The relationship between single nucleotide polymorphisms in ARRB2, KCNJ6 and BDNF genes and methadone response for pain management in palliative care

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Declaration of Originality

This work has not previously been submitted for a degree or diploma in any university. To the best of my knowledge and belief, the dissertation contains no material previously published or written by another person except where due reference is made in the dissertation itself.

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ABSTRACT

Background:

Pain has a negative impact on cancer patients' quality of life. It is highly prevalent within this vulnerable population, with an estimated 70 to 90% of patients with advanced cancer experiencing pain. It is the most feared symptom of advanced cancer. Opioids are recommended for moderate to severe pain in palliative care. Methadone has advantages over other opioids, but it is associated with significant interindividual variability and complex pharmacokinetic and pharmacodynamic parameters, which makes dosing challenging in practice. There is limited pharmacogenetic research on cancer pain. However, recent research on single nucleotide polymorphisms (SNPs) and pharmacodynamics has shown that SNPs contribute to interindividual variability in response to opioids.

The aim of this study was to investigate the relationship between SNPs in the three genes, namely KCNJ6, BDNF and ARRB2 and their influence on interindividual variability in methadone dosing requirements for pain management in advanced cancer.

Methods:

Fifty-five participants were recruited from the palliative and supportive care services at Mater Adults Hospital and St Vincent's Private Hospital, Brisbane, in a prospective multi-centre, open labelled, dose individualisation study. Patients were prescribed varying doses of oral methadone by specialist palliative care clinicians for the management of pain. Patient characteristics were collected at baseline, with pain scores recorded using the Brief Pain Inventory, on a numerical rating scale of 0 to 10. Genotyping was conducted using pyrosequencing for both BDNF and KCNJ6 and TaqMan assays were used for ARRB2.

Results:

Forty-six participants were included in the final study and received an average methadone dose of 17.7 mg. The mean pain score was 4.2 out of 10. The mean age of the population was 60.7 years. The patient characteristics measured in this study were not found to be covariates affecting methadone dose, response or pain scores. A patient was considered to be experiencing high pain if they had a pain score of $\geq$ 4/10. There was a significant association

between high pain scores and the following SNPs in BDNF and ARRB2: rs1491850 (p = 0.033), rs3786047 (p = 0.011), rs1045280 (p = 0.004) and rs2036657 (p = 0.05). SNPs in KCNJ6, BDNF and ARRB2 did not show significant associations with methadone dose.

Conclusion:

These findings suggest that specific SNPs in BDNF and ARRB2 may play a role in methadone response and that genetics may be an important factor in interindividual variability. In the future, the SNPs in these genes could be factored into a multimodal treatment algorithm for cancer pain.

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LIST OF ABBREVIATIONS

A	Adenine
Akt	Serine/threonine kinase
Alb	Albumin
ALP	Alkaline phosphate
ALT	Alanine transaminase
AMH	Australian Medicines Handbook
APS	Adenosine 5' phosphosulfate
ARRB2	*β-Arrestin2*
AST	Aspartate transaminase
BDNF	Brain-derived neurotrophic factor
BPI	Brief Pain Inventory
C	Cytosine
Ca	Calcium
CCD	Charge coupled device
CPCRE	Centre for Palliative Care Research and Education
CPD	Continuing professional development
CRF	Case report form
dATP	Deoxyadenosine triphosphate
dATPαS	Deoxyadenosine alfa-thio triphosphate
dNTP	Deoxyribonucleotide triphosphate
EGFR	Estimated glomerular filtration rate
eMR	Electronic medical record
ETG	Electronic Therapeutic Guidelines
G	Guanine
GGT	Gamma-glutamyl transferase
GP	General practitioner
GP	General Practitioner
GPCR	G-protein coupled receptor
HH	Homozygous dominant

Hh	Heterozygote
Hh	Homozygous recessives
HREC	Human Research Ethics Committee
HWE	Hardy-Weinberg wquilibrium
IBM	International Business Machines Corporation
KCNJ6	Potassium inwardly rectifying channels, subfamily J, member 6
LD	Linkage disequilibrium
LDH	Lactate dehydrogenase
MAF	Minor allele frequency
MAH	Mater Adults Hospital
MDT	Multidisciplinary team
MEDD	Morphine equivalent daily dose
MHR	My Health Records
MMT	Methadone maintenance treatment
MOR	*Mu*-opioid receptor
NCBI	National Centre for Biotechnology and Information
NHGRI	National Human Genome Research Institute
NMDA	*N*-methyl-*D*-aspartate
NRS	Numerical rating scale
NSAIDs	Non-steroidal anti-inflammatory
OST	Opioid substitution therapy
PD	Pharmacodynamics
PG	Pharmacogenetics
PK	Pharmacokinetics
PPi	Pyrophosphate
PPS	Palliative performance scale
SD	Standard deviation
SNPs	Single nucleotide polymorphisms
SPC	Specialist palliative care clinician
SPSS	Statistical package for the social sciences
SVPH	St. Vincent's Private Hospital

T	Thymine
UNICEF	The United Nations Children's Fund
VAS	Visual analogue scale
VRS	Verbal rating scale
VTA	ventral tegmental area
WBC	White blood cell
WHO	World Health Organization

LIST OF PUBLICATIONS AND OUTPUTS

Publications

- Advanced cancer pain – Are single nucleotide polymorphisms in ARRB2 (*β-Arrestin 2*) correlated with interindividual variability in methadone dosing? *Journal of Pain and Symptom Management* 2019 (In preparation).
- Association of single nucleotide polymorphisms in BDNF (*Brain-Derived Neurotrophic Factor*) and KCNJ6 (*Potassium Inwardly Rectifying Channels, Subfamily J, Member 6*) with methadone response for pain management in palliative care. *The Pharmacogenomics Journal* 2019 (In preparation).
- Genetic influence on methadone response for end-of-life care. *Progress in Palliative Care* 2019 (In preparation).

Conference presentations

- The effect of single nucleotide polymorphisms on methadone dose and pain in the palliative care population. *6th Brisbane Cancer Conference (BCC)*, Brisbane Convention and Exhibition Centre, 28-29 November 2019. Oral Presentation.
- Methadone for pain management in palliative care – Does genetics play a role in interindividual variability? *2019 Palliative Care in QLD Annual Summit*, Brisbane Convention and Exhibition Centre, 02 December 2019. Poster Presentation.

1 SIGNIFICANCE

Despite increased research and attention toward cancer pain, the prevalence of pain among cancer patients has not changed significantly over the last decade (1). Medications with narrow therapeutic windows, such as opioids, are used to treat moderate to severe pain in end stage palliative care patients. A recent systematic review of the prevalence of pain in the palliative care population revealed the need to overcome various barriers associated with effective pain management (2). Opioids provide substantial pain relief in most cases but are also associated with unwanted side effects. Dosing these medications, therefore, can be difficult. Even small dose changes can affect the therapeutic index and cause adverse events. While morphine is generally considered the first line opioid for cancer pain, methadone, has advantages over morphine, such as high oral bioavailability, rapid onset of effect, low cost and a lack of active metabolites, which makes methadone safe for patients with renal impairment (3, 4). However, methadone is also associated with high interindividual variability, in terms of response, dose and side effects (5). Integrated pharmacokinetic (PK), pharmacodynamic (PD) and pharmacogenetic (PG) studies are needed due to the wide genomic variability among patients (6).

Compared to research focused on patients with post-operative pain or those in the methadone maintenance treatment (MMT) program, little PG research has been done among the palliative care population (7). However, recent studies have examined the possible link between single nucleotide polymorphisms (SNPs) and interindividual variability in response to opioid analgesics (8-13). SNPs can change the clinical outcome of a drug by changing the amino acid sequence produced by a gene. Essentially, this change alters the structure or function of the enzyme, receptor or protein coded for by that gene by expression of the SNP in a region of a gene (exon), which is translated into the respective protein (14). There are conflicting results regarding the role of genetics in opioid response and dose, which highlights the need for more PG research to establish the link between genetics, opioid response and metabolism.

This PG study focused on genetic variations affecting drug response (15) due to pharmacodynamics, which is the 'study of how drugs have effects on the body' (16). The three

candidate genes ARRB2, KCNJ6 and BDNF were chosen because of their PD link to opioid response, which has been highlighted in previous literature. This study investigated whether the interindividual response to methadone could be explained by genetics. Participants from two palliative care centres were recruited in a prospective, multi-centre, open labelled, dose individualisation study. Patients' demographic and clinical data were recorded, including pain scores collected after each dose of methadone, using the Brief Pain Inventory (BPI), on a numerical rating scale of 0 to 10.

Due to the implementation of electronic medical records such as 'My Health Records' (MHR) and electronic medical records (eMR) in health care practices and nation-wide hospitals, incorporating genotype information into patient records will be easier than ever in the future. Thus, by increasing the ease of access of an individual's genetic data, healthcare providers will be able to view an individual's genetic make-up as a guideline to aid in the delivery of a safe and effective individualised dosing regimen, that is associated with the least side effects.

Overall, this study aimed to determine how an individual's genetic profile may influence their response to treatment, to provide adequate analgesia. It also sought to provide clear, succinct methadone dosing guidelines for specialist palliative care (SPC) clinicians. Providing individualised and personalised care by factoring in patients' genes when dosing opioids may optimise treatment. It may also provide data on whether patients can be categorised as responders or non-responders for specific therapies, which will improve care and quality of life for patients in palliative care.

1.1 Aims and objectives

The aim of this study was to determine whether single nucleotide polymorphisms in ARRB2, KCNJ6 and BDNF contribute to interindividual variability in methadone dosing requirements for pain management in advanced cancer. Specific objectives for the study were as follows:

- **Objective 1** - To assess whether patient characteristic variables affect methadone response and pain levels.
- **Objective 2** - To identify which single nucleotide polymorphisms in BDNF, KCNJ6 and ARRB2 are significantly associated with a high pain score.
- **Objective 3** - To identify which single nucleotide polymorphisms in BDNF, KCNJ6 and ARRB2 are significantly associated with high methadone dose requirements.

2 BACKGROUND AND LITERATURE REVIEW

2.1 Palliative care

Palliative care focuses on alleviating suffering and improving patients' quality of life (17). It is a holistic care service that incorporates treatments in the physical, spiritual, psychosocial and cultural domains (18). Palliative care is recognised as part of the human right to health and should be provided through integrated, person centred health services. However, many patients do not receive palliative treatment when required (19). End-of-life care is defined as the last few weeks of life in which an individual with a life-limiting illness is rapidly approaching death (18). Every year, around 40 million people require palliative care, 78% of whom live in low-to-middle income countries (19). Only 14% of people who require palliative care currently receive it (19). Palliative care is an underused but valuable service for patients requiring end-of-life care (19).

The World Health Organization (WHO) defines the aims of palliative care as (20):

- Providing pain relief
- Addressing other distressing symptoms such as anxiety and fear of death
- Providing psychological help
- Providing care that does not intend to hasten or postpone death
- Providing a support system to help patients to live as actively as possible until the final days of life
- Supporting families to cope during the end-of-life care and after the death of their loved ones
- Using a team approach to address the needs of a patient
- Increasing quality of life

Palliative care is becoming increasingly important as death from cancer is continuing to increase (21). It is estimated that globally, more than 15 million people will be diagnosed with cancer and annually, 10 million will die of cancer (21). Despite the increased interest in cancer research, the overall five-year survival rate from cancer is only around 50 to 60% (21). Thus, emphasising the importance and increased need of palliative care facilities to provide their services to the increasing population of individuals requiring end-of-life care (19). This is of

further significance as the global need of palliative care is on the rise due to the increasing ageing population and an increasing burden of non-communicable diseases (19).

Although palliative care is an integral component of the terminal health care system, it receives less than 1% of funding from the National Institute of Health in the United States (22). Research funding for palliative medicinal research has also been found to be lacking (22). Inadequate funding makes it more difficult to improve care for the terminally ill and their families (22). A study conducted by Temel *et al*. (23), with 151 participants, found that patients with terminal cancer in the palliative care intervention group survived 2.7 months ($p = 0.02$) longer than those receiving oncology care alone. It was concluded that palliative care significantly improves mood, quality of life and survival, despite the fact that palliative care patients receive less aggressive end-of-life care treatment compared to patients receiving standard oncology care alone (23).

Worldwide, there are numerous barriers that must be overcome to address the unmet need for palliative care (19). The WHO has identified the following barriers (19):

- National health policies and systems do not often include palliative care services
- Palliative care training for health professionals are often limited or non-existent
- Access to opioid pain relief is inadequate and fails to meet international conventions on access to essential medications
- Cultural and social barriers create a stigma around the palliative care system
- There is a misconception from the general public that providing access to opioid analgesics will lead to increased substance abuse
- There is a misconception that palliative care is only for patients with cancer, or for patients in the final weeks of life

Importantly, a study that investigated palliative care services in 234 counties, territories and regions, found that palliative care services were only integrated in 20 counties, while 42% of the counties and territories investigated had no palliative services available at all, and 32% had only isolated palliative care services (19, 24). In addition, the International Narcotics Control Board concluded that the provision of opioid pain relief in more than 121 counties were either ‘inadequate’ or ‘very inadequate’ to meet the basic medical needs of individuals

(19). Furthermore, an investigation in 2011 found that 83% of the world's population lived in counties with low to non-existent access to opioid analgesics (25). Thus, further highlighting the barriers that are preventing palliative care to reach its full potential, as it is also important to note that palliative care services are the most effective when considered early on in the course of the illness (19).

In 2014, the WHO developed this list of focus areas for strengthening palliative care (19):

- Integrating palliative care into all relevant global disease control and health system plans
- Developing guidelines and tools on integrated palliative care across disease groups and levels of care and addressing ethical issues related to the provision of comprehensive palliative care
- Improving access to palliative care essential medicines through improved national regulations and delivery systems, especially in developing countries
- Promoting increased access to palliative for children, in collaboration with the United Nations Children's Fund (UNICEF)
- Monitoring global palliative care access and evaluating progress made in palliative care programs
- Encouraging the adequate resourcing for palliative care programmes and research, especially in resource limited countries
- Building evidence for models of palliative care that are effective in low-and-middle income settings

The WHO's list emphasises the importance of palliative care programs in improving the quality of life for those with advanced cancer, whilst also highlighting the need to improve access and increase research in this filed.

In clinical practice, SPC clinicians ensure patients are treated with the upmost care, with the aim of improving quality of life and the quality use of medicines, which will be discussed in later sections. Performance status and symptom management focuses on the patient's comfort level. In palliative care, it is important to determine how well a patient is functioning,

as performance status is a significant predictor of survival and a vital tool used in clinical practice (26). Performance status, also referred to as the Palliative Performance Scale (PPS), can be divided in to the following three categories (27):

- Stable: 70 to 100%
- Transitional: 40 to 70%
- End-of-life: < 40%

The PPS is a validated tool (Table 1) that is used to assist clinicians in determining the appropriate time to introduce palliative care support services (28). The PPS can also help patients and their families to address personal and family matters, as it is focused on introducing care that is based on enhancing quality of life rather than introducing new therapies (26). However, most patients only receive palliative care services once they reach the end-of-life status, in most cases a couple of days prior to death (27, 28). This is typically due to insufficient assessment of performance status, which delays the integration of supportive services that can increase survival and quality of life (27, 28).

Table 1 Palliative Performance Scale definitions

PPS Level (%)	Ambulation	Activity and Evidence of Disease	Self-Care	Intake	Conscious Level
100	Full	Normal activity and work No evidence of disease	Full	Normal	Full
90	Full	Normal activity and work Some evidence of disease	Full	Normal	Full
80	Full	Normal activity with effort Some evidence of disease	Full	Normal or reduced	Full
70	Reduced	Unable to do normal job/work Significant disease	Full	Normal or reduced	Full
60	Reduced	Unable to do hobby/housework Significant disease	Occasional assistance necessary	Normal or reduced	Full or confusion
50	Mainly sit/lie	Unable to do any work Extensive disease	Considerable assistance required	Normal or reduced	Full or confusion
40	Mainly in bed	Unable to do most activity Extensive disease	Mainly assistance	Normal or reduced	Full or drowsy ± confusion
30	Totally bed bound	Unable to do any activity Extensive disease	Total care	Normal or reduced	Full or drowsy ± confusion
20	Totally bed-bound	Unable to do any activity Extensive disease	Total care	Minimal to sips	Full or drowsy ± confusion
10	Totally bed-bound	Unable to do any activity Extensive disease	Total care	Mouth care only	Drowsy or coma ± confusion
0	Dead	-	-	-	-

*Table 1 was adapted from Sutradhar *et al.*, 2013[27]

2.1.1 Specialist palliative care professionals and service types

Palliative care is a multidisciplinary approach that includes a diverse range of health practitioners to deliver the best patient centred care. Due to the complexity of cases seen at the end-of-life stage, a multidisciplinary approach is vital for both the patient and his/her caregivers (29). A multidisciplinary team (MDT) includes general practitioners (GP), specialist clinicians, nurses, pharmacists and other allied health professionals (29, 30). SPC clinicians have completed further specialised training in the care of people living with terminal illness and often deal with complex cases at hospitals, hospices and residential aged care homes in the palliative care unit (31).

Palliative care services are available in many forms. The following lists the types of specialist palliative care services available in Australia and was retrieved from the Better Health Channel, which is managed by the department of Health and Human Services based in Victoria, Australia (32).

- *Community palliative care*
    - Provides palliative care services and equipment from a multidisciplinary team that have access to medical reviews and assessment in patients' homes
- *Inpatient palliative care*
    - Provides care to patients who require complex symptom and pain management or end-of-life care in a specialist unit attached to a hospital, with some units also providing day care
- *Hospitals*
    - Provide consultancy to patients who require symptoms and pain management or end or life care within hospitals alongside other avenues of care
- *Outpatient clinics*
    - Provide assessment, care planning and interventions soon after an individual has been diagnosed with a life-limiting illness
- *Day hospices*
    - Provide psychological and emotional support for people with a life-limiting illness, and respite for their family and carers

2.1.2 Symptom management in palliative care

During the terminal phase of palliative care, major organs start to fail, accompanied by progressive and physical changes to the body (33). Although, the terminal phase is expected, it may present suddenly and escalate rapidly (33). Common symptoms observed in the terminal phase of palliative care include breathlessness, pain anxiety, terminal restlessness, nausea and/or vomiting (34) (Figure 1), each of which can present at varying intensities. The aim of terminal phase care is to relieve symptoms in the safest manner, with the least side effects, to ensure patient comfort (33).

Figure 1 shows the common symptoms that present in the terminal phase of palliative care. The figure also illustrates the class of medications frequently used to treat the associated symptoms. As it can also be seen in Figure 1, opioids are also used for symptoms other than pain, such as distress associated with breathlessness (33).

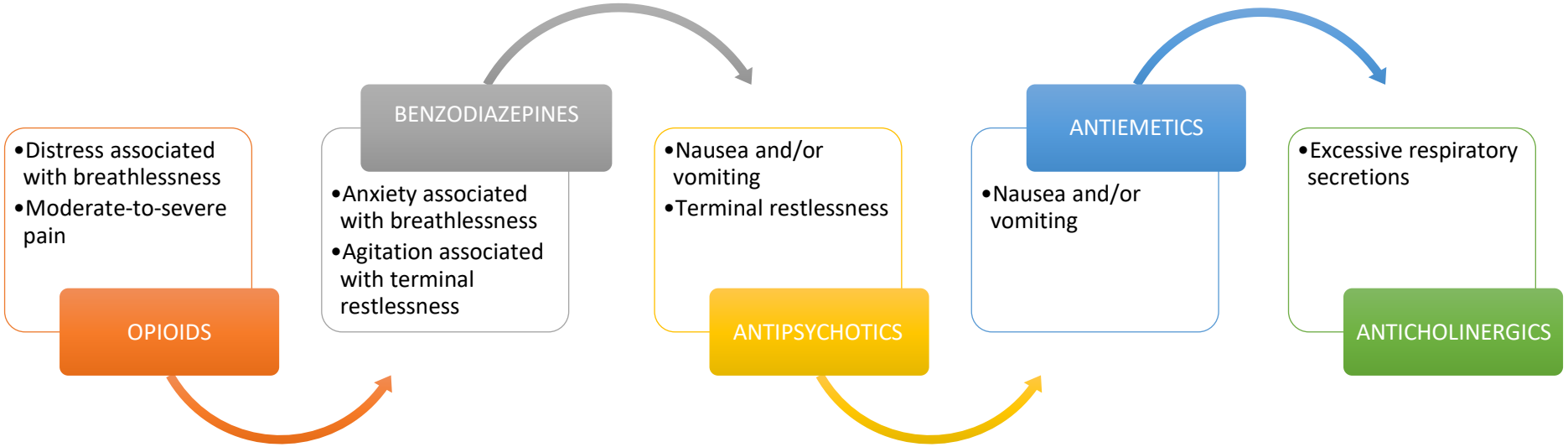

Figure 1 Medications used to treat common symptoms in the terminal phase

Figure 1 was adapted from the *Pharmaceutical Society of Australia LTD, 2019*[33]

2.2 Management of pain in palliative care

Pain is defined as an 'unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of actual damage' (35). Cancer pain can have a negative correlation with quality of life, with around 30 to 50% of patients with cancer experiencing moderate to severe pain (36). A further 70 to 90% of patients with advanced cancer experience pain (37). Cancer pain is often directly caused by a tumour pressing on the nerves, inflammation of the organs involved (38), or by the tumour invading surrounding tissue and causing damage leading to pain (3). Pain is associated with the most feared symptom in advanced cancer (36). Pain that is not controlled can have a significant impact on quality of life (39). Persistent pain impairs daily activities and function, as well as increasing the risk of anxiety, depression and suicidal ideation (39).

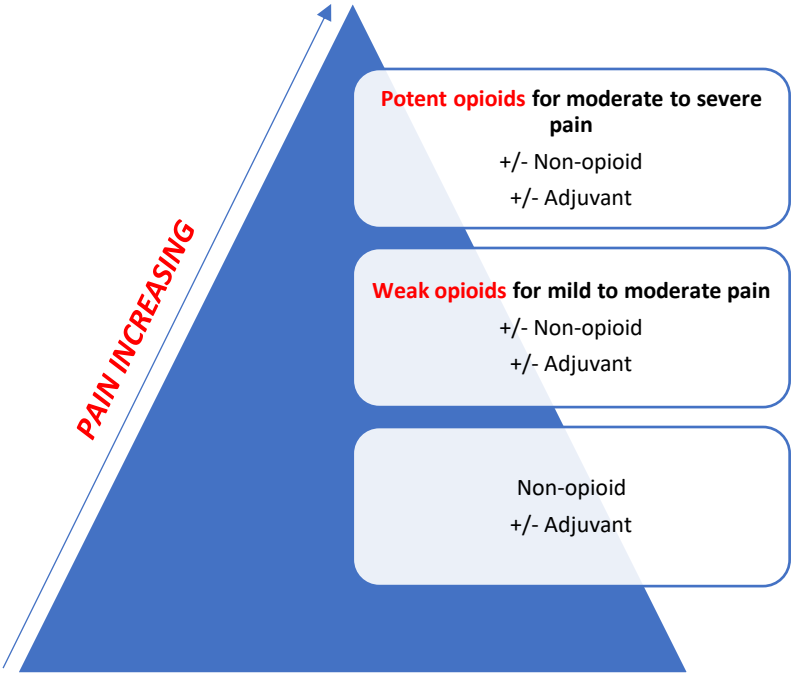

Figure 2 Pain relief ladder for adults, adapted from WHO [(40)].

Figure 2 was adapted from WHO guidelines (40). As shown on three-step ladder, it is evident that opioids are used in mild, moderate and severe pain (40). Examples of strong opioids include methadone, fentanyl and oxycodone (40). In advanced cancer, pain is managed with potent opioids, due to the severity of pain, which is part of the care provided in 'step 3' of the WHO pain relief ladder (Figure 1). Mild opioids include tramadol and codeine and are

commonly used in ‘step 2’ (40). Weak opioids are used in mild to moderate pain and strong opioids are utilised in moderate to severe pain.

Clinicians have suggested eliminating the second step of the pain ladder and replacing the use of weak opioids with low doses of morphine (40). Low doses of morphine have been found to reduce pain significantly compared to weak opioids, with similar tolerability and quick onset of action (40). Opioids are commonly combined with non-opioid alternatives, such as paracetamol and non-steroidal anti-inflammatory (NSAIDs) such as ibuprofen for additive analgesia (40). These pain relievers are commonly administered with adjuvant pain relief, in order to decrease anxiety and induce calmness (40). However, there is little evidence on the use of opioids for managing cancer pain, even though it has been reported that most patients with moderate to severe pain who are prescribed opioids will tolerate them and will achieve a reduction in pain intensity within two weeks (36). Furthermore, individuals receiving end-of-life care are experiencing pain levels that are off the chart (scale) and the dosing of opioids can become difficult when regular doses of opioid are no longer adequate to treat severe increasing pain levels.

2.2.1 *Assessment of pain*

The initial step in understanding cancer pain in diagnosis and treatment is through the classification of pain (41). Assessment of pain is required to identify its degree and intensity, to dose analgesics in a correct and suitable manner. However, pain assessment is subjective and is difficult to measure objectively (42). There are several validated assessment tools (Figure 2) that are frequently used to identify pain and analgesia requirements: visual analogue scale (VAS), verbal rating scale (VRS) and/or the numerical rating scale (NRS) (43). However, some medical professionals are reluctant to record pain in cancer patients, possibly due to a lack of knowledge and confidence to administer analgesia in an effective and safe manner (1). Poor pain assessment can, in turn, lead to poor pain management, poor medication management and poorer quality of life.

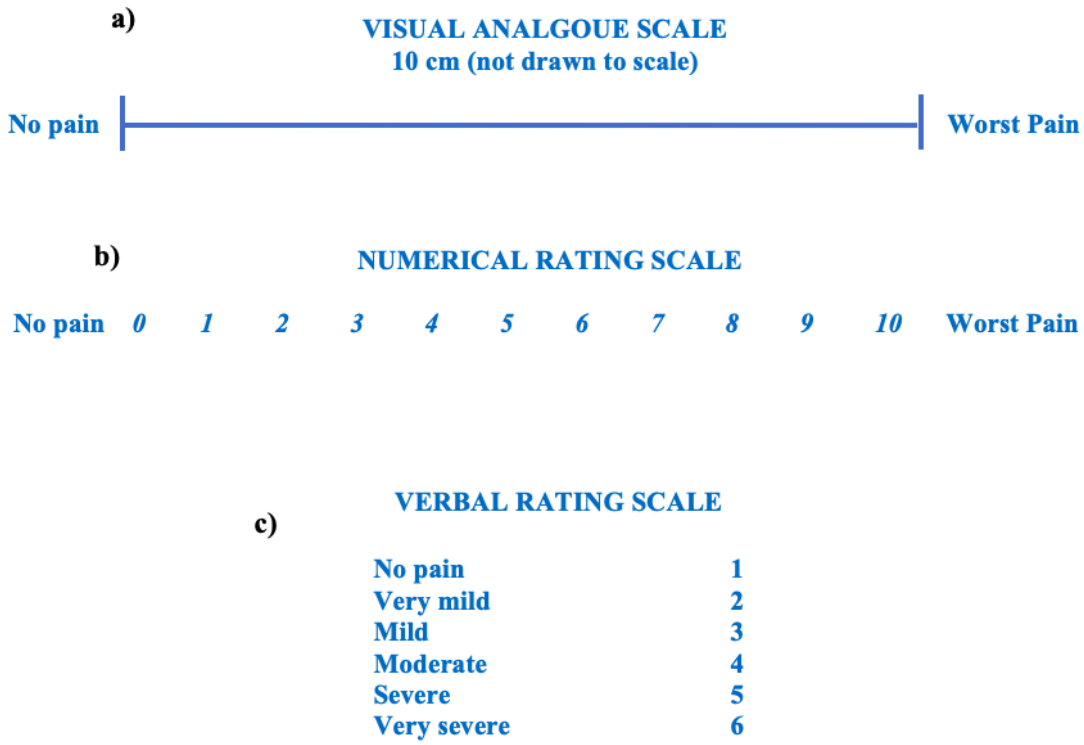

Figure 3 Commonly used pain assessment tools.

a) Visual analogue scale (VAS), b) Numerical rating scale (NRS) and c) Verbal rating scale (VRS)

Figure 3 shows examples of common pain assessment tools used in practice. It is also recommended to asses pain intensity and treatment outcomes via the VAS (a) or NRS (b) pain assessment scale by asking the following question: 'What has been your worst pain in the last

24 hours?'(43). However, in certain cases in palliative care, cognitive impairment can become a barrier in assessing pain effectively. When cognitive impairment is evident, observation of pain related behaviours and discomfort needs to be utilised in order to provide quality of care (44). This includes analysing facial expressions, body movements, changes in routine activity, verbalisation or vocalisation and changes in interpersonal interactions (43, 44). There are numerous pain assessment tools available, with the above three examples in Figure 3 being the most commonly used assessment tools.

2.2.2 Brief Pain Inventory (BPI)

Poorly managed cancer pain is a significant burden on the health system worldwide (45). Inadequate measurement and assessment of pain can lead to undertreatment of cancer pain and poor outcomes (45). The Brief Pain Inventory (BPI) is one of the most commonly used pain assessment tools in clinical practice (46). The BPI is widely used in clinical studies assessing severity of pain and the effectiveness of pain medications. Variations of the BPI have additional sections that asses how much pain interferes with daily activities, including its effect on general activity, walking, work, enjoyment of life, mood, relations with others and sleep (46). Pain is also measured on a scale of 0 to 10, as previously described above. The NRS that was used in this study is a shorter version of the BPI that has become the standard for use in clinical and research applications. The shorter version of the BPI is used in clinical studies as the longer version has proved to be lengthy and time consuming for repeated use in clinical monitoring, or as a repeated measuring tool in research.

2.3 Opioid analgesics

In its pain treatment ladder, the WHO recommends opioids to treat moderate to severe pain (36). Undertreatment is still common in the palliative care population, despite the availability of opioids in most developed countries (43). This can have a detrimental impact on patients receiving analgesia. Inadequate analgesia for patients with cancer pain is a serious concern because it is associated with frequent hospitalisation (47). The WHO has recognised the importance of opioid analgesics for managing cancer pain, including methadone, morphine and fentanyl patches, which are included in the WHO's list of essential medications (43). Despite this, a systemic review conducted in 2014 found that one-third of patients were not receiving appropriate analgesia proportional to the amount of pain they were experiencing (48).

Pharmacokinetic factors such as cytochrome P450 (CYP) enzyme polymorphisms can contribute to the variability in response to opioids by affecting the bioavailability of a drug, its elimination from the body and the production of active or inactive metabolites (49). Opioids undergo phase 1 metabolism by the CYP pathway (49). Methadone undergoes metabolism by CYP3A4 and CYP2B6. Other CYP enzymes such as, CYP2C8, CYP2C19, CYP2D6 and CYP2C9 contribute in varying degrees to the metabolism of methadone (50-52). The fact that methadone is metabolised by several CYP enzymes means the potential for interactions with other medications is more likely (49). Variations, known as polymorphism, in the CYP450 genes affect the function of the enzymes, making an individual either a slow or fast metaboliser (53). Slow metabolisers require a lower methadone dose compared to fast metabolisers (53).

There is a stigma of dependence concerning this class of effective analgesics. The role of the *mu*-opioid receptor (MOR) has been studied extensively in relation to opioid induced dependence (54-56). It has been identified that the MOR plays a central role in the rewarding effects of various pharmacological classes of abused drugs, one being opioids, via the disinhibition of the ventral tegmental area (VTA) of dopaminergic neurons (56). Thus, in order to reduce this stigma and use this class of medication in the most effective and safe manner, the development of individualised based protocols may hold the key in upholding the quality use of opioids.

2.3.1 Methadone

Morphine is a first line opioid of choice in palliative care because it is well tolerated and clinicians are familiar with its dosing (57). Morphine is associated with the following side effect profile: nausea and vomiting, respiratory depression, itching, increased pain sensitivity, tolerance, arrhythmias (QT interval associated) and constipation (58). Some studies have shown that switching from another opioid to methadone provides adequate pain relief and reduces the number of adjuvant analgesics needed to control pain (59, 60). When methadone is used in the palliative setting, it provides effective outcomes such as relieving anxiety, which is common among terminally ill patients and can lead to insomnia and reduced quality of life (61). Although adverse events are assumed to be common for methadone, withdrawal from treatment due to adverse events are rare (36).

Table 2 lists some of the advantages and disadvantages of methadone that have been seen out in clinical practice.

Table 2 Advantages [4, 9] and disadvantages [4] of methadone

Advantages	Disadvantages
Improved side effect profile	QT interval prolongation
Low cost	Arrhythmia
Relief of anxiety	Large interindividual variation in dosing
High oral bioavailability	
Rapid onset of action	
Less frequent dosing	
Can be used in renal impairment	
Lack of active metabolites	

The advantages and therapeutic clinical efficacy of methadone in pain management is hindered by its complex PK and PD profile (9). In addition, methadone is viewed as a second choice analgesic and is predominantly used in hard to control pain scenarios, when it is needed to improve the balance between analgesia and adverse effects (62). However, a systematic review conducted by Mercadante *et al.* (9), showed that methadone may be effective as a first-line analgesic in the management of cancer pain as it can provide similar

analgesia to other commonly used opioids, with a similar adverse effect profile, but with a more stable dosing regimen with a slow escalation index (9). Switching between opioids can be difficult, especially when working with high doses due to an unpredictable conversion ratio. However, using methadone first may be easier because the dose can be titrated against the effect with less unexpected clinical responses, which can occur when switching from another opioid to methadone (9).

A systematic review found that methadone dose tends to remain stable after a maintenance dose has been established, due to its prolonged half-life, which suggests that methadone's metabolic characteristics, extra opioid analgesic effects and anti-hyperalgesia properties are advantages over other commonly used opioids to treat pain (9). It has also been suggested by a study conducted by Cubero *et al*. (63), when comparing the quality of life of patients before and after switching to methadone, there was a significant improvement in some aspects of quality of life scales were found, specifically in the areas of symptomology and functional levels (63).

Patients on morphine can develop dose limiting side effects that are most likely due to the accumulation of active metabolites (4). Symptoms that suggest opioid toxicity from morphine due to accumulation of active metabolites include excessive sedation, confusion, hallucination, agitation, myoclonus, chronic nausea, hyperalgesia and allodynia, which are resolved when switching to an alternative opioid in most cases (58, 64-66). Methadone, due to its lack of active metabolites, is an ideal choice of opioid when patients start to experience adverse effects from morphine (64). Due to methadone being more lipophilic than morphine, the likelihood of experiencing nausea is reduced by providing a relative increase in centrally mediated antiemetic opioid effects, as opposed to peripheral emetic effects (64).

In practice, methadone is only considered in difficult to control pain scenarios (62). Caution is advised for the use of methadone in the following clinical settings:

1. Use in the geriatric population (3)
2. When pain is only partially responsive to methadone, where there is a risk of rapid dose escalation (3)

3. When a clinician believes that a patient's pain has a predominant psychological component (3)
4. Use in patients who have demonstrated sensitivity to low doses of opioids in the past (3)

Methadone acts mainly on the *mu*-opioid receptors located in the brainstem and thalamus, reducing transmission of the pain impulse, as well as modulating the descending inhibitory pathways from the brain, which are involved in pain perception and transmission (37, 67). Of all the opioids, methadone has the greatest efficacy on the *mu*-opioid receptor, which is the key mediator in supraspinal analgesia having a higher affinity to the delta-receptors, five times more than morphine, which is essential for spinal analgesia (64). Methadone is also associated with antagonist activity at the *N*-methyl-*D*-aspartate (NMDA) receptor (5). NMDA antagonism is associated with increased neuronal plasticity, reverse opioid analgesic tolerance and the alleviation of chronic pain states (68). The combination of opioid agonisms and NMDA receptor antagonisms provides added analgesia associated with fewer side effects, compared to other opioid analgesics (69).

Methadone is a racemic mixture of two isomers, laevorotatory, L-methadone (the *R*-enantiomer) and dextrorotatory, D-methadone (the *S*-enantiomer), with L-methadone being eight to fifty times more potent than D-methadone (6, 65). Oral absorption is linked with high gastrointestinal absorption levels, allowing plasma levels to be measured at 30 minutes. Peak plasma levels occur at four hours and start to decline at 24 hours, with high oral bioavailability at around 85% (3). Methadone is available in 10 mg tables (*Physeptone*), oral liquid (5 mg/ml, *Biodone Forte* and *Methadone Syrup)* and an injection (10 mg/ml, *Physeptone*) which are all racemic mixtures of the drug (4). The availability of the drug in both liquid, tablet and injection form allows clinicians to have better control with dose titration to achieve the desired effect. However, methadone dosing remains a challenge and is usually administered by experienced clinicians (specialised palliative care clinician), due to its variable pharmacokinetic and pharmacodynamic characteristics (9). There is also an increased urgency to find a balance between opioid efficacy, without causing patient harm (15).

Methadone offers potential cost savings compared to standard palliative pain management practices (70). In a study conducted in Philadelphia, methadone was associated with a cost of $2.76 USD per patient per day compared to $11.16 USD per patient per day for routine analgesics used in home-based hospice services (70). Additionally, the pharmaceutical costs of opioids are on the rise due to the increased cost of newer longer acting opioids like fentanyl and oxycodone (70). Thus, understanding the influence of genetics on methadone support specialist palliative care clinicians in dosing methadone in a clear and succinct manner, at the same time reducing the cost associated with monitoring patients to achieve these clinical goals (71).

When a drug is administered, it is absorbed, distributed (interaction with target receptors and enzymes), metabolised and excreted (72). Each step within this cascade of events can be affected by genetics, which can cause patients to respond differently to medications from one another even when they are given the same or similar doses, which all influence the interindividual variability seen in methadone (72).

Table 3 is a comparative table, comparing morphine to methadone. Morphine was chosen for comparison as it is the first line opioid used for pain. It should be noted that both the AMH (4) and Therapeutic Guidelines (eTG) (57), recommend that only a SPC practitioner prescribes methadone.

Table 3 Opioid comparative information.

Drug	Approximate dose equivalent to 10 mg IM/SC morphine	Approximate duration of action (hours)	Considerations
Morphine	• 30 mg oral	• 2-3; 12 or 24 (controlled release)	• Older and/or frail patients and those with moderate to severe kidney impairment, can be susceptible to adverse effects
Methadone	• Complex; discuss conversion with a pain or palliative care specialist	• 8-24 (chronic dosing) *palliative care dosing is usually different	• Complex PK properties • Time to reach steady state concentration following a change in dose can be unpredictable • Potency and half-life vary between patients

*Table 3 was adapted from AMH, 2019 [4, 58, 73] and ETG, 2019 [57]

2.4 Pharmacogenetics

Pharmacogenetics is the 'study of genetic variations affecting drug response' (15). The goal of PG is to develop tools to aid optimal dosing of medications and achieve adequate treatment with the least side effects by tailoring treatment based on a patient's individual genetics. An individualised approach will also streamline the clinician's prescribing process (74). Recent literature has explored the PG of opioids to see whether it can explain the wide inter-variability of response to these medications (75). Using genetics to determine the best dose of an opioid for a patients is of great importance when a patient can no longer communicate their pain levels (74).

A PG study was conducted on 121 sets of twins, who were given either a short acting opioid (alfentanil) or placebo (saline), to investigate the genetic and familial contribution to acute adverse effects and affective opioid response (76). The study found that individual genetics accounted for nausea (59%), respiratory depression (30%), opioid dislike (26%), sedation (29%), dizziness (32%) and pruritis (38%) (76). Covariates such as age, race, ethnicity, education, mood, age and sex were also of significant findings in the study (76). It was

concluded that genetic, environmental and demographic factors work together to influence adverse effects and reinforce opioid response, but contribute differently to specific responses (76).

2.4.1 Single nucleotide polymorphisms

Single nucleotide polymorphisms are variants of genetic sequence occurring at a position along a DNA strand where there is a change in a nitrogenous base (77) and are typically related to alternative nucleotide base pairings being referred to alleles (14). The four nitrogenous bases consist of the following: guanine (G), cytosine (C), adenine (A) and thymine (T). Therefore, as an example, a SNP may replace the nucleotide thymine (T) with cytosine (C). SNPs can change the clinical outcome of a drug through a change in the amino acid sequence produced by that gene, which in turn alters the structure or function of the enzyme, receptor or protein coded for by that gene by expression of the SNP in a region of the gene (exon) that is translated into the respective protein (14). However, SNPs can also occur in non-coding regions of a gene which can also result in various clinical outcomes (14). Genetic research has focused on select SNPs on certain candidate genes associated with the PK and PD of opioids and SNPs related to alternate pain pathways (78, 79). In a study conducted by Angst *et al.* (80) genetic effects accounted for 12-60% of overall observed interindividual variance to opioids and pain sensitivity (80).

2.4.2 Single nucleotide polymorphisms affecting pharmacodynamics

Pharmacodynamics is 'the study of a drug's mechanisms of action, encompassing the role of different receptors, second messengers and downstream effects of a drug once a receptor is activated' (81). Figure 4 depicts the G-protein coupled receptor, where opioids attach in order to elicit their effect. Essentially, PD encompasses 'what the drug does to the body'(81). Research into SNPs in the gene encoding the *mu*-opioid receptor have been linked to the variability in response to opioid analgesics (82). In the same way that SNPs within metabolic enzymes that process and eliminate opioids and their metabolites also have an important effect on an individual's response to opioid medications, so do SNPs that affect the bioavailability of opioids (82). SNPs in genes not only affect drug dose, but also the side effect profile exhibited by opioids (83). While early work in PG has focused primarily on SNPs

affecting the PK of drugs, recent research shows that SNPs may play a more significant role in PD. The genes and corresponding SNPs that affect analgesic responses associated with methadone and its PD profile are described below. Prior studies have identified an association between these SNPs and opioid dosing and response. The BDNF gene was chosen due to prior studies conducted by Levran *et al.* (11) and Nishizawa *et al.* (12), KCNJ6 was chosen based on the study conducted by Lotsch *et al.* (8) and ARRB2 was chosen based on the studies conducted by Oneda *et al.* (10) and Ross *et al*. (13).

2.4.3 ARRB2

β-Arrestin2 (ARRB2) is a component of the G-protein coupled receptor complex that regulates opioid signal transduction by promoting receptor desensitisation and internalisation (10). This gene regulates the number of functional receptors expressed on the cell surface at a given point in time (10). The gene spans around 11 kb of genomic DNA and consists of 14 exons and is expressed in multiple organs and tissues, especially in the brain (10). ARRB2 also functions as a signalling intermediate in response to dopamine receptor activation through a kinase phosphate scaffold (10). The gene is also involved in the *mu*-opioid receptor and dopamine receptor, D_2 receptor signalling, which are two central processes in methadone signal transduction (10).

The ARRB2 gene has also been found to be associated with the use of illegal drugs such as methamphetamine (84), nicotine dependence (85) and ethanol consumption in rats (86). The reward properties of commonly abused drugs are mediated by the *mu*-opioid receptor and genetic variations in this receptor and interacting proteins are involved in *mu*-opioid receptor signalling, which can increase the risk of drug dependence (56). Opioid tolerance occurs when an individual requires an increased dose of an analgesic to provide adequate pain relief, due to reduced responsiveness to the drug of choice (87). For opioids, tolerance is due to desensitisation of GPCR (87). This suggests that the regulation by the ARRB2 gene of the *mu*-opioid and D_2 receptors have a role in mediating the rewarding properties of drugs of abuse (88).

In addition, the ARRB2-mediated kinase/phosphate scaffolding of serine/threonine kinase (Akt) and protein phosphate 2A is responsible for the regulation of Akt by dopamine receptors

(10). Therefore, ARRB2 provides an alternative pathway by which D_2 class receptors lead to the expression of dopamine-associated behaviours (88). In a study conducted in rats (86), it was reported that ARRB2$^{-/-}$ and ARRB2$^{+/-}$ mutant rats consumed significantly less ethanol and showed reduced preference for ethanol compared to the control group, which were the rats' wild-type counterparts.

Oneda *et al.* (10) investigated the ARRB2 gene and its associated SNPs, rs3786047, rs1045280 and rs2036657 and found that these SNPs may contribute to variability and response to methadone maintenance treatment (MMT) program. In MMT, patients are treated with methadone to reduce their craving for illicit drugs and prevent addiction relapse and adverse reactions (10). Oneda *et al.* (10) hypothesised that genetic variation in the ARRB2 gene may be implicated in the reward pathway during MMT and influence response to treatment and/or may influence methadone requirements. The above SNPs were found to be significantly ($p < 0.02$) associated with response to methadone treatment in a the MMT population studied (10). Another study by Ross *et al. (13)* found an association between some SNPs - rs3786047 (1082 AG), rs1045280 (8622 CT), rs2271167 (8864 AG) and rs2036657 (11143 AG) – and cancer patients' clinical response to morphine, a *mu*-opioid receptor agonist, similar to methadone (13). It was hypothesised that genetic variation in the *β-arrestin2* gene may be a factor affecting response to morphine in their cohort of participants (13). In this cohort, all patients were receiving opioids for the relief of cancer pain and the investigation aimed to evaluate whether genetic variation affected how well an individual responded to morphine, versus those who had to switch (switchers) to alternative opioids due to unbearable side effects from morphine (13). Switchers were more likely to carry the common allele at 8622 TC and 11143 GA in the *β-arrestin2* gene ($p = 0.013$ and 0.043, respectively) (13). This was the first study linking SNPs in the ARRB2 gene in relation to analgesic doses of opioids in humans (13). It has also been reported in studies conducted on *β-arrestin2* knockout mice that these mice exhibited increased and prolonged analgesia (89).

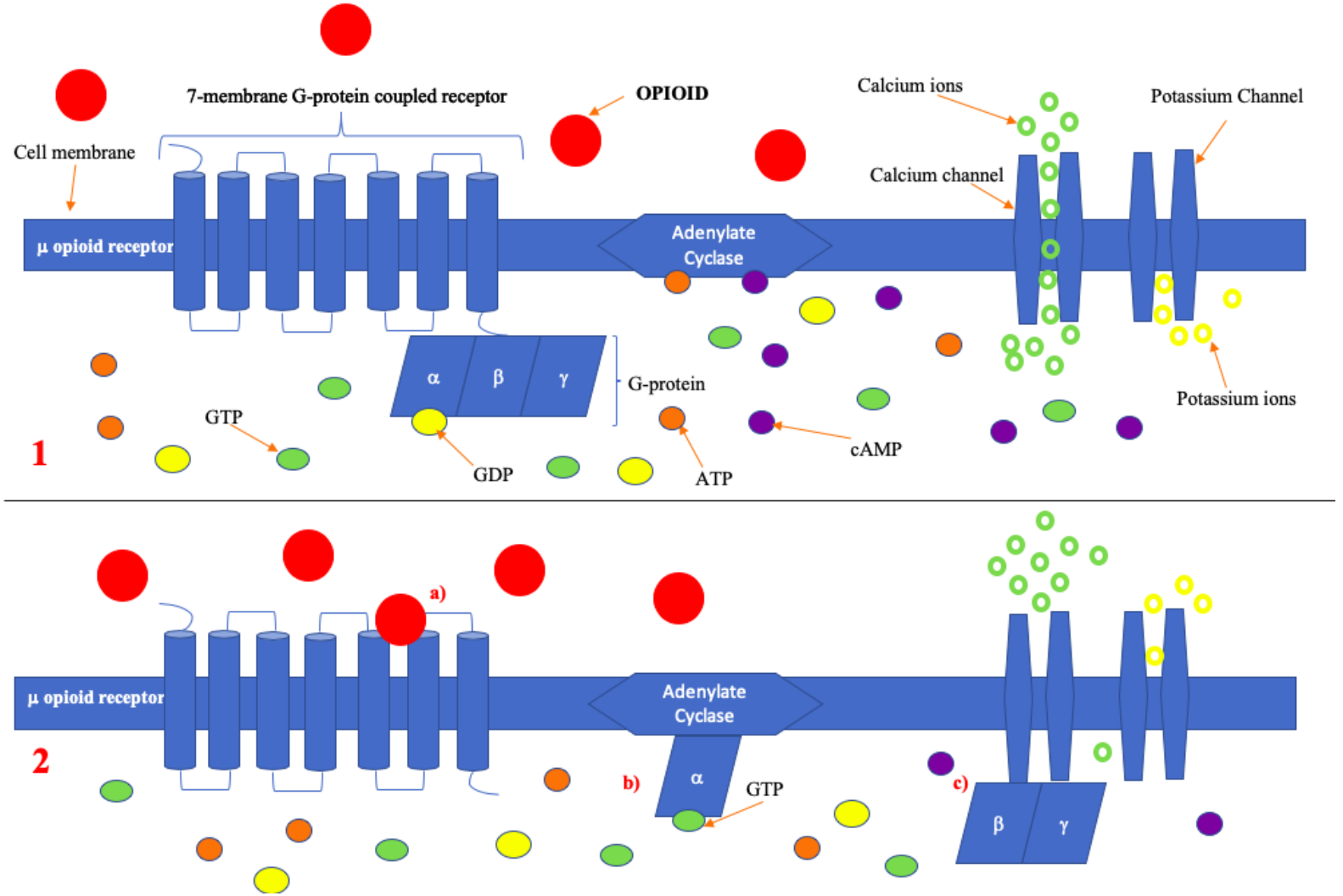

Figure 4 Opioid G-protein coupled receptor

Figure 4 represents the seven transmembrane structure of the opioid G-protein coupled receptor. The top image (1) represents the G-protein coupled receptor when an opioid has not yet attached to the μ receptor. The bottom image (2) depicts the following cascade of events that occur on the G-protein coupled receptor when an opioid binds to the μ receptor: a) opioid binding to the receptor causes GTP to replace GDP on the α subunit and break up the G-protein complex; b) The now GTP and α subunit complex inhibits adenylate cyclase decreasing cAMP levels; c) The βγ subunit of the G-protein binds to the calcium channel blocking the inward flow of calcium ions. It should be noted that methadone is also associated with antagonist activity at the N-methyl-D-aspartate (NMDA) receptor, which is not depicted in this diagram. *ATP: Adenosine triphosphate; cAMP: Cyclic adenosine monophosphate; GTP: Guanosine triphosphate; GDP: Guanosine diphosphate*.

2.4.4 KCNJ6

The KCNJ6 gene codes for potassium inwardly rectifying channels subfamily J member 6, (also known as Kir3.2 or GIRK2) (8). It is a G-protein coupled channel that is involved in opioid receptor transmission and eliciting opioid effects (90). The gene is also involved in postsynaptic inhibition of pain (90) and mediating components of the analgesic response (91, 92). Increased opioid analgesia requirements have been noted in Japanese patients after abdominal surgery with genetic variants in the KCNJ6 gene (12). In this cohort, patients who had an SNP on the KCNJ6 gene at rs2070995 G>A experienced decreased opioid effects and required increased doses of opioids (12).

Investigation into the KCNJ6 gene in humans on methadone and its effects on pain are limited. However, Lotsch *et al*. (8) found that Japanese patients with the AA (rs2070995) genotype required higher doses of opioids for both groups in the MMT cohort and those on opioids for analgesia. Those with the AA genotype also lacked opioid withdrawal symptoms (8). Participants in this study with the AA genotype in the first year of therapy required a methadone dose of 119.7 $\pm$ 49.6 mg/day, compared to 77.5 $\pm$ 26.2 mg/day (p = 0.003) for the other rs2070995 genotypes (8). The SNP in rs2070995 of the base change of a G>A is depicted in Figure 5.

A study conducted on infants it was found that those homozygous for the KCNJ6-1250G>A (rs6517442, c.-1787G>A) allele took lonfer to reach a level of no pain after being administered with opioids compared to infants with the AG or GG genotypes (93). This study suggested that preterm newborns with SNPs at the KCNJ6-1250A were predisposed to diminished opioid induced pain relief (93). Another study investigated the KCNJ6 gene and analysed the genetic contribution in lower pain (94). The aim of the study was to analyse opioid effectiveness in chronic lower back pain relief and opioid titration, to determine the impact of pharmacogenetics (94). It was found that patients with the A1032G-A allele (rs2070995) showed higher pain intensity that required larger doses of opioids (94). These individuals also experienced more side effects such as dizziness and dry skin (94). However, after implementing opioid titration according to patents' genetics, chronic lower back pain intensity, disability, anxiety and depression significantly decreased, while their quality of life increased (94).

Bruehl *et al.* (95) investigated a cohort of 311 patients undergoing total knee arthroplasty and the effects of eight SNPs in KCNJ6 on pain response. Overall, the effects of the KCNJ6 gene, which was gene set-based analysis, failed to reach significance (p = 0.054)

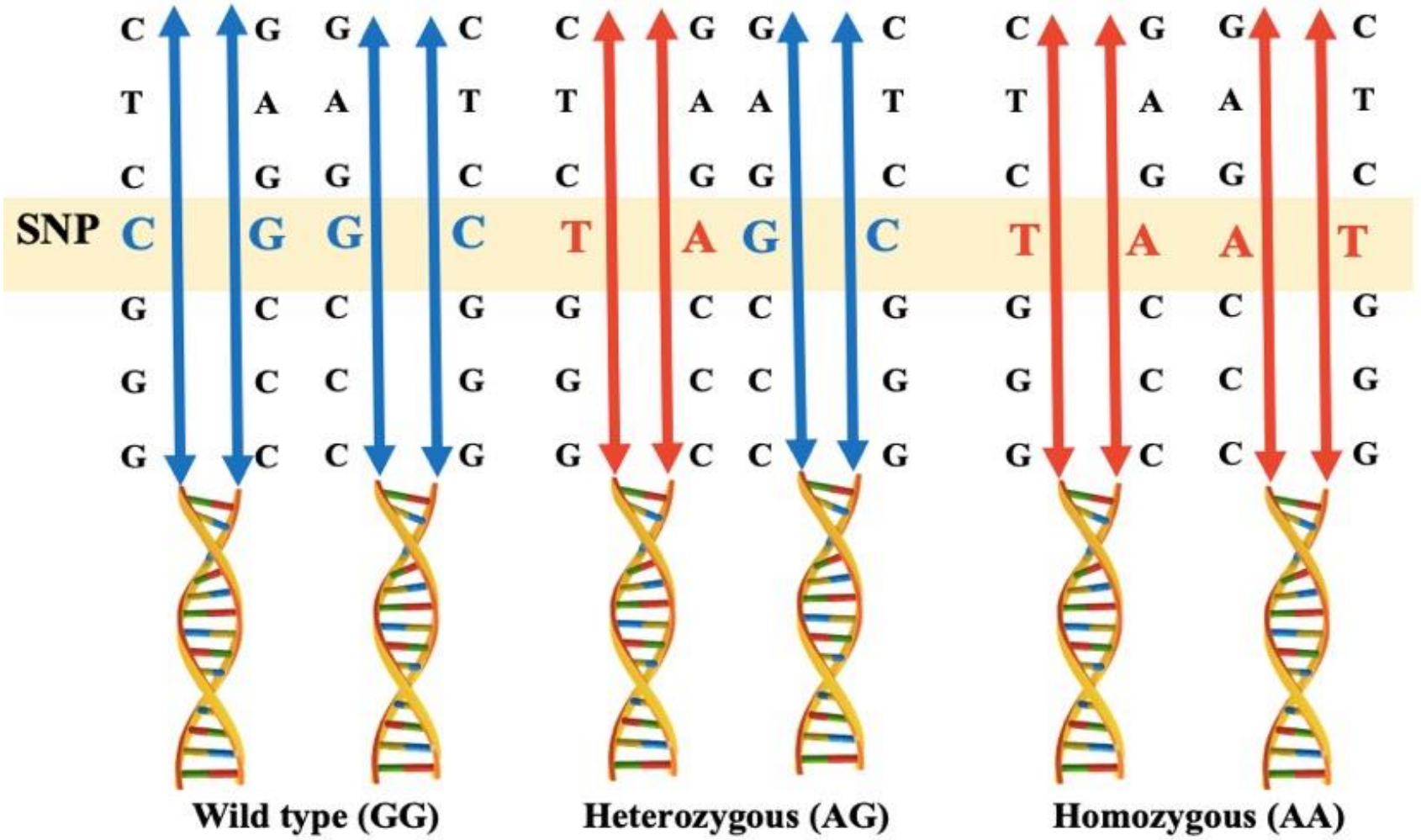

Figure 5 SNPS in the KCNJ6 gene

Figure 5 represents a single nucleotide polymorphism (SNP) (G>A) change along the KCNJ6 (rs2070995) gene.

A: Adenine; T: Thymine; G: Guanine; C: Cytosine

2.4.5 BDNF

Brain-derived neurotrophic factor (BDNF) is vital for developing and maintaining neuronal brain function (96). The BDNF gene encodes for the BDNF protein, which is found in the brain and spinal cord (97). The protein is responsible for the survival of neurons, by playing a role in growth, differentiation and maintenance of neuronal cells (97). This gene is involved in multiple pathways, such as neuroplasticity, rewards-related processes and the healing of neurons (96). BDNF is also a neuropeptide that is involved in the regulation of mid-brain dopamine release (98). Individuals with loss of BDNF function commonly have neurodegenerative or psychiatric diseases (96).

The BDNF gene is associated with several SNPs. The most common SNPs and associated functions are listed in Table 4.

Table 4 SNPs in BDNF and their associated functions.

	SNPs	Function	References
Brain-derived neurotrophic factor	rs10835210	Substance abuse, bipolar disorder, schizophrenia, internalizing disorders and phobic disorders	Levran *et al.* (11)
	rs1491850	Substance abuse, major depressive and obsessive compulsive	Levran *et al.* (11)
	rs7934165	Substance abuse, eating disorders and nicotine dependence	Levran *et al.* (11)

The effects of BDNF and its association with opioids has also been investigated, with it being linked to opioid induced plasticity (99), whereas, SNPs in rs10835210 have been associated with bipolar disorder and alcohol addiction (100). This SNP and its corresponding protein have also been associated with opioid use (101). However, there are many covariates, such as mood, antidepressants, psychostimulants, nicotine consumption and gonadal hormones, which can also affect the peripheral blood levels of BDNF (102).

A study conducted by Levran *et al.* (11) found an association between three SNPs in BDNF (rs10835210, rs1491850, rs7934165) and methadone dose, in the MMT program. Individuals who were carriers of two C alleles for rs10835210 required a higher methadone dose of 158.7

mg compared to individuals that were heterozygotes or non-carriers, who required only 130 mg and 134 mg of methadone, respectively (11). Participants in the study who were carriers of two T alleles of rs7934165 required a higher dose of 153 mg, compared to individuals who were carriers of one or two copies of the C allele, who required a mean dose of 137 mg (11). Carriers of two C alleles for the SNP rs1491850 required a lower mean methadone dose of 125 mg, compared to heterozygotes or non-carriers, requiring a mean dose of 139 mg and 149 mg, respectively (11). Linkage disequilibrium (LD) analysis also revealed a strong LD between SNPs rs10835210 and rs7934165 and only a moderately LD with rs1491850 and rs7931465, which did not occur at the expected frequencies (11).

Analysis of genes and SNPs associated with pain pathways and analgesic response may lead to the development of personalised treatment plans of opioid dosing. Recent research in this area suggests that individualisation when addressing pain is the key to successful opioid treatment (103). With the aid of PG, individualisation therapy may provide more effective pain relief with a lower side effect profile, increasing quality of life and quality use of medicines, both of which are vital in the palliative care setting. Individualisation therapy may solve the dilemma of undertreatment and overtreatment. However, further studies need to be conducted to determine the functional significance of KCNJ6, BDNF and ARRB2 genetic variants.

Figure 6 Illustrates the BDNF protein along a neuron. The BDNF protein is released from neuronal tissues under various stimuli.

Figure 7 illustrates the role of BDNF on neuronal tissues and its role in neuronal plasticity.

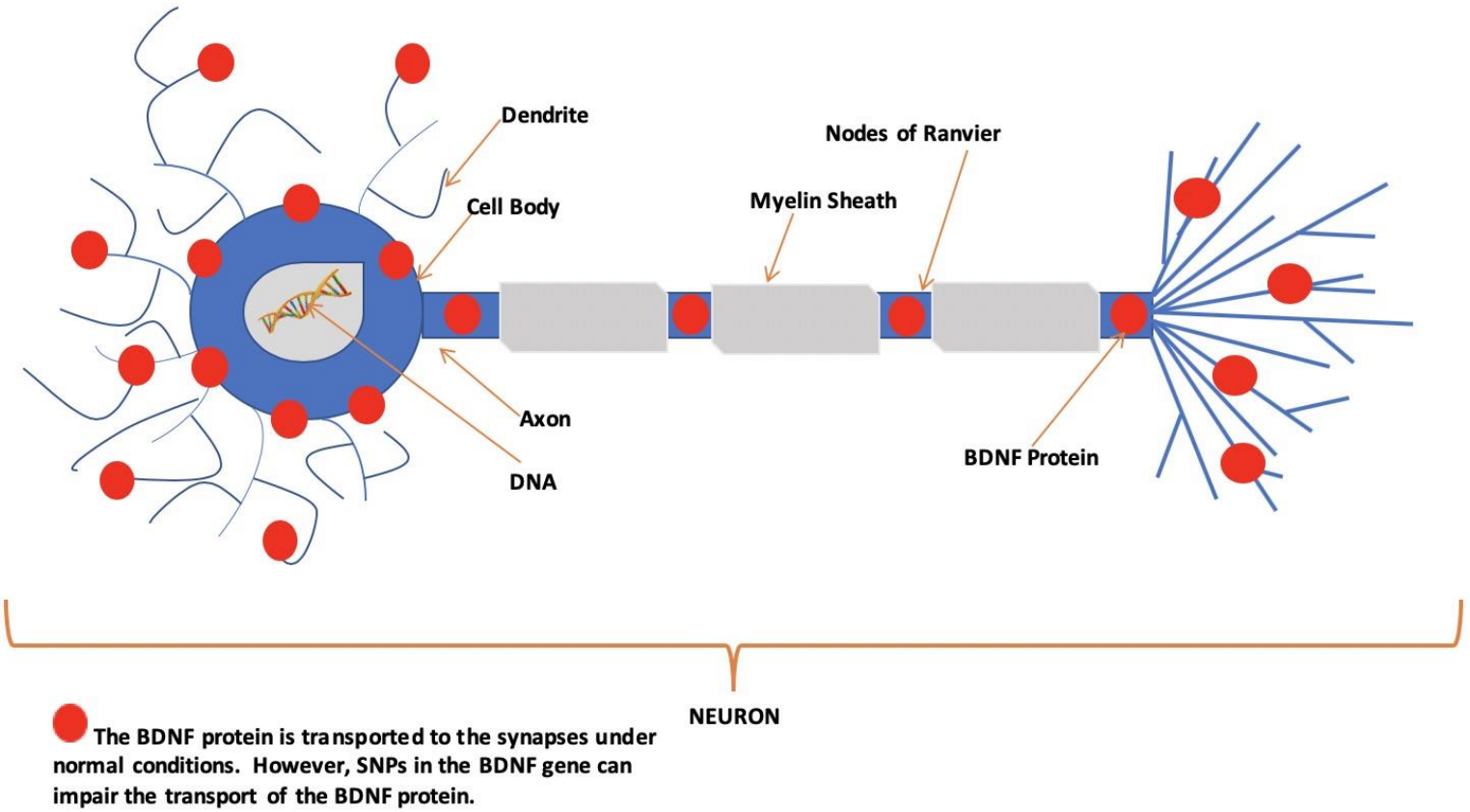

Figure 6 Neuron associated with BDNF protein

Figure 6 illustrates the BDNF protein along a neuron. The BDNF protein is released from the neuronal tissue under various conditions.

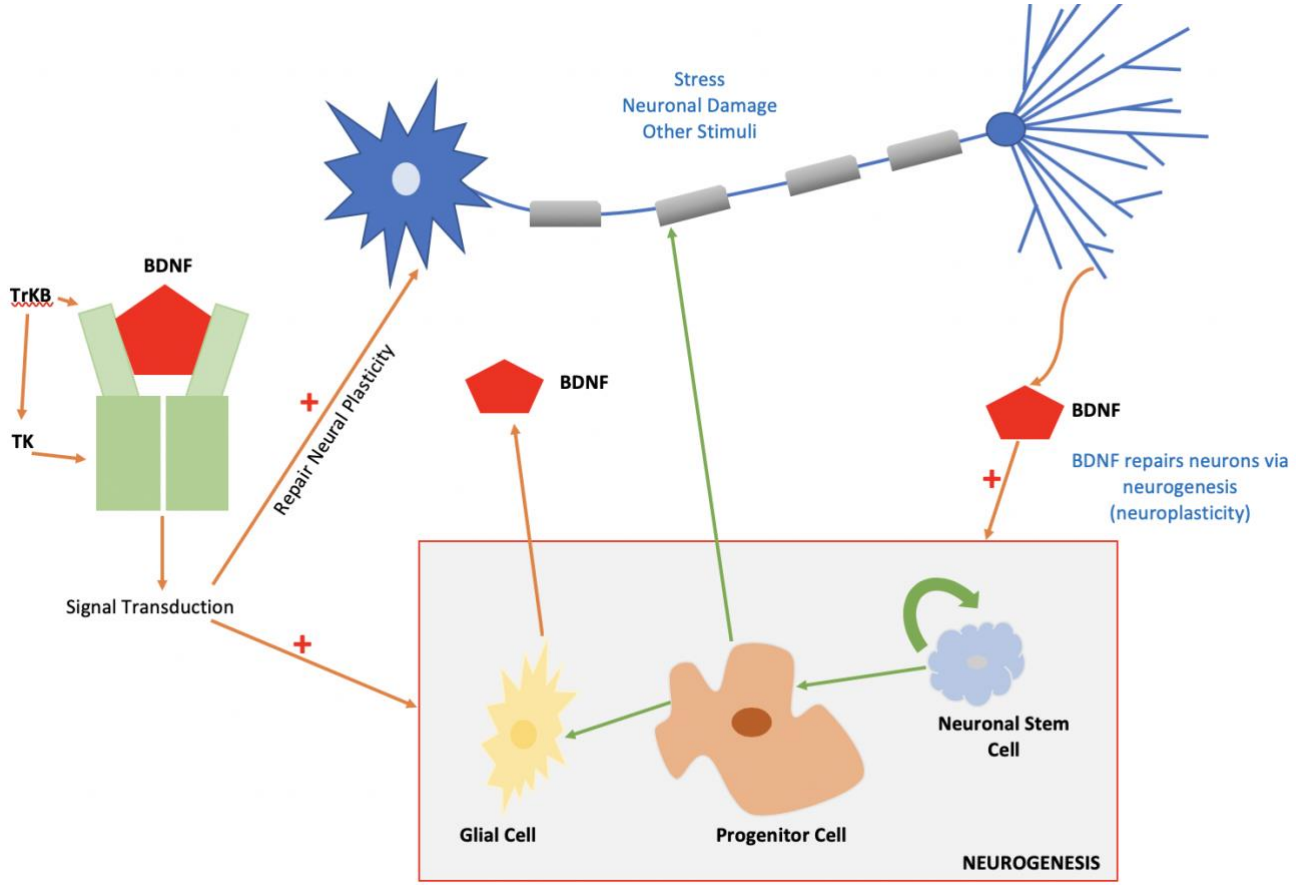

Figure 7 BDNF associated neuroregeneration and neuronal plasticity

Figure 7 was adapted from Habtemariam S. [104]

Figure 7 illustrates BDNF's role in neurogenesis. When neuronal tissue is damaged or due to other stimuli, such as stress, BDNF is released from neuronal tissue. BDNF then activates tropomyosin-related kinase B (TrkB) which then initiates signal transduction pathways via tyrosinekinase (TK) activity leading to neurogenesis and neuroregeneration.

2.4.6 *Gene, allele and genotype classification*

Multiple alleles with various SNPs have been investigated in this study, thus a classification of each allele has been developed. Table 5 is a list of the genes, alleles and SNPs being investigated in this study. The genotypes are further classified as either the homozygous dominant, heterozygous or homozygous recessive base pair.

Table 5 Genes and associated SNPs being investigated.

Gene	Allele	SNP	Genotype	Classification
KCNJ6	rs2070995	G > A	GG	Homozygous dominant
			GA	Heterozygote
			AA	Homozygous recessive
BDNF	rs7934165	C > T	CC	Homozygous dominant
			CT	Heterozygote
			TT	Homozygous recessive
	rs10835210	C > A	CC	Homozygous dominant
			CA	Heterozygote
			AA	Homozygous recessive
	rs1491850	T > C	TT	Homozygous dominant
			TC	Heterozygote
			CC	Homozygous recessive
ARRB2	rs34230287	C > T	CC	Homozygous dominant
			CT	Heterozygote
			TT	Homozygous recessive
	rs3786047	A > G	AA	Homozygous dominant
			AG	Heterozygote
			GG	Homozygous recessive
	rs1045280	C > T	CC	Homozygous dominant
			CT	Heterozygote
			TT	Homozygous recessive
	rs2036657	G > A	GG	Homozygous dominant
			GA	Heterozygote
			AA	Homozygous recessive

The most common SNPs studied in the genes ARRB2, KCNJ6 and BDNF that have an influence on opioid requirements have been investigated in this study. There are not many published PG studies specifically in the area of palliative care. The majority of studies have been conducted on MMT, chronic pain and cancer pain populations, or have investigated links to mental illness. Therefore, this is a novel study. To our knowledge, it is the only study conducted on the palliative care population where SNPs in BDNF, KCNJ6 and ARRB2 have been investigated only in association to their influence on methadone dose and pain scores.

2.5 Future potential of pharmacogenetics in palliative care

Dose individualisation is often practiced during therapeutic drug monitoring (TDM), which refers to the 'individualisation of dose by maintaining plasma or blood drug concentrations within a target range, which is also referred to as therapeutic range or therapeutic window' (105). Variability regarding drug response can be due to PK or PD. PK variability can be due to dose and plasma concentrations and PD variability can be due to drug concentrations at the receptor site and response (105). Previous research related to dose individualisation has focused on PK whereas this study focused on the novel field of PG. The practical aspect and aim of TDM allows clinicians to adjust a drug dose to treat an individual patient rather than to optimise the number on a TDM report (106). The following list retrieved from the Australian Medicines Handbook lists the characteristics of drugs for which TDM may be useful (106):

- A narrow therapeutic ratio
- A reasonably well-defined concentration-effect relationship
- An evidence-based therapeutic range
- Serious consequences if there is therapeutic failure
- No appropriate therapeutic direct measure of effect
- A suitable and accessible laboratory assay
- Non-linear pharmacokinetics

Dose individualisation is important for drugs that have wide interpatient variability, such as methadone. Other commonly used drugs that have been associated with TDM and dose individualisation commonly used in practice are digoxin, amiodarone, lithium, sodium valproate, theophylline and phenytoin (105). For example, digoxin is a cardioactive drug that is associated with a narrow therapeutic index and requires monitoring to ensure patient safety and reduced toxicity (107). Therapeutic drug monitoring is essential for this widely used cardiac drug in order to ensure efficacy and avoid toxicity (107).

Thus, dose individualisation is commonly used for narrow therapeutic drugs that require regular blood tests to adjust doses to ensure safety and efficacy. However, with the incorporation of PG, genotyping will be a once-off test that will ensure safety. In recent years,

PG has become relatively quicker and cheaper and can enhance patient care (108). Some pre-emptive PG tests are now being subsidised by Medicare for the monitoring of some medications such as azathioprine (108).

Pharmacists can also play an important role in providing genetic testing services in a community pharmacy. As for patient centred practice the genetic profiles of patients should be considered along with other factors (59). Further, evidence from international studies have proven that community pharmacists are able to offer professional genetic testing services to their clients if given suitable training, which would make it more accessible to the Australian community if pharmacogenomic testing becomes reimbursed through the Medicare Benefits Schedule (109). Pharmacogenetic tests in pharmacies are already available in the United States and Norway (110). With the integration of PG into clinical practice, pharmacists will also be able to direct clinicians, using a multidisciplinary approach for referral of genetic testing to ensure the safest and most effective dose has been prescribed for a patient.

The introduction and increase of electronic health records will allowed for the integration of PG research into practice faster than previously anticipated. Genotyping has become more accessible and more people are using genotyping facilities of their own accord, highlighting the importance of genetic research in practice and its role in TDM and the quality use of medications.

Palliative care forums are a great way to introduce new ideas and discuss important issues related to improving services and patients' quality of life, such as the importance of dose individualisation in clinical practice. The Centre for Palliative Care Research and Education (CPCRE) works collaboratively with many palliative care organisations like Care at the End-of-life Project and Palliative Care Queensland (111). CPCRE focuses on developing the capacity of the palliative care workforce, improving the links between research and practice, undertaking research and providing information, which supports the delivery of palliative care services (111). In the future, working with the CPCRE could provide an avenue for promoting PG in palliative care, to increase the quality care that is currently available. The effective integration of dose individualisation into healthcare will assist in improving the quality use of medicines in a safe and effective manner, as PG testing has been shown to optimise

pharmacotherapy whilst reducing drug-related toxicity and the healthcare costs associated with drug-related toxicity (109).

3 METHODOLOGY

This chapter describes the nature of the study, methods involved and the statistical analyses that were used to determine whether SNPs in the genes KCNJ6 (rs2070995), ARRB2 (rs34230287, rs3786047, rs1045280 and rs2036657) and BDNF (rs7934156, rs10835210 and rs1491850) are associated with high pain scores and high methadone dose requirements.

3.1 Study design

The study formed part of a larger prospective multi-centre, open labelled, dose individualisation study, of methadone for pain management in palliative care. The study was conducted at two clinical sites, namely Mater Adults Hospital, Brisbane (MAH) and St Vincent's Private Hospital, Brisbane (SVPH). Participants were administered individualised doses of methadone, depending on the severity of their pain at the time of dosing, according to clinical practice. The aim of this study was to investigate whether certain SNPs in the genes KCNJ6 (rs2070995), ARRB2 (rs34230287, rs3786047, rs1045280 and rs2036657) and BDNF (rs7934156, rs10835210 and rs1491850) affected methadone dose requirements and response. Participants completed a BPI to determine the level of their pain after each sampling point. Clinical characteristic variables, which are listed in Appendix 1 – Patient characteristic, were further analysed to see if these variables had any influence on pain management in palliative care.

3.2 Study population

3.2.1 Participants

Patients in the palliative care service at either MAH or SVPH located in Brisbane, who were taking oral methadone and met the eligibility criteria, were included in this study.

3.2.2 Eligibility criteria for participants

The inclusion and exclusion criteria for this study are listed below.

Inclusion criteria:

- ≥18 years
- Experiencing cancer related pain
- Receiving oral methadone
- Able and willing to provide blood samples
- Capable to read and understand the patient information sheet and provide written consent

Exclusion criteria:

- Receiving methadone for breakthrough pain

3.3 Study duration and location

The study was conducted at SVPH and MAH, Brisbane, between 2013 and 2016. SVPH is a 30-bed inpatient palliative care and symptom management unit for individuals requiring treatment at end-of-life illnesses (112). Whereas, MAH is considered an acute tertiary hospital that consists of a palliative care unit, which comprises of a 24-bed oncology and haematology inpatient unit (112).

3.4 Intervention

Participants were all considered to be in the 'intervention' group due to the nature of the study previously described. Participants were dosed with methadone, which is a racemic mixture of two isomers, laevorotatory, L-methadone (the *R*-enantiomer) and dextrorotatory, D-methadone (the *S*-enantiomer)(6, 65). The patients were dosed according to their pain levels and side effect profiles. Methadone was dosed via the oral route of administration. Methadone is available in Australia as a 10 mg tablet (*Physeptone*) or as a 5 mg/ml oral liquid

(*Biodone Forte*, *Methadone syrup*) (113). It is usual in clinical practice to start with a low dose and titrate upwards, due to the complex pharmacokinetics of the drug, 'start-low-go-slow' method (114). Methadone is also only prescribed by SPC clinicians, due to the complex PK parameters and the difficulty associated with adjusting the dose of methadone when switching from another opioid (115). The oral liquid is a convenient formulation that allows for more flexibility and control of dosing regimens.

3.5 Outcome measures

3.5.1 Patient characteristics

Patient characteristics were obtained from patient medical records and recorded to identify any other factors that may affect methadone response and dose (Appendix 1 – Patient characteristic). Patient characteristics and demographics are further explained and analysed in the results section of this study. Demographic and clinical characteristics for each patient were collected from patient charts, including gender, diagnosis, weight, BMI, BSA, performance status and liver and kidney function parameters were collected.

3.5.2 Methadone dose requirements

Methadone dose requirements were titrated according to pain levels by a SPC clinician at each site and recorded in a case report form (CRF), which can be viewed in Appendix 3 – Case report forms (CRF). Methadone dose was further categorised into high or low methadone dose requirements. A methadone dose of more than 10 mg was referenced as a patient requiring a high dose and a dose of $\leq$ 10mg was referenced as a patient requiring a low dose. The reasoning and evidence behind the chosen parameters to indicate a low and high dose are further explained in 3.8 Statistical methods.

3.5.3 Pain scores

Pain scores (Appendix 3 – Case report forms (CRF)) were collected to assess pain from a scale of 0 to 10. Zero pain indicates 'no pain at all' and a score of 10 indicates 'pain as bad as you can imagine'. Pain scores were averaged if the patient had more than one reading recorded.

3.5.4 Genotyping

Genotyping was performed using pyrosequencing or TaqMan assays, depending on availability of the test method for the gene being analysed

3.5.4.1 DNA extraction and genotyping

Blood samples (3-4 mL) were collected in standard 5 mL EDTA tubes without a serum separator plug and were stored frozen at -70°C until transferred and analysed at QUT Institute of Health and Biomedical Innovation (IHBI) for DNA extraction and genotyping. Genomic DNA was extracted from whole blood collected into EDTA tubes using a modified in-house salting out method (116). A NanoDrop™ ND-1000 spectrophotometer (ThermoFischer Scientific Inc., Waltham, MA, USA) was used to measure DNA concentration and purity before dilution to 15-20 ng/ μL and stored as stock gDNA at 4°C (117). Pyrosequencing was used to genotype SNPs in BDNF and KCNJ6. Genotyping TaqMan assays were used for ARRB2.

3.5.4.2 Pyrosequencing

Pyrosequencing is a method of DNA sequencing used to determine the order of nucleotides in DNA based on the 'sequencing by synthesis' principle, which involves taking a single strand of DNA to be sequenced and then synthesising its complementary strand enzymatically (118). This method involves amplification of the region of interest with primers and subsequent biotin-labelled sequencing primer with the two alternative nucleotides offered at the polymorphic site, followed by analysis of peak heights, which allows the genotype to be determined (118). A combination of three primers is used for this method of pyrosequencing: biotinylated primer for attachment to the Streptavidin-magnetic beads for specific amplicon selection and a sequencing primer (119).

Pyrosequencing was performed on a QSeq platform (Biomolecular Systems, QIAGEN) using Pyromark Gold Q24 reagents (QIAGEN) and the data was analysed to identify genotypes using QSeq software version (Biomolecular Systems, QIAGEN). The detailed steps involved in pyrosequencing are described below, which was retrieved from the QIAGEN webpage (120):

I. A DNA segment is amplified and the strand to serve as the Pyrosequencing template is biotinylated. After denaturation, the biotinylated single-stranded PCR amplicon is isolated and allowed to hybridise with a sequencing primer.

II. The hybridised primer and single-stranded template are incubated with the enzymes DNA polymerase, ATP sulfurylase, luciferase, apyrase, the substrates adenosine 5' phosphosulfate (APS) and luciferin.

III. The first deoxyribonucleotide triphosphate (dNTP) is added to the reaction. DNA polymerase catalyses addition of the dNTP to the sequencing primer, if it is complementary to the base on the template strand. Each incorporation event is accompanied by the release of pyrophosphate (PPi) in a quantity equimolar to the amount of incorporated nucleotide.

IV. ATP sulfurylase converts PPi to ATP in the presence of adenosine 5' phosphosulfate (APS). This ATP drivers the luciferase-mediated conversion of luciferin to oxyluciferin that generates visible light in amounts that are proportional to the amount of ATP. The light produced in the luciferase-catalysed reaction is detected by a charge coupled device (CCD) camera and observed in Pyrogram. The height of each peak is proportional to the number of nucleotides incorporated.

V. Apyrase, a nucleotide-degrading enzyme, continuously degrades unincorporated nucleotides and ATP. When degradation is complete, another nucleotide is added.

VI. Addition of dNTPs is performed sequentially. Deoxyadenosine alfa-thio triphosphate (dATPαS) is used a substitute for the natural deoxyadenosine triphosphate (dATP) and it can be used by DNA polymerase, but not recognised by luciferase. The complementary DNA strand is built up and the nucleotide sequence is determined from the signal peaks in the Pyrogram trace.

This technique was used for the genes BDNF (rs7934165, rs10835210 and rs1491850) and KCNJ6 (rs2070995). Pyrosequencing assay design software was used to design the pyrosequencing assays. The primers and sequences for these SNPs for BDNF and KCNJ6 are shown in Table 6 and Table 7.

Table 6 Primers for pyrosequencing – BDNF

SNP	Primer	Sequence
rs7934165	BDNF rs934165 G_A R	GGGAGCATGCCAGGAATTG
	bio-BDNF rs7934165 G_A L	biotin-GGAAGATGCCCAAGTAGATATGC
	BDNF rs7934165 G_A seq	TTGTGTCTTTGCACC
rs10835210	BDNF rs10835210 C_A L	TTGTCCTTCGGGTTATTTTTGCAT
	bio-BDNF rs10835210 C_A R	biotin-TGCCTTACTCGTGCTGTTGAAAT
	BDNF rs10835219 C_A seq	TGTAAAGCACAGGAAAGT
rs1491850	BDNF rs1491859 T_C L	CGCATATGAGACCTCAACATCTTC
	bio-BDNF rs1491850 T_C R	biotin-TTTCAGTTTCCCGAAAGCAT
	BDNF rs1491850 T_C seq	AATCATACAGATTTTACGTG

Table 7 Primers for pyrosequencing – KCNJ6

SNP	Primer	Sequence
rs2070995	KCNJ6 rs2070995 G_A_L	TTGACAATGGACCCCAACA
	bio-KCNJ6 rs2070995 G_A R	biotin-TGGTTATGGCTACCGGGTCA
	KNCJ6 rs2070995 G_A seq	TTAAGAGAAGAATAATTCCC

3.5.4.3 TaqMan assays

Genotyping TaqMan assays are designed for amplification, genotyping and detection of specific SNPs (ThemoFischer Scientific, Life Technologies) (121). This method was utilised for genotyping SNPs in ARRB2 (rs34230287, rs3786047, rs1045280 and rs2036657) and was conducted at the IHBI in Brisbane. The process included the addition of a 2.5 µL of two times TaqMan® Master Mix, 0.25 µL, twenty times Assay Working stock (TaqMan primer/probe mix), 1 µL of DNA (20 ng/µL concentration) and 1.25 of nuclease free water was added in each 5 µL reaction sample with each sample assayed and analysed in triplicate (59, 119).

Each assay contained a pair of unlabelled primers and two TaqMan probes on the 5' end and minor groove binders and nonfluorescent quenchers on the 3' end (121). TaqMan assays involve the three steps described below, retrieved from the ThemoFischer Scientific, Life Technologies webpage (121):

I. Genomic DNA is introduced into a reaction mixture consisting of TaqMan® Genotyping Master Mix, forward and reverse primers and to two TaqMan® minor groove binder probes.

II. Between the forward and reverse primer sites, each TaqMan minor groove binder probe anneals specifically to a complementary sequence. When the probe is intact, the proximity of the quencher dye to the reporter dye suppresses the reporter fluorescence.

III. The exonuclease activity of AmpliTaq Gold ® DNA Polymerase cleaves probes hybridized to the target. Cleavage separates the reported dye from the quencher dye, increasing fluorescence by the reporter. The increase in fluorescence will only occur if the amplified target sequence is complementary to the probe. The fluorescence signal generated by the polymerase chain reaction implication indicates which alleles are in the sample.

The cycling conditions for ARRB2 for the TaqMan assay are shown in Table 8.

Table 8 Cycling conditions for TaqMan assay – ARRB2

Step	Temperature	Duration	Cycles
AmpliTaq Gold, UP, Enzyme activation	95°C	10 minutes	Hold
Denaturation	95°C	15 seconds	40
Annealing/Extension	60°C	1 minute	40

3.6 Study procedures

3.6.1 Recruitment, screening and consent

Adult patients admitted into the palliative care unit at MAH and SVPH who gave consent (Appendix 2 – Patient consent form) and met the eligibility criteria were invited to partake in the study.

3.6.2 Participant involvement

Participants completed a questionnaire assessing their pain levels (Appendix 3 – Case report forms (CRF)) at the time of providing a blood sample. DNA was isolated from whole blood samples from participants for genotyping. Patients in the study were given the opportunity to withdraw from the study at any given time point.

3.6.3 Ethical considerations

Ethics approval for this study was obtained from Mater Human Research Ethics Committee (HREC), Griffith University HREC (Reference No: PHM/17/13/HREC) and SVPH HREC. This study was conducted in compliance with NHMRC National Statement on Ethical Conduct in Human Research, the Note for Guidance on Good Clinical Practice (CPMP/ICH-135/95), the Australian Code for the Responsible Conduct of Research and all other relevant guidance documents and legislation. Participation in this study was voluntary and no patient was obliged to take part if they did not wish to do so. Participants could withdraw themselves from the study at any stage.

3.6.4 Data management and storage

Participants were de-identified by being assigned a randomly selected participant identification number. None of the responses and results could be traced back to any specific participant. Quantitative and qualitative data collected from participants were stored in a password-protected computer and paper copies were stored in a locked and secured cabinet located in G16, Griffith University. When the report on this study finalised, all patient sensitive information will be destroyed in an appropriate manner according to the study protocol.

3.7 Sample size

The primary aim of the prospective multi-centre, open labelled, dose individualisation study was to investigate interindividual variability in response to methadone, by means of a population PK study, which does not use traditional statistical analyses (59). A sample size of 50 participants, providing two to four samples over the dosing period, was determined to be the minimum number necessary to generate satisfactory estimates of the structural parameters (e.g. clearance and volume of distribution) and the variance parameters (e.g. interindividual and inter-occasion variability) for the non-linear mixed effect modelling (POP-PK modelling) study. The current study investigated a secondary outcome, which was to determine if SNPs influence the variability in response seen in methadone treatment in advanced cancer. Therefore, genotyping for the current study was performed on the same sample of participants as the larger dose individualisation study.

3.8 Statistical methods

Data analysis was conducted using SPSS statistical analysis software by IBM (IBM, v25 2017) and Microsoft Excel (Microsoft ©, v16 2019). Inferential statistics were calculated to allow for extrapolation of the results from the small sample size, where significance will be considered if $p \leq 0.05$. Minor allele frequency (MAF) (i.e., the second most common allele occurring in a given population) for each SNP was obtained from the dbSNP database compiled by the National Centre for Biotechnology and Information (NCBI) (122).

Table 9 is a summary of the statistical methods that will be utilised for each objective.

Table 9 Statistical tests used for each objective

Objectives	Statistical Tests
1. To investigate whether patient characteristics affect methadone response and pain levels	• Regression analysis

2. To identify which SNPs in the genes being analysed are significantly associated with a high pain score	• Pain scores were treated as a categorical variable [123]
	  • Low pain (≤ 3/10)
	  • High pain (≥ 4/10)
	• Chi-square analysis

3. To identify which SNPs in the genes being analysed are significantly associated with high methadone requirements	• Methadone dose (continuous variable)
	  • Parametric test: ANOVA or non-parametric: Kruskal Wallis H test
	  • Genotypes were divided into three categories and methadone dose was measured on a continuous scale
	• Methadone dose (categorical variable)
	  • Methadone dose of > 10mg = high methadone requirements
	  • Chi-square analysis

3.8.1 Descriptive statistics

Data collected related to pain scores, methadone dose and patient characteristics will be described and introduced using descriptive statistics.

3.8.2 *Normality tests*

Normality tests were conducted to determine whether parametric or non-parametric tests should be used. When methadone dose was treated as a continuous variables for objective three, skewness and kurtosis, measures of normality, were calculated using SPSS statistical software (IBM, v25 2017), to determine whether the parametric ANOVA test should be used, or the non-parametric, Kruskal-Wallis H test should be used.

3.8.3 *Influence of patient characteristics on methadone response and pain levels*

Regression analysis is a statistical technique for determining the extent to which one variable is related to two or more other variables, and to predict the future outcome of the dependent variables (76). This method of statistical analysis was used to determine if any patient characteristics, listed in Appendix 1 – Patient characteristic, influenced methadone response and pain levels. This was conducted using SPSS statistical software by IBM technologies (IBM, v25 2017), with the aim of excluding patient characteristics as confounding factors on methadone response and pain levels.

3.8.4 *Influence of SNPs on pain scores and methadone dose when dependent variables are being treated as categorical variables*

Chi-square analysis is used for categorical variables when the association between nominal/dichotomous data is to be analysed (79). This statistical test is employed when the relationship between two categorical variables needs to be tested (124). In this study, significance was established if $p \leq 0.05$. The chi-square test was used to determine whether there was a significant difference between the frequency of genotypes for each gene being analysed and either pain scores or methadone dose. Again, SPSS statistical software (IBM, v25 2017) was again utilised to perform chi-square analysis.

Chi-square analysis was utilised for both objectives two and three. For objective two, pain scores were divided into two categories: low pain ($\leq$ 3/10) and high pain ($\geq$ 4/10). These categories were chosen based on recent studies by George *et al*. (59) and Bista *et al.* (78), which found that dividing pain scores into stricter categories provided increased quality of data analysis and an increased distinction between low and high levels of pain. In addition, other clinical studies, such as the one conducted by Gretton *et al*. (123) used the same pain

score parameters where pain that is ≤ 3/10 represents good pain control (123). Further, a pain score of ≥ 4/10 is when pain is said to interfere with normal functioning (125), and indicates mild to severe pain that is associated with increased analgesic requirements (40).

Methadone requirements (dose) were also divided into two categories: high methadone requirement (> 10 mg/day) and low-to-medium opioid requirement (≤ 10 mg/day). The further categorisation of methadone dose requirements was done in accordance after discussion with the SPC clinician involved in the study and what is also commonly acknowledged to be a high or low dose of the opioid, methadone, in the palliative care setting. In a systematic review conducted by Mercadante *et al*. (9) a low methadone dose was also considered to be that of ≤ 10 mg. Furthermore, a study by Lanken *et al*. (126) that overviewed palliative care for patients with respiratory and critical illness, also defined the initial dose of opioids to treat pain in opioid naïve patients, defining a starting dose of methadone for moderate to severe pain to be 5 to 10 mg (126). A higher dose was recommended for patients already established on opioids. Thus, the 5 to 10 mg dosing guideline is accepted as a low dose for methadone, which also supports the chosen parameters to depict a low and high methadone dose in the current study.

The SNPs for each gene being analysed were also a categorical variable, as each SNP was further divided into three groups: homozygous dominant, heterozygote and homozygous recessive. For example, BDNF (rs10835210) was divided into three groups: CC (homozygous dominant), CA (heterozygote) and AA (homozygous recessive).

3.8.5 Influence of SNPs on methadone dose requirements

The one-way analysis of variance (ANOVA) is a parametric test (127) and the Kruskal-Wallis H test is a non-parametric test used to determine significance between the means of two independent groups (128). The most appropriate statistical test was used to determine whether there is a significant difference in methadone dose requirements based on a patient's genotype. Each allele being investigated in this study was further categorised into three categories (genotypes) consisting of the homozygous dominant, heterozygous and the homozygous recessive group. Post hoc analysis will have to be conducted if any significance is found between genotypes and methadone dose. SPSS statistical software by IBM

technologies (IBM, v25 2017) was again used to determine significance at a level if $p < 0.05$, when conducting the ANOVA or Kruskal-Wallis H test.

3.8.6 Hardy-Weinberg equilibrium analysis

The SNPs investigated in this study were analysed to determine if they were in Hardy-Weinberg equilibrium (HWE). SPSS statistical software (IBM, v25 2017) was used to identify the observed genotypes in the data set and to determine significance by using the chi-square test. If $p > 0.05$, it signified that the SNPs in the selected population were all within HWE. Microsoft Excel (Microsoft ©, v16 2019) was used to determine observed allele frequencies and predicted genotype frequencies.

The HWE is used throughout genetic studies and can infer population stratification, inbreeding and issues in genotyping (72). The HWE states that in the absence of migration, mutation, natural selection and assortative mating, genotyping frequencies at any locus will remain constant from generation to generation (72). The equation for HWE is $p^2 + 2pq + q^2$. When this equation is equal to 1, the population is said to be in in HWE (73, 74). This equation is further explained as: if p (dominant allele) is the frequency of one allele and q (recessive allele) is the frequency of another allele for a biallelic locus, then p^2 represents the homozygous dominant genotype, $2pq$ will represent a heterozygous genotype and q^2 will represent the homozygous recessive genotype (73). Large significant deviations from the HWE suggest quality issues such as genotyping and laboratory errors. By checking the HWE, such errors can be ruled out as confounding factors (75).

The aim of the HWE test is to determine whether there is a significant difference ($p < 0.05$) between the observed genotypes and predicted genotype frequencies within a study. A detailed example of determining observed allele frequencies and predicted genotype frequencies is given in the results section of this study. The results for the HWE for each SNP being analysed in this study are also in the results section of this study under the heading 'Genotyping assay reliability'.

4 RESULTS

This chapter will be introduced by the HWE, which is used to ensure genotyping assay reliability. Patient characteristics will then be described. Performance status, BMI, cancer diagnosis, blood cell count, liver and kidney function tests of the cohort will also be described and presented in this section. Results from the logistic regression model, investigating the effect of patient characteristics on pain scores and methadone dose will be presented and described in the results. Further, results from the study of SNPs affecting methadone dose and pain scores have also been included in this section, where the results of this study have been compared to published literature. This section will end with MAFs of this study compared to both published literature and the NCBI database.

4.1 Genotyping assay reliability

The HWE test was applied to each SNP being analysed for the three genes BDNF, KCNJ6 and ARRB2. The HWE was conducted to determine if there was a significant difference between the expected and observed genotype frequencies. A significant deviation between the expected and observed values imply quality experimental errors, such a genotyping error (129). It is usually required to use the control cohort to test the HWE. However, the design of this study does not include a control group. The HWE equation is $p^2 + 2pq + q^2 = 1$, where 'p' signifies the dominant allele and 'q' signifies the recessive allele, thus, making '$2pq$' the heterozygous allele. Below is the process used to calculate the expected genotype frequencies.

The process for calculating the HWE is shown in Figure 8. The single nucleotide polymorphism, rs1491850, for the BDNF gene will be used as an example to explain, step-by-step, how the HWE was calculated for this study.

Process used to calculate expected genotype frequencies for HWE

1. Calculate the observed genotype frequencies. In this instance, the SPSS frequency function was used to yield the observed count.
2. Convert the observed genotype frequencies in to observed allele frequencies.

- Dominant allele: (dominant allele observed frequency * 2) + heterozygous observed frequency.
    - (18*2) + 22 = 58
- Recessive allele: (recessive allele observed frequency * 2) + heterozygous observed frequency
    - (15*2) + 22 = 34
- Total number of alleles genotyped:
    - 58 + 34 = 92
- Proportion of dominant allele:
    - (58/92) = 0.6304
- Proportion of recessive allele:
    - (34/92) = 0.3696

3. Convert observed proportions to predicted genotype frequencies.

- In this case there was 46 (18+22+6) individuals genotyped for this SNP.
- Homozygous *dominant* expected genotypes:
    - p^2 * total of individuals sampled
        - 0.6304^2 * 46 = 18.28
- Homozygous *recessive* expected genotypes:
    - q^2 * total of individuals sampled
        - 0.3696^2 * 46 = 6.28
- Heterozygous expected genotypes:
    - 2pq
        - (2*(0.6304*0.3696)) * 46 = 6.28

Chi-square analysis is then utilised to derive a *p* value (significance) to calculate if there is a significant difference between observed and predicted genotype frequencies.

Figure 8 Process for calculating the Hardy-Weinberg equilibrium

Table 10 Hardy-Weinberg equilibrium – BDNF

SNP	Observed genotypes				Observed allele frequency		Predicted genotype frequency			Allele		X^2 *p* value
	HH	Hh	hh	Frequency (total)	Dominant allele (%)	Recessive allele (%)	HH	Hh	hh	Dominant	Recessive	
rs7934165 C>T	13	28	5	46	58.70	30.43	15.85	16.43	4.26	54	38	0.08
rs10835210 C>A	15	27	4	46	61.96	38.04	17.66	21.68	6.66	57	35	0.10
rs1491850 T>C	18	22	6	46	63.04	36.96	18.28	21.44	6.28	58	34	0.86

X^2 = chi-squared test, HH = homozygous dominant, Hh = heterozygote, hh = homozygous recessive

In Table 10, the results of the HWE for BDNF (rs7931465, rs10835210 and ts1491850) are presented. Chi-square analysis was conducted using SPSS statistical software (IBM, v25 2017) to determine if there was any significance between observed and predicted genotype frequencies. There was no significant difference (p = 0.08, 0.10 and 0.86) between the observed and predicted genotypes. Thus, the genotype frequencies of the assessed polymorphisms met the HWE threshold for BDNF.

Table 11 Hardy-Weinberg equilibrium – KCNJ6

SNP	Observed genotypes				Observed allele frequency		Predicted genotype frequency			Allele		X^2 *p* value
	HH	Hh	hh	Frequency (total)	Dominant allele (%)	Recessive allele (%)	HH	Hh	hh	Dominant	Recessive	
rs2070995 G>A	26	20	0	46	78.26	21.74	28.17	15.65	2.17	72	20	0.06

X^2 = chi-squared test, HH = homozygous dominant, Hh = heterozygote, hh = homozygous recessive

In Table 11, the results of the HWE for KCNJ6 (rs2070995) is presented. There was no significant difference (p = 0.06) between the observed and predicted genotypes. Thus, the genotypes of the population, of 46 participants in this study, are in HWE.

Table 12 Hardy-Weinberg equilibrium – ARRB2

SNP	Observed genotypes				Observed allele frequency		Predicted genotype frequency			Allele		X^2 *p* value
	HH	Hh	hh	Frequency (total)	Dominant allele (%)	Recessive allele (%)	HH	Hh	hh	Dominant	Recessive	
rs34230287 C>T	31	15	0	46	83.70	16.30	32.23	12.55	1.22	77	15	0.19
rs3786047 A>G	2	20	24	46	26.09	73.91	3.13	17.74	25.13	24	68	0.39
rs1045280 C>T	11	10	25	46	34.78	65.22	5.56	20.87	19.57	32	60	*p*<0.05
rs2036657 G>A	2	19	25	46	25.00	75.00	2.88	17.25	25.88	23	69	0.49

X^2 = chi-squared test, HH = homozygous dominant, Hh = heterozygote, hh = homozygous recessive

All SNPs, except for rs1045280 C > T in the ARRB2 gene, were within the HWE, are shown in Table 12. This may suggest laboratory error, such as errors in genotyping, or more likely, it is due to the small sample size of the cohort. However, SNPs can also be out of the HWE as a matter of random chance (129). Overall, 46 participants were included in the final study, increasing the chances that alleles would not to be within the HWE. Thus, rs1045280 was still included for further analysis in this study. Deviation from the HWE was also found in the study conducted by Oneda *et al.* (10) on rs1045280 ($p = 0.04$), in the MMT cohort but not in the controls, suggesting that the SNP was associated with undergoing MMT. In the current study, this SNP could possibly be associated with having advanced cancer. The study design of the current study did not allow for a control group and the HWE test could not be conducted on healthy individuals.

4.2 Patient characteristics

Patient characteristics were analysed to exclude any confounding variables that may affect methadone response and pain levels. Figure 9 illustrates the study flow and the number of participants included in the data analysis, adapted from CONSORT (transparent reporting of trials) (115).

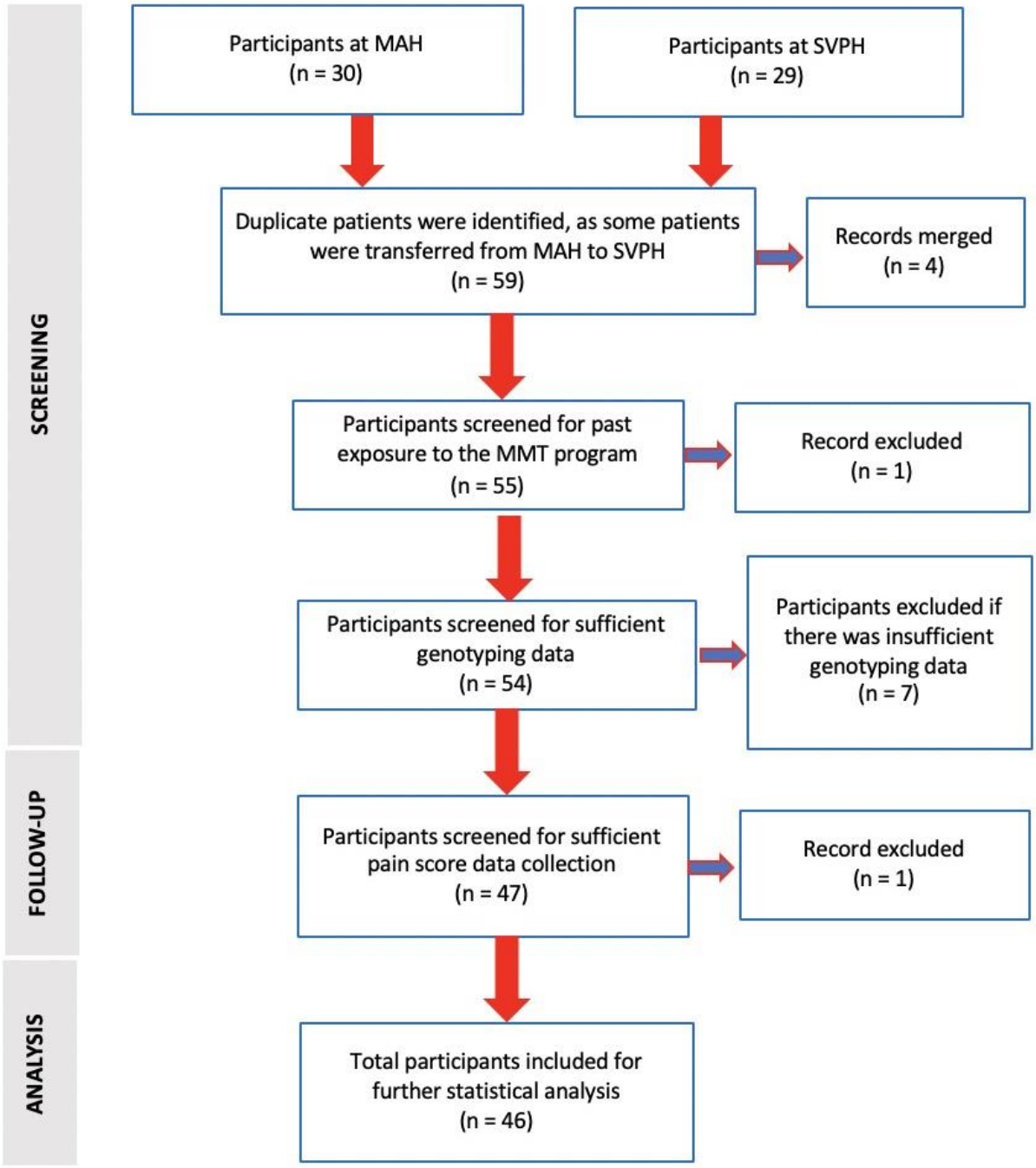

Figure 9 Study flow diagram – number of participants

Figure 9 is presented as a flow diagram as per the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (130).

Seven participants were excluded from the study (Figure 9) due to insufficient sample volume for the pharmacokinetic study (primary outcome) and genotyping (secondary outcome) to determine the SNP profile for the three genes being analysed. One participant was excluded due to being a past MMT patient and one other patient was excluded as no pain score information was available. The participant who was a prior MMT patient was excluded, as the PK of a prior MMT patient is different to opioid naïve patients, due to metabolic imbalances. It is believed that long-term heroin use causes permanent metabolic deficiency in the central nervous system and is associated with physiological disease, which requires regular administration of opiates to correct the metabolic deficiency (131). Participants who were receiving methadone for breakthrough pain were also excluded as the pervious study was a population PK study, which would have made it difficult for the investigators to study the effects of methadone as a treatment medication, rather than a breakthrough medication. It would have also changed the blood concentration of methadone and would have provided an inaccurate plasma concentration time curve. Overall, there were 46 participants that were included for data analysis as seen in Figure 9 Study flow diagram – number of participants.

Participants were either admitted to MAH or SVPH. Five participants were initially admitted to MAH then transferred to SVPH during the period of this study. MAH palliative care unit consist of a 24-bed oncology and haematology unit, which focuses more on acute cases and symptoms, whereas, SVPH is a 30-bed inpatient palliative care facility that focuses on symptom management of patients at the end of their life (112).

Table 13 describes patient demographic characteristics.

Table 13 Patient demographic characteristics

Patient Demographic Characteristics	
Number of patients	46
Age (years)	
Mean (range)	60.7 (32-83)
Gender	
Female, *n* (%)	27 (58.7%)
Male, *n* (%)	19 (41.3)
Performance Status Palliative Care	
Mean (range)	39.3 (1- 90)
Mean Methadone Dose	
Mean (SD)	18.1 (17.2)
Median (range)	15.0 (3.8-93.3)
Pain Score Average	
Mean (SD)	3.8 (2.0)
Median (range)	4.0 (0-8)

The mean age for the cohort was 60.7 years, with the ages ranging from 32 to 83 years. The majority of patients were female, at 58.7%, and the remaining 41.3% participants were male. The average performance status was at 39.3% (end-of-life). The mean methadone dose was at 18.1 mg, ranging from 3.8 mg to 93.3 mg. The whole cohort had a mean pain score of 4.2, which ranged from the scores of 0 to 8. The average weight of participants was 68.4 kg, ranging from 36 to 118 kg and the average BMI score was 24.6, ranging from 13 to 28.5.

4.2.1 Body Mass Index and cachexia

Body mass index is summarised in Table 14. Of the 46 participants, the BMI of one participant could not be collected. However, 42.2% were considered to be at a healthy weight, while, 33.3% were overweight and the remaining 6% were considered obese. Cachexia is a common occurrence in end-of-life situations, where there is a sudden loss of muscle mass and severe weight loss of more than 5% of body weight in twelve months or less (132). Among the study participants, 11.1% (5) of individuals were underweight, and thus, cachectic.

Table 14 Body Mass Index (BMI)

BMI (k/mg^2)	*n* (%)
Underweight (cachexia) (<18.5)	5 (11.1)
Healthy (18.5-24.9)	19 (42.2)
Overweight (25-29.9)	15 (33.3)
Obese (>30)	6 (13.3)

4.2.2 *Performance status*

Participants were further categorised by performance status. Most patients (45.7%) that participated in this study were considered to be 'transitional' when performance status was assessed.

Table 15 Performance status (palliative care)

Performance Status Palliative Care	*n* (%)
Stable (70 to 100%)	9 (19.6)
Transitional (40 to <70%)	21 (45.7)
End-of-life (<40%)	16 (34.8)

Table 15 shows the number of participants and the corresponding percentage of participants in the study who were either performing at a stable, transitional or at the end-of-life status, determined after calculating performance status.

Table 16 Performance status v. Palliative care unit

Palliative care unit	Performance status	*n* (%)
MAH	Stable *(70 to 100%)*	7 (31.8)
	Transitional *(40 to <70%)*	3 (13.6)
	End-of-life *(<40%)*	12 (54.5)
SVPH	Stable *(70 to 100%)*	3 (11.1)
	Transitional *(40 to <70%)*	20 (74.1)
	End-of-life *(<40%)*	4 (14.8)

Performance status was further split according to the palliative care unit patients were in at the time of the study, to further understand the patient demographics depending on which tertiary palliative care unit to which they were admitted to. A large proportion of participants (54.5%) admitted to MAH were classified as performing at an 'end-of-life' status. Whereas, the more than half of the participants (74.1%) at SVPH were considered to be at a 'transitional' status. MAH focuses on acute cases, compared to SVPH, which focuses more on patients that require symptom management, therefore, implying that in our study it seemed as SVPH focuses on patients performing at a transitional status, compared to MAH focusing on acute causes surrounding 'end-of-life'.

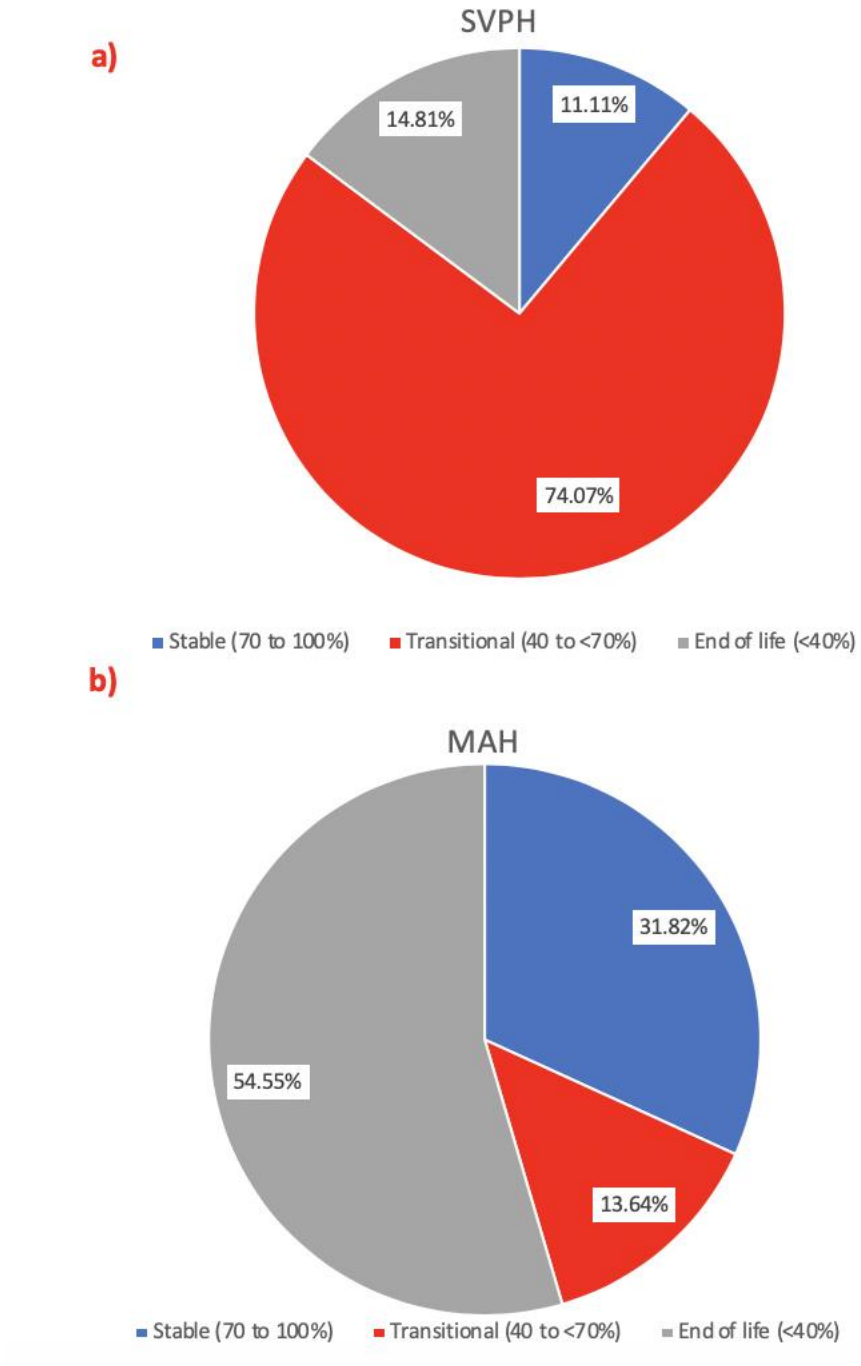

Figure 10 Performance status v. Palliative care unit

Figure 10 – a) SVPH, b) MAH.

Figure 10 shows that 54.55% of participants at MAH were performing at an 'end-of-life' status, 31.82% were performing at a stable status and 13.64% were performing at a transitional status. Whereas, 74.07% of participants were performing at a 'transitional' state at SVPH,

11.1% were performing at a stable state and the remaining 14.81% were performing at an end-of-life status.

4.2.3 Cancer diagnosis

As shown in Figure 11, there was a wide range of cancer diagnoses among study participants. Accordingly, patients were not further categorised in to 'cancer types', as the results would not have been significantly relevant. After conducting regression analysis, cancer diagnosis was found to have no effect on pain scores (p = 0.96) and methadone dose (p = 0.516).

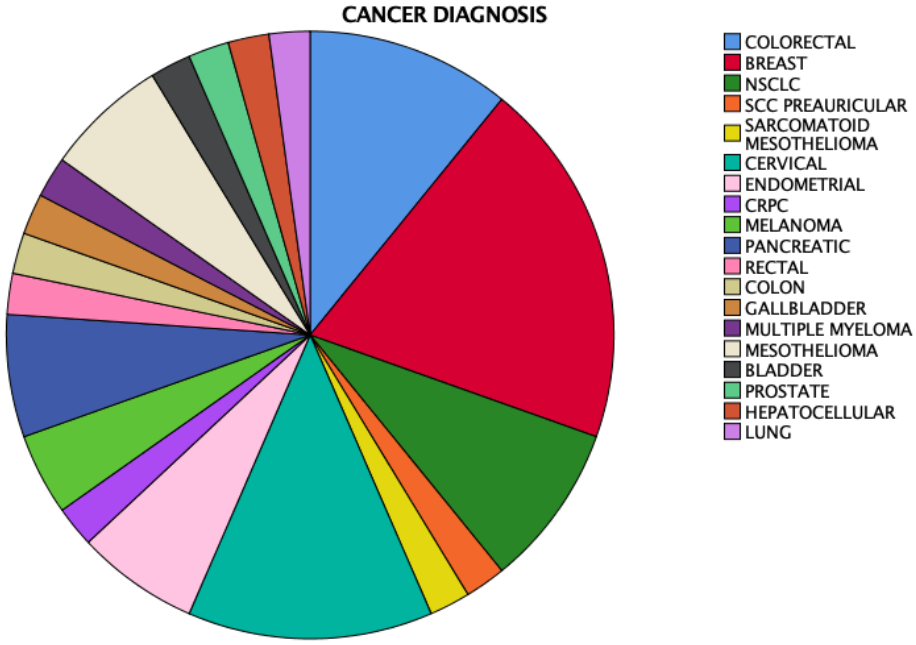

Figure 11 Cancer diagnosis

Figure 11 represents the wide range of cancer diagnoses among study participants.

4.2.4 Pain score

Pain scores were divided into low pain (≤ 3/10) and high pain (≥ 4/10) based from results from the Brief Pain Inventory.

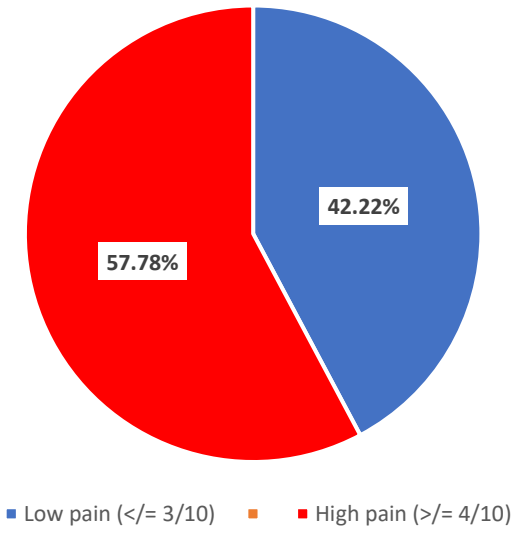

Figure 12 Pain score distribution

Of the 46 participants, 57.78% had a current pain score that was considered to be high (≥4/10), possibly indicating poor pain control associated with the complexities involved in dosing individuals with end-of-life illness, when pain is constantly increasing.

Multiple linear regression was used to determine whether patient characteristics influenced pain levels (pain scores) and methadone dose requirements. Patient characteristics included the following variables: height (cm), weight (kg), BSA, BMI, performance status and age.

Table 17 Multiple linear regression (pain scores) – patient characteristics

VARIABLES	B (95% Confidence interval)	*p*
HEIGHT (CM)	-0.12 (-0.905 to 0.665)	0.758
WEIGHT (KG)	-0.06 (-0.76 to 0.641)	0.864
BSA	8.928 (-62.408 to 80.265)	0.801
BMI	-0.15 (-1.642 to 1.341)	0.839
PERFORMANCE STATUS %	0.003 (-0.028 to 0.034)	0.845
AGE	-0.025 (-0.086 to 0.035)	0.400
GENDER	-0.49 (-2.765 to 1.786)	0.665

$r^2 = 0.037$

F =0.168, p=0.994

In Table 17, it can be seen that none of the variables (height, weight, BSA, BMI, performance status, age and gender) did not significantly ($p > 0.05$) influence a participants pain score in

this study (F=0.168, r^2=0.037, p=0.994). It should be noted that from the r^2 value, that the independent variables explain 6.6% of the variability of the pain scores.

4.2.5 Methadone dose requirements

The linear regression model was also used to determine the effect of patient characteristics on methadone dose, to examine if there were any other measurable factors affecting an individual's methadone dose requirements.

Table 18 Multiple linear regression (methadone dose) – patient characteristics

VARIABLES	B (95% Confidence interval)	*P*
PERFORMANCE STATUS %	0.032 (-0.166 to 0.229)	0.745
BMI	0.593 (-10.809 to 11.995)	0.916
BSA	-21.443 (-473.846 to 430.961)	0.924
WEIGHT (kg)	0.108 (-4.341 to 4.558)	0.961
HEIGHT (cm)	0.247 (-5.313 to 5.808)	0.929
AGE	-0.287 (-0.691 to 0.117)	0.158
GENDER	-5.334 (-20.073 to 9.404)	0.467

r^2 = 0.108
F =0.531, p=0.825

As with pain scores, none of the variables seen in Table 18, significantly (p > 0.05) influenced the average mean methadone dose (F=0.531, r^2=0.108, p=0.825). The r^2 value indicates that these variables only explain 10.8% the variability of the mean methadone dose.

4.2.6 Blood cell count

Blood cell counts have been used to predict length of survival in end-of-life care in the hospice setting. Total white blood cell count, platelet, creatinine, AST and ALT, to name a few, have been linked with prognostic factors. White blood cell count is also a strong indicator of infection and therefore, it was important to investigate whether these baselines characteristics may have had an effect on methadone dose or pain scores in this study.

Haemoglobin (Hb), white blood cell count (WBC) and platelets (PLTS) were included in the regression model, as they are commonly used as markers for infection. In this study, none of the participants had an infection, based on blood cell count. These factors were often used in

other studies to predict length of survival in a hospice centre (133). However, this was not an objective in this study.

Table 19 lists the significance values after conducting regression analysis to determine influence on pain scores and methadone dose.

Table 19 Regression analysis – blood cell count

Blood cell count	Pain scores (*p*)	Methadone dose (*p*)
Haemoglobin (Hb)	0.671	0.962
White blood cells (WBC)	0.692	0.902
Platelets (PLTS)	0.427	0.825

Table 19 indicates that no significance was found in relation to Hb, WBC and PLT and their influence on both pain scores and methadone dose. Thus, it could be concluded that these three factors could be excluded as other confounding variables that may influence an individual's methadone dose requirements or pain levels.

4.2.7 Liver function

Multiple linear regression was utilised to determine whether liver function influenced pain levels (pain scores) and methadone dose requirements. Liver function tests included the following variables: AST, ALT, ALP, GGT and LDH. Methadone dose can be influenced by liver function, potentially influencing pain management.

Table 20 lists the significance values from the linear regression model in relation to liver function tests and pain scores.

Table 20 Multiple linear regression (pain scores) – liver function

VARIABLES	B (95% Confidence interval)	*p*
Liver function tests		
AST	-0.265 (-1.58 to 1.05)	0.237
ALT	0.346 (-1.286 to 1.978)	0.226
ALP	-0.051 (-0.729 to 0.627)	0.514
GGT	0.014 (-0.204 to 0.232)	0.564
LDH	0.015 (-0.084 to 0.114)	0.310

Multiple linear regression conducted on liver function tests (AST, ALT, ALP, GGT, LDH,) were not significantly ($p > 0.05$) associated with pain scores

Table 21 lists the significance values from the linear regression model in relation to liver function tests and methadone dose.

Table 21 Multiple linear regression (methadone dose) – liver function

VARIABLES	B (95% Confidence interval)	*P*
Liver function tests		
AST	0.137 (-17.956 to 18.231)	0.939
ALT	0.457 (-21.994 to 22.908)	0.839
ALP	0.036 (-9.291 to 9.362)	0.969
GGT	-0.147 (-3.149 to 2.856)	0.646
LDH	0.022 (-1.34 to 1.383)	0.874

After conducting multiple linear regression, none of the liver function tests, listed above, after conducting multiple linear regression were found to influence the mean methadone dose requirements.

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Table 22 Liver function v. Pain scores

Liver function test	Pain score	Reference range	*n* (%)	*p*
AST	*LOW PAIN SCORE (≤3/10)*	Within range	12 (63.2)	0.258
		Outside range	7 (36.8)	
	HIGH PAIN SCORE (≥4/10)	Within range	11 (45.8)	
		Outside range	13 (54.2)	
ALT	*LOW PAIN SCORE (≤3/10)*	Within range	16 (84.2)	**0.010**
		Outside range	3 (15.8)	
	HIGH PAIN SCORE (≥4/10)	Within range	11 (45.8)	
		Outside range	13 (54.2)	
ALP	*LOW PAIN SCORE (≤3/10)*	Within range	8 (42.1)	0.606
		Outside range	**11 (57.9)**	
	HIGH PAIN SCORE (≥4/10)	Within range	12 (50)	
		Outside range	**12 (50)**	
LDH	*LOW PAIN SCORE (≤3/10)*	Within range	7 (43.8)	0.056
		Outside range	**9 (56.3)**	
	HIGH PAIN SCORE (≥4/10)	Within range	15 (75)	
		Outside range	**5 (25)**	
GGT	*LOW PAIN SCORE (≤3/10)*	Within range	8 (42.1)	0.131
		Outside range	11 (57.9)	
	HIGH PAIN SCORE (≥4/10)	Within range	5 (20.8)	
		Outside range	19 (79.2)	

Participants had their liver function tests further analysed to determine whether decreased function (outside reference range) influenced pain. It was a common trend among the five liver function tests, that individuals outside the reference ranges (poor liver function) experienced a high pain score, when compared to the population which exhibited a low pain score. For AST, 54.2% of individuals experienced high pain with AST levels outside the reference range, compared to 36.8% of individuals who were outside the reference range that exhibited a low pain score. For ALT, 54.2% of individuals experienced high pain levels that were outside the reference range, compared to only 15.8% who experienced low pain levels that were outside the range. ALT levels that were within range, were significantly (p = 0.010) associated with a low pain score. Thus, significance was found only for ALT out of all the other liver function tests investigated, which can be seen in Table 22. A similar trend was seen for GGT, where individuals were more likely to experience a high pain score if their levels were outside the range, compared to having levels inside the range (79.2% v. 57.9%), though this

was not significant (p = 0.131). However, for ALP and LDH, majority of individuals with levels outside the reference range experienced lower pain scores, which can be identified in Table 22.

4.2.8 Kidney function

Methadone exhibits high oral bioavailability and rapid onset of effect, therefore, making it safe to use in renal impairment (4). However, such patients with renal impairment will require longer dosing intervals (3). It is suggested according to the guideline, AMH, that due to the active/toxic metabolites that accumulate due to renal impairment, it is safest to dose methadone at longer dosing intervals (4).

Multiple linear regression was utilised to determine whether kidney function influenced pain levels (pain scores) and methadone dose requirements. Kidney function tests included the following variables: creatinine clearance, creatinine, urea, albumin and estimated glomerular filtration rate (EGFR). Methadone dose can be influenced by kidney function, because it can be influenced by liver function, potentially influencing pain management, which will be reflected on pain scores collected.

Table 23 Multiple linear regression (pain scores) – kidney function

VARIABLES	B (95% Confidence interval)	*P*
Kidney function tests		
CREAT CL	0.009 (-8.513 to 8.530)	0.992
CREAT	-0.119 (-12.286 to 12.047)	0.921
UREA	3.095 (-46.999 to 53.188)	0.576
ALB	0.037 (-59.349 to 59.423)	0.995
EGFR	0.346 (-8.892 to 33.91)	0.917

No significance was found regarding the effect of kidney function on pain scores.

Table 24 Multiple linear regression (methadone dose) – kidney function

VARIABLES	B (95% Confidence interval)	*P*
Kidney function tests		
CREAT CL	0.009 (-8.513 to 8.530)	0.992
CREAT	-0.119 (-12.286 to 12.047)	0.921
UREA	3.095 (-46.999 to 53.188)	0.576
ALB	0.037 (-59.349 to 59.423)	0.995
EGFR	0.346 (-8.892 to 33.91)	0.917

No significance was found regarding the effect of kidney function on methadone dose.

Table 25 Creatinine (kidney function) v. Pain score

Creatinine		
Pain Score	**Reference Range**	***n* (%)**
WITHIN RANGE	Low pain score (≤3/10)	10 (40)
	High pain score (≥4/10)	15 (60)
OUTSIDE RANGE	Low pain score (≤3/10)	9 (50)
	High pain score (≥4/10)	9 (50)

When pain scores were compared to kidney function in this cohort of participants, an identical percentage of patients who experienced low pain and high pain also had creatinine levels that were outside the range. Furthermore, there were more individuals (15 patients) with normal creatine levels who exhibited high pain levels, compared to 10 individuals who experienced low pain levels and had normal creatine levels. This observation suggests that having poor kidney function does not affect methadone dose and does not influence associated pain levels and scores.

Table 26 EGFR (kidney function) v. Pain score

Pain Score	EGFR Reference Range	n (%)
LOW PAIN SCORE (≤3/10)	Normal GFR (>90ml/min/1.73m^2)	12 (63.2)
	Mild decrease in GFR (60-89ml/min/1.73m^2)	4 (21.1)
	Moderate decrease in GFR (30-59ml/min/1.73m^2)	2 (10.5)
	Severe decrease in GFR (15-29ml/min/1.73m^2)	1 (5.3)
	Kidney failure (<15ml/min/1.73m^2)	0
HIGH PAIN SCORE (≥4/10)	Normal GFR (>90ml/min/1.73m^2)	8 (33.3)
	Mild decrease in GFR (60-89ml/min/1.73m^2)	13 (54.2)
	Moderate decrease in GFR (30-59ml/min/1.73m^2)	2 (8.3)
	Severe decrease in GFR (15-29ml/min/1.73m^2)	0
	Kidney failure (<15ml/min/1.73m^2)	1 (4.2)

As shown in Table 26, estimated glomerular filtration rate was divided into five categories depending on individual EGFR parameters. The five categories were: Normal GFR, mild decrease in GFR, moderate decrease in GFR, severe decrease in GFR and kidney failure. There were no participants in the study who had high a pain score and had either a severe decrease in GFR or kidney failure. However, 4.2% had kidney failure. The majority of participants who exhibited high pain levels had either normal GFR (33.3%) or a mild decrease in GFR (54.2%). Whereas, in the group that exhibited low pain levels, 63.2% had normal GFR, 21.1% had a

mild decrease in GFR, 10.5% had a moderate decrease in GFR, 5.3% had a severe decrease in GFR and no one had kidney failure.

4.3 Single nucleotide polymorphisms associated with pain scores

Chi-square analysis was used to analyse the influence of SNPs in BDNF, KCNJ6 and ARRB2 pain scores, as two categorical variables were being compared to one another. Pain scores were collected from patients after completing the Brief Pain Inventory, where pain was measured on a scale of 0 to 10. A low pain score was anything equal to or less than three and a high pain score was any score that was more than or equal to four. The decision to divide pain scores into these two categories was based on recent studies by George *et al*. (59) and Bista *et al.* (78), previously mentioned.

4.3.1 Normality test

The adequacy of each statistical test used for the study outcomes was assessed by examining residuals or heterogeneity and normality. Variables that were measured on a continuous scale were described using means and standard deviations (SD), if normally skewed. Non normally distributed continuous variables were presented via medians and interquartile ranges. Whereas, categorical variables were presented as frequencies and percentages.

Data is considered to normally distributed if skewness and kurtosis are within the range of ± 3. SPSS statistical software (IBM, v25 2017) was used to conduct normality tests on the variables in the study to determine normality.

Table 27 Normality check for pain scores

Pain score (continuous)	
Skewness	-0.326
Kurtosis	-1.984

Pain scores were normally distributed as skewness and kurtosis was within the ± 3 range, as seen in Table 27. However, chi-square analysis is a non-parametric test that does not require normality of variables, as the variables are required to be categorical and cannot be tested for normality. The only two assumptions required for chi-square analysis is that all variables are categorical and variables must consist two or more categorical, independent groups (124).

4.3.2 BDNF

Brain-derived neurotrophic factor is vital for developing and maintaining neuronal brain function (96). This gene is involved in multiple pathways, such as neuroplasticity, rewards-related processes and the healing of neurons (96). The following SNPs, rs7934165, rs10835210 and rs1491850, in BDNF have been associated with opioid requirements in the MMT program (11).

Table 28 Chi-square analysis – BDNF (rs7934165, rs10835210 and rs1491850)

BDNF	rs7934165			rs10835210			rs1491850		
	CC	**CT**	**TT**	**CC**	**CA**	**AA**	**TT**	**TC**	**CC**
Low pain score (≤3/10)	8 (42.1)	9 (47.4)	2 (10.5)	2 (10.5)	8 (42.1)	9 (47.4)	10 (52.6)	5 (26.3)	4 (21.1)
High pain score (≥4/10)	8 (19.2)	9 (73.1)	2 (7.7)	1 (3.8)	19 (73.1)	6 (23.1)	7 (26.9)	**17 (65.4)**	2 (7.7)
x^2	3.254			4.433			6.818		
p	0.197			0.109			**0.033**		

A significant association (x^2=6.818, *p*=0.033) was found between SNPs (TT, TC and CC) in the BDNF (rs1491859) gene and pain scores. Further analysis indicated that TC carriers were more likely to exhibit high pain scores. There were also more individuals with the TT genotype who experienced low pain compared to high pain. No significance was found regarding pain scores for rs7934165 and rs10835210.

4.3.3 *KCNJ6*

The KCNJ6 gene codes for potassium inwardly rectifying channels subfamily J member 6 (also known as Kir3.2 or GIRK2) (8). It is a G-protein coupled channel that is involved in opioid receptor transmission and eliciting opioid effects (90). The gene is also involved in postsynaptic inhibition of pain (90) and mediating components of the analgesic response (91, 92). The following SNP, rs2070995, in KCNJ6 were associated with opioid effectiveness and pain intensity (8, 94).

Table 29 Chi-square analysis – KCNJ6 (rs2070995)

KCNJ6	rs2070995		
	GG	**GA**	**AA**
Low pain score (≤3/10)	11 (57.9)	8 (42.1)	0
High pain score (≥4/10)	14 (53.8)	12 (46.2)	0
x^2	0.073		
p	0.787		

Chi-square analysis was conducted in order to determine whether the SNP in the KCNJ6 (rs2070995) gene were associated with either a low (≤ 3/10) or high (≥ 4/10) pain score. There was no significance (x^2=0.073, *p*=0.787) found between the three SNP groups (GG, GA and AA) and pain scores.

4.3.4 *ARRB2*

β-Arrestin2 (ARRB2) is a component of the G-protein coupled receptor complex and regulates opioid signal transduction through promotion of receptor desensitisation and internalisation (10). The gene is also involved in the *mu*-opioid receptor and dopamine receptor D_2 receptor signalling, which are two central process in methadone signal transduction (10). The following SNPs in ARRB2, rs34230287, rs3786047, rs10452180 and rs2036657, have been associated with variability and response to methadone in MMT program (10) and to morphine (13).

Table 30 Chi-square analyses – ARRB2 (rs34230287, rs3786047, rs1045280 and rs2036657)

ARRB2	rs34230287			rs3786047			rs1045280			rs2036657		
	CC	**CT**	**TT**	**AA**	**AG**	**GG**	**CC**	**CT**	**TT**	**GG**	**GA**	**AA**
Low pain score (≤3/10)	15 (78.9)	4 (21.1)	0	0	6 (31.6)	13 (68.4)	0	5 (26.3)	14 (73.7)	14 (73.7)	5 (26.3)	0
High pain score (≥4/10)	15 (57.7)	11 (42.3)	0	2 (7.7)	**14 (53.8)**	10 (38.5)	**11 (42.3)**	5 (19.2)	10 (38.5)	10 (38.5)	**14 (53.8)**	2 (7.7)
x^2	2.232			9.079			10.840			5.986		
p	0.135			**0.011**			**0.004**			**0.05**		

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There was a significant association (x^2=9.979, *p*=0.011) between SNPs (AA, AG and GG) in the ARRB2 (rs3786047) gene and pain scores. The AG genotypes were associated with a high pain score. There more individuals with the AA genotype (2 v. 0) that experienced high pain, as similar trend was seen in the AG genotypes. A different trend was seen in the homozygous recessive group, where individuals were less likely to be non-responders to methadone.

There was also significant association (x^2=10.84, *p*=0.004) between SNPs (CC, CT and TT) in the ARRB2 (rs1045280) gene and pain scores. There were significantly more individuals (11 v. 0) with the CC genotype who experienced high pain. The TT genotypes were more likely to experience low pain. TT carriers may respond better to methadone than CC carriers.

A significant association (x^2=5.986, *p*=0.05) was found between SNPs (AA, AG and GG) in the ARRB2 (rs2036657) gene and pain scores. The AG and the GG genotypes experienced higher pain scores, compared to the AA genotypes. However, the AA genotypes were significantly more likely to respond to methadone (low pain score). No significance (*p* = 0.135) was found regarding ARRB2 rs34230287 and low or high pain scores.

4.4 Single nucleotide polymorphisms associated with methadone dose

Methadone dose was treated as both a categorical and continuous variable for further statistical analysis. The Kruskal-Wallis H test was utilised when methadone dose was treated as a continuous variable. The non-parametric Kruskal-Wallis H test was chosen over the parametric ANOVA test, as after conducting normality tests, methadone dose was found to be not normally distributed.

4.4.1 Normality test

SPSS statistical software (IBM, v25 2017) was used to conduct normality tests on the variables in the study to determine normality.

Table 31 Normality check for mean methadone dose

Mean Methadone Dose (continuous scale)	
Skewness	2.418
Kurtosis	7.266

Data is considered to be normally distributed if skewness and kurtosis is within the range of ±3. The kurtosis value for mean methadone dose is 7.266, as this number is larger than 3, the data was not normally distributed and the non-parametric test, Kruskal-Wallis H test, was chosen over the ANOVA (parametric test).

Table 32 Kruskal-Wallis H test – KCNJ6 and BDNF

SNP		n	Mean Rank	Median (min, max)	Kruskal-Wallis H	*p*
KCNJ6						
rs2070995	GG	26	23.58	10.00 (4.0 to 58.3)	0.002	0.965
	GA	20	23.40	13.75 (3.8 to 93.3)		
	AA	0				
BDNF						
rs1491850	TT	18	22.47	11.25 (4.0 to 58.3)	0.198	0.906
	CT	22	24.36	12.50 (3.8 to 93.3)		
	CC	6	23.42	12.50 (6.3 to 31.3)		
rs7934165	CC	13	26.65	15.00 (5.0 to 43.3)	3.823	0.148
	CT	28	20.61	8.15 (3.8 to 93.3)		
	TT	5	31.50	16.70 (10.0 to 58.3)		
rs10835210	CC	15	27.03	15.00 (5.0 to 93.3)	3.031	0.220
	CA	27	20.67	8.60 (3.8 to 52.8)		
	AA	4	29.38	13.35 (10.0 to 58.3)		

The Kruskal-Wallis H test was conducted to determine if there was significant difference between the genotypes and methadone dose. No significance was found between SNPs in KCNJ6 and BDNF, after conducting the Kruskal-Wallis H test when methadone dose was analysed as a continuous variable.

Table 33 Kruskal-Wallis H test – ARRB2

SNP		n	Mean Rank	Median (min, max)	Kruskal-Wallis H	*p*
ARRB2						
rs34230287	CC	31	22.63	10.00 (3.8 to 93.3)	0.403	0.525
	CT	15	25.30	15.00 (5.0 to 58.3)		
	TT	0				
rs3786047	AA	2	43.25	49.15 (40.0 to 58.3)	5.529	0.063
	AG	20	24.78	12.50 (5.0 to 93.3)		
	GG	24	20.79	10.00 (3.8 to 25)		
rs1045280	CC	11	30.36	33.30 (3.8 to 93.3)	4.510	0.105
	CT	10	18.35	7.10 (5.0 to 40)		
	TT	25	22.54	12.50 (4.0 to 31.3)		
rs2036657	GG	2	43.25	49.15 (40.0 to 58.3)	4.562	0.102
	GA	19	22.68	9.20 (3.8 to 93.3)		
	AA	25	22.54	12.50 (4.0 to 31.3)		

No significance was found between SNPs in ARRB2, after conducting the Kruskal-Wallis H test when methadone dose was analysed as a continuous variable.

Methadone dose was also analysed as a categorical variable (low or high dose) and chi-square analysis was employed to determine if SNPs in BDNF, KCNJ6 and ARRB2 influence methadone dose, with the results presented in Table 34, Table 35 and Table 36.

Table 34 Chi-square analyses: methadone dose – BDNF

BDNF	rs7934165			rs10835210			rs1491850		
	CC	CT	TT	CC	CA	AA	TT	TC	CC
Low dose (≤10 mg)	5 (21.70)	16 (69.60)	2 (8.70)	6 (26.10)	15 (65.20)	2 (8.70)	9 (39.10)	11 (37.80)	3 (13.00)
High dose (>10 mg)	8 (34.80)	12 (52.20)	3 (13.00)	9 (39.10)	12 (52.20)	2 (8.70)	9 (39.10)	11 (37.80)	3 (13.00)
x^2		1.464			0.933			0	
p		0.481			0.627			1	

Table 35 Chi-square analyses: methadone dose – KCNJ6

KCNJ6	rs2070995		
	GG	GA	AA
Low dose (≤10 mg)	14 (60.90)	9 (39.10)	0
High dose (>10 mg)	12 (52.20)	11 (47.80)	0
x^2		0.354	
p		0.552	

Table 36 Chi-square analyses: methadone dose – ARRB2

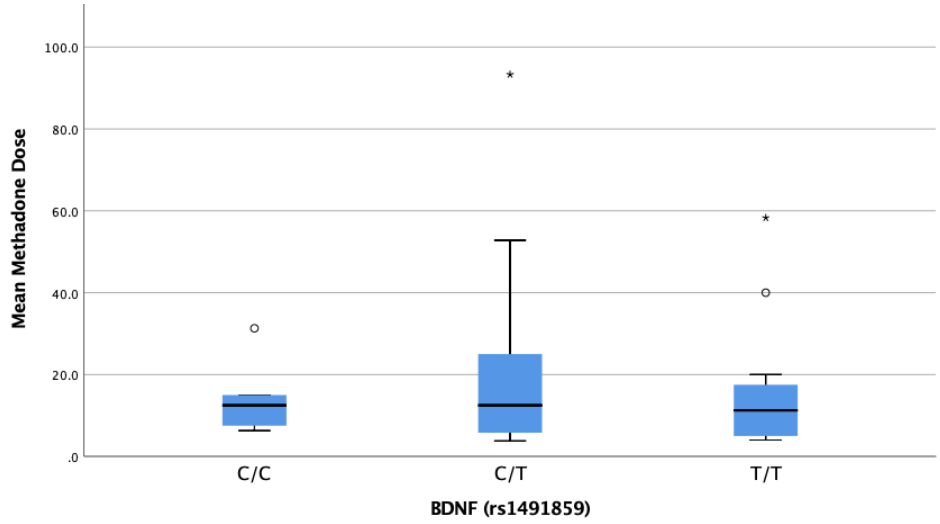
ARRB2	rs34230287			rs3786047			rs1045280			rs2036657		
	CC	CT	TT	AA	AG	GG	CC	CT	TT	GG	GA	AA
Low dose (≤10 mg)	16(69.60)	7(30.40)	0	0	10(43.50)	13(56.50)	4(17.40)	7(30.40)	12(52.20)	0	11(47.80)	12(52.20)
High dose (>10 mg)	15(65.20)	8(34.80)	0	2(8.70)	10(43.50)	11(47.80)	7(30.40)	3(13.00)	13(56.50)	2(8.70)	8 (34.80)	13(56.50)
x^2		0.099			2.167			2.458			2.514	
p		0.753			0.338			0.293			0.285	

No significance was found with SNPs in BDNF, KCNJ6 and ARRB2 and a low (≤ 10 mg) or high (> 10 mg) methadone dose, which can be seen in in Table 34, Table 35 and Table 36.

4.4.2 BDNF

4.4.2.1 rs1491850

Levran *et al*. (11) found a significant association between methadone dose in the MMT program and BDNF rs1491850. In that study, conducted with 121 Middle Eastern participants, the CC genotypes were associated with a lower dose. It was found that CC participants required a mean dose of 125 mg, which was significantly (p = 0.049) less than the other participants (11). In contrast, the CC participants in the current study required a higher mean dose of 23.42 mg compared to the TT participants, which required a mean dose of 22.47 mg. This suggests that the TT participants were better responders to methadone compared to the CC participants. However, only 21.1% of participants in the current study who experienced low pain scores carried the CC genotype for this SNP, whereas 52.6% who experienced low pain carried the TT genotype. Nonetheless, there were more CC carriers who exhibited low pain compared to high pain.



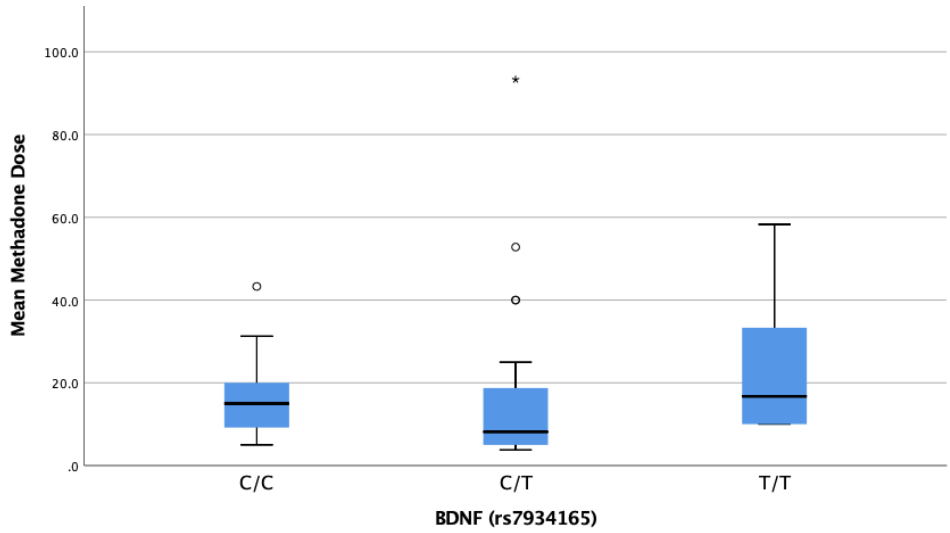
*°Outliers, * Extreme Outliers*

Figure 13 Methadone dose – BDNF (rs1491859)

Participants with the CT genotype had a higher median pain score, compared to the other genotype groups. This trend is illustrated in Figure 13, as the same group required a slightly higher methadone dose, although it was not to a significant level. Whereas, CC and TT genotypes exhibited similar methadone dosing patterns.

4.4.2.2 rs7934165

Levran *et al*. (11) also investigated the allele rs7934165 and the SNP C>T. It was found that the homozygous recessive (TT) individuals required a significantly (p = 0.036) higher mean dose of 152.7 mg, compared to the CC and TC participants (11). Again, however, the current study failed to show any significant association between methadone dose in the palliative care population and CC, CT and TT genotypes for the rs7934165 allele.



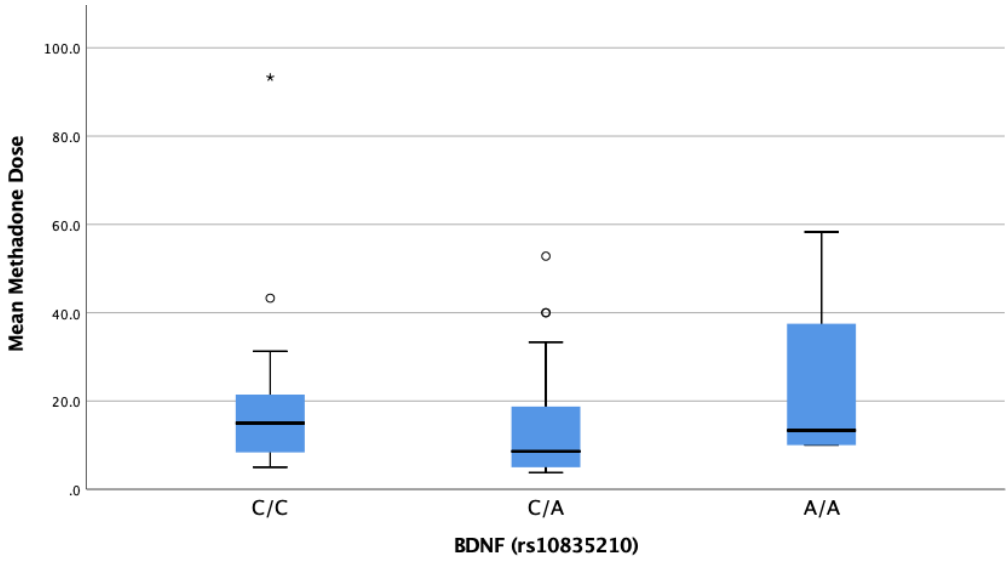
*° Outliers, * Extreme Outliers*

Figure 14 Methadone dose – BDNF (rs7934165)

All three genotypes, CC, CT and TT, had similar median pain score value, however, there was a wider spread of pain scores present for individuals that were carriers of the CT genotype. However, there was also a wider spread from the median score to quartile three in the TT genotype group. TT group exhibited a high median methadone dose, as well as highest maximum dose.

4.4.2.3 rs10835210

Levran *et al*. (11) also investigated the rs10835210 allele and found that the CC participants, significantly (p = 0.010) required a higher methadone dose in the MMT program, of 158.7 mg compared to the CA and AA individuals. In the current study, the CC subject required a lower dose of 27.03 mg compared to the AA participants that required a dose of 29.38 mg, however, again these findings were not significant, and we could not replicate the findings of Levran *et al (11)*.



*°Outliers, * Extreme Outliers*

Figure 15 Methadone dose – BDNF (rs10835210)

The CA group had the highest median pain score and a wider spread of pain scores compared to the other two groups, CC and AA. However, the CA group, as seen in Figure 15, also required a lower median methadone dose. Whereas, both the homozygous dominant and recessive groups had similar median doses, but the homozygous recessive had a wider distribution of doses and a higher minimum and maximum dose.

Table 37 is a comparative table comparing the results from this study to published literature.

Table 37 Comparison of results to published literature – BDNF

Gene	SNP/s		Literature	Current study
BDNF	rs10835210 C > A	**Levran *et al.*** [11] *Drug:* Methadone *Study population:* MMT *Sample size:* 227 Middle Eastern	The CC genotypes required a higher methadone dose of 158.7 mg.	There was no significance found for pain scores and methadone dose.
	rs1491859 T > C		The CC genotypes required a lower mean methadone dose of 125 mg.	Significant association with pain scores $p = 0.033$ where the TC genotypes had higher pain scores.
	rs7934165 C > T		Participants in the study who were carriers of the TT allele required a higher dose of 153 mg.	The TT carriers required a higher dose of 31.5 mg ($p = 0.148$).

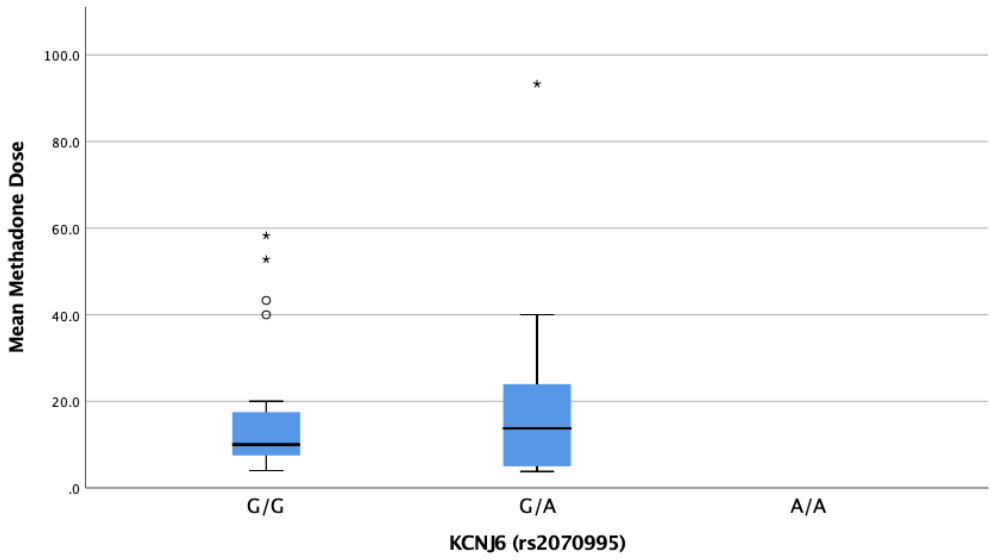
4.4.3 KCNJ6

4.4.3.1 rs2070995

This SNP in KCNJ6 was studied in 129 Japanese patients, regarding opioid dosage after abdominal surgery (12), unfortunately, the study did not include the opioid methadone. The final opioid doses were converted into a morphine equivalent daily dose (MEDD). The following were the mean MEDD for the following genotypes, AA, GA and GG, respectively, 20.45 mg, 10.84 mg and 13.07 mg (12). They found a significant difference regarding the dosing between the AA participants, compared to the GA and GG participants (12). The researchers concluded that the AA individuals required high opioid analgesics due to lower KCNJ6 gene expression, thus, leading to decreased analgesic effect (12).

Lotsch *et al*. (8) further examined the findings found in the Japanese cohort, but this time methadone was the choice of opioid used in context in the MMT program, investigated in 352 participants. It was found that the average daily methadone substitution dose in the first year of therapy was higher in the AA carriers (119.7 mg/day) compared to 77.5 mg/day for the other individuals (8). Thus, it was concluded that the association of the KCNJ6 rs2070995 AA individuals with increased opioid analgesic requirements extended from analgesia to opioids required in the MMT (8).

However, after conducting Kruskal-Wallis H test, a significant difference between the three genotypes regarding methadone dose was not found. Our results did not match the findings of Lotsch *et al*. (8) or that of the study conducted on Japanese patients (12). Our study did not unfortunately have any AA participants, therefore, not allowing for any comparisons to be made to published literature. The GG participants had a mean dose of 23.58 mg compared to 23.40 mg, for the GA carriers; however, this difference was not significant.



*°Outliers, * Extreme Outliers*

Figure 16 Methadone dose – KCNJ6 (rs2070995)

The GG genotypes had very similar pain score averages compared to the GA genotype, when it came to median pain score. However, there was a wider range of pain scores (minimum and maximum values) exhibited in the group of individuals with the GA genotype. Figure 16 illustrates that the GG genotypes had a lower median methadone dose compared to the GA genotypes. It is interesting to note that although both groups had similar pain scores, the GA genotypes required a higher dose though no statistical significance was found.

Table 38 is a comparison of the results yielded from the current study to published literature.

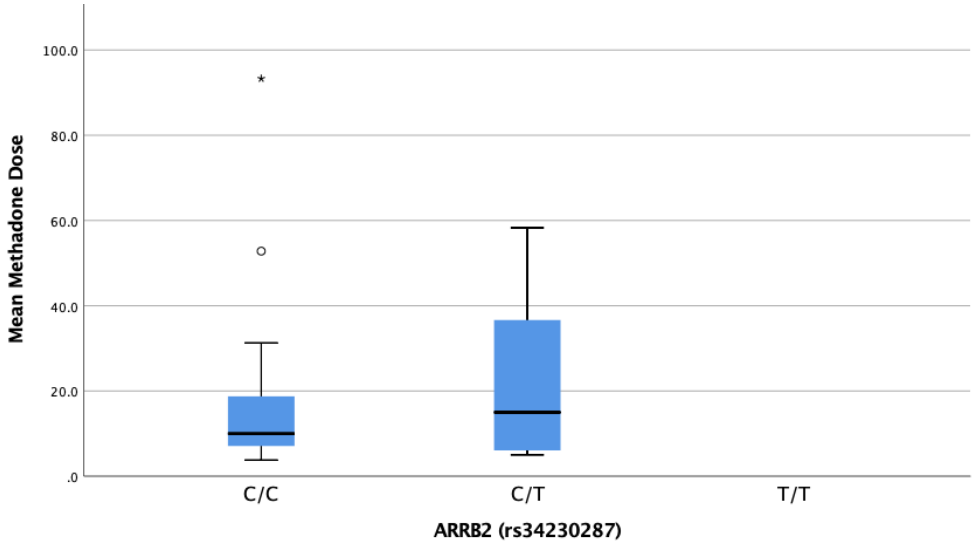
Table 38 Comparison of results to published literature – KCNJ6

Gene	SNP/s	Literature		Current study
KCNJ6	rs2070995 G > A	**Lotsch *et al.*[8]** *Drug:* Methadone *Study population:* MMT *Sample size:* 352 Caucasians	The AA genotypes were associated with high opioid requirements. They required a high dose of 119.7mg/day, compared to 77.5 mg/day (p = 0.003) compared to the other groups.	None of the participants in the current study were AA genotypes.
		Nishizawa *et al.*[12] *Drug:* Analgesics *Study population:* Post-operative analgesia after major abdominal surgery *Sample size:* 129 Japanese	The AA genotypes required higher doses of analgesics.	

4.4.4 ARRB2

4.4.4.1 rs34230287

Oneda *et al*. (10), investigated the allele rs34230287, in 278 Caucasian participants, in relation to response to methadone in MMT. It was found that rs34230287 did not affect how individuals responded to methadone in the MMT program, as there was no difference between the number of responders and non-responders between individuals (10). No significant difference in allele and genotype frequencies between MMT patients and in the control group was found in relation to opioid addiction (10). However, the results of the current study failed to imply any significance between methadone dose requirements and response between the three groups of participants, CC, CT and TT.



*°Outliers, * Extreme Outliers*

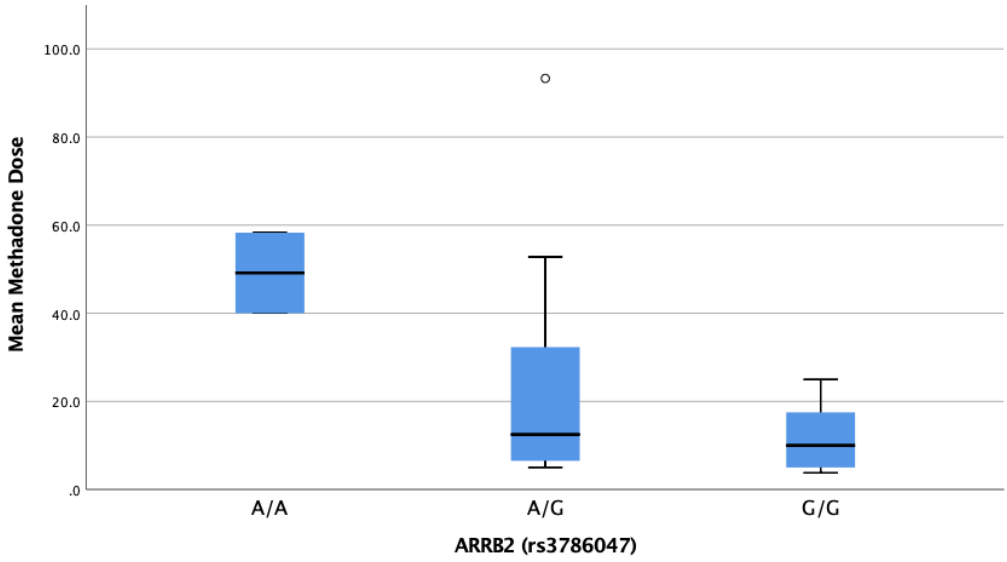
Figure 17 Methadone dose – ARRB2 (rs34230287)

The CC group exhibited a lower pain score average compared to the CT group. However, the homozygous dominant group also required a lower mean methadone dose and the heterozygous group required a higher median methadone dose, as well as exhibiting a higher dose at quartile three, with also a larger maximum dose.

4.4.4.2 rs3786047

Oneda *et al*. (10), found that patients who were homozygous for the variant allele in rs3786047, were significantly (p = 0.02), two and a half times more likely to be non-responders to methadone in the MMT program. Conversely, in the present study, there was no significant dosage difference between dose between the homozygous recessive group and

the heterozygous and homozygous dominant patients. Unlike the study conducted by Oneda *at al*. (10), the homozygous variant group (GG) in the present cohort had the lowest mean methadone dose of 20.79 mg compared to the AA genotypes, who required a mean dose of 43.25 mg. However, the difference in methadone doses between the groups did not reach statistical significance ($p = 0.063$). In relation to pain scores, significance ($p = 0.011$) was found for this particular SNP. Again, in the current study, the GG carriers were more likely to experience low pain, compared to the other two groups, again contradicting the results of Oneda *et al.* (10), that was conducted on individuals in the MMT program.



*°Outliers, * Extreme Outliers*

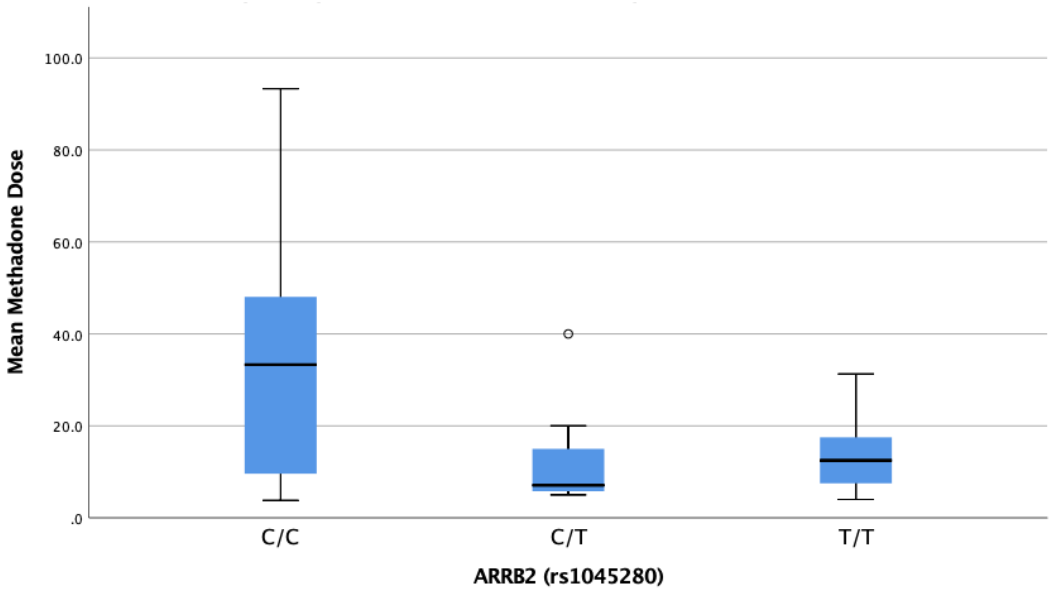
Figure 18 Methadone dose – ARRB2 (rs3786047)

The heterozygous dominant (AA) group exhibited a higher median dose and a third quartile pain score when compared to the AG and GG group. The AA group also required a higher methadone dose, which is evident in Figure 18. The AG and GG group also had very similar median scores, but the AG group had a wider distribution of doses and a higher maximum value.

4.4.4.3 rs1045280

Oneda *et al*. (10) found that CC participants with rs1045280 were significantly ($p = 0.02$), approximately three times more likely to be non-responders, which meant receiving higher doses of methadone in the MMT program. Whereas, when Ross *et al.* (13) investigated the clinical response to morphine and genetic variation in rs1045280, however, those who could

not tolerate morphine were more likely to carry the T allele (p = 0.013) (13). In the current study patients carrying the CC genotype received a higher mean methadone dose of 30.36 mg, compared to those carrying the CT and TT genotypes, who received a mean dose of 18.35 mg and 22.54 mg, respectively. Nonetheless, a significant (p = 0.004) association was found in relation to pain scores, a trend similar to that observed in relation to methadone dose. The CC group required a higher dose and were also more likely to experience higher pain compared to the other two groups.



*°Outliers, * Extreme Outliers*

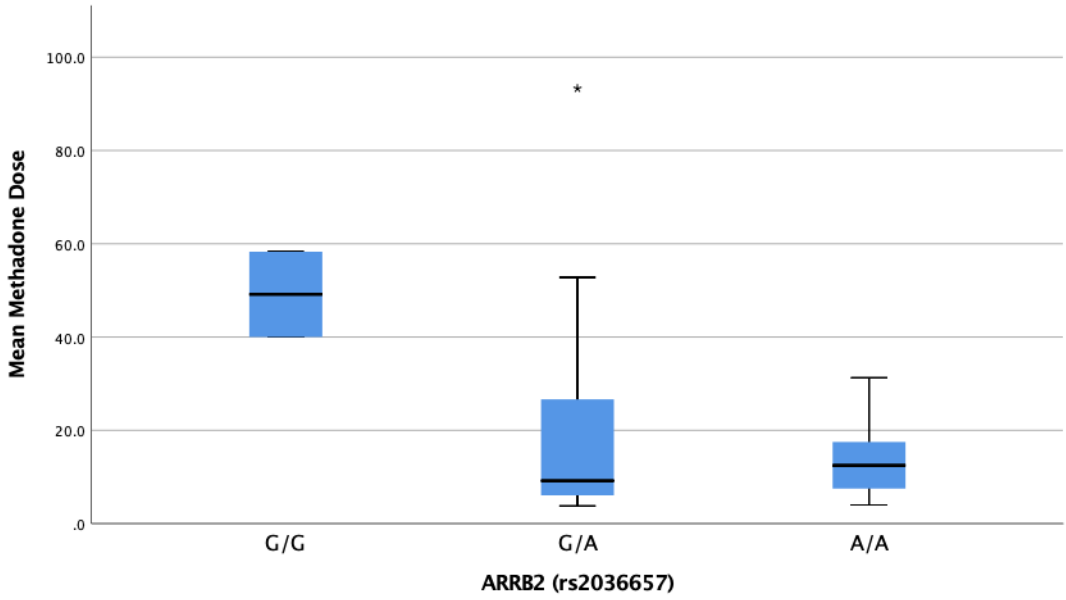
Figure 19 Methadone dose – ARRB2 (rs1045280

The CC genotypes had a higher median and third quartile pain score when compared to the CT and TT genotypes. Again, as shown in Figure 19, the CC group also required a higher methadone dose, compared to the other three groups. The TT group had a lower median pain score average but a higher median methadone dose, when compared to the heterozygous group.

4.4.4.4 rs2036657

Oneda *et al.* (10) found that in their studied cohort for rs2036657, non-responders to methadone were more likely to carry the common allele (G) and require a higher dose of the opioid to provide therapeutic effect. It was also identified that SNPs in rs2036657 were significantly (p < 0.02) associated with response to MMT treatment, contributing to 3% of MMT response variability (10). Ross *et al.* (13) investigated rs2036657 and found that those who could not tolerate morphine were more likely to carry the A allele (p = 0.043). A similar

trend was found within the cohort of the present study, where the GG carriers were more likely to experience high pain scores, suggesting reduced response to methadone, whereas, the AA carriers were more likely to experience low pain levels (p = 0.050). GG carriers required a higher mean methadone dose of 43.25 mg compared to the AA carriers, who required a dose of 22.68 mg. However, this difference did not reach statistical significance.



*°Outliers, * Extreme Outliers*

Figure 20 Methadone dose – ARRB2 (rs2036657)

The GG carriers had a higher median pain score, when compared to the other two genotype groups, GA and AA. A similar trend is apparent in Figure 20, regarding methadone dose, as the GG genotypes required a higher median methadone dose, even though it was not significant. Again, although the AA genotypes had a lower median pain score, the AA carriers exhibited a higher median methadone dose compared to the GA group.

Table 39 is a comparison of the results yielded in the current study compared to the published literature on the SNPs in ARRB2.

Table 39 Comparison of results to published literature – ARRB2

<table>
<tr><th>Gene</th><th>SNP/s</th><th></th><th>Literature</th><th>Current study</th></tr>
<tr><td rowspan="5">ARRB2</td><td>rs34230287
C > T</td><td rowspan="3">Oneda et al.[10]
Drug: Methadone
Study population: MMT
Sample size: 278 Caucasians</td><td>It was found that rs34230287 did not affect how individuals respond to methadone in the MMT program.</td><td>No significance was found regarding pain scores and dose and no participants were TT carriers.</td></tr>
<tr><td>rs3786047
A > G</td><td>The AA carriers were significantly (p = 0.02) 2.5 times more likely to be non-responders to methadone.</td><td>Significance was found regarding pain scores (p = 0.011) where the AG carriers had <u>higher pain scores</u>.</td></tr>
<tr><td>rs1045280
C > T</td><td>The CC participants were significantly (p = 0.02) three times more likely to be non-responders.</td><td rowspan="2">CC carriers had a significant (p = 0.004) association with a <u>high pain score</u>, compared to the other three groups.</td></tr>
<tr><td rowspan="3">rs2036657
G > A</td><td rowspan="3">Ross et al.[13]
Drug: Morphine
Study population: Cancer patients
Sample size: 162 Caucasians</td><td>Ross et al. (13), found that those who could not tolerate morphine were more likely to carry the T allele (p = 0.013)</td></tr>
<tr><td>Oneda et al. (10), found that the non-responders to methadone were more likely to carry the common allele (GG).</td><td rowspan="2">GA carriers had a significant (p = 0.05) association with a <u>high pain score</u>.</td></tr>
<tr><td>Ross et al. (13), found that those who could not tolerate morphine were more likely to carry the A allele (p = 0.043).</td></tr>
</table>

4.5 Minor allele frequencies

The NCBI's Single Nucleotide Polymorphism Database (dbSNP), in collaboration with the National Human Genome Research Institute (NHGRI) (122), reported MAFs from the 1000-genome population for the SNPs being analysed in the BDNF, KCNJ6 and ARRB2 gene. Table 40, Table 42 and Table 44 lists the MAFS from the NCBI database compared to the MAFs from our study.

The 1000 genome population was chosen for the comparation as it is large database that most closely resembles the population in this study. Australia is ethnically diverse and the MAFs in this study closely resembled the 1000 genome population, suggesting the results from this study have the potential to be generalised to the wider population. The 1000 genome study was an international research effort that included genome samples from around the world, including the following countries and continents: United States of America (USA), Africa, Bangladesh, United Kingdom (UK), Scotland, China, Colombia, Nigeria, Finland, China, Spain, Japan, Vietnam, Peru, Pakistan and Italy (134).

4.5.1 BDNF

Table 40 shows the MAFs gathered from the NCBI database (1000 genome population) and Table 41 shows the MAFs from the current study population.

Table 40 Minor allele frequency from dbSNP – BDNF

Allele (BDNF)	SNPs	MAF (NCBI)
rs7934165	C > T*	**0.43**
rs10835210	C > A*	**0.25**
rs1491850	T > C*	**0.37**

*Represents the SNP base associated with the MAF

Table 41 Minor allele frequencies for the study population – BDNF

SNPs	n	Frequency	Pain ≤ 3 n (%)	Pain ≥ 4-10 n (%)	*p*	Mean methadone dose	*p*
				rs7934165			
CC	13	0.28	8 (42.1)	8 (19.2)		26.65	
CT	28	0.61	9 (47.40)	9(73.1)	0.197	20.61	0.148
TT	5	0.11	2 (10.50)	2(7.7)		31.50	
				rs10835210			
CC	15	0.33	9(47.4)	6(23.1)		27.03	
CA	27	0.59	8(42.1)	19(73.1)	0.109	20.67	0.220
AA	4	0.09	2(10.5)	1(3.8)		29.38	
				rs1491859			
TT	18	0.39	10(52.6)	7(26.9)		22.47	
TC	22	0.48	5(26.3)	17(65.4)	0.033	24.36	0.906
CC	6	0.13	4(21.1)	2(7.7)		23.42	

The MAF for rs7934165 from the NCBI study was 0.43. However, in the current study, the frequency of the minor allele was 0.61. The TT allele required a higher methadone dose of 31.50 mg compared to the CC and CT allele groups. For rs10835210, the MAF from the 1000-genome project was 0.25. In the current study's population, it was 0.33. The MAF in the current study cohort was the homozygous dominant group (CC), which differed from the MAF in NCBI database, where CA was the minor allele. The CA genotype was more likely to experience high pain scores, but was associated with the lowest mean methadone dose of 10.67 mg. For rs1291850, the NCBI MAF was 0.37, whereas in the current study it was 0.39 and again, it was for the homozygous dominant group (TT), which differed from the MAF reported from the NCBI database. The TC genotypes were also more likely to be associated with a higher pain score and was associated with the highest mean methadone dose of 24.36 mg. It is apparent that the population in the current study differed from the 1000 genome project population data gathered by the NCBI.

The study by Levran *et al*. (11) was conducted with 227 Middle Eastern (Israeli) participants. The MAFs for that population were slightly different from the chosen 1000 genome population in the NCBI study (122). In Levran *et al's* (11) study, the MAFs for rs7934165, rs10835210 and rs1491850 were in a chronological order: 0.49, 0.44 and 0.37. Thus, our population yielded similar MAFs to that of Levran *et al.* (11) for rs7934165 and rs1491850,

but not that of rs0835210. Thus, the MAF for rs10835210 does not reflect that of our population in this current study.

4.5.2 KCNJ6

In the dbSNP database, the 1000 genome population's MAF for KCNJ6 rs2070995 was 0.18, (Table 42), indicating that 18% of the 1000 genome population have the minor allele for rs2070995.

Table 42 Minor allele frequency from dbSNP – KCNJ6

Allele (KCNJ6)	SNPs	MAF (NCBI)
rs2070995	G > A*	0.18

*Represents the SNP base associated with the MAF

Table 43 Minor allele frequencies for the study population – KCNJ6

SNP	N	Frequency	Pain ≤ 3 n (%)	Pain ≥ 4-10 n (%)	*p*	Mean methadone dose	*p*
				rs2070995			
GG	26	0.57	11(57.9)	14(53.8)		23.58	
GA	20	0.43	8(42.1)	12(46.2)	0.787	23.40	0.965
AA	0	0					

Table 43 lists the frequencies in the study population. In this study the frequency of the minor allele is 0.43, which in comparison to the 1000 genome study which is significantly greater than 0.18. There were no individuals in our study who were homozygous for the minor allele. In the study conducted by Lotsch *et al*. (8), the minor allele was 0.23 for healthy volunteers, 0.20 for chronic pain patients and 0.22 for methadone substituted patients, which was conducted on a Caucasian cohort undergoing MMT. Again, the MAF from our study population differed greatly to that of Lotsch *et al*. (8), where they found that the AA genotypes required a higher dose. However, in the current study there were no participants who were AA carriers. Whereas, the GG genotypes in our study required a higher mean dose compared to the heterozygous group, which required a slightly lower dose, which was not significantly different. All groups in our study were also experiencing similar pain levels.

4.5.3 ARRB2

In the dbSNP database, the MAFs in relation to the 1000 genome population for ARRB2 rs34230287, rs3786047, rs1045280 and rs2036657 were 0.08, 0.34. 0.33 and 0.33 respectively (Table 44)), indicating that 8% carry the minor allele for rs34230287, 34% for rs3786047 and 33% carry the minor allele for both rs1045280 and rs2036657.

Table 44 Minor allele frequency from dbSNP – ARRB2

Allele (ARRB2)	SNPs	MAF (NCBI)
rs34230287	C > T*	**0.08**
rs3786047	A* > G	**0.34**
rs1045280	C* > T	**0.33**
rs2036657	G* > A	**0.33**

*Represents the SNP base associated with the MAF

The minor allele frequencies for the current study population are listed below in Table 45.

Table 45 Minor allele frequencies for the study population – ARRB2

SNPs	n	Frequency	Pain ≤ 3 n (%)	Pain ≥ 4-10 n (%)	*p*	Mean methadone dose	*p*
				rs34230287			
CC	31	0.67	15(78.9)	15(57.7)		22.63	
CT	15	0.33	4(21.1)	11(42.3)	0.135	25.30	0.525
TT	0	0					
				rs3786047			
AA	2	0.04	0	2(7.7)		43.25	
AG	20	0.43	6(31.6)	14(53.8)	**0.011**	24.78	0.063
GG	24	0.52	13(68.4)	10(38.5)		20.79	
				rs1045280			
CC	11	0.24	0	11(42.3)		30.36	
CT	10	0.22	5(26.3)	5(19.2)	**0.004**	18.35	0.105
TT	25	0.54	14(73.7)	10(38.5)		22.54	
				rs2036657			
GG	2	0.04	14(73.7)	10(38.5)		43.25	
GA	19	0.41	5(26.3)	14(53.8)	**0.05**	22.68	0.102
AA	25	0.54	0	2(7.7)		22.54	

The minor allele for rs34230287 was 0.33, compared to 0.08 from the NCBI database. The CA group was also associated with a higher dose. However, no significance was found when compared to the findings from Oneda *et al.* (10), showing similar results. In reference to rs3786047, the MAF was 43% compared to 34% collected from the dbSNP database. The AG carriers had significantly higher pain scores (p = 0.011). Whereas, in the study conducted by

Oneda *et al.* (10), on the methadone maintenance treatment population, the MAF was 0.29 (Caucasian population), which is also different to that of our population. The MAF in the study conducted by Oneda *et al.* (10), for rs1045280 was 0.42, in our study it was 0.24 and 0.33 from the dbSNP database. However, the CC carriers had a significant (p = 0.004) association with high pain scores, thus more likely to be non-responders to methadone, which were similar findings to the study conducted by Oneda *et al* (10). For rs2036657, the MAF in our study was 0.41, compared to the 0.33 from dbSNP and 0.30 from the study conducted by Oneda *et al (10)*. In the current study, the GA carriers had a significant (p = 0.05) association with a high pain score, whereas Oneda *et al.* (10) found that the non-responders to methadone were more likely to carry the GG allele.

Overall, a significant association was found for rs1491850 (BDNF) and the three SNPs in ARRB2, rs3786047, rs1045280 and rs2036657 regarding low and high pain scores. Further comparison between the current study results and the literature was summarised previously in Table 37, Table 38 and Table 39.

5 DISCUSSION

5.1 Overview of study

The current study aimed to investigate the role of SNPs in ARRN2, KCNJ6 and BDNF to determine if they contribute to interindividual variability in methadone dose requirements for pain management in advanced cancer cases. There were three objectives identified in this study to determine the effects of SNPs on opioid response. The first objective was to assess whether patient characteristic variables affect methadone response and pain levels. Patient characteristic variables were analysed to test their influence on methadone dose and pain levels and to exclude any other factors, other than genetic variants, that may influence methadone dose and pain levels. The second objective was to identify which SNPs in the genes being analysed were significantly associated with high pain scores. The third objective was to determine if any of the SNPs were significantly associated with high methadone dose requirements. A total of 46 participants were included in the final study from two hospitals located in Brisbane, Queensland; MAH and SVPH. All SNPs, except for one (ARRB2 rs1045280 (C > T)), were found to be in HWE. Data was analysed using logistic regression, chi-square analysis and the Kruskal-Wallis H test.

Palliative care is a relatively new and rapidly growing field, as the global population is aging and living longer than previous generations (135). Pain in this vulnerable population has not been widely researched when compared to pain conditions not associated with end-of-life, such as chronic pain (135). Previous systematic reviews and international conferences have acknowledged and highlighted the gaps in scientific knowledge in this field and the fact that research in this area has been progressing slowly, in reference to other health fields (19, 135). This further implies that there are a variety of obstacles facing researchers who wish to undertake high quality studies with this vulnerable population (135). Some of the major obstacles that researchers face in this area are funding and ethical dilemmas that extend far beyond those involved in standard research trials (7). While ethical dilemmas are not necessarily a short coming in any field of research, the dilemmas are often magnified in the palliative care population (7, 136). Some of these ethical dilemmas include patient vulnerability, high rates of mental incapacity and emotional distress, which creates another challenge with respect to gaining informed consent. Conflicts of interest with dual roles of the

clinician–researcher, invasiveness, frequency of testing, the underlying question of scientific value and the need to balance the benefits and burdens of unproven interventions must also be considered, particularly in a population for whom comfort may be a priority (136).

However, genotyping is also becoming easier to incorporate into daily clinical practice. DoseMeRx, for example, is a software used in hospitals and other healthcare settings that utilises PK drug models, patient characteristics, drug concentrations and genetic data to guide dosing parameters for clinicians (137). Currently, DoseMeRx is based heavily on PK. However, with the ongoing growth in the field of PG, adding further genetic data into this software, or a similar product, could be another means of incorporating individual genotype into clinical practice to assist clinicians with dosing medications. The software could potentially simulate the outcomes of different dosing regimens to ensure the best possible decisions are made for every patient, which is one of the goals of DoseMeRx (137).

From another perspective, pain is highly prevalent and the most feared symptom of advanced cancer (36). Symptom management, including pain management, has been found to be sub-optimal from the point of view of patients and their families (138). Pain and other symptom management in palliative care have been less than ideal for patient comfort (138). A recent study on patients' and partners' views of care and treatment provided for metastatic castrate-resistant prostate cancer in the United Kingdom, found that pain and symptom management were not optimal and there was an increased need for specialist nurse provision and earlier palliative care referrals (138). This outcome further outlines the need for increased research in the field of palliative care, in order to develop new ways to manage symptoms and alleviate pain as effectively and safely as possible. It should become a priority for clinicians and other staff involved in the palliative care services to identify ways to optimise pain and symptom control, with the goal of improving patients' quality of life in the last years, months, weeks and days of life. The emerging field of PG offers exciting prospects in terms of dosing guidelines for opioids, especially methadone, which due to its complex PK and PD parameters, requires SPC clinicians to dose it in an appropriate setting.

The current study focused on the three genes and their associated genetic variants, BDNF (rs7934165, rs10835210 and rs1491850), KCNJ6 (rs2070995) and ARRB2 (rs34230287,

rs3786047, rs1045280 and rs2036657). The alleles and corresponding SNPs were chosen based on previous reports by Levran *et al* (11), Ross *et al* (13), Oneda *et al* (10), Nishizawa *et al.* (12), and Lotsch *et al* (8), indicating a possible link between these SNPs and methadone or opioid response. However, no research regarding these genes has been conducted in palliative care.

BDNF is involved with numerous pain relief functions in the body, especially that of opioid induced plasticity and regulation of mid-brain dopamine release (98, 99). KCNJ6 is a G-protein coupled channel that is involved in opioid receptor transmission, eliciting opioid effects, inhibition of pain (90) and mediating components of the analgesic response (91, 92). Whereas, ARRB2 is involved in the *mu*-opioid receptor and dopamine receptor D_2 signalling, which are two of the central processes in methadone signal transduction (10). The final results from this current study showed that SNPs are a predominant feature affecting pain scores, which suggests that SNPs affecting the PD parameters of methadone play a major role in dosing and the efficacy of the opioid.

5.2 Patient characteristics

A logistic regression model was conducted to determine if other covariates, such as, age, gender, cancer diagnosis, liver and kidney function had an influence on methadone dose and pain levels (scores). No significant association was found. However, after a closer inspection of the markers of liver function, significance (p = 0.010) was found for ALT and pain scores. Participants who had ALT levels that were within the range, were more likely to exhibit low pain scores ($\leq$ 3/10). There were also more patients in the current study who had ALT levels outside the range, and who experienced high pain ($\geq$ 4/10). As methadone is mostly metabolised by the liver, if the liver is not functioning at an optimal state, it is expected that the patient will not be able to metabolise methadone as efficiently as someone with normal liver function. A patient with impaired liver function, therefore, will not receive optimal analgesic effects from the drug and will be more likely to experience high pain. However, other liver function enzymes such as AST, ALP, LDH and GGT were not associated with high or low pain scores or methadone dose.

5.3 Single nucleotide polymorphisms associated with pain scores

A significant association was found between the following SNPs in BDNF and ARRB2, and pain scores: rs1491850 (p = 0.033), rs3786047 (p = 0.011), rs1045280 (p = 0.004) and rs2036657 (p = 0.05). The results of this study were compared to published literature, as shown in Table 37, Table 38 and Table 39. The study conducted by Ross *et al*. (13), investigated the clinical response to morphine in 162 Caucasian cancer patients and the association to genetic variation in candidate genes. It was found for ARRB2 rs1045280 (C > T), those who could not tolerate morphine were more likely to carry the T allele (p = 0.013) and 87% had symptom improvement when they switched to an alternative opioid (13). Patients who had the recessive allele were more likely to be non-responders to morphine and were not able to tolerate morphine due to side effects and therefore, benefit from opioid switching (13). However, the findings of the current study, which was conducted using methadone, were the opposite to the findings of Ross *et al*. (13). In the current cohort, CC carriers had a significant (p = 0.004) association with a high pain score ($\geq$ 4/10) compared to the other groups carrying at least one recessive allele, which suggests that the CC carriers were not responding to methadone efficiently, thus experiencing a high pain score. The results were more similar to the to the findings of Oneda *et al.* (10), who found that the CC carriers in a MMT population of 278 Caucasian participants were approximately three times more likely to be non-responders of methadone, which was significant (p = 0.02). Similarly, in the current study, CC carriers were also associated with high pain scores, indicating poor response to methadone.

These findings suggest that though morphine and methadone are both opioids, SNPs in the candidate genes have different clinical implications depending on the choice of opioid. An explanation for the difference in findings when comparing the findings of Levran *et al.* (11), and the current study to Ross *et al*. (13), may be due to methadone having a different mechanism of action to morphine, as it is also an NMDA antagonist (62). Due to antagonism at the NMDA receptor site, methadone has additional advantages compared to other opioids (62). Methadone is associated with decreased opioid tolerance (62) and is a valuable analgesic with fewer associated side effects when compared to other opioids (9), such as morphine. However, these advantages are not yet being used to their full potential. Clinicians shy away from using methadone in clinical practice due to the interindividual variability in dosing (62).

5.4 Single nucleotide polymorphisms associated with methadone dose requirements

While the association between the SNPs for the three genes, ARRB2, BDNF and KCNJ6 investigated in this study showed no significant influence on methadone dose, the possibility that the genetic variants in these genes do in fact influence both pain scores and methadone response cannot be ruled out.

An explanation for the lack of significant association found between genes and methadone dose may be explained by the fact that methadone reaches steady state levels in approximately five days (four to five half-lives) (139). Methadone treatment should begin at a low dose and increase gradually, monitored over days or weeks, until a stable daily dose can be reached (139). When patients were first admitted to either palliative care ward, MAH or SVPH, and were initially dosed on methadone, it would have taken approximately five days to titrate to maintenance dose, since methadone dosing should start at a low dose and titrate up slowly (114). However, due to the nature of palliative care environment, some participants unfortunately passed away before a stable dose could be established. Significant association may not have been found between the SNPs in BDNF, KCNJ6 and ARRB2 and methadone dose because the time required to reach a steady dose of methadone was longer than most study participants survived. Accordingly, significance could only be established in relation to pain scores and response.

Another explanation for this occurrence could be due to the small sample size, which is to be expected for in the field of palliative care. In addition, various other SNPs can occur along the three genes that have not been included in this study or any other study. Further comprehensive research focussing on genetic variants can be undertaken by conducting multi-gene analysis, to determine whether the SNPs in the three genes have a collective effect on methadone dose and pain scores. A larger sample size may also reveal that the genetic variants in the three genes investigated may significantly impact methadone dose, as the population in this study may have not been a true representation of the wider population.

5.5 Future potential of pharmacogenetics in palliative care

The findings from this study will hopefully influence further PD based genetic studies, with a future aim of supporting dose individualisation. Integrating PG into clinical practice is an ideal

route to implement the findings of this study and future studies aiming to promote dose individualisation. Moreover, with the introduction of the 'My Health Record' (MHR), earlier this year (2019) (140), and hospitals nationwide upgrading to electronic patient charts, such as EMR, including genetic data could into patient charts will be easier than it has ever been. At present the MHR include the following information: allergies, current medications, medication condition and pathology test results, such as blood tests (140). In the future, including genetic data could help clinicians to predict how a patient will respond to an analgesic. It should also be noted that many people are already genotyping on their own accord, from DNA ancestry tests to medical tests for determining if an individual carries the gene for breast cancer, for example. In light of this, it is likely that the community will welcome new PG-based advancements in medication dosing. Using PG to streamline dosage for a variety of complex medications, not just methadone, will help clinicians manage patient pain levels in a safer and much more efficient manner.

6 CONCLUSION AND FUTURE DIRECTIONS

6.1 Conclusion

Cancer is an ever-increasing burden on the health care system and the leading cause of death in Australia (141). Pain is highly prevalent in the palliative care population and administering analgesics, especially opioids, is necessary to relieve moderate to severe pain, when curative treatment is no longer an option. Methadone in particular is difficult to dose due high interindividual variability in response, which inhibits its use as an analgesic, especially in acute situations.

This study focused on the new emerging field of PG as a means of using a patient's genetics to dose methadone, an opioid with complex PK and PD parameters, which only specialist palliative clinicians can prescribe and administer. Specifically, the SNPs in BDNF, ARRB2 and KCNJ6 genes were investigates, which previous studies had identified as being associated with opioid dosing requirements and pain scores. The genes chosen for this study were either involved in opioid effect, through receptor desensitisation and internalisation, opioid receptor transmission, or had a role in the reward-related processes of the human brain. This study was conducted with a population of 46 participants and found significant associations in relation to high pain scores in BDNF rs1491850 (p = 0.033) and ARRB2 rs3786047 (p = 0.011), rs1045280 (p = 0.004) and rs2036657 (p = 0.05). However, no significance was found for methadone dose, possibly because the time required to reach a stable dose was longer than the average participant survival rate.

In conclusion, this was the first study to investigate the association between candidate SNPs in the three genes, KCNJ6, BDNF and ARRB2, and methadone response in palliative care. The aim of this study was to provide insight into the interindividual variability observed with methadone response, as an analgesic used in palliative care, using PG to explain it. To improve quality of life and relieve the suffering of patients at the end of their lives, it is vital to find ways to optimise the use of methadone. PG is an avenue that may allow clinicians to do so soon. The SNPs investigated in this study could be incorporated into a multimodal treatment algorithm for cancer pain, to provide insight into the interindividual variability of dosing methadone and alleviate suffering in the last months, weeks and days of life.

6.2 Future directions

In this study, SNPs in two genes related to PD factors, ARRB2 and BDNF, were found to be significantly associated with methadone response, suggesting that PG can play a major role in the interindividual variability observed in methadone response. Therefore, it is crucial to conduct further research to establish the association between SNPs in genes related to the PD of methadone response. Suggestions for future research and implications for practice are listed below.

6.2.1 Implications for future research

Future studies that focus on dosing methadone in a more predictive manner and improve palliative care dosing guidelines should consider the following suggestions for continuing this research:

- Increased sample size will provide a greater data yield, which will reduce the significance value of $p < 0.05$ to be reduced to $p < 0.01$. The small sample size in the current study limited generalisation of the results. These results, however, can be included in a larger meta-analysis, due to the rarity of DNA specimens from palliative care and the lack of prior studies conducted in this population receiving opioids, specifically that of methadone. A larger sample size will also allow for a better understanding of any variability due to genetic polymorphisms in palliative care patients receiving methadone.
- A suggestion for recruiting a larger sample size is to conduct large multi-centre studies. This study was a multi-centre study, however, it only included two hospitals in Brisbane, Queensland. Thus, a future recommendation would be to conduct a study that included more than two palliative care wards, such as a national study that included palliative care centres from other States, or international studies that include palliative care centres from other countries, which also allows for results to be extrapolated to the general population.
- Future studies that are able to recruit a larger sample size should analyse data according to ethnicity, as MAFs can differ between ethnicities and affect the data.

- Multi-gene analysis should be conducted because genes are known to affect one another. A possible gene-to-gene interaction with SNPs may have a more significant effect on methadone dose compared to one gene alone.

6.2.2 Implications for practice

It is hoped that this study will contribute to future dosing guidelines for methadone and support clinicians in providing care at end-of-life, while also enhancing the quality use of medicines. The findings of this study could be disseminated to professional palliative care support networks and dose individualisation software companies, such as DoseMeRx, to implement into clinical practice. The following suggestions for future directions for practice are below:

- Educational programs, such as continuing professional development (CPD) activities, should be developed and implemented to increase awareness of PG effects on dosing.
- Future projects should focus on practical, efficient ways to implement PG and genetics into clinical practice, such as using 'My Health Records' or other electronic systems.

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8 APPENDICES

8.1 Appendix 1 – Patient characteristics

Patient Characteristics
Gender
Age
Height (cm)
Weight (kg)
BSA (body surface area)
BMI (kg/m^2)
Performance status
Diagnosis (location of metastasis)
Liver Function Tests: *AST (aspartate transaminase)* *ALT (alanine transaminase)* *ALP (alkaline phosphate)* *GGT (gamma-glutamyl transferase)* LDH (*lactate dehydrogenase*)
Kidney Function Tests: *Creatinine* *Urea* *Alb (albumin)* *Creatinine Clearance* *EGFR (estimated glomerular filtration rate)*
WBC (white blood cell count)
Platelets (PLTS)
Haemoglobin (Hb)

8.2 Appendix 2 – Patient consent form

Patient Consent Form

Protocol Title:	Individualised Methadone Dosing for Cancer-Related Pain
Lay Title:	Methadone Study
Principal Investigator:	Dr Phillip Good Professor Janet Hardy
Address:	Mater Health Services Raymond Terrace South Brisbane, QLD, 4101 Australia
Telephone Number:	07 3163 8111
Research Nurse:	Angela Tapuni Phone: 07 3163 3884 Mobile: 0466 206 213

I have:

- Read and understood the information sheet;
- Had any questions or queries answered to my satisfaction;
- Been informed of the possible risks or side effects of the tests being conducted;
- Understood that the project is for the purpose of research and not for treatment;
- Been informed that the confidentiality of the information will be maintained and safeguarded;
- Given permission for access to my medical records, for the purpose of this research;
- Given permission for medical practitioners, other health professionals, hospitals or laboratories outside this hospital, to release information concerning my disease and treatment which is needed for this trial and understand that such information will remain confidential;
- Been assured that I am free to withdraw at any time without comment or penalty; and
- Agreed to participate in the project.

I agree to provide DNA sampling as part of the Methadone study: Yes □ No □

Signatures:

Participant _____ Signature _____ Date

_____ Print name

Investigator _____ Signature _____ Date

_____ Print name

8.3 Appendix 3 – Case report forms (CRF)

Case Report Forms

Individualised Methadone Dosing for Cancer-Related Pain

INVESTIGATOR NAME:

_____

CO-INVESTIGATORS:

_____

_____

RESEARCH NURSE:

_____

PARTICIPANT NUMBER:

_____

Date commenced:

_____

SCREENING

Participant Number: _____

Visit Date: _____

Inclusion Criteria

Inclusion criteria fulfilled?	**Yes**	**No***
1. Male or Female, at least 18 years or older	◯	◯
2. A diagnosis of malignant disease	◯	◯
3. Receiving, about to receive or about to discontinue Methadone	◯	◯
4. Understand the patient information sheet and provide written consent	◯	◯
5. Willing to provide up to 6 blood samples	◯	◯
6. Willing to provide up to 6 saliva samples	◯	◯
7. Willing to describe the experience of saliva sampling	◯	◯

* No leads to an exclusion of the participant in the study.

Exclusion Criteria

Exclusion criteria fulfilled?	**Yes***	**No**
1. Oral mucositis, infection and/or xerostomia that is painful or not possible to collect a saliva sample	◯	◯
2. Patients using Methadone for breakthrough medication/subcut infusion	◯	◯

* Yes leads to an exclusion of the participant in the study.

TRIAL PERIOD

Participant Number: _____

Visit Date: _____

Demographic details

Date of Birth: __/__/__ Age: ___ yrs

Gender: M or F

Height: _____ BSA _____ Weight: _____

Performance Status: _____

Diagnosis: _____ Sites of Mets: ___

Current Methadone dose: _____

Commencement of current dose: Start date: _____ Stop date: _____

Date of initial Methadone commencement: _____

Type and dose of breakthrough medication: _____

TRIAL PERIOD

Participant Number: _____

Commencement Date: _____

Methadone medication during trial

Date/time of dose change	Date/time sample taken	Time between dose and sample	Dose of methadone at time of sampling	Blood collection method	Pain score 0 – 10 as per pain scale Now	Pain score 0 – 10 as per pain scale Average	Sample Number and type (Se and/or Sa

Number of samples taken from participant: _____

- Se = blood sample Sa = saliva sample

Blood Results

Biochemistry and haematology screen	Date:	Date:	Date:	Date:	Date:	Date:
AST						
ALT						
ALP						
GGT						
LDH						
Ca Ca corr						
Creat						
Urea						
Alb						
eGFR						
Hb						
WBC						
Plts						
Creat Cl						

Brief Pain Inventory (BPI)

1. Please rate your pain by circling the one number that best describes your pain at its WORST in the last 24 hours?

No Pain at all	0	1	2	3	4	5	6	7	8	9	10	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?

No Pain at all	0	1	2	3	4	5	6	7	8	9	10	Pain as bad as you can imagine

3. Please rate your pain by circling the one number that best describes your pain at its AVERAGE?

No Pain at all	0	1	2	3	4	5	6	7	8	9	10	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?

No Pain at all	0	1	2	3	4	5	6	7	8	9	10	Pain as bad as you can imagine