

Tsholofelo Masiangwako

1473742

**Master of Science in Medicine (MSc Med,
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**“Genetic influences on methylation of the mu-opioid receptor
gene in black South African nyaope users.”**

A dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand,
in fulfilment of the requirements for the degree of Master of Science.

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Table of content

Declaration	i
Abstract	ii
List of figures	iii
List of Tables	iv
Acknowledgements	v
List of Abbreviations and symbols	vi
Chapter 1: Introduction and Literature review	1
1.The opioid problem	2
2. The impact of opioids on the brain	3
2.1 How opioids produce pleasure	3
2.2 Reward system activation and development of addiction	4
3. Molecular effects of heroin on the MOR	5
3.1 The structure of MOR	5
3.2 MOR signal transduction	6
3.3 Desensitisation, recycling and degradation of MOR	6
4. Variation in opioid responses	8
5. Epigenetic mechanisms: DNA Methylation	9
5.1 The impact of DNA methylation on gene transcription	9
5.2 DNA methylation patterns	10
5.3 Demethylation	12
5.1 Intermediate/partial methylation	12
5.1 DNA Methylation and addiction	13
6. Genetic influences: Single Nucleotide Polymorphisms	13
6.1 SNP rs1799971	14
6.2 SNP Haplotypes	16
7. Genetic-Epigenetic interactions	17
Chapter 2: Methods	20
1. Ethical Approval	20
2. Sample collection	20
3. Inclusion criterion	20
4. DNA extraction	20
5. Methylation-specific primer design	21
6. Bisulfite conversion and Polymerase Chain Reaction (PCR)	22
7. Sequencing and analysis	24
8. SNP Genotyping Assay	25
9. Data analysis	26

Chapter 3: Results	27
3.1 Methylation analysis	27
3.1.1 Total methylation levels	27
3.1.2 Methylation at individual CpG sites using the methylation assignment bias approach	28
3.1.3 Methylation at individual CpG sites using the partial methylation bias approach	30
3.2 SNP Frequencies	32
3.2.1 Genotype frequency of SNP rs1799971 and SNP rs3778150	32
3.2.2 Allele frequency distribution of SNP rs1799971 and SNP rs3778150	32
3.2.3 Allele frequency distribution of SNP rs3778150	34
3.3 SNP rs1799971 and SNP rs3778150 haplotype analysis	34
3.3 SNP rs1799971 and methylation levels	35
Chapter 4: Discussion	37
1. Methylation status of OPRM1 gene in nyaope users and healthy controls.	37
2. SNP rs1799971 and SNP rs3778150	40
2.1 Frequency	40
2.2 SNPs and nyaope association	40
2.3 SNP rs3778150 and rs1799971 genotypes correlation	41
2.4 Correlation of SNP genotype with the methylation status	41
Limitations	41
Conclusions	42
References	43

Declaration

I, Tsholofelo Masiangwako, declare that this dissertation is my own, unaided work. It is being submitted for the degree of Master of Science at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other university.

A handwritten signature in black ink, reading 'tmasiangwako' in a cursive script.

(Signature of candidate)

31 day of March 2022 in Midrand

Abstract

Background: Nyaope is a highly addictive heroin derivative that elicits its effects through interaction with the μ -opioid receptor. Genetic and epigenetic mechanisms, such as mutations and (cytosine-phospho-guanosine) CpG methylation, alter the function of the mu-opioid receptor gene (*OPRM1*) and protein. The single nucleotide polymorphisms (SNP) rs1799971 (118 A>G) and intronic rs3778150 (T>C) in the *OPRM1* have previously been associated with heroin use. The SNP 118 A>G can influence methylation levels of *OPRM1* and alter mRNA and protein expression. This study compared the degree of *OPRM1* methylation and frequency of SNPs 118 A>G and SNP rs3778150 in peripheral blood samples of black South African male nyaope users (n=200) to age-and-gender-matched screened controls (n=55) in a South African population.

Method: DNA was extracted from whole blood samples, bisulfite converted, amplified by methylation-specific PCR and sequenced to determine individual and total methylation levels across a total of 29 CpGs within the *OPRM1* promoter region (chr6: 154331689-154332224bp). The sequencing data was analysed by each of the two methods, methylation assignment bias and partial methylation bias methods.

Results: We found that the total methylation levels were lower in nyaope-users than in controls when using the methylation assignment bias method. Only CpG 29 sites showed substantial methylation in the control group in both approaches. In the partial methylation bias method, our main findings showed no differences in the total methylation levels between the two groups while most CpGs displayed significantly higher levels of partial methylation in nyaope-users while controls exhibited significantly higher unmethylation sites at some CpGs. The AG genotype frequency for SNP 118 A>G, was higher in controls than in nyaope-users while other genotypes (AA & GG) for SNP rs1799971 and (TT, CT, CC) SNP rs3778150 were broadly similar in both groups. While the TT genotype SNP rs3778150 occurred more frequently in the control group, there was no haplotype relationship between SNP rs1799971 and rs3778150. Neither was there any correlation observed between nyaope consumption and the presence of SNP rs1799971 and/or rs3778150 in the study populations.

Conclusion: This study provided evidence that methylation may not be associated with nyaope addiction and that SNPs rs1799971 and rs3778150 did not alter the methylation status of the *OPRM1* promoter or contribute to nyaope addiction in a black South African male population. The findings of this study are a novel contribution to the body of literature as, to the best of our knowledge, this is the first study to investigate *OPRM1* gene methylation patterns and the frequency of these specific SNPs in a South African population of nyaope users.

List of figures

Figure 1: Schematic representation of the μ opioid receptor protein.....	5
Figure 2:The effects of heroin on opioid receptor (MOR) signalling.....	8
Figure 3: DNA methylation pathways.	11
Figure 4: The gene sequence of 370bp present on human chromosome 19, 27722994- 27723372 in the 5' region of OPRM1..	22
Figure 5: Agarose gel electrophoresis for the determination of optimal annealing temperatures designed primers for OPRM1 gene in peripheral blood samples of heroin users' using gradient PCR.	23
Figure 6: A gene sequence from sample data.	27
Figure 7:Percentage methylation at 29 CpG sites within the OPRM1 promoter region in nyaope-dependent participants (A) and control participants (B) using the methylation assignment bias approach..	29
Figure 8: Percentage methylation at 29 CpG sites within the OPRM1 promoter region in nyaope-dependent participants (A) and control participants (B) using the partial methylation bias approach.	31
Figure 9:Allelic frequency distribution of SNP 118 A>G polymorphism in nyaope users and control participants.....	33
Figure 10: Allelic frequency distribution of SNP rs3778150 polymorphism in nyaope users and control participants.....	34
Figure 11: 95% confidence intervals for Mantel-Haenszel odds ratios for SNP rs1799971 and SNP rs3778150.	35
Figure 12: 95% confidence intervals for Mantel-Haenszel odds ratios for SNP rs1799971 and DNA methylation.....	36

List of Tables

Table 1: Summary of SNP 118A>G association with opioid addiction.	26
Table 2: Representation of analysis methods for DNA methylation.	32
Table 3: Genotype frequency distribution of SNP rs1799971 and SNP rs3778150 polymorphism in nyaope users and control participants..	39

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List of Abbreviations and symbols

μ	Mu opioid receptor
5mC	5-methylcytosine
BER	Base excision repair
Ca^{2+}	Calcium
cAMP	Cyclic adenosine monophosphate
CpG	Cytosine-phospho-guanosine
CREB	cAMP response element binding protein
DAG	Diacylglycerol
δ	Delta opioid receptor
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferases
GABA	Gamma aminobutyric acid
GPCR	G-protein-coupled receptor
GRK	G-protein receptor kinases
GTP	Guanosine-5'-triphosphate
HDAC	histone deacetylases
κ	kappa opioid receptor
K^{+}	Potassium
MAPK	Mitogen-activated protein kinases
MECP2	Methyl-CpG-binding protein 2
MOR	Mu-opioid receptor protein
mRNA	Messenger RNA
NAc	Nucleus accumbens
NaCl_2	Sodium chloride
<i>OPRM1</i>	Mu-opioid receptor gene
PFC	Prefrontal cortex
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulfate
SNP	Single nucleotide polymorphism
tRNA	Transfer RNA
μl	Microliters
VTA	Ventral tegmental area

Chapter 1: Introduction and Literature review

The endogenous opioid system modulates biological responses to pain and stress and mediates the reward pathway and addictive behaviour. Endogenous opioids such as endorphins, enkephalins and dynorphins are produced by neurons and have analgesic properties when they bind to opioid receptors in the brain (Zagon & McLaughlin, 2017; Holden *et al.*, 2005). Opioid analgesics are clinically prescribed to relieve pain through their interaction with the mu (μ also known as MOR), delta (δ) and kappa (κ) opioid receptors, which mimics the analgesic effects of endogenous opioid peptides (Zagon & McLaughlin, 2017). They are also used non-medically to activate the reward pathways (Zagon & McLaughlin, 2017).

There is a global epidemic of the non-medical use of opioids. In most first-world countries, the epidemic emerges from over-prescription of pain relief analgesics and the increased availability of low-cost synthetic opioids (Maclean *et al.*, 2020; Suzuki & El-Haddad, 2017). In the absence of significant pain, exogenous opioids activate the brain reward systems and promote repeated intake of the drug for pleasure (Kosten & George, 2002).

Chronic use of opioids results in a requirement for increases in the dosage to get the same pain relief and euphoric feelings that could previously be achieved by a lower dosage. This phenomenon is known as tolerance. Tolerance can be caused by repeated opioid exposure and its development is dependent on the frequency of exposure and the efficacy of opioid binding to the MOR as tolerance has been shown to be directly linked to the desensitization of opioid receptors (Allouche *et al.*, 2014). Pharmacokinetics, such as metabolic process up-regulation by drugs that stimulate their own elimination from the body, and pharmacodynamics, alteration of neural systems by drugs, can also result in tolerance (Hassan *et al.*, 2013). It is important to note that tolerance and dependence are not synonymous with addiction but are both progressive stages towards addiction, described as a mental disease that promotes intense drug cravings and compulsive drug intake despite the negative consequences (Barker *et al.*, 2015, Volkow *et al.*, 2010).

Managing patients who suffer from substance abuse continues to be a major challenge. Long-term frequent drug users usually experience withdrawal symptoms when they stop using the drug. These withdrawal symptoms can range from mild to life-threatening physical and mental symptoms and they are pronounced when users have developed dependence on the drug. As a result of the unpleasant withdrawal symptoms, sustained abstinence from drug intake is extremely difficult to achieve. Consequently, individuals who develop dependence to a

substance need to be weaned off it gradually to minimise the impact of the negative withdrawal symptoms. An alternative and more successful approach has been the application of opioid replacement therapy. As such the administration of long acting MOR agonists e.g. methadone MOR antagonists that block the euphoria induced by opioids have been very helpful in the management of the substance abuser.

1. The opioid problem

Patients who develop tolerance to- and dependence on- medicinal opioids often transition to cheaper, stronger and less regulated street or illicit opioids such as heroin (Banerjee *et al.*, 2016; Zacny *et al.*, 2003). Heroin, also known as diacetylmorphine, is the diacetylated form of morphine that was initially marketed in 1898 as an opiate to treat pain, asthma and coughs with no addictive properties. However, since then it has become the most used addictive recreational opioid (Darcq & Kieffer, 2018). In the brain, heroin is first converted to morphine. Interestingly, morphine converted from heroin is two-to-three-fold more potent in affecting the central nervous system than a similar dose of morphine administered intravenously. This is because heroin can cross the blood-brain barrier faster than morphine and therefore reaches the brain quicker.

In Sub-Saharan Africa, the opioid abuse epidemic has emerged primarily from drug experimentation, predominantly during early adolescence (Abuse, 2016; Acuda *et al.*, 2011). Studies have suggested that there are numerous environmental factors that drive individuals to use illicit/recreational drugs. These psychological factors include residing in areas that are hotspots for drug smuggling, the lack of recreational facilities, high rates of poverty, unemployment and underlying mental abnormalities such as depression (Khine & Mokwena, 2016; Fernandes, 2016).

In the northern provinces of South Africa, heroin is largely distributed as a street drug known as nyaope. Nyaope is a relatively affordable but high-quality heroin cocktail containing other ingredients such as antiretrovirals, cleaning detergents and rat poison (Mthembi *et al.*, 2018; Khine *et al.*, 2015). Nyaope is most commonly smoked together with cannabis (Morgan *et al.*, 2019). Historically, heroin availability, accessibility and distribution were restricted to white middle- and upper-class communities in South Africa. However, nyaope is predominantly used in black South African communities particularly in townships and rural areas because of its affordability (Khine & Mokwena, 2015; Harker *et al.*, 2020). Addiction to heroin as a result of increased access and availability of nyaope in disadvantaged communities, combined with an

underfunded and underserved healthcare system (Morgan *et al.*, 2019b), creates a high economic cost in crime, social care, and a massive burden on the health system (Fernandes, 2016; Khine and Mokwena, 2015).

2. The impact of opioids on the brain

2.1 *How opioids produce pleasure*

Drugs of abuse such as heroin hijack the brain communication channels and activate neurons by mimicking the chemical structure of natural neurotransmitters. Drug abuse is a repetitive stimulus that provides positive reinforcement, and these stimuli program the responses of the brain reward system which is central to experiencing pleasure (Volkow *et al.*, 2010). Fundamental to the reward system is the mesolimbic dopaminergic pathway between the ventral tegmental area (VTA) in the midbrain and limbic forebrain structures such as the nucleus accumbens (NAc) and prefrontal cortex (PFC). Drug addiction has been described as a disorder of learning and memory, implicating other brain structures involved in motivational behaviour (periaqueductal grey matter and thalamus), learning and memory (hippocampus) and emotion (amygdala), where opioid receptors are expressed highly (Le Merrer *et al.*, 2009, Volkow *et al.*, 2010). Heroin predominantly interacts with the MOR.

Opioids induce pleasurable effects by initially enhancing dopaminergic neurotransmission (van Steenbergen *et al.*, 2019). This occurs at two levels i.e., at the level of the NAc and at the level of the VTA. Heroin stimulates the release of dopamine, from the VTA in large quantities (Vargas-Perez *et al.*, 2014). Dopamine produced in the VTA, is released into synapses at the level of the NAc and PFC to process the reward-related stimuli. Under normal conditions presynaptic dopamine reuptake occurs rapidly after its functional release. However, in the presence of heroin, dopamine accumulation in the synapses is promoted by inhibiting dopamine reuptake, thereby prolonging its postsynaptic stimulatory effects (Powledge, 1999).

Dopaminergic neurons in the VTA receives inhibitory signals that produce gamma aminobutyric acid (GABAergic) interneurons, which reduce dopamine release. However, heroin can bind to opiate receptors on the GABA receptors located on these interneurons, blocking the release of GABA. This suppresses the inhibitory effects of GABA on dopamine neurotransmission with subsequent prolonging of the effects of dopamine in the reward circuitry (Johnson & North, 1992). Repetitive heroin consumption therefore induces a state of chronic dopamine release that produces a positive reinforcement behavioural pattern that triggers the desire to consume even more heroin (Volkow & Morales, 2015).

2.2 Reward system activation and development of addiction

Drugs that constantly stimulate dopamine release concurrently induce associative learning and memory (Volkow & Morales, 2015). Behavioural experiments show that rats develop a strong association between the chamber environment of a two-compartment place preference apparatus and the receipt and/or experience of drug intake (Ndlazi *et al.*, 2018; Subiah *et al.*, 2012). The environmental stimuli (such as places and people) associated with the drug become conditioned to such an extent that they can trigger the release of dopamine from the VTA without the presence of the actual drug. This increases the extracellular concentration of dopamine in the striatum, which houses the NAc, that drives the experience of anticipated reward and eventually manifests as compromised control of drug intake and compulsive drug seeking behaviour.

Environment-stimulated dopamine release is therefore linked to learning and memory processes in the brain and these processes play a critical role in the reinforcing effects of drugs. The rapid feeling of pleasure stimulated by opioid drugs is stored as memories by the hippocampus and the responsiveness to this repetitive stimulus is created by the amygdala while the salience attribution is created by the prefrontal cortex (Barker *et al.*, 2015; Volkow *et al.*, 2010). These conditioned associations (learning and memory mechanisms) determine how often dopamine is released, the intensity, abruptness and efficiency of the release, all factors that are foundational for the development of substance dependence (Volkow *et al.*, 2010; Drevets *et al.*, 2001).

Another important characteristic of addiction is the strong motivation to continue to consume rewarding stimuli, despite the serious harmful consequences. For natural reward stimuli, dopamine cells usually stop firing when such stimuli are consumed. However, with drugs of abuse, dopamine release continues to increase as they are consumed, resulting in the desensitization of dopaminergic receptors. Consequently, an individual experiences decreased sensitivity to natural stimuli during periods of withdrawal, thereby encouraging continued use of drugs to restore the dopamine deficit. This reinforcing process adjusts the compass of pleasure, while simultaneously compromises self-control. Furthermore, it promotes compulsive drug consumption and entraps the substance user in the cycle of addiction (Volkow *et al.*, 2002).

3. Molecular effects of heroin on the MOR

3.1 The structure of MOR

The MOR is a G protein-coupled receptor (GPCR) encoded by the *OPRM1*. The receptor has the typical GPCR structure consisting of an extracellular amino N-terminal domain with glycosylation sites, seven transmembrane helices with three extracellular and three intracellular loops and an intracellular carboxyl-terminus (C-terminal) (Manglik *et al.*, 2012) (Figure 1).

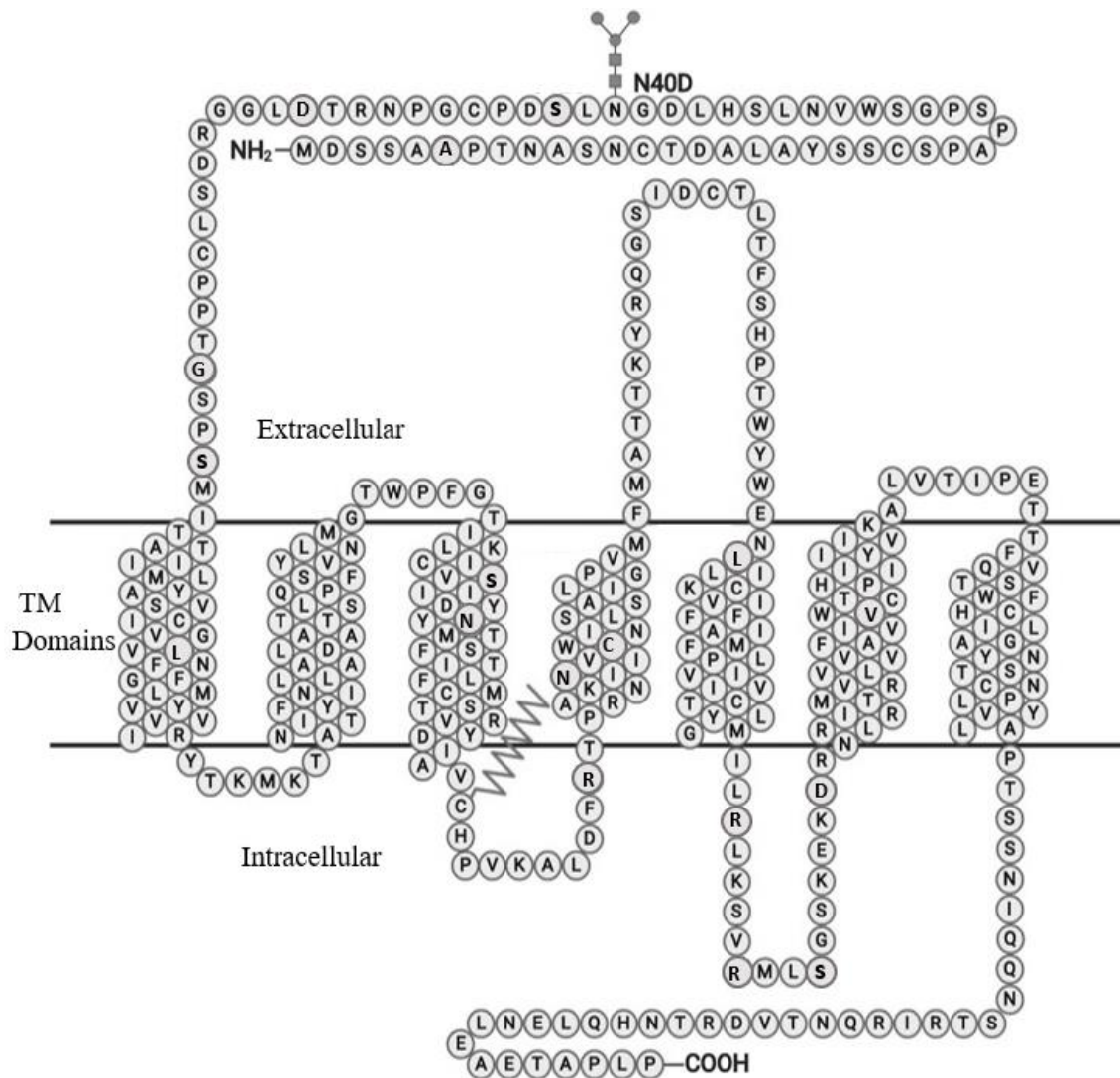


Figure 1: Schematic representation of the μ opioid receptor protein. Structure showing the extracellular N-terminal domain, seven transmembrane helices, three extracellular and three intracellular N-terminus loops and an intracellular carboxyl C-terminus. The SNP 118A>G which causes an amino acid substitution at position 40, replaces asparagine with aspartate (N40D) is also shown.

3.2 MOR signal transduction

It has been shown that opioid drugs bind to a pocket on the extracellular surface of MOR and trigger an intracellular cascade of chemical reactions (Manglik *et al.*, 2012). The binding of an agonist causes receptor conformational change that stimulates the dissociation of guanosine diphosphate (GDP) from the coupled G-protein. Guanosine-5'-triphosphate (GTP) readily replaces the GDP while the G-protein is separated into the α , β and γ subunits. GTP binds to the α subunit, forming an α -GTP complex that stimulates the mitogen-activated protein kinases (MAPK) pathways (Blume *et al.*, 1979). The dimer $\beta\gamma$ -subunits then dissociate from the α -GTP complex for initiation of intracellular signal transduction. This binding interaction leads to molecular alterations within the cell that inhibit activation of the G protein and stimulate internalization of the receptor protein (Williams *et al.*, 2013).

Calcium (Ca^{2+}) release from the endoplasmic reticulum activates the calcium signalling pathway. However, the G-protein subunits inhibit the opening of voltage gated calcium channels (VGCC), preventing its entry into the intracellular region thereby decreasing intracellular Ca^{2+} concentration (Moises *et al.*, 1994). The subunits simultaneously activate potassium channels. The inward rectification of potassium (K^+) channels activates K^+ to hyperpolarize the cell. The changes in Ca^{2+} and K^+ concentrations cause hyperpolarisation of the cellular membrane, reducing the membrane potential, excitability of the cell and release of several neurotransmitters such as glutamate, adrenaline, serotonin and substance P (Law *et al.*, 2000; North *et al.*, 1987).

Agonist binding to MOR activates the G_o/G_i family of G proteins, which is mediated by the intracellular loops II and III, and the C-terminal of the MOR. The G_o/G_i protein inhibits adenylate cyclase activity upon signal transduction; thereby suppressing the synthesis of cyclic adenosine monophosphate (cAMP) and activity of protein kinase A (Blume *et al.*, 1979; Zhang *et al.*, 2010). As a result, the cAMP response element binding protein (CREB) activity is suppressed, decreasing cellular responses that are stimulated by the cAMP pathway.

3.3 Desensitisation, recycling and degradation of MOR

Following activation, rapid G-protein receptor kinase (GRK) dependent phosphorylation occurs (Williams *et al.*, 2013). Phosphorylated receptors can therefore bind β -arrestin to block G-protein mediated signalling (Allouche *et al.*, 2014). This causes desensitization of the MOR as blocking G-protein binding sites inhibits MOR intracellular activity therefore lifting the inhibition on the adenylyl cyclase, modulation on ion channels and the associated signalling cascades (Williams *et al.*, 2013). β -arrestin also promotes endocytosis, a process that removes

ligand-receptor complexes from the cell surface (Berger & Whistler, 2010). The internalised MORs are recycled for re-expression on the cell surface. Recycling and endocytosis are important for agonist responsiveness recovery (Berger & Whistler, 2010).

Desensitisation is reversed by phosphorylation/dephosphorylation reactions when the agonist is removed. However, chronic use of opioids enhances desensitisation and impairs re-sensitisation (Allouche *et al.*, 2014). This leads to decreased responsiveness to MOR agonists and the development of tolerance (Williams *et al.*, 2013). In mice lacking BARR2, a β -arrestin encoding gene, there was a decrease in tolerance development to opioids while the analgesic properties of opioids were prolonged (Bohn, 1999; Bohn *et al.*, 2000). Transgenic mice expressing MOR showed that chronic morphine inhibits re-sensitisation and recycling (Quillinan *et al.*, 2011). Mice expressing a mutant MOR exhibited internalization of receptors that was associated with high morphine reward and analgesia while showing reduced tolerance, dependence and addiction (Kim *et al.*, 2008). In summary, these reports indicate that the molecular effects of heroin lead to alterations in membrane potential, reduced neuronal excitability and decreased neurotransmitter release.

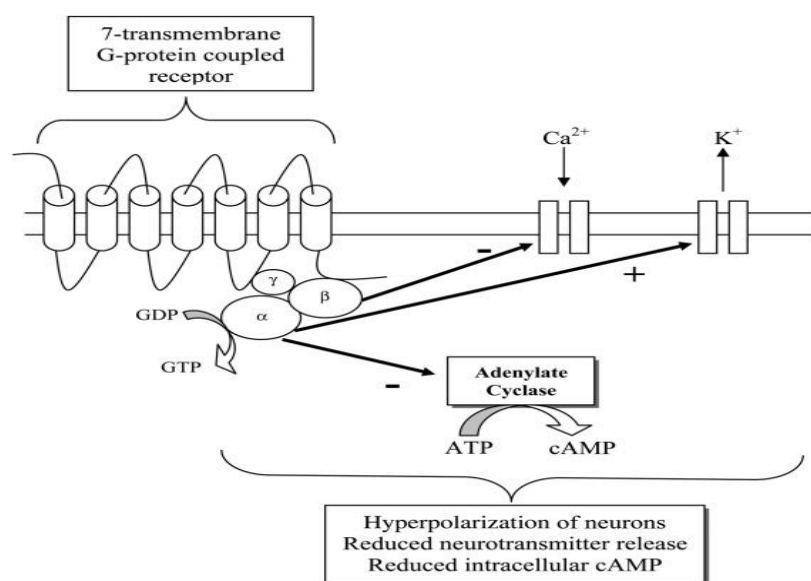


Figure 2: The effects of heroin on opioid receptor (MOR) signalling. Heroin binds to MOR to activate β -arrestin signaling, mitogen-activated protein kinases (MAPK) signalling and the G protein, which is composed of α , β and γ subunits. GTP binds to α -subunit and dissociates α from the $\beta\gamma$ leading to signal transduction. Soluble adenylate cyclase activity is inhibited, thereby reducing cAMP activity which is essential to activate cAMP response element binding protein (CREB) to regulate cellular processes that are stimulated by cAMP. The inward rectification of potassium (K⁺) channels is activated to hyperpolarize the cell. $\beta\gamma$ dimer reduces intracellular calcium (Ca²⁺) by blocking its channels. These events lead to hyperpolarization of neurons, reduction of neurotransmitter release, reduced intracellular cAMP. (Adapted from [https://www.bjaed.org/article/S2058-5349\(17\)30129-4/fulltext](https://www.bjaed.org/article/S2058-5349(17)30129-4/fulltext))

4. Variation in opioid responses

Opioid/Nyaope experimentation is usually voluntary at first. However, self-control is compromised by continued drug use and alterations of brain functions as described in section 2.2. Animal models of human addiction show that only 10% of animals develop addiction after being exposed to drugs of abuse (Seedall & Anthony, 2013). Although the use of prescription and illicit opioids has been increasing at alarming rates amongst teenagers, only a certain number of individuals exposed to these opioid drugs develop comorbid drug disorders and compulsive drug seeking behaviour (Leshner, 2001). In clinical settings, individual variations result in some individuals showing signs of adverse consequences (i.e., tolerance and dependence) or inadequate pain relief at standard opioid doses (Mura *et al.*, 2013).

In addition, some individuals can maintain abstinence while others have reoccurring drug cravings and relapses even after long drug-free periods. These variations in drug response may

stem from chronic stress exposure, pre-existing comorbid mental health issues or biological variability, and may be influenced by genetic variations and environmental factors (Leshner, 2001; Mura *et al.*, 2013). Better understanding of the biological factors underlying the variability in response to opioids will enable better understanding of addiction susceptibility, optimal dosage for analgesic therapy and development of individually tailored treatment for rehabilitation purposes.

5. Epigenetic mechanisms: DNA Methylation

Gene expression within the nervous system is regulated by transcription, which is modulated by epigenetic modifications such as DNA methylation, histone modification and non-coding RNA (Cecil *et al.*, 2015). Epigenetic modifications are heavily influenced by environmental factors and cause alterations to gene expression and phenotypes without altering the actual gene sequence (Nestler, 2014). DNA methylation is the most common type of epigenetic modification as it involves stable changes that are persistent in each generation of mammalian offspring, making it a heritable trait through cell division (Mitchellmore & Gede, 2014). DNA methylation involves the addition of a methyl group to the C-5 position of cytosine (5mC), mostly in the symmetric (methylation on both strands of the DNA) CpG dinucleotides within the promoter region of a gene (Klose & Bird, 2006).

5.1 The impact of DNA methylation on gene transcription

About 70-80 % of the CpG sites in the human somatic cells are methylated (Lister *et al.*, 2009; Ehrlich *et al.*, 1982). The methylation levels are unevenly distributed, lower methylation levels are common in the promoter regions and CpG islands (Ziller *et al.*, 2013). CpG islands are clustered in the promoter regions of a gene functioning as promoters and origins of replication and thereby regulate gene expression by recruiting proteins necessary for gene repression or by inhibiting the binding of transcription factors (Larsen *et al.*, 1992). The occurrence of DNA methylation in the gene body is positively correlated with gene expression (Yang *et al.*, 2014; Jjingo *et al.*, 2012). While some studies suggest that gene-body methylation levels increase consistently i.e., monotonic, some show that the relationship between methylation and gene expression are non-monotonic (Laurent *et al.*, 2010; Jjingo *et al.*, 2012). In contrast, DNA methylation decreases transcription and therefore protein expression when it occurs within the CpG islands in the promoter region of a gene.

Gene expression involves the binding of transcription factors and RNA polymerase to the promoter region of the DNA strand, forming a transcription complex (Alberts *et al.*, 2002).

This causes unwinding of the DNA double strand to provide a template for complementary messenger RNA (mRNA) synthesis (Alberts *et al.*, 2002). mRNA molecules carry information essential for synthesizing an amino acid chain. This genetic information is translated into a protein when a ribosome and a complementary tRNA, carrying an amino acid, bind to regions of mRNA strand, producing multiple proteins (Alberts *et al.*, 2002). This is a general process to produce proteins, including the *OPRM1* protein.

DNA methylation directly modifies the transcription process by interfering with the binding of certain transcriptional factors (Robertson, 2005). When the *OPRM1* promoter is hypermethylated (increased methylation levels), co-repressor complexes that block transcription factors from binding to mRNA, are recruited to the sites of methylation. This prevents the formation of transcription complexes, eventually causing a decrease in transcription of the *OPRM1* gene (Medvedeva *et al.*, 2013) and subsequent decreased MOR protein. Hypomethylation (decreased methylation) of the promoter results in increased mRNA levels. Even though the concepts underlying methylation are not well understood, methyl-CpG-binding protein 2 (MECP2) is thought to cause transcriptional repression by recruiting histone deacetylases (HDAC) after binding to a methylated cytosine and chromatin remodelling complexes (Mitchellmore & Gede, 2014). This results in decreased gene expression, changing long-term gene activity and possibly causes some functional changes such as imprinting disorders.

5.2 DNA methylation patterns

DNA methylation is catalysed by two types of enzymatic activities, *de novo* methylation and maintenance methylation. *De novo* methylation is the best studied type of methylation where a methyl group is added to an unmethylated CpG on both strands of the DNA (Figure 3A). *De novo* methylation is catalysed by DNA methyltransferases DNMT3A and DNMT3B enzymes, more especially DNMT3a which is expressed in the postnatal brain (Moore *et al.*, 2013; Okano *et al.*, 1999). In addition, DNMT3A and DNMT3B oppose the active demethylation of DNA thereby contributing to DNA methylation maintenance (Ginno *et al.*, 2020; Charlton *et al.*, 2020).

Maintenance of DNA methylation is tightly linked to DNA replication and mimics the original DNA methylation pattern of the parent strand (Hermann *et al.*, 2004, Bestor, 2000). DNA is double stranded, and a CpG on one strand has a complementary CpG on the other strand, hence methylation usually occurs on both strands as in *de novo* methylation (Hermann *et al.*, 2004, Li *et al.*, 1992). However, a new strand is formed during DNA replication which produces a

hemimethylation site for DNMT1, a different DNA methyltransferase, to act on (Li *et al.*, 1992, Bestor, 2000). The new strand synthesised in DNA replication is then methylated at the same CpG site as the parent strand to mimic the parent methylation pattern (Figure 3B). This mechanism makes methylation patterns heritable.

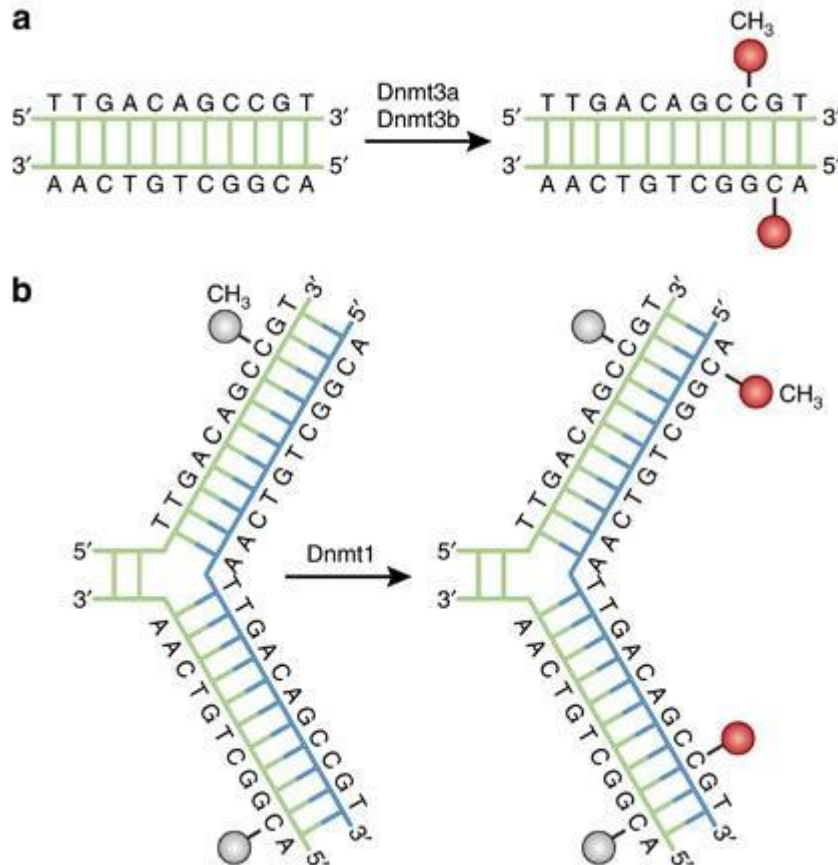


Figure 3: DNA methylation pathways. A family of DNA methyltransferases (Dnmts) catalyses the transfer of a methyl group to form 5-methylcytosine (5mC). (a) Dnmt3a and Dnmt3b are the de novo Dnmts and transfer methyl groups (red) on the unmethylated DNA to methylate the CpG. (b) Dnmt1 maintains DNA methylation pattern during replication. The parental DNA stand retains the original DNA methylation pattern (gray) when semiconservative replication takes place. Dnmt1 replicates the original DNA methylation pattern by adding methyl groups (red) onto the newly formed daughter strand (blue). (Adapted from <https://www.nature.com/articles/npp2012112>)

DNMTs are present in mature neurons in mammalian brains which suggests that DNA methylation might have a role in the brain post the mitotic stage (Feng *et al.*, 2005). The brain's communication channels, in response to environmental stimuli, entail the generation of membrane potentials and neuronal depolarisation processes. Interestingly, changes in gene expression as a result of epigenetic modifications that alter the DNA methylation pattern, often occur following depolarization (Guo *et al.*, 2011; Martinowich *et al.*, 2003). Therefore,

neuronal activity may alter DNA methylation patterns which is required for homeostatic and synaptic plasticity (Guo *et al.*, 2011, Feng *et al.*, 2010). Furthermore, it seems as if both DNA methylation and demethylation have to be active for the alteration of the DNA methylation pattern to be possible (Moore *et al.*, 2013).

5.3 Demethylation

The molecular mechanisms underlying DNA demethylation remain poorly understood. However, there is a growing body of evidence that suggests that DNA methylation might be a reversible process. Demethylation is suggested to occur either actively or passively. In active demethylation, the methylated cytosine residues are demethylated to form unmodified cytosines. In contrast passive demethylation occurs due to inactive DNMTs during cell division and is considered an error during maintenance DNA methylation (Gehring *et al.*, 2009; Métivier *et al.*, 2008). Demethylation requires enzymatic reactions that are needed to cleave the covalent carbon-to-carbon bonds that form the 5mC connection to produce an unmodified cytosine (Moore *et al.*, 2013). While the molecular mechanisms involved in demethylation are still unclear, deamination and oxidation reactions modify the 5mC to a product which the base excision repair (BER) pathway can recognise. BER pathway then replaces the modified 5mC product with an unmodified cytosine in the final step of DNA demethylation (Moore *et al.*, 2013).

5.1 Intermediate/partial methylation

The CpGs in homologous chromosomes of cell populations of a single cell type were initially thought to be uniformly methylated or unmethylated. This rendered DNA methylation to have a two-way control over gene expression, with a role of either increasing (unmethylated state) or decreasing (methylated state) gene expression. However, in reality the methylation status of a number of sites within the genome show significant variability with actual methylation status fluctuating between that of the methylated state and that of the unmethylated state. As such a state of intermediate methylation was coined to describe differences in allelic methylation, the occurrence of clusters of methylated or unmethylated CpG islands, or the variability in methylation status within a cell or between similar cells (Elliott *et al.*, 2015). Interestingly, intermediate methylation leads to intermediate gene transcription, suggesting that this methylation status may be important for context-dependent gene expression (Elliott *et al.*, 2015). Such a feature indicates that intermediate methylation may be relevant in cell differentiation and/or a cell's response to an external stimulus and may help explain variations in gene expression between similar cells.

5.1 DNA Methylation and addiction

Studies have shown that the use of opioids can alter gene expression and repeated use of the drug remodels the epigenome, causing long-term epigenetic changes that may underlie addiction (Nielsen *et al.*, 2012; Nestler, 2014). This view has been supported by studies reporting a strong link between the status of DNA methylation of *OPRM1* and heroin addiction in a cohort of Caucasians (Nielsen, 2009). Further evidence supporting this link was provided by Chorbov *et al.*, (2011) who demonstrated hypermethylation of *OPRM1* in sperm and blood samples of heroin users. However, it is not clear whether hypermethylation was a result of heroin abuse, or whether the hypermethylation predisposed individuals to heroin addiction. Nevertheless, it has been postulated that methylated CpG sites, overlapping with potential *Sp1* transcription factor binding sites, were able to reduce *OPRM1* expression and this observation was closely associated with opioid dependence (Nielsen, 2009; Chorbov *et al.*, 2011).

The association between methylation status of *OPRM1* and heroin use has not been investigated in a South African population. Hence in the present study, we wished to determine the *OPRM1* methylation status in South African male nyaope consumers, and subsequently investigate if an association exists between *OPRM1* methylation and heroin use, as has been reported in Caucasians.

Gene transcription and translation alterations are important for the formation and maintenance of new memories (Alberini, 2008). The interactions of active DNA methylation and DNA demethylation are important for the formation and maintenance of brain circuits (Feng *et al.*, 2010). Most addicts do not succeed at remaining abstinent and often relapse soon after initial days of attempts (Cecil *et al.*, 2015). This is because of the long-term impact that compulsive drug use may have on the brain circuit and neuronal mechanisms. Repetitive use of drugs that modify the brain rewards system can induce DNA methylation. DNA methylation patterns contribute to addiction pathology by altering expression of genes that regulate synaptic plasticity and memory consolidation (Cecil *et al.*, 2015). These neuroadaptations may contribute to failure to abstain and lead to dependence. This then lays the foundation for addiction pathology because addicts present with impaired memory, impaired movement coordination and other declining cognitive functions (Cecil *et al.*, 2015).

6. Genetic influences: Single Nucleotide Polymorphisms

SNPs are genetic variations where a single nucleotide in the promoter, exon or intron region of a gene is substituted by another nucleotide. Some SNPs alter protein synthesis and the function

of the encoded protein. The alterations caused by SNPs in exons depend on whether the SNP is synonymous or non-synonymous. Synonymous SNPs substitute a nucleotide without changing the encoded amino acid sequence while a non-synonymous alteration changes the encoded amino acid resulting in an altered protein sequence with subsequent malfunctioning of the protein (Mahmud *et al.*, 2016).

6.1 SNP rs1799971

SNP rs1799971 in the translated region, in exon 1 of the *OPRM1* gene, is a substitution of adenine in place of guanine at position 118 (SNP 118 A>G) (Bond *et al.*, 1998). SNP 118 A>G is non-synonymous and replaces asparagine with aspartate (N40D), which in turn causes changes in the extracellular region of the MOR protein (Figure 1) (Halikere *et al.*, 2019). N40D removes a potential site for N-linked glycosylation in the N-terminal domain of MOR. N-linked glycosylation is a post-transcriptional modification that promotes protein folding and cell membrane targeting and determines the structure, stability and function of the MOR (Huang *et al.*, 2012; Petrescu, 2004). The consensus sequence for N-linked glycosylation to occur is Asn-X-Thr/Ser motif, but SNP 118A>G changes the **Asn** of the consensus motif to **Asp**-X-Thr/Ser instead (Petrescu *et al.*, 2004). As a result, the substrate cannot be recognised by enzymes thereby preventing the motif to be glycosylated to glycoproteins (Huang *et al.*, 2012). This suggests that SNP 118 A>G changes the protein's structure and stability, as well its overall function.

SNP 118A>G is reported to be more frequent in Caucasians (17%) and Asians (48%) than in African Americans (2.2%) and sub-Saharan Africans (0.8%) (American National Centre for Biotechnology Information). Some studies have documented significant associations between SNP 118 A>G and vulnerability to addiction (Szeto *et al.*, 2001; Zhang *et al.*, 2007). Others have suggested that it may be protective against addiction (Bond *et al.*, 1998) while no significant association to the mutation has also been shown (Hancock *et al.*, 2015). The findings in literature are contradictory (as summarised in Table 1).

Due to population genetic relatedness and environmental modifiers, it is accepted that contradictory associations may exist across different ethnicities, hence the SNPs clinical relevance remains debatable for opioid addiction. Walter and Lotsch (2009), acknowledged that SNP 118 A>G may play an integral part in the complex system but cautioned against the use of SNP 118 A>G as a general clinical biomarker. Since SNP 118A>G introduces a new

methylation site we were interested to know whether it was present in a South African cohort of nyaope users and whether it was methylated in this population.

The transfection of human *OPRM1* coding region into Chinese hamster cells and the level of transcription in these cells showed that the presence of the SNP has no significant effects on the mRNA stability when compared to the 118A allele (Zhang *et al.*, 2005). However, the mRNA expression of the 118G variant was 2-fold lower than the wild-type 118A. The authors suggested that SNP 118 A>G is a *cis*-acting polymorphism that affects transcription, mRNA stability and processing and protein translation with no effect on the protein structure.

SNP 118A>G affects the *OPRM1* expression and the MOR receptor levels even though the SNP is located in the coding region and not the gene promoter. Human carriers of the (118G) SNP transcribe two-fold less mRNA and have ten-fold less MOR protein when compared to wild-type (118A) (Zhang *et al.*, 2005). This is because the SNP affects glycosylation which determines the structure, stability and function of the MOR. In addition, nicotine smokers expressing the heterozygous 118G allele have decreased receptor binding sites available on the cell surface when compared to the homozygous 118A allele (Ray *et al.*, 2011). The SNP has been suggested to alter three transcription factor binding sites within the *OPRM1* gene. This affects gene expression and therefore reduces the MOR receptor levels.

SNP 118A>G increases the binding affinity of β -endorphin (an endogenous opioid) three-fold but does not alter binding affinities of exogenous opioids (methadone, morphine, naloxone and fentanyl) (Bond *et al.*, 1998). The activation of G-protein coupled K^+ channels show three times more potency of β -endorphins in MOR (Bond *et al.*, 1998). Heroin users were found to be carriers of SNP 118A>G in a study that showed that heroin users who carry the 118G allele had significantly lower endogenous opioids (preproenkephalin and preprodynorphin) levels compared to 118A allele carriers (Drakenberg *et al.*, 2006). This suggests that MOR sensitivity to endogenous opioids is associated with SNP 118A>G and therefore variations to opioid drug response. In cells transfected with SNP 118A>G, morphine inhibited Ca^{2+} two times more than the wild-type (Margas *et al.*, 2007). These studies suggest an association of the SNP with increased effectiveness of endogenous opioids and decreased effectiveness of exogenous opioids. Carriers of this SNP may therefore require higher doses of exogenous opioids to achieve analgesia and euphoria. However, this may increase the risk of overdosing and tolerance development.

The changes resulting from N40D on receptor signalling transduction impacts how individuals respond to opioids exposure. In SNP 112A>G knock-out mice (analogous with human SNP 118A>G), the addictive properties of morphine were eliminated (Matthes, 1996). Furthermore, N40D has been suggested to decrease effectiveness of opioids by interfering with the binding property of MOR, thereby increasing the opioid dose demands. This suggests an association of SNP 118A>G with susceptibility to drug addiction (Kelly, 2013). Though SNP 118A>G has been extensively investigated, its functional implications have yet to be confirmed.

6.2 SNP Haplotypes

It has been suggested that a different SNP, SNP rs3778150, may account for the inconsistent associations of SNP 118 A>G with heroin addiction. SNP rs3778150 is in the non-coding intron 1 region of *OPRM1*. Although introns are excluded from translation, they may have functional effects on the encoded gene protein. Introns in the 5' of the untranslated region have been suggested to have *cis* regulatory sequences that initiate transcription (Bradman and Korf, 2008; Damgaard *et al.*, 2008).

SNP rs3778150 substitutes cytosine in place of thymine (T>C) and has been significantly associated with heroin addiction in European Americans, African Americans and Australians (Hancock *et al.*, 2015). SNP rs3778150 mutation has also been associated with a significant decrease in *OPRM1* expression. Haplotype analysis showed that SNP rs3778150 and rs1799971 are haplotypes, which are genetic variations that are inherited together and are located on the same chromosome (Hancock *et al.*, 2015; Roach *et al.*, 2011). The haplotype with SNP rs3778150 mutant allele (C) and SNP 118 A>G wildtype allele (A) was shown to be consistently associated with heroin addiction in African Americans and European Americans (Hancock *et al.*, 2015). This association was lost when a haplotype of SNP 118 A>G major allele occurred without the rs3778150 C allele (Hancock *et al.*, 2015). It was shown that individuals that carry the mutant allele for SNP 118 A>G in combination with other polymorphisms, required more opioid doses for the same analgesia effects than haplotype 118A carriers (Hayashida *et al.*, 2008).

The intronic SNP *OPRM1* T>C rs3778150 has been independently associated with the risk of heroin addiction (Nelson *et al.*, 2014). Studies have shown strong associations of SNP rs3778150 and the risk of opioid addiction in individuals of European ancestry and weaker associations in African descendants (Nelson *et al.*, 2014; Hancock *et al.*, 2015). In a South African population, the association of SNP rs3778150 with heroin addiction has not been

established. Furthermore, it remains unknown whether SNP rs3778150 and rs1799971 have a haplotype relationship that is associated with heroin addiction in a South African population.

7. Genetic-Epigenetic interactions

The 118 A>G SNP, which introduces a guanine (G) in place of an adenine (A) at site +118, introduces a CpG site at sites +117/+118 that would otherwise not be present (Oertel *et al.*, 2012; Zhang *et al.*, 2010). The introduced CpG site generates an additional methylation site, which may decrease *OPRM1* receptor expression. This extra methylation site has been shown to increase methylation levels at this position (+117) and downstream of this position (at +145, +150 and +169) in post-mortem brain tissues of Caucasian opiate addicts (Oertel *et al.*, 2012). Sites +145, +150 and +169 are potential binding sites for the *Sp1* transcription factor and methylation at these sites would prevent binding of *Sp1* and thereby alter *OPRM1* expression. Post-mortem brain tissue of heroin users with the 118AA genotype showed an increase in the number of MORs, a compensatory mechanism for the loss of signalling efficacy caused by chronic heroin use (Oertel *et al.*, 2012). However, this compensatory increase in MOR protein was not present in brains of carriers of the 118G SNP. Hence, it is likely that the presence of the SNP results in hypermethylation of *OPRM1* that prevents heroin-induced upregulation of MOR protein expression. Since it is not known whether the presence of SNP 118A>G affects methylation of the *OPRM1* gene in a South African cohort, the present study also addressed this gap in the current literature.

In summary, the analgesic and euphoric response to opioids amongst individuals varies greatly, and consequently the development of tolerance and transition to addictive behaviour are equally inconsistent between opioid consumers. DNA methylation, SNP 118A>G and haplotype relationships have been implicated in the development of addiction as molecular signatures that may determine vulnerability to the mental disorder. The SNP affects MOR structure and function by increasing DNA methylation levels and subsequently reducing MOR expression. It is important to understand the molecular concepts that lead to these functional changes in the MOR in order to predict addiction susceptibility, develop optimal dosage for analgesic therapy and individually tailor treatment for rehabilitation purposes. It is for this reason that the main aims of this study, were to assess

- i) the presence of methylation in the *OPRM1* gene,
- ii) the presence of SNP 118A>G,
- iii) the haplotype relationship between SNP rs3778150 and rs1799971, and

- iv) the effect of SNP 118A>G on methylation of the *OPRM1* gene in black South African descents.

Table 1: Summary of SNP 118A>G association with opioid addiction. Different ethnicities show different associations of SNP 118A>G with opioid addiction.

Drug	No of patients	Ethnicity	SNP 118A>G association with addiction	Reference
Heroin	123	Iranian	No association (absent)	Shakiba <i>et al.</i> , 2017
	126	Indian	Association present	Kapur <i>et al.</i> , 2007
	200	Chinese	Association = 118G allele associated with heroin dependence vulnerability	Szeto <i>et al.</i> , 2001
	336	Chinese	Association = 118G associated with positive responses to heroin	Zhang <i>et al.</i> , 2007
	96	Han Chinese	No association (absent)	Shi <i>et al.</i> , 2002
	473	Han Chinese	No association (absent)	Glatt <i>et al.</i> , 2007
	139	Swedish	Association	Bart <i>et al.</i> , 2004
	113	Multiple races	Association = Protective against addiction development	Bond <i>et al.</i> , 1998
	118	European Caucasians	Association	Drakenberg <i>et al.</i> , 2006
	224	European	No association	Hancock <i>et al.</i> , 2015
	129	European	No association	Levrán <i>et al.</i> , 2017

Chapter 2: Methods

1. Ethical Approval

This study formed part of a larger study that involved recruitment of the participants by Dr Nirvana Morgan in fulfilment of her PhD. Permission was granted for use of the blood samples in this study, which Dr Nirvana Morgan is a collaborator. The study was approved by the Human Research Ethics Committee of the University of the Witwatersrand (Johannesburg, South Africa). Ethical clearance number M 1704100 was assigned to the project. The study conformed to the standards set by the latest revision of the Declaration of Helsinki, whereby written informed consent was obtained from all participants.

2. Sample collection

Nyaope users between the ages of 18 to 45 years were previously recruited by Dr Nirvana Morgan (registered psychiatrist) from the Empilweni and Life Randfontein rehabilitation centers, Johannesburg, South Africa. The Opioid Treatment Index was used to assess their drug-dependence (Darke *et al.*, 1991). Venous blood (5ml EDTA tube) was collected from nyaope users (n=200), as well as healthy non-drug dependent controls (n= 55) who were age- and gender-matched. Both populations were recruited as per the inclusion criteria outlined below (n= 55).

3. Inclusion criterion

In order to be enrolled in the study, participants had to be older than 18 years and be willing and able to sign informed consent. Only males who reported to be of African ancestry and native to South Africa were included in the study.

4. DNA extraction

DNA was extracted from the peripheral blood cells of the participants using the “salting-out” method (Longmire *et al.*, 1987). Triton X-sucrose buffer (10 ml 1M Tris-HCl pH 8, 5ml 1M MgCl₂, 10ml Triton X, 109.5g Sucrose) was added to the blood samples to lyse the white blood cells. The mixture was centrifuged (Avanti J-26S Series, Beckman Coulter) for 10 minutes at 2400rpm at 4 °C. This formed a pellet that was washed using the wash buffer (10 mM Tris-HCl pH 8, 100 mM NaCl, 2.5 mM EDTA, 55% ethanol) and centrifuged (Avanti J-26S Series, Beckman Coulter) at 2400 rpm at 4 °C for 5 minutes. T20E5 (20Mm 1M Tris-HCl pH 8, 10ml 0.5M EDTA), 0.2ml of 10% sodium dodecyl sulphate and 0.5mL proteinase K mix (400ul 10%SDS, 16ul 0.5M EDTA, 2.8mL distilled water and 800ul Proteinase K) were added and

incubated overnight at 42°C. Saturated NaCl₂ (1mL of a 40g NaCl in 100ml distilled water solution) was then added to precipitate the proteins. A white pellet was visible following centrifugation (Avanti J-26S Series, Beckman Coulter) at 2400rpm at 4 °C for 30 minutes. The supernatant was then discarded and two volumes of cold absolute ethanol were added to the pellet to precipitate the DNA. Visible DNA was collected by fishing it out with a pipette and subsequently dissolved in Tris-EDTA buffer (50µl, 1M, pH of 7.4. 10 mM Tris-HCl pH 8, 1 mM EDTA).

DNA concentration (Absorbance 260nm/Absorbance 280nm) and DNA quality (Absorbance 260nm/Absorbance 230nm) in a 2-µl drop were determined using a Nanodrop 2000 Spectrophotometer (Thermo Scientific, Wilmington, DE, United States) and stored at 4 °C.

5. Methylation-specific primer design

The UCSC genome browser (Santa Cruz Biotechnology, University of California, United States, RRID: SCR_005780) was used to identify the *OPRM1* gene located on chromosome 6, the region of interest was mapped in exon 1 (chr6: 154331689-154332224bp) of human genome 19 and 29 CpG sites were identified within this gene segment. Methylation-specific primers were designed manually to flank this region of interest. We previously identified that the use of a single primer set that flanks a large amplicon (>350bp) may result in PCR product sequencing difficulty as the template DNA is first subjected to bisulfite conversion, which can be damaging to the DNA causing it to become fragmented. Therefore, two primer sets were designed to produce two shorter, overlapping amplicons that cover the region of interest (Figure 4) rather than a single long amplicon, which may have been subject to fragmentation.

For each DNA sample 20ng/µl of the primer was used. The melting temperature of the designed primers was calculated using the following formula: $T_m = 4 (G + C) + 2 (A + T)$. Optimal annealing temperatures for each primer pair were determined by means of gradient PCR.

5' **CCAGGACTGGTTTCTGTAAAGAAACAG**CAGGAGCTGTGGCAG**CGGCG**AAAGGAA
Forward primer 1: 5' TAGGATTGGTTTTTGTAAAGAAATAG3' 1 2
CGGCTGAGGCGCTTGAACCCGAAAAGTCT**CGGTGCTCCTGGCTACCTCG**CACA
3 4 5 6 7
CGGTGCCC**CGCCCG**GC**CGT**CAGTACC**ATG**GACAGCAG**CGCTGCCCCC****CGAACCG**
8 9 10 11 12 13 14
Reverse primer 1: 3' CCAAAAACCCAATTAAACAAAATAAATC5'
CAGCAATTGCACTGATGCCTTGG**CGTACTCAAGTTGCTCCCCAGCACCCAGCCCC**
15 16
Forward primer 2: 5' TATTTAAGTTGTTTTTTAGTATTTAG3'
GGTTCCTGGGTCAACTTGTCCCACTTAGATGGCA**A**ACCTGTCC**CG**ACCCATG**CGGTC**
17 18
CGAACCGCACCGACCTGGG**CGG**GAGAGACAGCCTGTGCCCTC**CGACCGG**CAGTCC
19 20 21 22 23 24
CTCCATGATCA**CGGCCATCA****CGATCATGGCCCTCTACTCCATCGTGTGCGTGGTG**
25 26 27 29
GGGCTCTTC**CGAAACTTCCTGGTCATGTATGTGATTG**3'
29 **Reverse primer 2:** 3' CCTTTAAAAACCAATACATACTAATC

Figure 4: The gene sequence of 370bp present on human chromosome 19, 27722994-27723372 in the 5' region of OPRM1. The target region used for the DNA methylation analysis consists of CpG sites 1-27 which are in bold font and numbered. The binding sites for forward and the reverse primer set 1 have been labelled and shaded in yellow and the second set is labelled and underlined in blue and the two primers sets are labelled. Three possible Sp1 transcription factor binding sites are underlined. SNP 118 A>G has been highlighted in pink. The ATG translation start site has been highlighted in red.

6. Bisulfite conversion and Polymerase Chain Reaction (PCR)

Bisulfite treatment causes deamination of unmethylated cytosine residues facilitating conversion to uracil whilst leaving the methylated cytosines unchanged. This allows for distinction between methylated and unmethylated cytosine residues. Taq polymerase recognizes uracil residues as thymine residues and methylated cytosine residues as cytosine residues during the subsequent PCR process (Li & Tollefsbol., 2011). The Epijet bisulfite conversion kit (Thermo Scientific) was used as per manufacturer's instructions. The kit denatures the DNA sample (250 ng) while simultaneously performing the bisulfite conversion reaction. Subsequently, the DNA is applied to the membrane of a micro-column to be hydrolysed by sulfonic acid and then eluted by the kit elution buffer (50µl of Tris-EDTA buffer).

To make a 25µl PCR product, I combined 12µl Hotstart Taq polymerase, 0.5µl forward primer, 0.5µl reverse primer, 2µl bisulphite treated DNA and 8µl distilled water. Gradient PCR, which uses a defined set of varying annealing temperatures, was performed using a Thermocycler (Biorad, T100) to determine optimal annealing temperatures for each primer set (Figure 5). The gradient was set from 45 °C to 50°C for both primer sets. Products with 259bp (primer set 1) were amplified across all the set temperatures but the optimal annealing temperatures were at 50°C while those with 244bp were amplified best at and 45.7° C and the actual annealing temperature for subsequent PCR reactions was 46°C.

PCR amplification comprised of a step of 3 min at 95°C, 35 cycles of 30 seconds at 95°C, a cycle at 50 °C (for primer set 1) and 46°C (for primer set 2) for 40 seconds for annealing and a cycle at 72°C for 1 minute. The final extension step of 7 minutes at 72°C was run and the PCR product was then kept at 4°C Each PCR product (5µl) was visualized using a 2% agarose gel (70V, 1hr) and successful PCR was identified by presence of bands at the calculated amplicon fragment lengths of 259bp and 244bp for primer set 1 and primer set 2.

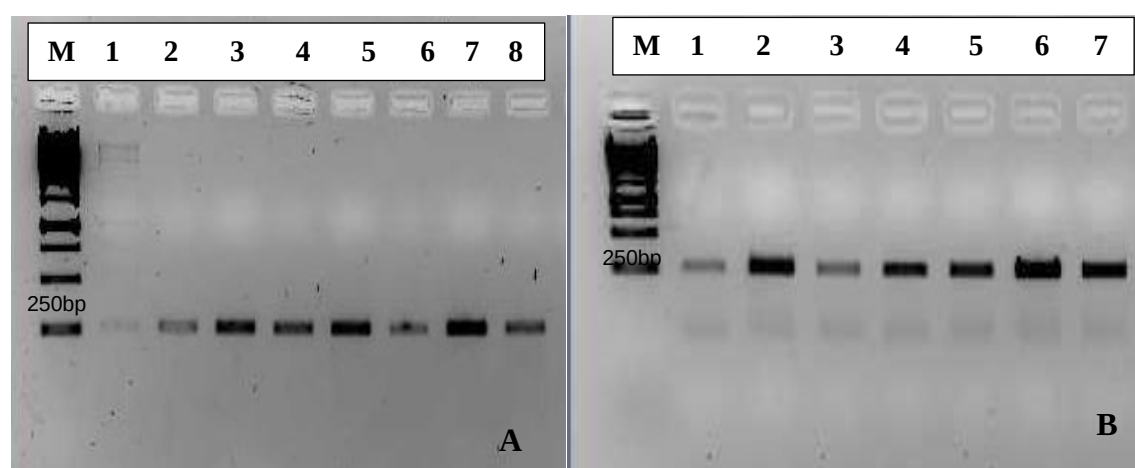


Figure 5: Agarose gel electrophoresis for the determination of optimal annealing temperatures designed primers for OPRM1 gene in peripheral blood samples of heroin users' using gradient PCR. The gradient was set as 45°C (lane 1), 45.3 °C (lane 2), 45.9°C (lane 3), 47°C (lane 4), 48.1 C (lane 5), 49°C (lane 6), 49.6°C (lane 7) and 50°C (lane 8) (A). Products with 259bp (primer set 1) were amplified across all the set temperatures but the optimal annealing temperatures were at 50°C. The gradient was set from 45.7 (lane 7), 46.9 (lane 6), 48.9 (lane 5), 47.9 (lane 4), 48.1 (lane 3), 49.6°C (lane 2) and 50°C (lane 1) (B). Products with 244bp (primer set 2) were amplified across all the set temperatures but the optimal annealing temperatures were at 48.1°C, 46.9 °C and 45.7°C. The actual annealing temperature for subsequent PCR reactions was 46°C. M, molecular weight marker.

7. Sequencing and analysis

PCR products that displayed the correct amplicon lengths in agarose gel electrophoresis were sent to Inqaba Biotec for post-PCR processing and DNA (Sanger) sequencing. Both strands of the PCR products were sequenced in order to obtain high quality sequence data over the full lengths of the amplicons. FinchTV software (FinchTV Version 1.5.0, Digital World Biology, Geospiza Inc) was used to visualize the sequencing chromatograms of the DNA methylation abi files generated by the sequencer. The forward and reverse DNA sequences were aligned with the original *OPRM1* sequence from UCSC genome browser through pairwise sequence alignment using EMBOSS Matcher (EMBL-EBI, Wellcome Genome Campus, Hinxton, Cambridgeshire, CB10 1SD, UK.) for each sample.

The CpG sites were then analysed manually from the aligned sequences, identifying the methylation status, as **methyalted (C)** or **unmethyalted (T)** when compared with the original *OPRM1* sequence. Many sequences showed an ambiguous nucleotide “Y”, which represents a mixture of pyrimidines (>25% C or T) for some CpG sites that the sequencer could not resolve as either C or T. The Y nucleotides were present only in CpG sites and not for conversion of C to T outside of CpG sites. This indicated that our sequencing was reliable and the presence of both C and T residues in the PCR products showed that some copies of the DNA in the patient samples were methyalted at specific CpG sites and other copies were not methyalted at those sites i.e., the CpG sites in the patients’ DNA samples were partially methyalted (intermediate methylation).

We used two different manual decision algorithms to assess (potential) partial methylation (see Table 2). In the first approach (methylation assignment bias) we sought to use the bidirectional sequencing to positively assign the methylation state of each CpG site, minimizing the numbers of CpG sites identified as ambiguous or partially methyalted. In this approach, if one sequence (forward or reverse) showed a Y and the other sequence showed a T or C, the T or C was accepted, and the appropriate methylation state assigned to that GpG site for that patient sample. CpG sites were identified as partially methyalted only if both sequences showed Y (Table 2). In the second approach (partial methylation bias) we used the presence of Y to identify all possible partially methyalted CpG sites. In this approach, if either sequence showed Y, the CpG site was identified as partially methyalted, and C or T was only assigned if both sequencing reactions showed the same base (Table 2).

Table 2: Examples of analysis methods for partial DNA methylation. If the Y nucleotide in one strand corresponded with a t in another strand it was recorded as unmethylated and it was recorded as methylated if it corresponded with a c in the other strand. In partial methylation bias, the Y nucleotide in the forward sequence was recorded as a P- partially methylated if there was a corresponding T or a C in the reverse sequence. The two results for each sample are for the forward (top) and reverse (bottom) sequencing.

Sample	Sequencing Result	Methylation Assignment bias		Partial Methylation bias	
		Decision	Interpretation	Decision	Interpretation
1	y	T	Unmethylated	P	Partially methylated
1	t				
2	t	T		T	Unmethylated
2	t		Methylated		
3	y	C		P	Partially methylated
3	c				
4	c	C		C	Methylated
4	c				
5	y	P	Partially methylated	P	Partially methylated
5	y				
6	c	A	Ambiguous	A	Ambiguous
6	t				

8. SNP Genotyping Assay

Genotyping for SNPs 118 A>G and rs3778150 was performed using TaqMan® SNP Genotyping Assays (Assay ID: C__8950074_1 and C__29641067_10, ThermoFischer Scientific) in a 96 well plate format. Genomic DNA with a minimum concentration of 20 ng/μl was combined with TaqMan® genotyping mastermix (ThermoFischer Scientific) and amplified using a StepOnePlus™ real-time PCR thermocycler (ThermoFischer Scientific) according to the manufacturer's instructions. PCR amplification consisted of a polymerase activation step of 10 min at 95°C, 40 cycles of 15 seconds at 95°C for denaturation and 60°C for 40 seconds for annealing and extension. TaqMan® Genotyper Software (v1.6, QuantStudio™) was used to analyze data and generate allelic discrimination curves. The allelic discrimination curves indicate individuals that are homozygous wild type (AA), homozygous mutant (GG) or

heterozygous (AG) for SNP rs1799971 and homozygous (TT), homozygous (CC) or heterozygous (TC) for SNP rs3778150.

9. Data analysis

Data analysis was performed using Graphpad Prism Version 7 (Graphpad software Inc, San Diego, USA). Results were considered statistically significant if $P < 0.05$. The Chi-square test was used to compare the methylation status of *OPRM1* in nyaope users and healthy controls and to determine whether an association existed between *OPRM1* methylation and heroin use. Fisher's exact test was used to determine the frequency of SNP *OPRM1* rs1799971 and SNP rs3778150 in nyaope users and compared to that of healthy controls. The Cochran-Mantel-Haenszel test was used to identify the level of correlation between the SNP genotype data to the methylation status data. The Cochran-Mantel-Haenszel test was also used to correlate SNP rs3778150 and rs1799971 genotypes in nyaope users and compared to healthy controls to test for haplotype relationships.

Chapter 3: Results

The sequences showed a large number of ambiguous nucleotides designated “Y”, which resulted in inability to assign methylation status for many CpG sites using conventional methylation analysis software (Figure 6). The participants CpG methylation status data was presented graphically and analysed statistically if their methylation status was detected as methylated, unmethylated or partially methylated using the methylation assignment bias approach (Figure 7) and the partial methylation bias approach (Figure 8).

```
TATTTAAGTTGTTTTTTAGTATTTAGTTTCG GTTTTTGGGTTAATTTGTTTTATT
-----TAGTTTTGGTTTTTGGGTTATTTTGTTTTATT
TATTTAAGTTGTTTTTTAGTATTTAGTTTCG GTTTTTGGGTTAATTTGTTTTATT
TAGATGGTAATTTGTTTCGATTTATGCGGTTCGAATCGTATCGATTGGGCGGGAG
TAGATGGTAATTTGTTTCGATTTATGCGGTTCGAATCGTATCGATTGGGCGGGAG
TAGATGGTAATTTGTTTCGATTTATGCGGTTCGAATCGTATCGATTGGGCGGGAG
AGATAGTTTGTGTTTTTCGATCGGTAGTTTTTTTATGATTACGGTATTACCGATT
AGATAGTTTGTGTTTTTCGATCGGTAGTTTTTTTATGATTACGGTATTACCGATT
AGATAGTTTGTGTTTTTCGATCGGTAGTTTTTTTATGATTACGGTATTACCGATT
ATGGTTTTTTTATTTTATCGTGTGCGTGGTGGGGTTTTTCGGAAATTTTTTGGTTA
ATGGTTTTTTTATTTTATCGTGTGCGTGGTGGGGTTTTTCGGAAATTTTTTGGTTA
ATGGTTTTTTTATTTTATCGTGTGCGTGGTGGGGTTTTTCGG-----
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Figure 6: A gene sequence from sample data. The bisulphite converted parent strand (black), sense strand (purple) and complementary strand (gold) are shown in the figure. CpGs are highlighted in yellow, unmethylated CpG are represented as TG and are highlighted in blue. There were a large number of ambiguous nucleotide “Y”, which represents a mixture of pyrimidines (>25% C or T) indicative of partial methylation.

3.1 Methylation analysis

3.1.1 Total methylation levels

The DNA methylation levels of CpG islands in the promoter region of *OPRM1* were compared between 200 nyaope consuming participants and 55 healthy non-drug consuming controls using Fishers exact test. Using the methylation assignment bias approach, we found that there was a statistically significant difference in the total methylation levels between the two groups with significantly lower levels observed in nyaope-consuming participants ($p = 0.0275$). The partial methylation bias approach showed no significant differences in the total methylation levels between the two groups ($p = 0.2017$).

3.1.2 Methylation at individual CpG sites using the methylation assignment bias approach

Using the methylation assignment approach, most CpG's were unmethylated (Figure 7). All CpG sites in the promoter region (CpGs 1 to 14) were predominantly unmethylated and there were no significant differences between nyaope users and controls ($p > 0.05$). Nyaope-consuming participants had significantly lower methylation levels when compared to control participants at CpG sites 15 ($p < 0.001$), 21 ($p = 0.0047$), 23 ($p = 0.0082$), 24 ($p = 0.0148$), 26 ($p = 0.0243$) and 28 ($p < 0.0001$) (Figure 8). CpG 26 also showed significantly higher partial methylation in participants that used nyaope when compared to controls ($p = 0.0053$, Figure 7). The methylation levels were significantly higher at CpG 29 in control participants ($p = 0.0052$) when compared to nyaope users.

3.1.3 Methylation at individual CpG sites using the partial methylation bias approach

Using the partial methylation bias approach, CpG sites were mostly partially methylated (Figure 8). In comparison, control participants had higher partial methylation levels while nyaope-consuming participants had higher unmethylation levels. Partial methylation was significantly higher at CpG sites 8 (0.048), 18 ($p = 0.0370$), 19 ($p = 0.0034$), 20 ($p = 0.0007$), 21 (0.0097), 23 ($p = 0.0003$), 24 ($p < 0.0001$), 25 ($p < 0.0001$), 26 (0.0008), 27 ($p = 0.0006$) and 28 ($p = 0.0003$) in control participants in comparison to nyaope users. Nyaope-consuming participants' methylation levels were different from those of control participants, showing higher unmethylation levels at CpG 1 ($p < 0.0001$), 2 ($p = 0.004$), 3 ($p < 0.0001$), 4 ($p < 0.0001$), 7 ($p = 0.0003$), 11 ($p = 0.0022$), 16 ($p = 0.0189$), 22 ($p = 0.0257$), 25 ($p = 0.0015$). CpG 15 (0.0025) and 29 ($p = 0.0053$) showed substantial methylation in control participants when compared to nyaope-consuming participants.

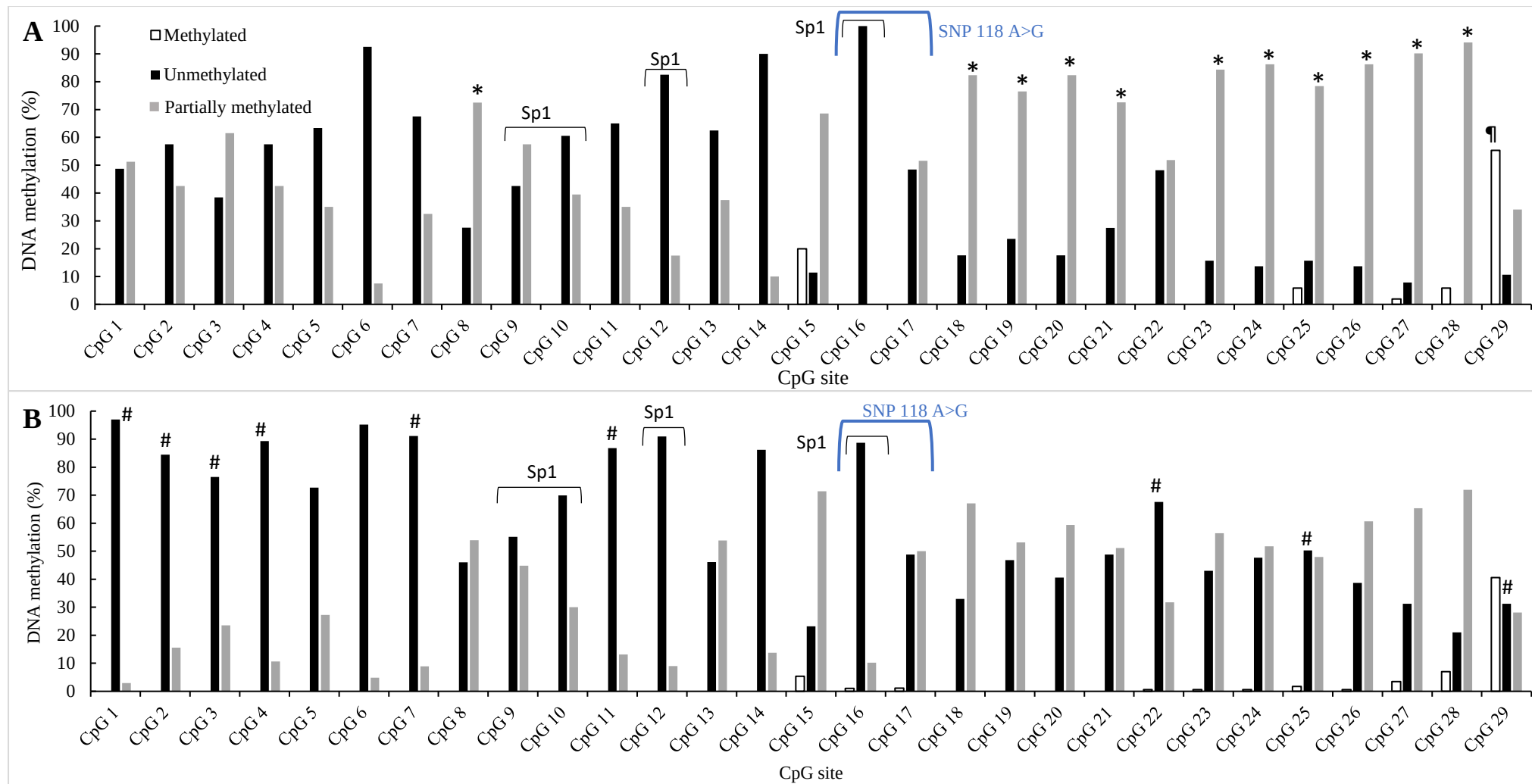


Figure 8: Percentage methylation at 29 CpG sites within the OPRM1 promoter region in nyaope-dependent participants (A) and control participants (B) using the partial methylation bias approach. The methylation status is labelled as methylated, unmethylated or partially methylated. Sp1, potential binding site for Sp1 transcription activator and SNP 118 A>G locations are indicated. ‡ indicates methylation, # indicates unmethylation and * partial methylation respectively (p < 0.05).

3.2 SNP Frequencies

3.2.1 Genotype frequency of SNP rs1799971 and SNP rs3778150

The AA genotype of SNP rs1799971 was predominant in both nyaope-consuming participants and control participants (Table 3). There was a significant difference ($\chi^2=18.55$; 1, $p < 0.0001$) between the genotype frequency of nyaope consumers (100% for AA, 0% for AG and GG) and controls (90.9% for AA, 9.1% for AG and 0% for GG) for SNP rs179997, with nyaope-consumers showing significantly greater frequency. The TT genotype of SNP rs3778150 intronic variant was predominant in both nyaope consumers and control participants (Table 3). The genotype frequencies of control participants (1.79% for CC, 21.82% for CT and 70.91% for TT) did not differ significantly ($\chi^2 = 1.469$; 2, $p = 0.4798$) from those of nyaope-consuming participants (1% for CC, 31% for CT and 68% for TT).

Table 3: Genotype frequency distribution of SNP rs1799971 and SNP rs3778150 polymorphism in nyaope-consuming participants and control participants. The data are represented as n (%), where n is the total number of participants genotyped.

	SNP rs1799971			Total	SNP rs3778150			Total
	AA	AG	GG		CC	CT	TT	
Controls	50 (90.9%)	5 (9.1) *	0 (0%)	55	1 (1.79%)	12 (21.82%)	39 (70.91%)	55
Users	200 (100%)	0 (0%)	0 (0%)	200	2 (1%)	62 (31%)	136 (68%)	200

For SNP rs1799971, $\chi^2 = 10.58$; 1, * $p = 0.0011$ and SNP rs3778150, $\chi^2 = 1.441$; 2, $p = 0.4864$.

3.2.2 Allele frequency distribution of SNP rs1799971 and SNP rs3778150

The variant G allele frequency distribution in the nyaope consumers group (1%) was lower than that of the control participants (8%) (Figure 9). Results from the chi-square test revealed significant differences ($\chi^2= 16.99$, 1, $p = 0.0001$) in allelic distribution of the mutant allele for SNP 118 A>G. The distribution of the wild-type allele in nyaope-consuming participants was similar to that of control participants and showed no significant differences ($p > 0.05$).

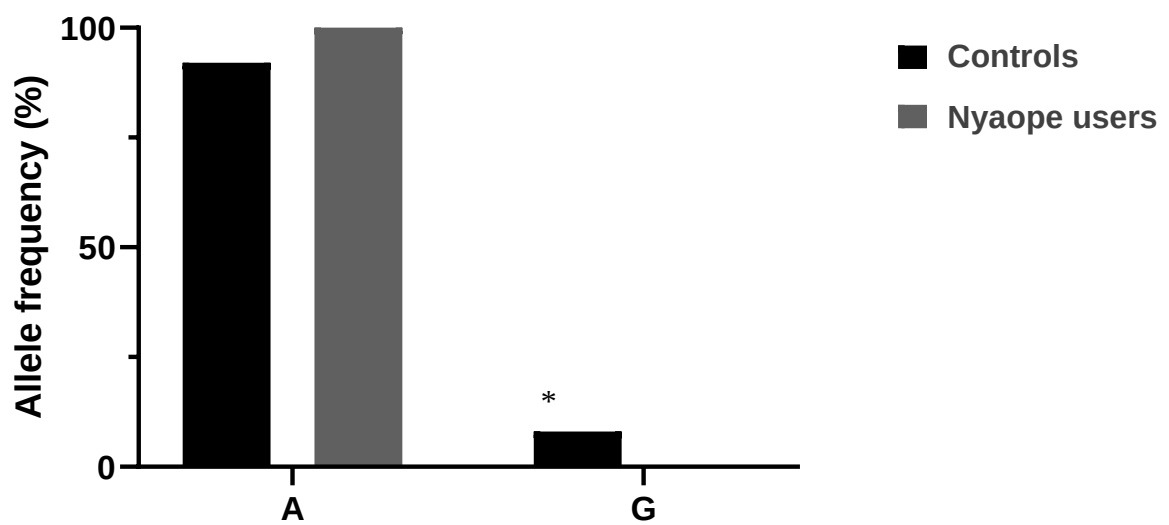


Figure 9: Allelic frequency distribution of SNP 118 A>G polymorphism in nyaope users and control participants. Control participants had higher G allele frequency when compared to nyaope users * indicates $p = 0.0001$.

3.2.3 Allele frequency distribution of SNP rs3778150

The distribution of the T allele was the same between the two groups of participants (24%) (Figure 10). Chi-square test analysis revealed no significant differences ($\chi^2 = 0.02049$; 1, $p = 0.8862$) in the allelic distribution for SNP rs3778150. The distribution of the wild-type allele in nyaope-consuming participants was similar to that of control participants and showed no significant difference ($p > 0.05$).

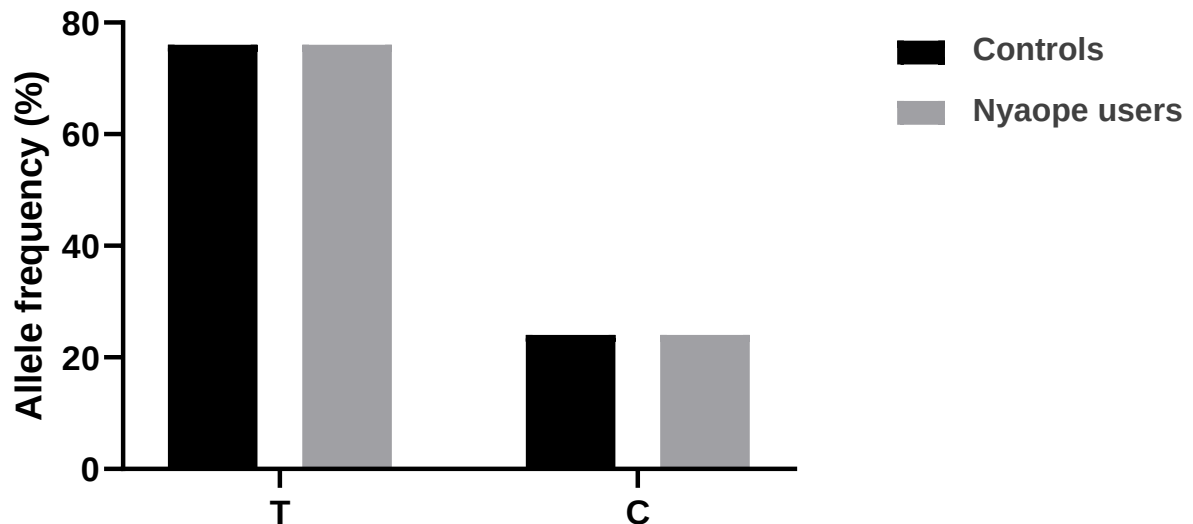


Figure 10: Allelic frequency distribution of SNP rs3778150 polymorphism in nyaope users and control participants. The allele distribution frequency was broadly similar between control and nyaope-consuming users.

3.3 SNP rs1799971 and SNP rs3778150 haplotype analysis

The haplotype relationship of SNP rs3778150 mutant allele (C) and SNP 118 A>G wildtype allele (A) was tested using Cochran-Mantel-Haenszel test. There was no correlation between the mutant allele for SNP rs3778150 and SNP rs1799971 ($\chi^2 = 0.23$; 1, $p = 0.631$).

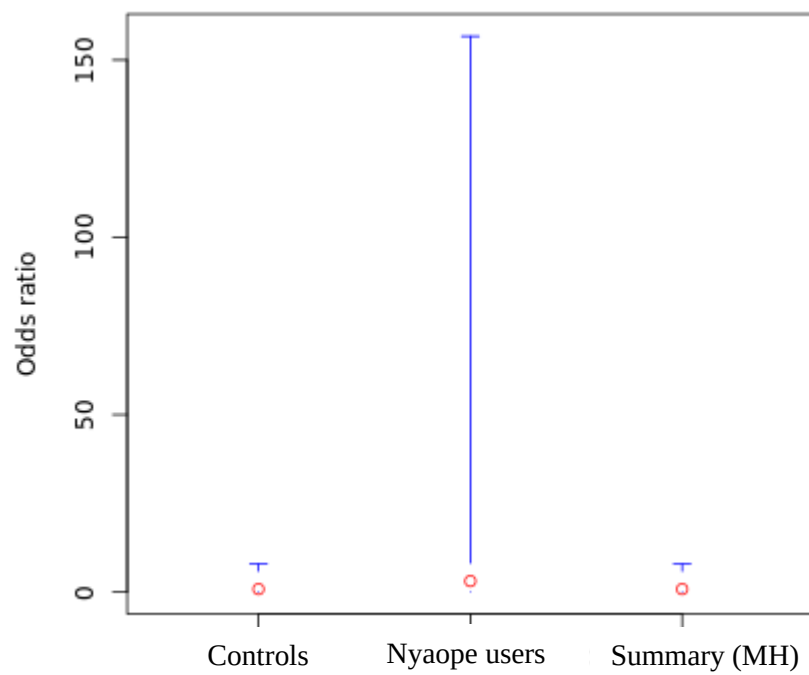


Figure 11: 95% confidence intervals for Mantel-Haenszel odds ratios for SNP rs1799971 and SNP rs3778150. Haplotype analyses of rs3778150 mutant allele C and SNP rs1799971 wild type allele A.

3.3 SNP rs1799971 and methylation levels

The data indicated that SNP 118 A>G does not correlate with the *OPRM1* methylation levels in black South African males ($\chi^2 = 0.13$; 1, $p > 0.05$) (Figure 12).

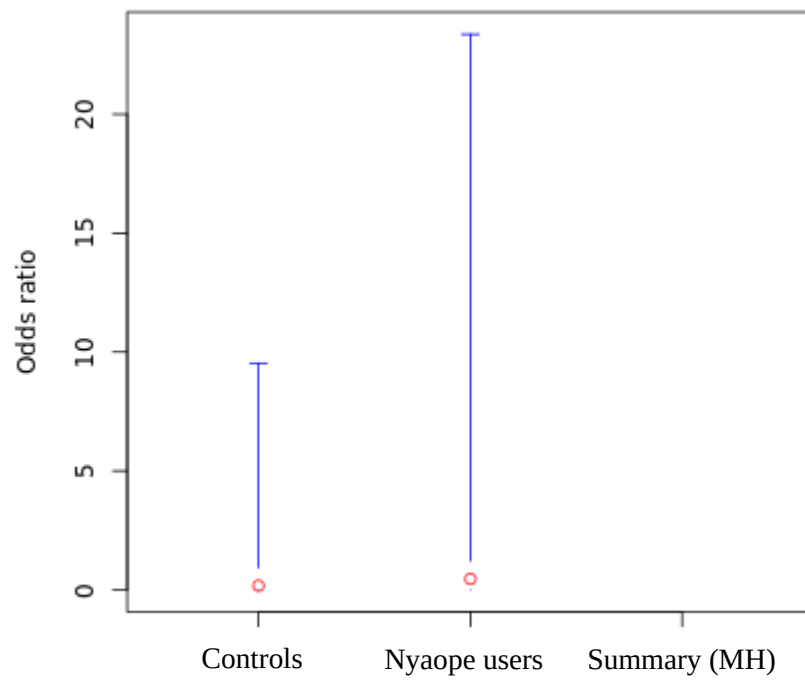


Figure 12: 95% confidence intervals for Mantel-Haenszel odds ratios for SNP rs1799971 and DNA methylation. No association shown between nyaope use and methylation levels.

Chapter 4: Discussion

The broad aim of this study was to investigate the epigenetic and genetic characteristics of the *OPRM1* gene in a black South African male population that uses nyaope, and to further assess if associations exist between any genetic variations observed and drug consumption. To the best of our knowledge, this is the first study to investigate *OPRM1* gene methylation pattern and the SNP genotyping in a South African population of heroin users.

Our data showed that overall, the total methylation levels were lower in nyaope-consuming participants than in controls when using the methylation assignment bias approach. Nyaope-consuming participants had significantly lower methylation levels when compared to control participants, with only CpG 29 showing substantial methylation in the control group. This observation suggests that the intake of nyaope could have induced a demethylation process at certain specific CpG sites. Such a mechanism is plausible as CpG sites at positions 15, 21, 23, 24, 26 and 28 were less methylated in nyaope-consumers than controls. Interestingly CpG 29 showed greater methylation in controls compared to nyaope-consumers and this may indicate a protective role in these participants.

In the partial methylation bias approach, our main findings showed no differences in the total methylation levels between the two groups. However, when further delineated, nyaope-consumers displayed significantly higher levels of partial methylation (at CpG sites 8, 18, 19, 20, 21, 23, 24, 25, 26, 27, 28), while control participants exhibited significantly higher unmethylation at sites (CpG sites 1, 2, 3, 4, 11, 16, 22, 25).

For SNP 118 A>G, the AG genotype frequency was higher in controls than in nyaope-consuming participants while other genotypes for both SNPS were broadly similar in both groups. While the frequency of SNP rs1799971 was greater in nyaope-consumers, SNP rs3778150 occurred more frequently in the control group. We could not find any haplotype relationship between SNP rs1799971 and rs3778150. Neither was there any correlation observed between nyaope consumption and the presence of SNP rs1799971 and/or rs3778150 in the study populations.

1. Methylation status of *OPRM1* gene in nyaope users and healthy controls.

DNA methylation is influenced by environmental factors such as temperature, nutrients and early life stress and factors such as age and gender (Burton & Metcalfe, 2014). Nyaope-consuming participants and controls were age and gender matched and composed of only black

South African males. Furthermore, recruitment of all participants was done from rehabilitation centres within the same location. This, together with factors such as similar cultural background, education levels and dietary behaviours, indicated that the nyaope-participants and the controls shared a comparable pool of environmental factors.

Evaluating the methylation status of all CpG sites in the promoter region of the *OPRM1* gene (using the methylation assignment bias approach), showed that most of these sites were unmethylated in both nyaope consumers and control participants. These results suggest that the intake of nyaope favoured the unmethylation of certain sites within the promoter region of the *OPRM1* gene that may lead to a state of hypomethylation in nyaope consumers. Other studies found methylation levels of *OPRM1* promoter to be higher in opioid addicts. For instance, hypermethylation of the *OPRM1* promoter was reported in a cohort of male opioid addicts (Chorbov *et al.*, 2011), as well as in a group of addicts that were undergoing methadone-maintenance treatment (Nielsen *et al.*, 2009). In a study that investigated DNA methylation of the *OPRM1* promoter across different ethnicities with former heroin users and controls, African Americans had the lowest methylation levels (hypomethylation) in comparison to the controls while other ethnicities such as Hispanics, had higher degrees of methylation in comparison to control participants (Nielsen *et al.*, 2010). Our findings were therefore in agreement with the results reported in the American study, showing that populations of African descendants have lower methylation levels.

Interestingly, only CpG 29 sites was substantially methylated in controls compared to nyaope-consuming participants in both approaches (methylation assignment bias and partial methylation bias approach). Hypermethylation in the promoter correlates to decreased *OPRM1* protein levels because methylation causes transcriptional silencing, resulting in decreased gene expression. Presumably, CpG 29 methylation may impede the binding of transcription factors such as *Sp1* and reduce *OPRM1* gene expression (Wang *et al.*, 2018). Hypermethylation has previously been associated with increased risk of heroin dependence as it may predispose individuals to the development of heroin addiction (Ebrahimi *et al.*, 2018; Doebling *et al.*, 2013; Oertel *et al.*, 2012; Nielsen *et al.*, 2009). That hypermethylation was suggested to prevent the upregulation of MOR, decreasing MOR expression and increasing the affinity of the MOR to endogenous opioids (Oertel *et al.*, 2012).

In a study conducted by Toskulkao and co-workers (2010), the authors reported a significant reduction in opioid receptor expression on the lymphocytes of heroin users on methadone-

maintenance treatment (i.e., opioid replacement therapy). This opioid-mediated down-regulation of MOR was confirmed by *in vitro* experiments where MOR expression was decreased on lymphocytes from naïve subjects following methadone treatment. This methadone effect was blocked by treating the lymphocytes with naloxone (Toskulkao *et al.*, 2010), an opioid antagonist that displaces bound opioids from their receptors (Mueller *et al.*, 2015). While the data of the study supported a heroin-induced decrease in MOR, it does not show that the reduction is mediated by methylation processes.

This concern has however been addressed by Hwang *et al.*, (2010) who used neuronal cells to demonstrate that methylation of CpGs which blocks the binding of the transcription factor *Sp1* to the promoter region of the *OPRM1* gene as well as enhancing the binding of the repressor MeCP2, to the promoter region. The authors suggested that MeCP2 and DNA methylation mediate the remodelling of *OPRM1* to a conformation state that allows transcriptional factors to access the promoter resulting in upregulation. There is therefore evidence, albeit indirect, that the hypomethylation, which does not promote MeCP2 binding, of the promoter region of the *OPRM1* gene observed in nyaope-consumers could have led to a decrease in MOR expression, thereby posing a mechanism to drive tolerance and subsequent addictive behaviour in these individuals.

DNMT3A and DNMT3B are two enzymes that oppose active demethylation thereby contributing to DNA methylation maintenance (Ginno *et al.*, 2020; Charlton *et al.*, 2020). These enzymes are responsible for the methylation of CpG sites that are usually 8 to 10 bp apart, or distances that are multiples of 9bp (Zhang *et al.*, 2009; Jia *et al.*, 2007). It was therefore not surprising that in the nyaope-consuming participants many CpG sites after CpG 16 that were about 8 to 10 bp apart, were mainly partially methylated using the partial methylation approach. This high degree of partial methylation was not seen in the control group. In addition, the demethylation of DNA induces the increase in the expression of the *OPRM1* gene which would be essential to mediate the effects of nyaope especially as a result of long-term use. Collectively these findings suggested that in some cases partial methylation may have significant physiological effects that may impact the function of cells. Its role in nyaope (or heroin) - induced consequences, however, remains to be elucidated.

2. SNP rs1799971 and SNP rs3778150

2.1 Frequency

Studies involving twins have pointed towards a genetic component that renders certain individuals vulnerable to opioid addiction (van de Bree *et al.*, 1998; Kendler *et al.*, 2003). These findings have spearheaded a series of investigations into the genome of persons suffering from substance use disorders. Subsequently a number of SNPs have been identified.

The prevalence of rs1799971 was greater in nyaope-consuming participants compared to controls, while rs3778150 was more prevalent in the control group of participants. SNP rs1799971 occurs in exon 1 of the *OPRM1* gene at position 118. It changes an adenine nucleotide to guanine (A>G) such that the corresponding amino acid is changed from aspartic acid to asparagine. Since the 118 position occurs on the N-terminal of the MOR (N40D), it modifies the functioning of this receptor that may include a reduction in downstream receptor signalling (Oertel *et al.*, 2012). SNP 118A>G has been reported to be more frequent in Caucasians (17%) and Asians (48%) than in African Americans (2.2%) and sub-Saharan Africans (0.8%) (American National Centre for Biotechnology Information). In our study cohort (black South African males), a frequency of 4,5% was recorded for SNP 118 A>G. This finding is in line with reports stating lower frequencies in African descendants (Hancock *et al.*, 2015). On the other hand, SNP rs3778150 frequency was reported at 18% for the African American population (Hancock *et al.*, 2015). In our study, an SNP rs3778150 frequency of 74% was obtained, this was much higher than that recorded by Hancock *et al.*, (2015).

2.2 SNPs and nyaope association

In our study, the SNP 118 A>G was more frequent in the control participants than in nyaope consumers. The results show that SNP 118 A>G is not associated with heroin addiction in South African black males. To date, SNP 118 A>G has not been conclusively associated with heroin addiction. The SNP has been significantly associated with predisposed vulnerability to addiction, protective properties against addiction and some studies have shown no significant association to the SNP (Szeto *et al.*, 2001; Zhang *et al.*, 2007; Bond *et al.*, 1998; Hancock *et al.*, 2015). The clinical relevance SNP 118 A>G in opioid addiction remains debatable even though it has biological plausibility. SNP rs3778150 has been associated with decreased *OPRM1* expression and therefore a role for this SNP in heroin addiction has been proposed, especially in population of European ancestry (Hancock *et al.*, 2015). Of note, the same authors found that rs1799971 was also associated with heroin addiction, but only when rs3778150 was

present. These results reflect the strong interaction between the two SNPs and their prominent role in the development of addictive behaviour.

2.3 SNP rs3778150 and rs1799971 genotypes correlation

In contrast to the study of Hancock *et al* (2015), our data showed no association between SNP 118 A>G and nyaope consumption despite the presence of rs1799971 and rs3778150. Our results underline the inconsistency of current data regarding SNP 118 A>G and its association with heroin addiction. While SNP rs1799971 has been significantly associated with predisposed vulnerability to addiction, as well as protective properties against addiction, other studies have shown no significant association to the SNP (Szeto *et al.*, 2001; Zhang *et al.*, 2007; Bond *et al.*, 1998; Hancock *et al.*, 2015).

2.4 Correlation of SNP genotype with the methylation status

SNP 118 A>G has been shown to introduce a new methylation site in the *OPRM1* gene between CpG 16 and CpG 17 as it converts a CA dinucleotide to CG (Oertel *et al.*, 2012). This new CpG site changed methylation patterns downstream from its position, increasing the methylation levels at the three subsequent CpG which overlap with the two *Sp1* transcription factor binding sites and thereby decreasing gene expression. This was not seen in our study as methylation levels as SNP 118 A>G have lower frequencies in our population.

Limitations

Notwithstanding the results obtained, a number of limitations in the study were also identified. For investigating population genetics, our sample size was comparatively small. Furthermore, the study population consisted of only South African black males. Since genetic and epigenetic profiles vary by populations, comparisons between studies were limited. Future studies should include other populations in the genetic-epigenetic investigations. While there was a focus on studying the impact of methylation on *OPRM1* gene expression, the actual measurement of *OPRM1* protein levels would have strengthened our finding and subsequent proposal of nyaope-induced hypomethylation of the promoter region of the *OPRM1* gene leading to MOR down-regulation. Lastly, only one gene was investigated, i.e., the *OPRM1* gene. Addiction is a complex disorder that involves many different structures and neurotransmitter systems in the brain. This complexity suggests that other genes may also participate in the development of addiction. It would therefore be beneficial to investigate multiple genes and epigenetic other epigenetic mechanisms in future.

Conclusions

In conclusion, the present study showed that the black South African male population have low frequencies of the mutant alleles of SNPs rs1799971 and rs3778150 and are therefore unlikely to be significant risk factors for the development of nyaope addiction in this population demographic. Contrary to some literature, which has been shown to associate SNP rs1799971 with addiction, the results of this study did not provide evidence for a haplotype relationship between SNP rs1799971 and SNP rs3778150 or an association of these SNPs with the development of nyaope addiction. The *OPRM1* promotor methylation was not influenced by the presence of SNP rs1799971. However, the *OPRM1* promotor methylation was low in nyaope consuming participants. This decrease in *OPRM1* promotor methylation suggests an upregulation of the MOR protein. This study provided evidence that SNPs rs1799971 and rs3778150 did not alter the methylation status of the *OPRM1* gene or contribute to nyaope addiction in this population. However, nyaope use may have influenced *OPRM1* promoter methylation such that there is downregulation of MOR protein, which should be investigated in future studies. I believe that the findings of this study are a novel contribution to the body of literature as, to the best of our knowledge, this is the first study to investigate *OPRM1* gene methylation patterns and the frequency of these specific SNPs in a South African population of nyaope users. Further investigation is required to define the precise link between genetic-and-epigenetic alterations and the development of opioid addiction.

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R14/49 Dr N Morgan, et al

**HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
CLEARANCE CERTIFICATE NO. M1704100**

NAME:
(Principal Investigator)
DEPARTMENT:

Dr N Morgan, et al
School of Clinical Medicine
Department of Psychiatry
Medical School
University

PROJECT TITLE:

A prospective cohort study of heroin users attending rehabilitation: assessing psychiatric co-morbidities, DNA methylation and treatment outcomes

DATE CONSIDERED:

05/05/2017

DECISION:

Approved unconditionally

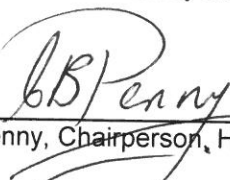
CONDITIONS:

Change of study title noted on 21/11/2019

SUPERVISOR:

Professors U Subramaney and W Daniels

APPROVED BY:


Dr CB Penny, Chairperson, HREC (Medical)

DATE OF APPROVAL:

28/06/2017

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Research Office Secretary on the 3rd Floor, Phillip Tobias Building, Parktown, University of the Witwatersrand, Johannesburg.
I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to submit details to the Committee. I **agree to submit a yearly progress report**. When a funder requires annual re-certification, the application date will be one year after the date when the study was initially reviewed. In this case, the study was initially reviewed in **April** and will therefore reports and re-certification will be due early in the month of **April** each year. Unreported changes to the application may invalidate the clearance given by the HREC (Medical).

Principal Investigator Signature

Date

PLEASE QUOTE THE CLEARANCE CERTIFICATE NUMBER IN ALL ENQUIRIES