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RESEARCH ARTICLE

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ALTERATIONS IN METHYLATION AND SNPS OF *OPRM1* GENE SHOW LACK OF ASSOCIATION WITH HEROIN (NYAOPE) CONSUMPTION IN SOUTH AFRICAN MALES OF AFRICAN ANCESTRY[1]

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SUMMARY

Background: Nyaope is a highly addictive heroin derivative that elicits its effects via the μ-opioid receptor. Gene mutations and (cytosine-phosphoguanosine) CpG methylation, alter the function of the μ-opioid receptor gene (*OPRM1*) and protein. The single nucleotide polymorphisms (SNPs) rs1799971 (118 A>G) and intronic rs3778150 (T>C) in the *OPRM1* have previously been associated with heroin use. The SNP rs1799971 can influence methylation levels of *OPRM1* and alter mRNA and protein expression. This study compared the degree of *OPRM1* methylation and allele frequency of SNPs rs1799 971 and rs3778150 in black South African male nyaope users (n=200) to age-and gender-matched controls (n=55).

Material/ Methods: DNA was extracted from whole blood samples, and analysed by methylation-specific PCR and sequencing to determine methylation frequencies across 29 CpGs within the *OPRM1*promoter gene region. SNPsrs1799971 (118 A>G) and rs3778150 (T>C) were assessed using SNP genotyping Taqman assays.

Results: Using a positive methylation assignment method, most CpGs were significantly more unmethylated in nyaope users than in controls. Using a partial methylation assignment method, most CpGs displayed significantly higher percentages of partial methylation in nyaope users than in controls. 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. There was no correlation observed between nyaope consumption and the presence of SNP rs1799971 and/or rs3778150 in the study populations.

Conclusions: This study provided evidence that methylation may not be associated with the intake of nyaope and that SNPs rs1799971 and rs3778150 did not alter the methylation status of the *OPRM1* promoter gene or contribute to nyaope addiction in a black South African male population.

Key words: genetics, epigenetics, addiction, opioid

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INTRODUCTION

The opioid system plays a vital role in mediating responses to pain, stress, and euphoria (Zagon & McLaughlin, 2017). Endogenous opioids, produced by neurons, alleviate pain by binding to specific brain receptors (Zagon & McLaughlin, 2017). Exogenous opioids are used both medically and recreationally to mimic these effects, inducing feelings of euphoria (Zagon & McLaughlin, 2017). Heroin, a potent opioid, binds to μ-opioid receptors (MORs), triggering the release of dopamine and resulting in euphoria (Johnson & North, 1992). However, prolonged heroin use leads to the development of tolerance, requiring higher doses to achieve the same effects, eventually culminating in addiction (Barker, Taylor, De Vries, & Peters, 2015). This cycle of addiction presents significant challenges, particularly in the context of substances like nyaope in South Africa, which is derived from heroin and poses serious social and economic implications (Fernandes & Mokwena, 2016).Tolerance and addiction involve a complex interplay of genetic and epigenetic factors. Epigenetic mechanisms, including DNA methylation, play a pivotal role in regulating gene expression (Nestler, 2014). In the case of the *OPRM1* gene, which encodes MORs, DNA methylation in its promoter region can decrease the expression of MORs (Nielsen et al., 2009). This link between DNA methylation and heroin addiction has been observed in certain populations, notably Caucasians (Chorbov, Todorov, Lynskey, & Cicero, 2011; Nielsen et al., 2009).Single nucleotide polymorphisms (SNPs) further contribute to individual variations in opioid response. SNP rs1799971 (118 A>G) in the *OPRM1* gene alters the sensitivity of MORs to endogenous opioids, potentially necessitating higher doses for the same effects (Bond et al., 1998). This SNP is more prevalent in Caucasians and Asians compared to other ethnic groups (“National Center for Biotechnology Information”). Another SNP, rs3778150, located in the intron region of *OPRM1* gene, has been linked to altered *OPRM1* gene expression and addiction vulnerability (Hancock et al., 2015). This SNP is of particular interest due to its association with addiction across different populations. The interaction between genetics and epigenetics adds another layer of complexity. The SNP rs1799971 (118 A>G) introduces a methylation site that affects MOR expression (Oertel et al., 2012). Methylation at this site impacts the binding of transcription factors, influencing gene expression levels (Oertel et al., 2012). Understanding these genetic-epigenetic interactions is crucial in comprehending addiction mechanisms. In this context, the current study investigated the epigenetic and genetic aspects of the *OPRM1* gene in a specific population of South African nyaope users. This study sought to determine the DNA methylation status of the *OPRM1* promoter in this population and explored whether associations exist between methylation patterns and heroin use. Additionally, the current study investigated the presence of SNPs, including the significant rs1799971 (118 A>G) and rs3778150 (T>C), and their potential impacts on addiction susceptibility.

MATERIAL AND METHODS

Participants and sample collection

This study was approved by the Human Research Ethics Committee (Medical) of the University of the Witwatersrand, clearance number M1704100. The study conformed to the standards set by the latest revision of the Declaration of Helsinki and written informed consent was obtained from all participants.

Male black South African nyaope users aged 18 to 45 years (n = 200) were recruited from in-patient programs at the Empilweni and Life Randfontein rehabilitation centers, Johannesburg, South Africa, as previously described (Morgan, Daniels, & Subramaney, 2019). Healthy non-drug dependent age-and gender-matched controls (n = 55) were recruited from the community in Soweto, South Africa. Venous blood (5ml EDTA tube) was collected from nyaope users and control participants.

DNA extraction

DNA was extracted from peripheral white blood cells of participants using the "salting-out" method (Miller, Dykes, & Polesky, 1988). Briefly, cells from anticoagulated blood (EDTA tubes) were collected and resuspended in 15 ml polypropylene centrifugation tubes along with 3 ml of lysis buffer (10 mMTris-HCl, 400 mMNaCl, and 2 mM Na_2EDTA, pH 8.2). The cell lysates underwent overnight digestion at 37°C with 0.2 ml of 10% SDS and 0.5 ml of a protease K solution (1 mg protease K in 1% SDS and 2 mM Na_2EDTA). After digestion, 1 ml of saturated 6M NaCl was added to each tube, followed by vigorous shaking for 10 seconds, and centrifugation (2500rpm, 4 ºC, 30 minutes, Avanti J-26S Series, Beckman Coulter). The protein pellet settled at the tube's bottom, and the DNA-containing supernatant was transferred to another 15 ml polypropylene

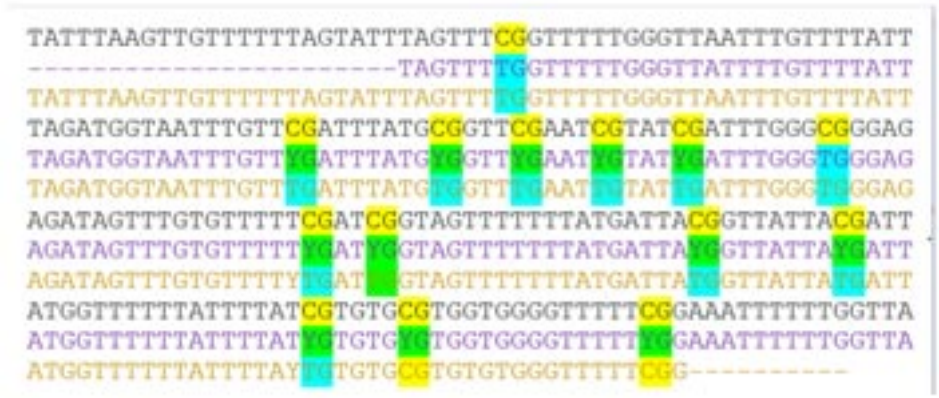


Fig. 1. A gene sequence snip from a patient sample. The bisulphite converted parent strand (black), forward strand (purple) and complementary strand (gold) are shown in the figure. CpG sites are highlighted in yellow, unmethylated CpG sites are represented as TG and are highlighted in blue. Ambiguous nucleotides assigned as "Y", which represent a mixture of pyrimidines (>25% C or T) were considered indicative of partial methylation

tube. To precipitate the DNA, exactly 2 volumes of cold (4°C) absolute ethanol were added and mixed by inverting the tubes. The resulting DNA strands were carefully transferred to a 1.5 ml microcentrifuge tube containing Tris-EDTA buffer (10 mMTris-HCl, 0.2 mM Na_2EDTA, pH 7.5). The DNA was allowed to dissolve for 2 hours at 37°C. DNA concentration and quality were determined using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Life Technologies, Carlsbad, USA) and stored at 4 ºC until further analysis.

Bisulfite conversion and methylation-specific polymerase chain reaction (PCR)

The overnight protocol of the Epijet bisulfite conversion kit (Thermo Scientific) was used, according to the manufacturer's instructions to convert unmethylated cytosine residues of DNA samples (250 ng) to uracil and the converted DNA was eluted with Tris-EDTA buffer (50 µl). 419bp at the 5' end of the *OPRM1*gene (Homo sapiens chromosome 6, GRCh38.p14; NCBI reference sequence: NC_000006.12) were amplified using two overlapping PCR reactions. Forward primer sequences for the bisulfite-converted DNA were 5'-TTAGGATTGGTTTTTGTAAGAAATAG-3' (primer set 1) and 5'-TATTTAAGTTGTTTTTTAGTATTTAG-3' (primer set 2) and reverse primer sequences were 5'-CTAAATAAAACAAATTAACC CAAAAACC-3' (primer set 1) and 5'-CAATCACATACATAACCAAAAAATTTCC-3' (primer set 2). The sequence contained 29 CpG sites. Bisulfite-converted DNA (2 µl) was mixed with Kapa HifiHotstart Taq polymerase (12 µl, Kapa Biosystems, Inc., Wilmington MA 01887, United States), forward primer (0.5 µl, 0.25 µM) and reverse primer (0.5 µl, 0.25 µM) in a final volume of 25 µl and amplified using 180 seconds at 95 ºC, followed by 35 cycles of 30 seconds at 95 ºC, 40 seconds at 46ºC and 60 seconds at 72ºC, followed by a final extension step of 7 minutes at 72ºC and holding at 4ºC. PCR products (5 µl) were visualized using 2% agarose gels and PCR reactions that showed presence of bands at 259 bp (primer set 1) or 244 bp (primer set 2) were sequenced (InqabaBiotec, Pretoria, South Africa). Both strands of the PCR products were sequenced in order to obtain high quality sequence data over the full lengths of the amplicons.

Analysis of PCR product sequences and methylation assignment

A manual method of analyzing the sequencing data was developed to overcome the ambiguous methylation assignments that were generated by the conventional BiQAnalyzer software (Max Planck Institute for Informatics, Saarland University, Germany). The software reports ambiguous base insertions within a CpG context as an unknown methylation state, which is not represented by the methylation analysis plots (Bock et al., 2005). Therefore, FinchTV software (Geospiza, 2012) was used to visualize the sequences of the forward and reverse strands of the PCR products. Each of the four DNA sequences for each blood sample was aligned with the *OPRM1*sequence using EMBOSS Matcher (Rice, Longden, & Bleasby, 2000). The CpG sites were then manually identified as methylated (C), unmethylated (T) or ambiguous (Y, >25% C or T), when com-

pared with the *OPRM1* sequence. The Y nucleotide assignments were present only in CpG sites and were not present for C residues outside of CpG sites, which can only be unmethylated (Bock et al., 2005). We deduced from this that bisulfite conversion was complete, that sequencing data was reliable and that the presence of both C and T residues at certain CpG sites in the PCR products showed that patient samples contained mixtures of DNA in which only some copies were methylated, i.e., many CpG sites were partially methylated (intermediate methylation).

We used two different manual decision processes to assess 'potential' partial methylation (table 1). In the first approach; positive methylation assignment, 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 methylated. 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 was assigned to that CpG site for that patient sample. CpG sites were only identified as partially methylated if both the forward and reverse sequences showed 'y' (Table 1). In the second approach; partial methylation assignment, we used the presence of 'y' to identify all possible partially methylated CpG sites. In this approach, if either sequence showed 'y', the CpG site was identified as partially methylated, and 'c' or 't' was only assigned if both sequencing reactions showed the same base.

SNP Genotyping Assay

Genotyping for SNPs rs1799971 and rs3778150 was performed using TaqMan® SNP Genotyping Assays (Assay ID: C___8950074_1 and C__29641067_10, Thermo Fisher Scientific, Life Technologies, Carlsbad, USA) in a 96 well

Tab. 1. Examples of manual analysis methods for DNA methylation. In the positive methylation assignment method; if the y nucleotide in one strand corresponded with a t in another strand it was recorded as unmethylated. In the positive methylation assignment method; if the y nucleotide in one strand corresponded with a c in the other strand it was recorded as methylated. In partialmethylation assignment method, the y nucleotide in one strand was recorded as a partiallymethylated if there was a corresponding t or a c in the other strand. The two results for each sample are for the forward and reverse sequencing calls

Example sequencing result	Nucleotide	Positive methylation assignment		Partial methylation assignment	
		Decision	Interpretation	Decision	Interpretation
1 forward	t	T	Unmethylated	T	Unmethylated
1 reverse	t				
2 forward	t	T	Unmethylated	Y	Partial methylation
2 reverse	y				
3 forward	y	C	Methylated	Y	Partial methylation
3 reverse	c				
4 forward	c	C	Methylated	C	Methylated
4 reverse	c				
5 forward	y	Y	Partial methylation	Y	Partial methylation
5 reverse	y				
6 forward	c	A	Ambiguous	A	Ambiguous
6 reverse	t				

plate format. Genomic DNA was combined with TaqMan® genotyping mastermix (Thermo Fisher Scientific, Life Technologies, Carlsbad, USA) and amplified using a StepOne Plus thermocycler (Thermo Fisher Scientific, Life Technologies, Carlsbad, USA) according to the manufacturer's instructions. 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 for the major allele (AA), homozygous for the minor allele (GG) or heterozygous (AG) for SNP rs1799971 and homozygous (TT), homozygous (CC) or heterozygous (TC) for SNP rs3778150.

Data analysis

Data analysis was performed using Graphpad Prism Version 7 (Graphpad software Inc, SanDiego, CA). The Chi-square test was used to compare the methylation status of *OPRM1* in nyaope users and healthy controls and to assess association between *OPRM1* methylation and heroin use. Fisher's exact test was used to determine the frequencies of *OPRM1* SNP rs1799971 and SNPrs3778150 in nyaope users and compared to that of the controls. Results were considered statistically significant if $p < 0.05$. The Hardy-Weinberg Equilibrium (HWE) principle was also used to assess the allele frequency of the two SNPs, where:

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

p is the frequency of the major allele.

q is the frequency of the minor allele.

p^2 is the frequency of individuals with the homozygous major genotype.

$2pq$ is the frequency of individuals with the heterozygous genotype.

q^2 is the frequency of individuals with the homozygous minor genotype.

The Cochran-Mantel-Haenszel test was used to identify the level of correlation between the SNP genotype data with 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 the controls to test for haplotype relationships.

RESULTS

The sequencing analysis data showed a large number of ambiguous nucleotides designated 'y', which resulted in an inability to assign methylation status for many CpG sites using the conventional methylation analysis BiQAnalyzer software (Max Planck Institute for Informatics, Saarland University, Germany). The participants CpG methylation status data was presented graphically and analysed statistically if their methylation status was detected as methylated, un-

methylated or partially methylated using the positive methylation assignment method and the partial methylation assignment method, as detailed above (table 1).

Methylation analysis

% Total methylated CpG sites

The DNA methylation percentages of CpG islands in the promoter region of *OPRM1* were compared between 200 nyaope users and 55 healthy non-drug consuming controls using Fishers exact test. Using the positive methylation assignment method, we found that overall, there was a significantly lower % of methylated CpG sites observed in nyaope users compared to controls ($p = 0.0275$). The partial methylation assignment method showed no significant differences in overall % methylated CpG sites between the two groups ($p= 0.2017$).

Methylation at individual CpG sites using the positive methylation assignment method

Using the positive methylation assignment method, most CpG sites were unmethylated across all samples in both groups. The % of unmethylated CpG sites was significantly higher in nayope users compared to controls; specifically at sites 15 ($p < 0.001$), 21 ($p= 0.0047$), 23 ($p= 0.0082$), 24 ($p= 0.0148$), 26 ($p= 0.0243$) and 28 ($p < 0.0001$, table 2). The % of partial methylation at CpG 26 was significantly higher in nyaope users when compared to controls ($p = 0.0053$, table 2). The % of methylated CpG sites was significantly higher at CpG 29 in control participants ($p = 0.0052$) when compared to nyaope users.

Methylation at individual CpG sites using the partial methylation assignment method

Using the partial methylation assignment method, the % of partially methylated CpG sites was significantly higher in nyaope users than controls, specifically at CpG sites 8 ($p = 0.048$), 18($p = 0.0370$), 19 ($p =0.0034$), 20 ($p= 0.0007$), 21 ($p = 0.0097$), 23 ($p= 0.0003$), 24 ($p< 0.0001$), 25 ($p< 0.0001$), 26 ($p = 0.0008$), 27 ($p= 0.0006$) and 28 ($p= 0.0003$, table 3). Controls showed higher % of unmethylated CpG sites than nyaope users, specifically at sites 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 ($p= 0.0015$, table 3). Nyaope users showed higher % of methylated CpG sites than controls, specifically at sites; CpG 15 (0.0025) and CpG 29 ($p= 0.0053$, table 3).

SNP frequencies

Genotype frequency of SNP rs1799971 and SNP rs3778150

The AA genotype of SNP rs1799971 was predominant in both nyaope users and control participants (Table 4). There was a significant difference ($\chi 2 =18.55$; 1, $p < 0.0001$) between the genotype frequency of nyaope users (100% for AA,

Tab 2. Percentage methylation at 29 CpG sites within the OPRM1 promoter region in nyaope users and control participants using the positive methylation assignment method

	% DNA methylation Nyaope users (n = 200)			% DNA methylation Controls (n = 55)		
CpG	Methylated	Unmethylated	Partially methylated	Methylated	Unmethylated	Partially methylated
1	0	97	3	0	97	3
2	0	99	1	0	98	2
3	1	96	3	1	97	2
4	0	100	0	0	100	0
5	0	98	2	0	100	0
6	0	99	1	0	100	0
7	0	100	0	0	99	1
8	5	93	2	0	91	9
9[a]	1	96	3	0	95	5
10[a]	2	97	1	0	98	2
11	0	99	1	0	100	0
12[a]	0	99	1	0	99	1
13	1	81	18	1	95	4
14	0	99	1	0	100	0
15	12	63[#]	25	25	35	40
16[ab]	1	90	9	0	100	0
17[b]	1	81	18	0	80	20
18	1	79	20	0	65	35
19	1	97	2	1	96	3
20	0	96	4	1	97	2
21	1	98[#]	1	0	85	15
22	1	97	2	0	99	1
23	1	83[#]	16	1	69	30
24	2	83[#]	15	10	66	24
25	2	90	8	4	87	9
26	1	79[#]	20[*]	3	84	13
27	12	70	18	20	62	18
28	30	51[#]	19	40	20	40
29	44[¶]	31	25	65	15	20

[a]1ndicates Sp1, potential binding site for Sp1 transcription activator; [b] indicates SNP 118 A>G locations; [¶] indicates methylated CpG sites; [#] indicates unmethylated CpG sites and * indicates partially methylated CpG sites, respectively ($p < 0.05$)

0% for AG and GG; HWE = 1: p =1, q= 0) and controls (90.9% for AA, 9.1% for AG and 0% for GG; HWE=1: p =0.91, q= 0.09) for SNP rs179997, with nyaope users showing significantly greater frequency of the major allele. The TT genotype of SNP rs3778150 intronic variant was predominant in both nyaope users and controls (table 4). The genotype frequencies of nyaope users (1% for CC, 31% for CT and 68% for TT; HWE = 1: p =0.32, q= 0.68) did not differ significantly ($\chi 2 = 1.469$; 2, p = 0.4798) from those of controls (1.82% for CC, 21.82% for CT and 76.36% for TT; HWE = 1: p = 0.24, q = 0.76).

Allele frequency distribution of SNP rs1799971

Results of the Chi-square test revealed that the G allele frequency, or allelic distribution of minor allele for SNP rs1700071, in the nyaope users (1%) was lower than that of the controls (8%) ($\chi 2= 16.99$, 1, p = 0.0001). There were no

Tab. 3. Percentage methylation at 29 CpG sites within the OPRM1 promoter region in nyaope users and control participants using the partial methylation assignment method

	% DNA methylation Nyaope users (n = 200)			% DNA methylation Controls (n = 55)		
CpG	Methylated	Unmethylated	Partially methylated	Methylated	Unmethylated	Partially methylated
1	0	48	52	0	98#	2
2	0	58	42	0	85#	15
3	0	38	62	0	88#	22
4	0	58	42	0	90#	10
5	0	62	38	0	75	25
6	0	92	8	0	97	3
7	0	68	32	0	91#	9
8	0	28	72*	0	45	55
9[a]	0	42	58	0	58	42
10[a]	0	60	50	0	70	30
11	0	64	36	0	88#	12
12[a]	0	82	18	0	92	8
13	0	62	38	0	45	55
14	0	90	10	0	88	12
15	21¶	9	70	5	22	73
16[ab]	0	100	0	2	88	10
17[b]	0	48	52	2	48	50
18	0	18	82*	0	32	68
19	0	25	75*	0	45	55
20	0	20	80*	0	40	60
21	0	28	72*	1	48	51
22	0	48	52	1	67#	32
23	0	18	82*	1	42	58
24	0	12	88*	1	48	51
25	5	18	77*	2	51#	47
26	0	12	88*	1	39	60
27	1	8	91*	3	31	66
28	5	0	95*	10	20	70
29	58¶	10	32	42	32#	26

[a] indicates Sp1, potential binding site for Sp1 transcription activator; [b] indicates SNP 118 A>G locations; ¶ indicates methylated CpG sites; # indicates unmethylated CpG sites and * indicates partially methylated CpG sites, respectively ($p < 0.05$).

differences in allelic distribution of the major allele between the two groups ($p > 0.05$). ***Allele frequency distribution of SNP rs3778150*** Results of the Chi-square test revealed that the T allele frequency, or allelic distribution of minor allele for SNP rs3778150, was the same between the two groups (24%) ($\chi 2 = 0.02049$; 1, $p = 0.8862$). There were no differences in allelic distribution of the major allele between the two groups ($p > 0.05$). ***SNP rs1799971 and OPRM1 methylation***The data indicated that no associations can be made between SNP rs1700071 (118 A>G), and the *OPRM1* methylation indices in black South African males ($\chi 2 = 0.13$; 1, $p > 0.05$, (figure 2). Figure 2: Mantel-Haenszel odds ratios (95% confidence intervals) for SNP rs1799971 and DNA methylation. No association shown between nyaope use and *OPRM1* methylation.

Tab. 4. Genotype frequency distribution of SNP rs1799971 and SNP rs3778150 polymorphism in nyaope users 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 (1.82%)	12 (21.82%)	42 (76.36%)	55
Users	200 (100%)	0 (0%)	0 (0%)	200	2 (1%)	62 (31%)	136 (68%)	200

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

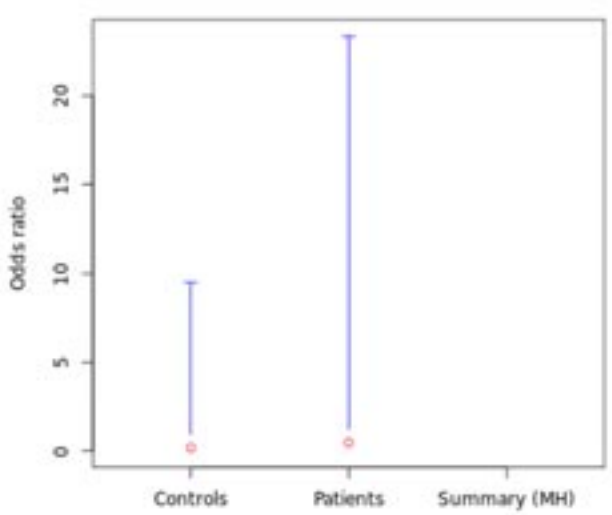


Figure 2: Mantel-Haenszel odds ratios (95% confidence intervals) for SNP rs1799971 and DNA methylation. No association shown between nyaope use and OPRM1 methylation

DISCUSSION

The overall aim of this study was to investigate several epigenetic and genetic characteristics of the *OPRM1*gene in a South African male population of African ancestry that uses nyaope, and to further assess if associations exist between any genetic variations observed and nyaope consumption. To the best of our knowledge, this is the first study to investigate *OPRM1* gene methylation patterns and the genotypes of SNPs rs1700071 and rs3778150 in a South African population of nyaope users.

Our data showed that when using the positive methylation assignment method, the % of unmethylated CpG sites was higher in nyaope users than in controls. Nyaope users had significantly less % of methylated CpG sites when compared to controls, with CpG 29 showing substantial methylation in the control group. This observation suggests that the intake of nyaope, which contains high levels of the opioid heroin, 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 showed a higher % of unmethylated CpG sites in nyaope users than controls. Interestingly CpG 29 showed a higher % of methylation in controls compared to nyaope users and this may indicate a protective role in these control participants, however these results will have to be validated by cloning. When using the partial methylation assignment method, our main findings showed that nyaope users displayed significantly higher % of partially methylated CpG sites than controls (CpG sites 8, 18, 19, 20, 21, 23, 24, 25, 26, 27, 28), while control participants exhibited significantly higher % of unmethylated CpG sites (CpG sites 1, 2, 3, 4, 11, 16, 22, 25).

For SNP rs1799971, the AG genotype frequency was higher in controls than in nyaope users, while other genotypes for both SNPS were broadly similar in both groups. Both SNPs alleles were in a state of Hardy-Weinberg Equilibrium (HWE). There was no correlation observed between nyaope consumption and the presence of SNP rs1799971 and/or rs3778150.

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 users and controls were age and gender matched and our study population comprised of only South African males of African descent. Furthermore, recruitment of all participants was conducted from rehabilitation centres within the same geographical area. This, together with factors such as similar cultural background, education levels and dietary behaviours, indicated that the nyaope users and the controls shared a comparable pool of environmental factors.

Evaluating the methylation status of 29 CpG sites within the promoter region of the *OPRM1* gene (using the positive methylation assignment method), showed that most of these sites were unmethylated in both nyaope consumers and control participants. These results suggest that the intake of nyaope favoured a lack of methylation across specific sites within the promoter region of the *OPRM1* gene that may lead to a state of hypomethylation in nyaope users. 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 site 29 showed a degree of substantial methylation across both the controls and nyaope users when using either methylation eval-

uation method (positive methylation assignment method and/or partial methylation assignment method). 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 (Ebrahimi 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 (Doehring, Oertel, Sittl, & Lötsch, 2013; Ebrahimi et al., 2018; Nielsen et al., 2009; Oertel et al., 2012). 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, Pornchai, Akkarapatumwong, Vatanatunyakum, & Govitrapong, 2010), an opioid antagonist that displaces bound opioids from their receptors (Mueller, Walley, Calcaterra, Glanz, & Binswanger, 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 CpGsthat 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 (Hwang et al., 2010). 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 users of the current study, 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 (Charlton et al., 2020; Ginno 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 9 bp (Jia, Jurkowska, Zhang, Jeltsch, & Cheng, 2007). It was therefore not surprising that in the nyaope users of the current study, many CpG sites after CpG 16 that were about 8 to 10 bp apart, were mainly partially methylated using the partial methylation assignment method. 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 find-

ings 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.

SNP rs1799971 and SNP rs3778150

Studies involving twins have pointed towards a genetic component that renders certain individuals vulnerable to opioid addiction (Lin et al., 1996; Tsuang, Bar, Harley, & Lyons, 2001). 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 the minor allele for SNP rs1799971 was greater in nyaope users compared to controls, while the minor allele for SNP 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 rs1799971 (118 A>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%). In the cohort of the current study of South African males, a frequency of 4.5% was recorded for the minor allele of SNP rs1799971 (118 A>G). This finding is in line with reports stating lower frequencies for the minor allele in African descendants (Hancock et al., 2015). Alternatively, the minor allele distribution of SNP rs3778150 was reported at 18% for the African American population. In the current study, the minor allele distribution of SNP rs3778150 was 74%, which was substantially higher than that recorded by Hancock *et al.* (2015).

In the current study, results show that SNP rs1799971 (118 A>G) is not associated with nyaope addiction in South African males of African lineage. To date, SNP rs1799971 (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 (Bond et al., 1998; Hancock et al., 2015; Szeto, Tang, Lee, & Stadlin, 2001; Taqi, Faisal, & Zaman, 2019). The clinical relevance of SNP rs1799971 (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 populations 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.

In contrast to the study of Hancock *et al* (2015), our data showed no association between SNP rs1799971 (118 A>G) and nyaope consumption. Our results

underline the inconsistency of current data regarding SNP rs1799971 (118 A>G) and its association with heroin addiction. While SNP rs1799971 (118 A>G) 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 (Bond et al., 1998; Hancock et al., 2015; Szeto et al., 2001; Taqi et al., 2019).

SNP rs1799971 (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). Previous studies reported that this new CpG site changed methylation patterns downstream from its position, increasing the methylation levels at the three subsequent CpG sites, which are said to overlap with two *Sp1* transcription factor binding sites, thereby causing a decrease in gene expression. This was not observed in the current study, possibly due to the lower genotypic distribution frequency of the minor allele of SNP rs1799971 (118 A>G) in this study 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 males of African ancestry, whom we cannot exclude as potential users of other harmful substances such as nicotine and alcohol. Neither can we exclude the possibility that these individuals suffered from mental disorders such as depression, which may also influence their DNA methylation profiles. 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 measurement of *OPRM1* protein levels would have strengthened our findings 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 other epigenetic mechanisms in future studies.

CONCLUSIONS

In conclusion, the present study showed that South African males of African descent may have low frequencies of the minor alleles of SNPs rs1799971 and rs3778150 and are therefore, unlikely to be significant contributing factors for the development of nyaope addiction in this population demographic. Contrary to some literature, which has been shown to associate SNP rs1799971 with ad-

diction, the results of this study did not provide evidence for an association of these SNPs with the consumption of nyaope. The *OPRM1* promotor methylation was not influenced by the presence of SNP rs1799971. However, the *OPRM1* promotor methylation was low in nyaope users. This decrease in *OPRM1* promotor methylation suggests an upregulation of the MOR protein. Finally, this study provided evidence that SNPs rs1799971 and rs3778150 did not significantly alter the methylation status of the *OPRM1* gene.

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