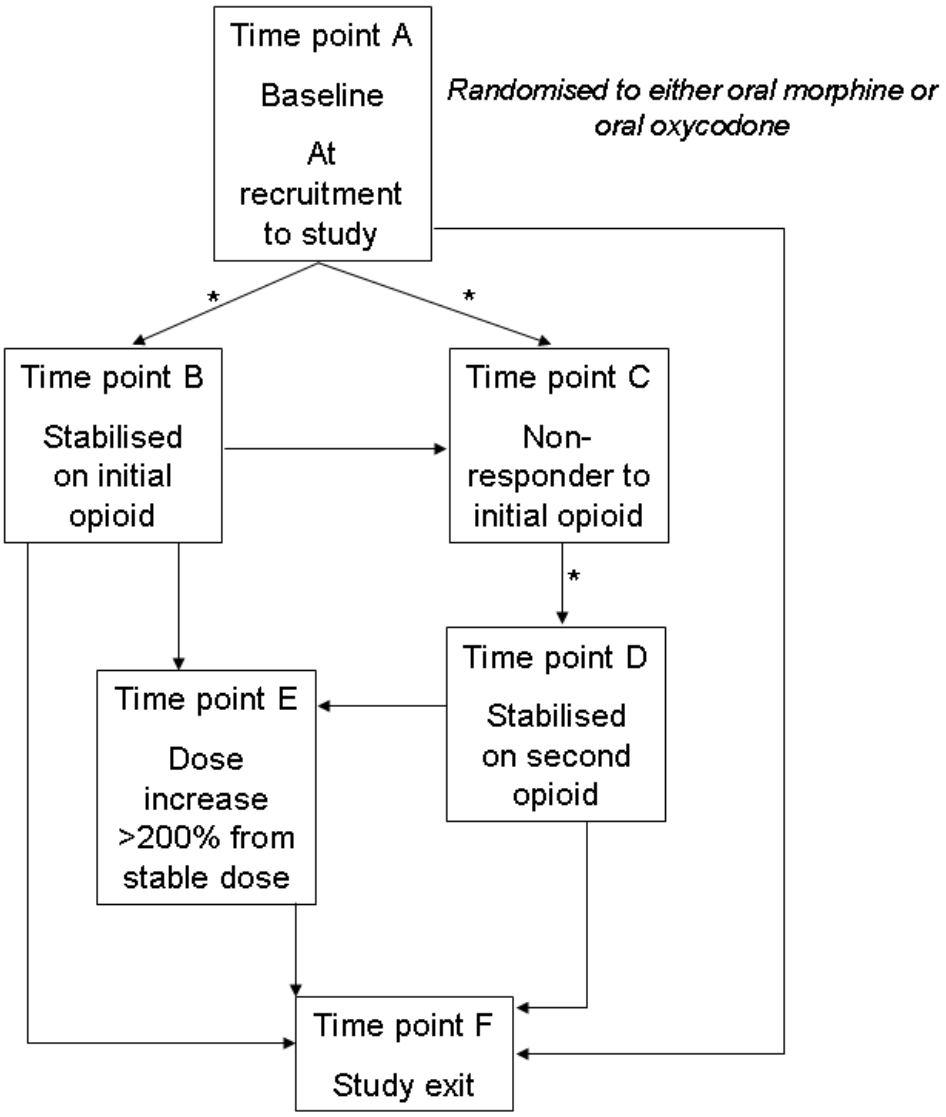

Figure 2.1 Study design for *A Prospective Randomized Controlled Trial of Morphine versus Oxycodone for Cancer Pain*.

* daily titration diaries collected

Reasons for study exit include:

- Non-response to both morphine and oxycodone
- Subjects converted to parenteral opioids on clinical grounds
- Subjects unable to take oral opioids on clinical grounds
- Development of renal failure of >1.5 upper limit of normal
- Patient dies
- Subject stops taking step 3 opioid on clinical grounds
- Subjects have reached one year post recruitment
- Needing to stop morphine and/or withdraw from the study on clinical grounds not specified above

2.2 Database construction and maintenance

Both the *Prospective Morphine Study* and *Prospective Randomized Controlled Trial of Morphine versus Oxycodone for Cancer Pain* have study specific databases which I was involved in designing and maintaining.

The procedures for collecting, storing and accessing the data collected in these trials are in keeping with GCP guidelines [281], the Data Protection Act [291] and Caldicott Principles [292]. Only members of the research team have direct access to data and documents pertaining to this study. The clinical data for each patient are recorded in a patient-specific Clinical Research Form (CRF) which is kept in a locked cabinet in a secure office in the palliative medicine research department. All patient identifiable data are removed from the electronic database. The subjects are pseudo-anonymised and a unique study number is assigned to each subject. The electronic databases have been constructed using Microsoft Excel. These are stored in a limited access, password protected encrypted network folder on the Royal Marsden Hospital Server.

Quality control measures to minimise data error include:

- Data entry onto the electronic database by two members of the research team
- The electronic database is checked against the source data (original CRFs / laboratory reports / genetic data)
- Data audit to identify outliers, missing data and unusual data points which require verification against source data. Data mining tools such as Knowledge Studio (www.Angoss.com) were used to audit the data for accuracy.

Work continues in the department to develop an integrated data management system to 1) cope with the vast quantities of data that are being generated by the studies and 2) aim for zero error in data fields. I was involved in the initial planning to develop an InForm Global Trial Management database [293]. This database is a web-based application which has an easy-to-use interface and simple navigation tools. Online edit checks are in place to reduce data entry errors and there is an online query process to facilitate efficient data discrepancy resolution. It also has an inbuilt audit facility to monitor data entry and data accuracy. This work is being continued by Dr Ruth Branford and will be presented as part of her PhD.

2.3 Laboratory methods

All biological samples (except for those sent to haematology and biochemistry) were labelled with the patient unique study identifier and not patient identifiable details.

2.3.1 DNA extraction from fresh blood

DNA was extracted from whole blood samples (9 mLs, tri-sodium citrate or di-sodium EDTA vacutainers) using a standard salting out method [294]. 9mls of whole blood was collected in an EDTA bottle. This was transferred to a 15ml Falcon tube and centrifuged at 2500 rpm at room temperature for 20 minutes. This resulted in separation of the white cells, red cells and plasma. The white cells were removed using a 3ml Pasteur pipette and placed in another Falcon tube. 13ml of Red Cell Lysis Buffer (144 mM NH_4Cl, 1 mM $NaHCO_3$) was added in order to remove any erythrocytes contaminating the sample and the sample shaken well. The sample was left at room temperature for approximately 20minutes until complete haemolysis was evident. The sample was centrifuged again at 2500 rpm for 20 minutes in order to separate out the white cells into a pellet at the bottom of the tube. The supernatant was discarded and the pellet was washed gently in Red Cell Lysis Buffer.
3mls Nuclear Lysis buffer (10 mM Tris-HCl pH 8.2, 400 mM NaCl, 2 mM Na_2EDTA pH 8.0) was added and the sample shaken in order to re-suspend the pellet. 1ml NaCl (Sodium Chloride) and 2mls 24:1 Chloroform / iso-amyl alcohol were then added in order to precipitate and separate proteins from the DNA. The sample was shaken well to form a milky solution which was then centrifuged at 3000 rpm for 30 minutes. This resulted in the separation of the DNA into a phase at the top of the sample, separated from the lower layer of debris by a layer of protein. The DNA layer was transferred into a Falcon tube and was topped up to 13mls with absolute ethanol. The mixture was inverted several times which allowed the DNA to precipitate out as a white filament. The DNA was removed from the ethanol and placed in a microfuge tube. The ethanol was allowed to evaporate in the fume cupboard and the DNA was re-suspended in sterile water (Baxter, UK). The sample was allowed to dissolve overnight in the fridge (4^o C) and then stored at -20^oC.

2.3.2 DNA amplification of genomic DNA

The yield of DNA from individual patient whole blood samples is limited and therefore it is often necessary to amplify existing DNA. This was carried out using Repli-g Midi kits and protocol (Qiagen, UK) [295].

2.3.2.1 Preparation of Genomic DNA for amplification

2.5µl template DNA was placed in a microcentrifuge tube. 2.5µl D1 buffer (9µl reconstituted DLB buffer and 32µl nuclease-free water per 15 reactions) was added to the DNA and mixed by vortexing and centrifuging briefly. The sample was incubated at room temperature for 3 minutes. This denatures the DNA: the double-stranded DNA unwinds and separates into single-stranded strands.
2.5µl buffer N1 (Stop solution 12µl and nuclease-free water 68µl per 15 reactions) was added to neutralise the reaction and again the samples were mixed by vortexing and centrifuging briefly. 10µl of the denatured DNA was placed in another microfuge tube to act as a template for amplification as detailed below (section 2.3.2.3).

2.3.2.2 DNA extraction from dried blood spots

Some of our collaborators collected blood on blood spot cards which only contain small amounts of DNA. Whole genome amplification from the dried blood spots was carried out using Repli-g Midi kits (Qiagen, UK)[295].

A 3mm dried blood spot was cut using a Harris unicore cutter™ and placed into a microfuge tube containing 500µl TE buffer (10mM Tris CL, 1ml EDTA, pH 8.0). The tube was inverted several times to wash the blood spot and was incubated at room temperature for 30 minutes. The sample was then centrifuged for 15 seconds and the supernatant discarded. The blood spot was rinsed again in 500µl TE buffer, centrifuged again for 15 seconds and the supernatant discarded. 40µl TE buffer was placed directly onto the blood spot in the microfuge tube. 500 µl Solution A was prepared by adding 40 µl 5 M KOH (potassium hydroxide) and 10 µl 0.5 M EDTA (pH 8) into 450 µl deionized water. 40µl denaturation buffer (D2) (10µl DTT 1M and 110µl Solution A per 3 reactions) was added to the tube in order to lyse the cells and

this was mixed by pipetting up and down 3 times before placing it on ice for 10 minutes. 40µl stop solution was added to neutralise the lysed cells and mixed by pipetting up and down 3 times. 10µl of the lysed blood spot solution (containing HCl and Tris HCl buffer) was placed in another microfuge tube to act as a template for DNA amplification (see below).

2.3.2.3 Amplification of Human Genomic DNA by MDA

Whole genome amplification by Multiple Displacement Amplification (MDA) was carried out using Repli-g Midi kits (Qiagen, UK)[296]. This high fidelity technique is reliable and has a low rate of amplification bias [297]. Genome amplification is carried out using DNA polymerase, an enzyme that catalyzes the polymerization of deoxyribonucleotides into a DNA strand. Random hexamer primers anneal to multiple sites along the target molecule and serve as initiation sites for the Phi29 DNA polymerase-mediated DNA replication. Phi29 DNA polymerase is stable, which facilitates many hours of performance, and it can replicate 100kb without dissociating from the template [297]. As the replication proceeds along the template molecule, it reaches the initiation site for other replication events that are processed in parallel. These upstream DNA strands are displaced to produce new templates and thus DNA replication continues exponentially to produce large numbers of copies of the original DNA. This process produces products that are greater than 10 kb in size, thus suitable for SSP-PCR genotyping [297;298]. The quality of the products of MDA is influenced by the quality of the original DNA.

The DNA polymerase was thawed on ice and a master mix was prepared (10µl nuclease free water (Baxter, UK), 29µl Repli-g Midi Reaction Buffer and 1µl DNA polymerase per reaction, kept on ice and used immediately). 40µl of the master mix was added to each microfuge tube containing the template DNA (as detailed above), vortexed for 10seconds and centrifuged briefly. The tube was then kept at 30^{o}C for 16hours in the thermal cycler for isothermal DNA amplification. The DNA polymerase was inactivated by heating the sample to 65 ^{o}C for 3 minutes and the DNA sample was stored at -20^{o}C.

2.3.3 DNA quantification

DNA concentration was measured using PicoGreen®, a fluorescent nucleic acid stain which binds selectively to dsDNA. It has an excitation maximum at 480 nm (lesser peaks in the short-wave UV range) and an emission peak at 520 nm. The unbound dye has practically no fluorescence [299].

A standard DNA concentration curve was constructed by serial dilution of stock DNA (A) as shown in table 2.1 and fig 2.2. The standard curve was generated by plotting the concentration of the DNA standards on the X-axis and the fluorescence reading from the microplate reader on the Y-axis.

Table 2.1 DNA dilution protocol to prepare the standard curve

Standard Concentration	**Volume of known standard**	**Volume of dH_2O**	**Final DNA standard concentration in PicoGreen assay***
(B) 2000 ng/ml	40µl of stock DNA (A) (100 µg/ml)	1960 µl	1000 ng/ml
(C) 1000 ng/ml	500µl of B (2000 ng/ml)	500 µl	500 ng/ml
(D) 500 ng/ml	500µl of C (1000 ng/ml)	500 µl	250 ng/ml
(E) 200 ng/ml	50µl of B (2000 ng/ml)	450 µl	100 ng/ml
(F) 50 ng/ml	50µl of D (500 ng/ml)	450 µl	25 ng/ml
(G) 5 ng/ml	50µl of F (50 ng/ml)	450 µl	2.5 ng/ml
(H) 0 ng/ml	0	500 µl	0 ng/ml

Stock DNA (A) is supplied at 100µg/mL.
* The final DNA standard concentration in PicoGreen assay is the standard concentration / 2 because the DNA standard is diluted 1:1 with the PG stock.

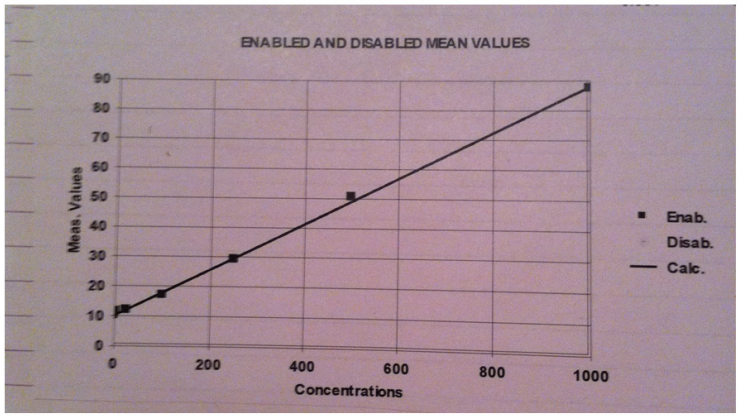

Figure 2.2 A DNA standard concentration curve

The PicoGreen dsDNA Quantitation Reagent (PG) is supplied as a 1mL concentrated dye solution in anhydrous dimethylsulfoxide (DMSO). 5µl PG was added to 100µl TE in 900µl dH2O (Baxter) to make up a 2 µg/mL PG stock solution (PG stock : 2XTE 1:200). This working PG solution was prepared in a plastic tube as the reagent may adsorb to glass surfaces. The tube was kept covered with foil to protect against light degradation.

DNA samples to be tested were prepared by diluting 2µl DNA in 500µl dH2O to make 1:250 dilution. (A high dilution of the experimental sample ensures that any contaminants are maximally diluted.)

10µl of each of the standard concentration DNAs was dispensed into the left lateral column (column 1) of wells of a 96-well plate (Griener Bio-One, UK) as demonstrated in figure 2.3. 10µl of the test DNA was dispensed into the other wells and a paper grid was labelled according to the position of the DNA samples. 10µl of the working PG solution was added to each well and the plate was centrifuged briefly.

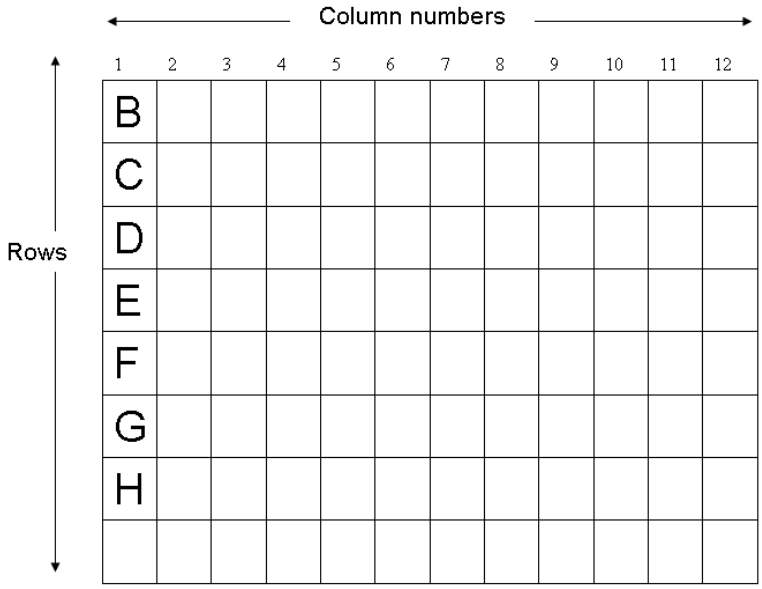

Figure 2.3 96 well plate set up for DNA quantification

10µl of each of the DNA standards are placed in the wells of column 1 as shown. The last well in column 1 is left empty as a negative control.

The fluorescence of the samples measured using a Lambda Fluoro 320 plus fluorescence microplate reader (MWG Biotech AG, Milton Keynes, UK). PicoGreen-stained samples were excited at 485nm and emission at 538nm was recorded. The concentration of each individual DNA sample was determined by plotting the fluorescence reading of the sample against the standard curve and reading the DNA concentration on the X-axis.

2.3.4 DNA quality control checks

The quality of DNA was checked in a number of ways:

- Electrophoresis of a small quantity of DNA (25 ng to 200 ng) on 1% agarose gel. Non-degraded DNA will run as a distinct homogenous band of high molecular weight (approximately 10-20kb). A smear of lower molecular weights that indicate degradation or shearing of the sample.

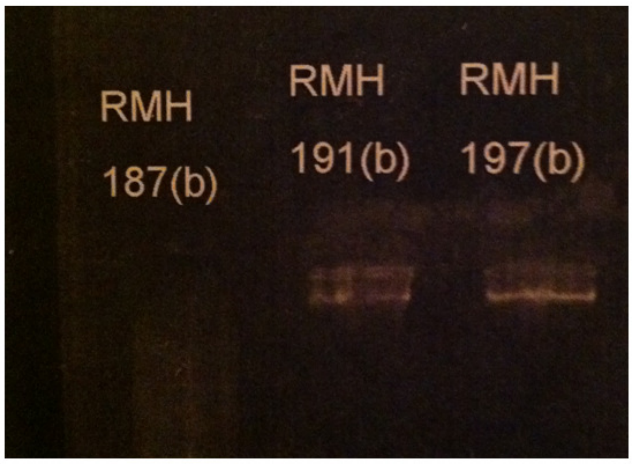

Figure 2.4 DNA quality control
RMH 187 (b) is degraded while RMH 191 (b) and RMH 197 (b) are non-degraded DNA.

- DNA quality was also checked using DNA-OK! (Microzone Ltd, UK). This is a multiplex PCR (polymerase chain reaction) which amplifies from 5 different regions of the genome. There is also a positive PCR control. The presence of 6 amplicons suggests good quality DNA. If the DNA is partially degraded, then the largest PCR fragments are lost and one or more of the amplicons are missing. If the DNA has been entirely degraded, only the positive control will amplify.
  3µl DNA OK primer mix, 5µl Megamix (containing recombinant *Taq* polymerase, dNTPs, reaction buffer and an enzyme stabiliser), 1µl water (Baxter, UK) and 1µl of the DNA to be tested were placed in a well of a 96-well plate. The plate was placed in a thermocycler and the cycling parameters were set as: 5 minutes at 95^0C, followed by 33 cycles of: 95^0C for 30 seconds, 63^0C for 30 seconds, and 72^0C for 45 seconds. The PCR products were then

electrophoresed on an agarose gel containing SYBR® Safe DNA gel stain (Invitrogen, UK) and visualised using a UV illuminator.

2.3.5 Sequence-Specific Primers and Polymerase Chain Reaction

In this thesis I used two methods to assess SNPs:

- SSP-PCR (Sequence-Specific Primers and Polymerase Chain Reaction)
- Genome-wide genotyping using the Affymetrix microarray platform.

I was involved in sample collection and preparation, assay design and testing and genotyping using SSP-PCR, the details of which are outlined below. I carried out the sample preparation and data analysis of the Affymetrix study but the genome-wide genotyping was carried out in Yale University, USA therefore I have only included a basic overview of this process in this thesis (section 2.3.6).

SSP-PCR is a technique which amplifies specifically selected DNA sequences, which can then be visualised on an agarose gel. A primer is a short oligonucleotide which serves as a starting point for DNA synthesis. The primer base-pairs to a specific sequence of DNA to allow initiation of a complimentary strand of DNA by a DNA polymerase. The DNA polymerase catalyses the synthesis of DNA by adding free nucleotides to the 3' end of the primer. Therefore the new strand of DNA is elongated in a 5'-3' direction.

In SSP-PCR, in order to test a bi-allelic SNP, two specific primers (known as allele specific / sequence specific primers) are designed so that their 3' ends are complimentary to the two allelic variants. Amplification will only take place if the 3' end base-pairs with the DNA sequence. A consensus primer is also designed which is a certain distance from the variant. The specific and consensus primers match the beginning and the end of the DNA sequence to be replicated and direct replication towards each other i.e. amplification of a known length of DNA sequence (amplicon) occurs between the two primers (specific and consensus). The newly synthesised stretch of DNA is complementary to the original template and therefore a double

stranded piece of DNA is recreated. SSP-PCR is a self-perpetuating process in which the DNA which is generated is used as a template for further amplification.

PCR consists of a series of heating and cooling cycles which are required for any DNA synthesis reaction:

- *Denaturation* of the template into single strands. This involves heating the DNA to 93-95^{o}C to melt the hydrogen bonds joining the two strands.
- *Annealing* of primers to each original strand for new strand synthesis. This occurs when the temperature is reduced to between 50-70^{o}C.
- *Extension* of the new DNA strands from the primers. This occurs between 70-75^{o}C as this is the optimal temperature at which *Taq*-polymerase works.

After 30 cycles of these three steps more than 100,000,000 copies of the original sequence are created.

When the PCR products are electrophoresed on an agarose gel, the presence of a PCR product of expected size indicates the presence of the specific allele. By using two reactions per bi-allelic SNP (one for each allele), the diploid genotype of each study subject can be determined.

SSP-PCR is a useful technique to identify SNPs as it is cheap, sensitive and fast.

2.3.5.1 SSP-PCR Primer Design

The DNA sequence around the SNP of interest must be known in order to design specific primers. Online databases such as NCBI (National Centre for Biotechnology Information) (http://www.ncbi.nlm.nih.gov) and CHIP (Childrens Hospital Bioinformatics Programme) (http://snpper.chip.org) [89] were used to identify the published genomic sequences for each gene. The location of the SNP of interest was identified from the literature and from these databases.

A number of design parameters were taken into consideration:

- Primer sequences were chosen to uniquely select for a region of DNA in order to avoid annealing to a similar sequence nearby. The NCBI BLAST (Basic Local Alignment Search Tool) function

(http://www.ncbi.nlm.nih.gov/BLAST/) was used to identify all the possible regions in the genome to which the primer may bind.

- Mononucleotide repeats were avoided, as loop formation can occur and contribute to mishybridization.
- The web-based Oligonucleotide properties calculator (http://www.basic.nwu.edu/biotools/OligoCalc.html) and FastPCR programme (http://www.biocenter.helsinki.fi/bi/bare-1_html/download.htm) were used to ensure that primers did not easily anneal with other primers in the mixture as this could lead to the production of 'primer dimer' products contaminating the mixture. Primers were also designed so that they did not anneal strongly to themselves, as internal hairpins and loops could hinder the annealing with the template DNA.
- The optimal GC content (the percentage of nitrogenous bases that are either guanine or cytosine) of the primer was 45-55%. A higher GC-content level indicates a higher melting temperature.
- The optimal length of each primer was 17-21 nucleotides. This length is long enough for adequate specificity and short enough for primers to bind easily to the template at the annealing temperature.
- The optimal salt adjusted melting point (Tm) was 58-61^{o}C. This was calculated using the Oligonucleotide calculator. Primer Melting Temperature is the temperature at which DNA denaturation into single strands occurs. This is therefore an indication of stability between the two DNA strands. Primers with melting temperatures above 65^{o}C have a tendency for secondary annealing.
- Tm is important in determining the annealing temperature (Ta). Too high Ta will produce insufficient primer-template hybridization resulting in low PCR product yield. Too low Ta may possibly lead to non-specific products caused by a high number of base pair mismatches.
- Amplicon length.

Control primers were used as positive controls in order to confirm that the amplification procedure was successful. These control primers were chosen depending on the length of the expected PCR product:

- Primers which amplify a constant region of the HLA-DRB1 gene on chromosome 6 were used if a PCR product of <450 base pairs was expected. These primers (63: 5′-TGC CAA GTG GAG CAC CCA A-3′ and 64: 5′-GCA TCT TGC TCT GTG CAG AT-3′) produce band sizes on a gel of approximately 650 and 1300 bp, with the former showing preferential amplification. Therefore 1 or 2 control bands are visible after electrophoresis.
- Primers amplifying a smaller product (250 bp) in the adenomatosis polyposis coli gene on chromosome 5 were used when the expected PCR was >450 bp, (210: 5′- ATG ATG TTG ACC TTT CCA GGG-3′ and 211: 5′- TTC ATC AGT TGC TGC CCC TC-3′).

2.3.5.2 SNP selection

When designing assays for SSP-PCR, within each of the genes polymorphisms were selected to cover alleleic diversity across the gene. Polymorphisms in regions most likely to have an impact on gene function were prioritised (promoter region, exons, intron-exon boundaries, 3-UTR), as were polymorphisms for which there exists published data for the Caucasian population. Intronic polymorphisms were selected at intervals spanning the gene to ensure full coverage of the region for haplotype analysis. Data on linkage disequilibrium, haploblocks and tag SNPs (see section 2.4.3) from the International HapMap Project (http://hapmap.ncbi.nlm.nih.gov) and the software programme Haploview (Whitehead Institute for Biomedical Research, USA) were used to limit the choice of SNPs.

2.3.5.3 SSP-PCR protocol

Control primer master mix was made up by adding 1ml deionised water (Baxter, UK) to 9µl of the loading dye cresol red (Sigma Ltd, Poole, UK) and either 2µl of each of the 63/64 control primers or 8µl of each 201/211 control primers. Cresol red undergoes a pH-dependent colour change from orange to purple on addition of DNA. 5µl primer stock (2000µg/ml) (Sigma Genosys Ltd, Poole, UK) (both allele-specific and consensus) was added to 1ml of control primer master mix. The mixture was vortexed well.

5µl of the primer mix (allele-specific and consensus primers + control primer) was placed in a well of a 96-well plate which contained 10µl mineral oil. To this 8µl a PCR reaction mix was added containing PCR buffer ($(NH_4)2SO_4$), $MgCl_2$ (50mM), dNTP mix (100mM), Taq DNA polymerase (Biotaq ^{TM}, Bioline Ltd, London, UK) and genomic DNA.

Each 1ml PCR reaction mix contained 749.1µl deionised water, 166.4µl ($(NH_4)2SO_4$ buffer, 65µl $MgCl_2$, 4.75µl each dNTP; deoxyadenosine triphosphate, deoxythymidine triphosphate, deoxyguanosine triphosphate, and deoxycytidine triphosphate and 6-8µl Taq DNA Polymerase. The dNTPs are the single units of DNA which are added to the primer by DNA polymerase. Magnesium acts as a cofactor for DNA ploymerase and the Mg^{2+} concentration can affect primer annealing; Tm of the

DNA template, product and primer-template associations and PCR product specificity. Each reaction in the 96-well plate required 0.08µg DNA.

Each 13µl reaction underwent PCR amplification in a MJ Research PTC200V thermal cycler (GRI, Braintree, UK). Cycling parameters were set at: 1 minute at 96^{o}C; followed by 5 cycles at 96 ^{o}C for 20 seconds, 70 ^{o}C for 45 seconds, and 72 ^{o}C for 25 seconds; followed by 21 cycles at 96 ^{o}C for 25 seconds, 65 ^{o}C for 50 seconds, and 72 ^{o}C for 30 seconds; followed by 4 cycles at 96 ^{o}C for 25 seconds, 55 ^{o}C for 60 seconds, and 72 ^{o}C for 120 seconds.

2.3.5.4 Gel electophoresis

When DNA amplicons (or other molecules) are placed in a gel connected to a power source, the DNA migrates from the negative towards the positive electrode. Larger molecules move more slowly through the gel while the smaller molecules move faster and therefore further.

To determine the presence or absence of the specific allele, the PCR amplified products were electrophoresed for 20 minutes on a 1.5% agarose gel made up with 500mls x 0.5% Tris Borate EDTA (TBE) (Sigma, Poole, UK) and 7.5g agarose (Bioline, UK). This was stained with 20µL SYBR® Safe DNA gel stain (Invitrogen, UK). 10mls of orange G loading buffer, a color marker for monitoring the process of agarose gel electrophoresis (150 mls glycerol (Sigma, Poole, UK), 350 mls 0.5 x TBE, 0.125 g Orange G (Sigma, Poole, UK)) was added to each sample before they were loaded into the wells of the gel. The PCR products were visualised using ultraviolet light and photographed. The presence of a band indicated the presence of that particular allele at that position. The presence of the control band for each reaction acted as a positive control.

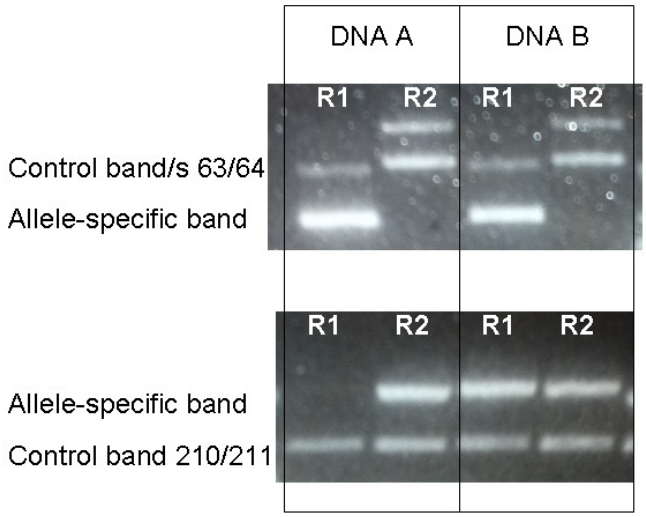

Figure 2.5 SSP-PCR products on an agarose gel using 63/64 and 210 / 211 control primers.

R1 (63/64) tests for the G allele, R2 (63/64) tests for the A allele of SNP X.

DNA A is positive for R1 (G) but negative for R2 (A), therefore the genotype is GG. Likewise the genotype for DNA B is GG.

R1 (210/211) tests for the C allele and R2 (210/211) tests for the T allele of SNP Y.

DNA A is negative for R1 (C) but positive for the R2 (T), therefore the genotype at this position is TT. DNA B is positive for both R1 and R2 therefore the genotype is CT.

2.3.5.5 SSP-PCR primer setup, titration and optimisation

The high sensitivity of PCR makes it susceptible to false positives from contamination. Such false-positives can be avoided through judicious laboratory set-up (e.g. maintaining a physical seperation between DNA samples and the PCR reaction area), using separate laboratory equipment and consumables (including disposable gloves) for pre- and post-SSP-PCR work and dividing the reagents into a number of aliquots [300].

False positive reactions (and false negatives) may also be seen in the presence of excess or inadequate primer. Titration of primers (specific and consensus) to

concentrations which minimise the likelihood of false positive and negative reactions is important in setting up the primer assay. In a false positive reaction, there is uneven intensity of the reaction products, whereby a true positive (heterozygous) reaction is indicated by the presence of both alleles with bands of equal intensity on electrophoresis.

2.3.5.6 Primer titration for primer concentration optimisation

5µl of the allele-specific and 5µl of the consensus primer stock (2000µg/ml) were added to 1ml of control primer master mix (as described above) and used to run PCR on 20 test DNA samples. Likely homozygous and heterozygous samples were identified. The allele-specific and consensus primers were titrated at different concentrations (table 2.2) and tested on the probable homo- and heterozygous DNA samples. 1ml primer mix containing control primer mix and either allele-specific or consensus stock primer in the volumes given below were made. 2.5µl of each allele-specific primer was dispensed along with 2.5µl of consensus primer, therefore the final primer volume was 5µl.

Table 2.2 SSP-PCR primer titration grid

Cons	**Cons**	**Allele 1**	**Allele 2**	**Allele 1**	**Allele 2**	**Allele 1**	**Allele 2**	**Allele 1**	**Allele 2**	**Allele 1**	**Allele 2**
Volume (µl) of stock primer to use in 1ml →		**2**	**2**	**4**	**4**	**6**	**6**	**8**	**8**	**10**	**10**
↓	**Final vol →**	**1**	**1**	**2**	**2**	**3**	**3**	**4**	**4**	**5**	**5**
2	**1** ↓	1/1	1/1	2/1	2/1	3/1	3/1	4/1	4/1	5/1	5/1
4	**2**	1/2	1/2	2/2	2/2	3/2	3/2	4/2	4/2	5/2	5/2
6	**3**	1/3	1/3	2/3	2/3	3/3	3/3	4/3	4/3	5/3	5/3
8	**4**	1/4	1/4	2/4	2/4	3/4	3/4	4/4	4/4	5/4	5/4
10	**5**	1/5	1/5	2/5	2/5	3/5	3/5	4/5	4/5	5/5	5/5

Cons = consensus primer
Final vol refers to the final volume of primer in 1ml of control primer mix.

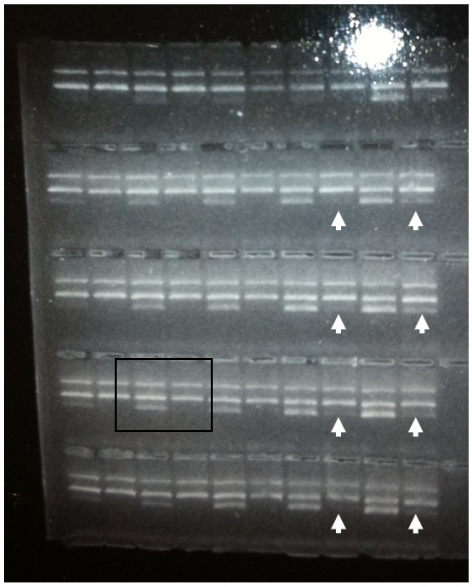

Figure 2.6 SSP-PCR primer titration

False positive reactions can be seen for allele 2 at the higher primer concentrations, as shown by the arrow heads. (The control primer for this experiment was 63/64). The combination of 2µl allele specific primer with 4µl consensus primer (highlighted) was chosen as the optimum primer concentration.

2.3.5.7 SSP-PCR for deletion-insertion polymorphism

The polymorphism in the promoter region of SLC6A4, (known as SERTPR), is a deletion-insertion (DIP) rather than a SNP. This DIP is a 20-23bp repeating unit. The allelic variants are named short (S) and long (L) depending on the presence or absence of the DIP. There are 3 possible allelic combinations: L (homozygous for the long form), S (homozygous for the short form) and LS (heterozygous). The L allele has 14 repeats and the S allele 16 repeats respectively [301]. Primers flanking this region which have been described in the literature [302;303] were used in this study to generate a 484bp[302] / 528bp[303] fragment (S allele) or a 528 bp[302] / 572bp[303] fragment (L allele). Therefore the two common alleles differ in length by 44 base pairs.

In order to visualise the SERTP insertion-deletion polymorphism (as detailed in chapter 5), the PCR products were electrophoresed for a longer period (60-90minutes) on a thicker 2% agarose gel. This ensured increased separation of the smaller DNA products. A 100bp DNA molecular weight standard ladder (Invitrogen, UK) was included during the gel electrophoresis.

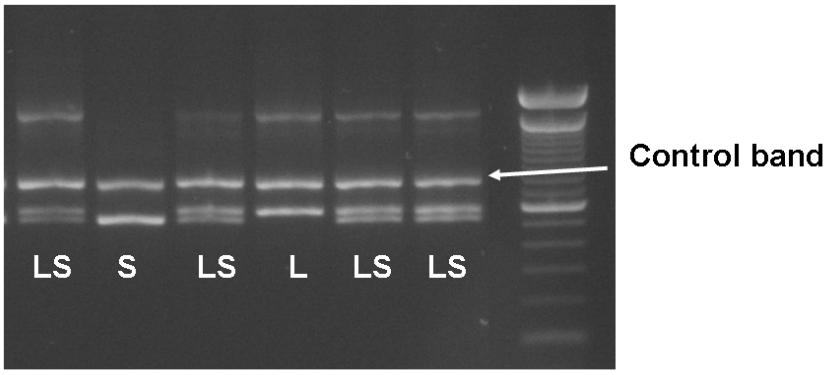

Figure 2.7 SERTP deletion-insertion polymorphism
This representative photo of an agarose gel shows the 3 possible allelic combinations for the SERTP deletion/insertion polymorphism. The 100bp DNA ladder is seen on the right hand side of the gel. On this ladder the 600bp band is 2-3 times brighter than the other bands.

2.3.6 Affymetrix genome wide SNP array

A genome-wide SNP array was carried out using the Affymetrix DNA micorarray 5.0, a multiplex assay that simultaneously tests for 440,794 SNPs.

Before the sample DNA can be added to the array it is digested using restriction enzymes (Nsp I and Sty I). Restriction enzymes cut double-stranded or single stranded DNA at specific recognition nucleotide sequences known as restriction sites. The digested DNA is then ligated to adaptors. These adaoptor-ligated DNA fragments are ampified using PCR, with preferential amplification of fragments in the 200-1,100 bp size range. The PCR products are purified and the amplified DNA is then fragmented, labeled, and hybridized to a Genome-Wide Human SNP 5.0 Array.

The SNP array consists of an orderly collection of microscopic spots (called features), each of which has a specific probe (a fragment of DNA, usually 100-1000 bases long of known sequence), which is attached to a solid surface (the chip). When these probes are exposed to labelled sample DNA, complementary strands will hybridise by forming non-covalent hydrogen bonds between complementary nucleic acids. Fluorescently labeled DNA sequences which bind to a probe sequence generate a signal which is used to generate the genotype data [304].

2.4 Preliminary genetic analyses and genetic data management

In order to be able to use genetic data in a genetic association study, there are a number of analyses and quality control checks which must be first carried out in order to ensure that the data are in the correct format for analyses and to minimise the likelihood that apparent SNP-phenotype associations are chance rather than true associations. These include:

- Calculating allele carriage, allele frequency and genotype frequency
- Hardy-Weinberg equilibrium testing
- Linkage disequilibrium analysis
- Inferrence of phase
- Haploblock and haplotype construction

A genetic database was set up in Microsoft Excel into which the genetic data was added in allele carriage format, as read from the presence or absence of bands on the agarose gels. The genotype was determined depending on the diploid allele carriage data at that position. Study subjects were considered heterozygous if they carried two different alleles at the same position or homozygous if they carried two copies of the same allele at that position.

Allele carriage and allele frequency were calculated.

Allele carriage is a sum of the number of subjects homozygous for that allele plus the number of heterozygotes, divided by the total number of individuals in the study.

Allele frequency is calculated by the following equation:

[(2 x number of homozygotes) + number of heterozygotes] / total number of alleles

Allele frequencies and genotype frequencies were used to compare genetic data in this study population to previously published data (e.g. HapMap CEU data) [90] as a quality control measure.

2.4.1 Genetic modelling

There are a number of genetic models under which a genetic variant may be assumed to influence phenotype:

- Dominant: Subjects who are either homozygous for the variant allele or heterozygous have the same phenotype i.e. subjects need only one copy of the variant allele to display the phenotype.

- Recessive: Subjects who are homozygous for the variant allele have a different phenotype to those who are homozygous for the alternative allele or heterozygous. In other words, under the recessive model, a subject requires both copies of the variant allele to demonstrate the phenotype.

- Codominant: This model assumes some quantitative measure of the phenotype. In a co-dominant model, the heterozygous mean differs from both homozygous means. The phenotype associated with each of the three genotypes is separated.

- Additive: This is a variant of the co-dominant model in which each copy of the variant allele has an effect. If the heterozygous mean is equal to the average of the homozygous means, then the model is co-dominant but also additive. In this situation a gene dose effect (where there is an intermediate phenotype when the heterozygous genotype may be demonstrated): AA vs AB vs BB

Ideally, the choice of statistical association test would depend on the genetic model underpinning the phenotype. It is rarely possible to know the likely genetic model in advance and therefore it is challenging to choose the best model to use in statistical analyses [305;306].

In this candidate gene association study I used two different approaches to test for SNP-phenotype associations: comparisons of allele carriage and genotype frequency. These analyses were exploratory, one of the aims being to establish the SNP-phenotype genetic model. Therefore no correction for multiple testing was included.

Allele carriage:

Using allele carriage assumes a dominant (or recessive) model of association [307]. When the phenotype being tested is a complex trait such as response to morphine, it is usually not known whether the variant allele is a risk-enhancing allele (increases the likelihood of a poor outcome) or a protective allele (increases the likelihood of a good outcome). Therefore assuming the dominant model for testing one allele in a bi-allelic SNP is the same as assuming the recessive model for the alternative allele, as shown below.

Table 2.3 Allele carriage in genetic association tests assumes a dominant / recessive mode of SNP-phenotype inheritance

Model	Genetic association test	Genotypes compared
Dominant for allele A	Carriage of A versus non-carriage of A	AA + AB vs BB
Recessive for allele A	Non-carriage of B versus carriage of B	AA vs AB + BB
Dominant for allele B	Carriage of B versus non-carriage of B	BB + AB vs AA
Recessive for allele B	Non-carriage of A versus carriage of A	BB vs AA + AB
Consider two alleles: A and B A subject is said to be "carrying" the A allele if the genotype is AA or AB. A subject is said to be "carrying" the B allele if the genotype is BB or AB.		

Genotype frequency:

The genotypes were recoded as 0 (homozygous for minor allele), 1 (heterozygous), 2 (homozygous for major allele) and a linear trend analysis was carried out which tested for a linear trend of increasing effect across the three genotype groups. The genotypes were entered into regression analyses as non-categorical data. This assumes a co-dominant additive model. This was the preferred model in this thesis because:

- When the allele under investigation is assumed to be in linkage disequilibrium (LD) with the true susceptibility allele, then testing genotype frequencies using an additive model is recommended, especially if the genetic effect is likely to be small, as LD is likely to "dilute" the true effect size [306].

- It is thought that the model of SNP-phenotype associations in complex traits is usually additive [305].

A "per-allele" analysis is often carried out in genetic analyses in which the allele frequencies are compared. In this type of analysis the sample size is doubled as each subject has two alleles, therefore increasing the power of the study. However this approach is prone to false-positives and false negatives, especially when Hardy-Weinberg deviations are present [307], see section 2.4.2. Furthermore it is difficult to interpret the results of per-allele analyses in terms of biological risks [306]. Therefore allele frequency was only used in this thesis to compare our data with published frequency data.

2.4.2 Hardy-Weinberg Equilibrium

The Hardy-Weinberg Equilibrium (HWE) states that alleles are independent and that the allele and genotype frequencies should remain constant (in equilibrium) from one generation to the next.

For any bi-alleleic SNP, consider the frequency of allele A = p, and the frequency of allele B = q. The frequency of genotype AA is p^2, AB is 2pq and BB is q^2. If the population is in HWE, then the sum of both allelic frequencies equals 100% i.e.

$p + q = 1$

and

$p^2 + 2pq + q^2 = 1$

The HWE model is based on a number of assumptions, departure from which can result in deviations from HWE:

- Large population. In a small population chance alone may alter allele frequencies in situations where there are only a few copies of a certain allele within that population (genetic drift).
- Random mating. Departures from random mating e.g. inbreeding causes an increase in the homozygosity of genes.

- Negligible mutations with little effect on allele frequencies. Mutations may have a greater effect on allele frequencies in smaller populations.
- No gene flow between populations (e.g. through migration).
- Natural selection is not operating on the population i.e. no selection of certain alleles over other alleles.

In order to test for whether the population is in HWE, the difference between expected genotype frequencies and observed genotype frequencies were compared using a Chi-squared test. Significant ($p < 0.05$) differences between expected and observed genotype frequencies indicate that the study population was not in HWE. If the population was not in HWE for a particular SNP, the raw data were rechecked for genotyping errors.

2.4.3 Linkage Disequilibrium and Haplotypes

Linkage disequilibrium (LD) is the non-random association of genetic variants within a population. LD refers to the idea that certain alleles occur on the same haplotype more often than is expected by chance. A haplotype is a combination of alleles that are transmitted together.

The human genome is thought to be comprised of areas of strong LD interspersed with areas of LD breakdown, presumably recombination hotspots. (Recombination is the generation of new combinations of genetic material as a result of crossover during meiosis). A haploblock (also known as a haplotype block) is a genomic region which is inherited without substantial historical recombination. The size of haplotype blocks in humans can range from a few kb to more than 100 kb [308]. Haplotypes are combinations of markers in LD together which are often defined within the haploblock structure. Within each haploblock there are only a few common haplotypes. Instead of analysing each SNP individually, these common haplotypes may be used to analyse genetic variation across sizable regions by testing only a small number of haplotype tag SNPS. This is the basis of the International HapMap Project [79;309]. Haplotype blocks may vary in different populations. The HapMap Project

characterised SNPs and LD in four different populations: 90 subjects of northern and western European origin (denoted CEU), 45 Han Chinese (CHB),
44 from Tokyo (JPN) and 90 subjects from the Yoruba in Ibadan, Nigeria (YRI). In Phase 1 of the International HapMap Project, over 1 million SNPs were genotyped [79]. In Phase 2, over 3.1 million SNPs have been included [309].The HapMap project has identified only approximately 1% of common variants which are "untagggable" as they lie within areas of low LD, usually recombination hotspots [309]. Another benefit of haplotype analysis is that it may provide information about the cumulative effect of a number of genetic variants on the study outcome [310].

LD can be affected by a number of genetic processes including selection and genetic drift. Changes in population size (e.g. population bottlenecks) and the exchange of individuals among populations with resultant mixing of individuals from subpopulations that have different allele frequencies can both alter LD [308]. The strength of LD between pairs of markers decreases as a function of the genetic distance between markers. LD around an allele decays due to recombination breaking down ancestral haplotypes [311]. However, LD patterns may be surprising with pairs of loci at considerable distances from each other being in complete LD [310]. These areas of long-range LD are thought to have occurred when the population was so small that the amount of genetic variation was limited e.g. during a population bottleneck (i.e. when there was a temporary reduction in the size of the population resulting in a loss of genetic variation) or due to an extreme founder effect. Long-range LD may also arise from population admixture, the mixing of genetically distinct populations [312].

There are many different measures of LD. Most measure the degree of LD between pairs of markers. For any two biallelic loci (Aa and Bb), there are four possible haplotypes: Ab, AB, aB and ab. The frequency of haplotype AB may be denoted as π_{AB} and the frequencies of the alleles π_A, π_B, π_a and π_b.

One of the early measures of LD was D, the coefficient of linkage disequilibrium. D is the difference between the frequency of gametes carrying the pair of alleles A and B at two loci (π_{AB}) and the product of the frequencies of those alleles (π_A and π_B).

$D_{AB} = \pi_{AB} - \pi_A\pi_B$ [313]

This measure is limited by its dependence on allele frequencies. To avoid (or at least minimise) this problem, D' was proposed as the coefficient of linkage disequilibrium (D), standardised to the maximum possible value of D at the given allele frequencies [313].

$D_{AB}' = D_{AB} / D_{AB, max}$ [311]

The absolute value of D', |D'| is usually used.
If all four haplotypes are present, historical recombination has occurred between the two loci and therefore |D'| < 1. If even one haplotype is not observed, |D'| =1 [314]. |D'| = 1 represents the situation where no recombination has occurred between the two markers. Therefore D' is also a measure of historical recombination. |D'| values may be inflated when rare alleles are examined or when the sample size is small. If a SNP has a low minor allele frequency, it is quite possible that it is carried on a rare haplotype which may not be observed in a small study population. Therefore, all four haplotypes will not be present in the study population. This results in |D'| =1, irrespective of the level of linkage disequilibrium.

r^2 is another commonly used marker of LD and represents the correlation coefficient between pairs of markers. Like D, r^2 is allele frequency dependent.

$r^2 = (D_{AB})^2 / \pi_A\pi_B\pi_a\pi_b = (\pi_{AB} - \pi_A\pi_B)^2 / \pi_A\pi_B\pi_a\pi_b$ [315]

|D'| and r^2 values range from 0 to1. |D'| or r^2 =1 represents complete LD while |D'| or r^2 =0 represents no LD.

In this study, LD (D' and r^2) was measured between pairs of alleles using the programme, Haploview [316].

Haploblocks were identified using Haploview which is based on the Confidence Interval algorithm described by Gabriel et al [315]. Each pair of markers was defined as being in "strong LD", "inconclusive" or "strong evidence for recombination"

depending on the confidence intervals (CI) for D'. For markers to be in "strong LD", the upper CI must be >0.98 and the lower CI >0.7. A haploblock was created if 95% of the informative markers (minor allele frequency >0.2) were in "strong LD". This method was chosen as it avoids the changes in absolute D' values which are associated with small numbers of samples.

The genotype of an individual will not tell us anything about the haplotype i.e. genotype data alone will not tell us about the pairwise relationship between alleles at each locus. Consider two bi-allelic loci on the same chromosome. If the first locus, A, has alleles p and q and the second locus B, alleles w and y, then the possible genotype combinations at locus A are pp, pq and qq and at locus B, ww, wy and yy. There are therefore nine possible configurations for the genotypes at these two loci. In order to resolve the haplotype phase (that is to determine haplotypes from genotype information), in a sample of unrelated individuals, statistical programmes are used which infer haplotypes from genotype data. The inferred haplotypes can then be treated as data. There are a number of such programmes available, all based on slightly different theoretical backgrounds. I used the software programme PHASE, a probability based Bayesian method, (http://www.stat.washington.edu/stephens/software.html) [317] to construct haplotypes within each haploblock which had been defined using Haploview.

When carrying out haplotype analysis, a very low rate of genotyping error is essential in order to avoid the identification of false rare haplotypes. Therefore if rare haplotypes were identified, the samples were regenotyped to ensure accuracy of genetic data.

Like allele frequencies, haplotype frequencies are calculated as the total number of haplotypes present in the population on both chromosomes [(2 x number of subjects with two copies) + number of subjects with one copy] divided by the total number of chromosomes in that population (total study population x 2). The sum of the haplotype frequencies adds up to 100%. Haplotype frequencies were calculated to allow comparisons with published data.

The combination of haplotypes in a pair of chromosomes is known as a diplotype. Haplotype carriage frequencies were calculated as the sum of the number of subjects with one or two copies of the haplotype, divided by the total number of subjects in the study. The haplotypes on each chromosome making up a diplotype may be dissimilar. In this way the sum of haplotype carriage frequencies in the study often add up to >100%. Haplotype carriage frequency was used in the association analyses.

2.5 Statistical analyses

Statistical analyses and plots were performed using SPSS (SPSS, Chicago, IL) and GraphPad. (GraphPad Prism version 4.02 for Windows, GraphPad Software, San Diego California USA).

Means and standard deviation (SD) values (or median and range if non-parametric) were used to describe continuous clinical data, with counts and percentages used for discrete variables. Normality of continuous data was checked by analysing skewness and kurtosis (both of which describe the shape of the data distribution) and by application of the Shapiro-Wilk test. Where appropriate, non-parametric data were transformed using logarithmic transformation.

Correlation coefficients may be calculated using a number of correlation coefficients. Spearman's correlation coefficient was used in this thesis as it is the test of choice if the data are non-parametric or if the sample size is small. Kendall-tau was used for non-parametric and non-interval scaled ordinals. A correlation coefficient of +/- 0.1 represents a small effect, +/- 0.3 a medium effect and +/- 0.5 a large effect [318].

Categorical variables were compared between using the Chi-square ($\chi 2$) or Fisher's exact test if the sample sizes were small. Continuous data were analysed using Student t-test / 1-way ANOVA for parametric data and Man-Whitney U / Kruskal-Wallis for non-parametric data. Paired data were compared using paired t-test (parametric) / Wilcoxon signed rank test (non-parametric).

2.5.1 Regression

The relationships between the clinical and genetic variables and the defined clinical phenotypes were determined using regression analyses; linear for continuous outcome variables and logistic for binary outcome variables. Regression analyses were used to demonstrate the level of change in the dependent variable (outcome variable) according to variance in each of the predictor variables, when all other predictor variables were held fixed.

Univariate regression was initially carried out to screen for an association between individual clinical and genetic variables and the outcome variables. Multiple regression was used to investigate the joint effect of predictor variables (clinical and genetic) on the clinical phenotype (dependent variable). The dependent variable was transformed to ensure parametric distribution when necessary for linear regression. Candidate clinical predictor variables included age, gender, time on morphine (days), dose of morphine (mg in 24 hours) and time to death (days), concomitant medications, biochemical and haematological parameters and tumour diagnosis. Clinical and genetic factors with $p<0.1$ on univariate analysis were included in the multivariate modelling. Variables with $p>0.1$ were excluded in order to reduce the number of predictor variables. Variable selection was carried out using a stepwise method, with $p<0.05$ and $p>0.1$ as criteria for entry and removal respectively. Only factors with $p<0.05$ were retained in the final model.

The following test statistics were generated:

Linear regression:

- R^2: The proportion of the variability in the outcome variable that can be explained by its relationship with the predictor variables. This value represents an assessment of goodness of fit.
- B (the regression coefficient), standard error of B, CI (B): This value represents the change in the outcome variable associated with a unit change in the predictor variable. A regression coefficient of 0 means that a unit change in the predictor variable is associated with no change in the outcome. In a multivariate regression analysis, the correlation coefficient provides information about the extent to which the predictor variable affects the outcome variable, if the effects of all of the other predictor variables are held constant [318]. The value of B is dependent on the unit of measurement of the variable. The relationship between the predictor and outcome variable may be positive or negative, as demonstrated by the sign of the B value. Confidence Intervals (CI) of B are provided in SPSS. If the model is a good model, the CI should not cross 0.

- T-test p-value. This is based on the null hypothesis that B = 0. If $p<0.05$, then the predictor variable is making a significant contribution to the model.
- β is a standardised version of B. This represents the change in the outcome variable (in terms of number of standard deviations) due to one standard deviation change in the predictor. In a multivariate regression model the different β values may be compared.
- ANOVA F-test: A significant result ($p<0.05$) indicates that there is a linear relationship between the outcome variable and at least some of the predictor variables i.e. that the model overall is a good predictor of the outcome variable.
- Variable Inflation Factor (VIF). This assesses whether the predictors are correlated together. An average VIF of >1 suggests that there may be multiple collinearity which may introduce bias into the model.

Logistic regression:

- Wald statistic: This provides information about whether the B value (regression coefficient) is significantly different from zero, i.e. whether a change in the predictor variable is associated with a significant change in the outcome variable.
- Exp (B) This represents a change in the odds associated with a unit change in the predictor variable. A value >1 suggests that as the predictor increases, then the odds of the outcome occurring increase and vice versa. (Therefore in a good model, the 95% CI for Exp(B) should not cross 1). In multivariate logistic regression, this value represents an estimate of the odds ratio for the predictor and outcome variable when all other predictor variables are held fixed.
- Nagelkerke's R^2: This value is used in logistic regression and is an adjusted square of the partial correlation between the outcome variable and each of the predictor variables,

2.5.2 Principal Components Analysis (PCA)

Principal components analysis was used to identify groups / clusters of variables within the dataset. Using this technique a number of uncorrelated artificial variables, known as components, are identified which account for most of the variance in the data. PCA is a variable reduction procedure. Although the number of components identified equals the number of variables, only the first few are retained in subsequent analyses as these account for most of the variance in the data. The first component extracted in a principal component analysis accounts for a maximal amount of total variance in the observed variables. The second component accounts for the maximum amount of variance in the data set that was not accounted for by the first component. In this study all components with eigenvalues >1 were retained [319]. The variables making up each component were determined by assessing the component loadings of each variable. The component loadings represent the importance of the variable to the component. The significance of a component loading depends on the sample size. A value >0.4 was considered significant in this thesis as the sample size was between 100 and 200 [318].

The appropriateness of PCA was determined using Kaiser-Meyer-Olkin measure of sampling adequacy (KMO). This generates a value between 0 and 1. Values above 0.7 suggest a statistically adequate sample size [318]. Individual variables with KMO values of <0.5 were excluded from further analysis. A significant value (<0.05) for Bartlett's test of sphericity suggests that there are relationships between the variables, which is required for PCA to be successful.

Individual component scores (log transformed) for each study subject were used as outcome variables in subsequent linear regression in the clinical and genetic association studies.

3 Chapter 3: Results from Prospective Morphine Study and Definition of the clinical phenotype

3.1 Summary of the rationale behind this study

- In the published literature there is little consensus with respect to definition of outcome measures in pain and analgesia studies. Therefore comparison and replication of results is challenging.
  Previously published reports have chosen one dimension of analgesic response or opioid side-effects as the outcome measure in genetic association studies.
- Response to morphine is determined by the subjective experience of pain control and side-effects. There may be many other clinical factors contributing to these subjective experiences.

3.1.1 Aim

The aim of this part of the study was to explore the clinical data to establish clinically relevant methods of defining the clinical phenotype of response to morphine. The resulting phenotype/s would then be used in the subsequent genetic association studies.

In this chapter I present two distinct clinical phenotypes of morphine response:

1. Composite scores of analgesic response and central side-effects as distinct outcome measures, defined by principal components analysis (PCA).
2. The daily dose of morphine required to achieve adequate analgesia.

A further clinical phenotype of constipation on morphine is presented in chapter 7.

3.2 Subjects and methods

Clinical data from the *Prospective Morphine Study* were used in these analyses.

The data were initially explored using descriptive statistics. Most continuous variables were not normally distributed and were described using medians and ranges. The reasons for morphine non-response were examined. The pain and side-effect scores for all study subjects were examined for correlation. Logistic regression with morphine non-responder (switcher) as the dependent variable was used to determine which pain / side-effects scores were predictors of morphine response / non-response.

Principal components analysis (PCA) was used to explore response to morphine morphine using a comprehensive assessment of five patient reported pain intensity scores and seven side-effect scores. PCA (or a similar factor analysis approach) has been used previously to examine relationships between different pain scores and thresholds [320;321]. This is the first time in which it has been used to identify clusters of variables defining the underlying dimensions of morphine response.

Principal components analysis (PCA) was carried out in order to examine the components of morphine response / non-response which account for the variability in the clinical data. All of the subjective symptom scores (pain and side-effects) were entered into the model as numeric data. As this part of the study represents a move away from the clinical phenotype of "morphine responder" versus "morphine non-responder", all subjects were included in the analysis together without making the distinction between responders and non-responders. Component scores were calculated using the regression method and rotation was carried out using an oblique method (direct oblimin). All components with eigenvalues >1 were retained [319].

The individual component loading scores were used as dependent / outcome measures in subsequent linear regression modelling. This was carried out to identify other clinical factors predictive of the outcome. The clinical factors included in the modeling included age, gender, dose of morphine, time on morphine, diagnosis, concomitant medications and haematological / biochemical parameters.

3.2.1 Maintaining a tight clinical phenotype

In order to maintain as tight a clinical phenotype as possible, the clinical data were examined in terms of 1) length of time on morphine at recruitment to the study and 2) pain scores in the morphine "responder" and "non-responder" groups.

Titration of morphine dose to achieve optimal pain control may take a variable length of time. One study demonstrated that the median time to achieve stable pain control ranged from 4 days for mild pain to 8 days for moderate pain to 22 days for severe pain [322]. In our study, subjects who had been on morphine for less than 1 week (N=19) were therefore excluded from analysis as it was felt that this may not have been adequate time to allow optimal dose titration.

Many sources agree that pain intensity of 4 or less on an 11 point NRS represents either mild pain or good analgesia [181;323-326]. An average pain of ≤ 4/11 has been considered to be adequate or acceptable pain control [92]. In this study there were a number of patients whose pain was well controlled according to both the patient and the clinical palliative care team but whose scores on the modified BPI did not reflect this. These patients were categorised clinically as "morphine responders" by the palliative care team yet their pain scores suggested that they were either inadequately titrated on their morphine dose or that they were simply unable to fill in the numerical rating scale accurately. In order to maintain a very tight clinical phenotype these patients (N=38) were also excluded from analysis.

89% (264) study subjects were Causacian, with 4% (13) Black / Black British, 2% (7) Asian / Asian British, and 5% (14) each Mixed or Other ethnicity. The study population was compared to all new patients attending the Royal Marsden Hospital in a defined 12 month period (2006-2007). The proportion of patients with different ethnicities in the study was representative of the ethnicities of patients attending the Royal Marsden Hospital (see figure 3.1).

The main aim of this study was to examine the genetic factors associated with opioid response. As the number of non-caucasian subjects was small in the study (N=34), in

order to avoid population stratification, these were also excluded from further analysis.

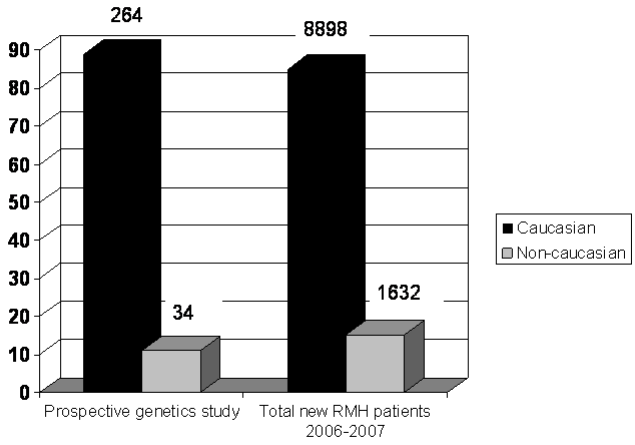

Figure 3.1 Ethnicity data

Number of patients shown on top of bars

A total of 207 study subjects were included in subsequent analyses.

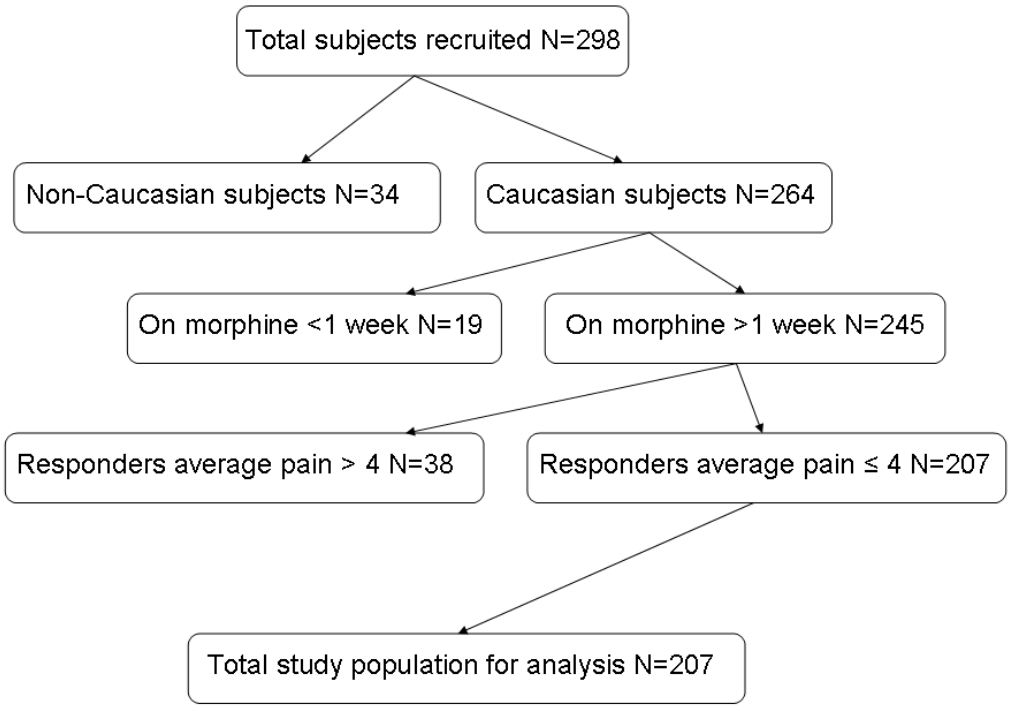

Figure 3.2 Study population used in thesis (Prospective Morphine Study)

3.3 Results

Prospective Morphine study: demographic data

The characteristics of the total study population are shown in table 3.1.

145 (70%) of the 207 subjects included in the analyses were morphine responders and 62 (30%) morphine non-responders.

3.3.1 Morphine responders versus morphine non-responders

The reasons for switching to an alternative opioid are presented in table 3.2. The most common reason for a patient to be considered a "morphine non-responder" was intolerable drowsiness (66%), followed by inadequate pain control (47%) and confusion or hallucinations (37%).

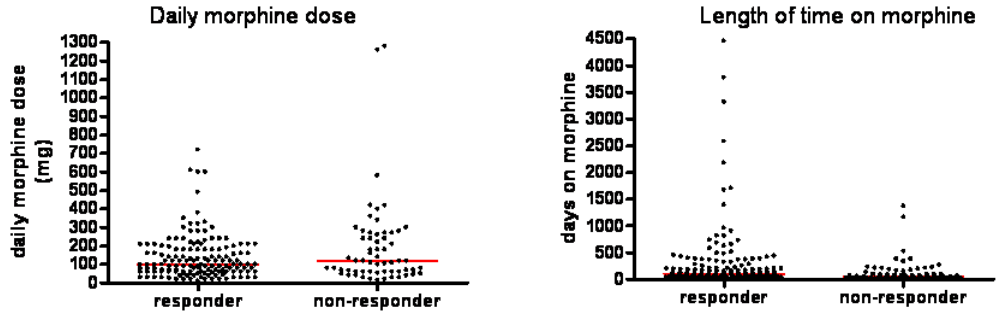

Figure 3.3 Morphine dose and Length of time on morphine

There was no statistical difference between the daily dose of morphine taken by non-responders (median 120mg, range 15-1280mg), compared to responders (median 100mg, range 10-720mg). Non-responders had been on morphine a shorter time than responders (median 59, range 8-1369 days and median 106, range 7-4455 days respectively, $p<0.0001$).

Table 3.1 Patient characteristics for total study population (N=207)

	Median (range)
Age (years)	60 (19-85)
Daily morphine dose (mg/24hours)	100 (10-1280)
White blood cell count ($x10^9$ /L)	8.6 (0.3-68.1)
Serum sodium (mmol/L)	136 (120-143)
Serum albumin (g/L)	30 (11-48)
Serum calcium (mmol/L, corrected for albumin)	2.24 (1.52-2.94)
Days to death (days)	131 (5-2147)
Days on morphine (days)	89 (7-4455)
	N (%)
Sex	
Male	108 (52)
Female	99 (48)
Diagnosis	
Gynaecological	18 (9)
Lung (including mesothelioma)	27 (13)
Breast	36 (17)
Urogenital (renal and bladder)	21 (10)
Upper gastrointestinal tract	16 (8)
Lower gastrointestinal tract	18 (9)
Head & Neck	12 (6)
Sarcoma	28 (14)
Prostate	17 (8)
Concomitant medications	
Antibiotic	54 (26)
Anticonvulsant	44 (21)
Anticoagulant	49 (24)
Anti-diabetic medication	11 (5.3)
Antiemetic	99 (48)
Aspirin	21 (10)
B Blocker	17 (8)
Diuretic	24 (12)
Laxative	136 (66)
NSAID	74 (36)
Paracetamol	83 (40)
Proton Pump Inhibitor	139 (67)
SSRI, SNRI antidepressants	23 (11)
Statin	11 (5.3)
Steroid	82 (40)
Tricyclic antidepressants	27 (13)

Table 3.2 Reason for morphine "non-response" (N=62)

	N (%)
Uncontrolled pain	29 (47)
Intolerable side-effects	
Nausea	18 (29)
Vomiting	5 (8)
Constipation	6 (10)
Drowsiness	41 (66)
Confusion / hallucinations	23 (37)
Nightmares	9 (15)
Pruritus	4 (6)
* Other	6 (10)

62 out of 207 patients were "morphine non-responders"
*Other reasons for switching included: myoclonus, fatigue, feeling "spaced out", lethargy, sweating and poor concentration. All of these symptoms were also accompanied by another reason for non-response.
Patients usually had >1 reason for morphine "non-response"

3.3.2 Prospective Morphine Study: Pain scores

There was a clear difference between the pain experienced by morphine responders and non-responders as shown in table 3.3.

Table 3.3 Pain scores, shown as median (range)

	All study subjects N=207	Responders N=145	Non-responders N=62	P value*
Worst pain	5 (0-10)	4 (0-9)	7 (2-10)	<0.0001
Least pain	0 (0-9)	0 (0-4)	2 (0-9)	0.01
Average pain	2.5 (0-9)	2 90-4)	5 (0-9)	<0.0001
Pain now	1.5 (0-9)	0 (1-6)	4 (0-9)	<0.0001
% pain relief	80 (0-100)	90 (30-100)	60 (0-100)	<0.0001
*Man-Whitney-U				

Correlations between the different pain scores are shown in table 3.4. The strongest correlation is between average pain and worst pain (r = 0.737). There is also moderate to strong correlation between average pain and all pain scores. Pain relief, average pain and worst pain were identified in this study as independent predictors of morphine non-response by multivariate regression analysis as shown in Table 3.5.

Table 3.4 Correlation between pain scores

	Worst pain	**Least pain**	**Average pain**	**Pain now**	**% pain relief in last 24hours**
Worst pain	1.000	0.495	0.737	0.574	-0.610
Least pain	0.495	1.000	0.654	0.675	-0.528
Average pain	0.737	0.654	1.000	0.675	-0.725
Pain now	0.574	0.675	0.675	1.000	-0.640
% pain relief in last 24hours	-0.610	-0.528	-0.725	-0.640	1.000
All pain scores NRS 0-10 % Pain Relief NRS 0-100					

Table 3.5 Pain scores predictive of morphine response

Multivariate logistic regression with morphine non-responder as dependent variable.

	B	S.E.	Wald	p value	OR (95% CI)
Worst pain	0.267	0.112	5.715	**0.017**	**1.306(1.049, 1.626)**
Least pain	-0.256	0.194	1.738	0.187	0.774(0.529, 1.133)
Average pain	0.568	0.198	8.219	**0.004**	**1.765(1.197, 2.603)**
Pain now	0.214	0.134	2.538	0.111	1.238(0.952, 1.611)
% Pain Relief	-0.024	0.011	4.837	**0.028**	**0.976(0.955, 0.997)**
Constant	-2.611	1.168	5.000	0.025	0.073

3.3.3 Prospective Morphine study: Side-effect scores

Drowsiness (80%) and constipation (73%) were the most common opioid side-effects experienced by the total study population. There were a higher proportion of subjects experiencing nausea, drowsiness, confusion, bad dreams and myoclonus in the "non-responder" group. However a substantial proportion of subjects in the "responder" group also experienced these symptoms. (Table 3.6: Side-effects on morphine: Morphine responders versus non-responders)

Table 3.7 shows the correlations between the side-effects of morphine. The highest correlations were between confusion/hallucinations and drowsiness (r = 0.38) and confusion/hallucinations and bad dreams (r = 0.309). Constipation was not significantly correlated with any other side-effect.

A multivariate logistic regression model defined drowsiness, confusion/hallucinations and bad dreams as independent predictors of morphine non-response (table 3.8).

Table 3.6 Side-effects on morphine.

Morphine responders versus non-responders **Total N= 207** **% (N)**						
Symptom	**Grade**	**Total study population**	**Responder (N=145)**	**Non-responder (N=62)**	**Chi-squared test* X^2**	**P value**
Nausea	All	49 (102)	44 (64)	61 (38)	**5.1**	**0.02**
	0	51 (105)	55.9 (81)	38.7 (24)		
	1	30 (63)	29.7 (43)	32.3 (20)		
	2	11 (23)	10.3 (15)	12.9 (8)		
	3	8 (16)	4.1 (6)	16.1(10)		
Constipation	All	71 (148)	71 (103)	73 (45)	0.8	0.05
	0	29 (59)	29.0 (42)	27.4 (17)		
	1	27 (55)	29.0 (42)	21.0 (13)		
	2	24 (50)	23.4 (34)	25.8 (16)		
	3	43 (20)	18.6 (27)	25.8 (16)		
Drowsiness	All	80 (166)	75 (109)	92 (57)	**7.7**	**0.006**
	0	20 (41)	24.8 (36)	8.1 (5)		
	1	31 (64)	37.9 (55)	14.5 (9)		
	2	29 (60)	31.0 (45)	24.2 (15)		
	3	20 (42)	6.2 (9)	53.2 (33)		
Confusion / hallucination	All	40 (83)	28 (40)	69 (43)	**32**	**<0.001**
	0	60 (124)	72.4 (105)	30.6 (19)		
	1	20 (41)	20.7 (30)	17.7 (11)		
	2	14 (30)	5.5 (8)	35.5 (22)		
	3	6 (12)	1.4 (2)	16.1 (10)		
Nightmares	All	23 (48)	14 (20)	45 (28)	**24**	**<0.001**
	0	77 (159)	86.2 (125)	54.8 (34)		
	1	13 (27)	9.7 (14)	21.0 (13)		
	2	9 (18)	4.1 (6)	19.4 (12)		
	3	1 (3)	0 (0)	4.8 (3)		
Dry mouth	All	12 (25)	10 (14)	18 (11)	2.7	0.1
	0	88 (182)	90.3 (131)	82.3 (51)		
	1	2 (4)	2.1 (3)	1.6 (1)		
	2	8 (16)	6.9 (10)	9.7 (6)		
	3	2 (5)	0.7 (1)	6.5 (4)		
Pruritus	All	4 (9)	3 (5)	6 (4)	-	0.5
	0	96 (198)	96.6 (140)	93.5 (58)		
	1	3 (7)	2.8 (4)	4.8 (3)		
	2	1 (2)	0.7 (1)	1.6 (1)		
	3	0 (0)	0 (0)	0(0)		
Myoclonus	All	4 (8)	1 (2)	10 (6)	**-**	**0.01**
	0	96 (199)	98.6 (143)	90.3 (56)		
	1	2 (4)	1.4 (2)	1.6 (1)		
	2	2 (4)	0(0)	6.5 (4)		
	3	0 (0)	(00)	0(0)		
* **Chi-squared test (or Fisher's exact test) performed on binary data: presence or absence of symptom**						

Table 3.7 Correlation between side-effects on morphine

		Nausea	Constipation	Drowsiness	Confusion	Bad dreams	Dry mouth	Itch	Myoclonus
Nausea	Correlation Coefficient	1.000	0.083	0.139	0.201	0.159	0.141	0.093	0.020
	P		0.164	**0.021**	**0.001**	**0.012**	**0.029**	0.154	0.765
Constipation	Correlation Coefficient	0.083	1.000	0.034	0.033	0.120	0.019	-0.006	0.033
	P	0.164		0.563	0.582	0.053	0.759	0.930	0.601
Drowsiness	Correlation Coefficient	0.139	0.034	1.000	0.380	0.227	0.071	-0.035	0.061
	P	**0.021**	0.563		**$1x\ 10^{-6}$**	**0.0002**	0.261	0.583	0.339
Confusion	Correlation Coefficient	0.201	0.033	0.380	1.000	0.309	0.081	0.016	0.122
	P	**0.001**	0.582	**$1x\ 10^{-6}$**		**$1.4\ x\ 10^{-6}$**	0.211	0.803	**0.064**
Bad dreams	Correlation Coefficient	0.159	0.120	0.227	0.309	1.000	0.017	0.091	0.025
	P	**0.012**	0.053	**0.0002**	**$1.4x\ 10^{-6}$**		0.793	0.178	0.711
Dry mouth	Correlation Coefficient	0.141	0.019	0.071	0.081	0.017	1.000	0.066	-0.068
	P	**0.029**	0.759	0.261	0.211	0.793		0.334	0.319
Itch	Correlation Coefficient	0.093	-0.006	-0.035	0.016	0.091	0.066	1.000	0.091
	P	0.154	0.930	0.583	0.803	0.178	0.334		0.190
Myoclonus	Correlation Coefficient	0.020	0.033	0.061	0.122	0.025	-0.068	0.091	1.000
	P	0.765	0.601	0.339	0.064	0.711	0.319	0.190	
Binary data (presence or absence of symptom) used in analysis									

Table 3.8 Side-effects predictive of morphine response

	B	S.E.	Wald	p value	OR (95% CI)
Nausea	0.199	0.209	0.915	0.339	1.221(0.811, 1.837)
Constipation	0.104	0.182	0.331	0.565	1.110(0.778, 1.585)
Drowsiness	**0.938**	**0.233**	**16.207**	**$5.7x10^{-7}$**	**2.554 (1.618, 4.032)**
Confusion	**0.733**	**0.224**	**10.754**	**0.001**	**2.082(1.343, 3.228)**
Bad dreams	**0.750**	**0.280**	**7.156**	**0.007**	**2.116(1.222, 3.666)**
Dry mouth	0.228	0.299	0.585	0.444	1.256(0.700, 2.255)
Itch	0.836	0.771	1.175	0.278	2.308(0.509, 10.464)
Myoclonus	2.064	1.237	2.785	0.095	7.880(0.698, 89.007)
Constant	-3.879	0.598	42.094	0.000	0.021

Multivariate logistic regression. Dependent variable: Morphine non-responder

3.3.4 Phenotype 1: Composite scores of analgesic response and central side-effects on morphine, as defined by PCA

The sample size was statistically adequate to carry out principal components analysis (Overall Kaiser-Meyer-Olkin (KMO) value 0.852, Bartlett's test of sphericity <0.001). Constipation, dry mouth and myoclonus were excluded from analysis as the individual KMO values were 0.404, 0.531 and 0.468, which are only borderline for sampling adequacy [318].

Two principal components emerged with eigenvalues >1 (Figure 3.4 and table 3.9). The first component, which carried high loadings from all the pain intensity scores, explained nearly 40% of the total variance. This component represents analgesic response to morphine. Component 2, accounting for 15% of the total variance, received high loadings from the central side-effects of confusion / hallucinations, drowsiness, bad dreams and nausea. Itch was not retained in either of the principal components. The final pattern matrix (table 3.9) demonstrates the loadings of each variable onto each component after rotation.

Component 1 (analgesia) and component 2 (central side-effects) were only mildly correlated, Spearman r = 0.18.

The principal components were transformed as they were not normally distributed. A higher $\log_{10}$Component 1 score was associated with higher average, least, worst and pain now scores and lower pain relief scores (correlation coefficients 0.889, 0.764, 0.803, 0.836and -0.834 respectively), as shown in Figure 3.5. Similarly a higher $\log_{10}$Component 2 score (central side-effect) score was associated with a higher individual symptom severity score (table 3.10). Confusion and hallucinations was associated with the highest loading of individual factors onto $\log_{10}$Component 2 (central side-effects). Therefore a higher $\log_{10}$Component 1 or $\log_{10}$Component 2 scores represents a worse outcome in terms of analgesia or central side-effects on morphine respectively.

Patients who were defined as being morphine responders had much lower analgesic and central side-effects components scores than morphine non-responders (p<0.0001).

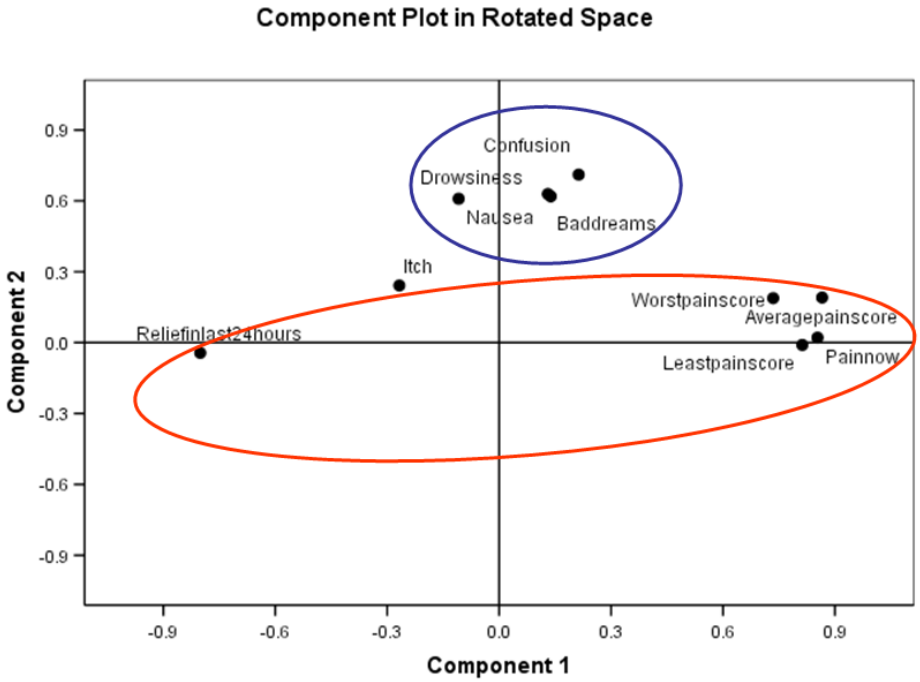

Figure 3.4 Principal Components of morphine response

The co-ordinates of the variables along the axes (components) represent a measure of the strength of the relationship between the variable and the component. Worst pain, average pain, least pain, pain now and pain relief scores correlate highly with component 1 (x-axis). Therefore component 1 appears to represent analgesic response. Confusion, drowsiness, nausea and bad dreams are correlated with component 2 (y-axis). Therefore component 2 appears to represent central side-effects. The variables which are highly correlated with component 1 (analgesic response: red circle) have a low correlation with component 2. Similarly variables which are highly correlated with component 2 (central side-effects: blue circle) have a low correlation with component 1.

Table 3.9 Pattern matrix showing the loading of each variable onto each component after rotation

	Component	
	1	**2**
Eigenvalue (after rotation)*	3.683	2.026
% variance explained	39.7	14.6
Average pain score	**0.865**	0.191
Pain now	**0.853**	0.021
Least pain score	**0.812**	-0.010
% Pain relief	**-0.801**	-0.043
Worst pain score	**0.734**	0.188
Itch	-0.267	0.242
Confusion**	0.213	**0.711**
Bad dreams	0.130	**0.629**
Drowsy	0.138	**0.619**
Nausea	-0.109	**0.609**
*Rotation Method: Direct oblimin with Kaiser Normalization. ** confusion includes hallucinations		

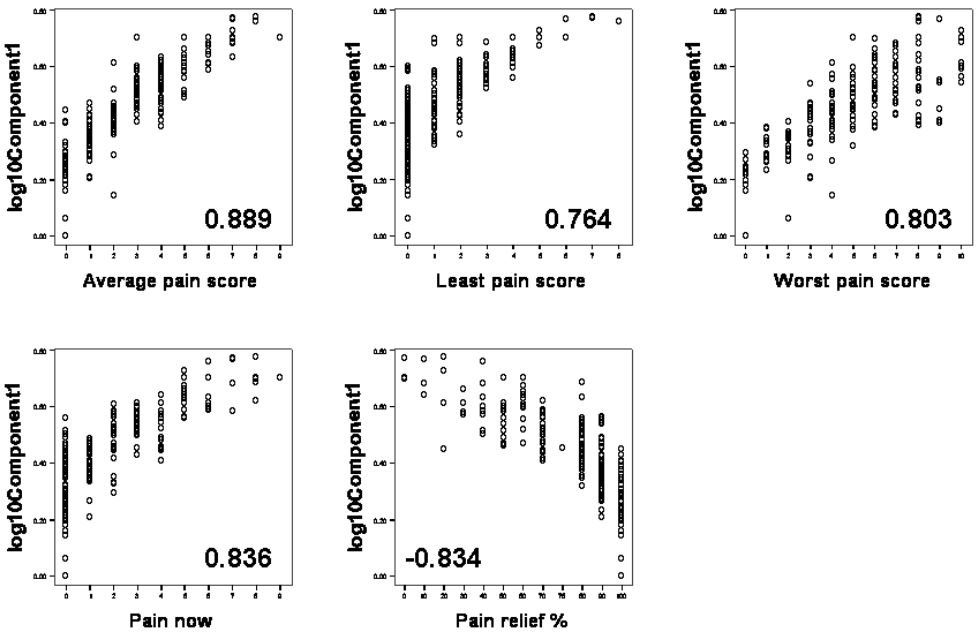

Figure 3.5 Correlations between pain scores and component analgesic response score

Component analgesic response scores are presented as $\log_{10}$Component 1. Spearman correlation coefficient presented for each pain score.

Table 3.10 Comparing component central side-effect scores with symptom severity scores

	Grade of severity	Total study population % (N)	Component central side-effect score Mean (SEM)
Nausea	0	51 (105)	0.3217 (0.013)
	1	30 (63)	0.4279 (0.016)
	2	11 (23)	0.5226 (0.024)
	3	8 (16)	0.6043 (0.028)
Drowsiness	0	20 (41)	0.2402 (0.02)
	1	31 (64)	0.3575 (0.016)
	2	29 (60)	0.4394 (0.014)
	3	20 (42)	0.5655 (0.017)
Confusion**	0	60 (124)	0.3124 (0.01)
	1	20 (41)	0.4628 (0.017)
	2	14 (30)	0.5742 (0.017)
	3	6 (12)	0.6347 (0.02)
Bad dreams	0	77 (159)	0.3476 (0.01)
	1	13 (27)	0.5253 (0.02)
	2	9 (18)	0.6044 (0.02)
	3	1 (3)	0.6908 (0.04)

* Component central side-effect score represents Log_{10}Component2 in PCA
** includes hallucinations
Total N = 207

The component scores were used in the subsequent genetic association studies (chapter 4 and 5). They were also used in linear regression analysis to determine the clinical factors predictive of variability in pain and central side-effect scores, as shown in tables 3.11 and 3.12.

Being on an antiemetic was associated with lower pain scores (log_{10}component1 scores) while being on a B-blocker, a diuretic or paracetamol was associated with higher pain scores. Diagnosis and length of time on morphine were not found to be predictive of pain scores on univariate analysis.

Length of time on morphine was inversely correlated with central side-effect scores (log_{10}component2 scores). Being on an antiemetic or a selective serotonin reuptake inhibitor (SSRI) was associated with higher central side-effect scores. Being on a steroid or a tricyclic antidepressant was associated with lower side-effect scores. Having a tumour of the pancreas or biliary tree was associated with higher central side-effect scores, but the numbers of patients in this group was very small so this variable was omitted from further analysis. Having prostate cancer was associated with lower central side-effect scores.

Table 3.11 Clinical factors predictive of variability in analgesic response

Univariate linear regression. Log_{10}Component1 dependent variable (pain scores)							
Variable	**B**	**Std. Error B**	**β**	**P value**	**Median (range)**	**Spearman's r**	
Age	0.001	0.001	0.065	0.358	60 (19-85)	0.013	
Daily morphine dose (mg)	<0.001	<0.001	0.131	0.062	102.5 (10-1280)	0.089	
Days to death	<-0.001	<0.001	-0.049	0.49	122.5 (5-2590)	-0.041	
Days on opioids	<-0.001	<0.001	-0.045	0.523	91 (7-4455)	-0.129	
Total White Cell Count	0.001	0.001	0.054	0.441	8.6 (0.3-68.1)	0.019	
					% (N)	**Mean**	
						Yes*	**No***
Gender	-0.027	0.02	-0.094	0.18	F 47.8 (99) / M 52.2 (108)	F 0.39 (0.049)	M 0.4 (0.048)
Diagnosis							
Upper gastrointestinal tract	0.011	0.038	0.021	0.765	7.7 (16)	0.31 (0.12)	0.4 (0.036)
Lower gastrointestinal tract	0.015	0.037	0.029	0.68	8.7 (18)	0.41 (0.123)	0.39 (0.036)
Gynaecological	-0.093	0.036	-0.18	0.10	8.7 (18)	0.22 (0.101)	0.41 (0.036)
Urogenital (renal and bladder)	-0.001	0.035	-0.001	0.988	10.1 (21)	0.4 (0.112)	0.39 (0.036)
Breast	-0.01	0.027	-0.027	0.707	17.4 (36)	0.36 (0.081)	0.4 (0.038)
Sarcoma	-0.03	0.03	-0.71	0.314	13.5 (28)	0.39 (0.094)	0.39 (0.037)
Lung (including mesothelioma)	0.007	0.031	0.016	0.826	13 (27)	0.38 (0.097)	0.39 (0.037)
Skin	0.119	0.066	0.127	0.071	2.4 (5)	0.8 (0.2)	0.38 (0.035)
Medications							
Anticonvulsant	0.024	0.025	0.066	0.347	21.3 (44)	0.44 (0.077)	0.38 (0.038)
Antiemetic	**-0.044**	**0.02**	**-0.149**	**0.033**	**49 (99)**	**0.43 (0.015)**	**0.47 (0.014)**
Aspirin	0.001	0.034	0.002	0.973	10.1 (21)	0.43 (0.111)	0.39 (0.036)
B Blocker	**0.089**	**0.037**	**0.169**	**0.016**	**8.2 (17)**	**0.71 (0.114)**	**0.36 (0.035)**
Diuretic	**0.066**	**0.032**	**0.146**	**0.037**	**11.6 (24)**	**0.54 (0.104)**	**0.37 (0.036)**
NSAID	0.011	0.022	0.037	0.604	35.7 (74)	0.39 (0.058)	0.39 (0.042)
Paracetamol	**0.046**	**0.021**	**0.155**	**0.027**	**40.1 (83)**	**0.48 (0.056)**	**0.34 (0.043)**
SSRI, SNRI antidepressants	0.045	0.032	0.097	0.168	11.1 (23)	0.48 (0.106)	0.38 (0.036)
Steroid	0.021	0.021	0.071	0.313	39.6 (82)	0.43 (0.055)	0.37 (0.044)
Tricyclic antidepressants	0.002	0.031	0.005	0.938	13 (27)	0.38 (0.097)	0.39 (0.037)

Other drugs included in analysis but non-significant: ACE-inhibitor, Antibiotic, Anticoagulant, Benzodiazepine, Brochodilator, H2 Antagonist, Proton Pump Inhibitor. Other diagnoses included but not significant: tumours of pancreas and biliary tree, haematological tumour, head and neck, prostate

*Yes / No represents being on the drug or not or having the diagnosis or not

Table 3.12 Clinical factors predictive of central side-effects on morphine

Univariate linear regression **Log_{10}Component2 as dependent variable (central side-effects)**							
Variable	**B**	**Std. Error B**	**β**	**P value**	**Median (range)**	**Spearman's r**	
Age	0.001	0.001	0.111	0.115	60 (19-85)	0.118	
Daily morphine dose (mg)	<0.001	<0.001	-0.043	0.542	102.5 (10-1280)	-0.013	
Days to death	<0.001	<0.001	-0.16	0.13	122.5 (5-2590)	-0.212	
Days on opioids	**<0.001**	**<0.001**	**-0.163**	**0.02**	**91 (7-4455)**	-0.294	
Total White Cell Count	0.002	0.001	0.083	0.238	8.6 (0.3-68.1)	0.016	
Plasma sodium	-0.006	0.003	-0.134	0.057	136 (120-143)	-0.137	
Plasma calcium	0.012	0.067	0.013	0.858	2.24 (1.52-2.94)	0.039	
						Mean	
					% (N)	**Yes***	**No***
Gender	0.01	0.022	0.032	0.65	F 47.8 (99) / M 52.2 (108)	F 0.29 (0.046)	0.35 (0.047)
Diagnosis							
Pancreas and biliary	**0.168**	**0.06**	**0.195**	**0.005**	**3.4 (7)**	**0.71 (0.184)**	**0.3 (0.033)**
Gynaecological	-0.015	0.039	-0.028	0.694	8.7 (18)	0.28 (0.109)	0.32 (0.034)
Haematological	0.028	0.057	0.035	0.621	3.9 (8)	0.25 (0.164)	0.32 (0.033)
Sarcoma	-0.061	0.032	-0.134	0.056	13.5 (28)	0.21 (0.079)	0.34 (0.036)
Head and Neck	-0.084	0.047	-0.126	0.072	5.8 (12)	0.17 (0.112)	0.33 (0.034)
Prostate	**-0.083**	**0.04**	**-0.146**	**0.037**	**8.2 (18)**	**0.29 (0.114)**	**0.32 (0.034)**
Medications							
Anticonvulsant	0.008	0.027	0.02	0.771	21.3 (44)	0.35 (0.074)	0.31 (0.037)
Antiemetic	**0.044**	**0.022**	**0.14**	**0.046**	**47.8 (99)**	**0.38 (0.049)**	**0.26 (0.043)**
Benzodiazepine	0.004	0.019	0.009	0.894	16.9 (35)	0.26 (0.075)	0.33 (0.036)
B Blocker	0.032	0.04	0.057	0.422	8.2 (17)	0.35 (0.119)	0.32 (0.034)
NSAID	-0.032	0.023	-0.098	0.163	35.7 (74)	0.23 (0.05)	0.37 (0.042)
SSRI, SNRI antidepressants	**0.078**	**0.035**	**0.157**	**0.025**	**11.1 (23)**	**0.43 (0.106)**	**0.3 (0.034)**
Steroid	-0.044	0.022	-0.136	0.053	39.6 (82)	0.27 (0.05)	0.35 (0.043)
Tricyclic antidepressants	**-0.065**	**0.033**	**-0.138**	**0.05**	**13 (27)**	**0.19 (0.079)**	**0.34 (0.036)**

Other drugs included in analysis but non-significant: Antibiotic, Anticoagulant, Aspirin, Benzodiazepine, Brochodilator, Diuretics H2 Antagonist, Paracetamol, Proton Pump Inhibitor. Other diagnoses included but not significant: tumours of skin,breast, lung, upper and lower gastrointestinal tract and urogenital tumours.

*Yes / No represents being on the drug or not or having the diagnosis or not

3.3.5 Phenotype 2: Daily morphine dose requirements

This phenotype of response to morphine examines the dose of morphine required to achieve adequate pain control i.e. the dose of morphine required to achieve an average pain of ≤ 4 in the preceding 24 hours. This method has been used previously in genetic association studies of response to morphine in cancer pain [92;135]. This phenotype was included in this thesis to allow some comparison with previous studies.

All patients who were "morphine responders" were included in this analysis (N=183). This was in order to stratify this phenotype to represent a well defined group of patients who had their dose of morphine titrated up until they achieved good pain control without intolerable side-effects. In the total Caucasian morphine-responder population (N=183), 79% (N=144) of patients achieved an average pain of ≤ 4 compared to 21% (N=38) who did not. (There was one patient with incomplete pain scores)

The clinical factors predictive of variability in morphine dose to achieve adequate pain control are shown in table 3.13. The strongest clinical predictor was being on an anticonvulsant medication. Patients who were taking anticonvulsants required much higher doses of morphine (median 195mg, range 30-610mg) compared to those not taking these drugs (median 90mg, range 30-610mg), $p= 4.6 \times 10^{-5}$. Being on an anticoagulant medication was also predictive of requiring a higher dose while being on a bronchodilator was predictive of a lower dose. Older subjects required lower doses of morphine.

Table 3.13 Clinical factors predictive of variability in daily morphine dose requirements

Univariate linear regression. Log_{10}daily morphine dose (mg) as dependent variable								
Variable	**B**	**Std. Error B**	**β**	**P value**	**R^2**	**% (N) / median (range)**	**Spearmans r**	
Age	**-0.005**	**0.002**	**-0.187**	**0.025**	**0.035**	**58 (19-85)**	**-0.186**	
Gender	-0.098	0.059	-0.138	0.098	0.019	F 49.3 (71) / M 50.7 (73)	-0.137	
Days to death	<-0.0001	<0.0001	-0.005	0.953	<0.0001	145.5 (6-2590)	0.109	
Days on opioids	<0.0001	<0.0001	0.026	0.761	0.001	104.5 (7-4455)	0.168	
Total White Cell Count	-0.003	0.005	-0.558	0.578	0.002	8.7 (0.3-37.6)	-0.117	
Serum albumin	0.005	0.005	0.084	0.317	0.007	29 (11-43)	0.074	
Serum calcium	0.179	0.175	0.088	0.307	0.008	2.2 (1.52-2.94)	0.11	
							Mean (log10dailymorphinedose)	
Diagnosis							**Yes***	**No***
Upper gastrointestinal tract	0.037	0.101	0.031	0.713	0.001	9.7 (14)	2.03 (0.08)	2.0 (0.03)
Lower gastrointestinal tract	0.034	0.112	0.025	0.764	0.001	7.6 (11)	2.03 (0.12)	2.0 (0.03)
Pancreas and biliary	0.034	0.209	0.014	0.871	<0.001	2.1 (3)	2.03 (0.16)	2.0 (0.03)
Gynaecological	-0.102	0.097	-0.087	0.298	0.008	10.4 (15)	1.9 (0.11)	2.01 (0.03)
Urogenital (renal and bladder)	0.044	0.108	0.034	0.684	0.001	8.3 (12)	2.04 (0.06)	2.0 (0.03)
Haematological	-0.198	0.148	-0.111	0.184	0.012	4.2 (6)	1.8 (0.13)	2.0 (0.03)
Breast	-0.098	0.077	-0.106	0.206	0.011	18.1 (26)	1.92 (0.06)	2.02 (0.03)
Sarcoma	0.128	0.079	0.134	0.108	0.018	16.7 (24)	2.11 (0.07)	1.98 (0.03)
Lung	0.065	0.095	0.057	0.496	0.003	11.1 (16)	2.06 (0.08)	1.99 (0.03)
Head and Neck	0.057	0.13	0.037	0.664	0.001	4.2 (6)	2.05 (0.1)	2.0 (0.03)
Prostate	-0.101	0.108	-0.078	0.35	0.006	8.3 (12)	1.91 (0.14)	2.01 (0.03)
Skin	0.073	0.209	0.029	0.728	0.001	2.1 (3)	2.07 (0.06)	2.0 (0.03)
Drugs								
Anticoagulant	**0.142**	**0.07**	**0.168**	**0.045**	**0.028**	**22.9 (33)**	**2.11 (0.06)**	**1.97 (0.03)**
Anticonvulsant	**0.308**	**0.073**	**0.333**	**4.6×10^{-5}**	**0.111**	**18.1 (26)**	**2.25 (0.06)**	**1.9 (0.03)**
Aspirin	0.114	0.108	0.088	0.293	0.008	8.3 (12)	2.1 (0.06)	2.0 (0.03)
B Blocker	0.057	0.149	0.032	0.701	0.001	4.2 (6)	2.06 (0.16)	2.0 (0.03)
Bronchodilator	**-0.225**	**0.102**	**-0.181**	**0.03**	**0.033**	**9 (13)**	**1.8 (0.08)**	**2.02 (0.03)**
NSAID	0.097	0.062	0.13	0.12	0.017	35.4 (51)	2.06 (0.05)	1.97 (0.04)
Paracetamol	0.003	0.062	0.004	0.961	<0.001	35.4 (51)	2.0 (0.05)	2.0 (0.04)
SSRI, SNRI antidepressants	0.081	0.097	0.07	0.406	0.005	10.4 (15)	2.07 (0.07)	2.0 (0.03)
Steroid	-0.027	0.061	-0.037	0.658	0.001	41 (59)	1.98 (0.04)	2.0 (0.04)
Tricyclic antidepressants	0.05	0.09	0.046	0.582	0.002	12.5 (18)	2.04 (0.06)	2.0 (0.03)

Other drugs included in analysis but non-significant: ACE-inhibitor, Antibiotic, Antiemetic Antihistamine, Haloperidol, Antiemetic 5HT4 agonist, Antiemetic 5HT3 antagonist, Antiemetic Phenothiazine, Antifungal, Benzodiazepine, Diuretic, H2 Antagonist, Proton Pump Inhibitor

*Yes / No represents being on the drug or not or having the diagnosis or not

3.4 DISCUSSION

3.4.1 Main Findings

The 2 phenotypes for response to morphine as presented here are:

- Phenotype 1: Composite scores of analgesic response and central side-effects as distinct outcome measures, defined by principal components analysis (PCA).
- Phenotype 2: Daily morphine dose required to achieve adequate pain control. The daily dose of morphine in morphine responders with an average pain of ≤4 is examined.

The main findings from this study are:

- There is inter-individual variation in response to morphine in terms of analgesia and side-effects and also in the dose of morphine required to achieve adequate pain control.

- Analgesic response, central side-effects on morphine and the dose of morphine required to achieve adequate pain control appear to be distinct clinical outcomes. There is little correlation between these outcomes. The clinical factors contributing to variability in these measures are dissimilar.

3.4.2 Study design

The design of the Prospective Morphine Study is such that response to morphine may be explored and defined. One of the main strengths of this study is the abundant clinical data that has been collected from each patient which allows in-depth analysis of individual factors contributing to morphine response / non-response.

Particular attention has been focused on maintaining an accurate clinical database in this study. This is why a number of subjects were excluded, including those who were categorised clinically as "morphine responders" by the palliative care team but whose pain scores suggested that they were either inadequately titrated on their morphine dose or that they were simply unable to fill in the numerical rating scale accurately. This finding reflects other studies which demonstrated that some patients, for a variety of reasons cannot complete pain assessment tools [327;328]. It was felt that inclusion of these patients in the analyses would permit unacceptable inaccuracy and bias. Unlike other studies in this area which rely solely on pain and side-effect scores and doses of morphine used, the additional clinical distinction of patients into morphine responders or non-responders in this study allowed refinement of the study population to include only those with as accurate clinical data as possible.

The wide-range of clinical, drug and laboratory data collected from each patient also allowed investigation of the other non-opioid factors which may influence response. Other studies have also included such data analysis [92;93;112]. In this study there were a wide number of different cancer diagnoses and the study subjects are on a variety of different concomitant medications.

3.4.3 Phenotype definition

In this study a validated tool, the brief pain inventory was used to collect pain scores [328]. This tool has also been used by other researchers in this field [92;112]. Although the BPI is a commonly used pain assessment tool, there is no consensus as to which of the five pain intensity variables is most clinically relevant. "Pain now" may be influenced by any number of factors including 1) the time since the last dose of morphine, 2) recent activity and 3) other psychological issues including desire to please the attending physician [329]. "Worst" and "least" pain scores may be altered by the presence or absence of incident pain i.e. the patient has pain which is exacerbated by activity. Average pain has been used in other genetic association studies of response to morphine [92;93;110;112]. Pain relief ratings have been shown to be sensitive to the effects of treatment [330]. Pain relief, average pain and worst pain were identified in this study as independent predictors of morphine non-response (Table 3.5). In a recent

large study investigating variables to include in an international pain classification system, average and worst pain and pain relief were chosen as dependent variables on the basis of expert opinion [331].

This is the first study that we are aware of in which a number of different pain intensity and side-effect scores have been included in the analysis. Instead of analysing each pain score (or side-effect score) separately, or instead of choosing one single dimension of pain intensity to study, in this study all actual pain and side-effect scores were included in the PCA modelling. PCA was used to develop a mathematical model of morphine response, incorporating as much clinical data on morphine response as possible. Two components, representing analgesic response and central side-effects respectively were identified which accounted for most of the variability in the data. The finding that pain and central side-effect scores loaded onto separate components and the lack of significant correlation between these two components suggests two separate dimensions of morphine response.

Principal components analysis has been used in validation studies of the Brief Pain Inventory and in most of these studies pain intensity scores loaded highly onto the same component [328;332]. In a more recent study, pain intensity and pain interference scores were explored using factor analysis (which is similar to PCA). In that study, like in ours, the pain intensity scores loaded onto the same factor [333].

Previous work in our department has used morphine responder versus morphine non-responder as the study outcome. In this study we found that although a higher proportion of morphine non-responders experienced morphine-related side-effects, a substantial proportion of subjects in the "responder" group also experienced these symptoms, especially constipation, drowsiness and nausea, although these appear not to have been dose-limiting. In order to capture the subjective experiences of all study subjects, we used all pain scores and side-effect scores in the PCA, regardless of whether the subjects were clinically defined as responders or non-responders.

In this study the different side-effects were separated out to examine which in particular were most problematic. Drowsiness, confusion/hallucinations and bad dreams were identified as being independent predictors of morphine non-responders.

These same variables loaded onto the same component in the PCA, along with nausea, making up a component representative of central side-effects.

The strongest correlations in this study were between the centrally mediated side-effects of drowsiness, confusion and bad dreams. In particular constipation was not well correlated with any of the other side-effects. Consistent with this was the fact that it did not meet the criteria for inclusion in the PCA modeling. Constipation was not included in the modelling, perhaps because it is mediated primarily by peripheral mechanisms [334]. The number of subjects with dry mouth or myoclonus was low, perhaps explaining why these factors were not included in the final model. Further consideration should be given to these side-effects in future studies.

In order to be able to compare our findings with other published genetic association studies, we also examined the variability of morphine dose required to achieve adequate pain control, as defined by average pain less than 4/10. This method has been used by the Norweigan group in some of their genetic association studies [92;135]. In some of their later studies they did not stratify according to pain scores and just examined variability in morphine dose, regardless of individual pain scores [110;112]. The Norweigan studies also did not take into account whether the patient had the opioid dose adequately titrated according to the individual level of pain. We adopted their initial approach as it provides a tighter model to explore individual variation in response to morphine. In this study we included only those patients who we knew to respond well to morphine. Thus these patients represent a well defined group of patients who had their dose of morphine titrated up until they achieved good pain control without intolerable side-effects. One of the main disadvantages to this approach was that the substratification required significantly reduced the numbers of study subjects, with the potential influence on the power of the study. The final number however (N=145) was larger that in the original Norweigan study (N=99).

This definition of morphine response potentially reflects variability in sensitivity to morphine for cancer pain. It may however also represent variability in pain perception. In clinical practice morphine is titrated according to individual patient response in terms of pain and side-effects. It is therefore unlikely that the dose of morphine for an individual patient will be predicted solely on such population data.

The PCA model of morphine response appears to be more clinically relevant and therefore more meaningful for use in genetic association studies.

3.4.4 Clinical factors influencing morphine response

Although to date no clinical factor except for renal impairment has been shown to consistently alter morphine response, it has been suggested that there are a number of factors which may influence opioid handling and pain perception. In this study different clinical factors appear to be associated with the different clinical phenotypes i.e. the clinical factors associated with pain scores (component 1 in PCA) are distinct to those associated with central side-effect scores (component 2) or those associated with morphine dose requirements. This further supports the proposal that pain, central side-effects and morphine dose requirements may be discrete end points in morphine response. This may be one reason why previous genetic association studies have failed to replicate in this field, namely because the outcomes were not the same.

In our study, gender was not associated with either pain or side-effect scores but there was a trend towards an association with variation in morphine dose ($p=0.098$), with men requiring a median dose of 120mg morphine per day to achieve adequate analgesia, compared to women who required a median dose of 90mg per day.

In our study there was an inverse relationship between age and dose of morphine: older patients required less daily morphine to achieve adequate analgesia. We found no association between age and analgesic or central side-effect scores.

We demonstrated an inverse relationship between central side-effects and length of time on morphine. This may suggest that tolerance to these central side-effects occurs with time, something which is recognized clinically[11] . Length of time on morphine did not influence either analgesic response to morphine or variability in morphine dose requirements.

Concomitant medications were found to account for some of the variability in morphine response. Our data show that cancer patients on morphine are often on a

large number of other medications. Many of these drugs increase the likelihood of opioid side-effects through a variety of mechanisms [335]. In our study drugs acting on beta-adrenergic receptors in particular were predictive of morphine response / non-response. Being on a bronchodilator (B-agonist) was associated with lower morphine requirements and being on a B-blocker was associated with less analgesia from morphine. There have been some reports previously of interaction between the adrenergic and opioidergic systems in terms of morphine response, tolerance and dependence [336;337]. The data presented here are in contrast to other studies in which beta-blockers have been shown to reduce analgesic requirements and improve pain scores in post-operative pain conditions [338;339]. One animal study however suggested that the effects of beta-blockade on opioid response was determined in part by the site of administration of the drugs [340]. Another study demonstrated that beta-blockers altered the morphine dose-response curve after chronic but not after acute morphine treatment [341]. There may be differential interactions between beta-blockers and opioids depending to the length of time on opioids and the method and frequency of opioid and beta-blocker administration. There have been some animal data suggesting specific interactions and even dimerisation between beta-adrenergic and opioid receptors [342-344]. Therefore it is biologically plausible that drugs which act on adrenergic receptors may alter morphine response. Antiemetics were associated with better analgesic response, which is plausible as some of these agents, especially phenothiazine antiemetics are purported to have intrinsic analgesic properties [345]. Being on an antiemetic may also facilitate improved analgesia because it may allow adequate dose escalation through avoidance of dose-limiting nausea.

Some of the findings are difficult to interpret. Antidepressants, paracetamol and NSAIDs are often used as adjunct analgesic agents when patients are on morphine. In this study however, being on paracetamol with higher pain scores i.e. a worse outcome in terms of analgesic response. It may be that patients taking these medications had more severe or complex pain. This finding mirrors another study in which paracetamol was an independent predictor for failure of analgesia for acute pain in a prehospital setting [346].

Anticonvulsants are used as analgesic agents, especially for neuropathic pain, therefore again, intuitively one would expect their use to be associated with decreased

opioid requirements. Instead, in this study, being on an anticonvulsant is associated with higher morphine dose requirements. This group of patients may represent those with pain which was not morphine responsive e.g. neuropathic pain. Patients with predominately neuropathic pain were excluded from the study on the basis that neuropathic pain is usually treated with non-opioid medications. However, in this study (unlike the subsequent Randomised Controlled Trial), a specific neuropathic pain assessment tool was not used. It is possible that there is a cohort of patients with mixed cancer pain with a significant neuropathic element which was not identified.

Antiemetics and SSRIs were associated with higher central side-effect scores. This is not surprising as these medications also have the potential to cause side-effects such as drowsiness. A previous study found that psychotropic medications (benzodiazepines, anxiolytics or SSRI antidepressants) were associated with higher morphine dose requirements post-operatively [50]. This was not replicated in our study. Tricyclic antidepressants, which are commonly associated with drowsiness, were associated with less central side-effects in our study.

It is unclear why certain tumour groups would be associated with differences in morphine response. There are two possible reasons why prostate cancer was found to be associated with lower central side-effect scores: 1) pain in prostate cancer is usually bone pain, which often responds well to opioid-sparing NSAIDs, and 2) prostate cancer is rarely associated with organ involvement, therefore there are less non-opioid reasons why this cohort would experience the symptoms of nausea, confusion and hallucinations or drowsiness. In a larger study, sub-stratification according to diagnosis would allow further exploration of this finding.

The data presented here suggest that the dose of the original opioid at which an opioid switch is required varies widely. Similarly the duration of the initial opioid therapy is variable. These data may suggest that there may be two broad groups of patients which undergo opioid switching. The first group require switching shortly after initiation of the initial opioid, when the opioid dose is relatively low. The second group appear to become non-responsive to the initial opioid at either higher doses or after some time has elapsed, perhaps due to changes in the underlying painful state, disease, tolerance or some other contributing factor. These data support a recent

proposal that there is a pharmacodynamic difference between initial failure of response to an opioid shortly after opioid initiation and alteration of response after initial good effect [35]. It was not possible to explore this potential difference further in this study.

3.4.5 Limitations

Although we attempted to account for as many clinical confounders as possible, it is likely that there are a large number of other clinical and environmental factors which may influence pain perception or opioid response, which are not included in this study including psychological state (anxiety and depression) [347], social status and diet[348]. The latter two factors have not been included in any study of opioid response, perhaps because of challenges in measuring and quantifying such variables. Psychological distress has been associated with pain intensity and treatment response [331] and this factor has been included in one previous opioid genetic study [124].

As this study was carried out at a single time point we were not able to control for factors such as pain intensity at initiation of morphine therapy, the development of morphine tolerance or the fact that cancer pain is likely to change in location, character and severity with time. Data pertaining to the exact cause of the cancer pain (treatment, tumour, metastases) were not collected.

This study (and further studies presented in this thesis) was carried out in Caucasian subjects. Further studies are needed to explore the applicability of these findings to other ethnic populations. This is discussed in more detail in chapter 8.

The findings presented in this chapter have not yet been replicated and we have not adjusted the results for multiple testing. Thus the PCA and regression modelling must be considered preliminary and hypothesis generating. However in terms of correcting for multiple testing, the use of PCA component scores in the genetic association study does reduce the multiplicity of analyses which would be necessary if each score was examined separately, while retaining as much clinical information as possible. In addition, although the findings presented here require testing in larger, prospective

studies, biologically sound hypotheses may be proposed to explain the association of most factors retained in the modelling.

3.5 Conclusion

Future work requires confirmation and validation of the applicability of these definitions or morphine response in other adequately powered prospective studies. Larger studies may facilitate more accurate sub-stratification according to diagnosis and concomitant medications. Prospective follow-up studies may allow exploration of the issues of tolerance and changing pain. A larger study would also facilitate examination of "extremes" i.e. subjects in both groups experiencing either no symptoms or severe symptoms of opioid side-effects.

4 Chapter 4: Results from candidate gene association study: Opioid receptors

4.1 Introduction and summary of the rationale behind this study

Morphine acts on opioid receptors, therefore the genes coding for opioid receptors are natural candidates for genetic association studies. Opioid receptors are found throughout the nervous system. Three different opioid receptors have been identified: mu (MOR), delta (DOR) and kappa (KOR). These receptors have been cloned [349-351] and are encoded by different genes: *OPRM (mu opioid receptor)*, *OPRD (delta opioid receptor)* and *OPRK (kappa opioid receptor)*.

Morphine acts primarily via the mu opioid receptor (MOR). In this study I tested the hypothesis that polymorphisms in any of the three opioid receptor genes may influence response to morphine for cancer pain because:

- Morphine also binds, albeit with a weaker affinity, to delta and kappa opioid receptors [24;158].
- Opioid receptors are thought to interact with each other and form heterodimers with altered function potentially affecting opioid response [159-162;352;353].
- Activation of MOR, DOR and KOR elicits different responses both in terms of response to morphine and also non-morphine effects. Although MOR are essential for morphine analgesia, as demonstrated by studies involving MOR knockout mice [354], DOR and KOR play a role in the modulation of certain aspects of morphine response and are also involved in nociception in general. MOR are involved in respiratory function and regulation of the gastrointestinal tract. DOR have been shown to play a role in morphine tolerance [163], depressive and anxiety-states [355], and in alcohol abuse [356]. KOR have been shown to mediate dysphoria to opioid and non-opioid drugs [356].
- MOR, DOR and KOR are all involved in nociception. There is well documented inter-individual variation in pain perception and pain sensitivity. Inter-individual variation in response to morphine is the result of a complex interplay between variability in nociception as well as sensitivity to the drug itself. MOR are involved in perception of thermal and irritant chemical pain [356;357]. DOR modulate mechanical, neuropathic and inflammatory pain [357;358]

and although the data are controversial, some authors suggest that KOR maybe involved in mediating chemical visceral (and thermal) pain [357;359]. More recent data have suggested that these opioid receptors may be distinct in both physical location and also mode of pain transmission, calling into question the idea of mu- delta dimers [360]. Cancer pain however is likely to be a mixed pain and therefore it remains biologically plausible that all three opioid receptors play a role in both cancer pain perception and morphine response.

The association of genetic polymorphisms in mu-opioid receptor gene, *OPRM* and variability in both pain perception and sensitivity to opioids has been a focus of a number of studies [83;87;92;94], as discussed in chapter 1. OPRM is located on chromosome 6 (6q52.2) and spans 79865 bp. There are 13 exonic SNPs located in the coding region of the gene but most have minor allele frequencies (MAF) of 1% or less. Over 800 polymorphisms in OPRM have been described [89]. *OPRD* is located on chromosome 1 (1p35.3) and spans 51556 bp. It consists of three exons and there are currently 344 known SNPs. The kappa opioid receptor gene, OPRK, is located on chromosome 8 (8q11.23) and spans 25919 bp.

Pain and response to morphine are complex traits and are likely to be under the influence of a number of genes and environmental factors. There have been some attempts to explore the influence of combinations of SNPs in OPRM and other genes on response to morphine for cancer pain. These studies have been limited to examining at most the interaction of two SNPs at a time. In each case these SNPs were chosen on the basis of previous candidate gene studies confirming a proposed clinical significance. This is the first study in cancer patients taking opioids in which multiple SNPs and multiple genes are systematically considered together, along with other clinical confounders which may influence the clinical outcome.

4.1.1 Aim

The aim of this study was to examine the influence of genetic variation in the opioid receptor genes on inter-individual variation in morphine response.

The clinical phenotypes defined in chapter 3 (analgesic response, central side-effects on morphine and variability in dose of morphine required to achieve adequate analgesia) were used in a hypothesis-generating genetic association study examining the effect of interaction between multiple polymorphisms in genes coding for the opioid receptors and other confounding clinical variables on morphine response.

4.2 Subjects and methods

Each of the genes were tested on all clinical phenotypes presented in chapter 3 i.e. analgesic response to morphine, central side-effects on morphine and variation in the daily morphine dose required to achieve adequate analgesic response.

The genetic assays were run on DNA from the *Prospective Morphine Study*.

4.2.1 Genetic Marker inclusion

Details of the primers used are found in appendix B. Seven polymorphisms in *OPRM (rs621029, rs1799971, rs589046, rs563649, rs9479757, rs2075572, rs533586)*, eight in *OPRK (rs10504151, rs7836120, rs6473799, rs1365098, rs7016778, rs7824175, rs16918875, rs963549)* and five in *OPRD (rs1042114, rs533123, rs419335, rs2236857, rs2234918)* were included in the study.
Primers for OPRK and OPRD were designed by Dr Sophy Gretton. I optimised and ran these primers on all 264 Caucasians. 7 SNPs across the OPRM gene were included in this study. 5 of these SNPs (*rs6912029, rs1799971, rs9479757, rs589046* and *rs2075572*) had been designed previously by Dr Joy Ross and run on a smaller cohort of this population (N=193). I ran these primers on the remaining patients in the final cohort (N=264). I designed, tested and optimised primers for 2 new SNPs in *OPRM rs563649* and *rs533586*. I ran these new primers and also *rs589046* on all 264

Caucasians. Details of the SNPs tested, including location on the gene, call rate and minor allele frequencies are found in tables 4.1 - 4.3.

4.2.2 Statistical analysis

Each SNP was tested individually using univariate regression analysis. Both the dominant and additive models were used to assess whether the association was due primarily to allele carriage or whether there was a gene dose effect. Univariate regression was used to screen for an association between individual clinical and genetic variables and the outcome variables.

Multivariate stepwise regression analysis was carried out to assess the effect of genetic and clinical factors on variability in the clinical phenotype. Clinical factors known from univariate analysis to predict the clinical phenotype (as detailed in chapter 3) were also included in this analysis. Clinical and genetic factors significant at the level of 10% ($p<0.1$) on univariate analysis were introduced simultaneously into the model using a stepwise approach.

No corrections for multiple testing are presented here. The data presented in the tables represents the raw unadjusted data as the data are considered exploratory until replication studies are performed. Tables containing significant results are presented in this chapter. Tables containing mainly non-statistically significant results are located in appendix c.

4.3 Results

4.3.1 Genetic marker data

All SNPs were in Hardy-Weinberg equilibrium.

All SNPs were comparable to published data from the HapMap northern and western European ancestry group (CEU) in term of allele and genotype frequencies. (Tables 4.1 - 4.3)

The haplotype data are presented in tables 4.4 - 4.6 and figures 4.1 – 4.3.

One haploblock was identified in *OPRM*, spanning 1670 bp and three haplotypes were constructed within this block.

Two haploblocks were identified across *OPRD*, consisting of six haplotypes in total. Three haploblocks comprised of two SNPs each were identified. The finding that *OPRD rs2234918* is not in LD with the other SNPs is similar to the *OPRD* LD pattern found in another study [361].

Three haploblocks were identified across *OPRK*. Nine haplotypes were constructed within the haploblocks. There was a region in the middle of the gene spanning approximately 10 kbp which was not included in any of these haploblocks

Table 4.1 OPRM marker data

N=249*								
SNP	**Position (bp)**	**Locus on gene**	**Allele Major:minor**	**MAF**	**Published MAF**	**HWp**	**Call rate**	**AA change**
rs6912029	154402201	Exon 1	G:T	0.05	0.05	1.0	1.0	Gln-His
rs1799971	154402490	Exon 1	A:G	0.15	0.167	0.2985	1.0	Asn-Asp
rs589046	154434831	Intron 1	C:T	0.27	0.246	0.5629	1.0	-
rs563649	154449660	Intron 1	C:T	0.1	0.08	0.21	1.0	-
rs9479757	154453037	Intron 2	G:A	0.11	0.117	0.9122	1.0	-
rs2075572	154453697	Intron 2	C:G	0.43	0.425	0.7319	0.996	-
rs533586	154455367	Intron 3	T:C	0.33	0.34 G	0.7819	1.0	-
Published genotype and allele frequencies from HapMap CEU * rs6912029, rs1799971, rs9479757, rs9479757 N=249 rs589046, rs563649, rs533586 N=243 Reference sequence NT_025741.14								

Table 4.2 OPRK marker data

N=244								
SNP	**Position (bp)**	**Locus on gene**	**Allele major:minor**	**MAF**	**Published MAF**	**HWp**	**Call rate**	**AA change**
rs10504151	54320793	Intron 2	T:C	0.11	0.05	0.27	1.0	-
rs7836120	54320092	Intron 2	A:G	0.18	0.108	0.42	1.0	-
rs6473799	54315676	Intron 2	T:C	0.22	0.192	0.84	1.0	-
rs1365098	54315236	Intron 2	G:T	0.31	0.212	1.0	1.0	-
rs7016778	54312658	Intron 2	T:A	0.14	0.1	0.23	1.0	-
rs7824175	54306727	Intron 3	G:C	0.11	0.092	0.56	1.0	-
rs16918875	54304707	Exon 4	C:T	0.08	0.018	1.0	1.0	Val-Val
rs963549	54304377	3UTR	G:A	0.18	0.133	0.8	1.0	-
Published genotype and allele frequencies from HapMap CEU Reference sequence NT_008183.18								

Table 4.3 OPRD marker data

N=243								
SNP	**Position (bp)**	**Locus On gene**	**Allele Major:minor**	**MAF**	**Published MAF**	**HWp**	**Call rate**	**AA change**
rs 1042114	29011562	Exon 1	T:G	0.15	0.12	0.779	1.0	Cys-Phe
rs 533123	29013742	Intron 1	A:G	0.21	0.15	0.5678	1.0	-
rs 419335	29024431	Intron 1	A:G	0.29	0.34	1	1.0	-
rs 2236857	29034196	Intron 1	T:C	0.24	0.27	0.7799	1.0	-
rs 2234918	29062184	Exon 3	T:C	0.42	0.5	1	1.0	Gly-Gly
Published genotype and allele frequencies from HapMap CEU Reference sequence NT_004610.18								

Table 4.4 OPRM haplotypes

						Haploblock 1		% (N)	
SNP	**rs 6912029**	**rs 1799971**	**rs589046**	**rs563649**	**rs 94797571**	**rs 2075572**	**rs533586**	**Frequency**	**Carriage frequency**
position	154402201	154402490	154434831	154449660	154453037	154453697	154455367		
1	-	-	-	-	-	C	T	0.57 (275)	0.81 (195)
2	-	-	-	-	-	G	C	0.32 (156)	0.53 (129)
3	-	-	-	-	-	G	T	0.11 (52)	0.2 (48)

Data shown for total Caucasian population (N=243)

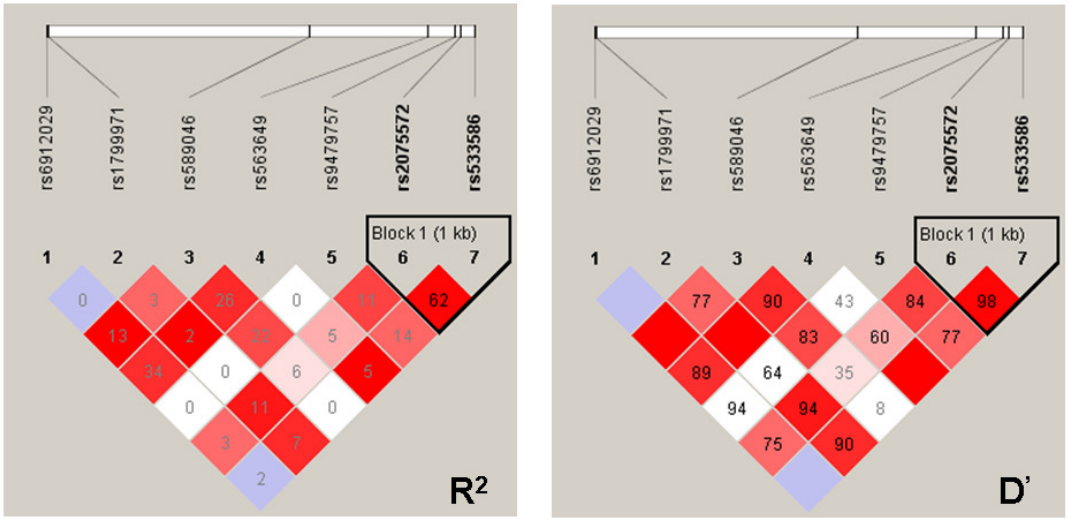

Figure 4.1 LD Plots for OPRM

(Haploview version 4.0, Broad Institute, Cambridge, USA). See List of Abbreviations for explanation of colour scheme.

Table 4.5 OPRD haplotypes

	Haploblock 1		Haploblock 2			% (N)	
SNP position	rs1042114 29011562	rs533123 29013742	rs419335 29024431	rs2236857 29034196	rs2234918 29062184	Frequency	Carriage frequency
1	T	A	-	-	-	0.79 (382)	0.95 (230)
2	T	G	-	-	-	0.06 (33)	0.13 (32)
3	G	G	-	-	-	0.15 (71)	0.28 (67)
1	-	-	A	T	-	0.7 (340)	0.91 (222)
2	-	-	G	T	-	0.06 (30)	0.12 (30)
3	-	-	G	C	-	0.23 (113)	0.4 (98)

Data shown for total Caucasian population (N=243)

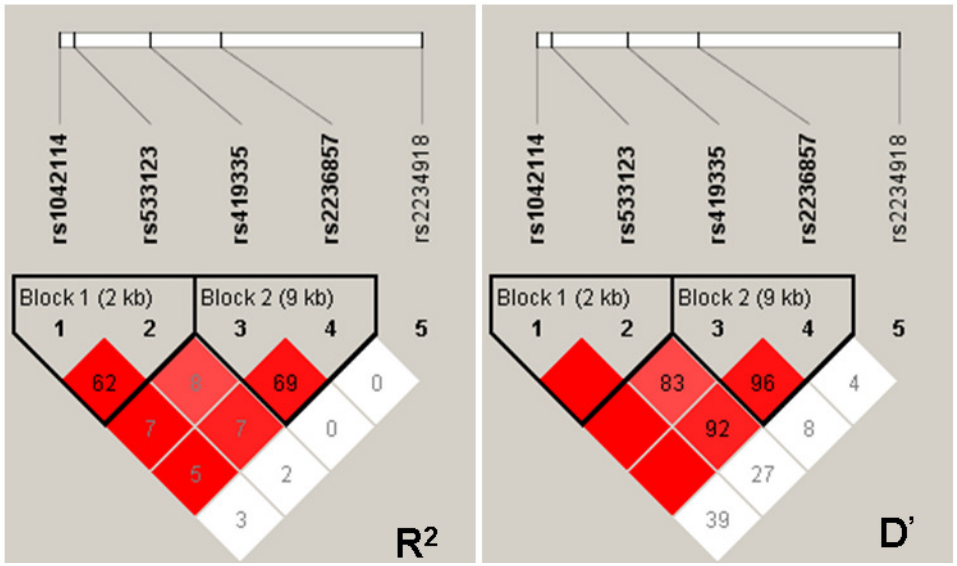

Figure 4.2 LD Plots for OPRD (Haploview version 4.0, Broad Institute, Cambridge, USA).

Table 4.6 OPRK haplotypes

	Haploblock 3		Haploblock 2				Haploblock 1		% (N)	
SNP	**rs 10504151**	**rs 7836120**	**rs 6473799**	**rs 1365098**	**rs 7016778**	**rs 7824175**	**rs 16918875**	**rs 963549**	**Frequency**	**Carriage frequency**
position	**54320793**	**54320092**	**54315676**	**54315236**	**54312658**	**54306727**	**54304707**	**54304377**		
1	-	-	-	-	-	-	C	G	0.82 (339)	0.96 (235)
2	-	-	-	-	-	-	C	A	0.1(50)	0.2 (49)
3	-	-	-	-	-	-	T	A	0.08 (39)	0.16(38)
1	-	-	T	G	-	-	-	-	0.69 (336)	0.9 (220)
2	-	-	T	T	-	-	-	-	0.09 (43)	0.17 (42)
3	-	-	C	T	-	-	-	-	0.2 (109)	0.4 (98)
1	T	A	-	-	-	-	-	-	0.82 (401)	0.96 (234)
2	T	G	-	-	-	-	-	-	0.07 (34)	0.13 (31)
3	C	G	-	-	-	-	-	-	0.11 (53)	0.2 (48)

Data shown for total Caucasian population (N=244)

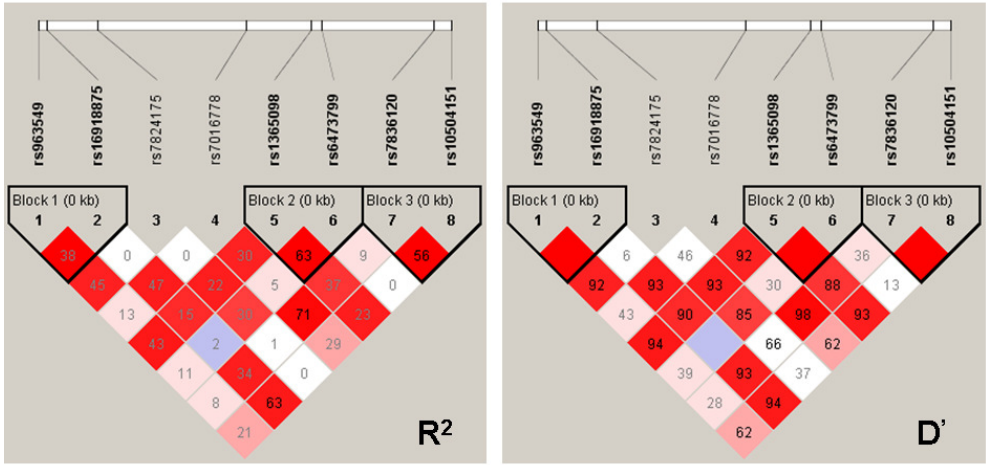

Figure 4.3 LD Plots for OPRK

(Haploview version 4.0, Broad Institute, Cambridge, USA).

4.3.2 Analgesic response

Univariate analysis

On univariate analysis, there was an association with analgesic response as defined by PCA component 1 (see chapter 3) with SNPs in OPRK, *rs1365098, rs7824175* and *rs963549*, (tables 4.8 and 4.9). These SNPs are not located in the same haploblock but they are in LD with each other (D' 0.92-0.94).

The strongest association was with intronic OPRK *rs7824175.* Carriers of the C allele (genotype CC or CG) at *OPRK rs7824175* had a worse outcome in terms of analgesic response i.e. higher pain scores (p=0.001, R^2 = 0.054). Conversely carriers of the G allele (genotype GG or CG) had lower pain scores (p=0.016, R^2 = 0.031). Although the numbers of patients who were homozygous for the variant allele (CC) was low (N=4), this SNP demonstrates a clear gene-dose effect in terms of analgesic response (p=0.0004, R^2 = 0.065), as demonstrated in figure 4.4.

Carriers of the A allele (genotype AA or GA) at *rs963549* had higher pain scores than non-carriers (GG p=0.005, R^2 = 0.042) and again there was a clear gene-dose effect (p=0.001, R^2 = 0.055). There was a similar pattern seen with *rs1365098* although the association was not as strong (p=0.021, R^2 = 0.023).

Haplotypes 1-2 in *OPRK* Haploblock 1 are significantly associated with variability in analgesic response (p=0.019 and 0.005 respectively) but this association is no stronger than the association with *OPRK rs963549 (*data not shown).

There was a weak association between two *OPRM* SNPs, *rs589046* (p=0.02, R^2 = 0.029) and *rs9479717* (p=0.034, R^2 = 0.024) and analgesic response. This association was found with allele carriage only, (table 4.7). There was no association between analgesic response and *OPRM* haplotypes or *OPRD* SNPs / haplotypes.

Table 4.7 Effects of OPRM on analgesic response to morphine (dominant model)

Allele carriage (Dominant model)									
		Carrying allele		**Not carrying allele**		**Univariate linear regression Log10Component1 (pain scores) as dependent variable**			
SNP name	**Allele**	**% (N)**	**Mean* (SEM)**	**% (N)**	**Mean* (SEM)**	**B**	**Std Error B**	**β**	**P value**
rs6912029	G	100 (194)	0.45 (0.01)	0	-	-	-	-	-
	T	8.2 (16)	0.45 (0.04)	91.8 (178)	0.45 (0.01)	-0.004	0.038	-0.008	0.911
rs1799971	A	95.9 (186)	0.44 (0.01)	4.1 (8)	0.49 (0.06)	-0.043	0.052	-0.06	0.411
	G	25.3 (49)	0.44 (0.02)	74.7 (145)	0.45 (0.01)	-0.008	0.024	-0.025	0.727
rs589046	**C**	**93.7 (177)**	**0.45 (0.01)**	**6.3 (12)**	**0.35 (0.04)**	**0.1**	**0.042**	**0.171**	**0.02**
	T	49.7 (94)	0.45 (0.01)	50.3 (95)	0.45 (0.02)	-0.002	0.021	-0.006	0.933
rs563649	C	97.9 (185)	0.45 (0.01)	2.1 (4)	0.45 (0.09)	<-0.001	0.073	<-0.001	0.998
	T	19.6 (37)	0.45 (0.02)	80.4 (152)	0.45 (0.01)	0.006	0.027	0.017	0.816
rs9479757	A	22.2 (43)	0.43 (0.02)	77.8 (151)	0.45 (0.01)	-0.026	0.025	-0.075	0.299
	G	**99 (192)**	**0.45 (0.01)**	**1.0 (2)**	**0.23 (0.01)**	**0.219**	**0.102**	**0.154**	**0.034**
rs2075572	C	81.9 (158)	0.44 (0.01)	18.1 (35)	0.46 (0.02)	-0.012	0.027	-0.032	0.659
	G	64.8 (125)	0.45 (0.01)	35.2 (68)	0.45 (0.02)	-0.002	0.022	-0.007	0.923
rs533586	T	89.4 (169)	0.45 (0.01)	10.6 (20)	0.44 (0.03)	0.01	0.034	0.021	0.78
	C	51.3 (97)	0.44 (0.01)	48.7 (92)	0.45 (0.02)	-0.006	0.021	-0.021	0.779

* mean (SEM) of log10Component1 scores
R^2 rs589046C = 0.029, rs9479757G = 0.024

Table 4.8 Effects of OPRK on analgesic response to morphine (dominant model)

SNP name	Allele	Carrying allele		Not carrying allele		Univariate linear regression Log10Component1 (pain scores) as dependent variable			
		% (N)	Mean* (SEM)	% (N)	Mean* (SEM)	B	Std Error B	β	P value
r10504151	T	97.9 (186)	0.45 (0.01)	2.1 (4)	0.51 (0.08)	-0.063	0.073	-0.034	0.385
	C	20 (38)	0.46 (0.02)	80 (152)	0.44 (0.01)	0.011	0.026	0.032	0.666
rs 7836120	A	96.3 (183)	0.45 (0.01)	3.7 (7)	0.48 (0.05)	-0.036	0.055	-0.048	0.513
	G	30 (57)	0.46 (0.02)	70 (133)	0.44 (0.01)	0.019	0.023	0.06	0.418
rs 6473799	T	94.7 (180)	0.45 (0. 01)	5.3 (10)	0.48 (0.05)	-0.03	0.047	-0.046	0.529
	C	40.5 (77)	0.47 (0.02)	59.5 (113)	0.43 (0.01)	0.036	0.021	0.123	0.093
rs 1365098	G	89.5 (170)	0.44 (0.01)	10.5 (20)	0.5 (0.03)	-0.054	0.034	-0.117	0.111
	T	**54.2 (103)**	**0.47 (0.01)**	**45.8 (87)**	**0.42 (0.02)**	**0.044**	**0.021**	**0.152**	**0.038**
rs 7016778	T	98.9 (188)	0.45 (0.01)	1.1 (2)	**0.57 (0.14)**	-0.119	0.102	-0.086	0.245
	A	26.3 (50)	0.47 (0.02)	73.7 (140)	0.44 (0.01)	0.032	0.024	0.098	0.182
rs 7824175	**G**	**97.9 (186)**	**0.44 (0.01)**	**2.1 (4)**	**0.62 (0.04)**	**-0.175**	**0.072**	**-0.176**	**0.016**
	C	**21.6 (41)**	**0.51 (0.02)**	**78.4 (149)**	**0.43 (0.01)**	**0.08**	**0.025**	**0.232**	**0.001**
rs16918875	C	99.5 (189)	0.45 (0.01)	0.5 (1)	-	-0.257	0.143	-0.131	0.074
	T	16.3 (31)	0.48 (0.03)	83.7 (159)	0.44 (0.01)	0.033	0.029	0.085	0.25
rs 963549	**G**	**96.3 (183)**	**0.44 (0.01)**	**3.7 (7)**	**0.57 (0.05)**	**-0.131**	**0.055**	**-0.173**	**0.018**
	A	**35.3 (67)**	**0.49 (0.02)**	**64.7 (123)**	**0.42 (0.01)**	**0.062**	**0.022**	**0.205**	**0.005**

* mean (SEM) of log10Component1 scores

R^2 rs1365098T = 0.023, rs7824175G = 0.031, rs7824175C = 0.054, rs963549G = 0.03, rs963549A = 0.042

Table 4.9 Effects of OPRK on analgesic response to morphine (additive model)

				Univariate linear regression Log10Component1 (pain scores) as dependent variable			
SNP name	**Genotype**	**% (N)**	**Mean* (SEM)**	**B**	**Std. Error B**	**β**	**P value**
rs10504151	TT	80 (152)	0.51 (0.08)				
	TC	17.9 (34)	0.45 (0.03)	-0.015	0.023	-0.047	0.52
	CC	2.1 (4)	0.44 (0.01)				
rs7836120	AA	70 (133)	0.44 (0.01)				
	AG	26.3 (50)	0.46 (0.02)	-0.017	0.019	-0.067	0.365
	GG	3.7 (7)	0.48 (0.05)				
rs6473799	TT	59.5 (113)	0.43 (0.01)				
	TC	35.3 (67)	0.47 (0.02)	-0.029	0.018	-0.119	0.104
	CC	5.3 (10)	0.78 (0.05)				
rs1365098	**GG**	**45.8 (87)**	**0.42 (0.02)**				
	GT	**43.7 (83)**	**0.46 (0.02)**	**-0.036**	**0.016**	**-0.169**	**0.021**
	TT	**10.5 (20)**	**0.5 (0.03)**				
rs7016778	TT	73.7 (140)	0.44 (0.01)				
	TA	25.3 (48)	0.47 (0.02)	-0.034	0.022	-0.111	0.131
	AA	1.1 (2)	0.57 (0.14)				
rs7824175	**GG**	**7834 (149)**	**0.43 (0.01)**				
	GC	**19.5 (37)**	**0.5 (0.02)**	**-0.777**	**0.021**	**-0.256**	**0.0004**
	CC	**2.1 (4)**	**0.62 (0.04)**				
rs16918875	CC	83.7 (159)	0.44 (0.01)				
	CT	15.8 (30)	0.47 (0.02)	-0.039	0.027	-0.105	0.152
	TT	0.5 (1)	-				
rs963549	**GG**	**64.7 (123)**	**0.42 (0.01)**				
	GA	**31.6 (60)**	**0.48 (0.02)**	**-0.06**	**0.018**	**-0.234**	**0.001**
	AA	**3.7 (7)**	**0.57 (0.05)**				

* mean (SEM) of log10Component1 scores

R^2 rs1365098 = 0.028, rs7824175 = 0.065, rs963549 = 0.055

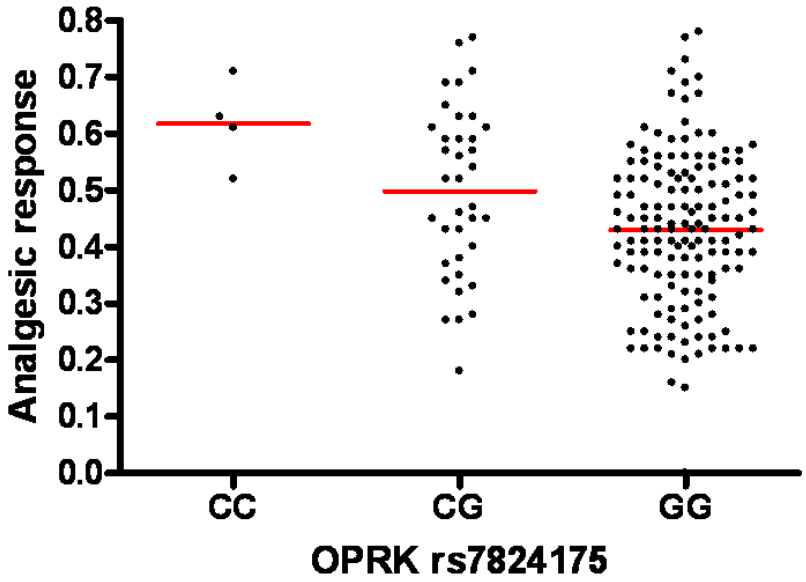

Figure 4.4 OPRK rs7824175 and analgesic response

The analgesic response scores on the y-axis are log10Component1 scores (ref. chapter 3) Higher scores represent worse analgesic response. There is a significant association between variation in analgesic response and *OPRK rs7824175* (genotype CC versus CG versus GG), p=0.0004, R^2 = 0.065. Red line demonstrates mean value. (Univariate linear regression uncorrected for other clinical and genetic factors)

Analgesic response: Gene-gene and gene-environment interactions

Using $\log_{10}$Component1as the dependent variable, multivariate logistic regression was used to model factors predictive of analgesic response to morphine (Tables 4.10 and 4.11).

Four factors were retained as independent predictors of analgesic response: *OPRK rs7824175*, use of a beta-blocker, taking an anti-emetic and the total daily morphine dose. Being on a beta-blocker was found to be associated with a worse outcome in terms of analgesic response (as evidenced by higher PCA scores), whereas being on an anti-emetic was associated with a better outcome (lower PCA scores). Daily morphine dose only contributes slightly to the model. On univariate analysis total morphine dose accounted for less than 2% of variability in analgesic response. Daily morphine dose and analgesic score are not correlated to any significant degree (r = 0.08). The multivariate model comprising *OPRK rs7824175*, being on a beta-blocker or an antiemetic and the total daily morphine dose accounted for 15.9% of variability in analgesic response to morphine.

The same four factors were retained in both the dominant (allele carriage) and additive (genotype) regression models.

Table 4.10 Clinical and genetic factors predictive of analgesic response to morphine (additive model)

Multivariate stepwise linear regression **Log10Component1 (pain scores) as dependent variable**				
	B	**Std. Error**	**Beta**	**P**
(Constant)	0.587 (0.509, 0.664)	0.039		<0.001
OPRK1 rs7824175	-0.080 (-0.121, -0.039)	0.021	-0.265	0.00015
B Blocker	0.118 (0.045, 0.192)	0.037	0.218	0.002
Antiemetic	-0.055 (-0.094, -0.016)	0.020	-0.192	0.006
Daily morphine dose (mg)	<0.001 (<0.001, <0.001)	<0.001	0.140	0.042
ANOVA F = 8.6, p=2.4 x 10^{-6} ,R^2 = 15.9 All genotype and clinical variables p<0.1 included in analysis				

Table 4.11 Clinical and genetic factors predictive of response to morphine, dominant model (allele carriage)

Multivariate stepwise linear regression **Log10Component1 (pain scores) as dependent variable**				
	B	**Std. Error**	**Beta**	**P**
(Constant)	0.426 (0.392, 0.460)	0.017		<0.001
OPRK1 rs7824175 CC / CG	0.083 (0.036, 0.131)	0.024	0.239	0.001
B Blocker	0.117 (0.043, 0.191)	0.038	0.215	0.002
Antiemetic	-0.054 (-0.093, -0.015)	0.020	-0.187	0.008
Daily morphine dose (mg)	<0.001 (<0.001,<0.001)	<0.001	0.140	0.045
ANOVA F = 7.7, p=9.8 x 10^{-6} ,R^2 = 14.6 All allele carriage and clinical variables p<0.1 included in analysis				

4.3.3 Central side-effects

Univariate analysis

The strongest association with central side-effects (log10Component2 scores) was with *OPRK rs1054151*. Carriers of the C allele (genotype CC or CT) had lower central side-effect scores than non-carriers (p=0.009, R^2 = 0.037). This finding remained significant on testing the individual genotypes (p=0.014, R^2 = 0.033) but the association is stronger for allele carriage (Tables 4.14 and 4.15).

There was also an association with *OPRM rs2075572*. Here carriers of the C allele (genotype CC or CG) had higher central side-effect scores (p=0.034, R^2 = 0.023). Again, this SNP demonstrated a gene-dose effect (Tables 4.12 and 4.13).

There was no association between SNPs in OPRD and central side-effects on morphine. There was no significant association with haplotypes in *OPRK, OPRM* or *OPRD*.

Table 4.12 Effects of OPRM on central side-effects of morphine (dominant model)

SNP name	Allele	Carrying allele		Not carrying allele		Univariate linear regression Log10Component2 (central side-effect scores) as dependent variable			
		% (N)	Mean* (SEM)	% (N)	Mean* (SEM)	B	Std Error B	β	P value
rs6912029	G	100 (194)	0.39 (0.01)	0	-	-	-	-	-
	T	8.2 (16)	0.39 (0.03)	91.8 (178)	0.39 (0.01)	-0.005	0.04	-0.01	0.894
rs1799971	A	95.9 (186)	0.39 (0.01)	4.1 (8)	0.46 (0.07)	-0.06	0.056	-0.078	0.28
	G	25.3 (49)	0.42 (0.02)	74.7 (145)	0.39 (0.01)	0.026	0.026	0.073	0.313
rs589046	C	93.7 (177)	0.4 (0.01)	6.3 (12)	0.38 (0.05)	0.014	0.045	0.023	0.754
	T	49.7 (94)	0.38 (0.020	50.3 (95)	0.41 (0.02)	-0.027	0.022	-0.09	0.224
rs563649	C	97.9 (185)	0.4 (0.01)	2.1 (4)	0.32 (0.08)	0.081	0.077	0.078	0.293
	T	19.6 (37)	0.36 (0.02)	80.4 (152)	0.4 (0.01)	-0.037	0.028	-0.095	0.197
rs9479757	A	22.2 (43)	0.38 (0.02)	77.8 (151)	0.4 (0.01)	-0.023	0.027	-0.062	0.391
	G	99 (192)	0.4 (0.01)	1.0 (2)	0.24 (0.09)	0.16	0.109	0.106	0.143
rs2075572	**C**	**81.9 (158)**	**0.4 (0.01)**	**18.1 (35)**	**0.35 (0.03)**	**0.061**	**0.029**	**0.154**	**0.034**
	G	64.8 (125)	0.38 (0.01)	35.2 (68)	0.42 (0.02)	-0.033	0.023	-0.102	0.162
rs533586	T	89.4 (169)	0.4 (0.01)	10.6 (20)	0.34 (0.04)	0.057	0.036	0.116	0.114
	C	51.3 (97)	0.39 (0.02)	48.7 (92)	0.4 (0.02)	-0.018	0.022	-0.058	0.428

* mean (SEM) of log10Component2 scores
R^2 rs2075572C = 0.023

Table 4.13 Effects of OPRM on central side-effects of morphine (additive model)

				Univariate linear regression Log10Component2 (central side-effect scores) as dependent variable			
SNP name	**Genotype**	**% (N)**	**Mean* (SEM)**	**B**	**Std. Error B**	**β**	**P value**
rs6912029	GG	91.8 (178)	0.39 (0.01)				
	GT	8.2 (16)	0.39 (0.04)	-0.005	0.04	-0.01	0.894
	TT	0	-				
rs1799971	AA	74.7 (145)	0.39 (0.01)				
	AG	21.1 (41)	0.41 (0.02)	0.025	0.021	0.088	0.225
	GG	4.1 (8)	0.46 (0.07)				
rs589046	CC	50.3 (95)	0.41 (0.02)				
	CT	43.4 (82)	0.38 (0.02)	-0.02	0.018	-0.082	0.264
	TT	6.3 (12)	0.38 (0.05)				
rs563649	CC	80.4 (152)	0.4 (0.01)				
	CT	17.5 (33)	0.37 (0.03)	-0.035	0.024	-0.106	0.15
	TT	2.1 (4)	0.32 (0.08)				
rs9479757	AA	1.0 (2)	0.24 (0.09)				
	AG	21.1 (41)	0.38 (0.03)	-0.028	0.025	-0.082	0.258
	GG	77.8 (151)	0.4 (0.01)				
rs2075572	**CC**	**35.2 (68)**	**0.42 (0.02)**				
	CG	**46.6 (90)**	**0.4 (0.02)**	**-0.033**	**0.016**	**-0.152**	**0.036**
	GG	**35.2 (68)**	**0.35 (0.03)**				
rs533586	TT	48.7 (92)	0.4 (0.02)				
	CT	40.7 (77)	0.4 (0.02)	0.022	0.017	0.097	0.186
	CC	10.6 (20)	0.34 (0.04)				

* mean (SEM) of log10Component2 scores
R^2 rs2075572 = 0.023

Table 4.14 Effects of OPRK on central side-effects of morphine (dominant model)

SNP name	Allele	Carrying allele		Not carrying allele		Univariate linear regression Log10Component2 (central side-effect scores) as dependent variable			
		% (N)	Mean* (SEM)	% (N)	Mean* (SEM)	B	Std Error B	β	P value
r10504151	T	97.9 (186)	0.4 (0.01)	2.1 (4)	0.34 (0.06)	0.052	0.077	0.049	0.505
	C	**20 (38)**	**0.33 (0.03)**	**80 (152)**	**0.41 (0.01)**	**-0.073**	**0.028**	**-0.19**	**0.009**
rs 7836120	A	96.3 (183)	0.39 (0.01)	3.7 (7)	0.42 (0.05)	-0.025	0.059	-0.032	0.666
	G	30 (57)	0.38 (0.02)	70 (133)	0.4 (0.01)	-0.023	0.024	-0.071	0.338
rs 6473799	T	94.7 (180)	0.4 (0.01)	5.3 (10)	0.34 (0.05)	0.055	0.05	0.082	0.264
	C	40.5 (77)	0.41 (0.02)	59.5 (113)	0.38 (0.14)	0.034	0.023	0.11	0.135
rs 1365098	G	89.5 (170)	0.4 (0.01)	10.5 (20)	0.35 (0.03)	0.053	0.036	0.107	0.145
	T	54.2 (103)	0.4 (0.02)	45.8 (87)	0.39 (0.02)	0.014	0.022	0.004	0.547
rs 7016778	T	98.9 (188)	0.39 (0.01)	1.1 (2)	0.36 (0.06)	0.031	0.109	0.021	0.777
	A	26.3 (50)	0.4 (0.02)	73.7 (140)	0.39 (0.01)	0.002	0.025	0.006	0.931
rs 7824175	G	97.9 (186)	0.39 (0.01)	2.1 (4)	0.42 (0.08)	-0.022	0.007	-0.021	0.771
	C	21.6 (41)	0.4 (0.02)	78.4 (149)	0.39 (0.01)	0.001	0.027	0.003	0.969
rs16918875	C	99.5 (189)	0.39 (0.01)	0.5 (1)	-	0.086	0.153	0.041	0.575
	T	16.3 (31)	0.35 (0.03)	83.7 (159)	0.4 (0.01)	-0.055	0.03	-0.132	0.072
rs 963549	G	96.3 (183)	0.4 (0.01)	3.7 (7)	0.33 (0.05)	0.064	0.059	0.079	0.28
	A	35.3 (67)	0.38 (0.02)	64.7 (123)	0.4 (0.01)	-0.03	0.023	-0.093	0.206

* mean (SEM) of log10Component2 scores
R^2 rs10504151 = C 0.036

Table 4.15 Effects of OPRK on central side-effects of morphine (additive model)

				Univariate linear regression Log10Component2 (central side-effect scores) as dependent variable			
SNP name	**Genotype**	**% (N)**	**Mean* (SEM)**	**B**	**Std. Error B**	**β**	**P value**
rs10504151	**TT**	**80 (152)**	**0.41 (0.01)**				
	TC	**17.9 (34)**	**0.33 (0.03)**	**0.059**	**0.024**	**0.179**	**0.014**
	CC	**2.1 (4)**	**0.34 (0.06)**				
rs7836120	AA	70 (133)	0.4 (0.01)				
	AG	26.3 (50)	0.37 (0.02)	0.013	0.02	0.048	0.513
	GG	3.7 (7)	0.42 (0.05)				
rs6473799	TT	59.5 (113)	0.38 (0.14)				
	TC	35.3 (67)	0.42 (0.02)	-0.015	0.019	-0.06	0.418
	CC	5.3 (10)	0.34 (0.05)				
rs1365098	GG	45.8 (87)	0.39 (0.02)				
	GT	43.7 (83)	0.41 (0.02)	0.004	0.017	0.017	0.822
	TT	10.5 (20)	0.35 (0.03)				
rs7016778	TT	73.7 (140)	0.39 (0.01)				
	TA	25.3 (48)	0.4 (0.02)	<0.001	0.024	-0.001	0.984
	AA	1.1 (2)	0.36 (0.06)				
rs7824175	GG	7834 (149)	0.39 (0.01)				
	GC	19.5 (37)	0.39 (0.02)	-0.003	0.024	-0.009	0.902
	CC	2.1 (4)	0.42 (0.08)				
rs16918875	CC	83.7 (159)	0.4 (0.01)				
	CT	15.8 (30)	0.35 (0.03)	0.052	0.029	0.133	0.069
	TT	0.5 (1)	-				
rs963549	GG	64.7 (123)	0.4 (0.01)				
	GA	31.6 (60)	0.38 (0.02)	0.029	0.02	0.106	0.147
	AA	3.7 (7)	0.3 (0.05)				

* mean (SEM) of log10Component2 scores
R^2 rs10504151 C = 0.032

Central side-effects: Gene-gene and gene-environment interactions

Using $\log_{10}$Component2as the dependent variable (representing central side-effect scores), multivariate logistic regression was used to model clinical and genetic factors predictive of central side-effects on morphine. A better outcome in terms of central side-effect was represented by lower PCA component 2 scores while a worse response was represented by higher scores.

Two SNPS (*OPRK rs10504151* and *OPRM rs2075572*) and a number of clinical factors (tumour diagnosis and concomitant medications) were retained in the multivariate model as independent predictors of developing central side-effects on morphine. (Tables 4.16 and 4.17) The same SNPs remained significant in both the additive and dominant model but the clinical variables varied slightly. In the dominant model (allele carriage), being on a tricyclic antidepressant was independently predictive of lower central side-effect scores. In the additive model however, being on a tricyclic antidepressant did not remain independently significant. Instead, having a sarcoma diagnosis or being on steroids was predictive of lower scores. These models of genetic and clinical factors account for 9-10% of variability in central side-effects on morphine.

Table 4.16 Clinical and genetic factors predictive of central side-effects on morphine (dominant model)

Multivariate stepwise linear regression Log10Component2 (central side-effect scores) as dependent variable				
	B (95% CI)	**Std. Error**	**Beta**	**P**
(Constant)	0.364 (0.314, 0.413)	0.025		<0.001
OPRK1 rs10504151 CC / CT	-0.074 (-0.128, -0.020)	0.027	-0.194	0.007
OPRM rs2075572 CC / CG	0.067 (0.013, 0.122)	0.028	0.174	0.016
Tricyclic antidepressant	-0.072 (-0.135, -0.010)	0.032	-0.163	0.024
ANOVA F = 5.7, p=0.001 ,R^2 = 8.7 All allele carriage and clinical variables p<0.1 included in analysis				

Table 4.17 Clinical and genetic factors predictive of central side-effects on morphine (additive model)

Multivariate stepwise linear regression Log10Component2 (central side-effect scores) as dependent variable				
	B (95% CI)	**Std. Error**	**Beta**	**P**
(Constant)	0.325 (0.238, 0.412)	0.044		<0.001
OPRK1 rs10504151	0.069 (0.023, 0.115)	0.023	0.211	0.004
OPRM rs2075572	-0.035 (-0.065, -0.006)	0.015	-0.167	0.020
Sarcoma	-0.066 (-0.128, -0.003)	0.031	-0.148	0.039
Steroid	-0.045 (-0.089, -0.001)	0.022	-0.144	0.045
ANOVA F = 4.9, p=0.001 ,R^2 = 9.9 All allele carriage and clinical variables p<0.1 included in analysis				

4.3.4 Variation in daily morphine dose to achieve adequate analgesia

Univariate analysis

Variation in daily morphine dose was associated with SNPs in *OPRM, OPRD* and *OPRK*. The strongest association was with *OPRM rs1799971*. Carriers of the G allele (genotype GG or GA) required higher morphine doses to achieve adequate pain control (p=0.027, R^2 = 0.036). Although the number of subjects homozygous for the minor allele was low (N=4), this association remained significant when the additive model was used (p=0.017, R^2 = 0.042 (figure 4.5). *OPRM rs589046* and *rs2075572* were also associated with variation in daily morphine dose (p=0.02 and 0.046 respectively), (Tables 4.18 and 4.19). These SNPs are not located on the same haploblock.

There were also associations with *OPRK rs7016778* (p = 0.045)*, OPRK rs7836120* (p = 0.031)*, OPRD rs419335* (p = 0.019) and *OPRD rs2236857* (p = 0.038), (Tables 4.20 and 4.21). None of these exhibited a gene-dose effect. There was a statistically significant association between *OPRK* haploblock 3 haplotype 1 (*rs10504151 T, rs7836120* A, p=0.031) but this is no stronger than the association with the individual SNPs. The same is true for *OPRD* haploblock 2 hapolotype 1 (*rs419335 A, rs2236857 T*, p=0.019).

Table 4.18 Effects of OPRM on daily morphine dose (dominant model)

SNP name	Allele	Carrying allele		Not carrying allele		Univariate linear regression log10 Daily morphine dose (mg) as dependent variable			
		% (N)	Mean* (SEM)	% (N)	Mean* (SEM)	B	Std Error B	β	P value
rs6912029	G	100 (135)	2.0 (0.03)	0	-	-	-	-	-
	T	8.9 (12)	2.08 (0.11)	91.1 (123)	2.0 (0.03)	0.087	0.105	0.071	0.410
rs1799971	A	97 (131)	1.99 (0.03)	3 (4)	2.26 (0.14)	-0.268	0.176	-0.131	0.130
	G	**23.7 (32)**	**2.12 (0.06)**	**76.3 (103)**	**1.95 (0.03)**	**0.156**	**0.069**	**0.191**	**0.027**
rs589046	**C**	**93.3 (125)**	**2.01 (0.03)**	**6.7 (9)**	**1.7 (0.09)**	**0.277**	**0.118**	**0.2**	**0.02**
	T	5.07 (68)	2.0 (0.04)	49.3 (66)	2.0 (0.04)	0.001	0.06	0.001	0.987
rs563649	C	97 (130)	2.0 (0.28)	3 (4)	2.0 (0.03)	0.011	0.204	0.005	0.957
	T	19.4 (26)	1.9 (0.08)	80.6 (108)	2.0 (0.03)	-0.089	0.075	-0.104	0.234
rs9479757	A	23.7 (32)	1.99 (0.05)	76.3 (103)	2.0 (0.04)	0	0.071	0	0.998
	G	98.5 (133)	2.0 (0.03)	1.5 (2)	1.55 (0.23)	0.455	0.245	0.159	0.066
rs2075572	C	81.3 (109)	2.02 (0.03)	18.7 (25)	1.89 (0.06)	0.136	0.077	0.152	0.08
	G	**64.9 (87)**	**1.95 (0.03)**	**35.1 (47)**	**2.1 (0.06)**	**-0.126**	**0.062**	**-0.172**	**0.046**
rs533586	T	88.8 (119)	2.0 (0.03)	11.2 (15)	1.9 (0.07)	0.09	0.095	0.082	0.344
	C	5.07 (68)	1.97 (0.04)	49.3 (66)	2.02 (0.05)	-0.057	0.060	-0.082	0.347

* mean (SEM) of Log10 Daily morphine dose(mg)
R^2 rs1799971G = 0.036, rs589046C = 0.04, rs2075572G = 0.03

Table 4.19 Effects of OPRM on daily morphine dose (additive model)

				Univariate linear regression log10 Daily morphine dose (mg) as dependent variable			
SNP name	**Genotype**	**% (N)**	**Mean* (SEM)**	**B**	**Std. Error B**	**β**	**P value**
rs6912029	GG	91.1 (123)	1.99 (0.03)				
	GT	8.9 (12)	2.06 (0.12)	0.087	0.105	0.071	0.41
	TT	0	-				
rs1799971	**AA**	**76.3 (103)**	**1.95 (0.03)**				
	AG	**20.7 (28)**	**2.1 (0.07)**	**0.141**	**0.058**	**0.205**	**0.017**
	GG	**3 (4)**	**2.3 (0.14)**				
rs589046	CC	49.3 (66)	2.0 (0.04)				
	CT	44 (59)	2.03 (0.05)	-0.045	0.049	-0.08	0.357
	TT	6.7 (9)	1.74 (0.09)				
rs563649	CC	80.6 (108)	2.01 (0.03)				
	CT	16.4 (22)	1.89 (0.08)	-0.067	0.064	-0.09	0.299
	TT	3 (4)	1.99 (0.29)				
rs9479757	AA	1.5 (2)	1.55 (0.23)				
	AG	22.2 (30)	2.02 (0.05)	0.03	0.064	0.041	0.639
	GG	76.3 (103)	2.0 (0.04)				
rs2075572	**CC**	**35.1 (47)**	**2.08 (0.06)**				
	CG	**46.3 (62)**	**1.97 (0.04)**	**-0.096**	**0.042**	**-0.198**	**0.022**
	GG	**18.7 (25)**	**1.89 (0.06)**				
rs533586	TT	49.3 (66)	2.02 (0.05)				
	CT	39.6 (53)	1.98 (0.04)	0.05	0.044	0.099	0.257
	CC	11.2 (15)	1.92 (0.07)				

* mean (SEM) of Log10 Daily morphine dose(mg)
R^2 rs1799971 = 0.042, rs2075572 = 0.039

Table 4.20 Effects of OPRD on daily morphine dose (dominant model)

SNP name	Allele	Carrying allele		Not carrying allele		Univariate linear regression log10 Daily morphine dose (mg) as dependent variable			
		% (N)	Mean* (SEM)	% (N)	Mean* (SEM)	B	Std Error B	β	P value
rs 1042114	T	97.7 (130)	1.99 (0.03)	2.3 (3)	2.04 (0.16)	-0.48	0.204	-0.20	0.815
	G	28.6 (38)	2.02 (0.05)	71.4 (95)	1.98 (0.04)	0.39	0.067	0.05	0.566
rs 533123	A	94.7 (126)	1.99 (0.03)	5.3 (7)	2.1 (0.08)	-0.104	0.135	-0.067	0.442
	G	38.3 (51)	1.97 (0.05)	61.7 (82)	2.0 (0.04)	-0.035	0.062	-0.05	0.571
rs 419335	**A**	**90.2 (120)**	**1.97 (0.03)**	**9.8 (13)**	**2.21 (0.1)**	**-0.238**	**0.100**	**-0.204**	**0.019**
	G	54.1 (72)	2.01 (0.05)	45.9 (61)	1.97 (0.04)	0.041	0.061	0.058	0.504
rs 2236857	C	42.9 (57)	2.01 (0.05)	57.1 (76)	1.98 (0.04)	0.033	0.061	0.048	0.587
	T	**94 (125)**	**1.98 (0.03)**	**6 (8)**	**2.24 (0.15)**	**-0.263**	**0.125**	**-0.181**	**0.038**
rs 2234918	C	66.9 (89)	2.02 (0.04)	33.1 (44)	1.94 (0.05)	0.08	0.064	0.109	0.212
	T	81.2 (108)	1.99 (0.03)	18.8 (25)	2.0 (0.07)	-0.007	0.078	-0.008	0.924

* mean (SEM) of Log10 Daily morphine dose(mg)
R^2 rs419335A = 0.042, rs2236857T = 0.033

Table 4.21 Effects of OPRK on daily morphine dose (dominant model)

SNP name	Allele	Carrying allele		Not carrying allele		Univariate linear regression log10 Daily morphine dose (mg) as dependent variable			
		% (N)	Mean* (SEM)	% (N)	Mean* (SEM)	B	Std Error B	β	P value
r10504151	T	98.5 (132)	2.0 (0.03)	1.5 (2)	1.5 (0.24)	0.463	0.245	0.162	0.061
	C	21.6 (29)	1.98 (0.08)	78.4 (105)	2.0 (0.03)	-0.019	0.073	-0.023	0.792
rs 7836120	**A**	**96.3 (129)**	**2.0 (0.03)**	**3.7 (5)**	**1.5 (0.13)**	**0.341**	**0.156**	**0.187**	**0.031**
	G	30.6 (41)	1.97 (0.06)	69.4 (93)	2.0 (0.03)	-0.021	0.065	-0.028	0.751
rs 6473799	T	94.8 (127)	2.0 (0.03)	5.2 (7)	2.0 (0.12)	-0.003	0.136	-0.002	0.985
	C	38.1 (51)	1.92 (0.05)	61.9 (83)	2.04 (0.04)	-0.120	0.061	-0.169	0.052
rs 1365098	G	90.3 (121)	2.0 (0.03)	9.7 (13)	1.89 (0.11)	0.121	0.101	0.103	0.236
	T	52.2 (70)	1.95 (0.05)	47.8 (64)	2.0 (0.04)	-0.77	0.060	-0.111	0.202
rs 7016778	**T**	**99.3 (133)**	**2.0 (0.03)**	**0.7 (1)**	**-**	**0.700**	**0.345**	**0.174**	**0.045**
	A	25.4 (34)	1.96 (0.07)	74.6 (100)	2.0 (0.03)	-0.036	0.069	-0.045	0.606
rs 7824175	G	99.3 (133)	1.99 (0.03)	0.7 (1)	-	-0.485	0.348	-0.121	0.165
	C	18.7 (25)	1.97 (0.07)	81.3 (109)	2.0 (0.03)	-0.028	0.077	-0.032	0.715
rs16918875	C	100 (134)	1.99 (0.03)	0	-	-	-	-	-
	T	16.4 (22)	1.97 (0.1)	83.6 (112)	2.0 (0.03)	0.03	0.081	-0.032	0.712
rs 963549	G	97.8 (131)	1.99 (0.03)	2.2 (3)	2.1 (0.25)	-0.059	0.204	-0.025	0.774
	A	32.8 (44)	1.98 (0.06)	67.2 (90)	2.0 (0.03)	-0.022	0.064	-0.03	0.733

* mean (SEM) of Log10 Daily morphine dose(mg)
R^2 rs7016778T = 0.03

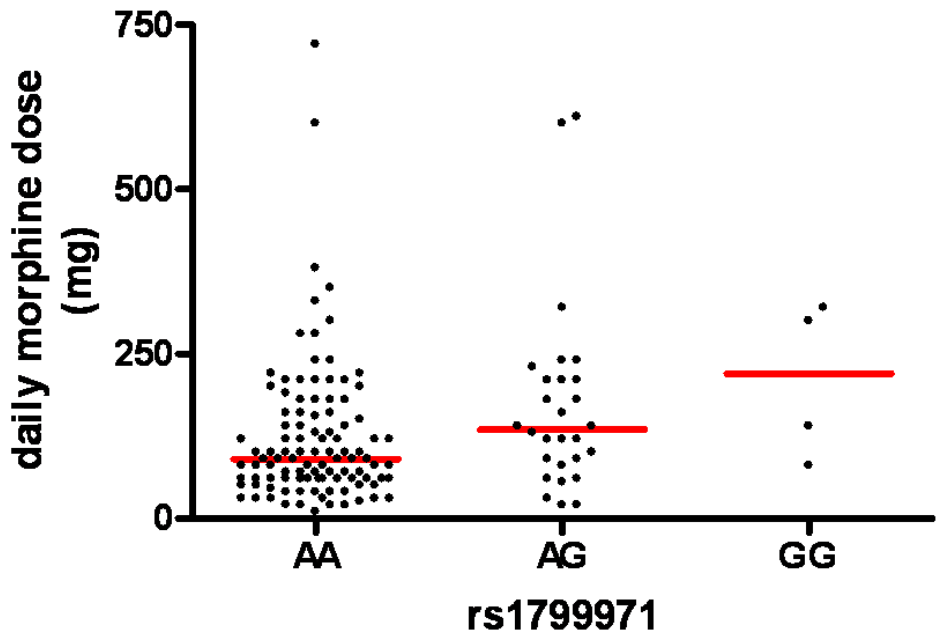

Figure 4.5 OPRM rs1799971 and morphine dose requirements

There is a significant association between analgesic response and *OPRM rs1799971*, p=0.017, R^2 = 0.042. Red line represents median value. (Univariate linear regression uncorrected for other clinical and genetic factors)

Variation in daily morphine dose to achieve adequate analgesia: Gene-gene and gene-environment interactions

Using $\log_{10}$ (daily morphine dose (mg)) as the dependent variable, multivariate logistic regression was used to model factors predictive of variation in morphine dose required to achieve adequate analgesia (Tables 4.22 and 4.23).

Similar to central side-effects, when multivariate regression analysis was carried out using the dominant and additive models, the resulting models were slightly different. The additive model identified *OPRM rs1779971*, being on an anticonvulsant or a bronchodilator as being independent predictors of variation in morphine dose requirements. Together these factors accounted for 22% of variability in morphine dose. When the dominant model was used, *OPRM rs1779971* was not retained. Instead *OPRM rs207572*, *OPRM rs589046* and *OPRK rs7836120* were included as independent predictors. Being on an anticonvulsant and taking a bronchodilator remained independent predictors of variability in the dose of morphine required to achieve adequate pain control in both models.

Table 4.22 Clinical and genetic factors associated with variability in daily morphine dose requirements (dominant model)

Multivariate stepwise linear regression **Dominant model (allele carriage)**				
	B (95% CI)	**Std. Error**	**Beta**	**P value**
(Constant)	1.437 (1.052, 1.821)	0.194		<0.001
Anticonvulsant	0.317 (0.174, 0.459)	0.072	0.339	2.26×10^{-5}
Brochodilator	-0.198 (-0.376, -0.020)	0.090	-0.169	0.029
OPRK rs7836120 AA/AG	0.406 (0.098, 0.714)	0.156	0.200	0.010
OPRM rs2075572 GG / GC	-0.119 (-0.230, -0.008)	0.056	-0.164	0.035
OPRM rs589046 CC/CT	0.222 (0.010, 0.434)	0.107	0.161	0.041
ANOVA F = 9.5, p = 1×10^{-7} , $R^2 = 0.27$ All clinical and genetic factors p<0.1 on univariate analysis included in analysis				

Table 4.23 Clinical and genetic factors associated with variability in daily morphine dose requirements (additive model)

Multivariate stepwise linear regression **Additive model (genotypes)**				
	B (95% CI)	**Std. Error**	**Beta**	**P value**
(Constant)	1.918 (1.85, 1.985)	0.034		<0.001
Anticonvulsant	0.344 (0.202, 0.486)	0.072	0.374	4.57×10^{-6}
Brochodilator	-0.191 (-0.372, -0.010)	0.091	-0.163	0.039
OPRM rs1799971	0.138 (0.033, 0.244)	0.053	0.202	0.010
ANOVA F = 12.1, p = 4.7×10^{-7} , $R^2 = 0.22$ All clinical and genetic factors p<0.1 on univariate analysis included in analysis				

4.4 Discussion

4.4.1 Main findings

The results of this study suggest that:

1. SNPs in the genes coding for the mu and kappa (and possibly delta) opioid receptors are associated with variability in morphine response.

2. Analgesia, central side-effects and variability in morphine dose are influenced by different genes / SNPs. This supports the hypothesis presented in Chapter 3 i.e. that these are distinct dimensions of morphine response.

3. Genetic variation in the genes coding for the opioid receptors account for some, but certainly not all of the variability in clinical response to morphine.

Previous studies of the influence of genetic variation in opioid receptors on morphine response have tended to focus exclusively on *OPRM*. Although some non-cancer studies have examined the association between SNPs in all three opioid receptor genes and alcohol dependence [362;363], this is the first study to include a comprehensive analysis of SNPs in *OPRM, OPRK* and *OPRD* in cancer patients taking morphine for pain.

4.4.2 Analgesic response to morphine

The dominant factor predicting analgesic response to morphine was a SNP in *OPRK* (*rs7824175*). This SNP has not been studied in terms of opioid response or pain sensitivity previously. Concomitant β-blocker therapy or the use of anti-emetics was also found to be independently predictive of analgesic response (as discussed in Chapter 3), with beta-blocker use associated with higher pain scores and anti-emetic use associated with lower pain scores. It is biologically plausible that drugs which act on adrenergic receptors may alter morphine response in association with genetic variation in *OPRK*.

OPRK rs963549 was also significantly associated with analgesic response on univariate analysis. It was not retained in multivariate analysis possibly because it is in LD with *OPRK rs7824175* (D'0.92, R^2 0.45). Previous studies have suggested that *OPRK rs963549* may be of functional importance [362;364]. Its location in the 3-UTR suggests that may be involved in regulating translational efficacy. One study has suggested differential OPRK expression associated with this region [365].

4.4.3 Central side-effects on morphine

Central side-effects were predicted by a model comprised of two polymorphisms (*OPRM rs2075572* and *OPRK rs10504151*), diagnosis and concomitant medications. *OPRK rs10504151* has not been studied in terms of opioid response or pain sensitivity previously. Haplotypes containing *OPRM rs2075572* have been found to be associated with variability in experimental pain sensitivity [148] but this SNP has not previously been associated with response to opioids.

Only 10% of the variability in the central side-effects on morphine is explained by the regression model. This suggests that central side-effects as a clinical phenotype may be quite complex, perhaps confounded by phenotypic variability (i.e. variability in onset / severity of symptoms) which is not captured by the study data or simply difficulty in subjectively scoring these symptoms. Complexity may also arise from the fact that a number of these symptoms may also be contributed to by other non-opioid factors. Therefore although nausea, confusion, drowsiness and bad dreams are central side-effects of morphine, the other clinical factors confounding this phenotype may be quite varied.

4.4.4 Morphine dose required to achieve adequate pain control

Modeling of the genetic and environmental factors associated with variability in daily morphine dose requirements is not conclusive. *OPRM rs1799971* which has been associated with this phenotype in other cancer and non-cancer pain studies,[82;83;92] remained an independent predictor when the additive model was used but not the dominant model. This finding may be reflective of the relatively weak associations between dose and *OPRM rs1799971*, both in our study and in other studies in this area. The findings may also suggest that since the SNPs and indeed genes are different

in both models, that the only true associations with morphine dose are the clinical variables which remain consistent i.e. being on a bronchodilator or an anticonvulsant medication.

4.4.5 OPRM, OPRK and OPRD

Although the primary site of action of morphine is the mu-opioid receptor, in this study no SNP in *OPRM* remained independently significant for analgesic response in the multivariate analysis. Instead a SNP in *OPRK* appeared to be more influential in determining analgesic response to morphine. In humans there is much less evidence regarding the influence of polymorphisms in *OPRD* and *OPRK* than *OPRM* in pain sensitivity or opioid response. Quantitative Trait Loci (QTL) analysis influencing morphine antinociception in four populations of mice revealed four significant QTLs. One of these mapped to the same region as *OPRM* and another mapped to the same region as *OPRK* [366]. In a study of 500 normal participants, a polymorphism in *OPRD rs2234918* was associated with a gender specific difference in thermal pain sensitivity [108]. However *OPRD* haplotype analysis in a similar study by the same researchers did not show any association [367]. *OPRD* did not appear to influence analgesia or central side-effects in our study.

In our study the *OPRK* and *OPRM* SNPs which appear to be predictive of analgesic response and central side-effects on morphine are found in intronic regions of the genes. Although the exact role of these intronic SNPs in opioid response is not yet known, it is possible that they may alter protein function through linkage disequilibrium, whereby these polymorphisms may represent markers for true susceptibility polymorphisms.[80] Alternatively these intronic SNPs may be involved in the production of functionally variable opioid receptor subtypes through the mechanism of alternative pre-mRNA splicing.

A recent study has analysed the impact of SNPs on the mRNA and translational activity of *OPRM* isoforms. [148] An intronic SNP, *OPRM* rs563649 was found to be located within an internal ribosomal binding site in an alternative exon of *OPRM*. The authors suggest that allelic variation at this site results in altered expression of a

corresponding mu-opioid receptor isoform, MOR-1K and differential pain sensitivity but not analgesic response to morphine [148]. I did include *OPRM* rs563649 in this analysis but it was not found to be associated with any of the clinical phenotypes tested.

4.5 Conclusion

The data presented in this study suggest that SNPs across the opioid receptor genes do appear to play a role in determining variability in response to morphine, be it analgesic response, the development of central side-effects or variation in the daily morphine dose required to achieve adequate analgesia. Further studies with larger numbers are required to facilitate in-depth analysis of these factors and also to validate these findings. However, even the multivariate regression models only explain at a proportion of this variability. 16% of variation in analgesic response was explained by our model in this study and 10% of variation in central side-effects. It is highly likely that there are other genes and biological pathways which play a role in this phenomenon of inter-individual variation in response to morphine.

These data also support the hypothesis presented in Chapter 3, that analgesia and side-effects on morphine and morphine dose requirements to achieve adequate pain control appear to be distinct dimensions of response to morphine. Analgesic response and side-effects on morphine scores load onto separate components in PCA, and all three outcome variables are associated with different genetic and clinical predictors.

In Chapter 5 the analysis of genes other than those coding for the opioid receptors is presented. These data have been used to build on the regression models presented here to build up a more comprehensive model of clinical and genetic factors predictive of response to morphine.

5 Chapter 5: Results from candidate gene association study: Other genes

5.1 Introduction and Summary of the rationale behind this study

There are significant animal data to support the hypothesis that pain and to morphine may be under genetic control. To date research in this area has focused on a small number of genes with mixed results. The analysis of polymorphisms in the genes coding for the opioid receptors is described in Chapter 4. Like other complex traits, it is likely that there are many genes involved in pain perception and analgesic response and that the clinical outcomes are polygenic, resulting from multiple gene-gene and also gene-environment interactions [172].

The hypothesis tested in this part of the study was that polymorphisms in genes coding for proteins and enzymes involved in biological pathways which interact with opioids and their receptors play a role in inter-individual variation in morphine response in cancer patients.

For this part of the study a number of candidate genes were chosen for investigation based on known biological pathways involved in pain and morphine response and also on previously published animal studies in this area.

The following genes were chosen for inclusion in this study: *TRPV1* (capsaicin receptor gene), *ADORA1* (A1-adenosine receptor gene), *ADRA2A* (α_{2A} adrenergic receptor gene) and *ALOX12* (arachidonate 12-lipoxygenase gene), *ARRB2* (β-arrestin2) and *SLC6A4* (serotonin reuptake transporter gene). For each of the genes presented in this thesis there is a biologically plausible association with pain perception and response to morphine. This is discussed in detail in the following sections.

5.1.1 Aim

The aim of this part of the study was to examine the influence of genetic variation in genes other than those coding for opioid receptors on inter-individual variation in morphine response.

- Initially the influence of single SNPs / genes was tested.

- Combining these data with date presented in chapters 3 and 4 pertaining to clinical factors and opioid receptors, gene-gene and gene-environment interactions were explored.

5.2 Subjects and methods

Each of the genes were tested on all clinical phenotypes presented in chapter 3 i.e. analgesic response to morphine, central side-effects on morphine, variation in the daily morphine dose required to achieve adequate analgesic response.

The genetic assays were run on DNA from the *Prospective Morphine Study*.

Details of the primers used are found in Appendix C. The assays for ADORA1, ADRA2A, TRPV1 and ALOX12 were designed by Dr Sophy Gretton. (REF) I tested and optimised each reaction and ran the assays on the study samples and carried out the analyses. Details of the SNPs tested, including location on the gene, call rate and minor allele frequencies are presented.

Each SNP was tested individually using univariate regression analysis. Both the dominant and additive models were used to assess whether the association was due primarily to allele carriage or whether there was a gene dose effect.

Where there was a biologically plausible link between two genes (including the opioid receptor genes), e.g. heterodimer formation, multivariate stepwise regression analysis was carried out to assess the effect of SNPs in both genes on variability in the clinical phenotype. Clinical factors known from univariate analysis to predict the clinical phenotype were also included in this analysis. Clinical and genetic factors significant at the level of 10% ($p<0.1$) on univariate analysis (as presented in chapter 3) were introduced simultaneously into the model using a stepwise approach.

No corrections for multiple testing are presented here. The data presented in the tables represents the raw unadjusted data as the data are considered exploratory until replication studies are performed. Tables containing significant results are presented in this chapter. Tables containing mainly non-significant results are located in Appendix D.

5.3 ADORA1

Adora1, the gene coding for the A1-adenosine receptor is located on chromosome 1 (1q32.1) and spans 76752bp. It is made up of 6 exons, containing 383 known SNPs [89] (http://snpper.chip.org, accessed 05/01/2010). This gene codes for a seven transmembrane G-protein coupled receptor. The second extracellular loop is important in ligand binding [368;369]. Two separate promoters, 600bp apart regulate A1-adenosine receptor expression [370].

Adenosine acts on a number of different adenosine receptors, A1, A_{2A}, A_{2B} and A_3. The activation of A1-adenosine receptors in particular is thought to play a role in modulation of pain transmission [371]. A1-adenosine receptors are found on the cell bodies of the dorsal root ganglion and on central terminals of afferent nociceptors [372]. They are highly expressed in the brain, especially the cortex, cerebellum and hippocampus [370]. Activation of these receptors inhibits adenylate cyclase in the spinal cord [373].

Adenosine agonists have antinociceptive properties. Intrathecal adenosine was studied in a small number of patients with neuropathic pain with good effect, although intraveneous adenosine was not effective [374]. A link between adenosine and opioid systems has been established. Animal studies have demonstrated that A1-adenosine agonists increase while caffeine (an adenosine receptor antagonist) decreases morphine analgesia, morphine tolerance and dependence [375;376]. There is some contradictory evidence however that caffeine (perhaps at varying doses and in varying species) may actually enhance morphine analgesia [377;378]. There is also debate about the influence of adenosine agonists on morphine tolerance and dependence. There may be differences in terms of acute and chronic drug exposure [372]. Theophylline, an adenosine receptor antagonist was shown to inhibit the analgesic but not the gastrointestinal effects of morphine administered intrathecally or intraventricularly [379].

Mu-opioid receptor knockout mice have reduced binding to A1-adenosine receptors, again providing evidence for a functional interaction between the opioid and adenosine receptor systems [380]. A1-adenosine receptor knockout mice have reduced analgesia from morphine administered intrathecally, but not systemically [381].

5.3.1 ADORA1 Results

Table 5.1 ADORA1 marker data

Name	Position	Location in gene	Allele major: minor	MAF	Published MAF	HWpval	Call rate	AA change
rs2364571	201327167	Promoter	C:G	0.305	0.32	0.7896	100	-
rs9660662	201335343	Intron 2	T:C	0.32	0.32	0.8376	100	-
rs11590405	201346887	Intron 2	T:C	0.318	0.31	0.7645	100	-
rs903361	201357897	Intron 2	A:G	0.322	0.32	0.9122	100	-
rs10920568	201364898	Exon 5	T:G	0.311	0.34	0.7748	100	Ala-Ala
rs10800901	201377927	Intron 5	G:A	0.332	0.28	0.7154	100	-
rs3766560	201385017	Intron 5	C:T	0.174	0.19	0.0626	99.2	-
rs10920576	201395802	Intron 5	C:T	0.148	0.19	1	100	-
Reference sequence NM_000674 Published frequencies HapMap CEU								

There are 5 validated SNPs occurring in coding exons, however only one of these was polymorphic in Caucasians, *rs10920568*. This SNP is located in exon 5 of the gene and was included in the analysis. Eight SNPS across *ADORA1* were investigated in this study. All SNPs were in Hardy-Weinberg equilibrium and the allele frequencies were similar to those published (table 5.1).

Three haploblocks were identified and haplotypes were constructed within each of these blocks (table 5.2 and figure 5.1).

In our study SNPs in *ADORA1* were found to be predictive of both variation in analgesic response to morphine and also in dose of morphine required to achieve adequate analgesia. There was no significant association between SNPs in ADORA1 and central side-effects on morphine.

In terms of variation in morphine dose, exonic SNP *rs10920568* and intronic *rs903361* had the strongest association, both p=0.004., R^2 =0.06. These SNPs are in close LD together, R'0.91 and are found on the same haploblock. Carriers of the *rs10920568* T allele (genotype TT or TG) required higher daily morphine doses than individuals with genotype GG, median 100mg versus 80mg respectively (figure 5.2). Intronic SNPs *rs2364571, rs9660662, rs11590405* which are also in LD with the two

previously mentioned SNPs were also associated with such variation. All of these intronic SNPs are located in the 5' untranslated region of the gene. A further intronic SNP, *rs10800901*, located in the coding region of the gene, and not in tight LD with any of the other SNPs tested was also associated with variation in morphine dose, p=0.014, R^2 0.045 . There was no gene dose effect for any of the associations (Table 5.3 and 5.4).

Haplotype carriage which was also associated with variability in daily morphine dose, however this was no stronger than the association with individual SNPs (figure 5.2).

ADORA1 *rs903361* was also associated with variability in overall analgesic response to morphine (p= 0.031) with patients homozygous for the minor allele (genotype GG) having lower pain scores. This association demonstrates a gene-dose effect. There was also a trend towards significance with a number of the SNPs tested, including exonic *rs10920568*, p=0.09 (Appendix D).

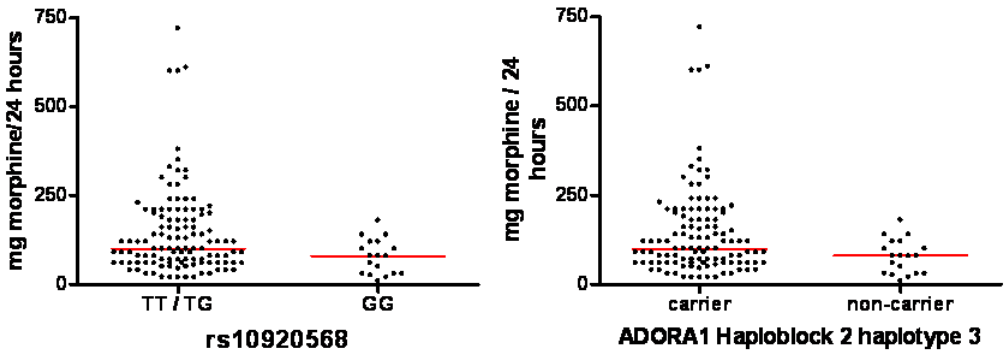

Figure 5.1 ADORA1 rs10920568 and daily morphine dose

There is a significant association between ADORA1 rs10920568 and haploblock 2 / haplotype 3 and variation in morphine dose required to achieve adequate analgesia. Red line represents median value.

Table 5.2 ADORA1 Haplotypes

	Haploblock 1			Haploblock 2			Haploblock 3		% (N)	
	rs2364571	rs9660662	rs11590405	rs903361	rs10920568	rs10800901	rs3766560	rs10920576	Frequency	Carriage frequency
1	C	C	C	-	-	-	-	-	1 (7)	2.5 (6)
2	C	T	T	-	-	-	-	-	68 (328)	89.3 (216)
3	G	C	C	-	-	-	-	-	31 (148)	51.2 (124)
1	-	-	-	G	T	-	-	-	1 (7)	2.5 (6)
2	-	-	-	G	G	-	-	-	31 (150)	52.1 (126)
3	-	-	-	A	T	-	-	-	67 (325)	88.8 (215)
1	-	-	-	-	-	-	C	C	83 (400)	95 (230)
2	-	-	-	-	-	-	T	C	2 (12)	5 (12)
3	-	-	-	-	-	-	T	T	15 (72)	27.7 (67)

Data shown for total Caucasian population.

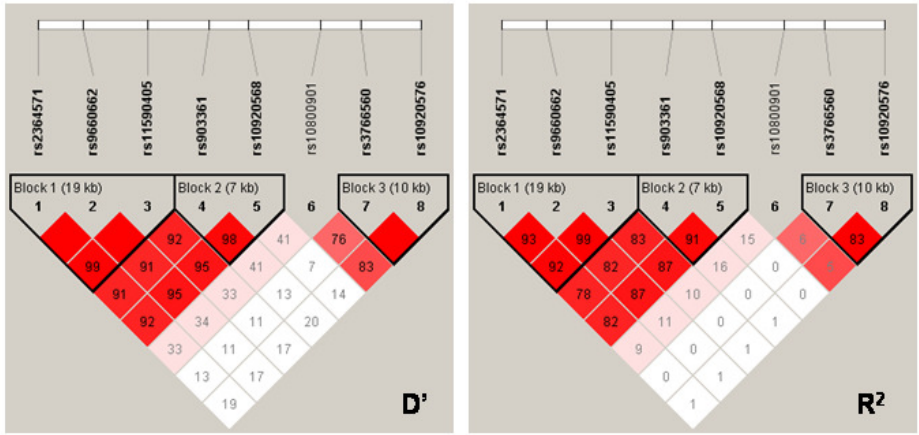

Figure 5.2 LD Plots for ADORA1

(Haploview version 4.0, Broad Institute, Cambridge, USA). See List of Abbreviations for explanation of colour scheme.

Table 5.3 Effects of ADORA1 on morphine dose (dominant model)

SNP name	Allele	Carrying allele		Not carrying allele		Univariate linear regression log10 Daily morphine dose (mg) as dependent variable			
		% (N)	Mean* (SEM)	% (N)	Mean* (SEM)	B	Std Error B	β	P value
rs 2364571	**C**	**86.6 (116)**	**2.026 (0.032)**	**13.4 (18)**	**1.787 (0.084)**	**0.241**	**0.086**	**0.237**	**0.006**
	G	56.7 (76)	1.994 (0.04)	43.3 (58)	1.994 (0.047)	-0.005	0.061	-0.007	0.932
rs 9660662	**T**	**85.1 (114)**	**2.023 (0.032)**	**14.9 (20)**	**1.828 (0.081)**	**0.197**	**0.083**	**0.202**	**0.019**
	C	59 (79)	1.999 (0.034)	41 (55)	1.986 (0.049)	0.007	0.061	0.01	0.904
rs 11590405	**T**	**85.1 (114)**	**2.023 (0.032)**	**14.9 (50)**	**1.828 (0.081)**	**0.197**	**0.083**	**0.202**	**0.019**
	C	58.2 (78)	1.998 (0.039)	41.8 (56)	1.988 (0.048)	0.005	0.061	0.007	0.938
rs 903361	**A**	**85.8 (115)**	**2.028 (0.032)**	**14.2 (19)**	**1.787 (0.078)**	**0.244**	**0.084**	**0.245**	**0.004**
	G	58.2 (78)	2.011 (0.04)	41.8 (56)	1.968 (0.047)	0.037	0.061	0.052	0.547
rs 10920568	**T**	**86.6 (116)**	**2.027 (0.318)**	**13.4 (18)**	**1.78 (0.082)**	**0.249**	**0.086**	**0.245**	**0.004**
	G	56 (75)	2.021 (0.041)	44 (59)	1.958 (0.046)	0.057	0.061	0.082	0.348
rs 10800901	**G**	**89.6 (120)**	**2.019 (0.031)**	**10.4 (14)**	**1.787 (0.096)**	**0.239**	**0.096**	**0.211**	**0.014**
	A	61.9 (83)	1.988 (0.037)	38.1 (51)	2.004 (0.05)	-0.022	0.062	-0.031	0.724
rs 3766560	C	95.5 (126)	1.985 (0.031)	4.5 (6)	2.171 (0.095)	-0.185	0.146	-0.111	0.206
	T	31.1 (41)	1.964 (0.05)	68.9 (91)	2.007 (0.037)	-0.043	0.066	-0.057	0.514
rs 10920576	C	99.3 (133)	1.992 (0.031)	0.7 (1)	-	-0.152	0.35	-0.038	0.666
	T	28.4 (38)	1.961 (0.056)	71.6 (96)	2.007 (0.036)	-0.049	0.067	-0.063	0.466

* mean (SEM) of log10 Daily morphine dose (mg)
rs2364571 C ANOVA P= 7.878, R^2 = 0.056
rs9660662 T ANOVA P= 5.635, R^2 = 0.041
rs11590405 T ANOVA P= 50635, R^2 = 0.041
rs903361 A ANOVA P= 8.046, R^2 = 0.06
rs10920568 T ANOVA P= 8.44, R^2 = 0.06
rs10800901 G ANOVA P= 6.167, R^2 = 0.045

Table 5.4 Effects of ADORA1 on morphine dose (additive model)

				Univariate linear regression log10 Daily morphine dose (mg)as dependent variable			
SNP name	**Genotype**	**% (N)**	**Mean* (SEM)**	**B**	**Std. Error B**	**β**	**P value**
rs 2364571	CC	43.3 (58)	1.994 (0.047)				
	CG	43.3 (58)	2.058 (0.043)	0.061	0.043	0.122	0.159
	GG	13.4 (18)	1.787 (0.084)				
rs 9660662	TT	41 (55)	1.986 (0.049)				
	TC	44 (59)	2.057 (0.042)	0.047	0.043	0.095	0.272
	CC	14.9 (20)	1.828 (0.081)				
rs 11590405	TT	41.8 (56)	1.987 (0.048)				
	TC	43.3 (58)	2.056 (0.043)	0.048	0.043	0.098	0.261
	CC	14.9 (20)	1.828 (0.081)				
rs 903361	AA	41.8 (56)	1.968 (0.047)				
	AG	44 (59)	2.084 (0.043)	-0.043	0.043	-0.086	0.324
	GG	14.2 (19)	1.787 (0.048)				
rs 10920568	TT	44 (59)	1.958 (0.046)				
	TG	42.5 (57)	2.097 (0.042)	0.031	0.043	0.062	0.476
	GG	13.4 (18)	1.78 (0.082)				
rs 10800901	GG	38.1 (51)	1.781 (0.096)				
	GA	51.5 969)	2.029 (0.041)	-0.067	0.047	-0.124	0.152
	AA	10.4 (14)	2.004 (0.05)				
rs 3766560	CC	68.9 (91)	2.007 (0.037)				
	CT	26.5 (35)	1.928 (0.058)	0.004	0.054	0.006	0.945
	TT	4.5 (6)	2.171 (0.095)				
rs 10920576	CC	71.6 (96)	2.007 (0.036)				
	CT	27.6 (37)	1.956 (0.058)	0.04	0.064	0.054	0.536
	TT	0.7 (1)	0				

* mean (SEM) of log10 Daily morphine dose (mg)
No statistically significant associations found

5.4 ADRA2A

ADRA2A is located on chromosome 10 (10q25.2) and spans 3651 bp. (http://snpper.chip.org, accessed 09/01/2010)[89]. It is an intronless gene and contains 1 exons and 101 known SNPs. Of these there are 6 validated SNPs occuring in the coding exon but four of these are non-polymorphic and for two, frequency data are not known.

ADRA2A codes for the α_{2A} adrenergic receptor (α_{2A} AR), one of three α_2 adrenergic receptors. These are seven transmembrane G-protein coupled receptors which play a role in regulating neurotransmitter release (including noradrenaline and adrenaline) from sympathetic and central adrenergic neurons [382].

The α_{2A} AR mediates analgesic effects of α_2 adrenergic receptor agonists [383]. α_2 adrenergic agonists, e.g. clonidine have analgesic properties [384;385].

α_{2A} AR agonists act synergistically with either MOR or DOR agonists, including morphine [383;386;387]. The mechanism for this synergy is poorly understood. Mice lacking α_{2A} AR demonstrate elevated concentrations of plasma noradrenaline [382]. Mice carrying a point mutation D79N, in which functional α_{2A} AR binding is reduced by 80%, exhibit a markedly increased ED_{50} for spinally administered morphine, suggesting that α_{2A} ARs may mediate some of the effects of morphine [383]. This point mutation also blocks the synergy between α_2 adrenergic agonists and either μOR or δOR agonists [383].

Knockout studies have shown that α_{2A} AR mediates the sympathetic inhibition of intestinal motility. α_{2A} AR knockout mice have reduced gastrointestinal transit time as compared to wild-type animals [388].

α_{2A} AR and DOR have been shown to colocalise in the superficial dorsal horn and also in peripheral nerve terminals, leading to the hypothesis that these receptors may form heterodimers together with altered function [387]. There is further evidence

suggesting that this synergy is mediated by protein kinase C [389]. Physical receptor-receptor interactions have also been proposed for α_{2A} AR and MOR [390].

5.4.1 ADRA2A Results

Table 5.5 ADRA2A marker data

Name	Position	Location in gene	Alleles major: minor	MAF	Published MAF	HWpval	Call rate	AA change
rs638019	112821819	promoter	G:A	0.238	0.354	0.993	99.6	-
rs11195418	112825149	promoter	A:G	0.045	0.02	1	99.6	-
rs1800544	112826493	promoter	C:G	0.266	0.205	0.4302	99.2	-
rs11195419	112829358	3UTR	C:A	0.098	0.142	0.3496	98.8	-
rs3750625	112829591	3UTR	C:A	0.048	0.067	0.8443	99.6	-
rs602618	112833075	Downstream	A:C	0.261	0.283	0.7488	100	-
Reference sequence NM_000681 Published frequencies HapMap CEU except rs638019 (AFD Eur Panel)and rs1800544 (PGA Ceph)								

Six SNPs across *ADRA2A* were investigated. All SNPs were in Hardy-Weinberg equilibrium and the allele frequencies were similar to those published, (table 5.5).

Within the *ADRA2A* region covered by the SNPs in this study a single haplotype block spanning 4674bp and including the three SNPs in the promoter region of the gene was identified (Table 5.6 and figure 5.3). This haploblock definition is different to that published by Belfer et al which includes a number of the same SNPs and which identifies a single haploblock spanning the entire gene. There were much fewer study subjects in that study (N=96 Caucasians) [391]. There was no association between the haplotypes and any of the clinical phenotypes in this study.

There was a weak association between a SNP in the promoter region, *rs11195418* and variation in daily morphine dose, p=0.039, R^2 0.032. Subjects carrying the G allele (genotype GG or GA) required less morphine to achieve adequate pain control than non-carriers of G (genotype AA). In this study no subject was homozygous for the minor allele (Appendix C).

There was no association between SNPs in *ADRA2A* and either pain scores or central side-effects on morphine (Appendix C).

Table 5.6 ADRA2A haplotypes

	Haploblock 1						Frequency	Carriage frequency
	rs638019	**rs11195418**	**rs1800544**	**rs11195419**	**rs3750625**	**rs602618**	% (N)	
1	A	A	G	-	-	-	19 (92)	36 (85)
2	A	G	G	-	-	-	4 (20)	8 (20)
3	G	A	C	-	-	-	74 (352)	95 (225)
4	G	A	G	-	-	-	2 (12)	4.6 (11)
Data shown for total Caucasian population.								

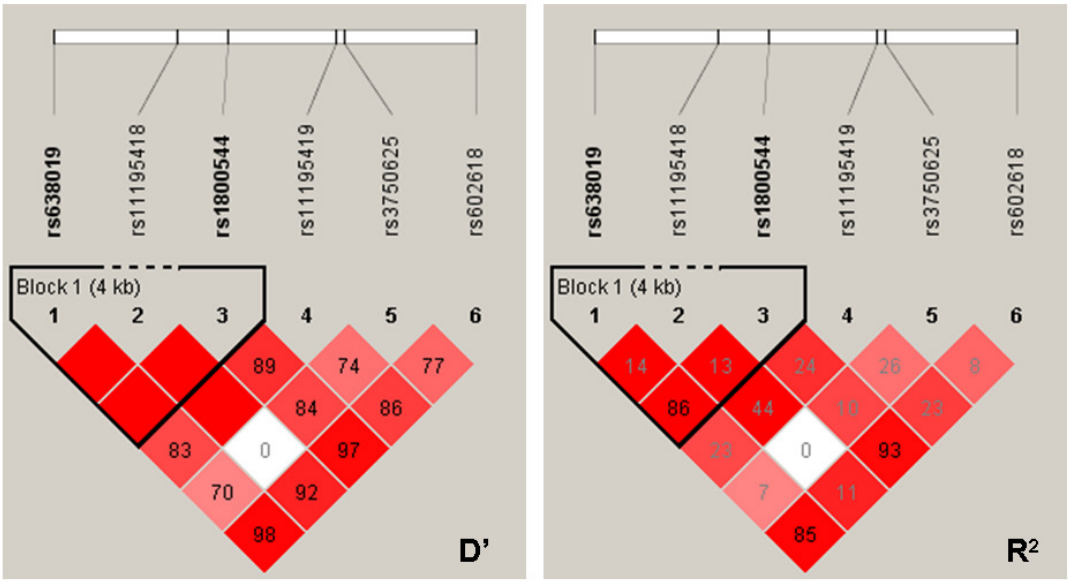

Figure 5.3 LD plot for ADRA2A

(Haploview version 4.0, Broad Institute, Cambridge, USA).

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5.5 TRPV1

TRPV1 codes for transient receptor potential cation channel, subfamily V, also known as the vanilloid or capsaicin receptor. This gene is located on chromosome 17 (17p13.3) and spans 43964 base pairs. It contains 426 known SNPs. (http://snpper.chip.org, accessed 10/01/2010) [89]. One of the SNPs included in this analysis, *TRPV1 rs222747*, occurs in a region of the gene which codes for a part of the receptor involved in protein-protein interactions[392].

The capsaicin receptor is a non-selective cation channel. It is expressed in the dorsal root ganglion and peripheral nociceptors. It is activated by capsaicin (the active ingredient of hot chilli peppers), protons and noxious heat [393]. Capsaicin receptors are also activated by 12-lipoxygenase metabolites including 12(S)-HPETE. 12(S)-HPETE and capsaicin are very similar structurally [394].

There are many studies demonstrating the role of the capsaicin receptor in pain sensation and also response to opioids. Mice lacking the capsaicin receptor have impaired responses to painful heat [395]. As well as causing pain, prolonged exposure to capsaicin results in analgesia due to desensitisation of nociceptors [393;396].

MOR and capsaicin receptors co-localise on the dorsal root ganglion (DRG) [397]. Chronic morphine treatment is associated with an increase in TRPV1 expression in the DRG. TRPV1 blockade has been shown to attenuate morphine tolerance in animal studies [397]. Knockout studies have shown that TRPV1 is involved in morphine-induced nociception and also in the development of morphine-induced hyperalgesia [398;399]. The use of TRPV1 antagonists potentiates the analgesic effects of morphine in some pain states [400].

5.5.1 TRPV1 Results

Table 5.7 TRPV1 marker data

SNP	Position	Location	Allele major:minor	MAF	Published MAF	HW p value	Call rate	AA change
rs877610	3422240	Exon 15	G:A	0.054	0.026	1	98.8	-
rs2277679	3427524	Intron 12	C:G	0.334	0.42	0.3285	100	-
rs222745	3435620	Intron 9	C:T	0.087	0.09	0.2849	99.2	-
rs161393	3436263	Intron 8	A:G	0.337	0.25	0.5747	99.6	-
rs222747	3439949	Exon 7	C:G	0.264	0.18	0.6389	100	Met-Ile
rs3744683	3460211	Promoter	A:G	0.025	0.004	0.2628	98.4	-
Published allele frequencies from HapMap CEU Reference sequence NM_080704								

TRPV1 is comprised of 17 exons and there are ten known SNPs located in coding exons. Of these, four have either unknown frequency data or else the frequency of the minor allele is less than 0.05. Four others were included for investigation initially, *rs877610, rs222747, rs224534* and *rs222748*. The latter two however were not in Hardy Weinberg equilibrium and were not included in the final analysis.

Six SNPs across TRPV1 were included in the final analysis. All were in Hardy-Weinberg equilibrium.

One haploblock was identified, spanning 643 base pairs (Table 5.8 and figure 5.4). There was no significant association between the haplotypes and any of the clinical phenotypes tested.

In this study there was no association between any of the TRPV1 SNPs and analgesic response or central side-effects on morphine. Two SNPs, *rs877610* (exonic) and *rs2277679* were associated with variability in daily morphine dose. Carriers of the A allele of rs877610 (genotype AA or AG) had a lower dose requirement than non-carriers (genotype GG), p=0.047, R^2 0.03. Carriers of the G allele of rs2277679 (genotype GG or CG) also required less morphine to achieve adequate analgesia, p=0.026, R^2 0.037 (Tables 5.9 and 5.10).There was no gene dose effect for either of these SNPs.

Table 5.8 TRPV1 haploblocks and haplotypes

			Haploblock 1				Frequency	Carriage frequency
	rs877610	**rs2277679**	**rs222745**	**rs161393**	**rs222747**	**rs3744683**		**% (N)**
1	-	-	C	A	-	-	66 (314)	88 (204)
2	-	-	C	G	-	-	25 (120)	44 (104)
3	-	-	T	G	-	-	8 (40)	17 (40)
Data shown for total Caucasian population.								

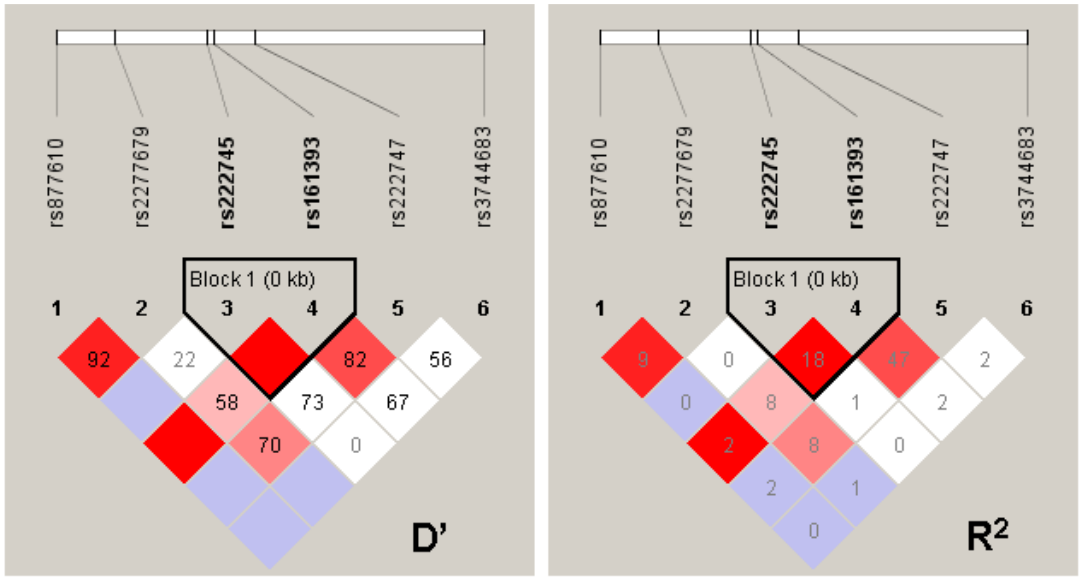

Figure 5.4 LD Plots for TRPV1

(Haploview version 4.0, Broad Institute, Cambridge, USA).

Table 5.9 Effects of TPRV1 on morphine dose (dominant model)

Univariate linear regression **Log10 Daily morphine dose(mg) as dependent variable**									
		Carrying allele		**Not carrying allele**					
SNP name	**Allele**	**% (N)**	**Mean* (SEM)**	**% (N)**	**Mean* (SEM)**	**B**	**Std Error B**	**β**	**P value**
rs877610	G	99.2 (131)	1.99 (0.031)	0.8 (1)	-	-0.33	0.352	0.082	0.35
	A	12.9 (17)	1.838 (0.086)	87.1 (115)	2.016 (0.033)	**-0.181**	**0.09**	**-0.174**	**0.047**
rs2277679	C	85.8 (115)	1.999 (0.035)	14.2 (19)	1.950 (0.063)	0.053	0.086	0.053	0.54
	G	53 (71)	1.931 (0.039)	47 (63)	2.063 (0.048)	**-0.133**	**0.059**	**-0.192**	**0.026**
rs222745	C	100 (133)	1.992 (0.031)	0	-	-	-	-	-
	T	15.8 (21)	1.996 (0.067)	84.2 (112)	1.992 (0.035)	0.017	0.083	0.018	0.839
rs161393	A	92.5(123)	1.998 (0.032)	7.5 (10)	1.923 (0.121)	0.069	0.115	0.053	0.548
	G	52.6 (70)	1.984 (0.044)	47.4 (63)	2.002 (0.045)	-0.017	0.061	-0.024	0.783
rs222747	C	94.8 (127)	2.0 (0.032)	5.2 (7)	1.832 (0.127)	0.125	0.135	0.08	0.357
	G	44.8 (60)	1.971 (0.52)	55.2 (60)	2.01 (0.038)	-0.041	0.061	-0.059	0.498
rs3744683	A	100 (131)	1.992 (0.031)	0	-	-	-	-	-
	G	3.1 (4)	1.774 (0.274)	96.9 (126)	1.999 (0.031)	-0.226	0.178	-0.111	0.207
* mean (SEM) of Log10 Daily morphine dose rs877610 A ANOVA F = 4.036, R^2 = 0.03 rs2277679 G ANOVA F = 5.058, R^2 = 0.037									

Table 5.10 Effects of TPRV1 on morphine dose (additive model)

Univariate linear regression **Log10 Daily morphine dose(mg) as dependent variable**							
SNP name	**Genotype**	**% (N)**	**Mean* (SEM)**	**B**	**Std. Error B**	**β**	**P value**
rs877610	GG	87.1 (115)	2.016 (0.033)				
	GA	12.1 (16)	1.807 (0.086)	0.134	0.083	0.14	0.11
	AA	0.8 (1)	-				
rs2277679	CC	47 (63)	2.063 (0.048)				
	CG	38.8 (52)	1.924 (0.049)	0.079	0.049	0.161	0.063
	GG	14.2 (19)	1.950 (0.063)				
rs222745	CC	84.2 (112)	1.992 (0.035)				
	CT	15.8 (21)	1.996 (0.067)	0.017	0.083	0.018	0.839
	TT	0	-				
rs161393	AA	47.4 (63)	2.002 (0.045)				
	AG	45.1 (60)	1.993 (0.047)	0.023	0.049	0.041	0.636
	GG	6.9 (10)	1.923 (0.121)				
rs222747	CC	55.2 (74)	2.01 (0.038)				
	CG	39.6 (53)	1.987 (0.056)	-0.046	0.051	-0.079	0.362
	GG	5.2 (7)	1.832 (0.127)				
rs3744683	AA	96.2 (126)	1.999 (0.031)				
	AG	3.5 (5)	1.774 (0.274)	0.158	0.16	0.087	0.324
	GG	0	-				
* mean (SEM) of Log10 Daily morphine dose No statistically significant associations found							

5.6 ALOX12

ALOX12, the gene which codes for arachidonate 12-lipoxygenase is located on chromosome 17 (17p13.1) and spans 14650 base pairs. It contains 178 known SNPs. (http://snpper.chip.org, accessed 10/01/2010)[89]. This enzyme is found in a number of regions including platelets and human leucocyte cells. A non-expressed 12-lipoxygenase pseudogene also exists on chromosome 17 [401].

Arachidonate 12-lipoxygenase is a key enzyme in arachidonic acid metabolism, producing 12(S)-HPETE (12(S)-hydroperoxy-5,8,10,14-eicosatetraenoic acid). Arachidonic acid and its metabolites, including 12(S)-HPETE are involved in cell signalling and act as second messengers [402]. In the brain, suppression of the inhibitory effects of GABA (*gamma*-Aminobutyric acid) on the descending antinociceptive pathways by opioids has been shown to occur through a pathway involving arachidonic acid and its metabolites [403].

12-lipoxygenase knockout mice have enhanced analgesia from morphine in the acute stage, but this effect diminished with chronic morphine treatment. This suggests that with long-terms morphine use, alternative second messangers that are not dependent on 12-lipoxygenase may be involved [404]. Furthermore, spinal inhibition of 12-lipoxygenase attenuates morphine withdrawal in animals treated with naloxone [405]. There is also evidence that 12-lipoxygenase metabolites activate TRPV1, the capsaicin receptor, which is implicated in nociception.

5.6.1 ALOX12 Results

Table 5.11 ALOX12 marker data

Name	Position	Locus on gene	Alleles major:minor	MAF	Published MAF	HWpval	Call rate	AA change
rs6502999	6833363	Promoter	A:G	0.373	0.45	0.6911	100	-
rs1126667	6843484	Exon 6	G:A	0.382	0.44	0.798	100	Gln-Arg
rs434473	6845658	Exon 8	A:G	0.384	0.43	0.4891	100	Asn-Ser
Published frequencies HapMap CEU Reference sequence NM_000697								

ALOX12 contains 14 exons,[406] in which there are 12 SNPs in coding regions. Of these 8 are non-polymorphic in Caucasians or have a very low minor allele frequency. Of those exonic SNPs which were not included for investigation, *rs1042356* was in tight LD with *rs1126667* (which was included) and *rs1042357* was not included in Hapmap data.

Three SNPs were included in the final analysis. These were all in Hardy-Weinberg equilibrium. One haploblock which included all three SNPs was identified (Table 5.12 and figure 5.5). There was no association between the ALOX12 SNPs or haplotypes and any of the clinical phenotypes tested.

Table 5.12 ALOX12 haplotypes

				Frequency	Carriage frequency % (N)
	rs6502999	**rs1126667**	**rs434473**		
1	A	A	G	2 (8)	3 (8)
2	A	G	A	60 (299)	84 (205)
3	G	A	G	36 (178)	59 (145)
Data shown for total Caucasian population.					

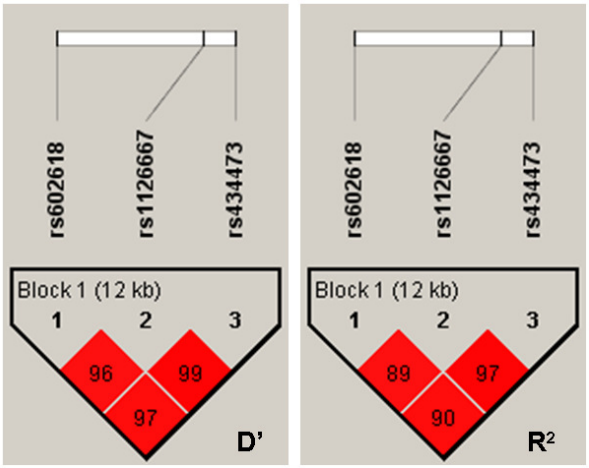

Figure 5.5 LD plots for ALOX12

(Haploview version 4.0, Broad Institute, Cambridge, USA).

5.7 SLC6A4

Serotonin is a key signaling molecule in both the central nervous system and the gastrointestinal tract. Serotonin is inactivated by SERT, the serotonin reuptake transporter, which is coded for by SLC6A4, (solute carrier family 6, member 4). SLC6A4 is located on chromosome 17 (17q11.2) and spans 37798 base pairs.

SERT has been associated with variability in morphine response. This involvement is somewhat complicated however and is dependent on a number of factors including the site and dose of morphine used [407]. One study demonstrated that SERT knockout mice have 64% reduction in morphine-induced analgesia, as compared to wild-type animals, $p = 0.014$ [408]. Furthermore, the use of SSRIs appears to attenuate naloxone-induced morphine withdrawal in rats [409]. There is some evidence that serotonin receptors, specifically 5-HT(1A) inhibit endogenous opioid release in the spinal cord [410].

The data pertaining to SERT and nociception are less clear. Serotonergic pathways are involved in both pronociception and antinociception [407]. One study demonstrated that SERT -/- mice developed reduced inflammatory pain after inflammation [411]. Another study however showed no difference in nociception (thermal, mechanical or formalin-induced) between SERT -/- and wild-type animals [412].

The 5' promoter region of SLC6A4 contains a 44-base pair deletion-insertion polymorphism (DIP) known as SERTPR or 5HTTLPR [413]. The allelic variants are named short (S) and long (L) depending on the presence or absence of the DIP. The short allele (S) has been associated with reduced transcriptional activity, a lower level of SERT-P expression and therefore reduced re-uptake and inactivation of serotonin [413;414].

5.7.1 SLC6A4 Results

Table 5.13 SLC6A4 marker data

Name	Chr Position (bp)	Locus on gene	Alleles major:minor	MAF	Published MAF	HWpval	Call Rate	AA change
rs4583306	25500441	Intron 9	A:G	0.43	0.4	0.884	99.6	-
rs140700	25405115	Intron 6	C:T	0.108	0.1	0.2452	98.4	-
rs6354	25411612	5UTR	T:G	0.215	0.21	0.0978	98.8	-
rs25528	25411692	Intron 1	T:G	0.224	0.18	0.305	96.7	-
rs7214991	25433987	promoter	A:G	0.363	0.35	0.1493	98.4	-
SERTP**		DIP	L / S	0.16	0.18*	0.2	97.5	-

Reference sequence NW_926750
Published allele frequencies from HapMap CEU
*refers to published genotype frequencies for 193 white US subjects [415]
**Contig position SERTP 3292128-3292610 [302]

SLC6A4 contains 15 exons and 348 known SNPs. (http://snpper.chip.org, accessed 10/01/2010)[89]. It contains nine validated SNPs in coding exon regions. Of these eight are non-polymorphic in Caucasians. The remaining exonic SNP *rs2228673* was included in this study but was found to be non-polymorphic in this study population and was therefore omitted from the final analysis.

Five SNPs and one deletion-insertion polymorphism were tested. All were in Hardy-Weinberg equilibrium.

One haploblock was identified which included two SNPs, *rs6354* and *rs25528*, spanning 80 base pairs (Table 5.14 and figure 5.6).

Table 5.14 SLC6A4 haploblock and haplotypes

	Haploblock 1					% (N)	
	rs 7214991	**rs25528**	**rs6354**	**rs 140700**	**rs4583306**	**Frequency**	**Carriage frequency**
1	-	T	T	-	-	0.77 (352)	0.93 (212)
2	-	G	G	-	-	0.22 (99)	0.37 (85)

Data shown for total Caucasian population.

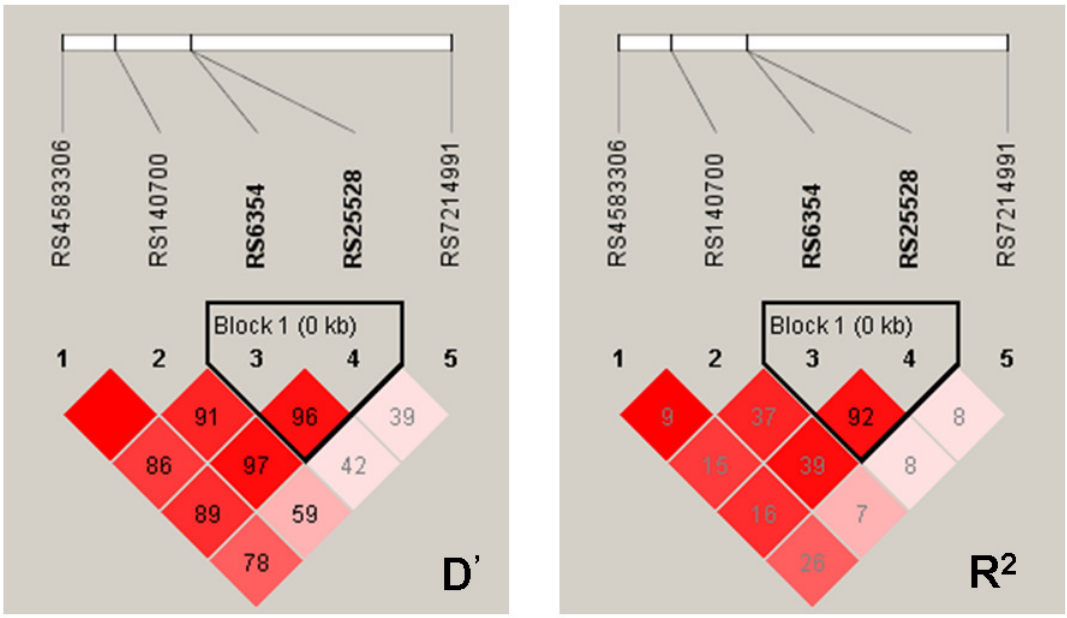

Figure 5.6 LD Plots for SLC6A4

Haploview version 4.0, Broad Institute, Cambridge, USA

Intronic SNP, *SLC6A4 rs140700*, was associated with analgesic response to morphine on univariate analysis. Carriage of the C allele (genotype CC or CT) at this position was associated with lower pain scores than non-carriage (genotype TT) p=0.009, R^2 0.037 (figure 5.7). This SNP exhibited a clear gene dose effect (Tables 5.15 and 5.16, figure 5.7).

Rs22528 was also associated with variation in pain scores, carriers of the T allele (genotype TT or TG) having lower pain scores than non-carriers (genotype GG), p=0.02, R^2 0.03. Again there was a gene dose effect, but this was borderline significant, p=0.052.

SNPs in SLC6A4 were not found to be associated with variation in daily morphine dose or central side-effects on morphine.

The deletion-insertion polymorphism SERTP was not statistically associated with any of the clinical phenotypes tested.

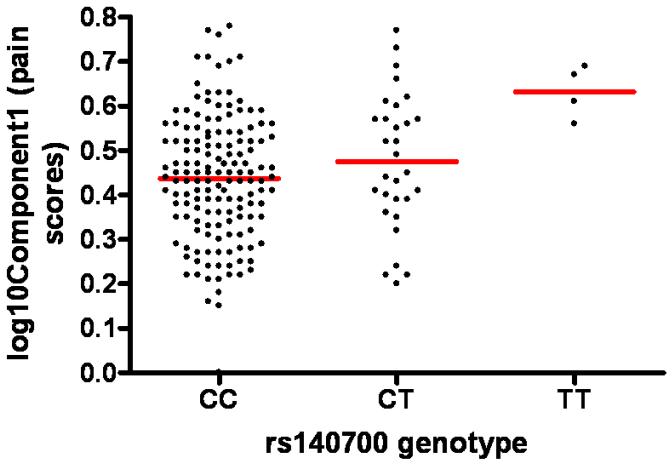

Figure 5.7 SLC6A4 and analgesic response

SLC6A4 *rs140700* is associated with variation in analgesic response to morphine. The analgesic response scores on the y-axis are log10Component1 scores (ref chapter 3) Higher scores represent worse analgesic response. The red line represents the mean value.

Table 5.15 SLC6A4 and analgesic response to morphine (dominant model)

Univariate linear regression **Log10Component1 (pain scores) as dependent variable**									
SNP name	**Allele**	**Carrying allele**		**Not carrying allele**		**B**	**Std Error B**	**β**	**P value**
		% (N)	**Mean* (SEM)**	**% (N)**	**Mean* (SEM)**				
rs 7214991	A	90 (171)	0.45 (0.012)	10 (19)	0.39 (0.035)	0.06	0.035	0.125	0.088
	G	59.8 (113)	0.44 (0.014)	40.2 (76)	0.46 (0.017)	-0.025	0.022	-0.084	0.253
rs 22528	T	**94.6 (174)**	**0.44 (0.011)**	**5.4 (10)**	**0.55 (0.034)**	**-0.108**	**0.046**	**-0.173**	**0.02**
	G	36.2 (67)	0.47 (0.018)	63.8 (118)	0.44 (0.014)	0.029	0.022	0.099	0.184
rs6354	T	93.7 (177)	0.44 (0.011)	6.3 (12)	0.52 (0.033)	-0.082	0.043	-0.14	0.057
	G	34.2 (65)	0.47 (0.018)	65.8 (125)	0.43 (0.014)	0.035	0.022	0.117	0.111
rs 140700	C	**97.9 (183)**	**0.44 (0.011)**	**2.1 (4)**	**0.61 (0.032)**	**-0.189**	**0.071**	**-0.193**	**0.009**
	T	**18.2 (34)**	**0.49 (0.026)**	**81.8 (153)**	**0.44 (0.012)**	**0.057**	**0.027**	**0.154**	**0.037**
rs 4583306	A	78.9 (150)	0.44 (0.012)	21.1 (40)	0.46 (0.027)	-0.012	0.026	-0.033	0.649
	G	71.1 (135)	0.45 (0.013)	28.9 (55)	0.45 (0.021)	-0.008	0.023	-0.025	0.731
SERTP	S	69 (126)	0.46 (0.012)	31 (56)	0.42 (0.2)	-0.03	0.028	-0.081	0.275
	L	82 (149)	0.44 (0.012)	18 (33)	0.47 (0.023)	0.43	0.023	0.139	0.062
* mean (SEM) of log10Component1 scores									

Table 5.16 SLC6A4 and analgesic response to morphine (additive model)

Univariate linear regression **Log10Component1 (pain scores) as dependent variable**							
SNP name	**Genotype**	**% (N)**	**Mean* (SEM)**	**B**	**Std. Error B**	**β**	**P value**
rs 7214991	AA	40.2 (76)	0.46 (0.017)				
	AG	49.7 (94)	0.45 (0.016)	-0.028	0.016	-0.123	0.094
	GG	10.1 (19)	0.39 (0.035)				
rs 22528	TT	63.9 (117)	0.44 (0.014)				
	TG	30.6 (56)	0.45 (0.019)	-0.035	0.018	-0.145	0.052
	GG	5.5 (10)	0.55 (0.034)				
rs 6354	TT	66.1 (125)	0.43 (0.014)				
	TG	27.5 (52)	0.46 (0.02)	**-0.035**	**0.017**	**-0.147**	**0.045**
	GG	6.3 (12)	0.52 (0.033)				
rs 140700	CC	81.8 (153)	0.44 (0.012)				
	CT	16 (30)	0.48 (0.028)	**0.061**	**0.023**	**0.193**	**0.009**
	TT	2.1 (4)	0.61 (0.032)				
rs 4583306	AA	28.9 (55)	0.45 (0.021)				
	AG	50 (95)	0.44 (0.015)	0.001	0.015	0.003	0.968
	GG	21.1 (40)	0.46 (0.027)				
SERTP	SS	17.8 (33)	0.48 (0.024)				
	LS	51.4 (95)	0.45 (0.015)	-0.023	0.015	-0.139	0.062
	LL	30.8 (57)	0.42 (0.022)				
* mean (SEM) of log10Component1 scores							

5.8 ARRB2

ARRB2 is the gene coding for intra-cellular B-arrestin 2 proteins. These proteins act as inhibitors to receptor coupling and are negative regulators of opioid receptor signalling [125]. B-arrestin2 knockout mice have been shown to experience prolonged analgesia from morphine, even at doses which are normally subanalgesic [126]. Desensitization and tolerance to morphine does not occur in these knock-out animals [416]. Morphine analgesia is also improved by inhibition of B-arrestin 2 expression in the periaqueductal grey matter of mice [417]. Conversely rats with over-expression of B-arrestin2 have little or no analgesia from morphine [418]. B-arrestin2-knockout mice also demonstrated reduced constipation and respiratory depression on morphine [419].

Opioid receptors are thought to interact and dimerise with each other and with other receptors in regulation of receptor function [420]. Mu-delta opioid receptor heterodimers can alter B-arrestin recruitment and so modulate opioid receptor signalling [421].

5.8.1 ARRB2: Results

Table 5.17 ARRB2 marker data

Name	Position (bp)	Locus on gene	Alleles major:minor	MAF	Published MAF	HWpval	Call Rate	AA change
rs1973555	4552636	Promoter	C:T	0.321	0.29	0.7661	100	-
rs3786047	4561847	Intron 1	G:A	0.366	0.283	0.3746	99.3	-
rs4522461	4568522	Intron 9	C:T	0.223	0.217	0.6781	99.6	-
rs1045280	4569387	Exon 11	T:C	0.361	0.31	0.2448	96.8	Ser-Ser
Published allele frequencies from HapMap CEU								

This gene is located on chromosome 17p13.2 and spans approximately 11007bp. It is comprised of 15 exons and contains 226 SNPs (http://snpper.chip.org, accessed 02/01/2010) [89]. Of these, three are located in coding exons, *rs1045280, rs9915175* and *rs34750256*. The latter two are non-polymorphic and were not included. Dr Joy Ross originally tested seven SNPs across this gene on the smaller subset of the study population [279]. A number of these were in tight LD together and therefore four were chosen for further investigation: *rs1973555 rs3786047, rs4522461, rs1045280.* The primers for these SNPs were designed by Dr Joy Ross [279] who also ran them on 199

caucasian patients who were included in this study. I ran these primers on the remaining 66 Caucasian patients. All were in Hardy-Weinberg equilibrium.

One haploblock was identified which included two of the SNPs and spanned 6675 base pairs (table 5.18 and figure 5.8).

Carriers of the T allele of *rs4522461* (genotype TT or TC) also had lower central side-effect scores compared to non-carriers (genotype CC), p=0.024. There was no gene dose effect for this SNP. There was also an association between Haplotype 2 and central side-effects, p=0.03. There was an association between exonic SNP *rs1045280* and central side-effects on morphine, p=0.039 (Tables 5.19 and 5.20).

None of the SNPs tested were associated with variation in analgesic response or morphine dose requirements.

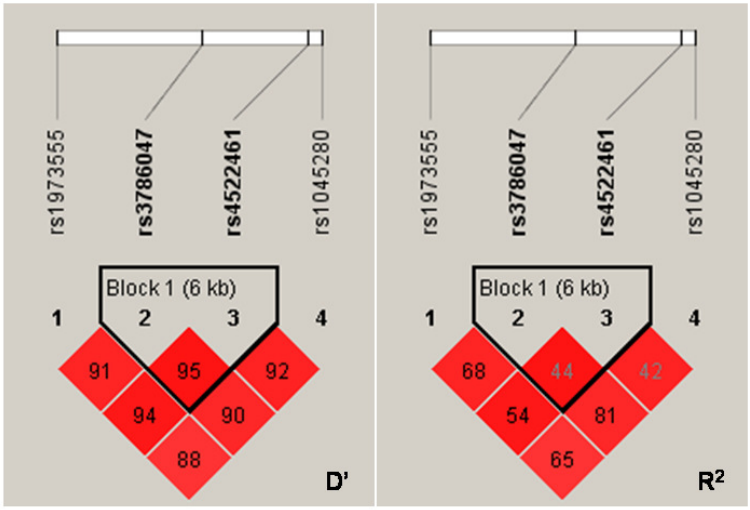

Figure 5.8 LD plots for ARRB2

Haploview version 4.0, Broad Institute, Cambridge, USA

Table 5.18 ARRB2 haplotypes

	rs1973555	rs3786047	rs4522461	rs1045280	% (N) Frequency	% (N) Carriage frequency
			Haploblock 1			
1	-	A	C	-	23 (53)	14 (66)
2	-	A	T	-	42 (98)	22 (105)
3	-	G	C	-	84 (199)	63 (298)
4	-	G	T	-	1 (3)	1 (3)

Data shown for total Caucasian population.

Table 5.19 ARRB2 and central side-effects on morphine (dominant model)

Univariate linear regression **Log10Component2 (central side effect scores) as dependent variable**									
SNP name	**Allele**	**Carrying allele**		**Not carrying allele**		**B**	**Std Error B**	**β**	**P value**
		% (N)	**Mean* (SEM)**	**% (N)**	**Mean* (SEM)**				
rs1973555	C	92.1 (176)	0.4 (0.012)	7.9 (15)	0.4 (0.041)	<0.001	0.043	<0.001	0.997
	T	52.9 (101)	0.38 (0.016)	47.1 (90)	0.42 (0.017)	-0.042	0.022	-0.135	0.065
rs3786047	G	87.8 (166)	0.4 (0.013)	12.2 (23)	0.39 (0.027)	0.009	0.035	0.018	0.805
	A	57.9 (110)	0.42 (0.018)	42.1 (80)	0.38 (0.015)	-0.035	0.023	-0.112	0.128
rs4522461	C	95.8 (182)	0.4 (0.012)	4.2 (8)	0.44 (0.052)	-0.033	0.06	-0.041	0.578
	T	**41.6 (79)**	**0.37 (0.018)**	**58.4 (111)**	**0.42 (0.015)**	**-0.052**	**0.023**	**-0.165**	**0.024**
rs1045280	T	89.2 (165)	0.4 (0.012)	10.8 (20)	0.36 (0.032)	0.048	0.037	0.096	0.2
	C	54.6 (101)	0.38 (0.015)	45.4 (84)	0.42 (0.017)	-0.045	0.023	-0.144	0.052
* mean (SEM) of log10Component2 scores									

Table 5.20 ARRB2 and central side-effects on morphine (additive model)

Univariate linear regression **Log10Component2 (central side-effect scores) as dependent variable**							
SNP name	**Genotype**	**% (N)**	**Mean* (SEM)**	**B**	**Std. Error B**	**β**	**P value**
rs1973555	CC	47.1 (90)	0.42 (0.017)				
	CT	45 (86)	0.38 (0.017)	0.027	0.018	0.108	0.14
	TT	7.9 (15)	0.4 (0.041)				
rs3786047	GG	42.3 (80)	0.42 (0.018)				
	GA	45.5 (86)	0.38 (0.017)	0.021	0.017	0.09	0.222
	AA	12.2 (23)	0.39 (0.027)				
rs4522461	CC	58.4 (111)	0.42 (0.015)				
	CT	37.4 (71)	0.36 (0.019)	0.035	0.02	0.13	0.077
	TT	4.2 (8)	0.44 (0.052)				
rs1045280	TT	45.4 (84)	0.42 (0.017)				
	TC	43.8 (81)	0.39 (0.018)	**0.036**	**0.017**	**0.153**	**0.039**
	CC	10.8 (20)	0.36 (0.032)				
* mean (SEM) of log10Component2 scores							

5.9 Gene-gene interaction / gene-environment interaction

The main positive findings from the univariate analyses are summarized in table 5.21.

Table 5.21 Summary of positive associations of genes with clinical outcomes

Gene	Analgesic response	Central side-effects	Morphine dose (mg/24 hours) to achieve adequate pain control
OPRM	√		√
OPRK	√	√	√
OPRD			√
ADORA1	√		√
ADRA2A			√
TRPV1			√
ALOX12			
SLC6A4	√		
ARRB2		√	

As discussed earlier in this chapter, there is evidence to suggest interaction between the following genes:

- OPRM / ADORA1
- OPRM / ADRA2A
- OPRM / ALOX12
- OPRM / TRPV1
- OPRM / ARRB2
- ALOX12 / TRPV1
- ADRA2A / OPRD
- ADORA1 / ADRA2A / OPRM
- Opioid receptors/SLC6A4

Multivariate regression modeling was used to examine these gene-gene interactions and also gene-environment interactions. Clinical and genetic factors significant at the level of 10% ($p<0.1$) on univariate analysis (as presented in chapter 3) were introduced simultaneously into the model using a stepwise approach.

(For some of these combinations there were no genetic factors with p<0.1 therefore it was not possible to carry out multivariate regression analyses.)

5.9.1 Analgesic response to morphine

5.9.1.1 SLC6A4 and opioid receptor genes

In chapter 4, I described the results of a multivariate regression analysis which identified *OPRK rs7824175*, daily morphine dose, being on a b-blocker and taking an antiemetic as independent predictors of variability in analgesic response to morphine, accounting for approximately 16% of variability in the clinical phenotype.

The main association with variation in analgesic response to morphine in this chapter was with SNPs in *SLC6A4*. Therefore SNPs in the opioid receptor genes together with SNPs in *SLC6A4* and clinical factors which were significant on univariate analysis (p<0.1) were entered into a multivariate regression model.

When SLC6A4 SNPs were added, *OPRK rs7824175*, *SLC6A4 rs140700*, SERTP and being on a B-blocker, antiemetic and taking paracetamol were included in the final model. Daily morphine dose was no longer included. This new model predicted 23.4% of variance in analgesic response to morphine (Table 5.22). In this model, the association between analgesic response and OPRK rs7824175 became stronger, with the p value increasing from 0.00015 to 7.68×10^{-5}. When the dominant model was used, a similar result was obtained. In the dominant model SERTP was not included and SLC6A4 rs25528 rather than SLC6A4 rs140700 was included. These latter SNPs are in LD together (D' 0.97, R^2 0.39).

Table 5.22 OPRM, OPRD, OPRK and SLC6A4 and analgesic response (additive model)

Multivariate regression model (using log10Component 1 (Pain scores) as dependent variable) of clinical and genetic factors associated with analgesic response to morphine.

	B (95% CI)	Std. Error	Beta	P
(Constant)	0.610 (0.527, 0.693)	0.042		<0.001
OPRK1 rs7824175	-0.083 (-0.123, -0.043)	0.020	-0.280	7.68×10^{-5}
SLC6A4 rs140700	0.062 (0.017, 0.107)	0.023	0.194	0.007
SERTP	-0.035 (-0.064, -0.006)	0.015	-0.171	0.020
Antiemetic	-0.043 (-0.084, -0.003)	0.020	-0.151	0.035
B Blocker	0.124 (0.048, 0.200)	0.039	0.222	0.002
Paracetamol	0.044 (0.005, 0.084)	0.020	0.152	0.029
Additive (genotype) model All SNPs (OPRM, OPRK, OPRD, SLC6A4)and clinical variables p<0.1 on univariate analysis included ANOVA F = 8.3, $p = 8 \times 10^{-8}$, $R^2 = 23.4$				

Table 5.23 OPRM, OPRD, OPRK and SLC6A4 and analgesic response (dominant model)

	B (95% CI)	Std. Error	Beta	P
(Constant)	0.514 (0.426, 0.601)	0.044		<0.001
OPRK1 rs7824175 CC/CG	0.087 (0.040, 0.135)	0.024	0.253	0.0004
SLC6A4 rs25528 TT/TG	-0.096 (-0.182, -0.010)	0.043	-0.156	0.028
Antiemetic	-0.050 (-0.090, -0.009)	0.020	-0.171	0.016
B Blocker	0.121 (0.042, 0.199)	0.040	0.213	0.003
Paracetamol	0.041 (0.000, 0.082)	0.021	0.140	0.048
Dominant (allele carriage) model All SNPs (OPRM, OPRK, OPRD, SLC6A4) and clinical variables p<0.1 on univariate analysis included ANOVA = 7.6, $p = 1.7 \times 10^{-6}$, $R^2 = 18.7$				

5.9.1.2 ADORA1 and opioid receptor genes

ADORA1 was also associated with variability in analgesic response to morphine on univaraite analysis. When SNPs in *ADORA1, OPRM, OPRD* and *OPRK* and clinical variables were included in the multivariate analysis, the final model (additive) consisted of four independent variables including *ADORA rs903361. OPRK rs7824175,* being on a B-Blocker and taking an antiemetic remained independent predictors of variation in analgesic response to morphine. This model however only explained 16% of variability in the clinical outcome, similar to the model which did not included the ADORA1 SNPs (data not shown).

5.9.2 Variation in central side-effects on morphine

The only gene with any association with central side-effects on morphine in this chapter was ARRB2. When this gene was added to the multivariate analysis described in chapter 4, no ARRB2 SNPs remained independent predictors of the clinical outcome.

5.9.3 Variation in daily morphine dose to achieve adequate analgesia

5.9.3.1 ADORA1 and opioid receptor genes

In chapter 4, I described the results of a multivariate regression analysis which identified *OPRK rs7836120*, being on a bronchodilator and taking an anticonvulsant and *OPRM rs2075572* and *OPRM rs589046* as independent predictors of variability in analgesic response to morphine, accounting for approximately 27% of variability in the clinical phenotype.

This model was built on to incorporate SNPs in *ADORA1*. Multivariate analysis of *ADORA1* and *OPRM* and *OPRK* with clinical factors associated with the clinical phenotype resulted in a regression model consisting of four independent variables which also predicted 27% of variability in morphine dose (table 5.23). *OPRM rs2075572, OPRK rs7836120* and being on an anticonvulsant were retained in the model, along with *ADORA rs10920568.* Being on a bronchodilator and *OPRM rs589046* were no longer included in the model.

Carriage of the T allele at ADORA rs10920568 (genotype TT or TG), or carriage of the OPRK1 rs7836120 A allele (genotype AA or AG) or being on an anticonvulsant are predictive of a need for higher daily morphine dose to achieve adequate analgesia. Carriage of the G allele OPRM rs2075572 (genotype GG or GC) is predictive of lower morphine dose requirements. This model is similar to that developed using clinical factors and SNPs in the opioid genes alone.

When the additive model was used (genotype) no ADORA1 SNPs were retained in the final model.

Table 5.24 OPRM, OPRD, OPRK and ADORA1 and daily morphine dose (additive model)

Stepwise multivariate linear regression model (using Log10 Daily morphine dose as dependent variable) of clinical and genetic factors associated with variability in daily morphine dose requirements.

	B	Std. Error	Beta	P value
(Constant)	1.467 (1.136, 1.797)	0.167		<0.001
Anticonvulsant	0.325 (0.183, 0.467)	0.072	0.348	1.3×10^{-5}
OPRK rs7836120 AA/AG	0.394 (0.086, 0.701)	0.155	0.194	0.012
ADORA1 rs10920568 TT/TG	0.208 (0.054, 0.363)	0.078	0.205	0.009
OPRM rs2075572 GG/GC	-0.137 (-0.247, -0.028)	0.055	-0.189	0.015
ANOVA F = 11.6, $p = 5 \times 10^{-8}$, $R^2 = 0.267$ All clinical and genetic factors OPRM, OPRK and ADORA1 p<0.1 on univariate analysis included				

5.9.3.2 ADRA2A and opioid receptor genes

Multivariate analysis of *ADRA2A* and *OPRM, OPRK* and *OPRD* with clinical factors associated with the clinical phenotype resulted in a regression model consisting of three independent variables which predicted 26% of variability in morphine dose (Tables 5.24 and 5.25). Both the additive and dominant models are similar including being on an anticonvulsant and *OPRM rs1799971* and *ADRA2A rs11195418*. The dominant model also includes a SNP in *OPRK* . These models do not predict any more variance in this clinical phenotype than the model including the opioid receptor genes alone.

5.9.3.3 Other gene combinations

When multivariate analysis of ADORA1, ADRA2A, OPRM, OPRD and OPRK was carried out, no ADRA2A genetic data was retained. The final model resembled that for OPRM, OPRD, OPRK and ADORA1 alone (table 5.23).

When multivariate analysis of ALOX12 and OPRM, ARRB2 and OPRM and TRPV1 and OPRM was carried out, no ALOX12 / ARRB2 / TRPV1 genetic data were retained.

Table 5.25 OPRM, OPRD, OPRK and ADRA2A and daily morphine dose (dominant model)

In tables 5.24 and 5.25 stepwise multivariate linear regression model, using Log10 Daily morphine dose as dependent variable was carried out to determine the influence of clinical and genetic factors on variability in daily morphine dose requirements.

	B (95% CI)	Std. Error	Beta	P
(Constant)	1.477 (1.171, 1.783)	0.155		0.000
Anticonvulsant	0.324 (0.176, 0.471)	0.075	0.340	$2.9x10^{-5}$
OPRK rs7836120 AA/AG	0.456 (0.145, 0.767)	0.157	0.225	0.004
OPRM rs1799971 GG/GA	0.171 (0.042, 0.300)	0.065	0.205	0.010
ADRA2A rs11195418 GG/GA	-0.225 (-0.422, -0.028)	0.100	-0.178	0.026
All SNPs (OPRM, OPRD, OPRK, ADRA2A) and clinical variables p<0.1 on univariate analysis included ANOVA F = 10.9, p = 13×10^{-7}, $R^2 = 25.9$				

Table 5.26 OPRM, OPRD, OPRK and ADRA2A and daily morphine dose (additive model)

	B (95% CI)	Std. Error	Beta	P
(Constant)	1.912 (1.844, 1.979)	0.034		<0.001
Anticonvulsant	0.348 (0.198, 0.498)	0.076	0.365	1×10^{-5}
OPRM rs1799971	0.159 (0.050, 0.269)	0.055	0.229	0.005
ADRA2A rs11195418	-0.213 (-0.414, -0.012)	0.101	-0.169	0.038
ANOVA F = 11.9, p = 6.2×10^{-7} , $R^2 = 22.1$ All clinical and genetic factors OPRM, OPRK, OPRD, ADRA2A p<0.1 on univariate analysis included				

5.10 General Discussion

5.10.1 Main findings

The main findings from the analyses presented in this chapter are:

- Response to morphine in terms of pain, central side-effects and morphine dose requirements are mediated by different polymorphisms in different genes with little overlap. This supports the argument that morphine response is a heterogeneous clinical outcome and adds more weight to the hypothesis presented in chapter 3 that these clinical phenotypes are separate and discreet.

- *SLC6A4* appears to play a role in analgesic response.

- *OPRK (rs7824175), SLC6A4 rs140700* and being on a B-blocker/anti-emetic/paracetamol are independent predictors, accounting for 23% of variability in analgesic response to morphine.

- Being on an anticonvulsant remains the strongest predictor of variability in morphine dose requirements on multivariate analysis, regardless of the genes included in the modeling.

- *OPRM, OPRK* and *ADORA1* also appear to play a role in variability in the dose of morphine required to achieve adequate pain control.

In chapter 4, models predicting response to morphine in terms of clinical factors and the opioid receptor genes were presented. The aim of this chapter was to explore the influence of other genes and to use these data to build up more comprehensive predictive models. Although each SNP was studied individually, one of the main aims of this chapter was to build multivariate regression models to explore the influence of gene-gene and gene-environment interactions on morphine response.

5.10.2 Models predicting analgesic response to morphine

In this study *SLC6A4* appears to be associated with variability in analgesic response. Two SNPs in *SLC6A4* were included in the final additive multivariate regression model, *SERTP* (the DIP in the promoter region) and *rs140700*. The *SERTP* short allele (S) has been associated with reduced transcriptional activity, a lower level of SERT-P expression and therefore reduced re-uptake and inactivation of serotonin. [414] [413]. There has only been one small study investigating this DIP in pain. In that study 43 healthy volunteers found that remifentanil, a short acting opioid, was more effective in subjects carrying the short (S) allele at this position [422]. *SLC6A4 rs140700* is an intronic SNP. This SNP may be in LD with a true marker SNP which may alter function of the serotonin reuptake transporter protein, thus altering response to opioids.

A multivariate regression model predicting 16% of variability in analgesic response to morphine had been described in chapter 4 in which clinical factors and SNPs in the opioid receptor genes were included. When SNPs in *SLC6A4*, the serotonin reuptake transporter gene were added, the model got significantly stronger, predicting 23% of variance in this clinical phenotype. *OPRK rs7824175* and being on a B-blocker / antiemetic were retained in the new model but daily morphine dose was not, suggesting that the former three factors are more likely to be true predictors of analgesic response to morphine. The fact that morphine dose was removed from the multivariate model is consistent with 1) the finding in this study that morphine dose only accounted for a very small proportion of the variance in analgesic response on univariate analysis and 2) the clinical finding of significant variation in morphine dose requirements for similar painful stimuli.
Therefore the model presented here suggests that increased serotonin levels in addition to variation in OPRK and being on paracetamol/β-blockers and antiemetic medications are predictive of analgesic response to morphine in cancer patients.

5.10.3 Models predicting central side-effects on morphine

The genetic associations with central side-effects are less significant. *ARRB2 rs1045280*, in the B-arrestin 2 gene, was previously found to be associated with morphine non-responders [95]. In that study (which was carried out on a subset of this study population) morphine non-responders were more likely to carry the T allele at this position (p=0.036). In this study this SNP was weakly associated with central side-effects but it is unlikely that these results would withstand any corrections for multiple testing.

In chapter 4 a model predicting 8-10% of variability in central side-effects on morphine was presented including SNPs in *OPRM* and *OPRK* and clinical variables. Despite testing more genes, *OPRK rs10504151* remains the strongest predictor on univariate analysis (p=0.009). *ARRB2* SNPs were not retained on multivariate testing.

5.10.4 Models predicting morphine dose requirements

Variability in morphine dose requirements to achieve adequate analgesia was associated with genetic variation in *ADORA1, ADRA2A* and *TRPV1* on univariate analysis. The most significant association was with SNPs in *ADORA1,* the gene coding for the A1-adenosine receptor. In chapter 4 a multivariate model consisting of SNPs in OPRK and OPRM and being on a bronchodilator or anticonvulsant medication was developed which predicted 27% of variance in daily morphine dose requirements. In this chapter SNPs in ADORA and ADRA2A were added to the model. Different models were generated depending on which genes were included in the analysis. However, throughout all, being on an anticonvulsant medication remains the single most consistent and significant predictor of variability in morphine dose requirements.

The lack of consistency among the SNPs in each of the multivariate models is likely to suggest that these models need further testing to eliminate false positive and false negative findings. However the models presented are biologically plausible.

In the spinal cord morphine produces a dose-dependent release of adenosine which is thought to mediate some of the antinociceptive effects of morphine [373;423]. MOR (much more so than DOR or KOR) activation increases adenosine release [424]. There also appears to be a synergistic antinociceptive action when adenosine receptor agonists are administered with delta or kappa opioid receptor agonists [425]. This supports the multivariate model of morphine dose requirements which includes SNPs in OPRM, OPRK and ADORA1.

ADORA1 rs10920568 is the most significant *ADORA1* SNP and remains an independent predictor of variation in morphine dose on multivariate linear regression with *OPRM, OPRK* and clinical factors. This SNP is located in exon 5, which is in the coding region of the gene. It is not associated with an amino acid change. There was also an association with a SNP in intron 5, *rs10800901* which is downstream to and not in LD with *rs10920568*. The LD plot for this gene demonstrates a disintegration of LD within intron 5. Intron 5 interrupts the coding sequence in a region which corresponds to the second intracellular loop of the receptor [370;426]. The second intracellular loop is involved in G-protein coupling [427]. The region of the gene upstream and including this region of LD disintegration (represented by haploblocks 1 and 2 in this study) is associated with the clinical phenotype of variation in morphine dose requirements and the region downstream to this (haploblock 3) is not. This LD block structure is similar to that presented in HapMap [90]. There were also associations with intronic SNPs located in the untranslated 5' region of the gene. Alternative splicing in A1-adenosine receptors is thought to occur in this region [426]. The data presented in this study suggests that the SNPs included may represent marker SNPs for the true susceptibility SNP which is located somewhere upstream to haploblock 3 and that the DNA variation may influence receptor function through post-transcriptional modification.

ADRA2A, the α_{2A} adrenergic receptor gene was also associated with variability in morphine dose. Although the association on univariate analysis was weak (p=0.039), a SNP in *ADRA2A rs11195419* remained an independent predictor of morphine dose when multivariate regression was carried out using clinical factors and SNPs in *OPRM, OPRK* and *OPRD* as co-variables. A multivariate predictive model was built

which included OPRM, and ADRA2A and being on an anticonvulsant in both the additive model and dominant model. Therefore, although this model again accounts for approximately 22-26% of variance in the clinical phenotype, it may represent a more robust model to that including ADORA1 or just the opioid receptor genes as the genetic data are more consistent regardless of the method of modelling used. This SNP was also significantly associated with morphine dose requirements in the test group (N= 1475) in the large EPOS study, but did not remain significant in the validation study [135].

In animal models the analgesic effects of clonidine (α2-adrenoceptor agonist) were blocked by PACPX and naloxone, which act as antagonists at the A1-adenosine receptor and mu-opioid receptors respectively [428]. This suggests that A1-adenosine receptors may exist as multi-receptor complexes with mu-opioid receptors and α2-adrenoceptors. All three receptors are located on the same primary afferent neurons and physical coupling may be involved in antinociception. Alternatively agonism of all three receptors may result in nociception through a common second messenger system [428]. In this study, when SNPs in *OPRM, OPRK, ADORA1* and *ADRA2A* were included in a multivariate regression analysis however, *ADRA2A* SNPs were not retained.

An exonic SNP in *TRPV1, rs877610* was also associated with variability in daily morphine dose but this SNP was not retained in any multivariate modeling including other genes. It is disappointing that there was no stronger association between our clinical phenotypes and TRPV1, especially since MOR in *TRPV1*-expressing neurons are thought to be involved in thermal nociception [360]. It may be that the pain experienced by our patients was mediated by different mechanisms or that this analysis failed to identify the associated polymorphisms. Two exonic SNPs in *TRPV1* were not investigated in this study, *rs8065080* and *rs222749*, both of which are associated with an animo acid change. The MAF of the former is 0.36 and of the latter is 0.06 [89]. Neither of these SNPs is in LD with the SNPs included in this analysis. TRPV1 *rs8065080* is thought to affect the area of the receptor which is involved in response to capsaicin and *rs 222749* affects the intracellular amino tail [392]. Neither of these SNPs however has been associated with variability in pain perception or response to opioids.

Multivariate regression to explore gene-gene and gene-environment interactions

The concept of epistasis (gene-gene interaction) is discussed in chapter 8.

5.11 Conclusion

Analgesia, central side-effects on morphine and variability in dose of morphine required to achieve adequate pain control appear to be distinct clinical phenotypes. This hypothesis is supported by the fact that the clinical and genetic variables associated with these clinical outcomes are dissimilar.

The data presented are exploratory and serve to present a method of defining clinical outcomes and approaching genetic analyses in studies of response to opioids. The findings from this study have not yet been replicated and as the findings are merely hypothesis-generating, we have not adjusted the results for multiple testing.

6 Chapter 6: Results from a Genome-Wide Association Study

6.1 Introduction and Summary of the rationale behind study

- Inter-individual variation in response to morphine is likely to be influenced by multiple genes.

- The choice of genes to include in candidate gene studies is limited by current understanding of biological and genetic pathways.

- Analysis of SNPs across the entire genome may identify novel genes which are associated with response to morphine.

6.1.1 Aim

The aim of this study was to use DNA micro-array technology to carry out a Genome-Wide Association Study (GWAS) to explore inter-individual variation in response to morphine. The following data represent the first genome wide association study in cancer patients taking morphine.
This study is a preliminary hypothesis-generating study.

6.2 Subjects and Methods

This study was carried out on a subset of patients from the *Prospective Morphine Study*.

6.2.1 Clinical Phenotype / outcome

This study was carried out before PCA was used to build a mathematical model of response to morphine. Therefore the clinical phenotypes used in this study are different to those presented in chapters 3-5.

In this study the variables "average pain" and "pain relief from morphine" in the past 24 hours were used to define the clinical outcomes. These parameters were chosen to best reflect what is clinically thought to be an acceptable analgesic response (see section 3.4.3). These parameters were subsequently included in a different study as the most clinically relevant pain intensity variables, based on the opinion of 16 experts. [331]

As discussed in chapter 3 (section 3.2.1), an average pain of ≤ 4/11 is considered to be adequate or acceptable pain control. Using average pain score ≤4 as the dependent variable, two clusters of scores for pain relief in the preceding 24hours were identified: pain relief <70% and pain relief ≥70%.Therefore 2 binary clinical phenotypes were used in the association analysis:

- Average pain ≤ 4
- Pain relief ≥ 70%

6.2.2 Experimental design

Figure 6.1 shows the experimental design for the genetic association study.

This study was carried out in a series of sequential stages.

Stage 1: **Genome-wide-association study:** A subset of samples from the Prospective Genetics Study was sent to Yale for genotying using Affymetrix Genome-Wide Human SNP Array 5.0. technology. In subsequent discussion, this dataset is known as the "Affy dataset". These genetic data were analysed for associations with clinical outcomes i.e. average pain ≤ 4 or pain relief $\geq 70\%$.

Stage 2**: Replication study using SSP-PCR**: Significant SNPs / genes from the "Affy dataset" were identified and these were run using SSP-PCR on a second, independent data set comprised of the remaining samples in the Prospective Genetics Study ("SSP-PCR dataset"). This second set were also analysed for associations with the relevant clinical phenotypes.

Stage 3**:** The "Affy" and "SSP-PCR" datasets were compared in terms of

- Accuracy of the data generated with both methods
- Significance of the associations with clinical outcomes
- Structure of the both datasets (genetic and clinical)

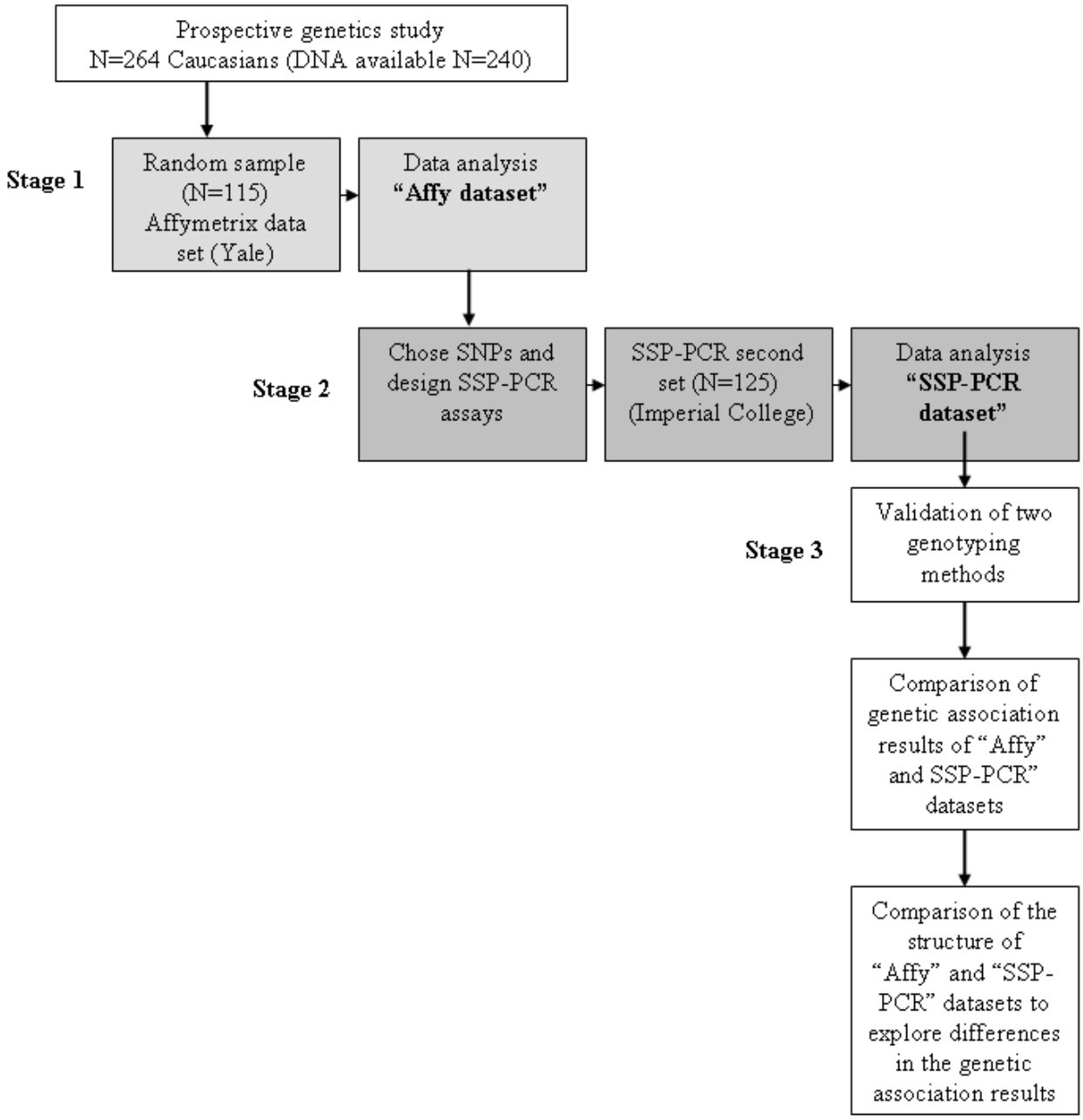

Figure 6.1 Experimental design for genetic association study

*132 samples in total run on Affymetrix5.0 chip but only Caucasians (N=115) included in analysis (see results section).

6.2.2.1 Stage 1: Genotyping using Affymetrix 5 gene chip and analysis of the resulting "Affy dataset"

132 DNA samples from the Prospective Genetics Study were genotyped using the Affymetrix Genome-Wide Human SNP-Array 5.0. The DNA was sent on ice to Yale University for this to be carried out under the supervision of Professor Josephine Hoh, Professor of Epidemiology and Public Health, and Professor A. DeWan, Assistant Professor of Epidemiology, Yale School of Medicine, Yale University, US.

DNA samples were chosen for analysis using Affymetrix on the basis of

- DNA quality checks:
    - Amplified samples were excluded (although genomic DNA amplified with the QIAGEN Repli-G Kit has been tested successfully with the Genome-Wide Human SNP 5.0 Assay).
    - 100ng of genomic DNA was run on a 1% agarose gel. Samples lacking a significant band of >10kb were excluded as this suggested that the sample was of insufficient quality for Affymetrix SNP genotyping. (Personal correspondence with Professor A. DeWan). Ligation and successful PCR will not occur in degraded DNA lacking intact restriction sites [304].
    - DNA quality was also checked using DNA-OK! (Microzone Ltd, UK) as detailed in Chapter 2.

- DNA quantity:
    - At least 3ug/sample of DNA was required at a minimum concentration of 50ng/ul. The DNA was quantified using picogreen (Invitrogen) and the concentration adjusted with Baxter H_20.

Before analysis, the GWAS data also underwent quality checks. SNPS were excluded from further analysis if:

- The Minor Allele Frequency < 0.05
- They were not in Hardy-Weinberg equilibrium
- There were a lot of missing genetic data i.e. the call rate < 95%.

440,794 SNPs were included in the Affymetrix Genome-Wide Human SNP-Array 5.0, of which 348,477 were included in the genetic association analysis.

Three data files were used to analyse the Affymetrix data:

1. The Affymetrix genetic data (.txt)
   This is the data file provided by Yale University. The Affymetrix output data was provided as a .txt file in which the genetic data was presented in a non-delimited form i.e. AA,AB,BB. Missing values were represented by NC (No Call). This data set is a tall data set i.e. the individual patient IDs are in columns and the SNP data are in rows.
   In order for this file to be used in R and STATA, the genetic data was split up into chromosomes and the format was changed from AA, AB, BB to 0, 1 and 2 respectively, where the numbers represent allele counts for the total number of B alleles. NC cells were changed to blank cells. The data were then split up into chromosomes using R to allow easier data manipulation.

2. The "Annotation data set" (Affy5 SNP list) which was downloaded from the Affymetrix website [429]. This file contains details about the each of the SNPs including chromosome, base pair position and rs number and facilitates alignment with the individual study subject data using the unique Affymetrix id.

3. The clinical dataset. This had a column which corresponded to the Affy ID and also had the corresponding clinical trial patient study number.

The association analysis was carried out using logistic regression. The additive / co-dominant model (using genotype frequency) was used for the genetic data and the analyses were carried out using R and STATA (www.r-project.org and www.stata.com). Gender was postulated as a possible influence on pain perception and analgesic response and was thus included as a predictor variable in the initial analysis. This initial analysis was run by Dr Saffron Willis-Owen, Research Associate, Molecular Genetics, National Heart and Lung Institute, Imperial College, London.

6.2.2.2 Stage 2: Chosing Affy SNPs to genotype on the second population using SSP-PCR

In order to choose SNPs from the Affy dataset to run on the SSP-PCR dataset, the results were examined in terms of:

- SNPs with the highest p values in the genome-wide association study.

  In many multi-stage genetic association studies, SNPs are chosen from the first stage for replication in the second stage on the basis of the strength of statistical association. A p-value threshold of $<5.67 \times 10^{-8}$ was calculated for this study using the conservative Bonferroni multiple test correction (440,794 SNPs x 2 tests). In the "Affy dataset", the most significant p value was 1×10^{-5}. Taking into account the exploratory nature of this study and the fact that the study size was relatively small, in order to minimise the chance of false negatives, a threshold for significance was not used to define the SNPs to be included in subsequent analysis. Instead SNPs with $p<0.001$ (i.e. $-\log_{10}P$ values >3) on genotype-phenotype analysis of the "Affy" dataset were considered for SSP-PCR genotyping.

- Genes in which there was >1 SNP associated with the clinical outcome.

- SNPs which were located in genes with a biologically plausible association with pain and opioid response.

For each gene in which there was a SNP which was chosen for inclusion in the SSP-PCR dataset, the genetic data for all of the SNPs included on the Affy array with $p<0.01$ ($-\log_{10}p > 2$) were examined for linkage disequilibrium using Haploview (Whitehead Institute for Biomedical Research, USA). This was used to identify and choose other SNPs in haploblocks surrounding the SNPs of interest. SNPs were chosen for inclusion in the SSP-PCR dataset on the basis of p value, whether they were in LD ($R^2>0.8$) with other SNPs, and whether they were included in a haploblock. The methodology used to design SSP-PCR assays is described in Chapter 2.

Analysis of the second SSP-PCR dataset (Stage 2)

Logistic regression and Chi-squared tests were used to assess odds ratios, confidence intervals and significance of association with the clinical outcome. As for stage 1, an additive inheritance model was assumed. Genotype data were examined for evidence of a gene-dose effect.

Adjustment for multiple testing was carried out using the Bonferroni formula. 9 SNPs in total were tested on a maximum of 2 clinical phenotypes therefore $p < 0.0027$ was considered significant in the SSP-PCR dataset (stage 2). The p values presented in the tables and results sections represent unadjusted p values.

6.2.2.3 Stage 3: Comparison of the "Affy" and SSP-PCR methods and datasets

- Correlations between the data generated with the Affymetrix 5.0 array and the SSP-PCR data were carried out in order to check validity and accuracy of both techniques.
    - All of the new SSP-PCR SNP assays were run on 16 DNA samples which were initially genotyped using the Affymetrix 5.0 array.
    - The SSP-PCR *PRKD3 rs1158219* assay was run on all samples i.e. both the "Affy" set and "SSP-PCR" set (Total N for comparison =115).

- As there were some differences in the genetic associations with the clinical phenotype between the "Affy" and "SSP-PCR" datasets, the datasets were

compared in terms of genotype frequencies, pairwise LD and clinical factors in order to examine why these differences might have occurred. Logistic regression was used to assess the contribution of clinical factors to the clinical outcome.

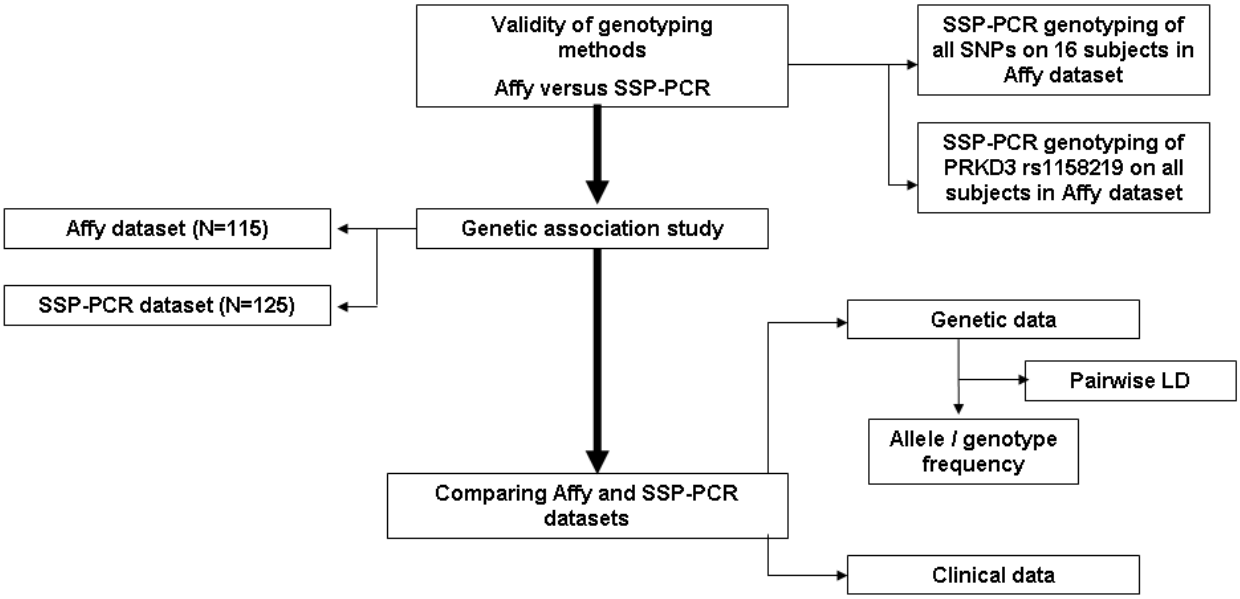

Figure 6.2 Comparing methods and results from different datasets

6.3 Results

Refining analysis dataset according to ethnicity

132 samples were analysed using Affymetrrix5.0 array, of which 115 were Caucasian. Principal components analysis demonstrated clear separation between Caucasians and non-Caucasians. In order to minimise population stratification bias [430], only Caucasians were included in further analysis. (Figure 3)

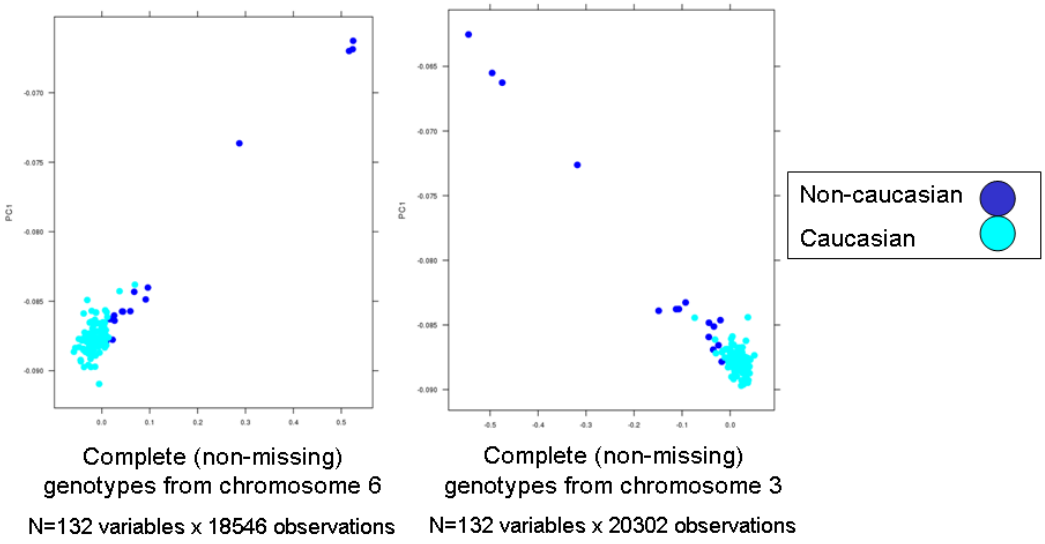

Figure 6.3 PCA demonstrating separation of genotypes according to ethnicity in two chromosomes

6.3.1 Stage 1: Genome-wide-association study results (N=115)

Many of the SNPs which were found to have an association with either of the clinical phenotypes were intragenic. 3 genes, *ZDHHC14, PRKD3 and NTRK3*, were associated with the clinical outcomes on analysis of the "Affy" data. These genes were therefore chosen to take forward to the second step in the study i.e. genotyping using SSP-PCR.

6.3.1.1 Average pain ≤ 4

The strongest association with average pain (highest p value / highest –log10p value) for the phenotype "average pain ≤ 4" was with a SNP, *PRKD3 rs1158219* on chromosome 2, p = 9.7×10^{-5} (figure 6.4). *PRKD3* codes for *protein kinase D3*, which is activated by G-protein coupled receptors and is involved in signal transduction and membrane trafficking. Therefore *PRKD3* is a gene which has a biologically plausible role in pain and opioid response. The next strongest associations were with SNPs in PWP2H (periodic tryptophan protein homolog) and TMEM1 (Transmembrane protein 1), neither of which are not known to be associated with pain or response to opioids and also with SNPs on Chromosomes 4, 5, 7, 8, 18 and 21 which were intragenic. A SNP in *NTRK3 (neurotrophic tyrosine kinase, receptor, type 3) rs8026216* on chromosome 15 has also one of the strongest associations with average pain in this dataset (p=0.0003). NTRK3 is a nerve growth factor receptor. These receptors are important in regulating the development of the central and peripheral nervous systems.

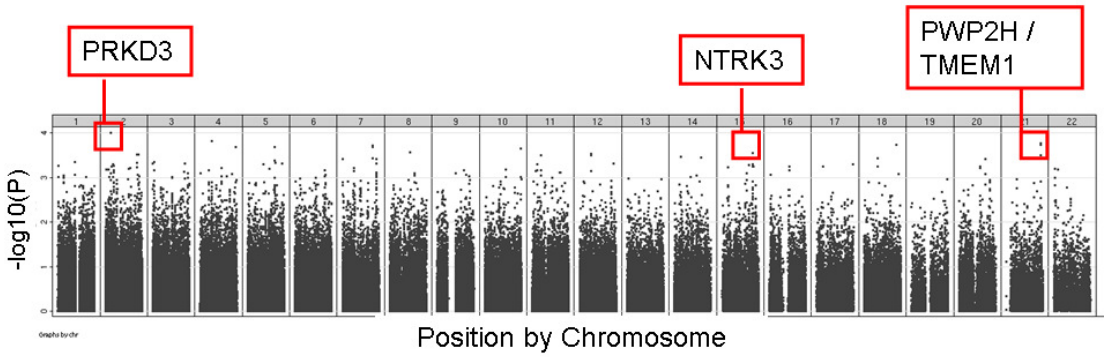

Figure 6.4 Results for genome-wide association testing with the clinical outcome, average pain ≤4

Manhattan plot for 348,477 SNPs from the step 1 genome-wide association analysis.

6.3.1.2 Pain Relief ≥ 70%

The strongest association with pain relief ≥70% was with a SNP, *ZDHHC14 rs17165436* on chromosome 6, p = 0.000013 (figure 6.5). The function of this gene, *Zinc finger, DHHC-type containing 14,* is as yet unknown. The next strongest associations were with SNPs in *ST6GALNAC3 (alpha-N-acetyl-neuraminyl-2,3-beta-galactosyl-1,3)-N-acetylgalactosaminide alpha-2,6-sialyltransferase 3*), *FBN2 (Fibrillin 2)* and *NRXN1 (Neurexin 1). ST6GALNAC3* is associated with smoking cessation, *FBN2* with Marfan's syndrome and *NRXN1* with autism. None of these genes has been associated with pain sensation or response to opioids. *NTRK3 (neurotrophic tyrosine kinase, receptor, type 3)* was also associated with pain relief ≥70%, p = 0.0003. Although *NTRK3* (neurotrophic tyrosine kinase receptor type 3) did not have the highest p values, it has a biologically plausible role in pain and opioid response and was found to be associated with both clinical phenotypes, therefore was included for SSP-PCR genotyping of the second step of the study.

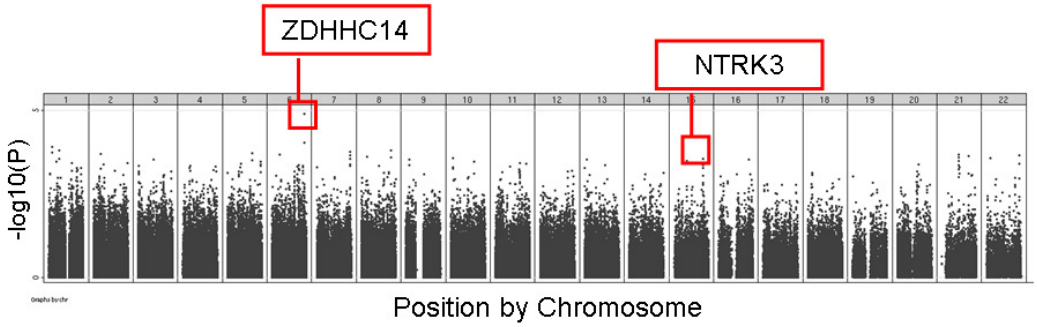

Figure 6.5 Results for genome-wide association testing with the clinical outcome, pain relief ≥70%.

Manhattan plot for 348,477 SNPs from the step 1 genome-wide association analysis.

6.3.2 Stage 2: Replication study using SSP-PCR

6.3.2.1 Chosing SNPS and designing primers for SSP-PCR set (N=125)

The primer details are found in Appendix E``.

PRKD3 primers for SSP-PCR

There are 17 *PRKD3* SNPs on the AFFY 5.0 array. There were 2 SNPs with p<0.001, *rs1158219* and *rs11124574*). There were 2 SNPS with p<0.01, *rs10177970* and *rs6730503* (Table 6.1). Three of these 4 SNPs made up one haploblock. The other SNP, *rs1158219,* lay outside this haploblock (figure 6.6).

Of these 4 SNPs, only 1, *rs1158219* was retained for analysis. It was not possible to design SSP-PCR primers for *rs10177970* as the GC content was too high. *Rs11124574* and *rs6730503* were omitted from analysis because the SSP-PCR assay failed.

Table 6.1 *PRKD3* SNPS (p<0.01) chosen from the "Affy dataset" as candidates for SSP-PCR

"Affy" marker	rs number	MAF	Call rate	HW p value	P value*
snp_a2257376	rs11124574	37.06897	100	0.319915	0.0008
snp_a4193911	rs10177970	37.93103	100	0.692834	0.003
snp_a1818533	rs6730503	37.5	100	0.552423	0.004
snp_a2037703	rs1158219	36.84211	98.27586	0.841542	9.75×10^{-5}
* association with average pain ≤ 4 on analysis of the "Affy" data set					

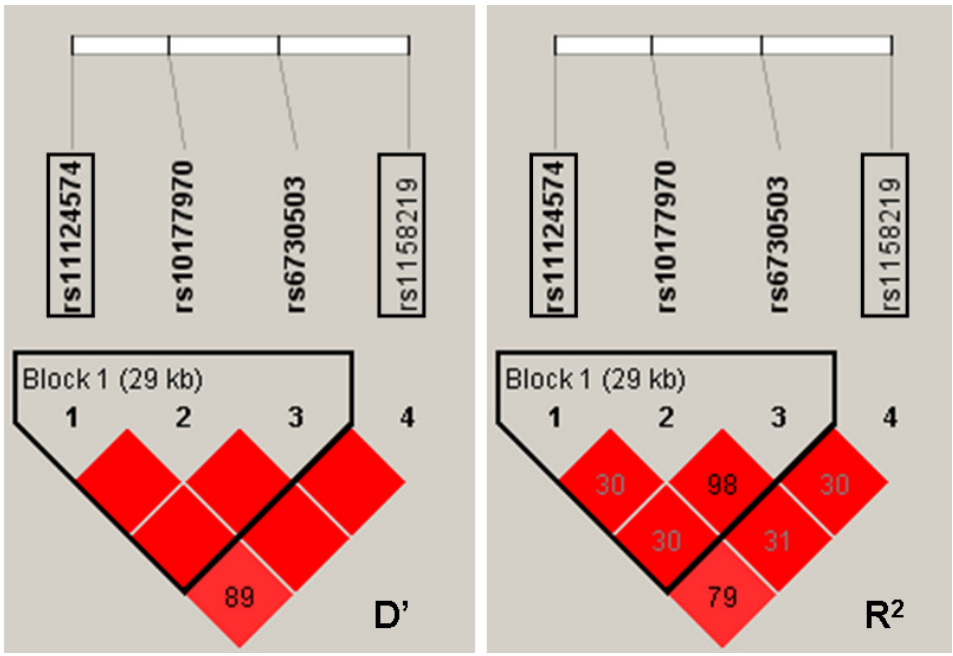

Figure 6.6 LD plots for PRKD3 in the "Affy" dataset
SNPs with p<0.001 with clinical phenotypes are highlighted

(Haploview version 4.0, Broad Institute, Cambridge, USA). See List of Abbreviations for explanation of colour scheme.

ZDHHC14 primers for SSP-PCR

There are 63 *ZDHHC14* SNPs on the AFFY 5.0 array. There were 2 SNPs with p<0.001, *rs4463281* and *rs17165436*. There were 8 SNPS with p<0.01 (Table 5). Both *rs4463281* and *rs17165436* were in the same haploblock (Figure 6.7). Therefore one other SNP in this block was chosen for SSP-PCR analysis, *rs17165429* (Table 6.2)

Of these 3 SNPs, *rs4463281* and *rs17165436* were retained for analysis. *Rs17165429* was omitted from analysis because the SSP-PCR assay failed.

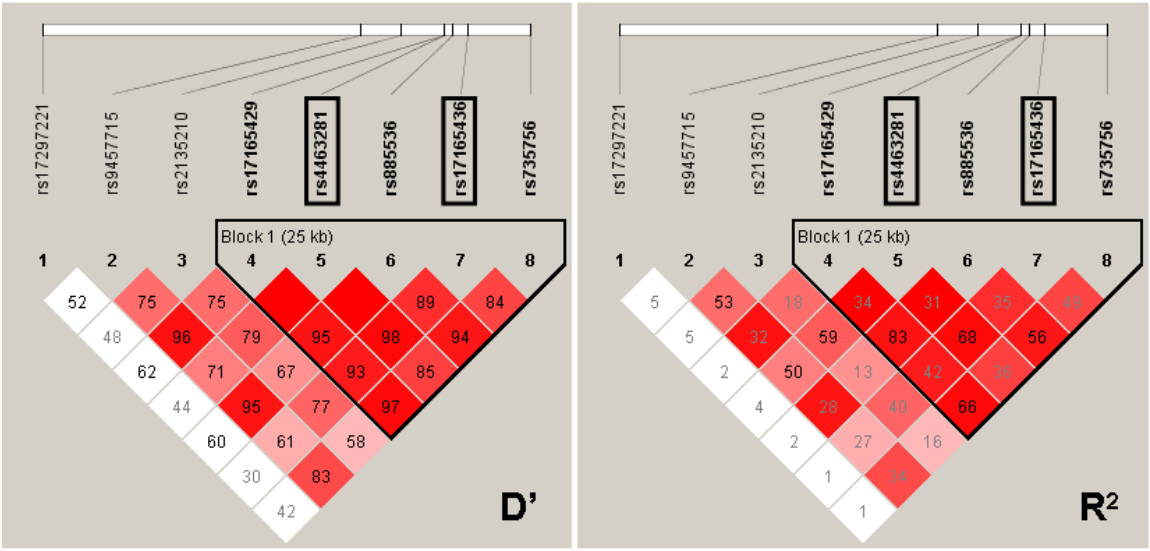

Figure 6.7 LD plots for ZDHHC14 in the "Affy" dataset

SNPs with p<0.001 with clinical phenotypes are highlighted

(Haploview version 4.0, Broad Institute, Cambridge, USA). SNPS with $-\log_{10}P>3$ highlighted

Table 6.2 ZDHHC14 SNPS (p<0.01) chosen from the "Affy dataset" as candidates for SSP-PCR

marker	rs_number	MAF	Call rate	HW p value	P *
snp_a1838804	rs9457715	47.41379	100	0.71307	0.003
snp_a1881342	rs17165436	45.17544	98.27586	0.707516	1.32×10^{-5}
snp_a1921155	rs735756	35.34483	100	0.545133	0.004
snp_a1927701	rs4463281	48.27586	100	0.35188	9.25×10^{-5}
snp_a2049841	rs17165429	28.01724	100	0.488516	0.002
snp_a2255670	rs17297221	14.22414	100	1	0.003
snp_a4202034	rs885536	26.57658	95.68965	0.470077	0.002
snp_a4246797	rs2135210	44.78261	99.13793	0.344902	0.002

* association with pain relief ≥70% on analysis of the "Affy" data set

NTRK3 primers for SSP-PCR

There are 166 *NTRK3* SNPs on the AFFY 5.0 array. There were 2 SNPs with p<0.001, *rs8026216* and *rs1075891*, for both clinical phenotypes (average pain ≤ 4 and pain relief ≥70%). There were 2 further SNPs (*rs1559822* and *rs17755717*) associated with pain relief ≥70%, p<0.001. . There were 13 SNPS in total with p<0.01 from the "Affy" set for either pain relief or average pain.

Rs8026216 and *rs1075891* made up one haploblock and *rs1559822* was part of a second haploblock. These 3 SNPs were included in stage 2 of the study using SSP-PCR. *Rs4887383* and *rs1863482* were also chosen as they were part of the latter haploblock. *Rs17755717* was also included as it was not in either of these haploblocks (Fig 6.8).

All NTRK3 SNPs which were chosen to run using SSP-PCR were analysed using both clinical phenotypes.

Table 6.3 NTRK3 SNPS (p<0.01) chosen from the "Affy dataset" as candidates for SSP-PCR

marker	rs_number	MAF	Call rate	HW p value	P value*	P value**
snp_a2173892	rs1559822	9.292035	97.4138	0.594337	0.02	0.0009
snp_a2303210	rs17755717	19.8275	100	0.042478	0.05	0.0004
snp_a2303391	rs8026216	11.63793	100	0.648292	0.0003	0.0005
snp_a2303394	rs1075891	11.73913	99.13793	1	0.0006	0.0003
snp_a4204914	rs4887382	10.43478	99.13793	0.607016	0.003	0.002
snp_a430356	rs1863482	9.91379	100	0.598687	0.02	0.003

6	4
*association with average pain ≤ 4 on analysis of the “Affy” data set **association with pain relief ≥70% on analysis of the “Affy” data set	

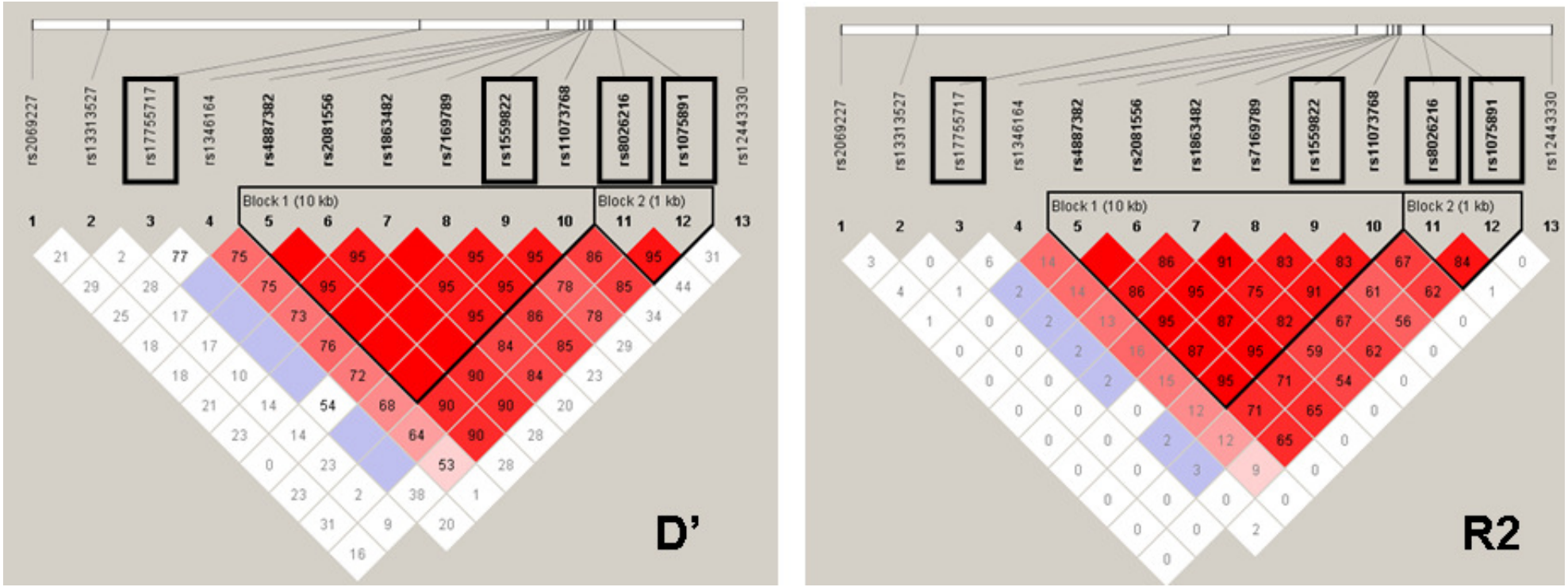

Figure 6.8 L D plots for NTRK3 in the "Affy" dataset

SNPs with p<0.001 with clinical phenotypes are highlighted.

(Haploview version 4.0, Broad Institute, Cambridge, USA). SNPS with $-\log_{10}P>3$ highlighted.

6.3.2.2 Stage 2: Analysis of "SSP-PCR" dataset

All SSP-PCR SNPs were in Hardy-Weinberg equilibrium. The call rates using SSP-PCR were 100% except for PRKD3 rs1158219 which was 99.2%.

As can be seen from tables 6.4 – 6.7 below, none of the associations between SNPs and clinical outcomes in the "Affy dataset" were replicated in the "SSP-PCR" datasets.

Three SNPs in *NTRK3* remained significantly associated with average pain in the SSP-PCR datasets; *rs1559822, rs4887382* and *rs1863482*. However, the direction of the genetic association with the clinical phenotype is the opposite i.e. the odds ratio changes direction / crosses 1 between the "Affy" and the "SSP-PCR" datasets. This change in odds ratio is mirrored by differences in genotype frequencies between the "Affy" and "SSP-PCR" datasets (Figure 6.9 and table 6.6).

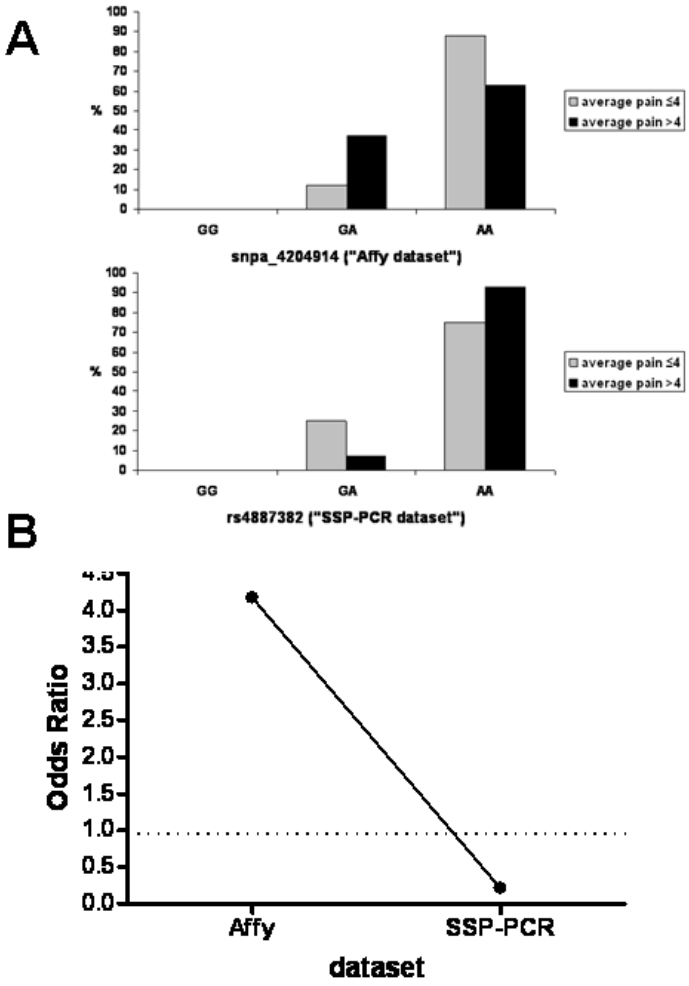

Figure 6.9 NTRK3 Flip-Flop

Figure A demonstrates the apparent "flip-flop" phenomenon in *NTRK3*. The association between *rs4887382* with average pain is the opposite of that between *snpa_4204914*, even though these are the same genetic variant. In the "Affy" dataset (*snpa_4204914),* a higher proportion of patients with average pain ≤ 4 have the AA genotype than patients with average pain> 4. In the "SSP-PCR" population (*rs4887382)*, a lower proportion of patients with average pain ≤ 4 have the AA genotype than patients with average pain > 4.

Figure B demonstrates the difference in Odds Ratio between the two datasets for the same SNP.

Table 6.4 ZDHHC14 and Pain relief

Data presented as % (N)									
		"Affy" dataset (N=115)				SSP-PCR dataset (N=125)			
rs number /genotype		**Pain relief ≥ 70% (N=75)**	**Pain relief < 70% (N=39)**	**P**	**OR**	**Pain relief ≥ 70% (N=88)**	**Pain relief < 70% (N=34)**	**P**	**OR**
rs17165436	CC	0.16 (12)	0.59 (23)			0.19 (17)	0.29 (10)	-	-
	CT	0.57 (43)	0.36 (14)	1.47×10^{-5}	4.9 (2.41-10.4)	0.58 (51)	0.47 (16)	-	-
	TT	0.27 (20)	0.05 (2)			0.23 (20)	0.24 (8)	-	-
rs4463281	GG	0.09 (7)	0.44 (17)			0.11 (10)	0.15 (5)	-	-
	GT	0.59 (44)	0.46 (18)	9.4×10^{-5}	4.108 (2.02-8.35)	0.57 (50)	0.5 (17)	-	-
	TT	0.32 (24)	0.1 (4)			0.32 (28)	0.35 (12)	-	-
- represents non-significant association Unadjusted p values presented.									

Table 6.5 PRKD3 and average pain ≤ 4

Data presented as % (N)									
		"Affy" dataset (N=115)				SSP-PCR dataset (N=125)			
rs number / genotype		**Average pain ≤ 4 (N=74)**	**Average pain > 4 (N=41)**	**P**	**OR**	**Average pain ≤ 4 (N=93)**	**Average pain > 4 (N=30)**	**P**	**OR**
rs1158219	AA	0.04 (3)	0.32 (13)			0.11 (10)	0.23 (7)	-	-
	AT	0.45(33)	0.46 (19)	7.7×10^{-5}	3.7 (1.94-7.1)	0.46 (43)	0.43 (13)	-	-
	TT	0.51(38)	0.22 (9)			0.43 (40)	0.33 (10)	-	-
- represents non-significant association Unadjusted p values presented.									

Table 6.6 NTRK3 and Average Pain

Data presented as % (N)									
rs number / genotype		**"Affy" dataset (N=115)**				**SSP-PCR dataset (N=125)**			
		Average pain ≤ 4 (N=74)	**Average pain > 4 (N=41)**	**P**	**OR**	**Average pain ≤ 4 (N=93)**	**Average pain > 4 (N=31)**	**P**	**OR**
rs17755717	GG	0.55 (41)	0.73 (30)	-	-	0.67 (62)	0.61 (19)	-	-
	GA	0.45 (33)	0.27 (11)	-	-	0.31 (29)	0.36 (11)	-	-
	AA	0(0)	0(0)	-	-	0.02 (2)	0.03 (1)	-	-
rs1559822	AA	0 (0)	0.07 (3)			0.01 (1)	0 (0)		
	AG	0.12 (9)	0.29 (12)	0.002	4.0 (1.7-9.6)	0.24 (22)	0.07 (2)	0.043	0.22 (0.05-0.96)
	GG	0.88 (65)	0.63 (26)			0.75 (70)	0.93 (29)		
rs8026216	TT	0 (0)	0.05 (2)			0.02 (2)	0 (0)		
	TG	0.11 (8)	0.37 (15)	0.0003	5.6 (2.2-14.1)	0.22 (20)	0.1 (3)	-	-
	GG	0.89 (66)	0.58)24)			0.76 (71)	0.9 (28)		
rs1075891	AA	0.01 (1)	0.02 (1)			0.02 (2)	0 (0)		
	AG	0.12 (9)	0.39 (16)	0.003	3.6 (1.6-8.5)	0.22 (20)	0.1 (3)	-	-
	GG	0.87 (64)	0.59 (24)			0.76 (71)	0.9 (28)		
rs4887382	GG	0 (0)	0 (0)			0 (0)	0 (0)		
	GA	0.12 (9)	0.37 (15)	0.003	4.2 (1.6-10.7)	0.25 (23)	0.07 (2)	0.043	0.21 (0.05-0.95)
	AA	0.88 (65)	0.63 (26)			0.75 (70)	0.94 (29)		
rs1863482	TT	0 (0)	0 (0)			0 (0)	0 (0)		
	TC	0.14 (10)	0.32 (13)	0.023	2.97 (1.2-7.6)	0.75 (70)	0.94 (29)	0.043	0.21 (0.05-0.95)
	CC	0.86 (64)	0.68 (28)			0.25 (23)	0.07 (2)		
- represents non-significant association Unadjusted p values presented.									

Table 6.7 NTRK3 and Pain Relief

Data presented as % (N)									
rs number / genotype		**"Affy" dataset (N=115)**				**SSP-PCR dataset (N=125)**			
		Pain relief ≥ 70% (N=74)	**Pain relief < 70% (N=41)**	**P**	**OR**	**Pain relief ≥ 70% (N=88)**	**Pain relief < 70% (N=34)**	**P**	**OR**
rs17755717	GG	0.49 (37)	0.85 (33)			0.63 (55)	0.74 (25)	-	-
	GA	0.51 (38)	0.15 (6)	0.001	0.177 (0.066-0.472)	0.35 (31)	0.23 (8)	-	-
	AA	0 (0)	0 (0)			0.02 (2)	0.03 (1)	-	-
rs1559822	AA	0.03 (2)	0.03 (1)			0.01 (1)	0 (0)	-	-
	AG	0.09 (7)	0.36 (14)	0.009	2.992 (1.312-6.821)	0.22 (19)	0.12 (4)	-	-
	GG	0.88 (66)	0.62 (24)			0.77 (68)	0.88 (30)	-	-
rs8026216	TT	0 (0)	0.05 (2)			0.02 (2)	0 (0)	-	-
	TG	0.12 (9)	0.36 (14)	0.001	4.896 (1.977-12.122)	0.21 (18)	0.12 (4)	-	-
	GG	0.88 (66)	0.59 (23)			0.77 (68)	0.88 (30)	-	-
rs1075891	AA	0.01 (1)	0.03 (1)			0.02 (2)	0 (0)	-	-
	AG	0.12 (9)	0.41 (16)	0.002	3.982 (1.689-9.386)	0.21 (18)	0.12 (4)	-	-
	GG	0.87 (65)	0.56 (22)			0.77 (68)	0.88 (30)	-	-
rs4887382	GG	0 (0)	0 (0)			0 (0)	0 (0)	-	-
	GA	0.12 (9)	0.39 (15)	0.002	4.583 (1.774-11.843)	0.23 (20)	0.12 (4)	-	-
	AA	0.88 (66)	0.62 (24)			0.77 (68)	0.88 (30)	-	-
rs1863482	TT	0 (0)	0 (0)			0 (0)	0 (0)	-	-
	TC	0.12 (9)	0.36 (14)	0.004	4.107 (1.579-10.678)	0.23 (20)	0.12 (4)	-	-
	CC	0.88 (66)	0.64 (25)			0.77 (68)	0.88 (30)	-	-
- represents non-significant association Unadjusted p values presented.									

6.3.3 Stage 3: Comparing "Affy" and "SSP-PCR" datasets

6.3.3.1 Assessing validity of genotyping methods

Validating "Affy" results against SSP-PCR data for 16 samples

All of the new SSP-PCR SNP assays were run on 16 DNA samples which were initially genotyped using the Affymetrix 5.0 array. The genotype data generated with the Affymetrix 5.0 array was compared to SSP-PCR data using Kendall's correction.

There was one data point out of 144 (0.7%) which differed between the "SSP-PCR" data and the "Affy" data (See table 6.8). In this instance the genotype for *PRKD3 rs1158219* was heterozygous using SSP-PCR but "Affy" designated it homozygous. This resulted in a correlation coefficient of 0.897.

Table 6.8 Comparing "Affy" results against SSP-PCR data for 16 samples

Gene	SNP name	"Affy" name	Correlation coefficient (Kendall)
ZDHHC14	rs17165436	SNP_A.1881342	1.0
	rs4463281	SNP_A.1927701	1.0
PRKD3	rs1158219	SNP_A.2037703	0.897
NTRK3	rs17755717	SNP_A.2303210	1.0
	rs1559822	SNP_A.2173892	1.0
	rs8026216	SNP_A.2303391	1.0
	rs1075891	SNP_A.2303394	1.0
	rs4887382	SNP_A.4204914	1.0
	rs1863482	SNP_A.4303566	1.0

Validating "Affy" results against SSP-PCR data for PRKD3 rs1158219

PRKD3 rs1158219 was run on all Caucasian subjects in the Prospective Morphine study to check validity of the "Affy" data against the SSP-PCR data. There was one case in which SSP-PCR data was not available. Out of 114 samples, there were 4 samples in which the "Affy" data did not matchup with the SSP-PCR data (correlation coefficient 0.959). In this instance, the genotype is clearly heterozygous on SSP-PCR (rerun twice) but on "Affy", 2 patients are designated homozygous for the major allele and 2 homozygous for the minor allele.

Affy / SSP-PCR	TT	TA	AA
TT	45	0	0
TA	2	51	2
AA	0	0	14

Figure 6.10 Comparing Affymetrix and SSP-PCR genotype results
PRKD3 rs1158219 genotypes generated by Affymetrix 5.0 compared to SSP-PCR in 114 study subjects.

6.3.3.2 Comparing "Affy" and "SSP-PCR" datasets

Because there were differences in the genetic associations with the clinical phenotypes between the "Affy" and SSP-PCR datasets in terms of significance of the findings and direction of association, the underlying structure of these datasets were compared in terms of genetic data (frequencies and LD) and clinical factors.

Comparing genotype frequencies

The overall genotype frequencies of the "Affy" and "SSP-PCR" datasets were compared (Table 6.9). The overall direction of allele frequencies were similar i.e. the minor allele was the same in all datasets and corresponds to the minor allele in previously published data in the HapMap CEU population.

Although there were no statistically significant differences between these overall genotype frequencies (tested using Chi^2 test), there were some apparent trend differences, especially in ZDHHC14 rs4463281. For this SNP, there was a higher proportion of patients homozygous for T and a lower proportion of patients homozygous for G in the SSP-PCR dataset than in the "Affy" dataset.

Table 6.9 Comparing genotype frequency data

Rs number	Genotype	Published genotype frequency (HapMap CEU)	"Affy" dataset (N=115)	SSSP-PCR dataset (N=125)
ZDHHC14				
rs17165436	CC	0.383	0.3 (35)	0.23 (29)
	CT	0.433	0.5 (58)	0.54 (68)
	TT	0.183	0.19 (22)	0.22 (28)
rs4463281**	GG	0.217	0.21 (24)	0.13 (16)
	TG	0.483	0.55 (63)	0.54 (68)
	TT	0.3	0.24 (28)	0.33 (41)
PRKD3				
rs1158219	TT	0.417	0.41 (47)	0.4 (50)
	TA	0.383	0.45 (52)	0.45 (56)
	AA	0.2	0.14 (16)	0.15 (18)
NTRK3				
rs17755717	AA	0.017	0 (0)	0.02 (3)
	AG	0.283	0.38 (44)	0.33 (41)
	GG	0.7	0.62 (71)	0.65 (81)
rs1559822	AA	0.033	0.03 (3)	0.01 (1)
	AG	0.183	0.18 (21)	0.2 (25)
	GG	0.783	0.79 (91)	0.79 (99)
rs8026216	GG	0.767	0.78 (90)	0.79 (99)
	TG	0.2	0.2 (23)	0.19 (24)
	TT	0.033	0.02 (2)	0.02 (2)
rs1075891	AA	0.017	0.02 (2)	0.02 (2)
	GA	0.203	0.22 (25)	0.19 (24)
	GG	0.78	0.76 (88)	0.79 (99)
rs4887382	AA	0.783	0.79 (91)	0.79 (99)
	GA	0.183	0.21 924)	0.21 (26)
	GG	0.033	0 (0)	0 (0)
rs1863482	CC	0.783	0.8 (92)	0.79 (99)
	TC	0.183	0.2 (23)	0.21 (26)
	TT	0.033	0 (0)	0(0)
**rs4463281 ""Affy"" vs "Second set" p=0.15				

Comparing Linkage disequilibrium

Pairwise linkage disequilibrium (LD) was calculated between SNPs in each gene using Haploview. (Whitehead Institute for Biomedical Research, USA).
The main differences in LD are seen between:

- *ZDHHC14 rs4463281* and *rs17165436*. The % LD is less in the "SSP-PCR" dataset than in the "Affy" dataset. (R^2 51% vs 71% and D' 88% vs 97% respectively)
- *NTRK3 rs17755717* and *rs8026216*. The D1 between these markers in the "Affy" dataset is 100% but in the "SSP-PCR" dataset is 2.4%.
- NTRK3 *rs1863482* and *rs17755717*. The D' between these markers in the "Affy" dataset is 100% but in the "SSP-PCR" dataset is 60%.

There are also other differences between NTRK3 SNPs in terms of D' (Table 6.10). The haploblock structure however, as defined by CI for D' [315], is similar in both the "Affy" and "SSP-PCR" datasets, as shown in figure 6.11.

Table 6.10 Pairwise LD between SNPs in NTRK3

		"Affy" dataset		"SSP-PCR" dataset	
SNP 1	**SNP 2**	**D'**	**R^2**	**D'**	**R^2**
rs17755717	rs4887382	1	0.028	0.603	0.01
rs17755717	rs1863482	1	0.026	0.603	0.01
rs17755717	rs1559822	0.546	0.009	0.718	0.014
rs17755717	rs8026216	1	0.031	0.024	0
rs17755717	rs1075891	0.383	0.005	0.024	0
rs4887382	rs1863482	0.951	0.862	1	1
rs4887382	rs1559822	1	0.876	1	0.959
rs4887382	rs8026216	0.903	0.715	0.912	0.765
rs4887382	rs1075891	0.901	0.656	0.912	0.765
rs1863482	rs1559822	0.95	0.753	1	0.959
rs1863482	rs8026216	0.847	0.599	0.912	0.765
rs1863482	rs1075891	0.844	0.548	0.912	0.765
rs1559822	rs8026216	0.786	0.617	0.873	0.731
rs1559822	rs1075891	0.782	0.563	0.873	0.731
rs8026216	rs1075891	0.957	0.844	1	1

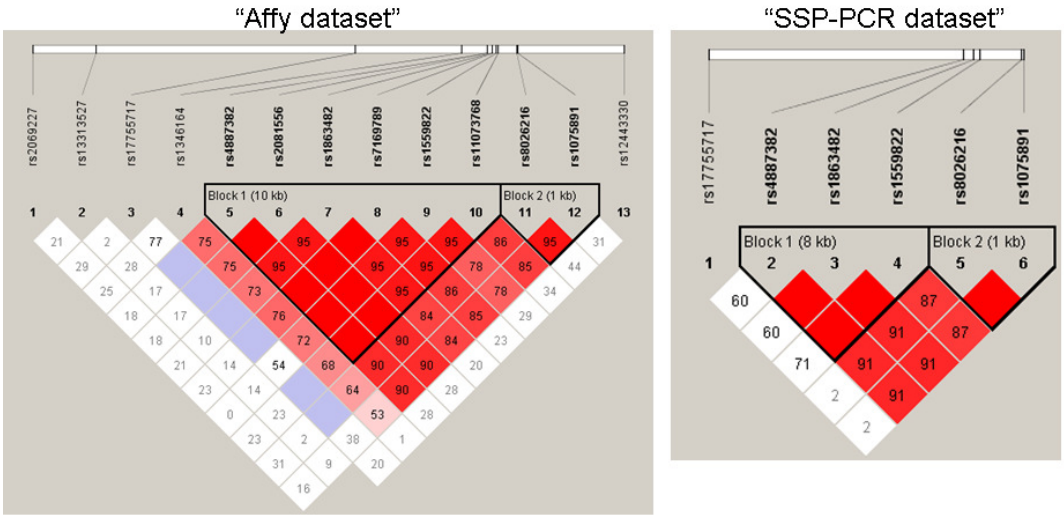

Figure 6.11 Comparing pairwise LD and haploblock structure in NTRK3

D' values are shown.

Comparing clinical factors

The “Affy” and SSP-PCR clinical datasets were compared (table 6.11). The study subjects in the “Affy” set had been on morphine for a shorter time than those in the SSP-PCR datasets, p=0.01. There was a higher proportion of morphine non-responders in the “Affy” dataset, p=0.003 and the “Affy” dataset had higher central side-effect scores, drowsiness and confusion/hallucinations, p=0.002 and 0.0001 respectively. There was a slight difference in albumin levels and the proportion taking a non-steroidal anti-inflammatory (NSAID) but the latter was not statistically significant. There was no difference in overall pain scores (analysed as both continuous and binary categorical data) between the “Affy” and “SSP-PCR” datasets.

Logistic regression was carried out on both “Affy” and “SSP-PCR” datasets to take into account any variability in the clinical phenotype which might have been accounted for by time on morphine, serum albumin or gender (the latter as this was included in the original analysis of the “Affy” dataset”). This however did not make any appreciable difference to the genetic association results (data not shown).

Table 6.11 Comparing Affymetrix and SSP-PCR data sets in terms of clinical factors

	Median (Range)	
	Affymetrix dataset (N=115)	**SSP-PCR second set (N=125)**
Age	60 (29-89)	59 (23-85)
Daily morphine dose (mg)	100 (10-1280)	100 (15-820)
Days to death	108 (5-1926)	182.5 (7-2147)
Days on morphine	**65 (1-2178)***	**105 (2-4455)***
Total White Cell Count	8.9 (0.3-68.1)	8.75 (2.8-30.6)
Serum albumin level	**29 (14-48)****	**31.5 (11-43)****
Serum urea level	4.5 (0.5-19.7)	5.05 (0.8-20.7)
Serum calcium level	2.23 (1.81-2.94)	2.24 (1.52-2.83)
Side-effect scores (0-3)		
Drowsiness	**2 (0-3)+**	**1 (0-3)+**
Confusion/hallucination	**1 (0-3)++**	**0 (0-3)++**
Bad dreams	0 (0-3)	0 (0-3)
Pain scores (NRS 0-10)		
Worst pain score	5 (0-10)	5 (0-10)
Least pain score	1 (0-8)	0 (0-8)
Average pain score	3 (0-9)	3 (0-8)
Pain now score	2 (0-10)	1.5 (0-9)
Pain relief	80 (0-100)	80 (0-100)
	% (N)	
Pain scores (categorical)		
Average pain ≤ 4/10	0.64 (74)	0.75 (93)
Pain relief ≥ 70%	0.66 (75)	0.72 (88)
Average pain ≤ 4/10 and Pain relief ≥ 70%	0.54 (62)	0.63 (77)
Gender		
Male	0.55 (63)	0.44 (55)
Female	0.45 (52)	0.56 (70)
Morphine response		
Morphine responder	**0.61 (70)**[¥]	**0.78 (98)**[¥]
Morphine non-responder	**0.39 (45)**[¥]	**0.22 (27)**[¥]
Diagnosis		
Upper gastrointestinal tract	0.14 (16)[¥¥]	0.06 (8)[¥¥]
Lower gastrointestinal tract	0.1 (11)	0.06 (8)
Pancreas and biliary	0.02 (3)	0.04 (4)
Gynaecological	0.05 (6)[¥¥]	0.12 (15)[¥¥]
Urogenital (renal and bladder)	0.09 (10)	0.1 (12)
Haematological	0.08 (9)	0.03 (4)
Breast	0.17 (19)	0.18 (23)
Lung (including mesothelioma)	0.11 (13)	0.17 (21)
Other medications		
B Blocker	0.06 (8)	0.08 (9)
Brochodiator	0.08 (9)	0.1 (12)
NSAID	0.3 (34)[¥¥]	0.42 (52)[¥¥]
Paracetamol	0.38 (44)	0.41 (51)
Steroid	0.37 (43)	0.34 (42)

Other drugs and diagnoses included in analyses but not significant: Aspirin Anticonvulsant Anticoagulant ACE Inhibitor, Antibiotic, Haloperidol, antiemetics, Benzodiazepines, Proton Pump Inhibitor, Tricyclic, SSRI and SNRI antidepressants, H2 Antagonist , Tumours of Skin, Unknown primary, Prostate, Head and Neck, Sarcoma.

* Days on morphine p=0.01

** Serum albumin level p=0.049

+drowsy score p=0.002, ++ confusion/hallucinations score p=0.0001

[¥]morphine responder / non-responder p=0.003, X^2 8.77

[¥¥]Trend towards significance 0.5< p<0.1

6.4 Discussion

6.4.1 Main Findings

- *NTRK3* and *PRKD3* were found to be associated with variability in average pain while *NTRK3* and *ZDHHC14* were associated with pain relief in the genome-wide association study. These findings were not replicated in the second patient sample using SSP-PCR.

- No single "pain" or "morphine response" gene was identified in this study.

- Genotype data generated by Affymetrix is comparable to that generated by SSP-PCR, thus validating both techniques.

6.4.2 Identification of novel candidate genes

To date studies examining association between genetic variation and response to morphine have focused on specific candidate genes which are known to play a biologically relevant role in morphine pharmacodynamics and kinetics including *OPRM*, the gene coding for the mu-opioid receptor and *COMT* (Catechol-O-methyltransferase gene) which codes for an enzyme involved in catecholamine metabolism. This is the first genome-wide association study (GWA) examining response to morphine in cancer pain. Neither *OPRM* nor *COMT* were identified in this study as being significantly associated with analgesic response to morphine. As discussed in chapter 3, variability in phenotype classification is a common reason for replication failure in genetic association studies [431]. It is not surprising therefore that commonly studied genes including *OPRM* and *COMT* were not found to be significant in this study. The phenotypes used in the *OPRM* and *COMT* studies have tended to be variability in daily morphine dose requirements, which, as demonstrated in chapters 3-5, is a distinct clinical phenotype to pain and side-effect scores.

In our study three novel genes were identified from the initial GWA as being possible candidates for association with analgesic response: *ZDHHC14, PRKD3* and *NTRK3*.

Both *PRKD3* and *NTRK3* are biologically plausible genes. *PRKD3* (protein kinase D3) codes for one of the protein kinase C family which is involved in cell signalling. [432]. *NTRK3* (Neurotrophic tyrosine kinase 3 receptor) is involved in the development of proprioceptive sensory neurones [433]. *NTRK3* mRNA levels change with chronic opioid exposure and withdrawal [434]. *NTRK3* is also thought to play a role in opioid-plasticity of the locus coeruleus, which in turn is involved in opioid dependence [434]. The function of *ZDHHC14* is as yet unknown. It is expressed throughout the body but has a higher expression in some areas including the spinal cord [435]. It is thought to probably code for palmitoyltransferase [436]. Palmitoylation is a process which increases the hydrophobicity of proteins and thus impacts on subcellular protein trafficking and protein-protein interactions [437]. Many proteins are known to undergo palmitoylation, including some G-protein coupled receptors [438]. *ZDHHC14* was identified as being possibly involved in the pathogenesis of post-transplant lymphoproliferative disorders and B-cell lymphoms [435]. *ZDHHC14* per se has not to date been associated with morphine response. However a recent mouse model of opioid tolerance demonstrated that repeated administration of morphine resulted in increased activity of spinal cord serine palmitoyltransferase [439]. This paper suggests that ceramide is involved in the development of morphine antinociceptive tolerance. One of the ways in which ceramide is generated involves serine palmitoyltransfease [439]. Palmitoylation has further been linked to pain through the identification of mutations in *SPTLC1*, the gene coding for serine palmitoyltransferase, in patients with hereditary sensory neuropathy [440].

Despite these three genes being associated with variability in the clinical outcome in the genome-wide-association analysis, these findings were not replicated in the validation SSP-PCR study. Therefore overall our study fails to identify a definitive "morphine response gene". What this study does do however is highlight a number of challenges associated with all genetic association studies, challenges which are worth considering and taking into account if further research into the pharmacogenomics of morphine is to be carried out.

The most likely reasons why this study failed to identify any single gene associated with morphine response are 1) inadequate sample size and 2) the complexity of morphine response as a clinical trait.

As discussed in chapters 3-5, pain perception and analgesic response to opioids are complex phenomena, influenced by not only by genetic factors but also by multiple physical, psychological, environmental and social factors. Similar to other complex diseases / traits, it is likely that pain perception and analgesic response are oligogenic, resulting from multiple gene-gene interactions. In this study we focused on identification of single independent associations. Epistasis was neither accounted for nor examined. The importance of epistasis is discussed in detail in chapter 8.

6.4.3 Sample size influences power to detect associations

A formal power calculation was not carried out for this genetic association study as the genotyping was carried out on samples that had been previously collected. The post hoc analysis of the genetic factors associated with clinical outcome variability in the GWA presented in this thesis can only be considered hypothesis generating [441]. It is likely that the sample size used in this study was simply too small to identify true associations with the clinical outcome. Such small numbers have been used previously and have been considered adequate, but in each case the clinical phenotype was well defined rather than the complex trait presented here. For example, in a study of statin-induced myopathy, a GWA of 318,237 SNPs was performed on 85 subjects and 90 controls using Illumina BeadChip. 2 SNPs were then chosen for replication in a cohort of 16,664 patients, of which 21 had myopathy (cases) and 16,643 did not (controls).

Some of the factors influencing sample size for a genetic association study include:

- The frequency of the susceptibility allele and the allelic odds ratio[442].
- The prevalence of the disease / trait in the general population.
- The number of SNPs to be tested.

These are discussed in more detail in chapter 8.

We used a multi-stage approach in our genetic association study as this has previously been proposed as a method to minimise required sample size [443]. In the first sample of patients in a multi-stage study, all SNPs are tested and a liberal p value is used to identify possible susceptibility SNPs. These SNPs are then tested in another population of similar or larger size, using a more stringent p value. A multi-stage approach is associated with little loss of power because of the low false-negative rate at stage 1 [444]. This multi-stage approach has been used successfully in a number of settings but there is no standard method to define significance thresholds. Some authors have proposed $p< 10^{-8}$ or $p<10^{-9}$ as evidence of strong association in GWAs [445;446]. It has been acknowledged that a strict Bonferroni correction, as was used in our study, may be too conservative, because it does not take into account the LD between SNPs. Therefore such a correction may dictate impossibly low p-values [443].

In the study of statin-induced myopathy, p-values $< 5 \times 10^{-7}$ were considered to provide strong evidence and $p< 5 \times 10^{-5}$ moderate evidence of association in the first stage. The significance of the association in the initial study set was $p = 4 \times 10^{-9}$ but in the second set was p=0.004 [447]. Another study examining genetic variants associated with height used a meta-analysis of GWA data on 13,665 individuals to choose 39 SNPs to genotype in a second population of 16,482 individuals. The data from the first and second groups were combined for final analysis. The significance threshold for the first group was set at $p < 1 \times 10^{-5}$. However SNPs were also included for genotyping in the second set if $p < 1 \times 10^{-4}$ and the SNP was in a region previously shown to be associated with either length in mouse studies or extreme changes in stature in human studies [448]. We adopted this approach in our study.

In general it is thought that the threshold for significance in the first sample should be set to ensure that 1) the power to detect genetic variants which account for a small proportion of the phenotypic variance is maintained, 2) genetic markers which do not meet significance are unlikely to achieve significance in the whole sample and 3) true positives are not rejected [443;444]. In our study, since 1×10^{-5} was the most significant p value in the initial screen of the first stage of the study ("Affy" dataset), we did not set a defined p value threshold for the first stage. This is a limitation of this study as it increased the risk of false-positive findings. Instead we chose SNPs to genotype in the second stage (SSP-PCR dataset) on the basis of:

- The lowest P values
- Occurring in genes in which there was more than one SNP with $p < 0.001$
- Occurring in genes in which there was a plausible biological explanation for involvement in morphine response
- Genes which appeared to have significant associations with >1 clinical phenotype

In multi-stage studies, there are no defined rules as to the genotyping techniques to be used in the second stage. It has been recommended that alternative methods to GWA be used because 1) these might be more suitable for genotyping smaller SNP sets and 2) this ensures that errors from technical genotyping issues are not replicated [443]. We used SSP-PCR to genotype the patients in the second sample set. The results of both methods demonstrated near 100% correlation in our data and allowed us to conclude that data generated from these methods was valid and comparable.

6.4.4 The genetic association results fail to replicate in the "Affy" and the "SSP-PCR" datasets

In our data there were differences in the strength of associations found in the first ("Affy") and the second ("SSP-PCR") datasets. Apart from the possibility that the initial findings were type 1 errors, other possible explanations for these disparities include a) differences in allele frequency and LD between the two populations and b) sample stratification bias due to some other factor.

We have previously mentioned how the power of a study is determined by the frequency and effect of the susceptibility allele [442]. One of the underlying premises of genetic association studies is that the observed allelic association may often represent a marker which is in some degree of LD with the true susceptibility allele.

Therefore power of the study to identify an association may be influenced by effect size of the marker allele. The effect size (odds ratio) of the marker allele in turn depends on 4 factors [80]:

- The effect size (OR) of susceptibility allele

- The extent of LD between the marker and susceptibility allele
- Marker allele frequency (mAF)
- Susceptibility allele frequency (sAF)

When mAF = sAF, the marker allele effect size is approximately equal to the susceptibility allele effect size multiplied by the LD between the 2 [80]. Therefore when mAF = sAF and when the LD between the 2 is complete, the effect size of the marker allele is similar to that of the susceptibility allele. However, if the susceptibility allele is either uncommon (frequency <0.1) or very frequent, no matter what the LD between the 2 markers, the dominant factor affecting marker effect is marker allele frequency. In this case even slight differences between the mAF and sAF can result in more marked differences between the effect size of the marker and susceptibility allele [80]. A change in mAF of <0.1 can result in a drop in power to detect the true susceptibility allele from 80 to 20% [431].

In terms of statistical power to detect an association, variation in LD between the marker and susceptibility alleles and discrepancies between mAF and sAF can be influential. If the effect size of the susceptibility allele is moderate, an association may only be detected if the susceptibility allele is common (>0.1) and either a) mAF is similar to SAF and / or b) the marker allele is in high LD with the susceptibility allele. [80]. If the effect of the susceptibility allele is high, these factors are less influential. If the effect size of the susceptibility allele is very low, the power to detect an association will remain low, despite high LD and similar mAF and SAF.

In our study we know neither the OR of the true susceptibility allele nor the true susceptibility allele frequency. By assuming that these remain stable, we can presume that if the effect size of the susceptibility allele is moderate or low (as is likely to be associated with common variants [80]), then a change in the marker allele frequency between the first and second datasets could certainly influence the power to detect an association with the clinical phenotype (Figure 6.13). This in turn could plausibly explain the perceived loss in significance of association with the clinical phenotype between the “Affy” and “SSP-PCR” datasets.

The change in LD between *ZDHHC14* and *NTRK3* markers in the "Affy" and SSP-PCR datasets might reflect differences in LD with the true susceptibility allele which may impact on the power of the study.

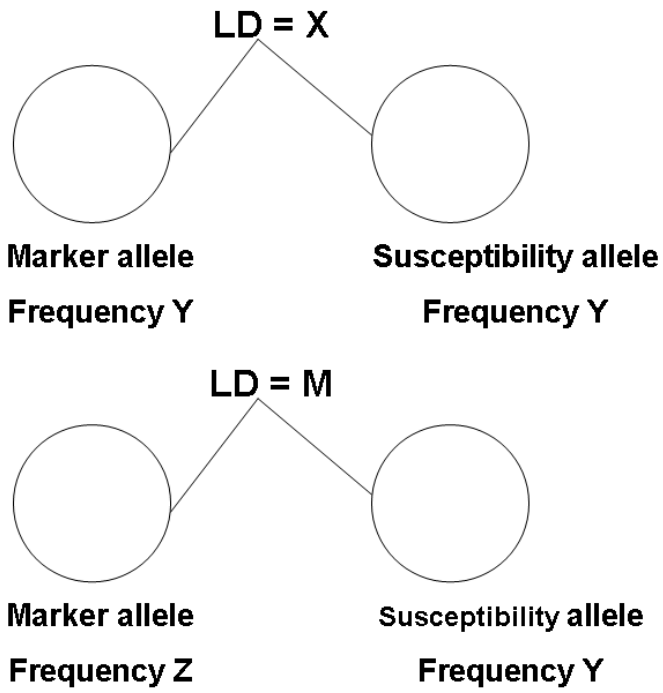

Figure 6.12 LD values may be determined by marker frequency values
A change in the frequency of the marker allele can change the LD between the marker allele and the susceptibility allele therefore changing the chances of detecting an association using the marker allele. (This is discussed in detail in chapter 2).

6.4.4.1 NTRK3 appears to be associated with a flip-flop

The most striking difference between the data for the *NTRK3* SNPs in the "Affy" dataset and the "SSP-PCR" datasets is the occurrence of an apparent "flip-flop" phenomenon when these datasets are examined according to clinical phenotype. For most SNPs in this gene the direction of the genetic association with the clinical phenotype appears to change between the two datasets. Conflicting genetic associations are not uncommon in the published literature. As discussed in Chapter 1, the G allele of OPRM A118G has usually been associated with higher opioid requirements for pain relief. One study however suggested that the A allele was associated with higher opioid dose requirements [85]. Variation in influences on

outcome by other non-characterised variants has been proposed as an explanation for this flip-flop phenomenon [449]. This occurs in two situations:

- When the SNPs being tested are merely markers for the true susceptibility SNP. Differences in correlation of the observed marker allele (which may or may not play a role in determining outcome) and another unobserved susceptibility allele (which may be either a protective or risk allele) may cause a change in the direction of allelic association. This is especially true if the susceptibility allele is common and there is weak LD between the unobserved susceptibility allele and the observed marker allele [449]. Flip-flop in genetic association may also occur when LD remains apparently stable [450]. This occurs because it is a change in the frequencies of haplotypes designated by the observed and unobserved susceptibility variants that brings about the flip-flop, rather than a change in LD itself.

- When multiple variants act together to bring about a particular outcome. The flip-flop may represent varying effects of the causal susceptibility SNP due to differences in genetic background and environment [449]. This is particularly relevant in genetic association studies of pain and analgesia since these traits are so complex and multi-factorial in nature.

Other common reasons for the flip-flop phenomenon include:

- Genotyping errors. This is unlikely in our study as the phenomenon is observed across multiple markers. In addition, the genetic data data was exactly the same when two genotyping platforms (Affymetrix and SSP-PCR) were used.
- Differences in LD patterns across different ethnic groups. Again this risk has been minimised as much as possible by only including white Caucasians in the study. However it is likely that even defining ethnicity in this manner results in inclusion of a somewhat heterogenous group of subjects because of population drift.
- Sampling variation. The “Affy” and “SSP-PCR” datasets were examined and compared in terms of clinical factors which might influence or be associated with pain and analgesia, but no striking differences were seen. However it is

extremely likely that there are other untested genetic and environmental factors contributing to sampling variation, of which we are simply unaware. (This is discussed further in chapter 3).

6.5 Conclusions

This study has identified a number of challenges in carrying out genetic association studies in pain and analgesic response, namely study size, study design, phenotype definition and data analysis. These challenges are not unique to studies of pain and neither are they insurmountable. Collaborations of investigators researching the same outcome would improve study power. Such collaboration has been proved possible in the EPOS study (European Pharmacogenetic Opioid Study) in which over 2000 cancer patients were recruited from 17 centres in 11 countries [135]. These factors must be considered when designing any genetic association study. Exploration of underlying biological interactions and epistasis will enrich the search for genetic associations. Correlation of genetic data with functional studies will be a further step towards validation of genetic findings. Identification of and focused study of biologically plausible interactions between genes and environment might yield more significant and useful results [451]. Hypothesis-free testing of hundred of thousands of genetic polymorphisms brings us closer to finding the true "pain genes" but do not negate the findings of other hypothesis-driven studies.

7 Chapter 7: Constipation in cancer patients taking morphine

7.1 Summary of the rationale behind this study

- Constipation is a significant problem in cancer patients.
- Opioids including morphine are a common cause of constipation in cancer and palliative care patients. 50-100% on patients taking opioids experience constipation [189;190].
- There has been relatively little study to date of assessment, management and risk factors for constipation on opioids.

7.1.1 Aims

The aims of this chapter were:

- To explore the prevalence and management of constipation in cancer patients taking morphine
- To present a clinically meaningful classification and definition of the phenotype of constipation on morphine
- To explore the clinical and genetic factors associated with constipation on morphine in cancer patients
- To examine the clinical assessment of constipation in cancer patients taking morphine
- To begin to develop a constipation assessment tool

The following chapter consists of a series of studies carried out in cancer patients taking morphine. The subjects and methods for each study are described briefly, as well as the main clinical findings. There is an overall discussion of all of the data at the end of the chapter.

7.2 Study 1: Examination of the prevalence and management of constipation in cancer patients taking morphine

7.2.1 Subjects and Methods

This part of the study was carried out using data collected as part of the Prospective Genetics Study (see chapter 2). In this study, patient subjective assessment of constipation during the preceding week was carried out using a four point adjectival scale: "not at all" (grade 0), "a little" (grade 1), "quite a bit" (grade 2), and "very much" (grade 3). This constipation assessment question is similar to that used in the validated European Organisation for Research and Treatment of Cancer Quality of Life Questionnaire [452]. Current medications including morphine and laxatives (type and dose) were also recorded.

Laxative dose groups (LDGs):
Patients who reported constipation were analysed according to their laxative use. In this study laxatives taken by the study subjects were classified in terms of Laxative Dose Groups (LDGs) (Table 7.1). The concept of LDGs is based on current recommendations that laxatives should be titrated according to individual patient response [453]. As with any other medication, individuals may vary in their sensitivity and tolerance of laxatives [195]. In some patients dose-limiting side-effects may necessitate rotation to an alternative laxative [454].

It is well known that palliative care patients are often prescribed medications at different (usually higher) doses that many other groups of patients. Taking this into account, LDGs were devised for each laxative using dosing information obtained from therapeutics manuals [455], reviews of laxatives [453] and symptom control guidelines specific for this patient group [456;457]. It was acknowledged that in general guidelines for the management of constipation in cancer patients are based on expert opinion as to date there is currently no evidence to support the use of one laxative over another. It was noted that in some studies and reports of laxatives, higher doses than those in the LDGs were used [212;458]. These higher doses were not incorporated into the LDGs because they were either being used under experimental conditions or

they are greater than those commonly used in cancer patients. It is possible that the use of vast doses of laxatives may be associated with an increased risk of side-effects. Similarly if the pill or liquid volume of laxatives is too great there is an increased risk of non-compliance in a palliative care and cancer population [459].

In this study laxative use by constipated patients was analysed in terms of LDGs in order to determine:

- The proportion of patients with inadequately treated constipation. These were defined as those patients who were constipated but were not on maximum recommended doses of laxatives. It is suggested that these patients could have their dose of laxatives increased in an attempt to alleviate the symptom of constipation. These patients were thought to have sub-optimally treated constipation.
- The proportion of patients who were constipated despite being on high / maximal laxative treatment. It is suggested that increasing the dose of that particular laxative in these patients would probably offer little added benefit. These patients were thought to have constipation which was resistant to that particular laxative.

Inter-individual response to the constipating effects of opioids was explored by looking for associations with the cancer diagnosis, factors associated with opioid therapy and concomitant factors which may be hypothesised to play a role in the pathogenesis of constipation.

Table 7.1 Laxative Dose Groups (LDG) for each laxative

For application to patients reporting constipation only
All doses are per 24 hours

Laxatives			Milpar (ml)	Senna (mg)	Danthron (mg)	Docusate (mg)	Bisacodyl (mg)	Movicol (Sachets)	Lactulose (ml)	
0	None		0	0	0	0	0	0	0	**Suboptimally treated constipation**
1	Subtherapeutic	Below usual starting dose	1-10	1 – 7.5	1 - 25	≤100	<5	1/2	1-10	
2	Low therapeutic	Within recommended dose	>10 ≤20	> 7.5 ≤15	> 25≤50	>100≤200	5 - 10	1	>10≤20	**Laxative dose could be increased**
3	Moderate therapeutic		>20 ≤40	>15 ≤ 30	>50 ≤100	>200 ≤400	>10 ≤20	2	>20 ≤ 40	
4	High therapeutic		>40 ≤60	> 30≤67.5	>100≤300	>400 < 600	>20 ≤ 40	3-4	> 40 ≤60	**Resistant onstipation**
5	Above usual therapeutic dose	Above normal recommended dose	>60	>67.5	>300	≥600	> 40	5-8	> 60	**Uncertain benefit to increasing laxative dose**

7.2.2 Results

274 patients were included in this study. 72% (197 / 274) of patients had mild to severe constipation (grade 1 – 3); 28% (77/274) had no constipation.
Of those who were not constipated, 53% (41/77) were not taking any laxatives.
Of the patients who said that they were constipated, 73% (144/197) were already taking laxatives but 27% (53/197) were not taking any laxatives.

Table 7.2 shows the proportion of patients with different degrees of constipation and the proportion of each group which is on laxatives.

Laxatives taken by the study population varied widely (Table 7.3). These were divided into predominately softening and predominately stimulating laxatives. Of the constipated patients in the study, 71% (139/197) were taking a softening agent and 53% (104/197) were taking a stimulating laxative. Only 50% (99/197) of constipated patients were taking a combination of a softener and a stimulant. (Figure 7.1)

75% (104/139) of constipated patients taking a stool softener and 77% (80/104) of those taking stimulant laxatives were on suboptimal laxative doses (LDG 0 to 3). 78% (77/99) of constipated subjects taking combination laxatives were also on a suboptimal dose. Therefore only 11% (22/197) of the study subjects who reported constipation had been titrated onto maximum recommended doses of combination laxatives (LDG 4 or 5).
In the total study population, 8% (22/274) were constipated despite being on high doses of laxatives. These were clinically deemed to have “resistant constipation” i.e. constipation that had not resolved on maximally titrated doses of that particular laxative / combination of laxatives.

89% (175/197) of constipated patients in this study group were on inadequate treatment for their constipation (no laxatives or suboptimal doses of laxatives).

Overall the results of this study demonstrate a distinct inter-individual variation in constipation on opioids with three main subgroups:

- Patients who do not experience constipation and do not need to take laxatives **(No constipation No laxatives).** This group comprises 15% (41/274) of study population.
- Patients who do not experience constipation *because* they are being adequately treated with laxatives **(No constipation On laxatives).** This group comprises 13% (36/274) of study population.
- Patients who experience constipation *despite* being on high dose combination laxatives **(Constipation On laxatives).** This group comprises 8% (22/274) of study population

Table 7.2 Proportion of patients on laxatives and not on laxatives according to the reported severity of constipation

% (N)					
	Grade 0 "Not at all"	**Grade 1 "A little"**	**Grade 2 "Quite a bit"**	**Grade 3 "Very much"**	**Total**
Not on laxatives	53 (41)	41 (28)	31 (21)	6 (4)	34 (94)
On laxatives	47 (36)	59 (40)	69 (46)	94 (58)	66 (180)
Total	(77)	(68)	(67)	(62)	(274)

Table 7.3 Laxatives used by study population

N (%)			
	All study subjects (N=274)	**Constipated study subjects (N=197)**	**Non constipated study subjects on laxatives (N=36)**
Predominantly softening			
Milpar *(magnesium hydrochloride and liquid paraffin emulsion)*	57 (21)	49 (25)	8 (22)
Docusate Sodium	12 (4)	11 (6)	1 (3)
Lactulose	14 (5)	12 (6)	2 (6)
Movicol *(polyethylene glycol)*	37 (14)	28 (14)	9 (25)
Predominantly stimulating			
Senna	57 (21)	43 (22)	14 (39)
Danthron containing laxatives (codanthramer, co-danthrusate)*	72 (27)	61 (31)	11 (31)
Bisacodyl	3 (1)	3 (2)	0

* docusate is classified as a stimulant in some refenences [455] and a softener in others [456].

** codanthramer and codanthrusate are combination of softener and stimulant laxatives

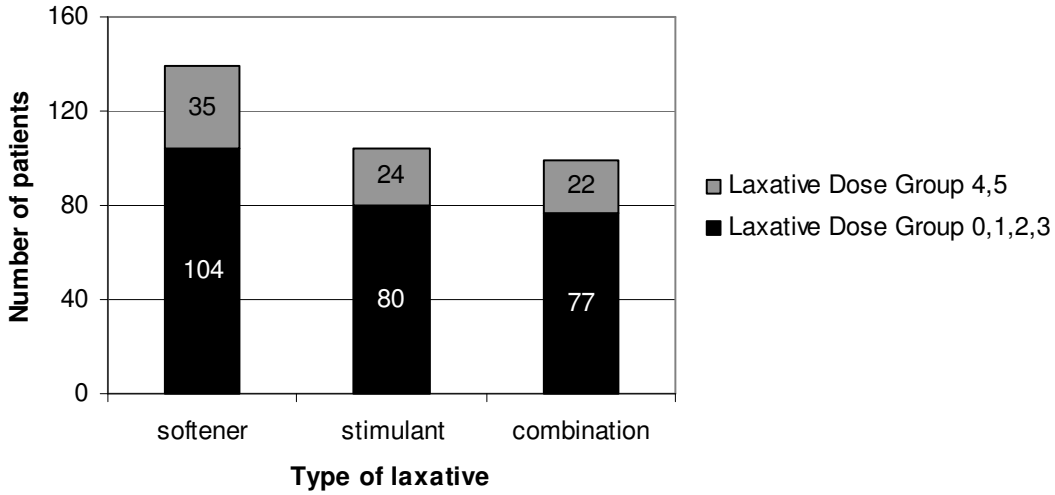

Figure 7.1 Type of laxatives taken by patients reporting constipation (N = 197)

- **Laxative Dose Group 0,1,2,3:** Sub-optimally treated constipation:
  Laxative dose could be increased
- **Laxative Dose Group 4,5:** Constipation resistant to that laxative / combination of laxatives:
  Uncertain benefit to increasing laxative dose

7.3 Study 2: Constipation on morphine in cancer patients: Clinical phenotype definition

7.3.1 Subjects and Methods

As previously, this part of the study was carried out using data collected as part of the Prospective Genetics Study (see chapter 2). Details about a number of other clinical variables were collected including demographic data, cancer diagnosis, concurrent medications (including laxatives), daily dose of morphine and length of time on morphine.

The study population was refined in order to define a tight clinical phenotype. Patients taking morphine for less than 1 week were excluded from the analysis because 1) most definitions of constipation are based around the period of a week and 2) the questionnaire about constipation in the study related symptoms experienced during the preceding week. Nine patients had incomplete data about concurrent laxatives and were therefore excluded from analysis. As the clinical phenotype was defined for use in the genetic association study, only Caucasian patients were included in order to reduce the risk of population stratification (discussed in chapter 8) in the genetic association analysis.

In our previous study, we identified three distinct groups of cancer patients taking morphine: 1) patients who did not experience constipation and who were taking no laxatives, 2) patients who did not experience constipation because they were on adequate laxative treatment and 3) patients who did experience constipation despite laxative treatment. The third group were further subdivided into those who were on suboptimal laxative treatment and those who remained constipated despite high dose combination laxatives.

In this study we re-classified these groups to allow comparison between subjects with bowel problems on morphine (N=199) those with no bowel problems (N=37). Patients who reported being constipated were included in the "bowel problems" group, whether they were on laxatives or not. There were a number of patients who felt that

they were not constipated but were taking laxatives. It was assumed that these patients required laxatives to maintain normal bowel function on morphine thus these patients were also included in the "bowel problems" group. The small group of patients (N=37) who were defined as having no bowel dysfunction on opioids were those who were not subjectively constipated and did not need to take any laxatives (Figure 7.2).

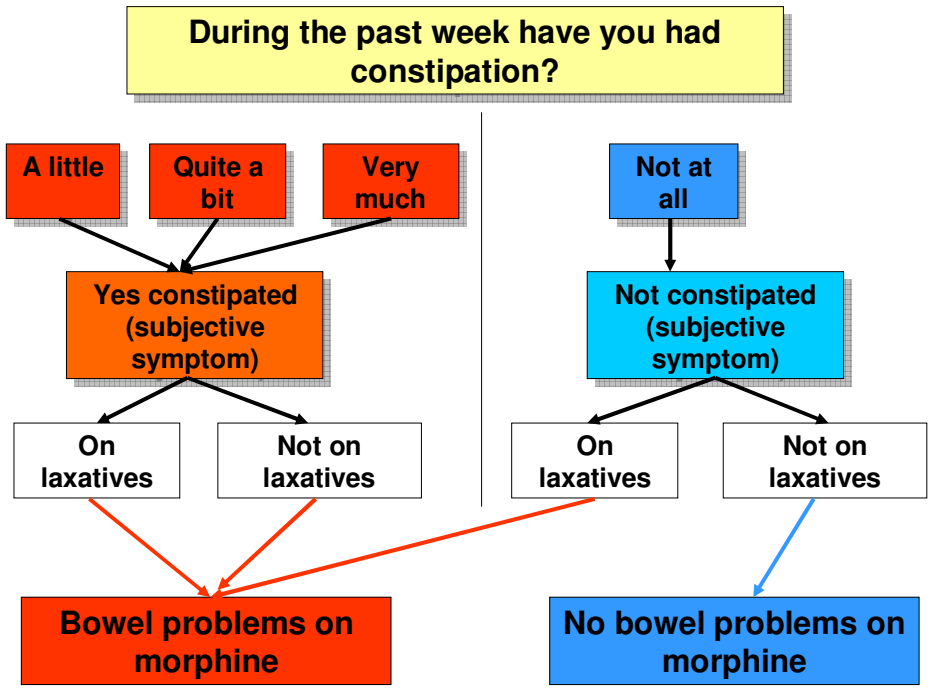

Figure 7.2 Defining the "Constipation on opioids" clinical phenotype

7.3.2 Results

236 Caucasian patients who had been taking oral morphine for at least one week were included in the analysis.

The subjective patient-reported severity of constipation is shown in table 7.4 together with details about laxative use.

The two clinical groups (those with bowel problems on morphine and those without) were compared in terms of clinical variables which might be associated with constipation (Table 7.5). There was no difference in terms of age, gender, time on morphine or dose of morphine between those who had bowel problems on morphine and those who did not. There was no difference in the proxy measure of disease status (time to death) between the 2 groups. Proxy measures of hydration (urea and haematocrit) did not differ between the 2 groups. There was no difference between the two groups in terms of cancer diagnosis except for tumours of the lower gastrointestinal tract. A smaller proportion of patients with bowel problems on morphine had a diagnosis of a tumour of the lower gastrointestinal tract compared to those without bowel problems (p=0.008). There was no difference between the groups however when all tumours involving the abdominal cavity were assessed together. The two groups were compared in terms of proportion of subjects taking medications (including chemotherapy within the preceding 4 weeks) which might alter bowel function but again there was no significant difference between the 2 groups.

Table 7.4 Severity of constipation and laxative use in study population

% (N)	**Grade 0 "Not at all"**	**Grade 1 "A little"**	**Grade 2 "Quite a bit"**	**Grade 3 "Very Much"**	**Total**
Not on laxatives	54 (37)	45 (27)	26 (15)	4 (2)	81
On laxatives	46 (31)	55 (33)	74 (42)	96 (49)	155
Total	68	60	57	51	236

Note: this table differs from table 7.2 as the study population has been refined to define a tight clinical phenotype for use in the genetic association study.

Table 7.5 Other clinical factors associated with constipation

Data presented as N (%) or median (range)		
	No bowel problems on morphine (n=37)	**Bowel problems on morphine(n=199)**
Male	19 (51.4)	98 (49.2)
Female	18 (48.6)	101 (50.8)
Morphine dose (mg / 24 hours)	95 (10-720)	110 (15-1280)
Time to death (days)	151 (18 – 2126)	121 (5 – 2590)
Days on morphine	120 (10 – 4455)	84 (7 – 4319)
Age	61 (29-83)	59 (19-85)
Urea	4.2 (1.1-11.1)	4.8 (0.5-20.7)
Haematocrit	0.341 (0.264-0.504)	0.344 (0.237-0.518)
Serum calcium (corrected for serum albumin)	2.25 (1.52 – 2.56)	2.23 (1.61 – 2.94)
Other constipating medications*	9 (24)	48 (24)
Chemotherapy that may cause constipation **	6 (16)	29 (15)
Chemotherapy that may cause diarrhoea***	8 (22)	32 (16)
Diagnosis ¥¥		
Upper gastrointestinal tract	4 (11)	17 (9)
Lower gastrointestinal tract ¥	**7 (19)**	**12 (6)**
Pancreas and biliary	2 (5)	5 (3)
Gynaecological	2 (5)	20 (10)
Urogenital (renal and bladder)	2 (5)	20 (10)
Haematological	2 (5)	12 (6)
Breast	5 (14)	37 (19)
Sarcoma	2 (5)	26 (13)
Lung (including mesothelioma)	6 (16)	24 (12)
Head and Neck	4 (11)	14 (7)
Melanoma	0 (0)	6 (3)
Prostate	2 (5)	15 (8)
Other	1 (3)	6 (3)
Lower gastrointestinal tract / gynaecological / pancreas	11 (30)	37 (19)

* includes 5HT3 antagonists, antimotility / antispasmodic / antimuscarinic agents, tricyclic antidepressants

** Vinca alkaloids, platinums, thalidomide

***5-fluorouracil, methotrexate, irinotecan, taxanes

¥¥ Some patients have more than 1 tumour diagnosis

¥ p=0.008, X^2 7

7.4 Study 3: Constipation on morphine: genetic association study

This is the first study of pharmacogenetic factors associated with constipation as a side-effect of an opioid. Other genetic association studies of response to morphine for cancer pain have tended to focus primarily on variation in the level of pain control or the dose of morphine required [92;94;124]. Some of these studies did include a constipation assessment score in secondary analysis however the number of genetic variants tested has been limited and no genetic association was found [92;110]. In this study the follwing genes were tested against the clinical phenotype of constipation in cancer patients taking morphine: OPRM, OPRD, OPRK, ADRA2A, ADRB2 and SLC6A4. Full details about these genes are found in Chapters 4 and 5.

Since morphine and other opioids cause constipation by acting as agonists at peripheral opioid receptors, the genes coding for the opioid receptors were the most obvious candidates for inclusion in this study. Mu, delta and kappa opioid receptors are found throughout the gastrointestinal tract and are involved in gastrointestinal function [202;203].

ARRB2, the gene coding for intra-cellular B-arrestin 2 proteins was also chosen. These proteins act as inhibitors to receptor coupling and are negative regulators of opioid receptor signalling [125]. B-arrestin2 knockout mice have reduced constipation on morphine [460]. These data suggest that B-arrestin2 plays an important role in mediating constipation on opioids [461].

ADRA2A codes for the α_{2A} adrenergic receptor (α_{2A} AR). Knockout studies have shown that α_{2A} AR mediates the sympathetic inhibition of intestinal motility. α_{2A} AR knockout mice have reduced gastrointestinal transit time as compared to wild-type animals [388].

Serotonin is synthesized in enterochromaffin cells in the GIT and plays a number of important roles in gut function. It is a key signalling molecule in GI motility, secretion and sensation. A study examining human colonic samples demonstrated reduced availability of serotonin in the mucosa of patients with Ulcerative Colitis (UC) and Irritable Bowel Syndrome (IBS) compared to healthy controls [462]. Serotonin is

inactivated by SERT, the serotonin reuptake transporter, encoded by the gene SLC6A4. All enterocytes express SERT. Treatment with mice with paroxetine, a known inhibitor of SERT, resulted in delayed GI transit and reduced stool output [463]. SERT -/- mice demonstrate increased gut water and motility and have alternating diarrhea and constipation [464].

High serotonin levels can cause increased bowel motility and secretion [465]. Cisapride and tegaserod are 5HT4-receptor agonists which are effective in the treatment of constipation through prokinetic activity. Unfortunately both these drugs are associated with cardiovascular side-effects. Prucalopride is a newer 5HT4-receptor agonist which does not appear to be limited by these side-effects [466;467]. Prucalopride has been recently granted UK marketing authorisation for the symptomatic treatment of chronic constipation in women in whom laxatives fail to provide adequate relief [468]. Due to some conflicting functional data however, some authors have suggested that in fact increased synaptic serotonin levels can result in a down-regulation in serotonin receptors with reduced serotonergic effect therefore slowing gut motility [302].

There have been a number of studies examining the role of this polymorphism in IBS but the results have been conflicting, especially in terms of the association either diarrhoea or constipation predominant disease. In one study the presence of the S/S genotype was associated with constipation-predominant IBS and the L/L genotype with the diarrhoea-predominant variant [302]. In other studies the S/S genotype was associated with diarrhoea-predominant IBS [303 301]. This polymorphism did not withstand a recent meta-analysis in this area [465]. This polymorphism has also been studied in relation to anxiety [414].

7.4.1 Subjects and methods

This part of the study was carried out using data collected as part of the *Prospective Genetics Study*. Subjects with bowel problems on morphine (N=199) were compared to those with no bowel problems (N=37) as defined in the previous section.

7.4.2 Results

Overall there were no highly significant genetic associations between constipation on morphine and SNPs in *OPRM, OPRD* or *OPRK*. There was a weak association with an intron in *OPRD, rs2236857*. A higher proportion of patients with bowel problems on morphine carried the T allele at this position (genotype TT or CT), p=0.035. Logistic regression analysis was carried out to examine any possible association between the constipation phenotype and SNPs in the opioid receptor genes while controlling for the variability accounted for by a diagnosis of a lower GI tract tumour (Tables 7.6 – 7.8). *OPRD rs2236857* was also significant in this model. This association however does not remain statistically significant after correcting for multiple testing.

There were no significant associations with haplotypes in any of these genes (not shown).

There was no significant association between constipation on morphine and any of the polymorphisms or haplotypes in SLC6A4, ADRA2A or ADRB2 (Appendix F).

Table 7.6 OPRM and Bowel problems on morphine

		Allele carriage (dominant model)				Genotype frequency (additive model)				
		Bowel problems on morphine					Bowel problems on morphine			
SNP name	Allele	No (N=37)	Yes (N=199)	OR (95% CI)	P value	Genotype	No (N=37)	Yes (N=199)	OR (95% CI)	P value
rs6912029	G	1.0 (35)	1.0 (186)	-	-	GG	0.86 (30)	0.92 (171)		
	T	0.14 (5)	0.08 (15)	0.484 (0.161-1.46)	0.198	GT	0.14 (5)	0.08 (15)	2.065 (0.685-6.23)	0.198
						TT	0 (0)	0 (0)		
rs1799971	A	0.97 (34)	0.96 (179)	0.677 (0.077-5.963)	0.725	AA	0.77 (27)	0.74 (138)		
	G	0.23 (8)	0.26 (48)	1.014 (0.463-2.632)	0.823	AG	0.2 (7)	0.22 (41)	0.893 (0.435-1.833)	0.757
						GG	0.03 (1)	0.04 (7)		
rs589046	C	0.94 (32)	0.95 (172)	1.28 (0.265-6.187)	0.759	CC	0.44 (15)	0.54 (98)		
	T	0.56 (19)	0.46 (84)	0.708 (0.334-1.501)	0.368	CT	0.5 (17)	0.41 (74)	1.312 (0.712-2.419)	0.384
						TT	0.06 (2)	0.06 (10)		
rs563649	C	1.0 (34)	0.97 (177)	<0.001	0.999	CC	0.74 (25)	0.82 (149)		
	T	0.27 (9)	0.18 (33)	0.555 (0.232-1.326)	0.185	CT	0.26 (9)	0.15 (28)	1.385 (0.656-2.925)	0.393
						TT	0 (0)	0.03 (5)		
rs9479757	A	0.23 (8)	0.2 (37)	0.808 (0.334-1.954)	0.636	AA	0.03 (1)	0.005 (1)		
	G	0.97 (34)	0.995 (185)	6.407 (0.389-105.5)	0.194	AG	0.2 (7)	0.195 (36)	1.365 (0.611-3.049)	0.448
						GG	0.77 (27)	0.8 (149)		
rs2075572	C	0.74 (26)	0.83 (153)	1.711 (0.72-4.068)	0.224	CC	0.34 (12)	0.34 (62)		
	G	0.66 (23)	0.66 (123)	1.031 (0.475-2.239)	0.939	CG	0.4 (14)	0.49 (91)	1.179 (0.702-1.978)	0.534
						GG	0.26 (9)	0.17 (32)		
rs533586	T	0.88 (30)	0.89 (162)	1.077 (0.336-3.449)	0.9	TT	0.5 (17)	0.46 (84)		
	C	0.5 (17)	0.54 (98)	1.138 (0.539-2.402)	0.734	CT	0.38 (13)	0.43 (78)	1.057 (0.605-1.846)	0.847
						CC	0.12 (4)	0.11 (20)		

Logistic regression corrected for clinical factors (lower GI tumour)
Data are given as % (N)

Table 7.7 OPRK and Bowel problems on morphine

		Allele carriage (dominant model)				Genotype frequency (additive model)				
		Bowel problems on morphine					Bowel problems on morphine			
SNP name	Allele	No (N=37)	Yes (N=199)	OR (95% CI)	P value	Genotype	No (N=37)	Yes (N=199)	OR (95% CI)	P value
rs10504151	T	0.97 (33)	0.98 (179)	1.606 (0.173-14.93)	0.677	TT	0.85 (29)	0.79 (144)		
	C	0.15 (5)	0.21 (39)	1.546 (0.553-4.321)	0.406	TC	0.12 (4)	0.19 (35)	1.302 (0.541-3.136)	0.556
						CC	0.03 (1)	0.02 (4)		
rs7836120	A	0.97 (33)	0.96 (175)	0.784 (0.094-6.527)	0.822	AA	0.71 (24)	0.68 (124)		
	G	0.29 (10)	0.32 (59)	1.029 (0.454-2.333)	0.945	AG	0.26 (9)	0.28 (51)	1.05 (0.525-20.98)	0.891
						GG	0.03 (1)	0.04 (8)		
rs6473799	T	0.91 (31)	0.96 (175)	2.547 (0.632-10.27)	0.189	TT	0.62 (21)	0.59 (107)		
	C	0.38 (13)	0.42 (76)	1.191 (0.553-2.565)	0.655	TC	0.29 (10)	0.37 (68)	0.965 (0.512-1.818)	0.911
						CC	0.09 (3)	0.04 (8)		
rs1365098	G	0.85 (29)	0.91 (166)	1.682 (0.562-5.033)	0.352	GG	0.6 (19)	0.43 (80)		
	T	0.44 (15)	0.57 (103)	1.633 (0.77-3.46)	0.201	GT	0.29 (10)	0.47 (86)	1.172 (0.658-2.09)	0.59
						TT	0.15 (5)	0.09 (17)		
rs7016778	T	1.0 (34)	0.99 (181)	<0.0001	0.999	TT	0.73 (25)	0.73 (133)		
	A	0.27 (9)	0.28 (50)	0.964 (0.414-2.244)	0.932	TA	0.27 (9)	0.26 (48)	1.013 (0.451-2.275)	0.975
						AA	0 (0)	0.01 (2)		
rs7824175	G	1.0 (34)	0.98 (179)	<0.0001	0.999	GG	0.73 (25)	0.8 (146)		
	C	0.27 (9)	0.2 (37)	0.863 (0.356-2.09)	0.744	GC	0.27 (9)	0.18 (33)	0.973 (0.437-2.168)	0.947
						CC	0 (0)	0.02 (4)		
rs16918875	C	1.0 (34)	0.995 (182)	<0.0001	1.0	CC	0.88 (30)	0.83(152)		
	T	0.12 (4)	0.17 (31)	1.547 (0.507-4.882)	0.433	CT	0.12 (4)	0.165 (30)	1.591 (0.525-4.825)	0.412
						TT	0 (0)	0.005 (1)		
rs963549	G	0.97 (33)	0.96 (176)	0.531 (0.058-4.888)	0.576	GG	0.68 (23)	0.65 (119)		
	A	0.32 (11)	0.35 (64)	1.28 (0.571-2.868)	0.549	GA	0.29 (10)	0.31 (57)	1.285 (0.64-2.58)	0.48
						AA	0.03 (1)	0.04 (7)		

Logistic regression corrected for clinical factors (lower GI tumour)
Data are given as % (N)

Table 7.8 OPRD and Bowel problems on morphine

Allele carriage (dominant model)						Genotype frequency (additive model)				
		Bowel problems on morphine					Bowel problems on morphine			
SNP name	Allele	No (N=37)	Yes (N=199)	OR (95% CI)	P value	Genotype	No (N=37)	Yes (N=199)	OR (95% CI)	P value
rs1042114	T	1.0 (34)	0.98 (178)	<0.0001	0.999	TT	0.79 (27)	0.73 (131)		
	G	0.21 (7)	0.28 (51)	1.438 (0.581-3.562)	0.432	GT	0.21 (7)	0.25 (47)	1.534 (0.662-3.551)	0.318
						GG	0 (0)	0.02 (4)		
rs533123	A	0.97 (33)	0.96 (175)	0.529 (0.057-4.868)	0.574	AA	0.68 (23)	0.63 (114)		
	G	0.32 (11)	0.37 (68)	1.262 (0.57-2.795)	0.566	GA	0.29 (10)	0.33 (61)	1.27 (0.64-2.519)	0.494
						GG	0.03 (1)	0.04 (7)		
rs419335	A	0.82 (28)	0.92 (168)	2.577 (0.892-7.448)	0.08	AA	0.41 (14)	0.48 (91)		
	G	0.59 (20)	0.5 (91)	0.691 (0.324-1.473)	0.339	AG	0.41 (14)	0.42 (77)	0.65 (0.373-1.131)	0.127
						GG	0.18 (6)	0.08 (14)		
rs2236857	C	0.47 (16)	0.43 (78)	0.82 (0.387-1.735)	0.603	CC	0.15 (5)	0.05 (9)		
	T	**0.85 (29)***	**0.95 (173)***	3.544 (1.081-11.613)*	**0.037**	TC	0.32 (11)	0.38 (69)	0.683 (0.383-1.22)	0.198
						TT	0.53 (18)	0.57 (104)		
rs2234918	C	0.65 (22)	0.66 (120)	1.038 (0.474-2.273)	0.926	CC	0.21 (7)	0.18 (32)		
	T	0.79 (27)	0.82 (150)	1.164 (0.457-2.965)	0.751	CT	0.44 (15)	0.49 (88)	0.971 (0.574-1.642)	0.911
						TT	0.35 (12)	0.34 (62)		

Logistic regression corrected for clinical factors (lower GI tumour)
Data are given as % (N)
* Nagelkerke $R^2 = 0.079$

7.5 Study 4: Constipation assessment audit

As we previously demonstrated, the management of constipation in cancer patients is poor. We proposed that one of the reasons for such poor constipation management might be related to inadequate symptom assessment. We carried out a retrospective audit on a random sample of the patients who had been included in the original study to evaluate 1) the documentation of constipation *assessment* and 2) the documentation of constipation *management* in nursing and medical notes. We aimed to identify areas in which documentation of constipation assessment and management could be improved.

7.5.1 Subjects and methods

This retrospective audit was carried out on a random sample of patients who had originally participated in the constipation on morphine study (section 7.2). The audit was approved by the Royal Marsden Audit Committee.

We used information from the constipation on morphine study regarding whether or not the patient had reported being constipated at the time of recruitment to the study. We accessed the medical, nursing and electronic patient records to carry out the audit. The audit focused on the clinical episode during which the patient had been recruited onto the constipation on morphine study. These clinical episodes varied from 2002 to 2008. If the patient was recruited to the constipation on morphine study during an outpatient clinic, then the episode only lasted 1 day. However, for follow up questions, e.g. was the change in laxative reviewed, subsequent outpatient appointment letters were reviewed. For inpatients, the clinical episode lasted until discharge from the hospital or death of the patient, if that occurred during the same inpatient stay.

The audit standards were derived from the literature:

- Constipation assessment involves regular reassessment of usual bowel pattern, stool consistency, straining and frequency.
- Constipation is a significant problem in cancer patients and should be proactively assessed at every clinical episode.

In addition, adherence to the local nursing care standard of using a dedicated "bowel care plan" if the patient is known to have constipation, was assessed. An audit proforma was designed to assess documentation of constipation assessment and management (Appendix G). Data on three key areas were recorded: 1) evidence as to whether constipation was identified as a problem or not, 2) documented evidence of assessment of constipation and 3) documentation of laxative use.

In order to ensure accuracy of data collection, two data collectors completed the audit proforma on 5 patients initially and the results were compared.

We aimed to audit at least 33.3% of the records of patients who had participated in the original study.

Where the patient had attended an outpatient clinic, the electronic patient record and/or the medical notes were reviewed. In these cases there was no separate nursing assessment.

The data were analysed using descriptive statistics. Differences between patients known to the specialist palliative care team and those not known were assessed using chi-squared test. In most cases subgroup analysis was not feasible due to small numbers. In some cases there was missing data from the patient paper records, e.g. the nursing documentation from that period was not available. In these cases the missing cases were excluded and the total cases of non-missing data were used in the analysis. In each case the total numbers of cases available for analysis are presented in brackets.

7.5.2 Results

109 sets of patient records were analysed. Of these two were omitted because the patient was in bowel obstruction at the time. Therefore 107 patients' data were included in the audit.

74% (79/107) of episodes occurred in the inpatient oncology unit and 26% (28/107) in the outpatient setting.

There was documentation that the palliative care team were involved in 63% (68/107) cases.

Identification of constipation:

A substantial proportion of patients (37%) who stated that they were constipated in the *Prospective Morphine Study* were not identified in the medical records as being constipated by the medical or nursing teams (figure 7.3). Nearly 20% of these patients had severe (grade 3) constipation.

When constipation was correctly documented as having been identified as a problem, in most cases this was documented by the nursing staff or the palliative care team.

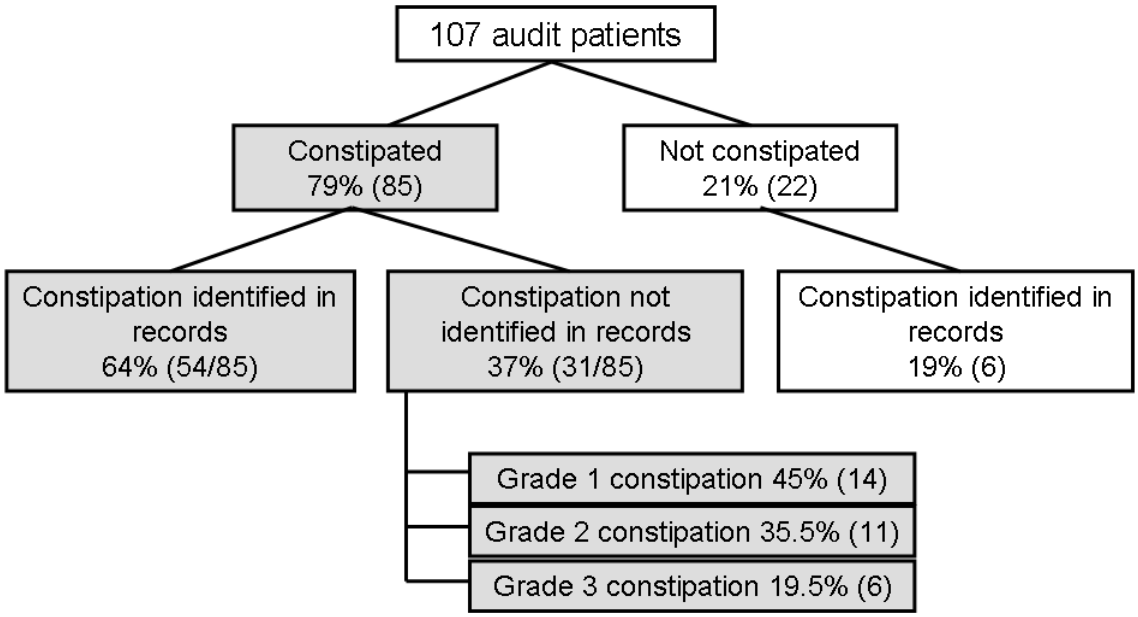

Figure 7.3 Identification of constipation in medical records
Documentation of constipation as an issue in the medical records was compared to whether the patient felt that he/she was constipated or not. The latter information was collected as part of the *Prospective Morphine Study.*

Documentation of constipation assessment:

62% (66/107) of patients had some documentation of bowel function within the first one – two days of the clinical episode. Of those who did not (41/107), 42% (17/41) had mild (grade 1) constipation, 17% (7/41) moderate (grade 2) and 17% (7/41) severe (grade 3) constipation.

Of those patients who did have documentation of bowel function within the first couple of days, in half of cases there was documentation about bowel frequency. Documentation about stool consistency or straining at stool was found in much fewer medical records (Figure 7.4). Only 1 patient had documented details about all three aspects of the constipation assessment. When the palliative care team were involved, stool consistency and straining were documented in a higher (but not statistically significant) proportion of cases.

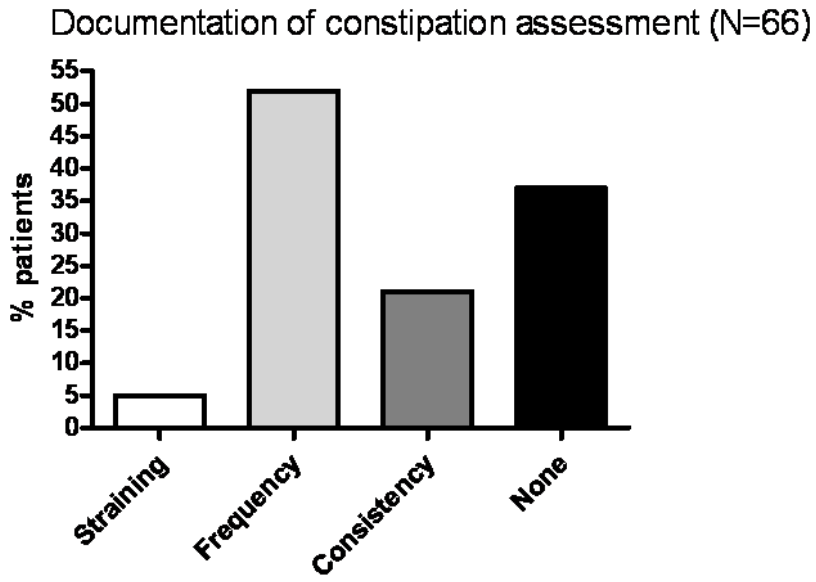

Figure 7.4 Documentation of identification and assessment of constipation.
Documentation of assessment of the three key constipation assessment questions (straining at stool, frequency of evacuation and stool consistency). There was documentation of bowel function in the patient notes within the first one-two days of the clinical episode in 66 cases. The graph details the proportion of these patients in whose records these assessment questions were documented.

There was documentation of the patient's usual bowel pattern in 33% (32/97) of patient records. (There was missing data on this question in 10 cases.) 53% (17/32) of these were recorded in the medical notes and in 69% (22/32) in the nursing notes.

60 patients were identified as being constipated, 51 of which were inpatient. Of these 39% (18/46) had evidence that the bowel care plan was being used. (The bowel care plan is an inpatient nursing paper document therefore patients who were only seen in the outpatient department at the time of the clinical episode were excluded from this analysis. There were missing data in five cases).

Documentation of constipation management:

50% (50/100) patients were on no laxatives at the start of the clinical episode. (These data were missing in 7 cases).

Of those who were constipated (according to the *Prospective Morphine Study* data), 45% (36/80) were on no laxatives at the start of the clinical episode. 58% (21/36) of these had either grade 2 or 3 constipation (figure 7.5). (There were data missing on this question in five cases.)

Of those who were constipated but on no laxatives at the start of the clinical episode, constipation was identified by the clinical team in 58% (21/36) cases. Laxatives were initiated in 86% (18/21) of these patients.

22 patients were not subjectively constipated. 70% (14/20) of those who were not constipated were on no laxatives (missing data on 2 patients) (figure 7.5).

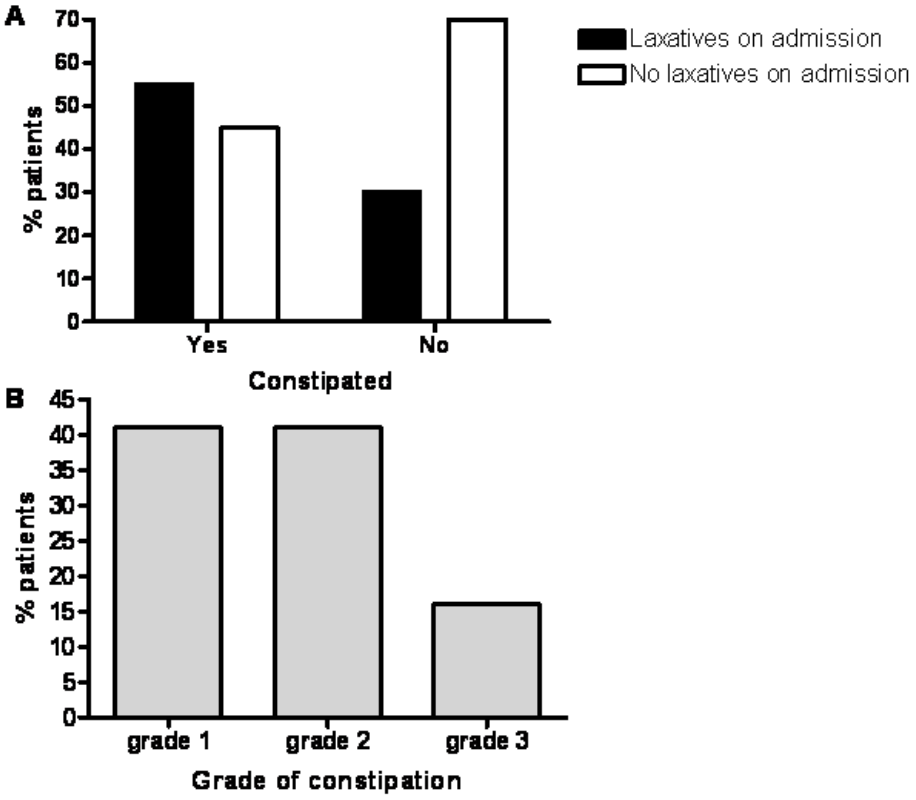

Figure 7.5 Documentation of constipation management

A: The proportion of patients who were subjectively constipated who were on laxatives on admission / at the start of the clinical episode.

B: The severity of constipation of patients who were subjectively constipated but who were on no laxatives at the start of the clinical episode (N=36)

Of those 60 patients who had been identified as being constipated, 46 patients in total had documented evidence that their laxative regimen was changed (type of laxative changed, laxative initiated or dose titrated), missing data in 3 cases. Of these, 40 were inpatients. In 90% of these inpatients (36/40) there was documentation that the response to the change was reviewed in either the medical notes.

Ninety two patients were known to be alive at the end of the episode. Of these 78% (72/92) had stated that they felt they were constipated in the Prospective Morphine Study. There was documentation that 51% (47/92) of patients who were still alive at the end of the clinical episode had been identified as being constipated in the patient notes. 43% (20/47) of these had follow-up plans documented.

7.6 Study 5: Constipation assessment tool

I designed a substantial amendment to the protocol of the *Randomised Controlled Trial of Oral Morphine versus Oral Oxycodone* which was approved by the MHRA, REC and CCR. The main objective of this amendment was to add the Constipation Assessment Tool in order to:

- Begin development of a clinically useful constipation assessment tool.
- Improve the definition of constipation as a phenotype to be used clinically and in trials.
- Promote improved rational management of this symptom with available laxatives according to the Royal Marsden symptom control guidelines [283].

The aims of this study were:

- Assessment of the patient's experience of constipation in a cancer.
- Comparison of subjective constipation assessment measures (e.g. 11 point numerical rating scale, categorical adjectival scale) that are often used in research and clinical practice
- Comparison of patient subjective assessment of constipation with objective assessment of constipation in a cancer and palliative care population
- Identify the items to be used in a composite constipation assessment score.

This study was not designed to determine the efficacy of the constipation assessment tool as there is no comparison group. Similarly a comparison of constipation scores between subjects on morphine versus those on oxycodone was not carried out as this will form part of the final study analysis.

The data presented here represent part of the interim analysis for the *Randomised Controlled Trial of Oral Morphine versus Oral Oxycodone*. The full data set will be analysed once the study is complete.

7.6.1 Subjects and Methods

The study population was comprised of patients recruited to the *Randomised Controlled Trial of Oral Morphine versus Oral Oxycodone*.

Each patient recruited to the study completed the Constipation Assessment Tool (Appendix B) at recruitment (Time Point A) and each week for four weeks afterwards. They also completed the questionnaire again if they reached another particular study time point. If the completed questionnaire suggested that the study subject was constipated, the research team liaised with either the clinical palliative care team, the oncology team or the community medical teams so that the patient's laxatives could be optimised according to the Royal Marsden symptom control guidelines [283].

The study subject was also given a copy of the daily Stool and Laxative diary to fill out to aid assessment over the phone. It was anticipated that not all patients would fill out this diary.

7.6.2 Constipation Assessment Tool Items

Items for the constipation assessment tool were identified through literature review and are detailed in Appendix A. The tool consisted of subjective and objective constipation scores.

All questions in the tool involved assessment of bowel function over the preceding week.

Subjective bowel assessment:

- The 11 point NRS (0 = no constipation and 10 = worst imaginable). This scale was in the original protocol for the *Randomised Controlled Trial of Oral Morphine versus Oral Oxycodone*. It has also been used in other studies assessing constipation [252;469].

- The four point categorical adjectival scale: “not at all” (grade 0), “a little” (grade 1), “quite a bit” (grade 2), and “very much” (grade 3). This assessment question was used by our research group in the previous study (*Prospective Morphine Study*) and is similar to that used in the EORTC Quality of Life Questionnaire, QLQ-C30 [274-276] and Rotterdam Symptom Checklist [277;278].
- A seven point adjectival scale in which the study subject rated their bowel function overall from “severe diarrhoea” to “severe constipation”. A bipolar scale has been recommended by Sykes [470] (and personal communication) as over-treatment of constipation may cause diarrhoea.
- A simple binary satisfaction question.

A free-text question about the subjective meaning of constipation was also included.

Objective bowel assessment:

In terms of the objective assessment of constipation, items relating to frequency of bowel movements, ease of defecation and stool consistency were included as the key questions in constipation assessment, as discussed in chapter 1. Questions relating to the subjects usual bowel pattern were also included.

- **Stool consistency**
  Study subjects were asked the question“Was your stool hard during the past week”? They were asked to grade their response on a four point adjectival Likert scale: Never (grade 0), rarely (grade 1), occasionally (grade 2), nearly always (grade 3).

- **Ease of evacuation of stool:**
  Study subjects were asked whether they had to strain to open their bowels in the past week. They were asked to score this on a four point adjectival Likert scale and this scale was graded numerically accordingly: Not at all (grade 0), a little (grade 1), quite a bit (grade 2), very much (grade 3).

- **Frequency in the past week**

Study subjects were asked about their usual frequency (usual number of bowel movements per week) and also how many times they had opened their bowels in the past week. The difference between the usual and the current frequency of bowel movements was calculated. A score was allocated to each patient as detailed in table 7.9.

Table 7.9 Stool frequency score

Frequency of bowel movements in the past week compared to usual frequency	Stool frequency score
>6 more than usual	-3
4-5 more than usual	-2
2-3 more than usual	-1
Usual bowel frequency per week ± 1	0
2-3 less than usual	1
4-5 less than usual	2
>6 less than usual	3

- Study subjects were also asked to about their stool consistency using the Bristol Stool Chart. The responses were then scored as detailed in table 7.10.

Table 7.10 Scoring using the Bristol Stool Chart

Bristol Stool Chart	Bowel Stool Chart score
Type 1	3
Type 2	2
Type 3	1
Type 4	0
Type 5	-1
Type 6	-2
Type 7	-3

7.6.3 Data analysis

Initially all the data (regardless of time point) were analysed together as one large dataset in order to:

- Test the rates of incorrect / missing responses for each constipation assessment question
- Explore any clustering of the different scales and variables using principal components analysis
- Carry out psychometric analyses: validity (convergent, discriminant and concurrent) and internal consistency. This was done in accordance with recent US Food and Drug Administration (FDA) guidelines on Patient Reported Outcome Measures [471].
- Decide which assessments should be included in a Constipation Assessment Tool and to devise an overall composite constipation assessment score.

The data were then analysed per time point (week 0 and week 1) to analyse overall change in constipation scores over time.

7.6.3.1 Assessment of the rates of incorrect response

Correct and incorrect responses were defined as per Jensen et al [472]. A correct response was defined as a response within the stated scale guidelines. An incorrect response was one in which the study subject did not respond according to the scale guidelines or when the response was ambiguous e.g. not answered at all, more than one box ticked on the scale, the response fell between two marks on the scale or extended beyond the boundaries of the scale or presented a range of responses.
If the study subject presented a range of responses which were within one point from each other on the scale, then the higher point was taken. If the subject presented a range of responses which were more than one point from each other, the response was discounted. The proportion of incorrect and missing responses was calculated for each scale.

7.6.3.2 Psychometric analyses

The convergent validity, i.e. the extent to which the items in the assessment tool correspond with other items relationship was evaluated using Kendall's tau correlation coefficient. (Kendall's tau was chosen as the dataset was small and the results non-parametric).

Principal components analysis (PCA) was used to assess whether there was any clustering between the subjective and objective assessment questions of constipation. Component scores were calculated using the regression method. All components with eigenvalues >1 were retained [319].

Discriminant validity, the ability to distinguish between distinct patient populations was assessed by measuring the differences in each item between those who were satisfied with their bowel function and those who were not satisfied.

Concurrent validity was measured by assessing correlation between the subjective and objective items and the previously validated Bristol Stool Chart.

Internal consistency, the extent to which all items measured a similar construct was determined using Cronbach α. The subjective and objective items were assessed as subscales separately.

The study was not designed to assess test-retest reliability. Missing data was not considered in the analysis at this exploratory stage.

7.6.3.3 Comparison of constipation scores across time

Constipation scores were compared across time i.e. week 0 versus week 1 and week 0 versus weeks 1-4 using non-parametric paired tests (Wilcoxon signed rank and Friedman's ANOVA).

7.6.4 Results

Out of 53 eligible patients, 52 completed the initial assessment, 40 the week 1 assessment, 33 the week 2 assessment, 18 the week 3 assessment, 19 the week 4 assessment, 12 an extra assessment at a different study time-point. Together there were 174 constipation assessments. Initially these were considered all together in order to 1) compare the different constipation scales, 2) test correlations between the assessment scales, 3) test the reliability of the scales and 4) assess whether or not these scales were answered correctly by the patients.

In the free text question, "What does being constipated mean to you", 39.4% (41/104) of patients included something about altered stool frequency. (There was no response to this question in 70 assessments).

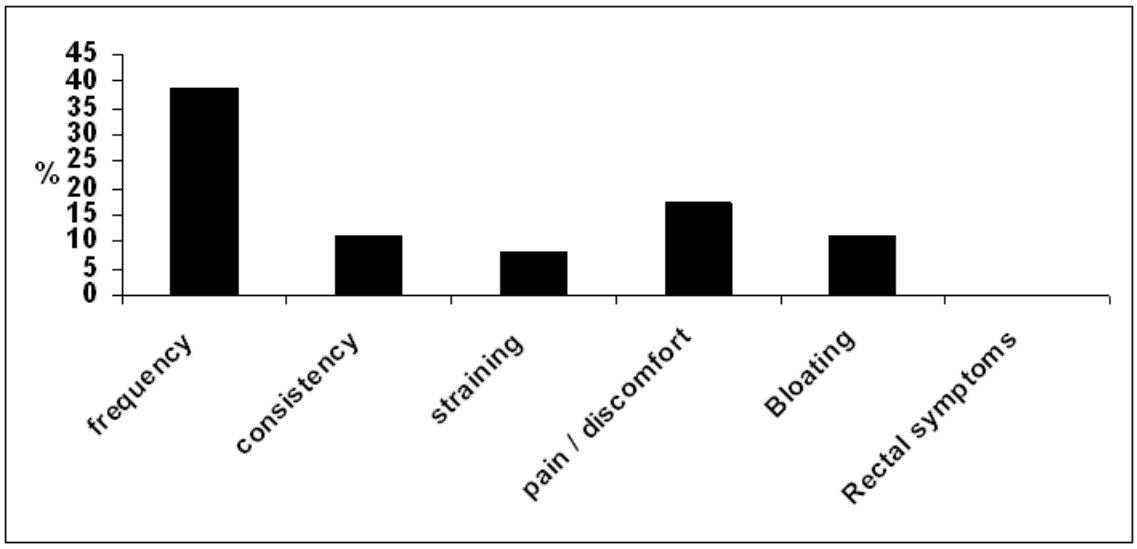

Figure 7.6"What does being constipated mean to you".

65% (113/173) felt that they were subjectively constipated, according to the question in which they were asked to grade their constipation on a four point adjectival scale. (This was not answered in one assessment). Of these, 62% reported opening their bowels less frequently than usual, 56% had hard stool and 73% had experienced some degree of straining.

61.5% (32/52) of patients at time of recruitment (initial assessment) felt that they were constipated yet 28% of these (9/32) were on no laxatives.

38.5% (20/52) patients at time of recruitment felt they were not constipated. In total 33% (17/52) were not constipated and were taking no laxatives.

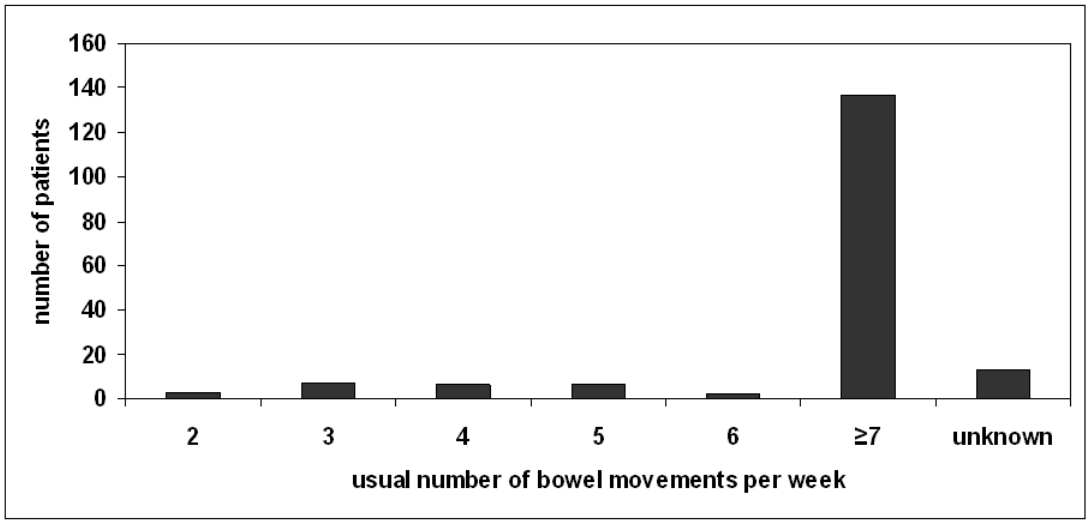

Figure 7.7 Usual bowel frequency

When asked about their usual bowel frequency, most study subjects (79%) reported opening their bowels once a day.

7.6.4.1 Assessment of the rates of incorrect response

The numbers of patients with incorrect or missing responses for each constipation assessment question are shown in table 7.11. There were fewer missing / incorrect responses for the subjective than for the objective constipation assessment questions. The highest rate of incorrect / missing responses was found with the Bristol Stool Chart, the second highest with the question about "usual frequency of bowel movements". When compared to the question with the highest correct responses (satisfaction with bowel function), these were highly significant.

Table 7.11 Proportion of patients who gave correct / incorrect responses to the constipation assessment scales

	Number of responses for each assessment scale N (%)			
Scale	**Total responses**	**Correct response**	**Incorrect response / No response**	**P value***
Subjective assessments				
Satisfaction with bowel function	173 (99.4)	173 (99.4)	1 (0.6)	
Grade of constipation	173 (99.4)	173 (99.4)	1 (0.6)	
Overall bowel function*	171 (98)	165(95)	9 (5)	0.02
NRS constipation	168 (96.5)	168 (96.5)	6 (3.5)	NS
Objective assessments				
Stool Frequency past week*	163 (94)	162 (93)	12 (7)	0.003
Usual Stool Frequency*	161 (92.5)	154 (88.5)	20 (11.5)	0.00001
Bristol Stool Chart score*	168 (96.5)	150 (86)	24 (14)	$7x10^{-7}$
Grade hard stool	171 (98)	171 (98)	3 (2)	NS
Grade need to strain	167 (96)	167 (96)	7 (4)	NS

* Proportion of correct versus incorrect / missing response compared to response rates for "satisfaction with bowel function" or "grade of constipation" question.

NS = not significant when compared to "satisfaction with bowel function" or "grade of constipation".

7.6.4.2 Psychometric analyses

Convergent and concurrent validity: Correlation of the subjective and objective constipation assessment scores:

Four subjective constipation assessment scores and four objective scores were compared using Kendall's correlation (Table 7.12)

Satisfaction with bowel function during the past week correlated most closely with grade and NRS of constipation severity, overall bowel function and frequency of bowel movements in the past week. Satisfaction correlated poorly with the score on the Bristol Stool Chart, the grade of straining or grade of stool hardness in the past week.

The other three subjective constipation assessments i.e. grade and NRS of constipation severity and overall bowel function were highly correlated (R=0.789 – 0.820). All of these correlations were significant suggesting shared variance among the assessment scales. These three subjective measures were moderately correlated with the grade of straining (R = 0.459 – 0.48), grade of stool hardness (R<0.4) and frequency (R=0.497-0.542)

There was moderate correlation between the three objective constipation assessments, frequency, straining and stool consistency.

The Bristol Stool Chart was most highly correlated with stool consistency (R = 0.573). There was only weak correlation between the subjective constipation assessment items and the Bristol Stool Chart (-0.153 - 0.358).

Table 7.12 Correlations between subjective and objective constipation assessment scores

	Subjective constipation assessments				Objective constipation assessments			
	Satisfied with bowel function	**Grade of constipation**	**Overall bowel function**	**NRS constipation**	**Stool Frequency**	**Grade hard stool**	**Grade need to strain**	**Bristol Stool Chart score**
Satisfied with bowel function	**1.000**	-0.548	-0.416	-0.484	-0.481	-0.201	-0.291	-0.153
Grade of constipation		**1.000**	0.796	0.820	0.526	0.377	0.480	0.249
Overall bowel function			**1.000**	0.789	0.542	0.358	0.465	0.358
NRS constipation				**1.000**	0.497	0.360	0.459	0.290
Stool Frequency					**1.000**	0.378	0.461	0.421
Grade hard stool						**1.000**	0.545	0.573
Grade need to strain							**1.000**	0.419
Bristol Stool Chart score								**1.000**

Comparison between objective measures of factors associated with constipation and subjective measures of constipation severity:

Principal components analysis was used to assess whether there was any clustering between the subjective and objective assessment questions of constipation.

Seven variables were included in this analysis: grade of constipation, NRS score of constipation severity, overall bowel function, difference in frequency of bowel motions pre week (usual frequency minus current frequency), score on Bristol Stool Chart, grade of straining and grade of stool hardness. The sample size was statistically adequate (Overall Kaiser-Meyer-Olkin value 0.853, Bartlett's test of sphericity <0.001). The individual KMO values ranged from 0.771 – 0.945 suggesting sampling adequacy.

Two components were identified with eigenvalues of 3.999 and 3.217 respectively (Table 7.13 and figure 7.8). After rotation component 1 was comprised primarily of the subjective constipation assessment questions i.e. grade and NRS of constipation severity and overall bowel function. Frequency of bowel movements per week was also included in this component but with a much lower loading than the others (table 7.13) Component 2 was comprised of the more objective constipation assessment questions i.e. the Bristol Stool Chart score, the grade of stool hardness and to a lesser degree, the grade of straining.

These components are not independent of each other, as evidenced by the component correlation score, R=0.528.

Table 7.13 Pattern matrix showing the loading of each variable onto each component after rotation

	Component	
	1	**2**
Eigenvalue (after rotation)	3.999	3.217
% variance explained	63.3	15.5
Grade of constipation	0.978	
Overall bowel function	0.975	
NRS constipation	0.916	
Stool Frequency	0.556	
Bristol Stool Chart score		0.921
Grade hard stool		0.918
Grade need to strain		0.557
Extraction Method: Principal Component Analysis. Rotation Method: Oblimin with Kaiser Normalization. Factors <0.4 are not shown here.		

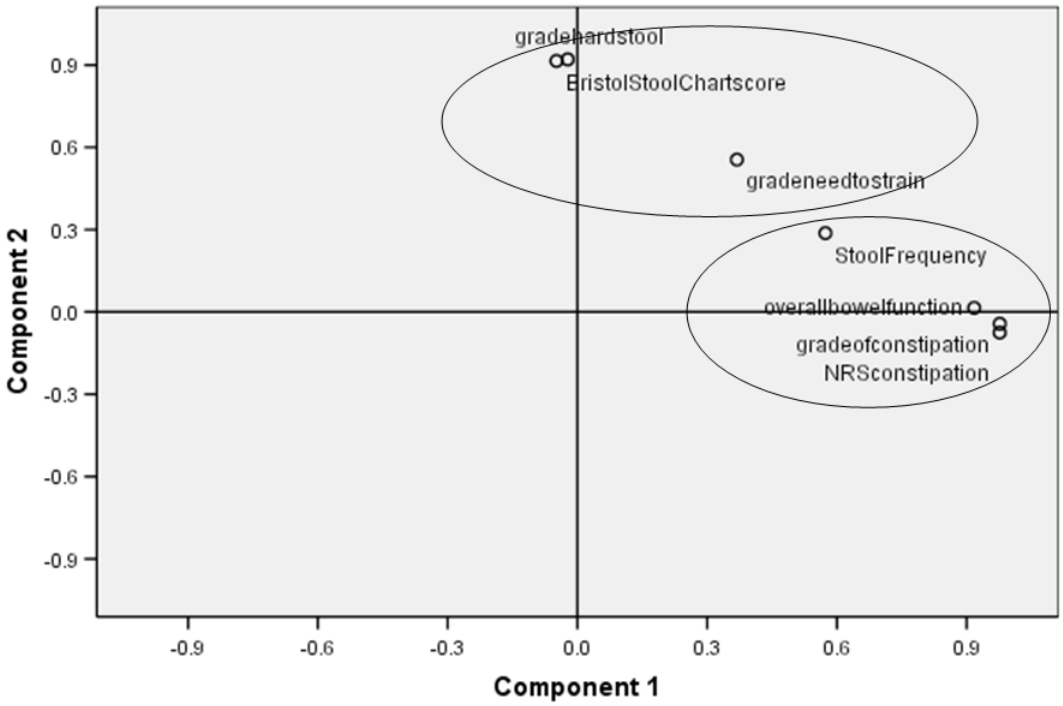

Figure 7.8 Principal component analysis for constipation assessment tool items
Component plot in rotated space demonstrating variables loading onto two main components.

Discriminant valididty (the ability to distinguish between distinct patient populations):

There was a significant difference between the proportions of patients scoring grades 0-3 on the 4-point categorical constipation scale depending on whether they were satisfied with their bowel function in the preceding week or not (table 7.14).

Table 7.14 Discriminant validity: Grade of constipation

	Grade of constipation				Total
	0	**1**	**2**	**3**	
Not satisfied	12.8 (10)	17.9 (14)	39.7 (31)	29.5 (23)	78
Satisfied	53.2 (50)	38.3 (36)	6.4 (6)	2.1 (2)	94
Data shown as %(N) Chi^2 70, $p=4x10^{-15}$					

There was also a significant difference between the scores using the NRS and the overall bowel function score ($p<0.0001$, Man-Whitney U), as shown in figure 7.9. Similarly there was a significant difference in the stool frequency score ($p<0.0001$), and the grades of straining and stool hardness (Chi^2 26, $p=8.1x10^{-6}$ and Chi^2 14.4, $p=0.02$ respectively), data not shown.

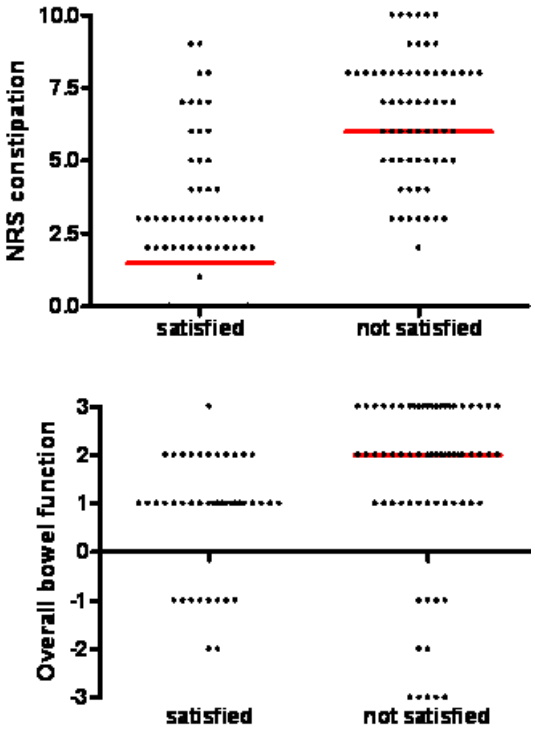

Figure 7.9 Discriminant validity

NRS and overall bowel function scale were compared in patients who were and were not satisfied with their bowel function in the preceding week.

Internal consistency:

When analysed together, the subjective assessment items had high internal consistency, Cronbach α 0.821.

Table 7.15 Reliability statistics for subjective constipation assessment items

	Corrected Item-Total Correlation	Cronbach α if Item Deleted
Grade of constipation	0.910	0.744
Overall bowel function	0.834	0.688
NRS constipation	0.885	0.888
Overall α 0.821		

The objective assessment items had slightly less internal consistency, Cronbach α 0.759, which is in keeping with the lower correlation coefficients for these items. This value for Cronbach α is still an acceptable value for a positive rating.

Table 7.16 Reliability statistics for objective constipation assessment items

	Corrected Item-Total Correlation	Cronbach's Alpha if Item Deleted
Stool Frequency Score	0.522	0.752
Grade hard stool	0.601	0.672
Grade need to strain	0.665	0.589
Overall α 0.759		

Comparing subjective severity scores and objective assessment scores across time:

There was no significant overall difference between the constipation scores at week 0 and those at week 1 (figure 7.10) or between weeks 0-4.

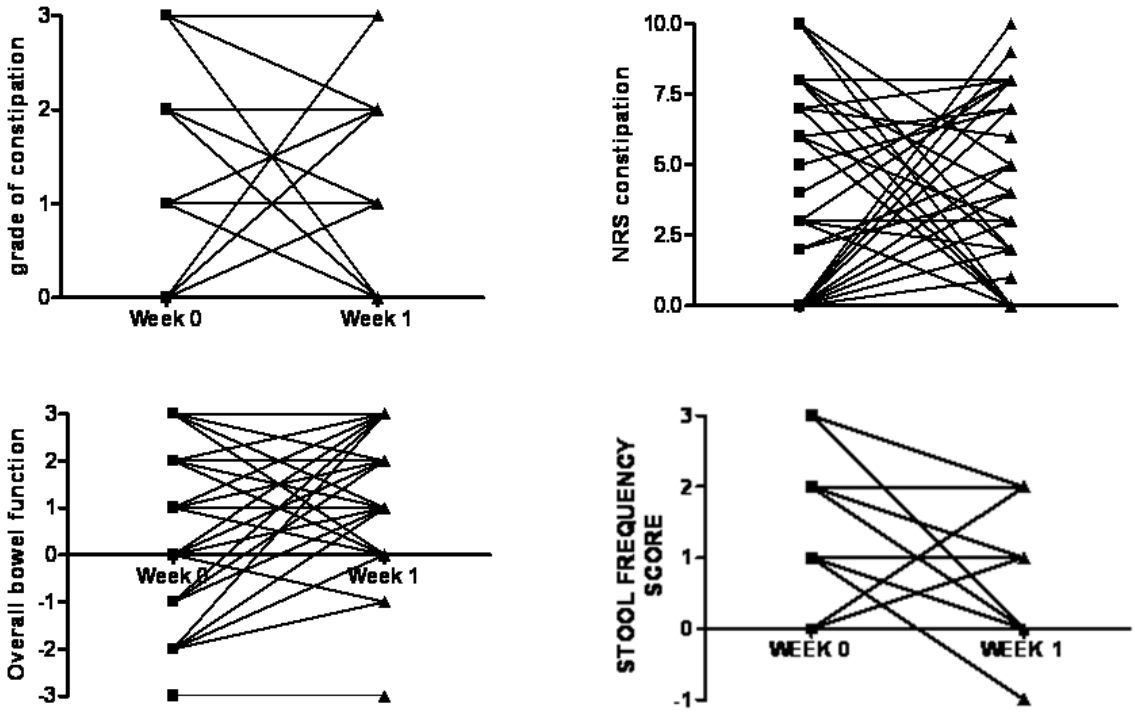

Figure 7.10 Constipation assessment items week 0-1

7.6.4.3 Developing a composite Constipation Assessment Score

Taking into account the statistical tests above, including accuracy of responses, subjective scoring of constipation severity using the 4-point adjectival Likert scale appears to be equivalent to using the 11-point Likert scale and preferable to using the bipolar overall bowel function scale.

The objective assessments of frequency (in the past week compared to usual), stool consistency and ease of evacuation are only moderately correlated, suggesting that

they represent different entities. Therefore it is proposed that a Constipation Assessment Tool should include either one of these subjective measures, along with these three objective scores.

An overall Constipation Assessment Score was devised by summing together one of the subjective scores (grade or NRS), together with stool frequency score, grade of stool consistency and grade of ease of evacuation. This composite score was devised based on two previously published scoring systems [196;260]. The higher the composite score, the worse the constipation. A score of 0 represented normal bowel function i.e. the subject does not feel that they are constipated and is opening his bowels with his usual frequency, with no straining and normal consistency. A negative score may represent diarrhoea or overflow diarrhoea.

When this composite score was used, there was a significant difference between subjects who were satisfied with their bowel function in the preceding week and those who were not, $p<0.0001$ (figure 7.11).

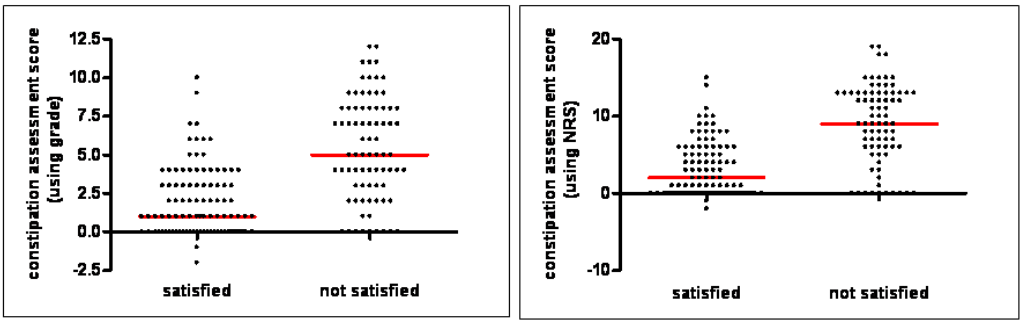

Figure 7.11 The Composite Constipation Assessment Score
This score can distinguish between subjects who are satisfied with their bowel function in the past week and those who are not, $p<0.0001$.

7.7 Overall Discussion

The main findings from the five studies presented in this chapter are:

- Constipation is a very common symptom, occurring in 72% in cancer patients on oral morphine.
- Constipation in this study population appears to be poorly managed. 89% of patients in this study were on inadequate treatment for constipation on morphine.
- There is marked inter-individual variation in bowel function in cancer patients on oral morphine: Some patients do not experience constipation and do not need to take laxatives. Some patients do not experience constipation *because* they are being adequately treated with laxatives. The final group are patients who experience constipation *despite* being on high dose combination laxatives. Overall there are two broad groups: patients with bowel problems on morphine and those without bowel problems on morphine.
- No clinical or genetic factors were identified as being associated with bowel problems on morphine in this patient group except for tumours of the lower gastrointestinal tract.
- The documentation of assessment of constipation in cancer patients on oral morphine is poor.
- Objective and subjective assessment questions about constipation are distinct and both should be included in a constipation assessment tool. Objective assessment of constipation may be assessed using an 11-point NRS or a 4-point adjectival Likert scale with equal merit.
- A Constipation Assessment Tool with a composite Constipation Assessment Score is presented comprised of a subjective score (4 point adjectival Likert scale), bowel frequency compared to usual frequency, consistency and straining.

7.7.1 Constipation management

In keeping with other published data [189;190], the data presented in this chapter suggest that constipation is a significant problem in cancer patients taking morphine. The results from both the *Prospective Morphine Study* and the *Randomised Controlled Trial* demonstrate that constipation in cancer patients is generally poorly treated. In the latter, one third of patients who felt they were constipated at time of recruitment were on no laxatives. Most, if not all of these patients were already taking weak opioids for cancer pain, therefore would have been known to be at risk of developing constipation. In the *Prospective Morphine Study*, overall ninety percent of the constipated study population was on inadequate laxative treatment. These findings have also been demonstrated in a recent large multicentre study of cancer patients on opioids. In that study 41% and 27% of patients reporting moderate or severe constipation respectively, had not been given any laxatives in the preceding 24 hours and overall 60% of patients were inadequately treated for constipation [473].

Documentation of constipation management in the Constipation Audit was also poor. Despite the fact that 80% of patients were subjectively constipated, only 50% were on laxative therapy at the start of the clinical episode. Although the evidence in this area is lacking, most guidelines, including local guidelines, recommend that most if not all patients who are on opioids should also be on oral laxatives [283;474]. The data from this study suggest that either these guidelines are not being adhered to or that patients are non-compliant with recommended regimens. Where the patient was on no laxatives at the start of the clinical episode but who was identified correctly as being constipated, laxatives were started in the majority of cases. In most cases there was documentation that the response was reviewed. There was a significant proportion of patients however who felt that they were constipated according to the morphine study data, who were on no laxatives but who were not identified as being constipated by the clinical teams. Similarly, documentation of follow up plans for those patients for whom constipation had been identified as a problem was poor.

There appear to be two main areas in which laxative prescribing in cancer patients on oral morphine is inadequate:

- Some patients who are constipated are on no laxatives at all
- Most patients who are constipated were taking laxative regimens which are clearly not effective as the patient was still experiencing the symptom.

The range of laxatives used to treat constipation in the *Prospective Morphine Study* reflects those commonly used in clinical practice. Previous studies have attempted to compare laxatives in terms of laxative units [195;214]. This study was *not* designed to compare laxatives in terms of efficacy. This study identified patients who were constipated despite taking laxatives and stratified them according to whether or not they were taking adequate laxative doses. Although the relationship between symptom intensity and symptom treatment has been used in other studies to determine adequacy of treatment [284], this is the first time in which the dose of laxative treatment has been taken into consideration. Since publication of the paper from this part of this thesis, this approach has been adopted to examine adequacy of treatment of other symptoms including nausea, depression and poor sleep [473].

There is much discussion in the literature about the use of novel opioid antagonists for the management of constipation on opioids. Using the results of this study, one might propose that that the small group of patients who have constipation that does not appear to be responsive to maximum recommended doses of combination laxatives may benefit from the use of such alternative measures. Similarly these agents may be efficacious in those patients who simply cannot tolerate laxative therapy. For most patients however, optimisation of laxatives and titration of laxative type and laxative dose according to patient symptoms would be the most obvious first step. Like all other drugs, rational laxative prescribing is essential to limit unnecessary and unsuccessful medication burden in the cancer patient population. Education to address this simple management strategy needs to be prioritised before newer, expensive and more complex treatments are added.

7.7.2 Inter-individual variation in constipation on morphine

It is well known that there is considerable inter-individual variation in analgesic response to opioids [11;95] A previous study in normal volunteers suggested variability in the constipating effects of opioids [212]. The data presented in this paper demonstrate that there is substantial inter-individual variation in constipation in patients on opioids for cancer pain. Some patients appear to be "constipation resisitant" i.e. they have no constipation and are taking no laxatives. This group of patients was defined in both the *Prospective Morphine Study* and the *Randomised Controlled Trial*. Another group of patients have no constipation *because* they are taking adequate laxatives. The final group are patients who remain constipated despite high dose combination laxatives.

In order to define a clinically meaningful phenotype which would be useful for subsequent studies, these subgroups were further redefined into two broad cohorts: those with bowel problems on morphine and those without. This definition was made possible with the information collected about laxative use. This approach will be applied to the data from the *Randomised Controlled Trial* once the study is complete.

7.7.2.1 Clinical factors associated with inter-individual variation in constipation on morphine

It has been suggested that approximately 25% of constipation in terminal illness is attributable to opioids [195]. It is difficult to determine what proportion of patients in this study are constipated solely because of the opioid and to what extent other constipating factors play a role. Constipation in cancer patients is known to be multi-factorial in nature [475]. Other factors which may contribute to constipation in cancer patients were explored in terms of this inter-individual variation in constipation on opioids.

Increasing age and female gender are thought to be associated with an increased prevalence of constipation [476]. In our study inter-individual variation in constipation on opioid was not age-dependant. Similarly there was no significant difference between the cohorts in terms of gender. In this study was no difference between the two groups in terms of the daily dose of morphine. These data support previous

suggestions that constipation on morphine is not associated with the dose of morphine [196]. There was no difference in terms of time on morphine either, which is in keeping with the widely accepted idea that tolerance to gastrointestinal effects of opioids does not usually occur[11].

The study subjects had a wide variety of cancer diagnoses but there was no association between most cancer diagnosis and inter-individual variation in constipation on opioids. This is in keeping with a larger study of 1840 cancer patients [477]. An increased prevalence of constipation and laxative use however has been associated with tumour involvement of the abdominal cavity [214]. In the study presented in this thesis, a larger proportion of patients *without* bowel problems had a diagnosis of a tumour in the lower intestinal tract. In the multicentre EPOS study of 1938 cancer patients on opioids, patients with a gastrointestinal tumour had a lower risk of being inadequately treated for constipation [473].

In this study performance status and diet were not measured. It has been shown that patients with lower performance status may be more predisposed to constipation in general [260]. Fluid balance is also important in the pathogenesis of constipation in cancer patients. Fluid intake was not recorded in these patients however there was no difference in biochemical parameters that reflect fluid balance and hydration (blood urea and haematocrit). There was also no difference in terms of serum calcium levels.

The data were also analysed in terms of potentially constipating concomitant medications. The numbers of patients taking these medications was small but there was no association found between the use of these medications and the inter-individual variation in constipation on opioids.

Therefore this study failed to identify any clinical factor associated with bowel problems on morphine with the exception of location of the tumour in the lower gastrointestinal tract. This prompted the hypothesis that perhaps genetic factors account for the clearly defined inter-individual variability in the constipating effects of morphine in cancer patients.

7.7.2.2 Inter-individual variation in constipation on morphine and variation in candidate genes

A large number of polymorphisms in *OPRM, OPRK* and *OPRD* were examined in this study. Despite the fact that opioid receptors play a key role in gastrointestinal function, only one SNP on *OPRD* was found to be associated with variability in the clinical phenotype. This finding did not remain significant after correcting for multiple testing. The effect of opioids on gastrointestinal function may be more complex than simply opioid receptor activation. Interactions between the serotonergic, adrenergic and opioid systems have been been postulated [208;478]. Polymorphisms in SLC6A4, ARRB2 and ADRA2A however were not found to be associated with the clinical phenotype of constipation in cancer patients taking opioids.

It is likely that there are other genes involved in opioid-induced constipation. These may include:

- CLCN2, the gene coding for the chloride channel protein 2. Lubiprostone is a prostaglandin which activates this chloride channel to stimulate gut lumen secretion. This indirectly counteracts the anti-secretory effects of morphine [479].
- MDR-1, the gene coding for p-glycoprotein. P-glycoprotein is expressed in the luminal membrane of enterocytes and limits gastrointestinal drug absorption. Inhibitors of p-glycoprotein (e.g. grapefuit juice and quinidine) have been shown to increase gastrointestinal morphine absorption in rats [117;480].

In order to fully explore inter-individual variation in the constipating effects of morphine, larger studies (to accommodate the relatively lower proportion of patients with no bowel problems on morphine) are required with analysis of multiple genes and environmental interactions.

7.7.3 Constipation Assessment

The data from the *Prospective Morphine Study* were used to examine why constipation is so poorly managed in this patient population. The results of the Constipation Audit highlight a number of concerning short-comings in daily clinical practice:

- Although constipation was a symptom for the large majority of patients, there was no documentation of constipation being a clinical problem in a significant proportion of cases.
- In those patients who were identified as being constipated, detailed assessment was often inadequate, as was documentation of rationale constipation management.
- Local, national and international standards as to the assessment and management of constipation were not being adhered to.

The data presented in this audit are consistent with other studies suggesting that the documentation of constipation assessment is sub-optimal. A retrospective chart review of 122 palliative care inpatients examined the patient charts for details about stool frequency, and symptoms associated with constipation. In many cases there was no reference at all to these symptoms [249]. In two Danish studies, comparison was made between the record of symptoms in medical and nursing records and the extent of symptoms as reported by patients in self-assessment questionnaires. With the exception of pain, both studies demonstrated that many patients experience symptoms which are not documented in medical or nursing notes. In terms of constipation, significant constipation (scored as "quite a bit" or "very much" on the EORTC Quality of Life Questionnaire, QLQ-C30) was recognised and documented by nurses in 38% of cases and by doctors in only 50% of cases [481;482].

Underestimation of symptom intensity by healthcare providers has been shown to be associated with inadequate symptom control [473]. Accurate and detailed symptom assessment is essential to facilitate rational symptom management. Rigorous symptom

assessment promotes 1) identification of the severity of the problem and 2) review of the impact of management plans.

In a study of 200 patients, 5.5% volunteered constipation as a current symptom they were experiencing when assessed using open ended questions, however 33.5% were identified as being constipated using a symptom checklist [483]. This suggests that certain symptoms are less likely to be volunteered by patients, perhaps because of cultural pressures, patient education or issues with time during the assessment period [483]. A study carried out in London suggested that patients are less likely to volunteer information about constipation as they believe that the doctor would not consider this important [484]. Many different constipation assessment tools exist however there is no such tool which has been designed, validated and is commonly used in cancer and palliative care patients. The data from the Constipation Audit suggest that since standard documentation of assessment and management of constipation is lacking, there is a need for a simple, brief, standardised constipation assessment tool for cancer and palliative patients.

When bowel function was assessed, the audit data demonstrate that the assessment process was patchy and inconsistent, mainly focused on bowel frequency. The data from this audit are consistent with previous data suggesting that objective assessment of bowel function is often based on bowel frequency [485]. In the *Randomised Controlled Trial*, stool frequency again appeared to be an important factor in an individual's perceptions of what it means to be constipated. Nearly 40% of patients mentioned stool frequency in the free text question of what constipation meant to them. The subjective assessment items in the *Randomised Controlled Trial* correlated highest with the stool frequency score as compared to the other objective assessment scores. Similarly the subjective measures and stool frequency loaded onto the same component in PCA. Stool frequency may however be an unreliable marker of constipation [486], mainly because most people have an irregular bowel habit. In the *Randomised Controlled Trial,* most subjects stated that they usually opened their bowels once a day. 12% of patients however, usually opened their bowels less frequently than this. A survey of 1897 people demonstrated that only 40% of men and 33% of women have a regular daily bowel movement [487]. As bowel function varies so widely, constipation assessment and management must take into account the patient's

individual usual bowel pattern. In the Constipation Audit, documentation of usual bowel pattern was poor.

The necessity to strain at stool is much more reliable constipation assessment than frequency with 94% sensitivity [488]. In the *Randomised Controlled Trial* very few patients cited straining or stool consistency when they were asked what being constipated means to them. This is despite the fact that a high proportion of study subjects were experiencing these symptoms on direct questioning. Similarly, in the Constipation Audit, details on straining and stool consistency were not often recorded. These results differ from two studies, one a survey of 1128 young adults and the other a survey of 209 older people, in which most defined constipation as straining and hard stools [489;490].

7.7.4 Constipation Assessment Tool

The constipation study in the *Randomised Controlled Trial* represents an initial attempt to define the items which best capture the patient's experience of constipation and to explore which assessment system is most applicable to this patient group. The data presented here represent an interim analysis of data in a larger clinical trial. Once the clinical trial is complete, full analysis including a longitudinal analysis of constipation and laxative use over time will be possible.

The items included in the Constipation Assessment Tool (bowel frequency, straining and consistency) were derived from the literature: from previous studies of constipation in other patient populations and in palliative care and cancer patients, from definitions of constipation and from data supporting the pathogenesis of constipation [196;251;255;260;491]. In some assessment tools used in other patient populations, abdominal symptoms are included as test items e.g. the Constipation Assessment Scale (CAS)[265], the Constipation Assessment Scale for Pregnancy[492], the Cleveland Clinic Constipation Scoring System[266], the Patient Assessment of Constipation Symptoms (PAC-SYM)[258], the Bowel Function Diary[263]. This item was not formally included in this constipation assessment tool as abdominal pain / bloating is a common symptom in cancer patients in general and was felt to be non-specific

and non-discriminatory in this patient group. The PAC-SYM also includes rectal symptoms but in this study no patient included rectal symptoms in their definition of what constipation meant to them

Subjective versus objective:
Constipation in patients taking morphine for cancer pain is usually a symptom or a secondary effect rather than a primary disorder. Thus the subjective assessment of the symptom of constipation is essential in defining constipation on opioids. Similarly the management of symptoms in cancer patients is generally dependant on the subjective experience of those symptoms. Therefore the subjective assessment of whether the patient felt he/she was constipated was chosen as an adequate outcome measure in the observational *Prospective Morphine Study*.

The constipation assessment question used in the *Prospective Morphine Study* is similar to that used in the validated European Organisation for Research and Treatment of Cancer Quality of Life Questionnaire [452]. The subjective assessment of constipation alone has some limitations however, as it does not take into account whether or not the patient is on treatment (or adequate treatment) for this symptom. Other clinical and genetic association studies of analgesic response to morphine have included this four point constipation scale as a secondary outcome without taking into account laxative use [92;110]. In this thesis, a novel method for defining bowel dysfunction on morphine is presented, in which clinical data about the patients' subjective assessment of constipation is combined together with data about their laxative use.

Although the phenotype definition presented in this study represents a more clinically meaningful classification of the constipating effects of morphine than just using the subjective assessment alone, future studies incorporating both subjective and objective analysis of constipation in these patients will allow even further definition.

The data from the *Randomised Controlled Trial* support the idea that subjective assessment of constipation does not measure the same things as objective assessment, as demonstrated from the lower correlation coefficients between subjective and objective measures. Also, there is quite marked disparity between the proportion of

patients citing altered frequency, straining and stool consistency as factors defining constipation for them when compared to the proportions of subjectively constipated patients who reported experiencing these symptoms. A previous study of 44 palliative care patients compared patient self-assessment of constipation with two diagnostic tools, i.e. compared the subjective and objective assessments of constipation. One objective diagnostic tool consisted of three items (frequency, ease of passing stool and stool consistency)[260] and the other two items (frequency and consistency)[196]. The diagnostic objective tools identified 23 and 45% of the study population as being constipated (with a sensitivity and specificity of 22% and 76% and 66% and 61% respectively[493]) but only 23% of patients themselves reported themselves as being constipated. The authors concluded that the results were probably due to patient's individual perceptions of constipation [254].

Overall there was not any great difference between the three subjective assessment items (grade, NRS and overall bowel function) in terms of psychometric analyses. There was strong correlation between all three scores and they all demonstrated significant discriminant validity. The overall Cronbach α score suggest that they all measure a similar construct and they all load onto the same component in PCA. Component 1 as derived by PCA represents a derived composite which may be considered as the "best possible" subjective constipation assessment. In previous studies, assessments which showed the closest association with the composite score, as judged by the loading value, could be deemed to be the most valid [472]. All three subjective constipation measures were significantly associated with the composite score (loadings 0.916 – 0.978), suggesting that they are equally valid. The only difference between the three subjective constipation assessments is in the rate of correct responses, which is a measure by which pain intensity scales have been judged[472]. The highest correct response rate was found with bowel satisfaction and grade of constipation. Study participants also scored highly using the NRS. There was a significant difference in correct responses when the overall bowel function question was used. Therefore one conclusion is that grading constipation severity on a four point adjectival Likert scale and scoring constipation severity on a 0-10 NRS are equally valid and accurate and either of these could be used in a constipation assessment scale. A bipolar scale "overall bowel function" maybe equally useful with the caveat that there may be a relatively lower rate of correct responses.

In terms of the objective constipation assessment items, the rate of correct responses was much less for straining, frequency and consistency compared to the subjective items. The highest incorrect response rate was found with the previously validated Bristol Stool Chart. The Bristol Stool Chart score was included as an item as this is one of the most commonly used bowel assessments in cancer and palliative care patients. The Bristol Stool Chart is thought to provide information on gut transit time [264]. In our study the Bristol Stool Chart and the question about stool hardness loaded highly onto the same PCA. This and the fact that it correlated best with stool hardness are not surprising as both these items measure stool consistency. The correlation between the Bristol Stool Chart and objective constipation measures was only moderate, and with subjective constipation measures was relatively weak. Therefore overall it is uncertain whether the Bristol Stool Chart is indeed a useful item in assessing constipation in cancer and palliative care patients.

Overall the interim data from the *Randomised Controlled Trial* suggest that a constipation assessment tool for use in cancer and palliative care patients should include:

- A subjective assessment of constipation, either the NRS or four point adjectival scale.
- An objective assessment of constipation including frequency (current as compared to usual), straining and consistency. None of the objective items correlate very strongly with each other suggesting that they do not measure the same construct and therefore that they may be combined in a composite score without redundancy.

The main differences between this tool and many other constipation assessment tools are:

- It was designed specifically for cancer and palliative care patients. Unlike other constipation assessment tools, such a tool would be brief and concise and may be useful in both the clinical and the research setting.
- Clinically, since laxatives may be broadly categorised into stool softeners and stimulants, information about frequency, straining and consistency could facilitate tailored management with currently available laxatives.

- Unlike other scores which focus on absolute numbers of bowel movements per week, this tool uses the number of bowel movements as compared to the patient's usual frequency. This captures the wide variation in normal frequency of bowel movements.

A composite constipation assessment score was developed using methods derived from two previous studies of constipation in cancer patients [196;260]. The composite constipation assessment score demonstrated significant discriminant validity in terms of satisfaction with bowel function. Such a score may be a useful outcome measure in clinical trials.

7.7.5 Limitations of these studies:

7.7.5.1 Limitations of the Prospective Morphine Study

This study has a number of limitations which may contribute to the negative associations between the clinical outcome and either clinical or genetic predictors. There are relatively small numbers of study subjects in the "No bowel problems on morphine" group. Correcting for multiple testing in such a small group may inadvertently result in type 2 errors. The clinical definition of patients with "no bowel problems on morphine" i.e. not constipated and not taking any laxatives, appears sound however as it is true in both the *Prospective Morphine Study* and the *Randomised Controlled Trial*.

The data in this study were collected from a single time point which does not reflect the dynamic nature of symptoms in cancer patients. Prospective follow-up studies are required. Analysis of the final constipation data set of the *Randomised Controlled Trial* will facilitate such longitudinal assessment.

7.7.5.2 Limitations of the Constipation Audit

One of the unique aspects of the data presented as part of the constipation audit is that the constipation status of every subject was known through independent documentation as part of the previous research study. Therefore retrospective analysis

of the clinical records was thought to be an appropriate method to analyse documentation of assessment and management of constipation in these patients.

There are two main limitations in analysis of the data from this audit. Firstly, the retrospective audit provides information on *documentation* of constipation assessment and management. A lack of documentation does not necessarily mean that the assessment did not take place. However, this raises further questions; if assessment and management plans are not documented, it is unclear how the clinical situation can be managed adequately. Secondly, as part of the audit, the entire clinical episode was reviewed, whereas the subjective assessment of constipation as documented in the morphine study was a single time point. Thus the subjective assessment of constipation documented in the morphine study does not take into account the dynamic nature of bowel function.

7.7.5.3 Limitations of the Randomised Controlled Trial

The data presented here do not show any difference in the individual constipation assessment items across time. These interim data however do not take into account laxative use in order to define bowel function / dysfunction and the numbers of patients when each time point is analysed independently are relatively small. Therefore it is not possible at this time to prospectively test the clinical phenotype of bowel problems on opioids as defined in the *Prospective Genetics Study.* Similarly the numbers are too small to prospectively validate our earlier findings of other clinical factors which may impact on constipation. These analyses will be possible once the full complete data set is available. Furthermore, this study was not designed to test the efficacy of the constipation assessment tool in terms of improving constipation management. Once the analysis on the final data set is completed, the items to be included in the final constipation assessment tool will be determined. A further study including a comparison group will be necessary to test the efficacy of this tool.

8 Chapter 8: General Discussion and Future work

8.1 Summary of thesis

The aim of this thesis was to examine the inter-individual variation in response to morphine for cancer pain. The definition of clinical response to morphine was explored using data from the *Prospective Morphine Study*. Clinical confounders influencing morphine response were included in the analysis. Assays were set up to study the pharmacogenetics of morphine using both a candidate gene and genome-wide approach. The interaction of clinical and genetic factors was investigated. Constipation as a common troublesome side-effect of morphine treatment was explored in detail using data from both the *Prospective Morphine Study* and the *Randomised Controlled Trial of Oral Morphine versus Oral Oxycodone*. A study was set up to begin the development of a constipation assessment tool.

The main findings from this thesis were:

- Pain and side-effect scores and also the dose of morphine required by individual patients vary widely.

- Analgesia, central side-effects on morphine and morphine dose requirements appear to be distinct dimensions of response to morphine. This hypothesis is based on a number of findings:

    - Principal Components Analysis demonstrated that pain and central side-effect scores loaded onto separate components with little correlation between them.
    - The clinical factors associated with analgesia, central side-effects on morphine and morphine dose requirements are different.
    - These three outcomes are associated with different polymorphisms in different genes with little overlap.

- Genetic variation in a number of genes studied (including the opioid receptor genes) may contribute to some of the variation in morphine response. The

genes studied however only account for a small proportion of response variability.

- Constipation is a common symptom in cancer patients taking morphine. In general, assessment and management of constipation appear to be inadequate.

8.2 Challenges highlighted by this thesis

It is disappointing, but perhaps not surprising, that no single definitive gene defining opioid response was identified. Many large genetic association studies examining highly heritable complex human traits have only been able account for a small proportion of variability in that trait. For example, 90% of variability in human height is thought to be due to genetic variation [448]. Studies involving thousands of individuals have identified multiple genetic associations with height but these only account for approximately 3-4% of this variance[448;494].

The data presented in this thesis highlight a number of challenges which need to be addressed if the genetic factors underlying inter-individual variability in morphine response are to be elucidated:

- The highly complex (clinical and genetic) nature of pain perception and analgesic response.
- Study size
- Phenotype definition and replication of results
- Recruitment to cancer and palliative care studies

8.2.1 Pain perception and response to morphine are complex traits, both clinically and genetically

In this thesis, 53 SNPs in 9 candidate genes were studied. Until publication of the EPOS data [135], which included 112 SNPs in 25 genes, this was the largest number of SNPs and genes examined (and published) in this field. However it is highly likely that there are many more genes which may play a role in differential pain perception and pain control. Furthermore, like other complex traits, it is likely that pain

perception and analgesic response are influenced by multiple gene-gene and also gene-environment interactions [172]. In order to explore response to morphine as a complex trait, in this thesis both the clinical and genetic factors contributing to the phenotype were explored. Clinical variables such as tumour diagnosis, concomitant medications and age were found to be associated with the various clinical phenotypes. These clinical factors were included in multivariate regression analyses with the genetic factors to build models of morphine response.

To date the genetic association studies of response to opioids for cancer (and indeed non-cancer) pain, have merely scraped the surface in terms of genetic complexities which may underpin this phenomenon. Challenges for future research include designing studies to explore epistasis, to account for population stratification and to identify and analyse genetic variation other then SNPs. Replication of results has traditionally been seen as the gold-standard for validation of genetic associations. However, previous candidate and genome-wide association studies (GWAs) in other areas have been disappointing, mainly because of failure of replication of findings. Less than 20% of genetic associations have been robustly replicated without bias [441]. Likewise, very few single SNP results are replicated even when there is good evidence for the real nature of the association. Potential reasons for these discrepancies include population stratification bias, population heterogeneity, inadequate statistical power [430;495], differences in phenotype definition and the fact that most genetic association analysis modelling uses a linear framework which only considers one SNP at a time, this ignoring gene-gene and gene-environment interactions [496].

8.2.1.1 Gene-gene and gene-environment interactions

In recent years there has been increased awareness of the concept of epistasis (gene-gene interaction) in disease pathogenesis and drug response [173;497]. There may be a number of genetic variants which have a slight or even a negligible effect when analysed independently, however in the presence of another genetic or environmental variant, may be quite significant. This notion can be demonstrated in the data presented in Chapter 5. For example, on univariate analysis, carriers of the SERTP short allele (SS) appeared to have higher pain scores than non carriers but this was not

statistically significant (p=0.062). This DIP was however included in the multivariate regression model as an independent predictor of analgesic response to morphine (p=0.02).Conversely, gene-gene interactions may be antagonistic [173]. Gene-gene interactions are likely to be much more important and informative than single independent associations alone, especially in complex traits such as pain and analgesia. Association studies which focus on the association of single variants with pain and analgesic response may produce ambiguous results and possibly miss clinically relevant genetic variants [449].

In this thesis, gene-gene and gene-environment interactions were examined, albeit in a relatively superficial manner. Even with the most robust statistical software, the sheer volume of data generated through combinations of polymorphisms is overwhelming. Examination of all possible two-gene interactions in a study of n genes would require n (n-1)/2 interactions [498]. Therefore comprehensive testing of all two-SNP interactions in my candidate gene study of 53 SNPs would yield 1378 interactions. Such analysis in the GWA would result in 9.7 x 10^{10} reactions! (This calculation is obviously over-simplistic and does not take LD into account). The possible number of gene-gene interactions in a complex trait such as opioid response is however as yet unknown and carries the additional requirements for multiple testing corrections [498]. It is easy to imagine how a comprehensive search for all interactions would increase the volume of data generated and the required study sizes exponentially [451]. At present it is computationally and biologically challenging to explore all possible SNP-SNP interactions in a study such as this. As a result, epistasis was not examined in the GWA in this thesis or in many other studies, including the large EPOS study. When epistasis is ignored, a linear relationship between genetics and the clinical outcome is assumed. Such an assumption is likely to be inaccurate when dealing with complex traits such as opioid response. More sophisticated data analysis tools are required to deal with the interactive complexity of epistasis.

A number of statistical methods for examining gene-gene interactions have been proposed including regression, classification and regression trees, neural networking, combinatorial partitioning and multifactor dimensionality reduction [173]. None of these methods are without limitations and indeed data analysis with each may produce dissimilar results [499]. In this study multivariate linear regression was used to analyse

interactions between genetic and clinical data. This method has a number of limitations:

- Firstly, as the number of predictor variables entered into the model increases, the distribution of data is reduced. This phenomenon is known as the "curse of dimensionality" and is particularly problematic when the number of predictor variables exceeds greatly the number of observations [500], there are missing data or when the loci have low minor allele frequencies [173]. To address some of these issues and to reduce the number of markers to a more manageable size, a two-step approach has been proposed [500]. In the first step a subset of the most important markers is chosen and in the second step interactions between these markers is explored [500]. A number of different approaches to this two-step model have been used.

  In this study the subset of best markers was chosen according to the p-value on univariate analysis. A threshold of $p<0.1$ was chosen as it is likely that some factors which are only borderline significant on univariate analysis will become more influential in the presence of other factors. A more generous threshold e.g. $p<0.2$, could also have been applied [93], but while this may have identified more significant factors which have negligible effects when tested independently, there would be a greater risk of type 1 error. The predefined threshold may be one reason why in some instances the multivariate additive and dominant models are dissimilar. Another explanation may be that the numbers in the study are relatively small especially when the additive model is used.

- This approach may also run the risk of type 2 error. One of the main limitations of stepwise regression model is that the main effects are determined before analysis for interaction effects between variables i.e. it requires that the predictor variables are only included if they fulfill predefined criteria, i.e. in this thesis being significant at a level of $p<0.1$ [173;500].

- A further limitation of regression modelling to explore epistasis is that if the predictor variable is merely a marker for the true susceptibility variable, then the result is likely to be diluted, depending on the extent of LD between the two markers [501]. This however is a factor which must be considered in all genetic association studies, regardless of the statistical test used.

Evidence of a statistical interaction does not necessarily imply a biological interaction [501], therefore any statistical modelling must be interpreted with caution. One of the major differences between physiological and statistical epistasis is that the latter is dependent on allele frequencies in the study population [502]. In this thesis a number of models were presented from the candidate gene analysis. In each of these models, a biologically plausible connection can be made between most of the predictor variables and the clinical phenotype. However each of the genes was chosen because of prior knowledge of an association with morphine response. In the GWA, genes which were not previously known to be associated with morphine response were identified. Before any conclusions are drawn about the biological or statistical relevance of these findings, replication in larger studies using the same clinical phenotypes is required.

It has been recently proposed that new computational and biostatistical approaches should be adopted in genetic association studies to include data mining and machine learning methods. This would facilitate modelling of the complex interactive networks of biomolecules which may / may not be controlled by environmental influences [496]. Incorporation of existing information about biological pathways into data analysis algorithms will enrich subsequent non-linear analyses [496]. In light of the negative findings of the EPOS study, Klepstad et al have suggested a move away from candidate gene association studies [135]. However, in order to maximise the potential of finding true associations, analysis of epistasis in genetic association studies (even genome-wide association studies) should be guided by existing knowledge of plausible underlying biological pathways [501;503]. In the candidate gene study presented in this thesis, combinations of genetic data pertaining to genes which are known to play a role in morphine response and pain perception were explored. Identification of and focused study of biologically plausible interactions between genes and environment might yield significant and useful results [451].

8.2.1.2 Population stratification

Although the number of pharmacogenetic studies of opioids in cancer pain has been low, the study populations have been quite distinct in terms of ethnicity and geographical location. This adds a further layer of complexity to any comparisons between studies, especially if the studies represent collaboration between different countries. The EPOS data included study subjects from 11 European countries. Although 61 patients in total were excluded from analysis so that the study population only included Caucasians, even among European counties marked genetic variation between different geographical regions differences has been demonstrated [504;505]. This was demonstrated in the GWA presented in this thesis, figure 6.3. These differences may lead to spurious results in genetic association studies if the difference in allele frequency between cases and controls is due to population stratification rather than to the outcome being measured [430]. Such genetic heterogeneity may also be another reason why many genetic association studies fail to replicate in independent samples [431]. Similarly population stratification makes replication in distinct geographic populations important before genetic association study findings can be applied in a clinical setting.

8.2.1.3 More complex genetic variation

As mentioned in Chapter 1, differences in response to drugs may be brought about by genetic variation at a DNA, RNA or protein level. To date all genetic association studies of response to opioids in cancer pain have focused on genetic variation in terms of SNPs. There have been no published studies examining the role of modifications at an RNA or protein level in this study population. The practical challenge of obtaining suitable sample tissue (e.g. nervous or brain tissue) has precluded much direct research in this area. However, there has been one small but very exciting study examining the influence of SNPs on the production of functionally significant splice variants [148]. Integration of gene expression data with SNP data will undoubtedly enhance our understanding of inter-individual variation in response to opioids.

8.2.2 Study size

One of the major limitations of the studies presented in this thesis is sample size. There are a number of factors which need to be taken into account when determining the optimum sample size for any genetic association study. These include:

- The frequency of the susceptibility allele and the allelic odds ratio (effect size)[442]. The lower the frequency of the susceptibility allele and the lower the allelic odds ratio (i.e. the weaker the strength of association), the larger the samples size required.
- The prevalence of the disease / trait in the general population. Rare traits often require larger sample sizes to identify adequate number of cases.
- The number of SNPs to be tested, to take into account corrections for multiple testing. This however, is less influential than the size of the allelic effect [506].
- Multifactorial diseases / traits are likely to be influenced by multiple incomplete penetrant genetic variants. Any risk-factor strongly associated with such a disease / trait is likely to be rare, resulting in a small increase in prevalence [80]. Genetic variability is usually associated with small or modest effect sizes (odds ratio) in common, complex, multifactorial diseases / traits such as response to opioids. Therefore larger studies tend to be preferable to smaller studies [443].

In the GWA presented in this thesis, the proportions of patients in each phenotype group are sizable i.e. in the whole study population 80% of patients achieve a good analgesic response compared to 20% who do not. This supports previous data which suggests that non-response to morphine is not a rare phenomenon, occurring in approximately one third of patients[11] . Depending on the clinical phenotype used, in the candidate gene study, up to 207 study subjects were included in the analyses. This is a relatively large number in terms of the previously published papers in this field. In order to minimise the sample size required, in this study, SNPs were only included if they had a minor allele frequency of > 10%. If the fact that response to morphine is a complex trait is ignored, and an (incorrect) assumption is made that response to morphine is determined by a single gene / SNP, then the number of patients in both

the GWA and the candidate gene studies were possibly adequate to allow identification of possibly true associations in common alleles (allele frequencies >10% [80]) with more significant effect sizes. The relatively small size of the sample population in these studies undoubtedly resulted in reduced power to identify low-risk susceptibility alleles with low minor allele frequencies. Such small sample sizes are unquestionably too small if multiple testing for joint gene effects in a complex trait was to be taken into account.

8.2.2.1 Multiple testing

The use of multiple endpoints and the application of multiple statistical tests in a study increase the likelihood of Type 1 error i.e. increases the likelihood that the test will reject a true null hypothesis. In genetic association studies therefore, a significant proportion of the positive associations will occur due to chance alone. A number of methods have been proposed to adjust for multiple analyses. Two commonly used methods include the Bonferroni correction and False Discovery Rate.

Bonferroni correction:
The Bonferroni correction adjusts the significance level (α) according to the number of tests performed. The adjusted α is often calculated as 0.05/n, where n = number of tests. In a genetic association study where 500,000 SNPs are tested against one clinical outcome, then true positive associations may be considered as those with a significance level (α) value of 10^{-7}. The Bonferroni correction has traditionally been popular as it is relatively simple to calculate. This was used in the only other GWA in pain [81] and was also applied in the GWA presented in this thesis. However this correction is often considered to be overly conservative as it assumes that each test is independent. In genetic association studies this correction does not take linkage disequilibrium into account [507]. Therefore in fact the Bonferroni correction may actually increase the likelihood of Type 2 errors.

False Discovery Rate (FDR):
The FDR was proposed by Benjamini and Hochberg in 1995 as an alternative method. Instead of adjusting according to the number of tests performed, this method controls for the expected proportion of falsely rejected null hypotheses [508]. Associations that

have a p value above the calculated FDR thresholds are considered to be true associations. In practice, instead of using the traditional p value, this approach presents a q-value associated with each test. This q value is a measure of statistical significance in terms of the false discovery rate [509]. This approach is commonly used in GWAs.

8.2.3 Accurate Phenotype to reduce required study size

One of the main reasons behind replication failure in genetic association studies is lack of consensus about phenotype definition.

Much of this thesis has been based on the need for phenotype definition of response to morphine. In terms of sample size, accurate phenotyping reduces the numbers of study subjects required. One of the earliest GWAs was carried out in 146 individuals (96 cases of advanced age-related macular degeneration (AMD) and 50 controls). 103,611 SNPs were analysed and a polymorphism in the gene coding for complement factor H was found to be significantly associated with the phenotype [510]. This phenotype was later tightened to include only wet AMD and a GWA conducted in 96 cases and 130 controls yielded very significant results ($p = 4.1 \times 10^{-12}$) which have been subsequently replicated [511]. In pharmacogenetic studies in other areas, when the phenotype can be precisely defined and where possible confounders are minimal, the study power is markedly lower. As discussed in chapter 6, a GWA carried out in only 85 subjects and 95 controls, yielding significant association with statin-induced myopathy which again stood up to replication [447]. In chapter 1 the role of UGT1A1 in irinotecan toxicity was described. One of the landmark studies identifying the genetic variant associated with irinotecan-induced neutropenia included only 63 patients, of whom six had the clinical outcome of interest (grade 4 neutropenia) [54]. Pain and response to opioids however is a very complex outcome with many possible confounding factors. Therefore it is reasonable to assume that the numbers required in pain and opioid studies are significantly higher.

Apart from accurately defining response to morphine as a clinical phenotype, one of the ways in which the genetics of pain and opioid response may be elucidated is

through collaboration with studies involving normal volunteers or non-cancer patients. These studies are "purer" in that the confounding factors are limited and the painful stimulus / pain relieving drug is usually standardised. The only GWA published in pain included only 221 patients. It is probable that, like our studies, this number is too small for any definitive conclusions to be drawn. However, unlike our study, this GWA was carried out in a defined population (European Americans, aged 17-35 years), all of whom underwent the same painful stimulus (third molar extraction). Therefore in this population the number of confounding factors contributing to pain and pain control were less than in our study of cancer patients.

8.2.4 Recruitment to palliative care studies

The practical and ethical challenges of conducting research in palliative care have long been debated [512;513]. It is widely acknowledged however that research in this area is essential in order to establish best practice based on good quality evidence [514;515]. Research in palliative care has been recognised as a key area of specialty and service development [516] and as recently as July 2008 has been highlighted as a priority agenda at a governmental level [517].

Two hundred and ninety eight patients were recruited to the *Prospective Morphine Study.* This was a relatively simple study in that it consisted of a single time point and no onerous patient follow-up. The *Prospective Randomized Controlled Trial of Morphine versus Oxycodone for Cancer Pain* is a more complicated study, as evidenced by the fact that recruitment is still ongoing. Carrying out research in palliative care is difficult, for a number of reasons:

- Potential study subject identification, recruitment and retention are often challenging because of patient frailty, high attrition rates and unequal access to palliative care services.
- Inclusion-exclusion criteria which stipulate minimum life-expectancy and performance status may discriminate against many palliative care patients [518], especially those at the end of life. This is confounded by the fact that for many palliative care patients performance status may vary according to treatment and disease trajectory.

- Inaccurate prognositication is well recognised and may result in patients being excluded from studies [519].
- Identification of potential study subjects is often carried out through the patient's health care provider. Although health professionals can advise about the potential suitability of patients for a particular study, over-enthusiastic protection is potentially damaging to palliative care research on two counts. Firstly potentially eligible and suitable patients may be denied the choice and opportunity to participate in research. Secondly this may introduce a selection bias which may influence the results [513].

Built into the study design of *the Randomised Controlled Trial* was the acknowledgement that patients would reach study endpoint early for a number of reasons including death or being too unwell to continue. Therefore in this study death was not considered to be a serious adverse event. In both studies, in order to minimise recall burden and bias, recall period was at most a week. Study questionnaires were kept to a minimum while trying to collect as much clinical data as possible.

The importance of research as a discipline in palliative care was recognised when research became a mandatory part of specialist registrar training in 2010 [520]. More and more palliative health care professionals are becoming interested and involved in research. Supportive and collaborative bodies have been established, including the Junior Forum of the European Association of Palliative Care Research Network (EAPC-RN) and the Palliative Care Research Society (PCRS), to promote research in this field.

8.3 Challenges for current and future research

In order to address some of the issues discussed above, the main challenges facing researchers in the area of genetics and response to opioids are:

- Increasing study size. The recent success of the EPOS study in which 2294 patients were recruited demonstrates the benefits and possibilities of

multicentre collaborations [135]. Such collaborations are vital especially if GWAs are to be carried out.

- Consensus on outcome measures for morphine response / non-response. The use of principal components to define morphine response may be useful if validated in other studies. There has been an international call for standardisation of assessment tools for symptoms in palliative care, a call which has been supported by the European Association of Palliative Care Research Network. Such standardisation will facilitate comparison between research studies.

- Partnership and collaboration with researchers in other settings. The importance and benefits of this are two-fold.

  Firstly some of the complexities of morphine response in cancer patients may be teased out through collaboration with researchers working with normal volunteers and chronic non-cancer pain patients and with basic scientists. Clinical and genetic association studies which are carried out under experimental conditions in otherwise healthy volunteers or in chronic non-cancer pain patients are less susceptible to the myriad of confounding factors which influence inter-individual variation in response to opioids. Therefore such studies may provide a "cleaner" platform from which to unpick this conundrum. Studies in healthy volunteers may facilitate the objective definition and classification of analgesic response. Studies in non-cancer chronic pain patients may assist in our understanding of the phenomenon of physiological tolerance and its impact on response to opioids. Collaboration with basic science units is important in order to validate the findings of genetic-association studies. For example, if a particular gene was found to be strongly associated with a particular response to morphine, then a rational next step in proving functional significance would be the development and testing of a knock-out model.

Secondly, evidence supporting ongoing study of inter-individual variation in pain and opioid response in cancer patients comes from a number of sources including healthy volunteer, familial and twin studies. Studies of monozygotic (MZ) and dizygotic (DZ) twins provide a classical approach to teasing apart the influence of genetic and environmental (shared or unique) factors on disease and traits. Twin studies have identified a number of painful conditions which appear to have some element of heritability, including back pain, dysmenorrhoea, sciatica, musculoskeletal pain, spinal pain, back pain and irritable bowel syndrome [521-523]. In the last five years a number of twin studies have been carried out which propose 26-60% heritability of response to experimental painful stimuli [524;525]. These studies prompted the first opioid response study in twins, the preliminary data from which were published in 2010. The enrolment target for this study was one hundred and twenty-five pairs of healthy twins (80 MZ and 45 DZ). Each study participant was randomised to receive infusions of saline and alfentanil. Experimentally induced pain sensitivity to heat and cold pressor pain, opioid analgesia and opioid side-effects (including nausea and respiratory depression) were assessed [526]. The final results of this study are awaited with interest. Informal reporting of these data however suggests that over 50% of variability in opioid analgesia and 40-60% of variance in opioid side-effects is due to genetic factors [527]. These findings add convincing weight to the hypothesis that pain and analgesic response are, partially at least, genetically mediated traits.

There are a number of heritable pain conditions in chronic non-cancer pain patients in which the underlying genetic mechanism has been identified[528]. One example includes the SCN9A gene, which codes for a peripheral sodium channel $Na_V1.7$. As mentioned in chapter 1, variation in this gene is associated with either a complete inability to sense pain or extreme pain disorders such as erythermalgia and paroxysmal extreme pain disorder [60;61]. Such rare disorders provide a fascinating insight into the mechanisms and pathophysiology of certain pain states. These findings have paved the way for further research into the association of the genes involved and other pain states, including cancer pain and opioid response.

- I have been working with researchers in Denmark (Professor Drewes, Laboratory for Experimental Pain Research , Aalborg Hospital, Aarhus University Hospital, Denmark) and also in London (Professor Qasim Aziz, Wingate Institute of Neurogastroenterology, Barts and the London School of Medicine & Dentistry, Queen Mary University of London, London, UK), both of whom have carried out pain studies in normal volunteers. The Danish studies consisted of subjects undergoing a number of different painful stimuli including somatic and visceral pain. Analgesic response to opioids including morphine and oxycodone was measured [529-533]. The London studies involved an attempt to define the pain phenotype through a number of modalities including personality testing, autonomic responses, functional magnetic resonance imaging and genetics [534;535]. I have been involved in genotyping the subjects of both groups using some of the assays already tested in the *Prospective Genetics Study*.

- Increasing recognition of the fundamental importance of post-DNA variation. Dr Ruth Branford, another PhD student in this department, is examining opioid receptor expression in white blood cells from patients recruited to the RCT. These data will be combined with SNP data and will hopefully expand our understanding of inter-individual variation in morphine response.

8.4 Closing Remarks

I have been extremely fortunate to have had the opportunity of participating in research which combined clinical trials and laboratory based techniques. The data presented in this thesis add to the growing body of knowledge in the field of opioids and cancer pain. The clinical implications of such data however are as yet unclear. The possiblity for further research in this area is enormous. The incorporation of validated symptom assessment tools with a comprehensive investigation of the genetic factors underlying inter-individual variation in response to opioids has the potential to revolutionize cancer pain management. Prospective prediction of choice and dose of opioid would facilitate targeted and expedited pain management and

promote rationale drug use for individualized patient care. Pharmacogenomics is a rapidly expanding field. The application of such advances in cancer pain is likely to be possible in the future, but not just yet.

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Appendix A Publications arising from the work of this thesis

Journal articles, Book Chapters

- Droney J, Ross J, Gretton S, Welsh K, Sato H, Riley J. Constipation in cancer patients on morphine, *Supportive Care in Cancer* 2008; May 16 (5) 453-9

- Stevens A, Droney J. Managing and Treating Opioid-induced Constipation in patients with Cancer. *Gastrointestinal Nursing* 2008 November 6 (9); 16-22

- Droney J, Riley J. Recent advances in the use of opioids for cancer pain. *Journal of Pain Research* 2009; 2 135-155

- Droney J, Riley J, Ross JR. Evolving knowledge of opioid genetics in cancer pain.*Clinical Oncology* 2011 May 23; [Epub ahead of print]

- Droney J, Riley J. Opioid switching and the genetics of opioid sensitivity Chapter in 'Anaesthesia, Intensive Care and Pain Management for the Cancer Patient' Oxford University Press 2011

- Droney J, Ross JR, Riley J. Opioid genetics in the context of opioid switching. Curr Opin Support Palliat Care 2012 Jan 5 (epub ahead of print).

Other publications

- Droney J, Stevens A. Understanding Constipation. Patient Booklet. Royal Marsden Hospital 2008

- Droney J, Sevens A, Constipation. Symptom Control Guidelines, Royal Marsden Hospital 2008.

- Droney J, Stevens A. Assessment of constipation in End of Life Care patients. NHS E-learning for Healthcare

- Droney J, Stevens A. Management of constipation in End of Life Care patients. NHS E-learning for Healthcare

Appendix B Case Report forms for the clinical trials presented in this thesis

This appendix contains the following documents:

Prospective Morphine Study Case Report Form

- Demographics
- Pain Assessment (modified Brief Pain Inventory)
- Morphine Toxicity Scores
- Opioid and breakthrough medication details
- Criteria for changing to an alternative opioid
- Concomitant medication details
- Reason for study withdrawal and details of death

Randomised Controlled Trial Case Report Form

- Demographics
- Pain Assessment (including modified Brief Pain Inventory, S-LANSS)
- Opioid Toxicity Scores
- Opioid history and concomitant medications
- Opioid and breakthrough medication details
- Criteria for switching to an alternative opioid
- Follow up log
- Clinical Assessment sheet
- Reason for reaching study endpoint
- Titration diary
- Constipation assessment tool

Other study documents used included the patient information sheet, the consent form and the GP information sheet (not shown here).

Prospective Morphine Study Case Report Form

A Study to assess the Genetic Determinants of Response to Morphine

Demographics

Name: **Hospital No:**

Diagnosis **DOB:**

Sex **Date:**

Height (cm): **Weight (Kg):**

Information on Ethnicity

Please select one of the following that most clearly resembles your ethnic origin.

White -

British	☐	1
Irish	☐	2
Any other White background	☐	3

Please specify……

Black or Black British -

Carribean	☐	4
African	☐	5
Any other Black background	☐	6

Please specify……

Asian or Asian British –

Indian	☐	7
Pakistani	☐	8
Bangladeshi	☐	9
Any other Asian background	☐	10

Please specify……

Mixed -

White and Black Carribean	☐	11
White and Black African	☐	12
White and Asian	☐	13
Any other Mixed background	☐	14

Please specify…….

Chinese	☐	15
Jewish - Sephardic	☐	16
Ashkenazi	☐	17
Any other Ethnic Background	☐	18

Please specify…….

Current alcohol consumption.

How many units of alcohol do you take, on average, per week?
(Please circle one of the following)

- 0 - 7 units?
- 7 - 14 units?
- 14 - 21 units?
- More than 21 units?

A Study to assess the Genetic Determinants of Response to Morphine

MODIFIED BRIEF PAIN INVENTORY (Short Form)

Name:

_____

Date: ____/____/____

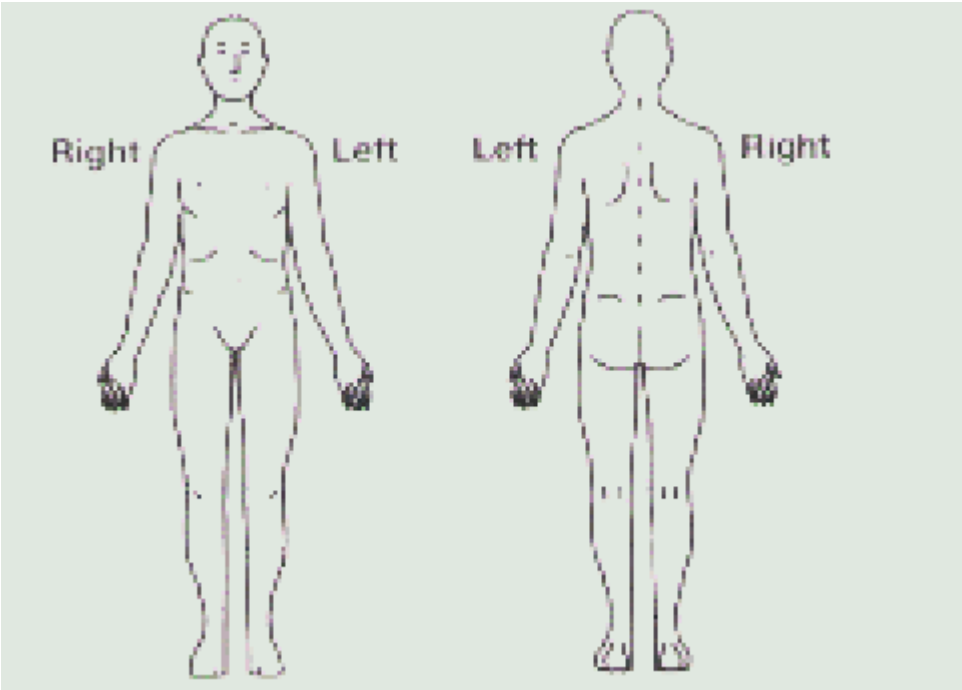
Circle as appropriate Control / Switch **(Patients who are switched need to have a pain score repeated 1 week after they are switched- appendix 5 and 6).**

Date pain and toxicity scores need to be repeated*:* ____/____/____

1. **Throughout our lives, most of us have had pain from time to time (such as minor headaches, sprains and toothaches). Have you h ad pain other than these everyday kinds of pain today?**

   1. Yes 2. No

2. **On the diagram, shade in the areas where you feel pain. Put an X on the area where you have pain.**



3. Please rate your pain by circling the one number that best describes your pain at its *worst* in the last 24 hours.

0	1	2	3	4	5	6	7	8	9	10
No pain										*Pain as bad as you can imagine*

4. Please rate your pain by circling the one number that best describes your pain at its *least* in the last 24 hours.

0	1	2	3	4	5	6	7	8	9	10
No pain										*Pain as bad as you ca imagine*

5. Please rate your pain by circling the one number that best describes your pain on the *average.*

0	1	2	3	4	5	6	7	8	9	10
No pain										*Pain as bad as you can imagine*

6. Please rate your pain by circling the one number that tells how much pain you have *right now.*

0	1	2	3	4	5	6	7	8	9	10
No pain										*Pain as bad as you can imagine*

***7.* In the last 24 hours, how much *relief* have pain treatments or medications provided for your pain? Please circle the one percentage that most shows how much relief you have received?**

0%	10%	20%	30%	40%	50%	60%	70%	80%	90%	100%
No relief										*Complete relief*

A Study to assess the Genetic Determinants of Response to Morphine

Toxicity Scores

Name:

_____

Date: ____/____/____

During the past week:

	Not at All	A Little	Quite a Bit	Very Much
1) Have you felt nauseous?	0	1	2	3
2) Did you vomit?	0	1	2	3
3) Have you had constipation?	0	1	2	3
4) Have you had diarrhoea?	0	1	2	3
5) Have you been drowsy?	0	1	2	3
6) Have you felt confused, disorientated or had hallucinations?	0	1	2	3
7)				
8) Have you had bad dreams?	0	1	2	3

Please record here any other adverse effects you have experienced with the study medication, and score appropriately as above:

Symptom	**Not at All**	**A Little**	**Quite a Bit**	**Very Much**
8)……	0	1	2	3
9)……	0	1	2	3

A Study to assess the Genetic Determinants of Response to Morphine

Opioids and Breakthrough Medication

Name:
_____

Date: _____/_____/_____

Current Regular Opioid	**Dose and Frequency**
Breakthrough Opioid	**Dose and Frequency**
No of breakthrough doses per day (Average daily requirements over past 3 days)	

- Time and Date of last dose of opioid .....
- Time and Date of study blood sample .....
- Time and Date of study urine sample .....

What date did you start taking opioids .....
(If you cannot remember the date, give an approximation in weeks and days of the length of time you have been on this dose).

What date did you start taking **this dose** of opioid .....
(If you cannot remember the date, give an approximation in weeks and days of the length of time you have been on this dose).

If you have changed your opioid medication in the last month, please tell us why you changed it :

.....
.....

- Do you take anything else for your breakthrough pain?

Describe: .....

A Study to assess the Genetic Determinants of Response to Morphine

Criteria for changing to an alternative opioid

Name:_____
___

Date*:* _____/____/____

Please give reason for changing to an alternative opioid

1) Uncontrolled Pain ☐

2) Opioid Toxicity
(Side effects which are intractable despite appropriate intervention and at a level unacceptable to the patient)

- Nausea ☐
- Vomiting ☐
- Constipation ☐
- Drowsiness ☐
- Confusion / Hallucinations ☐
- Nightmares ☐
- Pruritis ☐
- Myoclonus ☐
- Any other symptom ☐

Describe…….

Was patient already on medications to control this symptom? Y/N
If so, what (Medication and dose)?:-

.....
.....

A Study to assess the Genetic Determinants of Response to Morphine

Concomitant Medication

Name:
_____

Drug	**Date __/____/____** Enter dose and frequency

Date and Time last chemo given: .....

Date and Time of last Blood transfusion: .....

A Study to assess the Genetic Determinants of Response to Morphine

Reasons for withdrawal

Name:

_____

On what date did the patient withdraw from the study? .....

What was the reason for withdrawal?

patient requests to withdraw ? Y/N

patient too ill to continue? Y/N

patient unable to complete assessments? Y/N

other Y/N
please specify .....
.....

Date of Death .....

Certified Cause of Death Ia .....
Ib .....
Ic .....
II .....

Randomised Controlled Trial Case Report Form

Morphine versus Oxycodone for Pain Relief

Patient Study Number: RCT_____

Name:	**Hospital No:**
Diagnosis	**DOB:**
Sex	**Date:**
Height (cm):	**Weight (Kg):**
Postcode:	**NHS number:**

Information on Ethnicity

Please select one of the following that most clearly resembles your ethnic origin.

White -

British	☐	1
Irish	☐	2
Any other White background	☐	3

Please specify……

Black or Black British -

Carribean	☐	4
African	☐	5
Any other Black background	☐	6

Please specify……

Asian or Asian British –

Indian	☐	7
Pakistani	☐	8
Bangladeshi	☐	9
Any other Asian background	☐	10

Please specify……

Mixed -

White and Black Carribean	☐	11
White and Black African	☐	12
White and Asian	☐	13
Any other Mixed background	☐	14

Please specify…….

Chinese		☐	15
Jewish -	Sephardic	☐	16
-	Ashkenazi	☐	17
Any other Ethnic Background		☐	18

Please specify…….

<u>Current alcohol consumption.</u>

How many units of alcohol do you take, on average, per week?
(Please circle one of the following)

- 0 - 7 units?

- 7 - 14 units?

- 14 - 21 units?

- More than 21 units?

Is your natural hair colour red? Yes / No

Morphine versus Oxycodone for Pain Relief

MODIFIED BRIEF PAIN INVENTORY (Short Form)

Name: _____ **Date**: ___/____/____

Time points: (please circle)

A: At time of entry to the study (baseline).

B: When the patient is stabilised on first line opioid (approx one week).

C: If patients do not respond to the first line opioid and require switching to the alternative arm of the study (samples must be taken *before* receiving their first dose of alternative opioid).

D: If the patient is stabilised on second line opioid (approx one week post-switch).

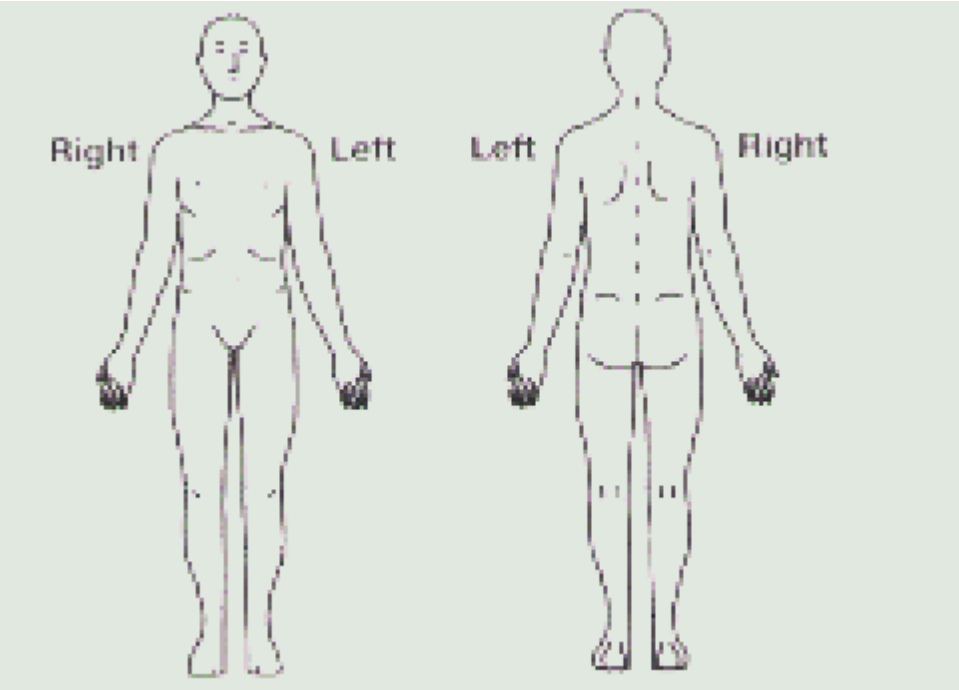
E: If patients' analgesic requirements have increased by 200% of their initial stable dose of opioid.

F: If patients do not respond to the second opioid and fit the criteria to exit from the study.

1. Throughout our lives, most of us have had pain from time to time (such as minor headaches, sprains and toothaches). Have you had pain other than these everyday kinds of pain today?

1. Yes 2. No

2. On the diagram, shade in the areas where you feel pain. Put an X on the area where you have pain.



What type of pain is the participant describing? (please circle)

Somatic	Body surface (usually sharper and may have a burning or pricking quality)or deep tissues (ie musculoskeletal)
Somatic (bony)	dull ache within bones or tender hot spot
Visceral	Pain due to infiltration, compression, extension or stretching of the thoracic, abdominal or pelvic viscera (internal organs within a cavity). Visceral pain is usually not well localized and described pressure-like/squeezing.
Neuropathic	Due to injury to the nervous system, usually burning or tingling.

3. **Please rate your pain by circling the one number that best describes your pain at its *worst* in the last 24 hours.**

0 1 2 3 4 5 6 7 8 9 10
No pain *Pain as bad as you can imagine*

4. **Please rate your pain by circling the one number that best describes your pain at its *least* in the last 24 hours.**

0 1 2 3 4 5 6 7 8 9 10
No pain *Pain as bad as you can imagine*

5. **Please rate your pain by circling the one number that best describes your pain on the *average.***

0 1 2 3 4 5 6 7 8 9 10
No pain *Pain as bad as you can imagine*

6. **Please rate your pain by circling the one number that tells how much pain you have *right now*.**

0 1 2 3 4 5 6 7 8 9 10
No pain *Pain as bad as you can imagine*

7. **In the last 24 hours, how much *relief* have pain treatments or medications provided for your pain? Please circle the one percentage that most shows how much relief you have received?**

0% 10% 20% 30% 40% 50% 60% 70% 80% 90% 100%
No relief *Complete relief*

S-LANSS

1. **In the area where you have pain, do you also have 'pins and needles', tingling or prickling sensations?**

   a) NO – I don't get these sensations (0)
   b) YES – I get these sensations (5)

2. **Does the painful area change colour (perhaps looks mottled or more red) when the pain is particularly bed?**

   a) NO – The pain does not affect the colour of my skin (0)
   b) YES – I have noticed that the pain does make my skin look different from normal (5)

3. **Does your pain make the affected skin abnormally sensitive to touch? Getting unpleasant sensations or pain when lightly stroking the skin might describe this.**

   a) NO – The pain does not make my skin in that area abnormally sensitive to touch (0)
   b) YES – My skin in that area is particularly sensitive to touch (3)

4. **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.**

   a) NO – My pain doesn't really feel like this (0)
   b) YES – I get these sensations often (2)

5. **In the area where you have pain, does your skin feel unusually hot like a burning pain?**

   a) NO – I don't have burning pain (0)
   b) YES – I get burning pain often (1)

6. **Gently <u>rub</u> 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?**

   a) The painful area feels no different from the non-painful area (0)
   b) I feel discomfort, like pins and needles, tingling or burning in the painful area that is different from the non-painful area (5)

7. **Gently <u>press</u> on the painful area with your finger tip then gently press in the same way on to a non-painful area (the same non-painful area that you chose in the last question). How does this feel in the painful are?**

   a) The painful area does not feel different from the non-painful area (0)
   b) I feel numbness or tenderness in the painful area that id different from the non-painful area (3)

Scoring: a score of 12 or more suggests pain of predominately neuropathic origin

TOXICITY SCORES

Name: _____ ***Date:*** ___/____/____

Current Opioid (please circle) Morphine / Oxycodone

Time point (please circle) **A B C D E F**

During the past 24 Hours:

Symptom	**Not at all**										**Worst imaginable**
Have you felt nauseous due to the opioid?	0	1	2	3	4	5	6	7	8	9	10
Have you vomited due to the opioid?	0	1	2	3	4	5	6	7	8	9	10
Have you been constipated due to the opioid?	0	1	2	3	4	5	6	7	8	9	10
Have you had diarrhoea due to the opioid?	0	1	2	3	4	5	6	7	8	9	10
Have you felt drowsy due to the opioid?	0	1	2	3	4	5	6	7	8	9	10
Have you felt confused, disorientated or experienced hallucinations due to the opioid?	0	1	2	3	4	5	6	7	8	9	10
Have you had bad dreams due to the opioid?	0	1	2	3	4	5	6	7	8	9	10
Have you had any other notable symptoms due to the opioid? …….	0	1	2	3	4	5	6	7	8	9	10
……..	0	1	2	3	4	5	6	7	8	9	10

OPIOID HISTORY AND CONCOMITANT MEDICATION

Name:…… **Date ….. / …… / …….**

Current Opioid: (please circle) Morphine/Oxycodone

Time Point: (please circle) **A B C D E F**

Opioid History: (Baseline data collection only)

Name of previous step 2 analgesia	**Approximate dates of administration (start – finish)**

Concomitant Medications:

Drug	**Dose and frequency**

Chemo: Date and time last given …….

Blood transfusion: Date and time last given ……

OPIOID DOSAGE AND BREAKTHROUGH MEDICATION

Name:…… **Date**: ……/……/……..

Time Point: (please circle) **A** **B** **C** **D** **E** **F**

Current Regular Opioid: (please circle) Morphine / Oxycodone	**Dose and Frequency**
Breakthrough Opioid	**Dose and Frequency**
Number of breakthrough doses per day (Average daily requirements over past 3 days)	

- Time and Date of last dose of opioid
  ……..

- Time and Date of study blood sample
  …….

- Time and Date of study urine sample ……

- What date did you start taking **this dose** of opioid
  ……
  (If you cannot remember the date, give an approximation in weeks and days of the length of time you have been on this dose).

- Do you take anything else for your breakthrough pain?

  Please specify……..

Morphine versus Oxycodone for Pain Relief

CRITERIA FOR CHANGING TO AN ALTERNATIVE OPIOID

Name: …… **Date**: ……./……../……..

Current Opioid: (please circle) Morphine / Oxycodone

Has the participant already switched once? (please circle) Y / N

Please give the primary reason for changing to an alternative opioid

1) Uncontrolled Pain ☐

2) Opioid Toxicity
(Side effects which are intractable despite appropriate intervention and at a level unacceptable to the patient)

- Nausea ☐
- Vomiting ☐
- Constipation ☐
- Drowsiness ☐
- Hallucinations ☐
- Nightmares ☐
- Pruritis ☐
- Myoclonus ☐
- Any other symptom ☐

Describe…….

Was patient already on medications to control this symptom? Y/N
If so, what (please specify medication and dose)

……
……

Morphine versus Oxycodone for Pain Relief

SOURCE OF REFERRAL

Name …… Date ……./……./……

Referring Team: (please circle)

Medical Oncology

Clinical Oncology

Surgical

Palliative Care

Geography of Patient: (please circle)

Outpatient Clinic

Inpatient

Ward (please specify)……

Morphine versus Oxycodone for Pain Relief

FOLLOW UP PROFORMA

Name ……

TELEPHONE CALLS

DATE	REASON	OUTCOME

OUTPATIENT CLINICS

CLINIC DATE	ROUTINE (Y / N)	TRANSPORT BOOKED (Y / N)	OUTCOME

CLINICAL ASSESSMENT FORM

Name…….. **Date** ……/……/……

Current Opioid (please circle) Morphine / Oxycodone

Initial Stable Dose Of Opioid …… **Present Dose Of Opioid** ……

1. Have there been any significant clinical events in last 4 weeks?

……

……

2. Is there any specific evidence of disease progression?
(blood tests/tumour markers, imaging)

……

……

……

3. Have there been any changes to concurrent medication? (please specify medication)

……

……

……

4. Is there any other relevant information?

……..

Morphine versus Oxycodone for Pain Relief

STUDY ENDPOINT

Name: …….

On what date did the patient reach the study endpoint? .....

Why has the subject reached the study endpoint?

1. Non-response to 2nd line opioid (ie reached Time Point F) ☐
2. Converted to parenteral opioids ☐
3. Unable to take oral opioids ☐
4. Renal impairment (>1.5 upper limit of normal) ☐
5. Patient requested to withdraw ☐
6. Patient has died ☐

7. Other

   …….

Date of Death .....

Certified Cause of Death

Ia…….

Ib…….

Ic……

II…….

Morphine versus Oxycodone for Pain Relief

OPIOID TITRATION DIARY

Name:…… **Today's date**:
……/……/……..

CURRENT OPIOID (please circle) Morphine / Oxycodone

4 hourly dose	**mg**
Number of 4 hourly doses taken in last 24hours	
Breakthrough dose	**mg**
Number of breakthrough doses in last 24hours	
Total dose (mg) of morphine/oxycodone in last 24hours	**mg**

SIDE EFFECTS: In the past 24hours…….

Symptom	**Not at all**										**Worst imaginable**
Have you felt nauseous?	0	1	2	3	4	5	6	7	8	9	10
Have you vomited due to the opioid?	0	1	2	3	4	5	6	7	8	9	10
Have you been constipated due to the opioid?	0	1	2	3	4	5	6	7	8	9	10
Have you had diarrhea due to the opioid?	0	1	2	3	4	5	6	7	8	9	10
Have you felt drowsy due to the opioid?	0	1	2	3	4	5	6	7	8	9	10
Have you felt confused, disorientated or experienced hallucinations due to the opioid?	0	1	2	3	4	5	6	7	8	9	10
Have you had bad dreams due to the opioid?	0	1	2	3	4	5	6	7	8	9	10
Have you had any other notable symptoms? ……	0	1	2	3	4	5	6	7	8	9	10

PAIN SCORES IN LAST 24 HOURS

1. **Please rate your pain by circling the one number that best describes your pain at its *worst* in the last 24 hours.**

0 1 2 3 4 5 6 7 8 9 10
No pain *Pain as bad as you can imagine*

---

2. **Please rate your pain by circling the one number that best describes your pain at its *least* in the last 24 hours.**

0 1 2 3 4 5 6 7 8 9 10
No pain *Pain as bad as you can imagine*

---

3. **Please rate your pain by circling the one number that best describes your pain on the *average*.**

0 1 2 3 4 5 6 7 8 9 10
No pain *Pain as bad as you can imagine*

---

4. **Please rate your pain by circling the one number that tells how much pain you have *right now*.**

0 1 2 3 4 5 6 7 8 9 10
No pain *Pain as bad as you can imagine*

---

5. **In the last 24 hours, how much *relief* have pain treatments or medications provided for your pain? Please circle the one percentage that most shows how much relief you have received?**

0% 10% 20% 30% 40% 50% 60% 70% 80% 90% 100%
No relief *Complete relief*

OTHER PAIN KILLERS TAKEN IN LAST 24 HOURS?
ie paracetamol, ibuprofen, gabapentin

……
……

Study number: _____ Date: _____

Constipation on Opioids Assessment

Are you satisfied with how your bowel has functioned during the PAST WEEK?

(Please tick) Yes☐ No☐

What does being constipated mean to you? _____

During the PAST WEEK how active have you been? (Please tick)

- ☐ Fully active, able to carry out all activities without restriction
- ☐ Fully mobile, able to carry out light work. Unable to carry out physically strenuous activity
- ☐ Mobile, capable of self-care, unable to carry out any work. Up and about more than 50% of waking hours.
- ☐ Capable only of limited self-care, confined to bed or chair more than 50% of waking hours.
- ☐ Unable to carry out self care. Totally confined to bed or chair

In the PAST WEEK have you been constipated? (Please tick)

Not at all ☐ A little☐ Quite a bit ☐ Very much ☐

In the PAST WEEK how has your bowel functioned OVERALL? (Please tick)

Severe diarrhoea	☐
Moderate diarrhoea	☐
Mild diarrhoea	☐
Normal	☐
Mild constipation	☐
Moderate constipation	☐
Severe constipation	☐

In the PAST WEEK please rate how constipated you been?

0=not at all, 10 = worst imaginable

(Please tick)

0 ☐ **1**☐ **2**☐ **3**☐ **4**☐ **5**☐ **6**☐ **7**☐ **8**☐ **9**☐ **10**☐

During the past week what has your food intake been like?

As usual☐ Less than usual☐ More than usual☐

During the past week what has your fibre intake been like?

As usual☐ Less than usual☐ More than usual☐

During the past week what has your fluid intake been like?

As usual☐ Less than usual☐ More than usual☐

Everyone's *usual* bowel activity is different.
During the PAST WEEK has your bowel activity been USUAL FOR YOU, MORE* CONSTIPATED *than usual or LESS CONSTIPATED than usual? (Please tick)

A little bit more constipated	☐	Usual for you	☐	A little less constipated	☐
Quite a bit more constipated	☐			Quite a bit less constipated	☐
A lot more constipated	☐			A lot less constipated	☐

During the PAST WEEK how many times did you open your bowels?
(Please tick)

More than once a day☐ _____times in the week☐ None at all ☐

Everyone's *usual* bowel activity is different. How many times a week do you USUALLY open your bowels? _____

During the PAST WEEK did you open your bowels MORE or LESS FREQUENTLY than usual? (Please tick)

A little bit less frequently	☐	Usual for you	☐	A little bit more frequently	☐
Quite a bit less frequently	☐			Quite a bit more frequently	☐
A lot less frequently	☐			A lot more frequently	☐

During the PAST WEEK what has your STOOL CONSISTENCY been like in general?
(See Bristol stool chart. Please tick)

Type 1☐ 2☐ 3☐ 4☐ 5☐ 6☐ 7☐

During the PAST WEEK have your stool been HARD (Please tick)

Never ☐ Rarely☐ Occasionally ☐ Nearly always ☐

During the PAST WEEK did you have to STRAIN to open your bowels? (Please tick)

Not at all ☐ A little☐ Quite a bit ☐ Very much ☐

Laxatives taken during the past week:

Laxative Name	Laxative dose and how often taken a day?	How many days did you take this medication?	Any side-effects with this medication?

Other current medications:

Medication Name	Dose and frequency

Planned change to laxative regimen:

The Royal Marsden NHS Foundation Trust

RCT 2586

Adverse / Serious Adverse Event Log

SAE Definition:
Hospitalisation / prolongation of hospitalisation
Death
Life-threatening event
Resulting in persistent / significant incapacity
Resulting in congenital abnormality

AE Definition:
Any untoward medical occurrence in a subject to whom a medicinal product has been administered, including occurrences which are not necessarily caused by or related to that product.

Patient Initials and Study Number:	
Date entered study:	**Date completed:**

AE No	**Adverse event** (Diagnosis or syndrome if known or signs / symptoms)	**Start date** DD/MM/YY	**Stop date** DD/MM/YY	**Ongoing** Tick if 'yes' Once stop date known enter date in stop date field and draw a line through ongoing box. Initial / date	**Severity** 1=mild 2=moderate 3 =Severe	**Expectedness** 1 = expected 2 = not expected	**Relationship to study drug** 1= probably 2= possibly 3=unrelated	**Action taken with study drug** 1=None 2=dose reduced temporarily 3=study med interrupted	**Outcome** 1=recovered 2= stabilized 3=recovered with sequelae 4= not yet recovered 5=fatal 6=unknown	**Serious Adverse Event** If serious complete SAE form as per SOP Yes / No	**CI/Delegated individual review** Please initial and date

Appendix C OPRM, OPRD and OPRK

This appendix contains details of the primer sequences for OPRM, OPRD and OPRK. Negative findings from the candidate gene association study are also presented here.

Primer sequences

Primer sequence for OPRD								
Gene / rs number	**Location**	**Absolute position (bp)**	**AA change**	**Nucleotide**	**5' – 3' specific primer sequence**	**Product length**	**Vol (uL)[2]**	**Control primer**
rs 1042114	Exon 1	29011562	Cys-Phe	G	GCCTACCCTAGCGCCT**G**	693	10	210/211
				T	CGCCTACCCTAGCGCCT**T**		10	
				Consensus	AAGCTCACACCAACAGATGAGC		10	
rs 533123	Intron 1	29013742		G	GTTGGCTGACTGAGCACA**G**	819	10	210/211
				A	TGGTTGGCTGACTGAGCACA**A**		10	
				Consensus	TCTGCTCTCTGAGGACTCTAA		10	
rs 419335	Intron 1	29024431		A	TCCCTGCCTCTCTCTCACTT**T**	702	10	210/211
				G	CCTGCCTCTCTCTCACTT**C**		10	
				Consensus	AGGCACCCTGCCTAGAGAT		10	
rs 2236857	Intron 1	29034196		T	TCCAACACTCAGACAGCATG**T**	267	10	63/64
				C	TCCAACACTCAGACAGCATG**C**		10	
				Consensus	ACACTCAACAAGAAGCAGGAC		10	
rs 2234918	Exon 3	29062184	Gly-Gly	C	TTGAGGCTGCTATTGGCGTA**G**	766	10	63/64
				T	TTGAGGCTGCTATTGGCGTA**A**		10	
				Consensus	TGTTGTCTGTCTTAGCAGTCC		10	
These primers were designed by Dr Sophy Gretton OPRD reference sequence NT_004610.18								

Primer sequence for OPRM								
Gene / rs number	**Location**	**Absolute position (bp)**	**AA change**	**Nucleotide**	**5' – 3' specific primer sequence**	**Product length**	**Vol (uL)[2]**	**Control primer**
rs6912029*	Exon 1	154402201	Gln-His	G	GGAGAATGTCAGATGCTCA**G**	520	10	210/211
				T	AGGAGAATGTCAGATGCTCA**T**		10	
				Consensus	CATTGAGCCTTGGGAGTTAG		10	
rs1799971*	Exon 1	154402490	Asn-Asp	A	TTGTCCCACTTAGATGGC**A**	670	10	210/211
				G	TGTCCCACTTAGATGGC**G**		10	
				Consensus	GTTGCCAAGGTCCATCTC		10	
rs589046*	Intron 1	154434831	-	C	ACAGACTGATGGTTAGCAAC**C**	310	4	63/64
				T	CACAGACTGATGGTTAGCAAC**T**		4	
				Consensus	CTGCCTACCTTGCTATGCC		2	
rs563649	Intron 1	154449660	-	C	GATCATGCAGGTCTATAACCAA**C**	647	5	210/211
				T	GATCATGCAGGTCTATAACCAA**T**		5	
				Consensus	GCTTCTGATTTACACGGTGCC		4	
rs9479757*	Intron 2	154453037	-	G	CATATCAGGCTGTGAACC**C**	530	10	210/211
				A	AACATATCAGGCTGTGAACC**T**		10	
				Consensus	AATAGCCAAGCTGATACTGG		10	
rs2075572*	Intron 2	154453697	-	G	CTCTGGTCAAGGCTAAAAAT**G**	750	20	210/211
				C	CTCTGGTCAAGGCTAAAAAT**C**		20	
				Consensus	CAATTTGCTTCCCCTCTTC		20	
rs533586	Intron 3	154455367	-	C	TGTGAGGACAGATGGCTC**C**	209	5	63/64
				T	ACTGTGAGGACAGATGGCTC**T**		5	
				Consensus	ATGACGGATGTATCTGGGCC		5	

*These primers were designed by Dr Joy Ross
OPRM reference sequence NT_025741.14

Prmer sequence for OPRK								
Gene / rs number	**Location**	**Absolute position (bp)**	**AA change**	**Nucleotide**	**5' – 3' specific primer sequence**	**Product length**	**Vol (uL)[2]**	**Control primer**
rs 10504151	Intron 2	54320793		T	CCTGACAGTTATGGCCACAC**A**	746	10	210/211
				C	CTGACAGTTATGGCCACAC**G**		10	
				Consensus	AGCTTCTTGGAGGAGTAATCC		10	
rs 7836120	Intron 2	54320092		A	GAGTATAAGGGAGGATCTGC**A**	679	10	210/211
				G	GAGTATAAGGGAGGATCTGC**G**		10	
				Consensus	TTAGAAGGACCAAGCTCTAG		10	
rs 6473799	Intron 2	54315676		T	ACTCATCTCTGACCATGCTCA**T**	673	10	210/211
				C	CTCATCTCTGACCATGCTCA**C**		10	
				Consensus	AGTAAAGGAACTCAGGCTTTC		10	
rs 1365098	Intron 2	54315236		G	CATTGTTTTGTCCATTGAGGG**G**	659	10	210/211
				T	CATTGTTTTGTCCATTGAGGG **T**		10	
				Consensus	TTCCTGCTGAGGAGTCACAG		10	
rs 7016778	Intron 2	54312658		T	CATTCCCCAAGCTTTGTCTCA**A**	279	10	63/64
				A	CATTCCCCAAGCTTTGTCTCA**T**		10	
				Consensus	TAGAAGGGCAGTAGTTTCGG		10	
rs 7824175	Intron 3	54306727		G	CAGTTCACATTCTGGATGGA**G**	278	10	63/64
				C	CAGTTCACATTCTGGATGGA**C**		10	
				Consensus	GTAGAAGAGTTTCCTTGGAGG		10	
rs 16918875	Exon 4	54304707	Val-Val	C	AGTCCAGCAGACGACGAA**G**	244	10	63/64
				T	GAGTCCAGCAGACGACGAA**A**		10	
				Consensus	ATTGAGTGCTCCTTGCAGTTC		10	
rs 963549	3UTR	54304377		G	TCATTGAACTCCTCTCTTCC**C**	261	10	63/64
				A	GATCATTGAACTCCTCTCTTCC**T**		10	
				Consensus	ACTTCTGCATCGCCTTAGGC		10	
These primers were designed by Dr Sophy Gretton OPRK reference sequence NT_008183.18								

Analgesic response

Effects of OPRM on analgesic response to morphine (Additive model)							
				Univariate linear regression Log10Component1 (pain scores) as dependent variable			
SNP name	Genotype	% (N)	Mean* (SEM)	B	Std. Error B	β	P value
rs6912029	GG	91.8 (178)	0.45 (0.01)				
	GT	8.2 (16)	0.45 (0.04)	-0.004	0.038	-0.008	0.911
	TT	0	-				
rs1799971	AA	74.7 (145)	0.45 (0.01)				
	AG	21.1 (41)	0.43 (0.02)	<0.001	0.02	0.002	0.981
	GG	4.1 (8)	0.49 (0.06)				
rs589046	CC	50.3 (95)	0.45 (0.02)				
	CT	43.4 (82)	0.46 (0.01)	-0.017	0.017	-0.073	0.319
	TT	6.3 (12)	0.35 (0.04)				
rs563649	CC	80.4 (152)	0.45 (0.01)				
	CT	17.5 (33)	0.45 (0.03)	0.005	0.023	0.015	0.842
	TT	2.1 (4)	0.44 (0.09)				
rs9479757	AA	1.0 (2)	0.23 (0.01)				
	AG	21.1 (41)	0.44 (0.02)	-0.034	0.023	-0.105	0.148
	GG	77.8 (151)	0.45 (0.01)				
rs2075572	CC	35.2 (68)	0.45 (0.02)				
	CG	46.6 (90)	0.44 (0.02)	0.003	0.015	0.013	0.861
	GG	35.2 (68)	0.46 (0.02)				
rs533586	TT	48.7 (92)	0.45 (0.02)				
	CT	40.7 (77)	0.45 (0.02)	0.005	0.016	0.025	0.734
	CC	10.6 (20)	0.44 (0.03)				
* mean (SEM) of log10Component1 scores No statistically significant associations found							

Effects of OPRD on analgesic response to morphine (Dominant model)									
		Carrying allele		Not carrying allele		Univariate linear regression Log10Component1 (pain scores) as dependent variable			
SNP name	Allele	% (N)	Mean* (SEM)	% (N)	Mean* (SEM)	B	Std Error B	β	P value
rs1042114	T	98.4 (186)	0.45 (0.01)	1.6 (3)	0.4 (0.03)	0.043	0.084	0.038	0.607
	G	26.5 (50)	0.45 (0.02)	73.5 (139)	0.45 (0.01)	0.001	0.024	0.002	0.98
rs533123	A	95.2 (180)	0.45 (0.01)	4.8 (9)	0.44 (0.03)	0.009	0.049	0.014	0.851
	G	34.9 (66)	0.44 (0.02)	65.1 (123)	0.45 (0.01)	-0.006	0.022	-0.02	0.79
rs419335	A	89.4 (169)	0.44 (0.01)	10.6 (20)	0.47 (0.04)	-0.023	0.034	-0.05	0.499
	G	55 (104)	0.44 (0.02)	45 (85)	0.46 (0.01)	-0.017	0.021	-0.807	0.42
rs2236857	C	45 (85)	0.44 (0.02)	55 (104)	0.45 (0.01)	-0.013	0.021	-0.046	0.533
	T	92.6 (175)	0.45 (0.01)	7.4 (14)	0.43 (0.05)	0.014	0.04	0.025	0.731
rs2234918	C	65.6 (124)	0.45 (0.01)	34.4 (65)	0.44 (0.02)	0.018	0.022	0.058	0.434
	T	81.5 (154)	0.45 (0.01)	18.5 (35)	0.44 (0.02)	0.011	0.027	0.029	0.693
* mean (SEM) of log10Component1 scores No statistically significant associations found									

Effects of OPRD on analgesic response to morphine (Additive model)							
				Univariate linear regression Log10Component1 (pain scores) as dependent variable			
SNP name	**Genotype**	**% (N)**	**Mean* (SEM)**	**B**	**Std. Error B**	**β**	**P value**
rs1042114	TT	73.5 (139)	0.45 (0.01)				
	GT	24.9 (47)	0.45 (0.02)	0.002	0.022	0.008	0.912
	GG	1.6 (3)	0.4 (0.03)				
rs533123	AA	65.1 (123)	0.45 (0.01)				
	GA	30.2 (57)	0.44 (0.02)	0.005	0.018	0.021	0.773
	GG	4.8 (9)	0.44 (0.03)				
rs419335	AA	45 (85)	0.46 (0.01)				
	AG	44.4 (84)	0.43 (0.02)	0.005	0.016	0.021	0.773
	GG	10.6 (20)	0.47 (0.04)				
rs2236857	CC	7.4 (14)	0.43 (0.05)				
	TC	37.6 (71)	0.44 (0.02)	0.011	0.017	0.047	0.527
	TT	55 (104)	0.45 (0.01)				
rs2234918	CC	18.5 (35)	0.44 (0.02)				
	CT	47.1 (89)	0.46 (0.01)	-0.005	0.015	-0.308	0.758
	TT	34.4 (65)	0.44 (0.02)				
* mean (SEM) of log10Component1 scores No statistically significant associations found							

Central Side-effects

Effects of OPRD on central side-effects of morphine (Dominant model)									
		Carrying allele		Not carrying allele		Univariate linear regression Log10Component2 (central side-effect scores) as dependent variable			
SNP name	Allele	% (N)	Mean* (SEM)	% (N)	Mean* (SEM)	B	Std Error B	β	P value
rs1042114	T	98.4 (186)	0.39 (0.01)	1.6 (3)	0.42 (0.03)	-0.023	0.089	-0.019	0.8
	G	26.5 (50)	0.39 (0.02)	73.5 (139)	0.4 (0.01)	-0.009	0.025	-0.027	0.716
rs533123	A	95.2 (180)	0.39 (0.01)	4.8 (9)	0.41 (0.05)	-0.015	0.052	-0.021	0.773
	G	34.9 (66)	0.38 (0.02)	65.1 (123)	0.4 (0.01)	-0.022	0.023	-0.07	0.341
rs419335	A	89.4 (169)	0.39 (0.01)	10.6 (20)	0.43 (0.03)	-0.035	0.036	-0.071	0.337
	G	55 (104)	0.41 (0.02)	45 (85)	0.38 (0.02)	0.035	0.022	0.116	0.115
rs2236857	C	45 (85)	0.41 (0.02)	55 (104)	0.38 (0.01)	0.03	0.022	0.098	0.182
	T	92.6 (175)	0.39 (0.01)	7.4 (14)	0.46 (0.03)	-0.067	0.042	-0.116	0.114
rs2234918	C	65.6 (124)	0.39 (0.01)	34.4 (65)	0.4 (0.02)	-0.006	0.024	-0.02	0.788
	T	81.5 (154)	0.4 (0.01)	18.5 (35)	0.38 (0.03)	0.022	0.029	0.055	0.454
* mean (SEM) of log10Component2 scores No statistically significant associations found									

Effects of OPRD on central side-effects of morphine Genotype (Additive model)							
				Univariate linear regression Log10Component2 (central side-effect scores) as dependent variable			
SNP name	**Genotype**	**% (N)**	**Mean* (SEM)**	**B**	**Std. Error B**	**β**	**P value**
rs1042114	TT	73.5 (139)	0.4 (0.01)				
	GT	24.9 (47)	0.39 (0.02)	0.0065	0.023	0.02	0.79
	GG	1.6 (3)	0.42 (0.03)				
rs533123	AA	65.1 (123)	0.4 (0.01)				
	GA	30.2 (57)	0.38 (0.02)	0.013	0.019	0.05	0.499
	GG	4.8 (9)	0.41 (0.05)				
rs419335	AA	45 (85)	0.38 (0.02)				
	AG	44.4 (84)	0.41 (0.02)	-0.027	0.017	-0.12	0.103
	GG	10.6 (20)	0.43 (0.03)				
rs2236857	CC	7.4 (14)	0.46 (0.03)				
	TC	37.6 (71)	0.4 (0.02)	-0.03	0.018	-0.126	0.087
	TT	55 (104)	0.38 (0.01)				
rs2234918	CC	18.5 (35)	0.38 (0.03)				
	CT	47.1 (89)	0.4 (0.02)	0.009	0.016	0.044	0.554
	TT	34.4 (65)	0.4 (0.02)				
* mean (SEM) of log10Component2 scores No statistically significant associations found							

Daily morphine dose

Effects of OPRD on daily morphine dose (mg) Genotype (Additive model)							
				Univariate linear regression log10 Daily morphine dose (mg) as dependent variable			
SNP name	**Genotype**	**% (N)**	**Mean* (SEM)**	**B**	**Std. Error B**	**β**	**P value**
rs1042114	TT	71.4 (95)	1.98 (0.04)				
	GT	26.3 (35)	2.02 (0.05)	-0.035	0.06	-0.58	0.563
	GG	2.3 (3)	2.04 (0.16)				
rs533123	AA	61.7 (82)	2.0 (0.04)				
	GA	33.1 (44)	1.95 (0.05)	0.009	0.051	0.015	0.861
	GG	5.3 (7)	2.1 (0.08)				
rs419335	AA	45.9 (61)	1.97 (0.04)				
	AG	44.4 (59)	1.97 (0.05)	-0.073	0.046	-0.137	0.115
	GG	9.8 (13)	2.2 (0.1)				
rs2236857	CC	6.0 (8)	2.24 (0.15)				
	TC	36.8 (49)	1.98 (0.05)	-0.062	0.05	-0.109	0.211
	TT	57.1 (76)	1.98 (0.04)				
rs2234918	CC	18.8 (25)	2.0 (0.07)				
	CT	48.1 (64)	2.03 (0.05)	-0.038	0.043	-0.077	0.377
	TT	33.1 (44)	1.94 (0.05)				
* mean (SEM) of Log10 Daily morphine dose(mg) No statistically significant associations found							

Effects of OPRK on daily morphine dose (mg) **Genotype (Additive model)**							
				Univariate linear regression **log10 Daily morphine dose (mg) as dependent variable**			
SNP name	**Genotype**	**% (N)**	**Mean* (SEM)**	**B**	**Std. Error B**	**β**	**P value**
rs10504151	TT	78.4 (105)	2.0 (0.03)				
	TC	20.1 (27)	2.02 (0.08)	0.049	0.066	0.064	0.464
	CC	1.5 (2)	1.54 (0.24)				
rs7836120	AA	69.4 (93)	2.0 (0.03)				
	AG	26.9 (36)	2.03 (0.07)	0.056	0.055	0.088	0.313
	GG	3.7 (5)	1.5 (0.13)				
rs6473799	TT	61.9 (83)	2.04 (0.04)				
	TC	32.8 (44)	1.91 (0.06)	-0.12	0.061	-0.169	0.052
	CC	5.2 (7)	2.0 (0.12)				
rs1365098	GG	47.8 (64)	2.0 (0.04)				
	GT	42.5 (57)	1.97 (0.05)	0.069	0.046	0.131	0.131
	TT	9.7 (13)	1.89 (0.11)				
rs7016778	TT	74.6 (100)	2.0 (0.03)				
	TA	24.6 (33)	1.98 (0.07)	0.058	0.066	0.076	0.384
	AA	0.7 (1)	-				
rs7824175	GG	81.3 (109)	2.0 (0.03)				
	GC	17.9 (24)	1.95 (0.07)	0.004	0.073	0.005	0.955
	CC	0.7 (1)	-				
rs16918875	CC	83.6 (112)	2.0 (0.03)				
	CT	16.4 (22)	1.97 (0.1)	0.03	0.081	0.032	0.712
	TT	0	-				
rs963549	GG	67.2 (90)	2.0 (0.03)				
	GA	30.6 (41)	1.98 (0.06)	0.013	0.058	0.02	0.822
	AA	2.2 (3)	2.05 (0.25)				
* mean (SEM) of Log10 Daily morphine dose(mg) No statistically significant associations found							

Appendix D ADORA1 / ADRA2A / TRPV1 / ALOX12 / ARRB2 / SLC6A4

This appendix contains details of the primer sequences for ADORA1, ADRA2A, TRPV1, ALOX12, ARRB2 and SLC6A4. Negative findings from the candidate gene association study are also presented here.

Primer sequences

Primer sequence for ALOX12							
rs number	**Location**	**Absolute Position (bp)**	**AA change**	**Primer Type**	**5'-3' primer**	**Vol (uL)[2]**	**Control primer**
rs6502999	Promoter	6833363	-	A	TTG CTG CTT CAC CTT GCA CT**T**	5	210/211
				G	TTG CTG CTT CAC CTT GCA CT**C**	5	
				CONSENSUS	CTT GGT GTG CTT CTC ACA GTA	5	
rs1126667	Exon 6	6843484	Gln-Arg	G	GTT CTT TCT CCA GTT GAG CC**C**	10	63/64
				A	AGT TCT TTC TCC AGT TGA GCC **T**	10	
				CONSENSUS	ATA TAC CTG AAC CCC TGG GGT	10	
rs434473	Exon 8	6845658	Asn-Ser	A	TCC CAG ATT CAG CCT CCC A**A**	10	63/64
				G	TCC CAG ATT CAG CCT CCC A**G**	10	
				CONSENSUS	ACA GTC TCC TCT TCA CTC ACA	10	
These primers were designed by Dr Sophy Gretton							

Primer sequence for ADRA2A							
rs number	**Location**	**Absolute Position (bp)**	**AA change**	**Primer Type**	**5'-3' primer**	**Vol (uL)[2]**	**Control primer**
rs638019	promoter	112821819	-	G	GAA AAC ACC TGG CTT AAT TTT CC**G**	10	63/64
				A	GAA AAC ACC TGG CTT AAT TTT CC**A**	10	
				CONSENSUS	CCT TGG GAA TCC CAT CGT TAT	10	
rs11195418	promoter	112825149	-	A	TGT ATC ACT GGT GCG GGC T**A**	10	210/211
				G	TAT CAC TGG TGC GGG CT**G**	10	
				CONSENSUS	TTG AGG ACC GAA AGC TAG ACT	10	
rs1800544	promoter	112826493	-	C	AGT TGG CCA TGC AGC T**C**	5	210/211
				G	AGT TGG CCA TGC AGC TCG**C**	5	
				CONSENSUS	AGT CTA GCT TTC GGT CCT CAA	5	
rs11195419	3UTR	112829358	-	C	ATC CCC AGT TGT TGG TTT GG**C**	15	63/64
				A	ATC CCC AGT TGT TGG TTT G**G**	15	
				CONSENSUS	GAG AGA GAG TTG GGA ATG GAAA	15	
rs3750625	3UTR	112829591	-	C	AAG AAA AAT GCT AAG GGC AGC **C**	10	63/64
				A	AAG AAA AAT GCT AAG GGC AGC **A**	10	
				CONSENSUS	GAA ATC TCA AAG CAG GTC CGT	10	
rs602618	Downstream	112833075	-	A	TTG ACA CAA AGC AGG CAC AG**T**	5	63/64
				C	TTG ACA CAA AGC AGG CAC AG**G**	5	
				CONSENSUS	TAG AGC CAG AAG CAT CCT CAT	5	
These primers were designed by Dr Sophy Gretton							

Primer sequence for SLC6A4									
rs number	**Location**	**Absolute Position (bp)**	**AA change**	**Primer Type**	**S/AS**	**5'-3' primer**	**Product length**	**Vol (uL)[2]**	**Control primer**
rs7214991	PROMOTOR	3300457	-	A	S	ACA ACT TCA GCC CAT TTC CCT **A**	486	5	63/64
				G	S	ACA ACT TCA GCC CAT TTC CCT **G**		5	
				CONSENSUS	AS	TAC CGA CCG GCT TGG TGA T		5	
	INTRON							5	63/64
rs 25528	BOUNDARY 1	3278162	-	T	S	TGA TGC TGG GGT GGT TGG T**T**	332		
				G	S	ATG CTG GGG TGG TTG GT**G**		5	
				CONSENSUS	AS	ATA AAC AGT CCA TCC CTG GC		5	
rs 6354	5UTR	3278082	-	T	AS	CGA CCT TGC TTG CCC TCT **A**	501	5	210/211
				G	AS	GAC CTT GCT TGC CCT CT**C**		5	
				CONSENSUS	S	ACT CGC TTC CCT TTG AGC CA		5	
rs 140700	INTRON 6	3271585	-	C	S	AAG ACC TTG AGA AAG GAG GG**C**	257	5	63/64
				T	S	AAG ACC TTG AGA AAG GAG GG**T**		5	
				CONSENSUS	AS	AAG ATT CCT TCA ACC CAC TGG		5	
rs 4583306	INTRON 9	32668911	-	A	S	GTA ACA GAT CCA AGA CCC AG**A**	565	5	210/211
				G	S	GTA ACA GAT CCA AGA CCC AG**G**		4	
				CONSENSUS	AS	AGT GGG TTT GCT TCC TGT GAT		4	
SERTP*[302]	PROMOTOR	3292128	-	FORWARD	S	GAG GGA CTG AGC TGG ACA ACC AC	484/528	5	63/64
				REVERSE	AS	GGC GTT GCC GCT CTG AAT GC		5	
SERTP*[303]	PROMOTOR	3292079	-	FORWARD	S	GGA G GAA CTG ACC CCT GAA AAC TG	484/528	5	63/64
				REVERSE	AS	GCC GCT CTG AAT GCC AGC AC		5	
Reference primer sequence NW_926750 *This denotes the reference paper from which the primer is taken									

Primer sequence for ADORA1							
rs number	**Location**	**Absolute Position (bp)**	**AA change**	**Primer Type**	**5'-3' primer**	**Vol (uL)[2]**	**Control primer**
rs2364571	Promoter	201327167	-	C	ACA CGG AGG GAA AGT AGA AGA **C**	5	63/64
				G	ACA CGG AGG GAA AGT AGA AGA **G**	5	
				CONSENSUS	AGT GAG GTT AAA CCT GGG AGC	5	
rs9660662	Intron 2	201335343	-	T	GTG AAC TCC ATA GCT GTG TG**A**	5	63/64
				C	GTG AAC TCC ATA GCT GTG TG**G**	5	
				CONSENSUS	TAG CTC TGC CAT TAA CCC GTA	5	
rs11590405	Intron 2	201346887	-	T	GTA TAA GGC TTA CCT CTC TGG G**T**	5	63/64
				C	AT AAG GCT TAC CTC TCT GGG **C**	5	
				CONSENSUS	ATC TAG CAT GGG CCA GTA AGA	5	
rs903361	Intron 2	201357897	-	A	GTC CCA GAT CTG CTC TGT AG**T**	5	63/64
				G	TCC CAG ATC TGC TCT GTA G**C**	5	
				CONSENSUS	CAC AGC GTC TTG GAT GAT CTT	5	
rs10920568	Exon 5	201364898	Ala-Ala	T	GGC CCT GCT GGC AAT TGC **T**	5	63/64
				G	TGC CCT GCT GGC AAT TGC **G**	5	
				CONSENSUS	AGT TCA CAG TAC AGT CCT CCA	5	
rs10800901	Intron 5	201377927	-	G	GCA CAT AGT AGG CAC TCC **G**	5	63/64
				A	TGG CAC ATA GTA GGC ACT CC**A**	5	
				CONSENSUS	CTT CCC AAC CTG GCT TAC TCA	5	
rs3766560	Intron 5	201385017	-	C	CCT TCT TCT CCT TAT CCC CA**C**	5	210/211
				T	CCT TCT TCT CCT TAT CCC CA**T**	10	
				CONSENSUS	CAC ATC TTG GTT GGC CTC ATT	5	
rs10920576	Intron 5	201395802	-	C	TGC TCC AAG CTT CCA TCC **C**	5	63/64
				T	CTG CTC CAA GCT TCC ATC C**T**	5	
				CONSENSUS	TCA ACC AGC GGG TAT AAT GCA	5	
These primers were designed by Dr Sophy Gretton							

Primer sequence for TRPV1							
rs number	**Location**	**Absolute Position (bp)**	**AA change**	**Primer Type**	**5'-3' primer**	**Vol (uL)[2]**	**Control primer**
rs877610	Exon 15	3422240	-	G	TTG CCT GAG CGG AAG GC**C**	5	63/64
				A	TTG CCT GAG CGG AAG GC**T**	5	
				CONSENSUS	GAT CTT GGC TGA CTG CAA CC	5	
rs2277679	Intron 12	3427524	-	C	TGG GAA GGG GCT GGT TTA **C**	5	63/64
				G	TGG GAA GGG GCT GGT TTA **G**	5	
				CONSENSUS	GGG ATG CGT CTT GAG AAC GT	5	
rs222745	Intron 9	3435620	-	C	CCC TGC CTC AGG CTG TA**C**	5	63/64
				T	CCC TGC CTC AGG CTG TA**T**	5	
				CONSENSUS	GGT TTC ACC ATG TTG GTC AG	5	
rs161393	Intron 8	3436263	-	A	GGG AGG GAG TTA TTA GAC ACA **A**	5	63/64
				G	GGA GGG AGT TAT TAG ACA CA**G**	5	
				CONSENSUS	CAT GTC GTG GCG ATT CTA GG	5	
rs222747	Exon 7	3439949	Met-Ile	C	TGC AGT TTG GCC CCC AG**C**	5	63/64
				G	TGC AGT TTG GCC CCC AG**G**	5	
				CONSENSUS	AGG TTT GGG CAG AGA CAG AG	5	
rs3744683	Promoter	3460211	-	A	GGC ATG GGG CAG TTC TCT **T**	5	210/211
				G	GCA TGG GGC AGT TCT CT**C**	5	
				CONSENSUS	ATA GCA GTC CCT GAG CCC A	5	
These primers were designed by Dr Sophy Gretton							

Primer sequence for ARRB2							
rs number	**Location**	**Absolute Position (bp)**	**AA change**	**Primer Type**	**5'-3' primer**	**Vol (uL)[2]**	**Control primer**
rs1973555	Promoter	4552636	C:T	C	CTGCATAATTGAGGGTGACAA**G**	5	63/64
				T	CTGCATAATTGAGGGTGACAA**A**	5	
				CONSENSUS	CTTGAGGCTGGGAAATGGAG	5	
rs3786047	Intron 1	4561847	G:A	G	GCCAAGTTAGCAGTCTCCTT**C**	10	63/64
				A	GCCAAGTTAGCAGTCTCCTT**T**	10	
				CONSENSUS	GAGGGTATCAGGAATAGGGAAT	10	
rs4522461	Intron 9	4568522	C:T	C	GAGGCTCCCCGTATCATT**C**	2	63/64
				T	CGAGGCTCCCCGTATCATT**A**	2	
				CONSENSUS	ACACACTGATGATGGGAACAG	2	
rs1045280	Exon 11	4569387	T:C	T	CCATAACCCCACTGCTCAG**T**	4	63/64
				C	CATAACCCCACTGCTCAG**C**	4	
				CONSENSUS	CCTTCATACTTGTCACTAAACC	4	
These primers were designed by Dr Joy Ross							

ADORA1

Effects of ADORA1 on analgesic response to morphine Allele carriage (Dominant model)									
		Carrying allele		Not carrying allele		Univariate linear regression Log10Component1 (pain scores) as dependent variable			
SNP name	**Allele**	**% (N)**	**Mean* (SEM)**	**% (N)**	**Mean* (SEM)**	**B**	**Std Error B**	**β**	**P value**
rs 2364571	C	89.5 (170)	0.45 (0.011)	10.5 (20)	0.4 (0.026)	0.054	0.034	0.116	0.115
	G	53.2 (101)	0.47 (0.015)	46.8 (89)	0.43 (0.015)	-0.031	0.021	-0.108	0.141
rs 9660662	T	88.4 (168)	0.46 (0.011)	11.6 (22)	0.39 (0.025)	0.063	0.032	0.141	0.053
	C	55.8 (106)	0.44 (0.015)	44.2 (84)	0.46 (0.015)	-0.021	0.021	-0.073	0.321
rs11590405	T	88.4 (168)	0.46 (0.011)	11.6 (22)	0.39 (0.025)	0.063	0.032	0.141	0.053
	C	55.3 (105)	0.44 (0.015)	44.7 (85)	0.46 (0.015)	-0.02	0.021	-0.069	0.345
rs903361	A	89.5 (171)	0.45 (0.011)	10.5 (20)	0.39 (0.025)	0.062	0.034	0.133	0.069
	G	56 (107)	0.43 (0.015)	44 (84)	0.47 (0.015)	-0.037	0.021	-0.126	0.084
rs10920568	T	90 (171)	0.45 (0.011)	10 (19)	0.39 (0.026)	0.06	0.035	0.126	0.086
	G	53.7 (102)	0.44 (0.015)	46.3 (88)	0.46 (0.015)	-0.025	0.021	-0.088	0.232
rs10800901	G	90 (171)	0.45 (0.011)	0.45 (0.026)	10 (19)	<0.01	0.035	<0.01	0.997
	A	57.4 (109)	0.44 (0.013)	42.6 (81)	0.46 (0.017)	-0.027	0.021	-0.092	0.21
rs3766560	C	95.7 (180)	0.45 (0.011)	4.3 (8)	0.39 (0.051)	0.064	0.052	0.091	0.218
	T	27.7 (52)	0.43 (0.017)	72.3 (136)	0.45 (0.013)	-0.021	0.024	-0.067	0.366
rs10920576	C	98.4 (187)	0.45 (0.011)	1.6 (3)	0.44 (0.098)	0.01	0.084	0.009	0.901
	T	25.3 (48)	0.43 (0.018)	74.7 (142)	0.45 (0.013)	-0.026	0.024	-0.079	0.284
* mean (SEM) of log10Component1 scores									

Effects of ADORA1 on analgesic effects morphine **Genotype (Additive model)**							
				Univariate linear regression **Log10Component1 (pain scores) as dependent variable**			
SNP name	**Genotype**	**% (N)**	**Mean* (SEM)**	**B**	**Std. Error B**	**β**	**P value**
rs 2364571	CC	46.8 (89)	0.47 (0.015)				
	CG	42.6 (81)	0.44 (0.017)	0.029	0.016	0.134	0.067
	GG	10.5 (20)	0.4 (0.026)				
rs 9660662	TT	44.2 (84)	0.46 (0.015)				
	TC	44.2 (84)	0.45 (0.017)	0.026	0.016	0.121	0.098
	CC	11.6 (22)	0.39 (0.025)				
rs 11590405	TT	44.7 (85)	0.46 (0.015)				
	TC	43.7 (83)	0.45 (0.017)	0.025	0.015	0.118	0.106
	CC	11.6 (22)	0.39 (0.025)				
rs 903361	**AA**	**44 (84)**	**0.47 (0.015)**				
	AG	**45.5 (87)**	**0.44 (0.017)**	**-0.034**	**0.016**	**-0.157**	**0.031**
	GG	**10.5 (20)**	**0.39 (0.025)**				
rs 10920568	TT	46.3 (88)	0.46 (0.015)				
	TG	43.7 (83)	0.45 (0.017)	0.027	0.016	0.124	0.09
	GG	10 (19)	0.39 (0.026)				
rs 10800901	GG	42.6 (81)	0.46 (0.017)				
	GA	47.4 (90)	0.43 (0.015)	-0.015	0.016	-0.07	0.339
	AA	10 (19)	0.45 (0.026)				
rs 3766560	CC	72.3 (136)	0.45 (0.013)				
	CT	23.4 (44)	0.44 (0.018)	0.023	0.019	0.088	0.234
	TT	4.3 (8)	0.39 (0.051)				
rs 10920576	CC	74.7 (142)	0.45 (0.013)				
	CT	23.7 (45)	0.43 (0.019)	0.022	0.022	0.074	0.313
	TT	1.6 (3)	0.44 (0.098)				
* mean (SEM) of log10Component1 scores							

Effects of ADORA1 on central side-effects on morphine Allele carriage (Dominant model)									
		Carrying allele		**Not carrying allele**		**Univariate linear regression Log10Component2 (central side-effect scores) as dependent variable**			
SNP name	**Allele**	**% (N)**	**Mean* (SEM)**	**% (N)**	**Mean* (SEM)**	**B**	**Std Error B**	**β**	**P value**
rs 2364571	C	89.5 (170)	0.4 (0.012)	10.5 (20)	0.39 (0.035)	0.003	0.036	0.006	0.933
	G	53.2 (101)	0.4 (0.017)	46.8 (89)	0.39 (0.015)	-0.007	0.022	-0.304	0.762
rs 9660662	T	88.4 (168)	0.4 (0.012)	11.6 (22)	0.38 (0.032)	0.012	0.035	0.025	0.733
	C	55.8 (106)	0.39 (0.015)	44.2 (84)	0.4 (0.017)	-0.004	0.023	-0.014	0.845
rs 11590405	T	88.4 (168)	0.4 (0.012)	11.6 (22)	0.38 (0.032)	0.012	0.035	0.025	0.733
	C	55.3 (105)	0.39 (0.015)	44.7 (85)	0.4 (0.017)	-0.003	0.023	-0.011	0.878
rs 903361	A	89.5 (171)	0.4 (0.012)	10.5 (20)	0.36 (0.029)	0.035	0.036	0.071	0.334
	G	56 (107)	0.39 (0.015)	44 (84)	0.4 (0.017)	-0.001	0.022	-0.005	0.949
rs 10920568	T	90 (171)	0.4 (0.012)	10 (19)	0.36 (0.031)	0.033	0.037	0.065	0.376
	G	53.7 (102)	0.39 (0.015)	46.3 (88)	0.4 (0.017)	-0.002	0.022	-0.006	0.938
rs 10800901	G	90 (171)	0.39 (0.012)	0.45 (0.026)	0.42 (0.033)	-0.025	0.037	-0.049	0.509
	A	57.4 (109)	0.38 (0.014)	42.6 (81)	0.41 (0.018)	-0.025	0.023	-0.082	0.262
rs 3766560	C	95.7 (180)	0.4 (0.012)	4.3 (8)	0.34 (0.046)	0.055	0.055	0.074	0.318
	T	27.7 (52)	0.38 (0.02)	72.3 (136)	0.4 (0.013)	-0.02	0.025	-0.058	0.433
rs 10920576	C	98.4 (187)	0.4 (0.011)	1.6 (3)	0.4 (0.101)	-0.01	0.089	-0.008	0.908
	T	25.3 (48)	0.37 (0.021)	74.7 (142)	0.4 (0.013)	-0.026	0.026	-0.073	0.318
* mean (SEM) of log10Component2 scores No statistically significant associations found									

Effects of ADORA1 on central side-effects on morphine Genotype (Additive model)							
				Univariate linear regression Log10Component2 (central side-effect scores) as dependent variable			
SNP name	**Genotype**	**% (N)**	**Mean* (SEM)**	**B**	**Std. Error B**	**β**	**P value**
rs 2364571	CC	46.8 (89)	0.4 (0.017)				
	CG	42.6 (81)	0.39 (0.017)	0.004	0.017	0.02	0.791
	GG	10.5 (20)	0.39 (0.035)				
rs 9660662	TT	44.2 (84)	0.4 (0.017)				
	TC	44.2 (84)	0.39 (0.017)	0.005	0.017	0.023	0.759
	CC	11.6 (22)	0.38 (0.032)				
rs 11590405	TT	44.7 (85)	0.4 (0.017)				
	TC	43.7 (83)	0.39 (0.017)	0.005	0.017	0.02	0.783
	CC	11.6 (22)	0.38 (0.032)				
rs 903361	AA	44 (84)	0.4 (0.017)				
	AG	45.5 (87)	0.4 (0.017)	-0.008	0.017	-0.037	0.618
	GG	10.5 (20)	0.36 (0.029)				
rs 10920568	TT	46.3 (88)	0.4 (0.017)				
	TG	43.7 (83)	0.4 (0.017)	0.008	0.017	0.034	0.643
	GG	10 (19)	0.36 (0.031)				
rs 10800901	GG	42.6 (81)	0.41 (0.018)				
	GA	47.4 (90)	0.38 (0.016)	-0.009	0.017	-0.04	0.586
	AA	10 (19)	0.42 (0.033)				
rs 3766560	CC	72.3 (136)	0.4 (0.013)				
	CT	23.4 (44)	0.39 (0.022)	0.02	0.02	0.074	0.315
	TT	4.3 (8)	0.34 (0.046)				
rs 10920576	CC	74.7 (142)	0.4 (0.013)				
	CT	23.7 (45)	0.37 (0.021)	0.021	0.023	0.065	0.38
	TT	1.6 (3)	0.4 (0.101)				
* mean (SEM) of log10Component2 scores No statistically significant associations found							

ADRA2A

Effects of ADRA2A on analgesic response to morphine Allele carriage (Dominant model)									
		Carrying allele		Not carrying allele		Univariate linear regression Log10Component1 (pain scores) as dependent variable			
SNP name	Allele	% (N)	Mean* (SEM)	% (N)	Mean* (SEM)	B	Std Error B	β	P value
rs638019	G	94.7 (179)	0.45 (0.011)	5.3 (10)	0.46 (0.049)	0.003	0.047	0.005	0.946
	A	40.2 (76)	0.45 (0.018)	59.8 (175)	0.45 (0.013)	-0.003	0.022	-0.011	0.881
rs11195418	A	100 (188)	0.45 (0.011)	0	-	-	-	-	-
	G	8.5 (16)	0.44 (0.048)	91.5 (172)	0.45 (0.011)	-0.018	0.038	-0.035	0.64
rs1800544	C	94.7 (177)	0.45 (0.017)	5.3 (10)	0.45 (0.014)	0.004	0.047	0.007	0.925
	G	46 (86)	0.45 (0.011)	54 (101)	0.46 (0.049)	-0.002	0.021	-0.007	0.922
rs11195419	C	98.4 (182)	0.45 (0.011)	1.6 (3)	0.52 (0.026)	-0.07	0.084	-0.062	0.405
	A	17.3 (32)	0.47 (0.027)	82.7 (153)	0.44 (0.012)	0.03	0.028	0.081	0.279
rs3750625	C	99.5 (187)	0.45 (0.011)	0.5 (1)	-	-0.022	0.145	-0.011	0.878
	A	9 (17)	0.46 (0.042)	91 (171)	0.45 (0.011)	0.011	0.037	0.022	0.77
rs602618	A	94.2 (178)	0.45 (0.011)	5.8 (11)	0.45 (0.046)	0.014	0.045	0.024	0.75
	C	45.5 (86)	0.45 (0.017)	54.4 (103)	0.45 (0.014)	-0.001	0.021	-0.003	0.964
* mean (SEM) of log10Component1 scores									

Effects of ADRA2A on analgesic effects morphine Genotype (Additive model)							
				Univariate linear regression Log10Component1 (pain scores) as dependent variable			
SNP name	**Genotype**	**% (N)**	**Mean* (SEM)**	**B**	**Std. Error B**	**β**	**P value**
rs638019	GG	59.8 (113)	0.45 (0.013)				
	GA	31.9 (66)	0.45 (0.02)	-0.003	0.018	-0.011	0.882
	AA	4.8 (10)	0.46 (0.049)				
rs11195418	AA	91.5 (172)	0.45 (0.011)				
	AG	8.5 (16)	0.44 (0.048)	-0.018	0.038	-0.035	0.64
	GG	0	-				
rs1800544	CC	54 (101)	0.45 (0.014)				
	CG	40.6 (76)	0.44 (0.018)	-0.002	0.018	-0.009	0.907
	GG	5.3 (10)	0.46 (0.049)				
rs11195419	CC	82.3 (153)	0.44 (0.012)				
	CA	16.1 (30)	0.47 (0.03)	-0.026	0.024	-0.079	0.287
	AA	1.6 (3)	0.52 (0.026)				
rs3750625	CC	91 (171)	0.45 (0.011)				
	CA	8.5 (16)	0.46 (0.045)	-0.01	0.034	-0.023	0.76
	AA	0.5 (1)	-				
rs602618	AA	54.5 (103)	0.45 (0.014)				
	AC	39.7 (75)	0.45 (0.018)	-0.003	0.017	-0.012	0.872
	CC	5.8 (11)	0.45 (0.046)				
* mean (SEM) of log10Component1 scores							

Effects of ADRA2A on central side-effects on morphine **Allele carriage (Dominant model)**									
		Carrying allele		**Not carrying allele**		**Univariate linear regression** **Log10Component2 (central side-effect scores) as dependent variable**			
SNP name	**Allele**	**% (N)**	**Mean* (SEM)**	**% (N)**	**Mean* (SEM)**	**B**	**Std Error B**	**β**	**P value**
rs638019	G	94.7 (179)	0.39 (0.012)	5.3 (10)	0.41 (0.038)	-0.022	0.05	-0.032	0.661
	A	40.2 (76)	0.41 (0.018)	59.8 (175)	0.38 (0.015)	0.028	0.023	0.09	0.223
rs11195418	A	100 (188)	0.39 (0.011)	0	-	-	-	-	-
	G	8.5 (16)	0.47 (0.039)	91.5 (172)	0.39 (0.012)	0.063	0.04	0.117	0.113
rs1800544 p	C	94.7 (177)	0.41 (0.038)	5.3 (10)	0.39 (0.012)	-0.024	0.05	-0.035	0.633
	G	46 (86)	0.41 (0.017)	54 (101)	0.38 (0.016)	0.025	0.023	0.081	0.277
rs11195419	C	98.4 (182)	0.39 (0.011)	1.6 (3)	0.44 (0.131)	-0.053	0.089	-0.044	0.552
	A	17.3 (32)	0.43 (0.026)	82.7 (153)	0.38 (0.013)	0.035	0.03	0.088	0.239
rs3750625	C	99.5 (187)	0.39 (0.011)	0.5 (1)	-	-0.089	0.153	-0.043	0.562
	A	9 (17)	0.39 (0.032)	91 (171)	0.39 (0.012)	-0.011	0.039	-0.02	0.785
rs602618	A	94.2 (178)	0.39 (0.012)	5.8 (11)	0.43 (0.043)	-0.048	0.047	-0.075	0.311
	C	45.5 (86)	0.41 (0.017)	54.4 (103)	0.38 (0.016)	0.029	0.022	0.096	0.194
* mean (SEM) of log10Component2 scores No statistically significant associations found									

Effects of ADRA2A on central side-effects on morphine Genotype (Additive model)							
				Univariate linear regression Log10Component2 (central side-effect scores) as dependent variable			
SNP name	**Genotype**	**% (N)**	**Mean* (SEM)**	**B**	**Std. Error B**	**β**	**P value**
rs638019	GG	59.8 (113)	0.38 (0.015)				
	GA	31.9 (66)	0.41 (0.02)	0.022	0.019	0.086	0.242
	AA	4.8 (10)	0.41 (0.038)				
rs11195418	AA	91.5 (172)	0.39 (0.012)				
	AG	8.5 (16)	0.47 (0.039)	0.063	0.04	0.117	0.113
	GG	0	-				
rs1800544	CC	54 (101)	0.38 (0.016)				
	CG	40.6 (76)	0.41 (0.018)	0.021	0.019	0.081	0.277
	GG	5.3 (10)	0.41 (0.038)				
rs11195419	CC	82.3 (153)	0.38 (0.013)				
	CA	16.1 (30)	0.43 (0.027)	-0.034	0.026	-0.096	0.195
	AA	1.6 (3)	0.44 (0.131)				
rs3750625	CC	91 (171)	0.39 (0.012)				
	CA	8.5 (16)	0.39 (0.033)	0.004	0.036	0.009	0.908
	AA	0.5 (1)	-				
rs602618	AA	54.5 (103)	0.38 (0.016)				
	AC	39.7 (75)	0.4 (0.018)	0.027	0.018	0.108	0.143
	CC	5.8 (11)	0.43 (0.043)				
* mean (SEM) of log10Component2 scores No statistically significant associations found							

Effects of ADRA2A on morphine dose **Allele carriage (Dominant model)**									
		Carrying allele		**Not carrying allele**		**Univariate linear regression** **log10 Daily morphine dose (mg) as dependent variable**			
SNP name	**Allele**	**% (N)**	**Mean* (SEM)**	**% (N)**	**Mean* (SEM)**	**B**	**Std Error B**	**β**	**P value**
rs638019	G	94.7 (126)	2.002 (0.032)	5.3 (7)	1.917 (0.138)	0.098	0.135	0.063	0.471
	A	39.1 (52)	1.95 (0.044)	60.9 (81)	2.028 (0.043)	-0.078	0.062	-0.11	0.209
rs11195418	A	100 (132)	1.998 (0.031)	0	-	-	-	-	-
	G	8.3 (11)	1.8 (0.119)	91.7 (121)	2.013 (0.032)	**-0.227**	**0.109**	**-0.18**	**0.039**
rs1800544	C	94.7 (124)	2.002 (0.032)	5.3 (7)	1.916 (0.138)	0.093	0.137	0.059	0.5
	G	45.8 (60)	1.965 (0.041)	54.2 (71)	2.025 (0.045)	-0.067	0.062	-0.096	0.276
rs11195419	C	98.5 (128)	1.991 (0.31)	1.5 (2)	2.433 (0.111)	-0.445	0.249	-0.156	0.076
	A	15.4 (20)	1.918 (0.079)	84.6 (110)	2.012 (0.034)	-0.109	0.086	-0.112	0.203
rs3750625	C	99.2 (131)	1.995 (0.031)	0.8 (1)	-	-0.329	0.352	-0.082	0.351
	A	9.1 (12)	2.021 (0.095)	90.9 (120)	1.99 (0.033)	0.05	0.106	0.041	0.642
rs602618	A	94.7 (126)	2.002 (0.032)	5.3 (7)	1.916 (0.138)	0.014	0.045	0.024	0.75
	C	45.1 (60)	1.965 (0.041)	54.9 (73)	2.025 (0.045)	-0.001	0.021	-0.003	0.964
* mean (SEM) of log10 Daily morphine dose (mg)									

Effects of ADRA2A on morphine dose **Genotype (Additive model)**							
				Univariate linear regression **log10 Daily morphine dose (mg)as dependent variable**			
SNP name	**Genotype**	**% (N)**	**Mean* (SEM)**	**B**	**Std. Error B**	**β**	**P value**
rs638019	GG	60.9 (81)	2.028 (0.042)				
	GA	33.8 (45)	1.955 (0.047)	-0.066	0.051	-0.114	0.192
	AA	5.3 (7)	1.916 (0.138)				
rs11195418	AA	91.7 (121)	2.013 (0.032)				
	AG	8.3 (11)	1.8 (0.119)	**-0.227**	**0.109**	**-0.18**	**0.039**
	GG	0	-				
rs1800544	CC	54.2 (71)	2.025 (0.045)				
	CG	40.5 (53)	1.971 (0.043)	-0.06	0.051	-0.102	0.245
	GG	5.3 (7)	1.916 (0.138)				
rs11195419	CC	84 (110)	2.012 (0.034)				
	CA	14.5 (19)	1.857 (0.074)	0.048	0.074	0.057	0.516
	AA	1.5 (2)	2.433 (0.111)				
rs3750625	CC	90.9 (120)	1.99 (0.033)				
	CA	8.3 (11)	2.057 (0.101)	-0.063	0.095	-0.058	0.506
	AA	0.8 (1)	-				
rs602618	AA	54.9 (73)	2.025 (0.045)				
	AC	39.8 (53)	1.971 (0.043)	-0.05	0.051	-0.085	0.328
	CC	5.3 (7)	1.916 (0.138)				
* mean (SEM) of log10 Daily morphine dose (mg)							

TRPV1

Effects of TPRV1 on analgesic response to morphine Allele carriage (Dominant model)									
		Carrying allele		Not carrying allele					
SNP name	**Allele**	**% (N)**	**Mean* (SEM)**	**% (N)**	**Mean* (SEM)**	**B**	**Std Error B**	**β**	**P value**
rs877610	G	99.5 (186)	0.45 (0.011)	0.5 (1)	-	0.038	0.145	0.019	0.795
	A	11.2 (21)	0.4 (0.027)	88.8 (166)	0.45 (0.012)	-0.041	0.033	-0.091	0.218
rs2277679	C	86.8 (165)	0.45 (0.012)	13.2 (25)	0.41 (0.025)	0.027	0.031	0.065	0.377
	G	51.1 (97)	0.44 (0.015)	48.9 (93)	0.45 (0.015)	-0.012	0.021	-0.041	0.581
rs222745	C	188 (100)	0.45 (0.011)	0	-	-	-	-	-
	T	15.4 (29)	0.44 (0.02)	84.6 (159)	0.39 (0.011)	-0.005	0.03	-0.013	0.857
rs161393	A	91.5 (173)	0.44 (0.011)	8.5 (16)	0.47 (0.038)	-0.017	0.039	-0.032	0.665
	G	53.4 (101)	0.43 (0.015)	46.6 (88)	0.46 (0.015)	-0.029	0.021	-0.1	0.173
rs222747	C	94.2 (179)	0.45 (0.011)	5.8 (11)	0.45 (0.054)	0.005	0.047	0.008	0.911
	G	46.8 (89)	0.44 (0.017)	53.2 (101)	0.45 (0.013)	-0.023	0.021	-0.079	0.284
rs3744683	A	186 (100)	0.45 (0.011)	0	-	-	-	-	-
	G	3.8 (7)	0.48 (0.04)	96.2 (178)	0.44 (0.011)	0.036	0.056	0.048	0.520
* mean (SEM) of log10Component1 scores No statistically significant associations found									

Effects of TPRV1 on analgesic response to morphine **Genotype (Additive model)**							
SNP name	**Genotype**	**% (N)**	**Mean* (SEM)**	**B**	**Std. Error B**	**β**	**P value**
rs877610	GG	88.8 (166)	0.45 (0.012)				
	GA	10.7 (20)	0.4 (0.028)	0.038	0.031	0.089	0.228
	AA	0.5 (1)	-				
rs2277679	CC	48.9 (93)	0.45 (0.015)				
	CG	37.9 (72)	0.45 (0.018)	0.012	0.015	0.06	0.412
	GG	13.2 (25)	0.41 (0.025)				
rs222745	CC	84.6 (159)	0.45 (0.012)				
	CT	15.4 (29)	0.44 (0.02)	-0.005	0.03	-0.013	0.857
	TT	0	-				
rs161393	AA	46.6 (88)	0.46 (0.015)				
	AG	45 (85)	0.43 (0.016)	0.015	0.017	0.065	0.374
	GG	8.5 (16)	0.47 (0.038)				
rs222747	CC	53.2 (101)	0.45 (0.013)				
	CG	41.1 (78)	0.43 (0.019)	-0.017	0.018	-0.069	0.349
	GG	5.8 (11)	0.45 (0.054)				
rs3744683	AA	95.7 (178)	0.44 (0.011)				
	AG	4.3 (8)	0.48 (0.04)	**-**	**-**	**-**	**-**
	GG	0	-				
* mean (SEM) of log10Component1 scores No statistically significant associations found							

Effects of TPRV1 on central side-effects on morphine **Allele carriage (Dominant model)**									
		Carrying allele		**Not carrying allele**					
SNP name	**Allele**	**% (N)**	**Mean* (SEM)**	**% (N)**	**Mean* (SEM)**	**B**	**Std Error B**	**β**	**P value**
rs877610	G	99.5 (186)	0.39 (0.11)	0.5 (1)	-	0.043	0.154	0.02	0.782
	A	11.2 (21)	0.4 (0.029)	88.8 (166)	0.39 (0.012)	0.012	0.036	0.025	0.737
rs2277679	C	86.8 (165)	0.39 (0.013)	13.2 (25)	0.38 (0.024)	-0.009	0.033	-0.02	0.791
	G	51.1 (97)	0.38 (0.015)	48.9 (93)	0.41 (0.017)	-0.024	0.022	-0.077	0.293
rs222745	C	188 (100)	0.39 (0.011)	0	-	-	-	-	-
	T	15.4 (29)	0.43 (0.025)	84.6 (159)	0.39 (0.013)	0.028	0.031	0.066	0.369
rs161393	A	91.5 (173)	0.4 (0.012)	8.5 (16)	0.33 (0.034)	0.065	0.041	0.117	0.112
	G	53.4 (101)	0.39 (0.016)	46.6 (88)	0.39 (0.017)	<0.001	0.002	<0.001	0.996
rs222747	C	94.2 (179)	0.4 (0.012)	5.8 (11)	0.31 (0.049)	0.051	0.05	0.075	0.307
	G	46.8 (89)	0.38 (0.017)	53.2 (101)	0.4 (0.015)	-0.019	0.022	-0.062	0.395
rs3744683	A	186 (100)	0.39 (0.011)	0	-	-	-	-	-
	G	3.8 (7)	0.42 (0.068)	96.2 (178)	0.39 (0.012)	0.029	0.059	0.037	0.624
* mean (SEM) of log10Component1 scores, No statistically significant associations found									

Effects of TPRV1 on central side-effects morphine Genotype (Additive model)							
SNP name	**Genotype**	**% (N)**	**Mean* (SEM)**	**B**	**Std. Error B**	**β**	**P value**
rs877610	GG	88.8 (166)	0.39 (0.012)				
	GA	10.7 (20)	0.4 (0.031)	-0.008	0.033	-0.019	0.8
	AA	0.5 (1)	0				
rs2277679	CC	48.9 (93)	0.41 (0.017)				
	CG	37.9 (72)	0.38 (0.018)	0.01	.0.16	0.045	0.537
	GG	13.2 (25)	0.38 (0.024)				
rs222745	CC	84.6 (159)	0.39 (0.013)				
	CT	15.4 (29)	0.43 (0.025)	0.028	0.031	0.066	0.369
	TT	0	0				
rs161393	AA	46.6 (88)	0.39 (0.017)				
	AG	45 (85)	0.41 (0.017)	0.012	0.018	0.051	0.493
	GG	8.5 (16)	0.33 (0.034)				
rs222747	CC	53.2 (101)	0.4 (0.015)				
	CG	41.1 (78)	0.39 (0.018)	-0.021	0.019	-0.081	0.273
	GG	5.8 (11)	0.31 (0.049)				
rs3744683	AA	95.7 (178)	0.39 (0.012)				
	AG	4.3 (8)	0.42 (0.068)	-0.021	0.055	-0.029	0.7
	GG	0	-				
* mean (SEM) of log10Component1 scores No statistically significant associations found							

ALOX12

Effect of ALOX12 on analgesic response Allele carriage (Dominant model)									
		Carrying allele		Not carrying allele					
SNP name	Allele	% (N)	Mean* (SEM)	% (N)	Mean* (SEM)	B	Std Error B	β	P value
rs6502999	A	83.8 (160)	0.45 (0.012)	16.2 (31)	0.44 (0.025)	0.015	0.028	0.038	0.602
	G	59.2 (113)	0.46 (0.014)	40.8 (78)	0.45 (0.015)	0.032	0.021	0.108	0.141
rs1126667	G	83.2 (159)	0.45 (0.012)	16.8 (32)	0.46 (0.025)	-0.015	0.028	-0.04	0.584
	A	60.7 (116)	0.46 (0.014)	39.3 (75)	0.43 (0.016)	0.036	0.021	0.123	0.092
rs434473	A	82.2 (157)	0.44 (0.012)	17.8 (34)	0.46 (0.026)	-0.017	0.027	-0.045	0.536
	G	60.7 (116)	0.46 (0.014)	39.3 (75)	0.44 (0.012)	0.036	0.021	0.123	0.092
* mean (SEM) of log10Component1 scores No statistically significant associations found									

Effect of ALOX12 on analgesic response Genotype (additive) model							
SNP name	Genotype	% (N)	Mean* (SEM)	B	Std. Error B	β	P value
rs6502999	AA	40.8 (78)	0.43 (0.015)				
	AG	42.9 (82)	0.47 (0.017)	0.011	0.015	0.054	0.461
	GG	16.2 (31)	0.44 (0.025)				
rs1126667	GG	39.3 (75)	0.43 (0.016)				
	AG	44 (84)	0.46 (0.016)	0.021	0.015	0.105	0.15
	AA	16.8 (32)	0.46 (0.025)				
rs434473	AA	39.3 (75)	0.43 (0.016)				
	AG	42.9 (82)	0.46 (0.016)	-0.021	0.014	-0.107	0.144
	GG	17.8 (34)	0.46 (0.026)				
* mean (SEM) of log10Component1 scores No statistically significant associations found							

Effect of ALOX12 on central side-effects
Allele carriage (Dominant model)

SNP name	Allele	Carrying allele % (N)	Carrying allele Mean* (SEM)	Not carrying allele % (N)	Not carrying allele Mean* (SEM)	B	Std Error B	β	P value
rs6502999	A	83.8 (160)	0.39 (0.012)	16.2 (31)	0.4 (0.025)	-0.004	0.03	-0.01	0.895
	G	59.2 (113)	0.4 (0.014)	40.8 (78)	0.38 (0.018)	0.02	0.023	0.072	0.328
rs1126667	G	83.2 (159)	0.39 (0.012)	16.8 (32)	0.4 (0.025)	-0.011	0.03	-0.027	0.714
	A	60.7 (116)	0.41 (0.014)	39.3 (75)	0.37 (0.019)	0.031	0.023	0.1	0.172
rs434473	A	82.2 (157)	0.39 (0.013)	17.8 (34)	0.4 (0.024)	-0.002	0.029	-0.006	0.939
	G	60.7 (116)	0.41 (0.014)	39.3 (75)	0.37 (0.019)	0.031	0.023	0.1	0.172

* mean (SEM) of log10Component1 scores
No statistically significant associations found

Effect of ALOX12 on central side-effects
Genotype (Additive model)

SNP name	Genotype	% (N)	Mean* (SEM)	B	Std. Error B	β	P value
rs6502999	AA	40.8 (78)	0.38 (0.018)				
	AG	42.9 (82)	0.4 (0.017)	0.012	0.016	0.054	0.46
	GG	16.2 (31)	0.4 (0.025)				
rs1126667	GG	39.3 (75)	0.37 (0.019)				
	AG	44 (84)	0.41 (0.017)	0.018	0.016	0.082	0.261
	AA	16.8 (32)	0.4 (0.025)				
rs434473	AA	39.3 (75)	0.37 (0.019)				
	AG	42.9 (82)	0.41 (0.017)	-0.015	0.015	-0.07	0.339
	GG	17.8 (34)	0.4 (0.024)				

* mean (SEM) of log10Component1 scores
No statistically significant associations found

Effect of ALOX12 on daily morphine dose (mg)
Allele carriage (Dominant model)

SNP name	Allele	Carrying allele % (N)	Carrying allele Mean* (SEM)	Not carrying allele % (N)	Not carrying allele Mean* (SEM)	B	Std Error B	β	P value
rs6502999	A	82.1 (110)	1.997 (0.33)	17.9 (24)	1.988 (0.076)	0.009	0.079	0.01	0.908
	G	56.7 (76)	1.997 (0.42)	43.3 (58)	1.995 (0.043)	0.002	0.061	0.002	0.98
rs1126667	G	82.1 (110)	2.002 (0.03)	17.9 (24)	1.965 (0.068)	0.037	0.079	0.041	0.637
	A	58.2 (78)	2.005 (0.4)	41.8 (56)	1.983 (0.046)	0.021	0.061	0.03	0.733
rs434473	A	81.3 (109)	1.995 (0.03)	18.7 (25)	1.998 (0.073)	-0.002	0.077	-0.003	0.975
	G	58.2 (78)	2.005 (0.04)	41.8 (56)	1.984 (0.046)	0.021	0.061	0.03	0.733

* mean (SEM) of log10Component1 scores
No statistically significant associations found

Effect of ALOX12 on daily morphine dose (mg)
Genotype (Additive model)

SNP name	Genotype	% (N)	Mean* (SEM)	B	Std. Error B	β	P value
rs6502999	AA	43.3 (58)	1.995 (0.43)				
	AG	38.8 (52)	2.000 (0.05)	-0.002	0.041	-0.004	0.966
	GG	17.9 (24)	1.988 (0.76)				
rs1126667	GG	41.8 (56)	1.984 (0.05)				
	AG	40.3 (54)	2.021 (0.05)	-0.001	0.041	-0.001	0.987
	AA	17.9 (24)	1.965 (0.07)				
rs434473	AA	41.8 (56)	1.984 (0.05)				
	AG	39.6 (53)	2.008 (0.05)	-0.01	0.041	-0.021	0.808
	GG	18.7 (25)	1.998 (0.07)				

* mean (SEM) of log10Component1 scores
No statistically significant associations found

SLC6A4

Effects of SLC6A4 on central side-effects on morphine Allele carriage (Dominant model)									
SNP name	**Allele**	**Carrying allele**		**Not carrying allele**		**B**	**Std Error B**	**β**	**P value**
		% (N)	**Mean* (SEM)**	**% (N)**	**Mean* (SEM)**				
rs 7214991	A	90 (171)	0.39 (0.012)	10 (19)	0.43 (0.039)	-0.036	0.037	-0.072	0.331
	G	59.8 (113)	0.4 (0.016)	40.2 (76)	0.39 (0.016)	0.016	0.023	0.051	0.487
rs 22528	T	94.6 (174)	0.39 (0.012)	5.4 (10)	0.42 (0.028)	-0.02	0.05	-0.03	0.685
	G	36.2 (67)	0.41 (0.0190	63.8 (118)	0.39 (0.015)	0.024	0.024	0.074	0.318
rs 6354	T	93.7 (177)	0.4 (0.012)	6.3 (12)	0.4 (0.032)	-0.01	0.046	-0.016	0.826
	G	34.2 (65)	0.42 (0.019)	65.8 (125)	0.38 (0.014)	0.035	0.023	0.11	0.134
rs 140700	C	97.9 (183)	0.4 (0.012)	2.1 (4)	0.41 (0.068)	0.006	0.077	0.006	0.934
	T	18.2 (34)	0.42 (0.027)	81.8 (153)	0.39 (0.013)	0.028	0.029	0.072	0.329
rs 4583306	A	78.9 (150)	0.4 (0.013)	21.1 (40)	0.38 (0.023)	0.026	0.028	0.07	0.338
	G	71.1 (135)	0.4 (0.014)	28.9 (55)	0.39 (0.022)	0.003	0.025	0.008	0.917
SERTP	S	69 (126)	0.38 (0.014)	31 (56)	0.4 (0.02)	-0.015	0.024	-0.047	0.529
	L	82 (149)	0.39 (0.013)	18 (33)	0.39 (0.024)	-0.004	0.029	-0.01	0.889
* mean (SEM) of log10Component1 scores, No statistically significant associations found									

Effects of SLC6A4 on central side-effects morphine Genotype (Additive model)							
SNP name	**Genotype**	**% (N)**	**Mean* (SEM)**	**B**	**Std. Error B**	**β**	**P value**
rs 7214991	AA	40.2 (76)	0.39 (0.016)				
	AG	49.7 (94)	0.39 (0.0170	0.017	0.017	0.072	0.332
	GG	10.1 (19)	0.43 (0.039)				
rs 22528	TT	63.9 (117)	0.39 (0.015)				
	TG	30.6 (56)	0.41 (0.022)	-0.021	0.019	-0.083	0.27
	GG	5.5 (10)	0.42 (0.028)				
rs 6354	TT	66.1 (125)	0.38 (0.014)				
	TG	27.5 (52)	0.42 (0.022)	-0.023	0.018	-0.093	0.209
	GG	6.3 (12)	0.4 (0.032)				
rs 140700	CC	81.8 (153)	0.39 (0.013)				
	CT	16 (30)	0.42 (0.029)	0.02	0.025	0.06	0.421
	TT	2.1 (4)	0.41 (0.068)				
rs 4583306	AA	28.9 (55)	0.39 (0.022)				
	AG	50 (95)	0.4 (0.017)	-0.008	0.016	-0.486	0.628
	GG	21.1 (40)	0.38 (0.023)				
SertP	SS	17.8 (33)	0.4 (0.0230				
	LS	51.4 (95)	0.39 (0.017)	0.006	0.016	0.026	0.731
	LL	30.8 (57)	0.41 (0.021)				
* mean (SEM) of log10Component1 scores No statistically significant associations found							

Effects of SLC6A4 on morphine dose **Allele carriage (Dominant model)**									
SNP name	**Allele**	**Carrying allele**		**Not carrying allele**		**B**	**Std Error B**	**β**	**P value**
		% (N)	**Mean* (SEM)**	**% (N)**	**Mean* (SEM)**				
rs 7214991	A	88.8 (119)	2 (0.03)	11.2 (15)	20.6 (0.12)	-0.068	0.097	-0.061	0.482
	G	62.4 (83)	2.01 (0.04)	37.6 (50)	1.98 (0.05)	0.026	0.064	0.035	0.687
rs 22528	T	94.6 (122)	2 (0.03)	5.4 (7)	2.07 (0.05)	-0.069	0.139	-0.044	0.619
	G	36.6 (48)	1.99 (0.05)	63.4 (83)	2 (0.04)	-0.015	0.065	-0.02	0.82
rs 6354	T	93.2 (124)	2 (0.03)	6.8 (9)	1.99 (0.1)	0.013	0.123	0.01	0.913
	G	34.3 (46)	1.99 (0.05)	65.7 (88)	2.01 (0.04)	-0.027	0.064	-0.036	0.68
rs 140700	C	99.2 (131)	2 (0.03)	0.8 (1)	-	0.049	0.358	0.012	0.891
	T	16.7 (22)	1.98 (0.07)	83.3 (110)	2.01 (0.03)	-0.022	0.083	-0.023	0.796
rs 4583306	A	80.6 (108)	2.01 (0.03)	19.4 (26)	1.96 (0.09)	0.055	0.077	0.062	0.475
	G	70.9 (95)	2.01 (0.04)	29.1 (39)	1.99 (0.05)	0.014	0.067	0.018	0.836
SERTP	S	67 (88)	2 (0.038)	33 (43)	2.01 (0.05)	-0.006	0.067	-0.008	0.924
	L	82 (108)	1.99 (0.03)	18 (23)	2.1 (0.08)	-0.07	0.082	-0.075	0.394
* mean (SEM) of log10Component1 scores									

Effects of SLC6A4 on morphine dose Genotype (Additive model)							
SNP name	**Genotype**	**% (N)**	**Mean* (SEM)**	**B**	**Std. Error B**	**β**	**P value**
rs 7214991	AA	3706 (50)	1.98 (0.05)				
	AG	51.1 (68)	2 (0.04)	0.031	0.048	0.057	0.511
	GG	11.3 (15)	2.06 (0.12)				
rs 22528	TT	63.6 (82)	2.01 (0.04)				
	TG	31 (40)	1.98 (0.05)	-0.002	0.053	-0.004	0.968
	GG	5.4 (7)	2.07 (0.05)				
rs 6354	TT	66.2 (88)	2.01 (0.04)				
	TG	27.1 (36)	1.98 (0.06)	0.02	0.05	0.034	0.695
	GG	6.8 (9)	1.99 (0.1)				
rs 140700	CC	83.3 (110)	2.01 (0.03)				
	CT	15.9 (21)	1.99 (0.07)	-0.021	0.078	-0.024	0.786
	TT	0.8 (1)	-				
rs 4583306	AA	29.1 (39)	1.99 (0.05)				
	AG	51.5 (69)	2.02 (0.04)	-0.012	0.044	-0.024	0.786
	GG	19.4 (26)	1.96 (0.09)				
SertP	SS	17.6 (23)	2.06 (0.08)				
	LS	49.6 (65)	1.98 (0.04)	-0.018	0.045	-0.035	0.688
	LL	32.8 (43)	2.01 (0.05)				
* mean (SEM) of log10Component1 scores No statistically significant associations found							

ARRB2

Effects of ARRB2 on analgesic response to morphine
Allele carriage (Dominant model)

SNP name	Allele	Carrying allele		Not carrying allele		B	Std Error B	β	P value
		% (N)	Mean* (SEM)	% (N)	Mean* (SEM)				
rs1973555	C	92.1 (176)	0.45 (0.011)	7.9 (15)	0.46 (0.042)	0.005	0.041	0.009	0.905
	T	52.9 (101)	0.45 (0.015)	47.1 (90)	0.46 (0.016)	-0.013	0.021	-0.043	0.557
rs3786047	G	87.8 (166)	0.45 (0.012)	12.2 (23)	0.47 (0.026)	-0.013	0.033	-0.029	0.698
	A	57.9 (110)	0.45 (0.015)	42.1 (80)	0.46 (0.016)	-0.013	0.022	-0.044	0.547
rs4522461	C	95.8 (182)	0.45 (0.011)	4.2 (8)	0.53 (0.05)	-0.045	0.056	-0.058	0.43
	T	41.6 (79)	0.44 (0.018)	58.4 (111)	0.46 (0.014)	-0.022	0.022	-0.075	0.304
rs1045280	T	89.2 (165)	0.45 (0.012)	10.8 (20)	0.45 (0.026)	0.002	0.036	0.005	0.95
	C	54.6 (101)	0.44 (0.015)	45.4 (84)	0.46 (0.015)	-0.024	0.022	-0.083	0.265

* mean (SEM) of log10Component1 scores
No statistically significant associations found

Effects of ARRB2 on analgesic response to morphine Genotype (Additive model)							
SNP name	**Genotype**	**% (N)**	**Mean* (SEM)**	**B**	**Std. Error B**	**β**	**P value**
rs1973555	CC	47.1 (90)	0.46 (0.016)				
	CT	45 (86)	0.44 (0.016)	0.009	0.017	0.038	0.603
	TT	7.9 (15)	0.46 (0.042)				
rs3786047	GG	42.3 (80)	0.46 (0.016)				
	GA	45.5 (86)	0.44 (0.018)	0.003	0.016	0.013	0.856
	AA	12.2 (23)	0.47 (0.026)				
rs4522461	CC	58.4 (111)	0.46 (0.014)				
	CT	37.4 (71)	0.43 (0.018)	0.012	0.019	0.046	0.532
	TT	4.2 (8)	0.53 (0.05)				
rs1045280	TT	45.4 (84)	0.46 (0.015)				
	TC	43.8 (81)	0.44 (0.018)	0.014	0.016	0.065	0.385
	CC	10.8 (20)	0.45 (0.026)				
* mean (SEM) of log10Component1 scores No statistically significant associations found							

Effects of ARRB2 on variability in morphine dose required to achieve adequate analgesia
Allele carriage (Dominant model)

		Carrying allele		Not carrying allele					
SNP name	**Allele**	**% (N)**	**Mean* (SEM)**	**% (N)**	**Mean* (SEM)**	**B**	**Std Error B**	**β**	**P value**
rs1973555	C	93.2 (123)	1.99 (0.032)	6.8 (9)	1.96 (0.149)	0.064	0.121	0.046	0.599
	T	55.3 (73)	1.98 (0.042)	44.7 (59)	2.01 (0.047)	-0.022	0.061	-0.031	0.721
rs3786047	G	87.8 (115)	1.99 (0.033)	12.2 (16)	2.01 (0.094)	0.006	0.094	0.006	0.949
	A	61.4 (81)	1.99 (0.039)	38.6 (51)	1.99 (0.053)	-0.003	0.063	-0.004	0.963
rs4522461	C	97.7 (128)	1.99 (0.031)	2.3 (3)	2.21 (0.308)	-0.046	0.205	-0.02	0.821
	T	43.5 (57)	1.98 (0.05)	56.5 (74)	2.0 (0.041)	-0.016	0.062	-0.022	0.801
rs1045280	T	88.2 (112)	1.98 (0.033)	11.8 (15)	2.06 (0.094)	-0.073	0.097	-0.067	0.451
	C	58.3 (74)	1.97 (0.04)	41.7 (53)	2.02 (0.05)	-0.05	0.063	-0.071	0.426

* mean (SEM) of log10Daily morphine dose
No statistically significant associations found

Effects of ARRB2 on variability in morphine dose required to achieve adequate analgesia Genotype (Additive model)							
SNP name	**Genotype**	**% (N)**	**Mean* (SEM)**	**B**	**Std. Error B**	**β**	**P value**
rs1973555	CC	44.7 (59)	2.01 (0.047)				
	CT	48.5 (64)	1.98 (0.044)	0.025	0.05	0.045	0.611
	TT	6.8 (9)	1.96 (0.149)				
rs3786047	GG	38.9 (51)	1.99 (0.053)				
	GA	48.9 (64)	1.98 (0.043)	0.002	0.046	0.005	0.958
	AA	12.2 (16)	2.01 (0.094)				
rs4522461	CC	56.5 (74)	2.0 (0.041)				
	CT	41.2 (54)	1.97 (0.05)	0.009	0.057	0.015	0.867
	TT	2.3 (3)	2.21 (0.31)				
rs1045280	TT	41.7 (53)	2.02 (0.05)				
	TC	46.5 (59)	1.95 (0.044)	0.01	0.047	0.02	0.824
	CC	11.8 (15)	2.06 (0.094)				
* mean (SEM) of log10Daily morphine dose No statistically significant associations found							

Appendix E GWA primers

This appendix contains details of the primer sequences for PRKD3, NTRK3 and ZDHHC14, used in the validation study in the GWA.

SSP-PCR primers designed for PRKD3 (chr2:37,331,152-37,397,726 2p22.2)						
Rs / "Affy" id	**Position (absolute bp)**	**Locus**	**Primer Type**	**S/AS**	**5'-3' primer**	**Product size**
rs 1124574	37,352,793	Intron 11	SPECIFIC	AS	GCC TTC TCT TTG AAC CAG TCT TA**T**	357
			SPECIFIC	AS	CCT TCT CTT TGA ACC AGT CTT A**C**	
			CONSENSUS	S	TAT CCT GTA GCC CAG GTG GT	
rs 6730503	37,381,982	Intron 1	SPECIFIC	S	CCT TTG TTC TTT GTT CTC CTG **G**	443
			SPECIFIC	S	CCT TTG TTC TTT GTT CTC CTG **A**	
			CONSENSUS	AS	CAA ATT GCT CTT TAT GGC GGT C	
rs 1158219	37400324	Promoter	SPECIFIC	S	TGA GAA CAC ATC AGC ATC CG**A**	286
			SPECIFIC	S	TGA GAA CAC ATC AGC ATC CG**T**	
			CONSENSUS	AS	CAG ATA GTT TGT ACC CCG TAG	
Reference sequences used for primer design: NW_001838769 CHIP Bioinformatics PRKD3 accessed 20/11/08						

SSP-PCR primers designed for ZDHHC14 (chr6:157,722,545-158,014,965 6q25.3)						
rs	**Position (absolute bp)**	**Locus**	**Primer Type**	**S/AS**	**5'-3' primer**	**Product size**
rs17165436	157907605	Intron 2	SPECIFIC	AS	CTG CTC TTT CAG GGT AGG TC**T**	406
			SPECIFIC	AS	TGC TCT TTC AGG GTA GGT C**C**	
			CONSENSUS	S	GCA TGG AGG CAC ATG CTT G	
rs4463281	157900894	Intron 2	SPECIFIC	S	GGC CTA TTT CAA CTT CTT TCA CA**T**	305
			SPECIFIC	S	GGC CTA TTT CAA CTT CTT TCA CA**G**	
			CONSENSUS	AS	AGC CAC CCT GCC ATC TTG AA	
rs17165429	157900789	Intron 2	SPECIFIC	S	TCT CTC TCT GTA AAT CCA GAC **A**	330
			SPECIFIC	S	CTC TCT CTG TAA ATC CAG AC**G**	
			CONSENSUS	AS	TTG CCT CTT TCC GTG GGC AT	
Reference sequences used for primer design: NW_001838991						

SSP-PCR primers designed for NTRK3 (chr15:86,220,992-86,600,665 15q25.3)						
rs	**Position (absolute bp)**	**Locus**	**Primer Type**	**S/AS**	**5'-3' primer**	**Product size**
rs17755717	86,425,100	Intron 12	SPECIFICG	AS	CTG AGG CCA TTC GAT CAG A**G**	384
			SPECIFIC A	AS	CTG AGG CCA TTC GAT CAG A**A**	
			CONSENSUS	S	GAG TCT CAC TTT GTC ACC CA	
rs1559822	86,564,157	Intron 2	SPECIFIC A	AS	GGT CAC ACA GCT CTC ACT G**A**	529
			SPECIFIC G	AS	GT CAC ACA GCT CTC ACT G**G**	
			CONSENSUS	S	AGT GGC AGA GCC TAG ATT TGA	
rs8026216	86,585,243	Intron 2	SPECIFIC T	S	CAGT AAA GAC CTG TGG ACT G**T**	278
			SPECIFIC G	S	AGT AAA GAC CTG TGG ACT G**G**	
			CONSENSUS	AS	GGA CCT GCC ATG TCA CTG T	
rs1075891	86,585,302	Intron 2	SPECIFIC A	S	TAC GTG CTC TTC CCA GGC **A**	247
			SPECIFIC G	S	TAC GTG CTC TTC CCA GGC **G**	
			CONSENSUS	AS	AAT GGA CGG AAT CTG CCG TG	
rs4887382	86,555,933	Intron 2	SPECIFICG	S	CTT TTC ACC TTA GCT GCC A**G**	515
			SPECIFIC A	S	CCT TTT CAC CTT AGC TGC CA**A**	
			CONSENSUS	AS	CTA TTG AGC AGT CAC ACT CCC	
rs1863482	86,560,748	Intron 2	SPECIFIC T	S	CTA ATT TGG GGT CGT GCA GAA **T**	324
			SPECIFIC C	S	TAA TTT GGG GTC GTG CAG AA**C**	
			CONSENSUS	AS	GAG CGA GTT GTT ATG TTG TAG C	
Reference sequences used for primer design: NM_002530 Chip Bioinformatics, accessed 21/11/08						

Appendix F Constipation on morphine: Genetic association study

Negative findings from the genetic association study of constipation on morphine are presented here.

ARRB2 and Bowel problems on morphine **Logistic regression corrected for clinical factors (lower GI tumour)** **% (N)**										
	Genotype frequency (additive model)					**Allele carriage (dominant model)**				
SNP	**Genotype**	**No bowel problems on morphine N=34**	**Bowel problems on morphine N=183**	**OR (95% CI)**	**P value**	**Allele**	**No bowel problems on morphine N=37**	**Bowel problems on morphine N=199**	**OR (95% CI)**	**P value**
rs1973555	CC	0.5 (17)	0.44 (21)			C	0.94 (32)	0.9 (165)	0.653 (0.143-2.975)	0.581
	CT	0.44 (15)	0.45 (83)	0.789 (0.431-1.444)	0.442	T	0.5 (17)	0.56 (102)	1.294 (0.611-2.739)	0.501
	TT	0.06(2)	0.1 (19)							
rs3786047	GG	0.47 (16)	0.41 (74)			G	0.91 (31)	0.84 (153)	0.616 (0.174-2.184)	0.453
	GA	0.44 (15)	0.43 (79)	1.32 (0.75-2.325)	0.336	A	0.53 (18)	0.6 (109)	1.386 (0.652-2.945)	0.396
	AA	0.9 (3)	0.16 (29)							
rs4522461	CC	0.65 (22)	0.57 (105)			C	1.0 (34)	0.96 (175)	<0.0001	0.999
	CT	0.35 (12)	0.39 (70)	0.664 (0.323-1.365)	0.265	T	0.35 (12)	0.43 (78)	1.407 (0.644-3.076)	
	TT	0 (0)	0.04 (8)							
rs1045280	TT	0.5 (17)	0.42 (74)			T	0.88 (30)	0.84 (148)	0.763 (0.246-2.37)	0.64
	TC	0.38 (13)	0.42 (74)	1.304 (0.75-2.266)	0.347	C	0.5 (17)	0.59 (103)	1.475 (0.692-3.143)	
	CC	0.12 (4)	0.16 (29)							
No statistically significant differences seen										

SLC6A4 and Bowel problems on morphine
Logistic regression corrected for clinical factors (lower GI tumour)

		Allele carriage (dominant model)				Genotype frequency (additive model)				
		Bowel problems on morphine					Bowel problems on morphine			
SNP name	Allele	No (N=37)	Yes (N=199)	OR (95% CI)	P value	Genotype	No (N=37)	Yes (N=199)	OR (95% CI)	P value
rs 7214991	A	91 (31)	90 (163)	0.81 (0.221-2.978)	0.752	AA	38 (13)	40 (72)		
	G	62 (21)	60 (109)	1.014 (0.47-2.189)	0.47	AG	53 (18)	50 (90)	0.947 (0.526-1.705)	0.855
						GG	9 (3)	10 (19)		
rs 22528	G	44 (14)	37 (67)	0.77 (0.353-1.670)	0.511	GG	3 (1)	6 (10)		
	T	97 (31)	94 (169)	0.516 (0.061-4.369)	0.544	GT	41 (13)	31 (56)	0.902 (0.477-1.707)	0.752
						TT	56 (18)	63 (112)		
rs6354	G	41 (14)	36 (65)	0.825 (0.385-1.768)	0.62	GG	3 (1)	7 (12)	0.969 (0.524-1.792)	0.92
	T	97 (33)	93 (170)	0.424 (0.052-3.461)	0.423	GT	38 (13)	29 (52)		
						TT	59 (20)	65 (118)		
Rs 140700	C	100 (34)	98 (177)	<0.0001	0.999	CC	77 (26)	83 (150)		
	T	24 (8)	17 (31)	0.744 (0.299-1.848)	0.524	CT	23 (8)	15 (27)	1.144 (0.509-2.57)	0.744
						TT	0 (0)	2 (4)		
rs 4583306	A	77 (26)	81 (149)	1.325 (0.542-3.24)	0.537	AA	32 (11)	31 (57)		
	G	68 (23)	69 (126)	1 (0.449-2.23)	1	AG	44 (15)	50 (92)	1.098 (0.648-1.858)	0.729
						GG	24 (8)	19 (34)		
SertP						SS	24 (8)	17 (31)		
						LS	41 (14)	54 (96)	0.965 (561-1.661)	0.561
						LL	35 (12)	29 (52)		

Data are given as % (N)
No significant associations found

ADRA2A and Bowel problems on morphine **Logistic regression corrected for clinical factors (lower GI tumour)**										
	Genotype frequency (additive model)					**Allele carriage (dominant model)**				
SNP	**Genotype**	**No bowel problems on morphine N=37**	**Bowel problems on morphine N=199**	**OR (95% CI)**	**P value**	**Allele**	**No bowel problems on morphine N=37**	**Bowel problems on morphine N=199**	**OR (95% CI)**	**P value**
rs638019	GG	57.6 (19)	60.1 (110)			G	100 (33)	94 (172)	<0.001	0.999
	GA	42.4 (14)	33.9 (62)	0.904 (0.477-1.713)	0.756	A	42.4 (14)	39.9 (73)	0.901 (0.425-1.909)	0.785
	AA	0	6 (11)							
rs11195418	AA	88.2 (30)	92.3 (167)			A	100 (34)	100 (181)	-	-
	AG	11.8 (4)	7.7 (14)	1.59 (0.49-5.162)	0.44	G	11.8 (4)	7.7 (14)	0.629 (0.194-2.04)	0.44
	GG	0	0							
rs1800544	CC	50 (17)	55 (99)			C	100 (34)	93.3 (168)	<0.001	0.999
	CG	50 (17)	38.3 (69)	0.955 (0.517-1.763)	0.882	G	50 (17)	45 (81)	0.818 (0.393-1.704)	0.592
	GG	0	6.7 (12)							
rs11195419	CC	76.5 (26)	84.4 (152)			C	97.1 (33)	98.3 (176)	1.778 (0.179-17.617)	0.623
	CA	20.6 (7)	13.9 (25)	0.652 (0.309-1.375)	0.261	A	23.5 (8)	15.1 (27)	0.577 (0.237-1.408)	0.227
	AA	2.9 (1)	1.7 (3)							
rs3750625	CC	82.4 (196)	92.8 (168)			C	100 (34)	99.4 (180)	<0.001	1
	CA	17.6 (6)	6.6 (12)	0.435 0.164 – 1.156)	0.095	A	17.6 (6)	7.2 (13)	0.361 (0.127-1.029)	0.057
	AA	0	0.6 (1)							
rs602618	AA	52.9 (18)	55.5 (101)			A	100 (34)	92.9 (169)	<0.001	0.999
	AC	47.1 (16)	37.4 (68)	0.881 (0.476-1.63)	0.687	C	47.1 (16)	44.5 (81)	0.902 (0.433-1.88)	0.784
	CC	0	7.1 (13)							
% (N) No statistically significant differences seen										

Appendix G Proforma for Constipation Audit

Constipation audit number_____
Patient initials_____

Constipated? Yes □ No □

Outpatient □ Inpatient □ (dates of inpatient admission_____ to _____)

1. Is there documentation of the patient's bowel function in the **medical notes/EPR** within the first 1-2 days of the current clinical episode

Yes □ No □

2. If the answer to question 1 is yes is there documentation of the following in the **medical notes/EPR**

Straining	Yes □	No □
Frequency	Yes □	No □
Consistency	Yes □	No □

3. Was there documentation of the patient's usual bowel function at any stage in the clinical episode in the:

Medical notes/EPR Yes □ No □

Nursing notes Yes □ No □

4. Who identified constipation as a problem?

Medical Oncologists	Yes □	No □
Surgeons	Yes □	No □
Palliative Care Team	Yes □	No □
Nursing Staff	Yes □	No □
Other	Yes □	No □Specify----------

5. If constipation was identified by medical/ nursing staff was the constipation core care plan being used?

Yes □ No □

6a. Was the patient on laxatives on admission / at the start of this clinical episode?

Yes □ No □

6b. Was there evidence that laxatives had been changed (changed, initiated or titrated) on the medication chart if constipation was identified?

Yes □ No □

7. Was there evidence of changes in laxative therapy based on the patient's symptoms?

Medical notes/EPR	Yes □	No □
Nursing notes	Yes □	No □

Describe_____

_____

8. If laxatives were changed/initiated was there evidence that the response was reviewed?

Medical notes/EPR	Yes □	No □
Nursing notes	Yes □	No □

9a. Did the patient die in hospital this admission? Yes □ No □

9 b. If not was there evidence either from the medical/nursing notes or on EPR that a follow up plan was in place for the management of constipation following this clinical episode (details in discharge letter etc)

Yes □ No □