THE RELATIONSHIP BETWEEN METHYLATION AND GENES ASSOCIATED WITH OPIOID RESPONSE IN HUMANS.

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ABSTRACT

"The relationship between methylation and genes associated with opioid response in humans."

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Opioids are used to alleviate pain however ~10–30% of the Caucasian population obtain ineffective pain relief and / or intolerable side effects. Genetic polymorphisms in opioid pharmacodynamic and pharmacokinetic important genes have been investigated however there has been a lack of conclusive results. Epigenetic gene regulation is another study field of interest. DNA methylation is a gene regulatory mechanism that has been associated with gene repression or expression. Thus it was hypothesised that variable opioid response was influenced by methylation alterations.

The promoter region methylation of *ABCB1/MDR1*, *CYP2D6* and *OPRM1* genes were analysed by pyrosequencing in smoking, opioid exposed and control pilot populations. Genetic variations within *ABCB1/MDR1, COMT, CYP2B6, CYP2D6* and *OPRM1* genes were also investigated. Tissue opioid concentrations were determined by HPLC-MS/MS and GC-MS/MS.

DNA methylation was influenced by chronic opioid exposure and / or the lifestyle associated with opioid dependency, but not by cigarette smoke exposure. An increase in DNA methylation was observed in the opioid exposed baby population but this was not indicative of development of neonatal abstinence syndrome (NAS). Associations between the *CYP2B6*6* genotype and NAS development were found. No relationship was observed between gene methylation and opioid response but variants in *OPRM1* and *ABCB1/MDR1* exhibited a relationship with opioid response in cancer pain.

This investigative pilot research revealed that some genetic variants can be used as diagnostic markers to predict susceptibility of methadone exposed babies to NAS development, and others are associated with variable opioid response in a cancer patient population. Although DNA methylation was not observed to be a contributory factor to opioid response, this research has ascertained the influence of chronic opioid exposure on DNA methylation of various biological samples. This finding is significant for future studies.

TABLE OF CONTENTS

Abstract **III**

Table of Contents **IV**

Table of Figures **IX**

Table of Tables **XII**

Acknowledgements **XIV**

Authors Declaration **XV**

List of Abbreviations **XVI**

CHAPTER 1.0 INTRODUCTION 1

1.1 Study rationale 3
1.2 Study aim 4
1.2.1 Objectives 4

CHAPTER 2.0 LITERATURE REVIEW 5

2.1 Opioids 5
2.1.1 Opioid adverse effects 9
2.1.1.1 Opioid tolerance 10
2.1.1.2 Opioid dependence 11
2.1.2 Opioid pharmacodynamics 12
2.1.2.1 μ-Opioid receptor distribution 12
2.1.2.2 μ-Opioid receptor structure 13
2.1.2.3 μ-Opioid receptor signal transduction 14
2.1.2.4 μ-Opioid receptor desensitisation, recycling and degradation 16
2.1.2.5 μ-Opioid receptor subtypes 18
2.1.2.5.1 Receptor splice variants 20
2.1.3 Opioid pharmacokinetics 23
2.1.3.1 Absorption and bioavailability 23
2.1.3.2 Metabolism 27
2.1.3.2.1 Morphine 27
2.1.3.2.2 Oxycodone 28
2.1.3.2.3 Methadone 29
2.1.3.3 Distribution 30
2.1.3.4 Elimination 32
2.1.4 Factors that influence opioid response 32
2.1.4.1 Age and opioid response 33
2.1.4.2 Organ dysfunction 34

2.1.4.3 Choice, dose and route of opioid administration 34
2.1.4.4 Drug-drug interactions 35
2.1.4.5 Prior opioid experience 36
2.1.4.6 Genetic variations 36
2.2 DNA methylation 42
2.2.1 Functions of DNA methylation 45
2.2.2 DNA methyltransferases (DNMTs) 48
2.2.2.1 *De novo* methylation and maintenance methylation 48
2.2.3 Mechanism of gene regulation by DNA methylation 51
2.2.4 Factors that influence DNA methylation 55
2.2.4.1 Diet 55
2.2.4.2 Age 58
2.2.4.3 Environmental and drug exposures 58
2.2.4.4 Smoking 60
2.2.5 DNA methylation and opioid genes 63
2.2.6 DNA methylation analysis 65

CHAPTER 3.0 GENERIC METHODS 69

3.1 Ethical considerations and patient recruitment 69
3.1.1 Does smoking affect buccal DNA methylation? 69
3.1.2 Methadone-prescribed opioid-dependant mothers and their new-born babies 70
3.1.3 Tissue specific DNA methylation 72
3.1.3.1 Dundee blood and tissue samples 72
3.1.3.2 Edinburgh tissue samples 72
3.1.4 Pilot trial: Personalising opioid therapy for cancer pain relief 73
3.1.4.1 Royal Bournemouth, Christchurch and Poole Hospitals 73
3.1.4.2 Royal Marsden Hospital 77
3.2 Sample collection 78
3.2.1 Does smoking affect buccal DNA methylation? 79
3.2.1.1 Oral swabs 79
3.2.2 Methadone-prescribed opioid-dependant mothers and their new-born babies 79
3.2.2.1 Oral swabs 79
3.2.2.2 Plasma samples 79
3.2.3 Tissue specific DNA methylation 80
3.2.3.1 Blood samples 80
3.2.3.2 Tissue samples 80
3.2.4 Pilot trial: Personalising opioid therapy for cancer pain relief 81
3.2.4.1 Oral swabs 81
3.2.4.2 Blood samples 81
3.3 Toxicological analysis 82

3.3.1 Methadone-prescribed opioid-dependant mothers and their new-born babies 82
3.3.2 Tissue specific DNA methylation 83
3.3.2.1 Determination of methadone – opioid overdoses 83
3.3.2.2 Determination of morphine – opioid overdoses 83
3.3.3 Pilot trial: Personalising opioid therapy for cancer pain relief 84
3.4 SNP and gene duplication/deletion analysis 86
3.4.1 DNA extraction 86
3.4.1.1 DNA from buccal swabs 86
3.4.1.2 DNA from blood and tissue 86
3.4.1.3 DNA from plasma 87
3.4.2 DNA quantification 87
3.4.3 DNA preparation and packaging for LGC 88
3.4.3.1 DNA quantity and volume 91
3.4.3.2 Packaging, transportation 92
3.5 DNA methylation analysis 93
3.5.1 Pyrosequencing method – trial 93
3.5.1.1 Primer design 93
3.5.1.2 Bisulfite conversion 94
3.5.1.3 PCR optimisation 95
3.5.1.4 ABI310 genetic analyser 98
3.5.1.5 Pyrosequencing 100
3.5.2 Designing methylation assays for *ABCB1/MDR1* and *CYP2D6* 102
3.5.2.1 Primers for *ABCB1/MDR1* and *CYP2D6* 103
3.5.2.2 Bisulfite conversion 104
3.2.2.3 PCR amplification 104
3.6 Statistical analysis 106
3.6.1 Normally distributed data 106
3.6.2 Non-normally distributed data 106

CHAPTER 4.0 DOES SMOKING INFLUENCE BUCCAL DNA METHYLATION? 107

4.1 Introduction 107
4.2 Materials and methods 107
4.2.1 Sample collection 107
4.2.2 Methylation analysis 107
4.3 Results 108
4.4 Discussion 111
4.4.1 Study limitations 112

CHAPTER 5.0 METHADONE-PRESCRIBED OPIOID-DEPENDANT MOTHERS AND THEIR NEW-BORN BABIES: Babies Born to Methadone-

Prescribed Opioid-Dependant Mothers have Elevated DNA Methylation on *ABCB1/MDR1*, *CYP2D6* and *OPRM1* 113

5.1 Introduction 113
5.2 Materials and methods 114
5.2.1 Sample collection 114
5.2.2 Toxicological analysis 114
5.2.3 DNA methylation analysis 115
5.3 Results 116
5.3.1 Methylation differences between methadone exposed and non-opioid exposed mothers and babies 116
5.3.2 Relationship between methylation and smoking / residential status 118
5.3.3 Relationship between methylation and NAS development 119
5.3.4 Methylation differences between paired plasma and buccal DNA samples 120
5.4 Discussion 121
5.4.1 Study limitations 126

CHAPTER 6.0 METHADONE-PRESCRIBED OPIOID-DEPENDANT MOTHERS AND THEIR NEW-BORN BABIES: The *CYP2B6*6* polymorphism protects babies exposed to methadone *in utero* from neonatal abstinence syndrome development 127

6.1 Introduction 127
6.2 Materials and methods 129
6.2.1 Sample collection 129
6.2.2 Toxicological analysis 129
6.2.3 SNP and gene duplication / deletion analysis 129
6.3 Results 130
6.3.1 Associations between *ABCB1/MDR1*, *COMT*, *CYP2B6*, *CYP2D6* and *OPRM1* genes and NAS development 131
6.3.2 Genetic variations and methadone dose and plasma concentrations 134
6.4 Discussion 136
6.4.1 Study limitations 137

CHAPTER 7.0 TISSUE SPECIFIC DNA METHYLATION: DNA methylation of *ABCB1/MDR1*, *CYP2D6* and *OPRM1* in individuals whose deaths were attributed to heroin toxicity 139

7.1 Introduction 139
7.2 Materials and methods 141
7.2.1 Sample collection 141
7.2.2 Toxicological analysis 141

7.2.3 SNP and gene duplication / deletion analysis 141
7.2.4 DNA methylation analysis 141
7.3 Results 142
7.3.1 Age and methylation 143
7.3.1.1 *ABCB1/MDR1* DNA methylation and age 143
7.3.1.2 *CYP2D6* DNA methylation and age 144
7.3.1.3 *OPRM1* DNA methylation and age 145
7.3.2 Gender and methylation 146
7.3.3 Intra-individual gene methylation tissue differences in opioid associated deaths 147
7.3.4 Intra-individual gene methylation tissue differences in controls 149
7.3.5 Opioid exposed tissues v non-opioid exposed tissues 151
7.3.6 Genetic variations v blood drug concentrations 154
7.4 Discussion 157
7.4.1 Methylation of *ABCB1/MDR1* gene 159
7.4.2 Methylation of *OPRM1* gene 160
7.5 Conclusion 162

CHAPTER 8.0 PILOT TRIAL: PERSONALISING OPIOID THERAPY FOR CANCER PAIN RELIEF. The influence of DNA methylation and SNPs on opioid response 163

8.1 Introduction 163
8.2 Materials and methods 164
8.2.1 Sample collection 164
8.2.2 Toxicological analysis 164
8.2.3 SNP and gene duplication / deletion analysis 164
8.2.4 DNA methylation analysis 164
8.3 Results 165
8.3.1 Methylation 170
8.3.1.1 Paired tissue gene DNA methylation comparison 170
8.3.1.2 Gene DNA methylation and effect of age in morphine / oxycodone responders and non-responders 170
8.3.2 SNP and gene duplication / deletion analysis 172
8.3.2.1 Morphine responders v morphine non-responders 172
8.3.2.2 Oxycodone responders v oxycodone non-responders 175
8.4 Discussion 179

CHAPTER 9.0 THESIS DISCUSSION 180

References

Appendices

TABLE OF FIGURES

Figure 2-1. Structures of naturally occurring alkaloids, semi-synthetic and synthetic opioids
Figure 2-2. World Health Organisation (WHO) pain step ladder
Figure 2-3. Diagrammatic representation of the μ-opioid receptor
Figure 2-4. Chromosomal location of *OPRM1* and layout of coding exons
Figure 2-5. Summary of opioid receptor signalling
Figure 2-6. Ligand-specific signalling complexes
Figure 2-7. Schematic of the human *OPRM1* gene and the MOR-1 splice variants
Figure 2-8. Vascular pathway of drugs absorbed from various systemic routes of administration and sites of first pass metabolism
Figure 2-9. Metabolism pathways of morphine
Figure 2-10. Metabolism pathways of oxycodone
Figure 2-11. Metabolism pathways of methadone
Figure 2-12. Schematic depiction of free drug movement through blood-tissue membranes
Figure 2-13. Genetic variations and DNA methylation
Figure 2-14. Positioning of methyl group on a cytosine base
Figure 2-15. Distribution of methylated and unmethylated CpGs within the mammalian genome
Figure 2-16. Functions of DNMTs
Figure 2-17. Proteins recruited to methylated CpGs
Figure 2-18. Simplified version of one-carbon metabolism showing some major metabolic intermediates, cofactors and dietary sources of methyl groups
Figure 2-19. Mechanisms by which smoking effects DNA methylation
Figure 2-20. Process of bisulfite conversion
Figure 2-21. Mechanism of pyrosequencing
Figure 3-1. Flow diagram of pharmacogenetic study
Figure 3-2. Location of 1236C>T, 2677G>T/A and 3435C>T SNPs in relation to the 28 exons that code for the *ABCB1/MDR1* transport protein
Figure 3-3. Location of -98A>G, 186C>T, 408C>G and 472G>A SNPs in relation to the 6 exons that code for the *COMT* metabolising enzyme
Figure 3-4. Location of 516G>T and 785A>G SNPs in relation to the 9 exons that code for the *CYP2B6* metabolising enzyme
Figure 3-5. Location of 1707T>delT, 1846G>A and 2459A>delA SNPs in relation to the 9 exons that code for the *CYP2D6* metabolising enzyme
Figure 3-6. Location of 118A>G SNP in relation to the 4 exons that code for the μ-opioid receptor
Figure 3-7. CpG sites located in the promoter region and exon 1 of *OPRM1*
Figure 3-8. Promoter region and exon 1 of *OPRM1* with CG regions of interest indicated
Figure 3-9. Temperature program for amplification of samples for sequencing
Figure 3-10. *OPRM1* +12 and -30 PCR optimisation
Figure 3-11. SureClean purification of OPRM1 -30 primer set
Figure 3-12. -30 primer concentration optimisation
Figure 3-14. Incubation temperature program
Figure 3-14. Pyrograms of the *OPRM1* promoter region from DNA samples that underwent either 35 or 40 PCR amplification cycles

Figure 3-15. Promoter region and exon 1 of *ABCB1/MDR1* with CG regions of interest
Figure 3-16. CpG sites located in the promoter region and exon 1 of *CYP2D6*
Figure 3-17. Temperature program for amplification of samples for sequencing
Figure 4-1. *CYP2D6* methylation in non-smokers, ex-smokers, frequent smokers (<15 or >15 cigarettes/day) and social smokers
Figure 4-2. *OPRM1* methylation in non-smokers, ex-smokers, frequent smokers (<15 or >15/day cigarettes) and social smokers
Figure 5-1. Methylation differences between methadone exposed babies and controls in *ABCB1/MDR1, CYP2D6* and *OPRM1*
Figure 5-2. Methylation differences between methadone exposed mothers and controls in *ABCB1/MDR1, CYP2D6* and *OPRM1*
Figure 5-3. Methylation differences between methadone exposed mothers – baby dyads and control mother – baby dyads in *ABCB1/MDR1, CYP2D6* and *OPRM1*
Figure 5-4. MPOD mother buccal and plasma methylation
Figure 7-1. Relationship between *CYP2D6* methylation and liver, muscle and thalamus from non-opioid exposed samples
Figure 7-2. Relationship between age and *OPRM1* DNA methylation in thalamus samples of opioid exposed individuals
Figure 7-3. Median *ABCB1/MDR1* methylation (%) in blood, liver, muscle and thalamus obtained from opioid associated deaths
Figure 7-4. Average *CYP2D6* methylation (%) in blood, liver, muscle and thalamus obtained from opioid associated deaths
Figure 7-5. Average *OPRM1* methylation in blood, liver, muscle and thalamus obtained from opioid associated deaths
Figure 7-6. Median *CYP2D6* methylation (%) in liver, muscle and thalamus obtained from non-opioid associated deaths
Figure 7-7. Average *OPRM1* methylation (%) in liver, muscle and thalamus obtained from non-opioid associated deaths
Figure 7-8. *CYP2D6* methylation (%) in liver, muscle and thalamus from opioid and non-opioid associated deaths
Figure 7-9. *OPRM1* methylation in liver, muscle and thalamus from opioid and non-opioid associated deaths
Figure 7-10. Average methylation (%) of a) *ABCB1/MDR1, OPRM1* and b) *CYP2D6* in blood obtained from opioid exposed and non-opioid exposed donors
Figure 8-1. Responders and non-responders to morphine and oxycodone in an opioid naïve cancer population
Figure 8-2. Average pain score / 24 hours over an 11 day period of morphine responders and non-responders
Figure 8-3. Drowsiness in morphine responders and non-responders over study period
Figure 8-4. Average pain score / 24 hours over an 11 day period of oxycodone responders and non-responders
Figure 8-5. Dry mouth and vomiting in oxycodone responders and non-responders over study period
Figure 8-6. Relationship between age and OPRM1 methylation in morphine responders and non-responders

Figure 8-6. *OPRM1* 118AA v AG/GG genotype in morphine responders and non-responders
Figure 8-8. a) *ABCB1/MDR1* 1236 CC v CT/TT, b) *ABCB1/MDR1* 3435 CC v CT/TT and c) *CYP2B6* GG v GT/TT genotype in oxycodone responders and non-responders

TABLE OF TABLES

Table XVI-1. PhD candidate's involvement at each stage within the study process
Table 2-1. Impact of methylated DNA on transcription factor binding site recognition
Table 3-1. Tissue samples collected from each study population for genetic, epigenetic and toxicological analyses
Table 3-2. Table of SNPs analysed by LGC genomics
Table 3-3. Table of *CYP2D6* variants analysed using Hy-Beacons assay
Table 3-4. *OPRM1* primers; reconstitution and optimum annealing temperature
Table 3-5. Sample preparation of bisulfite converted DNA for PCR amplification
Table 3-6. Primers for PCR and pyrosequencing
Table 3-7. Master mix for amplification of *OPRM1*, *ABCB1/MDR1* and *CYP2D6*
Table 4-1. Age of smoking and non-smoking study participants
Table 5-1. No relationship was observed between smoking exposure or residential status and *ABCB1/MDR1*, *CYP2D6* and *OPRM1* gene DNA methylation
Table 5.2 No relationship was observed between the development of NAS and *ABCB1/MDR1*, *CYP2D6* and *OPRM1* gene DNA methylation
Table 6-1. Summary of demographic and methylation data of methadone exposed babies (n=20) treated or not treated for Neonatal Abstinence Syndrome
Table 6-2. Baby genotype frequencies (n=20)
Table 6-3. Association between genotype and NAS development as determined by Fisher's exact test
Table 6-4. Association between maternal genotype and baby NAS development as determined by Fisher's exact test
Table 6-5. Association between maternal genotype and baby NAS development as determined by Fisher's exact test
Table 7-1. Age, gender and gene methylation of opioid exposed and control populations
Table 7-2. Correlation between *ABCB1/MDR1* gene DNA methylation and age in opioid exposed and opioid naïve individuals
Table 7-3. Correlation between *CYP2D6* gene DNA methylation and age in opioid exposed and opioid naïve individuals
Table 7-4. Pearson correlation between *OPRM1* gene DNA methylation and age in opioid exposed and opioid naïve individuals
Table 7-5. Gender differences in *ABCB1/MDR1*, *CYP2D6* and *OPRM1* gene methylation in opioid exposed and opioid naïve populations
Table 7-6. Average morphine and metabolite concentrations in different genotypes
Table 7-7. Morphine and metabolite concentrations vs tissue specific methylation
Table 8-1. Relationship between gene DNA methylation in paired blood and buccal samples
Table 8-2. Relationship between age and gene blood and buccal DNA methylation in opioid responders and non-responders
Table 8-3. Gender, age, dose, drug concentration, genotype and methylation differences between morphine responders and morphine non-responders
Table 8-4. Frequency of haplotypes in oxycodone responders and non-responders

Table 8-5. Gender, age, dose, drug concentration, genotype and methylation differences between oxycodone responders and oxycodone non-responders
Table 8-6. Frequency of haplotypes in oxycodone responders and non-responders

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AUTHORS DECLARATION

In the course of completing this thesis, the genotype *CYP2B6*6* was observed to be associated with apparent protection from the development of neonatal abstinence syndrome. As such a patent was submitted to protect the finding.

The protocols, supporting documents and ethics applications were created and submitted to the relevant ethical bodies by the PhD candidate for the smoking, tissue and cancer study (Table 1). As the participants of the methadone and tissue study were located in Scotland sample collection was undertaken by a third party, however sample collection for the smoking and cancer study were completed by the PhD candidate. DNA methylation of *ABCB1/MDR1*, *CYP2D6* and *OPRM1* were analysed by the PhD candidate using primers designed by the PhD candidate (with the exception of *ABCB1/MDR1*) and analysed by assays developed by the PhD candidate. The SNPs to be investigated were chosen, and samples prepared for SNP analysis by the PhD candidate however an external company undertook the SNP analysis for cost and time efficiency reasons. Drug analysis within study samples were undertaken using already developed methods but the drug extraction and instrument operation was undertaken by the PhD candidate.

Table 1 PhD candidate's involvement at each stage within the study process

Study	Ethical Considerations	Sample Collection	DNA Methylation Analysis	SNP Analysis	Drug Analysis
Smoking[a]	Y	Y	Y	na	na
Methadone[b]	N	N	Y	N	Y
Tissue[c]	Y	N	Y	N	N
Cancer[d]	Y	Y	Y	N	Y

Full study titles: [a]Does smoking affect DNA methylation?; [b]Methadone-prescribed opioid-dependant mothers and their newborn babies; [c]Tissue specific DNA methylation; [d]Pilot trial: Personalising opioid therapy for cancer pain relief. Y = yes, PhD candidate undertook the activity; N = no, the activity was undertaken by a third party; na = not applicable.

LIST OF ABBRIEVIATIONS

A	Adenine
ABCB1/MDR1	ATP-Binding Cassette, Sub-Family B (MDR/TAP), Member 1
BBB	Blood Brain Barrier
BCSFB	Blood Cerebrospinal Fluid Barrier
BMI	Body Mass Index
C	Cytosine
CNS	Central Nervous System
COMT	Catechol-O-Methyltransferase
CpG	Cytosine - Guanine Dinucleotide
CYP2B6	Cytochrome P450, Family 2, Subfamily B, Polypeptide 6
CYP2D6	Cytochrome P450, Family 2, Subfamily D, Polypeptide 6
DDIs	Drug Drug Interactions
DNA	Deoxyribonucleic Acid
DNMT	DNA Methyltransferase
EDDP	2-Ethylidene-1,5-Dimethyl-3,3-Diphenylpyrrolidine
EM	Extensive Metaboliser
G	Guanine
GC-MS	Gas Chromatography Mass Spectrometry
GI	Gastrointestinal
IRAS	Integrated Research Application System
LC-MS/MS	Liquid Chromatography–Tandem Mass Spectrometry
M	Mole
M3G	Morphine-3-Glucuronide
M6G	Morphine-6-Glucuronide
MDT	Multidisciplinary Team Meeting
mL	Millilitre
MPOD	Methadone-Prescribed Opioid-Dependant
MTA	Material Transfer Agreement
NAS	Neonatal Abstinence Syndrome
NHS	National Health Service
NMDA	N-Methyl-D-Aspartate Receptor
non. syn	Nonsynonymous Substitution
OIH	Opioid Induced Hyperalgesia
OPRM1	Opioid Receptor, Mu 1
PCR	Polymerase Chain Reaction
PH	Poole Hospital
PIS	Patient Information Sheet
PM	Poor Metaboliser
R&D	Research and Development
RBCH	Royal Bournemouth and Christchurch Hospitals
RBH	Royal Bournemouth Hospital
REC	Research Ethics Committee

RMH	Royal Marsden Hospital
SAH	S-Adenosylhomocysteine
SAM	S-Adenosylmethionine
SNP	Single Nucleotide Polymorphism
SSI	Site Specific Information
stat6	Signal Transducer and Activator of Transcription 6
syn	Synonymous Substitution
T	Thymine
UGT2B7	UDP Glucuronosyltransferase 2 Family, Polypeptide B7
UM	Ultra-Rapid Metaboliser
WHO	World Health Organisation
β-arrestin2	Beta-Arrestin-2
δ	Delta
κ	Kappa
μ	Mu
μL	Microlitre

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1.0 INTRODUCTION

Opioids have been used for centuries, either medicinally to alleviate pain or recreationally for their euphoric properties (Kritikos and Papadaki 1967; Brownstein *et al.* 1993; Dikotter *et al.* 2004; Chou *et al.* 2009). Despite the long history of opioid use / abuse, individual response to opioids is difficult to predict. One individual may take a dose of an opioid and obtain adequate analgesia and no side effects, conversely another individual on the same dose may obtain only the adverse effects of the opioid and none of the benefits (Galer *et al.* 1992; Skorpen *et al.* 2008). Clinically, and for the purpose of this study, a patient perceived score of ≤ 4 on an 11 point scale (0 = "no pain", 10 = "worst pain imaginable") is considered a good response to opioids, as long as the side effect experienced are "none" or "a little".

The variable response to opioids has been explained in part by age (Wilder-Smith 2005; Mercadante 2010), organ dysfunction (King *et al.* 2011; Neerkin *et al.* 2012), concurrent drug use (Nielsen *et al.* 2007; Gaertner *et al.* 2012; Tetrault and Fiellin 2012), prior opioid experience (Hay *et al.* 2008; Alford *et al.* 2013), route of opioid administration (Katz *et al.* 2011; Daoust *et al.* 2015), as well as genetic variations in genes associated with opioid pharmacokinetics (Branford *et al.* 2012; Somogyi *et al.* 2015); however these factors do not fully account for the variable response.

Another factor that may contribute to variable opioid response is the epigenetic mechanism, DNA methylation. Gene expression is, in part, regulated by DNA methylation (Bell *et al.* 2011; Jones 2012) therefore aberrant methylation in genes involved in opioid pharmacokinetics may alter drug response. The aim of this study was to recruit pilot populations to help determine the influence of gene DNA

methylation on the response to opioid analgesics, as cancer patients who are prescribed either morphine or oxycodone do not always obtain the desired effects and on occasion obtain severe adverse side effects.

As DNA methylation is a dynamic mechanism influenced by environmental exposures and cellular stress (Doehring *et al.* 2013; Hammoud *et al.* 2013; Nestler 2014) control populations to determine the effect of smoking and chronic opioid exposure on selected opioid genes were investigated. Female smokers and non-smokers between the ages of 18 and 50 were recruited from a student and staff population at Bournemouth University (n = 96). Once the influence of smoking was ascertained the effect of chronic opioid exposure on gene methylation was ascertained in 30 methadone-prescribed opioid-dependant (MPOD) mothers, who were also smokers. The babies of the MPOD mothers provided a unique opportunity to determine the effect of in utero methadone exposure on DNA methylation of the opioid related genes.

DNA methylation has been reported to be tissue specific (Davies *et al.* 2012; Sliekar *et al.* 2013; Lokk *et al.* 2014) so to determine whether methylation changes in peripheral tissues were relevant to gene methylation in tissues where the opioids exert their action or are bio-transformed, intra-individual tissue DNA methylation differences in blood, liver, psoas muscle and thalamus samples was ascertained in ~30 opioid associated deaths. Finally, taking into account the findings of the prior studies, the influence of DNA methylation on opioid response was determined using a population of opioid naive cancer patients (n = 147).

The selected genes were the transport protein encoded by the *ABCB1/MDR1* gene, the metabolising enzyme encoded by the *CYP2D6* gene, and the μ-opioid receptor

encoded by the *OPRM1* gene. *ABCB1/MDR1, CYP2D6* and *OPRM1* were chosen as genetic variations within these genes have been associated with variable drug response by previous studies (detailed in *2.1.4.6 Genetic Variations*) thus it was plausible that gene methylation may also play a role. As genetic variations, such as single nucleotide polymorphisms (SNPs) have been associated with variable opioid response, mutations within *ABCB1/MDR1, COMT* (gene that encodes for the metabolising enzyme of endogenous opioids), *CYP2B6* (gene that encodes for a xenobiotic metabolising enzyme), *CYP2D6* and *OPRM1*, it was necessary to investigate these variations alongside the gene DNA methylation analysis.

1.1 Study rationale

Genetic variations have been associated with variable drug response. Warfarin is the common example – SNPs in *CYP2C9* and *VKORC1* and demographic characteristics enable appropriate doses to be prescribed. The Human Genome Project was established to map the human genome to gain a better understanding of genes in the body. Following its completion in 2003 it was hoped that individuals could be prescribed selected drugs at appropriate doses based upon their genetic traits. For disorders caused by single gene-defects, such as clotting disorders this has been achieved, as with warfarin (Feero *et al.* 2010; Brenner 2012). However the majority of diseases are polygenic which makes predicting drug response more difficult (Motulsky and Qi 2006). Not only are multiple genes involved but also body mass index (BMI), diet, exercise, concomitant drug use and the epigenetic mechanism DNA methylation has been shown to influence the response of drugs, such as tamoxifen. Therefore the effect of *ABCB1/MDR1, CYP2D6* and *OPRM1* methylation on opioid response, in conjunction with commonly investigated SNPs was investigated.

1.2 Study aim

The aim of this work was to explore the DNA methylation on the *ABCB1/MDR1*, *CYP2D6* and *OPRM1* genes to determine whether DNA methylation affected response to opioid analgesics.

1.2.1 Objectives

- Determine the effect of smoke exposure on DNA methylation of *ABCB1/MDR1*, *CYP2D6* and *OPRM1* in buccal DNA of female adults.
- Determine the effect of chronic opioid exposure on DNA methylation of *ABCB1/MDR1*, *CYP2D6* and *OPRM1* in buccal DNA of female adults and new-born babies exposed to opioids *in utero*.
    - Determine the effect of drug dose, mother plasma methadone concentrations, gene methylation and polymorphisms on the development of neonatal abstinence syndrome (NAS).
- Determine the methylation profile of *ABCB1/MDR1*, *CYP2D6* and *OPRM1* in blood, liver, muscle and thalamus samples obtained from opioid exposed and opioid naïve adults.
- Determine the methylation status (and genotype) of opioid responding and opioid non-responding cancer patients.

2.0 LITERATURE REVIEW

Genetic and epigenetic variations of the P-glycoprotein transporter (encoded by *ABCB1/MDR1*), the CYP2D6 opioid metabolising enzyme and the μ-opioid receptor gene (*OPRM1*) were investigated in relation to opioid response. In addition, genetic variations were also investigated in the *COMT* and *CYP2B6* metabolising enzyme genes. To understand the importance of these genes, an introduction to opioids is provided including opioid pharmacodynamics and pharmacokinetics.

2.1 Opioids

The term "opioid" refers to any chemical with a morphine-like structure and activity that exerts its action via opioid receptors (Martin 1967). Activation of the opioid receptors, mu (μ), delta (δ) and kappa (ϰ) results in analgesia and decreased gastrointestinal (GI) motility; hence the historic use of opioids as a remedy for the relief of pain and diarrhoea (Pasternak and Pan 2013). The efficiency of analgesia and euphoria, as well as the occurrence and severity of side effects experienced varies between different opioids and routes of opioid administration (Benyamin *et al.* 2008; Wolff *et al.* 2012; Mori *et al.* 2013; Daoust *et al.* 2015).

Substrates of opioid receptors include 1) naturally occurring alkaloids obtained from the unripe seed capsules of the opium poppy (*Papaver somniferum*), 2) semi-synthetic drugs, 3) fully synthetic drugs and, 4) endogenous opioid peptides (Trescot *et al.* 2008). The earliest known record of the poppy plant being used and cultivated for its pharmacological properties dates back to c.4500 BC (Norn *et al.* 2005; Gussow *et al.* 2013). However the first naturally occurring pharmacologically active alkaloid was not isolated from raw opium until 1805. This alkaloid was named morphine after Morpheus, the Greek god of sleep (Sertürner 1817) and was first sold commercially

by Merck in 1827. The profound addictive qualities of morphine were soon realised and the search for non-addictive alternatives began (Pasternak and Pan 2013).

The discovery of morphine led to the identification of other alkaloids within opium, the most predominant alkaloids (after morphine) being codeine, noscapine, papaverine and thebaine (Spinella 2001). In an attempt to develop drugs with the analgesic properties of the opium but without the adverse side effects and addictive properties, the chemical structures of the alkaloids were modified producing a wide range of semi-synthetic opioids such as diamorphine, oxycodone, oxymorphone, buprenorphine and naloxone (Rosenblum *et al.* 2008) (Figure 2-1). Ironically diamorphine, AKA heroin, was initially marketed as the opioid to treat morphine addiction; however heroin has a quicker onset of opioid action and shorter duration of effects resulting in greater addiction (Twycross 1973; Olivieri *et al.* 1986; Katz *et al.* 2007).

During the 20th century numerous fully synthetic opioids were developed for use as analgesics but with less abuse / dependence potential (Armstrong *et al.* 2009; Pathan and Williams 2012). Similarly to the semi-synthetic opioids, the majority of synthetic opioids have equivalent, or greater abuse potential as the archetypal opioid morphine; e.g. meperidine (pethidine), ketobemidone and tramadol (Trescot *et al.* 2008). Synthetic opioids, e.g. methadone and buprenorphine have slower onset of action and longer duration and as such are currently used to treat opioid withdrawal (Silverman 2015a; 2015b); but these opioids can also be misused (Cicero *et al.* 2014).

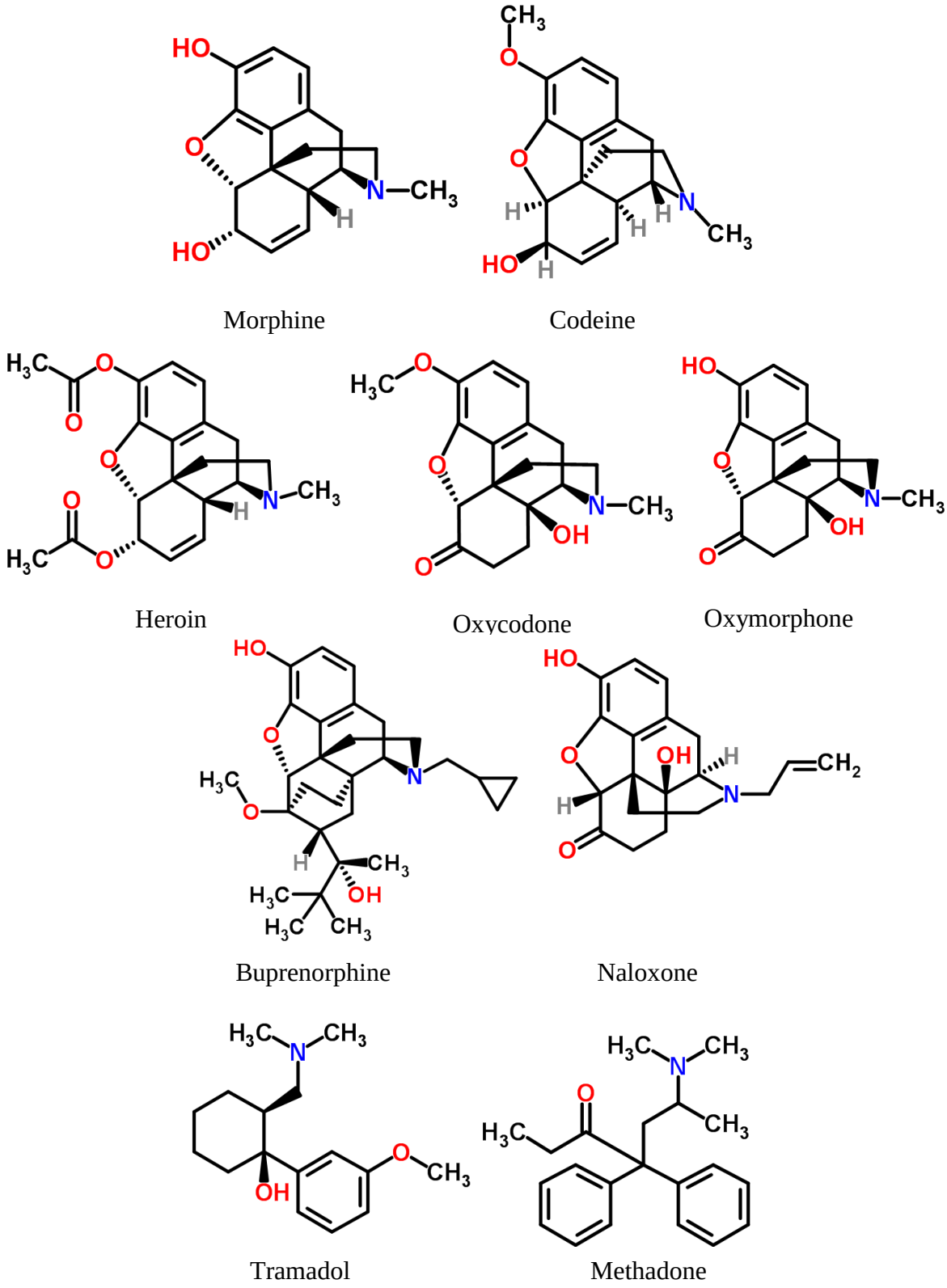

Figure 2-1. Structures of naturally occurring alkaloids, semi-synthetic and synthetic opioids
Morphine and codeine are naturally occurring alkaloids; heroin, oxycodone and oxymorphone, buprenorphine and naloxone are semi-synthetic opioids; tramadol and methadone are fully synthetic opioids. Chemical structures obtained from ChemSpider (http://www.chemspider.com/).

The current guidelines, suggested by the World Health Organisation (WHO), to alleviate pain depends on the extent of the pain. A pain relief step ladder (Figure 2-2) directs the pharmacological management of pain from the use of non-opioids (e.g. paracetamol and aspirin) for mild pain (Step I), adding weak opioids (e.g. codeine) for moderate pain (Step II), and using regular strong opioids such as morphine, oxycodone, hydromorphone, fentanyl and alfentanil for moderate to severe pain (Step III) (WHO 1986; WHO 1996). Traditionally morphine is the prototype analgesic to alleviate pain because of its availability, cost and familiarity (Hanks *et al.* 2001; Caraceni *et al.* 2012). However alternative Step III opioids, e.g. oxycodone and hydromorphone have similar efficacy and tolerability profiles (Reid *et al.* 2006; Wiffen and McQuay 2007; Hanna and Thipphawong 2008; Caraceni *et al.* 2011) so are plausible first-line analgesics.

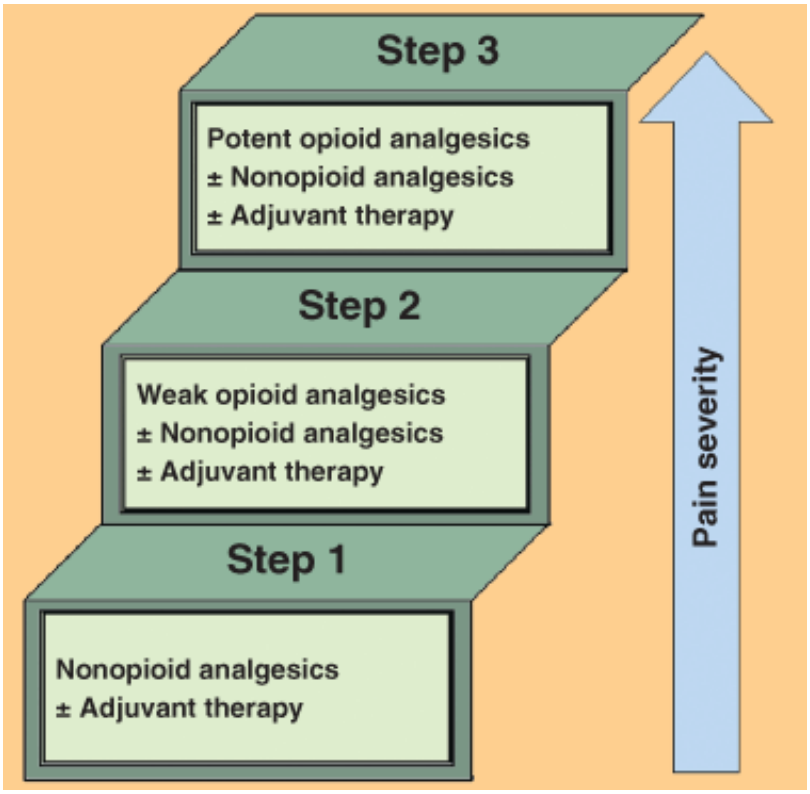

Figure 2-2. World Health Organisation (WHO) pain step ladder
WHO proposed the use of non-opioid analgesics for the relief of mild pain, Step I; mild opioids in conjunction with non-opioids for mild pain that cannot be relieved by non-opioid analgesics alone, Step II; strong opioids in conjunction with non-opioids if necessary to relieve severe pain, Step III. Figure obtained from Davis *et al.* (2007).

2.1.1 Opioid adverse effects

Alongside the advantageous properties of opioids, adverse side effects have been reported throughout history, as have the effects of inappropriate dosing (Emmanuel and Tschirc 1923; Tamsen *et al.* 1979; EAPC 1996; Argoff 2010). For example, up to 30% of cancer patients on oral morphine for pain are known as "morphine non-responders" (Cherny *et al.* 2001; Ross *et al.* 2005; Oertel *et al.* 2006). These "morphine non-responders" present in a number of different ways (Mercadante and Bruera 2006):

- Patients who achieve good analgesia but with intolerable side-effects.
- Patients who do not achieve good analgesia because of dose-limiting side-effects.
- Patients who do not achieve good analgesia but do not experience side-effects either, despite escalating morphine doses.

Although most work in this area had been carried out in patients on morphine, inter-individual variation in response to other strong opioids for pain relief also exists. Emerging evidence suggests that there may be two broad groups of opioid non-responders:

- Patients who do not achieve an adequate clinical response to the initial opioid shortly after initiation, when the opioid dose is relatively low, reflecting that not all drugs are efficacious in all patients. "Heterogeneity of treatment effects" is seen with most pharmaceutical medications and may be explained in part by individual pharmacokinetic or pharmacodynamic factors.

- Patients who appear to become non-responsive to the initial opioid at either higher doses or after chronic opioid therapy. This may be attributable to the development of physical tolerance to the initial opioid (Slatkin 2009).

Recognition of this substantial inter-individual variation in response to morphine for pain relief has resulted in the emergence of "opioid switching" as a clinical manoeuvre to redress the balance between analgesia and side-effects. If patients are considered to be “non-responders” to the initial opioid, it is common clinical practice to switch them to an alternative strong opioid (Quigley *et al.* 2003; Dale *et al.* 2011).

Common opioid side effects include constipation, nausea, vomiting, dry mouth, drowsiness, pruritus and dizziness that can have an adverse effect on quality of life (Benyamin *et al.* 2008; Pizzi *et al.* 2012). Classic μ-opioid receptor agonists such as morphine and oxycodone induce nausea and vomiting in approximately 15-30% of chronic or acute pain patients (Cherny *et al.* 2001; Kalso *et al.* 2004; Moore and McQuay 2005). Nausea and vomiting are considered the least desirable side effects of opioids from a patient’s perspective (Strassels *et al.* 2005). The precise mechanism of nausea / vomiting induction is unknown however activation of the μ-opioid receptor is postulated to be a predominant factor as naloxone, a μ-opioid receptor antagonist, can alleviate nausea (Smith and Laufer 2014). Severity of side effects influences opioid continuation as an analgesic and fear of side effects can lead to under-dosing and inadequate analgesia (Morley-Forster *et al.* 2003; Arnold 2004; Bhamb *et al.* 2006; Benyamin *et al.* 2008).

2.1.1.1 Opioid tolerance

Individuals can be, or can become tolerant to opioids as a result of predetermining genetics or acquired through repeated opioid exposure, respectively (Collett 1998;

Cepeda-Benito *et al.* 2005). Tolerance can be a result of pharmacokinetic changes such as the up-regulation of metabolic processes by a drug that stimulates its own elimination from the body or as a result of pharmacodynamics, when responses within the neural system are altered by drugs (Hassan *et al.* 2013). As a result of the development of tolerance, increasing doses of opioids are required to obtain effective analgesia (Angst and Clark 2006; Benyamin *et al.* 2008; Chu *et al.* 2008). The occurrence of tolerance has been shown to be dependent upon the method of administration (repeated or continuous) and the efficacy of the opioid on the μ-opioid receptor (Pawar *et al.* 2007; Kumar *et al.* 2008; Madia *et al.* 2009). It has been postulated that agonists that cause μ-opioid receptor internalisation produce less behavioural tolerance than low efficacy agonists (Williams *et al.* 2013). Morphine is a low-efficacy agonist of the μ-opioid receptor and as such tolerance is more likely to occur than following administration of high-efficacy agonists, e.g. DAMGO, sufentanil, etorphine, methadone (Stevens and Yaksh 1989; Duttaroy and Yoburn 1995; Madia *et al.* 2009; Enquist *et al.* 2012).

2.1.1.2 Opioid dependence

Prolonged opioid exposure can lead to physical dependence for the drug (Benyamin *et al.* 2008) and arises in virtually all chronically opioid exposed individuals (Ballantyne 2007). Abrupt cessation of drug results in unpleasant withdrawal symptoms (Angst and Clark 2006; Kauer and Malenka 2007; Drdla *et al.* 2009; Heinl *et al.* 2011) and therefore opioid users consume more drug to avoid the prospect of withdrawal symptoms (Juurlink and Dhalla 2012). Additionally opioid users may consume opioids for their pleasurable effects caused by stimulation of reward centres in the brain (Juurlink and Dhalla 2012). Prolonged dependence to chronic opioid exposure can cause neurochemical and structural neuronal changes that cause

hypersensitivity in the brain's reward system (Tetrault and Fiellin 2012). This hypersensitivity causes the individual to crave more opioid and leads to compulsive drug-seeking and drug-taking behaviour (Cami and Farrer 2003). To reduce the symptoms of opioid withdrawal, tapering strategies are employed. A slower rate of tapering has been demonstrated to reduce the symptoms of opioid withdrawal, e.g. initially a 10% reduction per week followed by a 25-50% reduction in opioid (Chou *et al.* 2009). Treatments for opioid dependence include long acting μ-opioid receptor agonists such as methadone and buprenorphine, and the antagonist naltrexone that block opioid induced euphoria and stabilise drug seeking behaviour (Tetrault and Fiellin 2012).

2.1.2 Opioid pharmacodynamics

Morphine and other clinically used exogenous opioids preferentially bind to the μ-opioid receptor and have poor affinity towards δ and ϰ receptors (Börner *et al.* 2008). Therefore, the μ-opioid receptor is the main focus for the remainder of the thesis.

2.1.2.1 μ-Opioid receptor distribution

Opioids exert their pharmacological effects by binding to receptors primarily in the brain and spinal cord but also in peripheral tissues (Oertel *et al.* 2008). Various brain regions involved in sensory and motor function and central control of nociceptive transmission have opioid receptors at their neurons (McDonald and Lambert 2005). The expression and distribution of μ-opioid receptors varies between different organs. Peripheral organs containing opioid receptors include heart, lungs, liver, gastro-intestinal (GI) tract (stomach, duodenum, jejunum, ileum and colon) and reproductive tracts (Wittert *et al.* 1996; Villemagne *et al.* 2002; Bigliardi-Qi *et al.* 2004; Holzer 2004; Gray *et al.* 2005). In addition the μ-opioid receptor is also expressed in immune cells such as macrophages and lymphocytes (Bidlack 2000; Vousooghi *et al.* 2009).

The stimulation of peripherally located receptors, whilst playing a small role in the analgesic effect of opioids, are mostly associated with producing the side effects of opioids such as reduced GI motility (McDonald and Lambert 2005; Holzer 2012).

2.1.2.2 μ-Opioid receptor structure

The μ-opioid receptor is a member of the G-protein-coupled receptor (GPCR) superfamily. The μ-opioid receptor is encoded by the *OPRM1* located on 6q24-q25 (Figure 2-3). The receptor consists of seven transmembrane domains, an extracellular N-terminus with several glycosylation sites, three intracellular loops, 3 extracellular loops and an intracellular C-terminus (Figure 2-3). Exon 1 codes for the first transmembrane domain, and exon 2 and 3 code for the second through to seventh transmembrane domains (Pasternak 2004) (Figure 2-4).

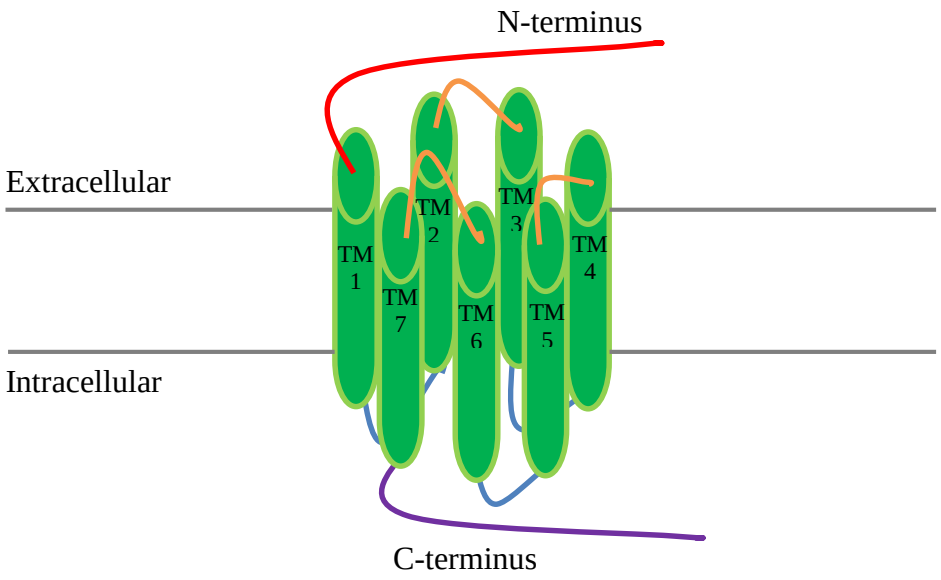

Figure 2-3. Diagrammatic representation of the μ-opioid receptor
The μ-opioid receptor consists of 7 transmembrane domains that span the cell membrane. N-terminus resides in the extracellular space whereas the C-terminus is in the intracellular space. Extracellular loops in orange; intracellular loops in blue; TM = transmembrane domain.

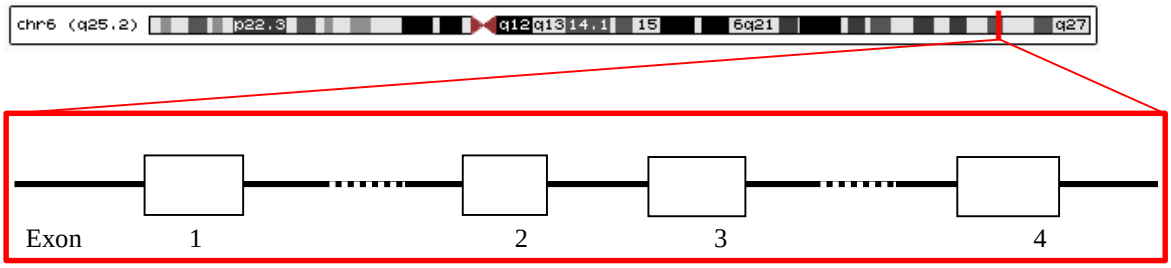

Figure 2-4. Chromosomal location of *OPRM1* and layout of coding exons

2.1.2.3 μ-Opioid receptor signal transduction

Once opioids interact with the receptor, an intracellular cascade of chemical reactions takes place. The binding of a μ-opioid receptor agonist, such as morphine or methadone causes a receptor conformational change that stimulates the disassociation of guanosine diphosphate (GDP) from the coupled G-protein. The GDP is readily replaced by guanosine-5'-triphosphate (GTP) and the G-protein separates into two components, α subunit and the β,γ subunit, that stimulate intracellular changes. Coupled to μ-opioid receptor is the pertussis toxin sensitive protypical G heterotrimeric protein (G_i/G_o) that inhibits adenylate cyclase activity, modulates neurotransmitter release via ion channel alterations and stimulates signal transduction pathways via secondary messengers (Mostany *et al.* 2008; Al-Hasani and Bruchas 2011) (Figure 2-5).

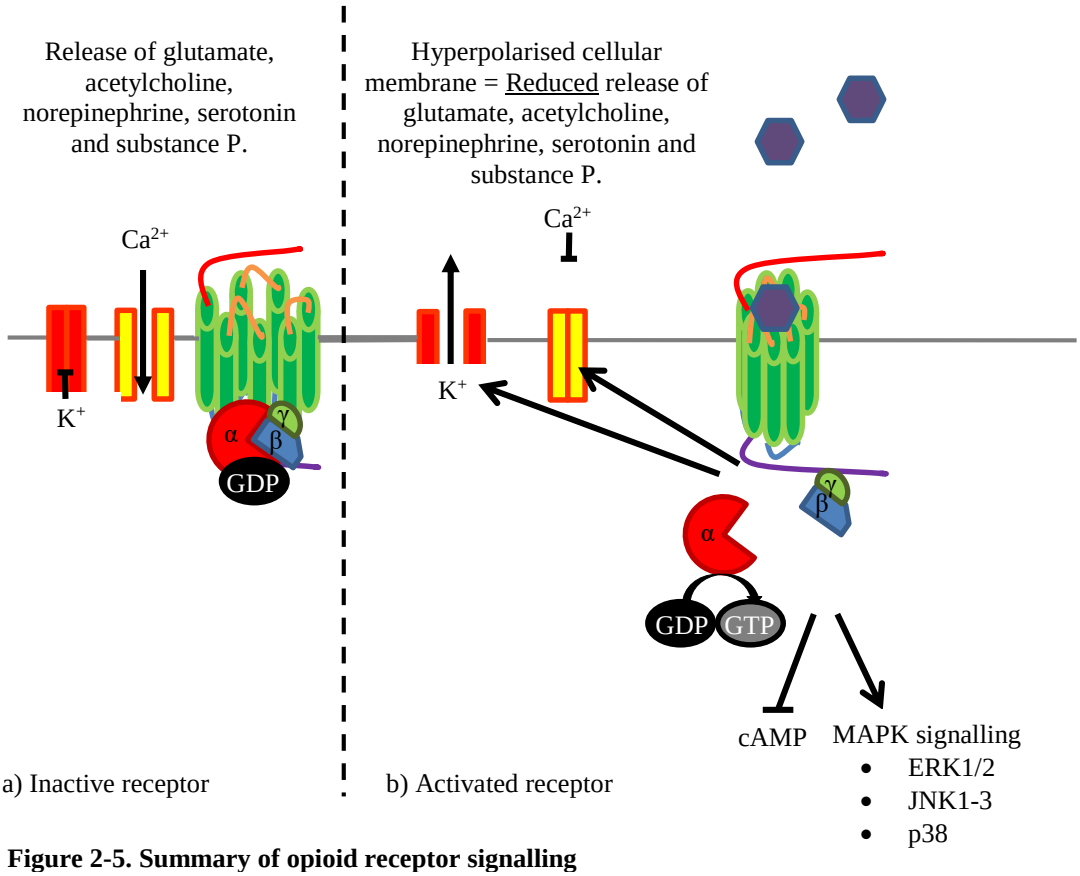

Figure 2-5. Summary of opioid receptor signalling
Inactive opioid receptors (a) are coupled to a G-protein made up of three subunits (α, β,γ). In an inactive state the receptor is in an open conformation free to bind opioids, calcium channels (yellow and red rectangles) are open enabling calcium (Ca^{2+}) to enter the cell and potassium channels (red rectangles) are closed inhibiting the extracellular release of potassium (K^{+}). When an opioid (purple hexagon) binds the conformation of the receptor alters stimulating the release and disassociation of the coupled G-protein and simultaneous conversion of GDP to GTP. The disassociated G-protein stimulates the release of potassium from the cells and inhibits the entry of calcium into the cell effectively hyperpolarising the cell membrane. The hyperpolarisation inhibits the release of neurotransmitters. The disassociated G-protein also inhibits the cAMP pathway and activates MAPK signalling pathways.

The G-protein subunits inhibit N-type and P/Q type calcium channels and activate potassium channels preventing calcium ions entering the intracellular region and allowing potassium ions into the extracellular region, respectively (Currie and Fox 1997; Zamponi and Snutch 2002; Altier and Zamponi 2004; Pérez-Garci *et al.* 2013). The change in ion concentrations causes cellular membrane hyperpolarisation that decreases the release of neurotransmitters such as glutamate, acetylcholine, norepinephrine, serotonin and substance P. The reduction of neurotransmitters in the synaptic cleft reduces the excitability of the nociceptive pathways and brain regions involved in pain perception producing an analgesic effect (Oertel and Lotsch 2008).

The disassociated G-protein also inhibits adenylyl cyclase activity and therefore the cAMP dependant pathway. The $G\alpha_i$ subunit stimulates mitogen-activated protein kinases (MAPK) pathways within 5-10 minutes of agonist binding. The MAPK pathways are fundamental for cell proliferation, differentiation, channel phosphorylation, apoptosis and transcription factor regulation (Raman *et al.* 2007). Members of the MAPK family include: extracellular signal-related kinases 1 and 2 (ERK1/2), c-Jun N-terminal kinases (JNK1-3) and p38 (α, β, γ, δ) stress kinases. The ERK1/2 cascade is the most commonly studied opioid-induced MAPK pathway (Al-Hasani and Bruchas 2011). Opioid stimulated ERK1/2 pathways modulate and direct cell fate determination in embryonic stem cells (Kim *et al.* 2006; Hahn *et al.* 2010) and inhibit axon/dendrite and synapse formation in astrocytes (Ikeda *et al.* 2010). Activation of the JNK pathway results in increased gene expression of c-Jun which is a component of the transcription factor, activator protein-1 (AP-1). The p38 pathway is important for response to chemical stimulus and cell proliferation and has been shown to play a role in neuropathic pain response (Watkins *et al.* 2001; Xu *et al.* 2007).

2.1.2.4 μ-Opioid receptor desensitisation, recycling, degradation

Simultaneously to the ion channel modulations, adenylyl cyclase inhibition and stimulation of MAPK pathways; the intracellular domain of the μ-opioid receptor undergoes G-protein receptor kinase (GRK)-dependent phosphorylation (Figure 2-6). GRK phosphorylation is rapid, saturating the receptor in less than 20 seconds (Williams *et al.* 2013) producing binding sites for arrestin molecules (β-arrestin 1 and β-arrestin 2). These arrestin molecules bind to the receptor resulting in desensitisation as the G-protein binding sites are blocked. Blocking of the G-protein binding sites inhibits the μ-receptor intracellular activity, therefore adenylyl cyclase is no longer

inhibited, ion channels are not modulated and associated signalling cascades are not continued (Dang *et al.* 2012; Williams *et al.* 2013).

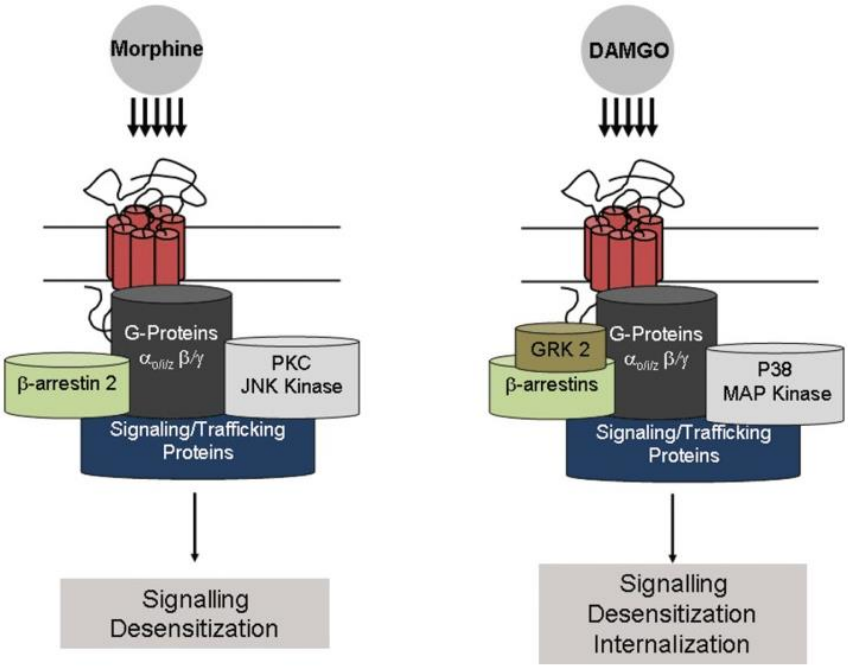

Figure 2-6. Ligand-specific signalling complexes
Different opioids affect the signalling and trafficking of opioid receptors. Morphine, a low internalising opioid is postulated to desensitise the receptor via β-arrestin 2 and PKC-dependant pathway; whereas DAMGO, a high internalising agonist recruits GRK2, β-arrestin 1 and 2, and p38 MAPKs. Figure obtained from Pradhan *et al.* (2012).

Non-GRKs such as JNK, PKC and p38 MAPK can also phosphorylate the μ-receptor (Koch and Hollt 2008). The recruitment of phosphorylating agents is postulated to be ligand specific. For example morphine is thought to desensitise the μ receptor via β-arrestin 2 and PKC-dependant pathways, whereas DAMGO recruits GRK2, β-arrestin 1 and 2, and p38 MAPKs (Tan *et al.* 2009a; Groer *et al.* 2011; Pradhan *et al.* 2012). The desensitisation process is reversed by cellular phosphatases upon removal of the agonist. However, after continued exposure to opioids (hours to weeks), μ-receptor desensitisation becomes enhanced and the re-sensitisation process impaired leading to a lack of agonist responsiveness and development of tolerance (Dang *et al.* 2012; Williams *et al.* 2013).

The phosphorylating agents recruited by the different opioids can alter the receptor's fate. For example as mentioned above, morphine desensitises the receptor via β-arrestin 2 and PKC-dependant pathway whereas DAMGO and fentanyl phosphorylate the receptor more than morphine by GRK2, β-arrestin 1 and 2, and p38 MAPK (Tan *et al.* 2009a; Groer *et al.* 2011; Pradhan *et al.* 2012). The binding of GRK2, β-arrestin 1 and 2, and p38 MAPK complex results in receptor conformational changes enabling receptor internalisation, therefore DAMGO and fentanyl have better opioid internalisation than morphine (Evans 2004; Kelly *et al.* 2008). In addition, the opioid receptor–arrestin complex is not inactive; the complex stimulates signal transduction cascades such as mitogen-activated protein kinase (MAPK) pathways (Lefkowitz and Shenoy 2005), as discussed in *2.1.2.3 μ-Opioid receptor signal transduction*.

Knock-in mice expressing a mutant μ-opioid receptor were able to internalise and had increased morphine analgesia and reward effects, reduced tolerance, dependence and addictive behaviour (Kim *et al.* 2008; Berger and Whistler 2011). Effects remained unchanged in wild-type and mutant mice exposed to methadone (Kim *et al.* 2008). Methadone already has high internalisation properties so analgesic efficacy and duration were unchanged, whereas morphine that has poor internalisation properties has increased analgesic effect with facilitated internalisation (Pradhan *et al.* 2012). Abuse potential and tolerance towards morphine may in part be due to lack of receptor internalisation (Whistler *et al.* 1999; Finn and Whistler 2001; Koch *et al.* 2005). Agonists of receptor internalisation therefore represent valuable molecular targets for more effective analgesics (Berger and Whistler 2010).

2.1.2.5 μ-Opioid receptor subtypes

Although clinically used opioids such as morphine, oxycodone and methadone preferentially bind to the same receptor (μ), the mechanism of action is different for

these different opioids (Crews *et al.* 1993; Mercadante 1999). In the event of an adverse response, a switch to an alternative μ-opioid receptor agonist can alleviate the negative effect even though the same receptor is being activated. In addition, individuals can develop tolerance to one opioid after prolonged exposure but have incomplete cross-tolerance to other opioids (Pasternak 2004; Riley *et al.* 2006; Dumas and Pollack 2008).

Clinical experiences have reflected the different responses to opioids observed in animal studies. CXBK mice with a low number of opioid receptors do not obtain analgesic activity from morphine; however their analgesic response to M6G and fentanyl is not affected (Rossi *et al.* 1996; Chang *et al.* 1998). Also mice tolerant to morphine have complete cross-tolerance to codeine but obtain normal analgesic response to M6G, heroin and fentanyl (Rossi *et al.* 1996). A possible explanation of this variation in opioid response is the existence of μ receptor subtypes (review by Cox *et al.* 2015).

Subtypes of the μ-opioid receptor were suggested in the 1980s with the use of opioid antagonists (Pasternak *et al.* 1980; Wolozin and Pasternak 1981). The μ-receptor antagonist naloxonazine binds irreversibly and reversibly to the subtypes named μ_1 and μ_2 respectively (Hahn and Pasternak 1982; Chaijale *et al.* 2013). The presence of naloxonazine blocked the analgesic effects of morphine but not the side effects which suggested that analgesia was achieved through the μ_1 subtype, and side effects via the μ_2 subtype (Ling *et al.* 1985; Paul and Pasternak 1988; Andoh *et al.* 2008). However knockout of the individual subtypes eliminates all the effects associated with the μ-receptor implying there was only one μ-receptor gene (Kitchen *et al.* 1997; Simonin *et al.* 2001; Weibel *et al.* 2013). Therefore, rather than the existence of μ-receptor subtypes Dietis *et al.* (2011) suggested that, 1) splice variants, 2) receptor

dimerization, and 3) receptor-protein complexes contribute to variable opioid response.

2.1.2.5.1 Receptor splice variants

Three major splice variants can occur in the μ-opioid receptor increasing protein diversity (Figure 2-7):

1) **Full-length (7 transmembrane domains) with 3' splice variant**

This variant has the same 7 transmembrane domains as the wild type receptor (Figure 2-3) and so has the same opioid-binding pocket. However a splicing at the C-terminal yields a unique amino acid sequence that alters agonist-induced G-protein coupling, receptor phosphorylation, internalisation and postendocytic sorting (Abbadie and Pasternak 2001; Tanowitz *et al.* 2008; Pasternak 2014).

2) **Truncated variant (6 transmembrane domains)**

The truncated variant lacks exon 1 which encodes the first transmembrane domain therefore the variant has only 6 transmembrane domains (Pasternak 2014). The expression of this splice variant is controlled by the exon 11 promoter (Pan *et al.* 2009; Majumdar *et al.* 2012). Mouse knockout studies have shown that the analgesic effect of morphine is completely lost when exon 1 is disrupted but full analgesic response can be obtained with heroin and M6G if dose is increased. Lack of exon 11 significantly reduces heroin and M6G activity but not morphine or methadone (Schuller *et al.* 1999; Pan *et al.* 2009; Xu *et al.* 2013).

3) Single transmembrane domain

With the loss of exon 2 or the loss of exon 2 and 3 only a single transmembrane domain is produced. They do not bind opioids directly however modulate opioid activity by increasing expression of the full-length 7 transmembrane variants through a chaperone like action (Xu *et al.* 2013; Pasternak 2014).

The binding affinities of endogenous and exogenous opioids to the splice variants have been investigated and different binding efficiencies and potency of effects have been observed. However, the efficiency of binding is not related to potency as opioids that have similar binding properties can have very different potencies (Bolan *et al.* 2004; Pasternak 2004; Xu *et al.* 2009).

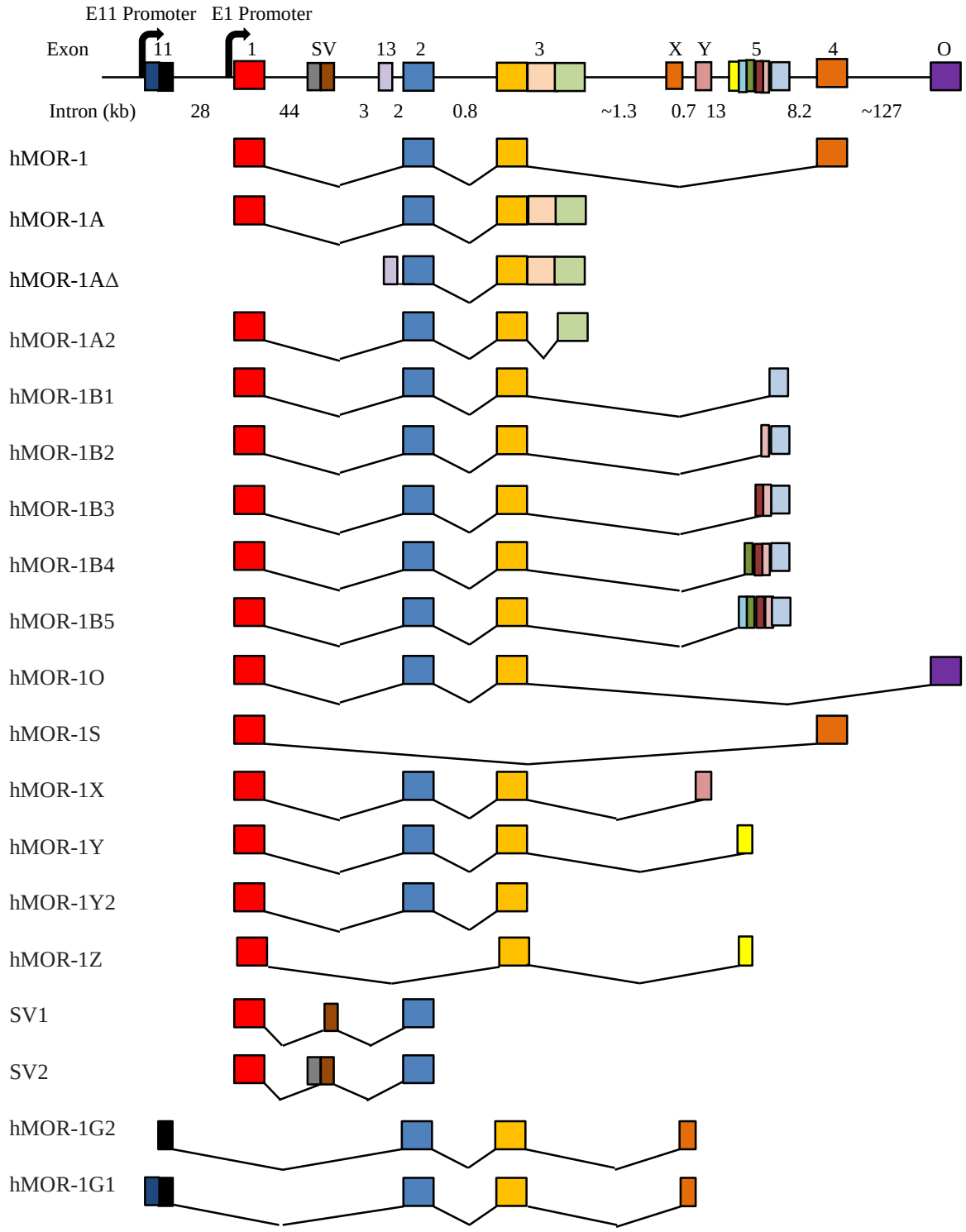

Figure 2-7. Schematic of the human *OPRM1* gene and the MOR-1 splice variants
Exons and introns are shown by boxes and horizontal lines, respectively. Exons are numbered in the order in which they were identified. The approximate sizes of introns (in kb) are indicated. The locations of the promoters upstream of exons 11 and 1are indicated. Image adapted from Andersen *et al.* (2013) and Xu *et al.* (2013).

2.1.3 Opioid pharmacokinetics

2.1.3.1 Absorption and bioavailability

For opioids to exert their effects they must first enter the systemic circulation and permeate blood-tissue membranes to reach the opioid receptors (Figure 2-8). The concentration and speed at which the opioid reaches its target sites depends on the route of administration, the physiology of the opioid and the extent of first pass metabolism. The most common route of opioid administration to alleviate pain is orally (Leppert *et al.* 2013) as it is considered non-invasive, easy to administer and has a high patient acceptance (Linardi *et al.* 2012). However, it is one of the routes with the poorest bioavailability, i.e. the proportion of opioid that enters the systemic circulation able to exert its effects.

The poor bioavailability results from incomplete absorption and first pass metabolism. Orally administered opioids must first dissolute, cross the gastrointestinal epithelial membrane and enter the hepatic portal vein that flows to the liver. The rate of absorption through the epithelial membrane is limited by the opioids ionisation, lipophilicity and formulation. Morphine, oxycodone and methadone are weak bases, pKa 8.0, 8.9 and 8.9 respectively (Moffat *et al.* 2011), that disassociate into ionised (BH+) and non-ionised (B) fractions, the proportions of which depend on the surrounding pH. In the stomach, that has a pH of ~2, opioids are highly ionised so are poorly absorbed. The pH of the small intestine however is more alkaline increasing the proportion of non-ionised fraction. The non-ionised opioids are more lipid soluble so can diffuse across the epithelial membrane down a concentration gradient.

The lipophilicity of an opioid is measured by their octanol-water partition ratio that measures the solubility of the opioid in two immiscible phases, i.e. octanol and water.

Opioids with a high octanol-water partition ratio are hydrophobic and therefore cross the epithelial membrane more readily than opioids with a low octanol-water partition ratio. For example morphine and fentanyl have similar pKa values (pH at which the proportion of BH+ = B), 8.0 and 8.4 respectively. However fentanyl has a much higher octanol-water partition ratio than morphine (Log *P* = 2.3 and -0.1, respectively (Moffat *et al.* 2011)), which is postulated to explain the shorter time taken for fentanyl to exert its effects compared to morphine (Aronoff *et al.* 2005). Oxycodone and methadone have higher octanol-water partition ratios than morphine, 0.7 and 2.1 respectively (Moffat *et al.* 2011).

The formulations of the opioid preparations can also affect the rate of absorption. Morphine is available in immediate release (IR) or sustained release (SR) formulations. The sustained release formulations have polymer coatings that encase the morphine (capsules) or trap the morphine within the pores of a hydrophilic polymer matrix (tablets). For the morphine to be released from the tablets and capsules the GI fluids must first breakdown the hydrophilic layers delaying drug absorption and therefore prolonging pharmacological response (Amabile and Bowman 2006). Opioids in solution have the fastest rate of absorption, followed by drug emulsions, suspensions, capsules, tablets, coated tablets, enteric coated tablets and finally sustained release tablets.

Even once the opioid is released from formulation, not all of the absorbed opioid will enter the systemic circulation as a result of drug efflux from the enterocytes by P-glycoproteins (P-gp) and first-pass metabolism that occurs in the gut wall and primarily the liver (Smith 2009). Morphine, oxycodone, fentanyl and methadone are substrates of P-gp efflux (Henthorn *et al.* 1999; Dagenais *et al.* 2004; Hamabe *et al.* 2006; Hassan *et al.* 2007; Ortega *et al.* 2007; Hassan *et al.* 2009a, 2009b; Fujita-

Hamabe *et al.* 2012) however the extent of interaction with the P-gp varies between opioids (Dagenais *et al.* 2004; Metcalf *et al.* 2014). Hassan *et al.* (2009a) incubated morphine analogs with a known excess of ATP and recombinant human P-gp. Morphine had greater ATP consumption than etorphine and codeine, but all 3 opioids had greater ATP consumption than the control demonstrating that they were P-gp substrates (Hassan *et al.* 2009a).

The opioid antagonists, naloxone and naltrexone however are classified as P-gp non-substrates (Hassan *et al.* 2009a). Despite absorption of naloxone and naltrexone not being inhibited by P-gp efflux they have poorer bioavailability than the P-gp substrates morphine, oxycodone and methadone as a result of extensive hepatic metabolism. Approximately 2% of naloxone and naltrexone enters the systemic circulation following oral administration (Smith *et al.* 2012; Goonoo *et al.* 2014) whereas the average bioavailability of morphine, oxycodone and methadone is ~30%, ~60% and ~75% respectively (Poyhia and Kalso 1992; Hanks *et al.* 2001; Eap *et al.* 2002; Moffat *et al.* 2011; Pathan and Williams 2012).

Opioids that are administered intravenously however have 100% bioavailability as the opioid is injected directly into the systemic circulation (Figure 2-8). Sublingual, intramuscular, subcutaneous, transdermal (patches and gels), intranasal and rectal (by-passes 50% of first-pass metabolism) administration of opioids have greater bioavailability than oral routes but do not have complete bioavailability as their absorption is limited by the ionisation and lipophilicity of the opioid (Narang and Sharma 2011; Inui *et al.* 2012; Krishnamurthy *et al.* 2012; Leppert *et al.* 2013; Patel *et al.* 2014).

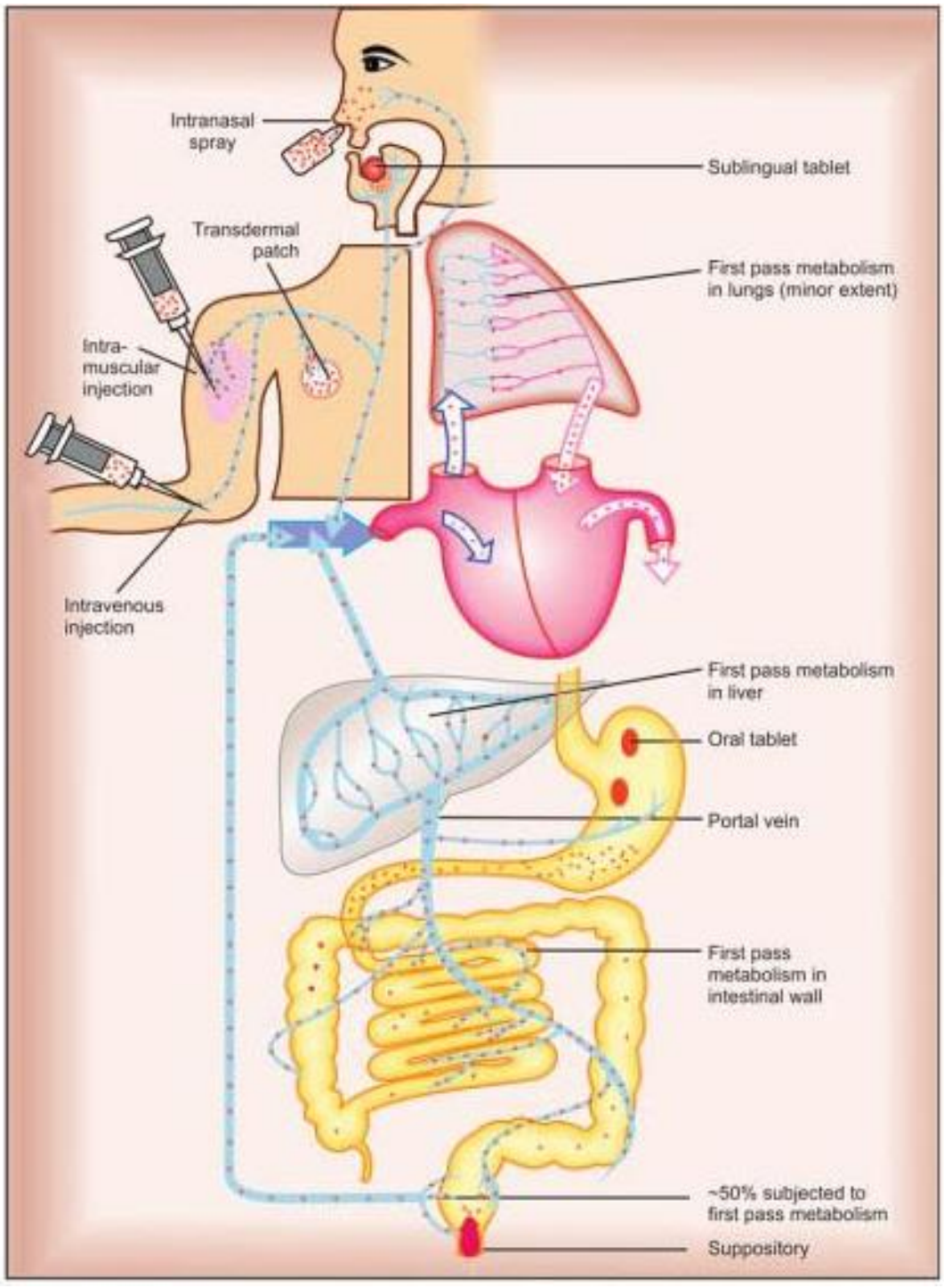

Figure 2-8. Vascular pathway of drugs absorbed from various systemic routes of administration and sites of first pass metabolism

Total drug absorbed orally is subjected to first pass metabolism in intestinal wall and liver, while approximately half of that absorbed from the rectum passes through the liver. Drug entering from any systemic route is exposed to first pass metabolism in lungs, but its extent is minor for most drugs. Image obtained from Tripathi (2013, p. 7).

2.1.3.2 Metabolism

Metabolism is a process of detoxification that converts a drug into a hydrophilic form enabling its excretion from the body. The primary site of opioid metabolism is in the liver where high densities of phase I and phase II enzymes are situated. CYP enzymes are phase I enzymes that metabolise opioids by oxidation and hydrolysis. Phase II enzymes metabolise opioids through conjugation with hydrophilic substances, the most common of which being glucuronic acid. The process of glucuronidation is undertaken by UGT enzymes. Opioids can undergo phase I metabolism, phase II metabolism or both. The products formed can be pharmacologically inactive or active, and some of the active metabolites can be more potent than the parent compound (Smith 2009), e.g. oxymorphone is more potent than oxycodone.

2.1.3.2.1 Morphine

Morphine is metabolised to the potent analgesic morphine-6-glucuronide (M6G) and non-analgesic morphine-3-glucuronide (M3G) by *UGT2B7*, and *UGT2B7* and *UGT1A3* respectively (Figure 2-9) (Christrup 1997; Coffman *et al.* 1997; Green *et al.* 1998). M3G is the major metabolite (45-55%), whereas 10-15% of morphine is metabolised to M6G and 5% is metabolised to the minor metabolites normorphine, normorphine 6-glucuronide, morphine-3, 6-diglucuronide, morphine ethereal sulfate and hydromorphone are also formed (Christrup 1997; Coffman *et al.* 1997; Cone *et al.* 2008; McDonough *et al.* 2008; Smith 2009).

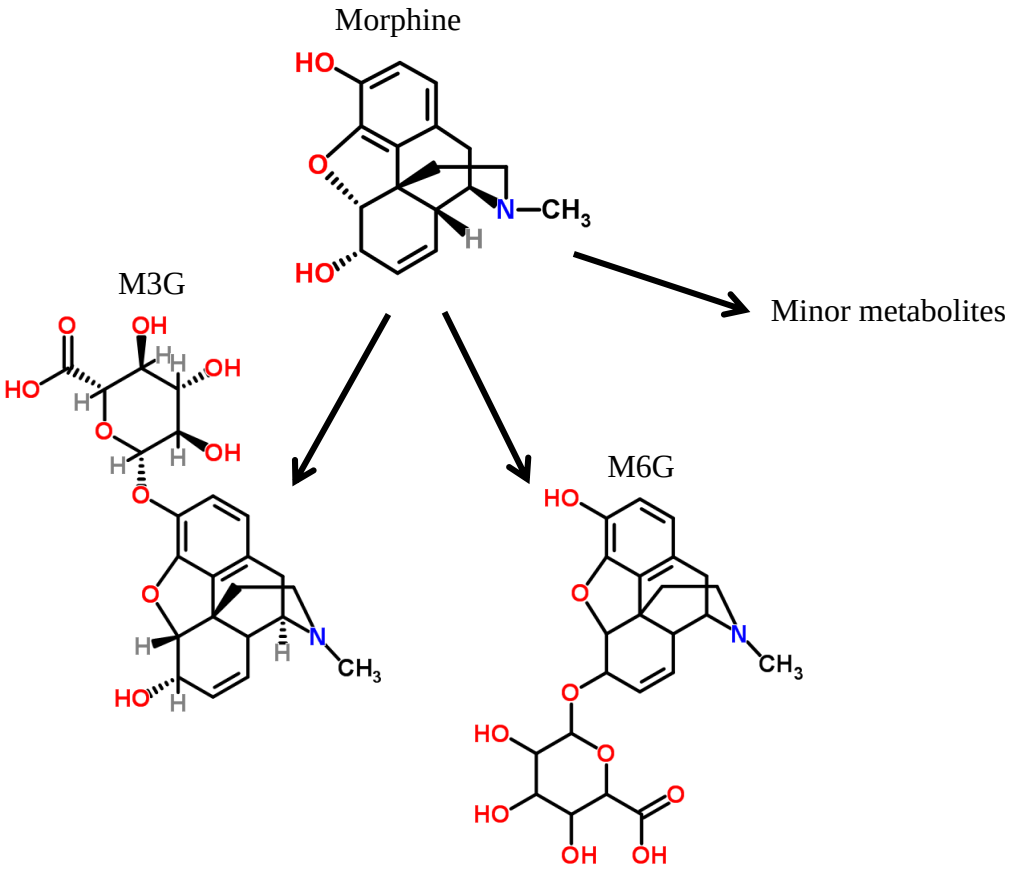

Figure 2-9. Metabolism pathways of morphine
Morphine is metabolised to the analgesically inactive metabolite M3G and the potent analgesic M6G, as well as minor metabolites.

2.1.3.2.2 Oxycodone

Oxycodone is a substrate for phase I CYP enzymes. CYP3A4 and CYP3A5 metabolise oxycodone to the major metabolite noroxycodone that has no analgesic activity (Figure 2-10). A small proportion of oxycodone is metabolised by CYP2D6 to oxymorphone that is a potent analgesic (Lalovic *et al.* 2004; Lalovic *et al.* 2006; Smith 2009). Oxymorphone is inactivated by glucuronidation (Smith 2009).

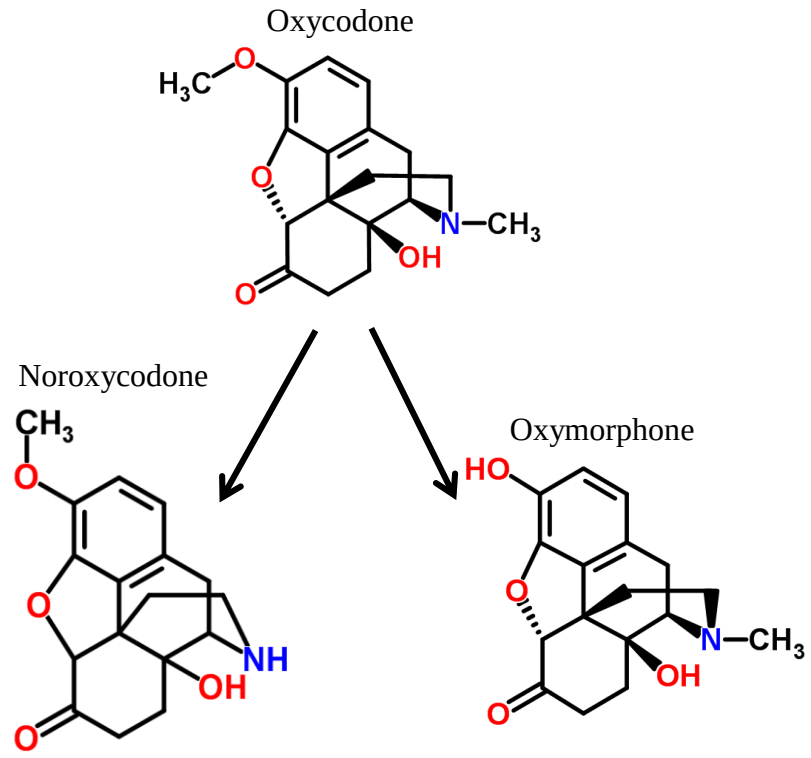

Figure 2-10. Metabolism pathways of oxycodone
Oxycodone is metabolised to the analgesically inactive metabolite noroxycodone and the potent analgesic oxymorphone.

2.1.3.2.3 Methadone

Methadone has no active metabolites; instead it undergoes *N*-demethylation by CYP3A4, CYP2B6, CYP2C19, CYP2D6 and CYP2C8 to the inactive metabolites 2-ethyl-1, 5-dimethyl-3, 3-diphenylpyrrolidine (EDDP) and 2-ethyl-5-methyl-3, 3-diphenylpyrroline (EDMP)(Figure 2-11) (Kharasch *et al.* 2004; Totah *et al.* 2008; Chang *et al.* 2011; Kharasch and Stubbert 2013).

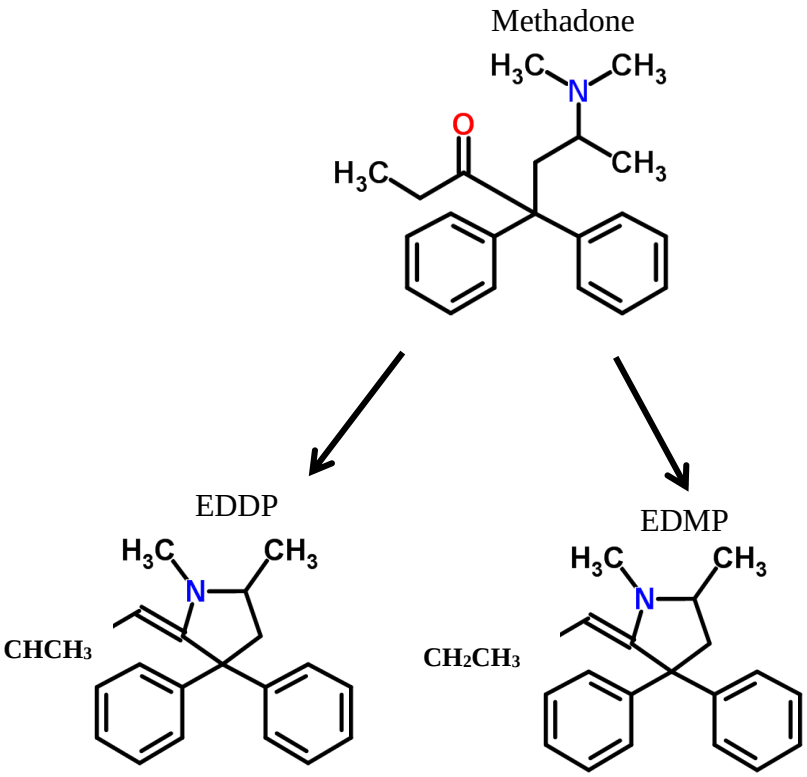

Figure 2-11. Metabolism pathways of methadone
Methadone is metabolised EDDP and EDMP by CYP450 enzymes.

2.1.3.3 Distribution

Opioids that enter the systemic circulation are transported around the body to organs to exert their pharmacological effects, to be stored, to be metabolised and to be excreted. The rate of blood flow to a tissue, the volume of the tissue and presence of protective barriers influence opioid distribution. Drugs are distributed first to the liver, kidneys, lungs, heart and brain that have a good blood supply, then to adipose tissues, bones and teeth that have a lesser blood supply (Sanders *et al.* 2011, p. 287). However the concentration and rate at which opioids distribute into tissues such as the brain, cerebrospinal fluid, placenta and the vitreous humor is influenced by protective barriers. The blood-brain barrier (BBB) and blood-cerebrospinal fluid barrier (BCSFB) lack pores or gaps between the endothelial cells and has an additional continuous basement membrane consisting of astrocytes that modulate tissue

permeability (Redzic 2011; Tournier *et al.* 2011). These barriers also contain transporters and enzymes that impede the free diffusion of drugs from blood into the brain and CSF (Redzic 2011). In particular, the P-gp ATP-binding cassette B1/multiple drug resistance 1 (*ABCB1*/*MDR1*) transports morphine, oxycodone and methadone from the endothelial cells back into systemic circulation (Wyman and Bultman 2004; Dumas and Pollack 2008; Hassan *et al.* 2009b).

The occurrence and rate of tissue penetration is dependent upon the physiology of the opioid, e.g. ionisation and lipophilicity, as discussed above (*2.1.3.1 Absorption and bioavailability*). Additionally the extent of opioid-protein binding influences tissue distribution. The protein bound fraction of opioid, be it within the plasma or binding to proteins within opioid permeable tissues, is too large to diffuse through cell membranes therefore inhibiting distribution. However a constant ratio between protein bound and unbound opioid is maintained within the plasma and between the tissues (Figure 2-12). When unbound (free fraction) opioid is redistributed into the tissues, metabolised or excreted, bound drug disassociates from plasma proteins to maintain the ratio (Lehman-McKeeman 2013, p. 169).

The ratio varies between different opioids, for example 15-35% of morphine is protein bound, predominately to albumin (Leow *et al.* 1993; Ederoth *et al.* 2004), oxycodone is 45% protein bound (Gallego *et al.* 2007) and methadone is highly bound to α_1-acid glycoprotein (70-87%) (Kharasch *et al.* 2009; Parmar and Parmar 2013). The greater the extent of protein binding, the longer the delay in onset of action and the longer the duration of drug action is observed. The action of methadone, which is highly protein bound, is experienced 30-60 minutes following administration and may last up to 48 hours whereas the action of immediate release formulations of morphine and oxycodone occur after 15-30 minutes and last 4-6 hours.

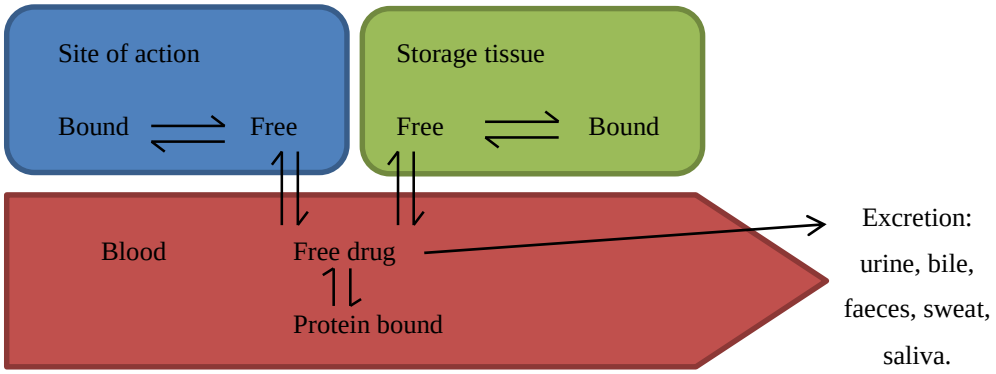

Figure 2-12. Schematic depiction of free drug movement through blood-tissue membranes
Only free drug can pass through blood-tissue membranes. However, as the concentration of free drug decreases (through metabolism, distribution and excretion) the ratio of free drug and protein bound drug is maintained by the disassociation of drug from proteins. This disassociation provides free drug that can pass through membranes. Image adapted from Tripathi (2013, p. 10).

2.1.3.4 Elimination

Opioids are removed from the body primarily through kidney secretion but also via the bile. Through the kidneys ~10% of the original dose of morphine, oxycodone and methadone is excreted as unchanged drug and the remainder as metabolites (Moffat *et al.* 2011). Following filtration from the glomerulus into the convoluted tubules of the kidney nephrons, opioids can be reabsorbed into the peritubular capillaries leading to the renal vein (Sim 2015). The proportion of opioids excreted via the kidneys is dependent upon urinary pH and the ionisation of the opioid. The reabsorption of morphine, oxycodone and methadone rises with increasing urine alkalinity (Nilsson *et al.* 1982). The increased reabsorption reduces the plasma clearance rate thereby increasing the half-life of the opioid prolonging its duration of effects. In addition to renal absorption, the prolonged effects of opioids are also attributable to the reabsorption of opioid from the gastrointestinal tract, referred to as enterohepatic circulation (Hanks and Wand 1989).

2.1.4 Factors that influence opioid response

Inter-individual variation in response to opioids may be a result of factors such as age, gender, disease and lifestyle that affect the pharmacodynamic and pharmacokinetic

properties of a drug. A recent study of over 2000 patients suggested that psychological issues and sleep deprivation may also play a role in opioid response (Knudsen *et al.* 2011). To date no clinical factors (with the exception of renal impairment) have been identified which can be used prospectively to predict opioid response in this cohort. Therefore there is a growing interest in the possibility that an individual's genetic makeup may influence opioid response.

2.1.4.1 Age and opioid response

Individuals greater than 60 - 70 are more sensitive to analgesics as a result of age related changes in pharmacokinetics and pharmacodynamics (Mercadante and Acuri 2007). Normal pharmacological changes associated with aging include slowing of the GI, reduction in total body water content and fat-free mass and decreased glomerular filtration and hepatic flow (Chau *et al.* 2008; Reisner 2011; Mercadante 2010). As a result of these pharmacological changes the absorption of opioids and elimination is reduced resulting in extended duration or action of drug (Mercadante 2010).

The opioid response in neonates is also different from older children and adults as a result of changes in body composition, liver mass, metabolic activity, renal function and CNS structure and function (Marsh *et al.* 1998; Tayman *et al.* 2011). The rate of drug absorption via enteral routes in neonates is slower than that of adults as a result of reduced rate of gastric emptying, slower GI transit time and reduced intestinal surface area (Dumont and Rudolph 1994; Carlos *et al.* 1997; Menard 2004). The rate of distribution however is increased because neonates have a higher body water percentage than adults increasing the volume of distribution of hydrophilic drugs (Kearns *et al.* 2003; Rakhmanina and van der Anker 2006). Neonates also have a larger fraction of free drug as the quantity of plasma proteins in neonate blood is less than that in an adult (Kearns *et al.* 2003). The quantity of free drug influences the clearance

rate, volume of distribution and half-life of the drug. Additionally protective tissue barriers, such as the BBB are not completely matured and therefore are permeable to drugs (Mahmood 2008). Biotransformation of drugs is also less than that of adults as the presence and activity of UGT enzymes and CYP450 enzymes is reduced in neonates (de Wildt *et al.* 1999; Gow *et al.* 2001; Johnson *et al.* 2001; Hines and McCarver 2002; Hines 2008). Finally, neonate kidneys are anatomically and functionally immature and therefore their elimination rate is lower than that of adults (Yared and Ichikawa 1994; Hua *et al.* 1997; Kearns 2003). As a result of reduced absorption rate, reduced elimination and greater distribution of drug to target sites, neonates experience extended duration of drug action (Tayman *et al.* 2011).

2.1.4.2 Organ dysfunction

Opioids such as morphine and oxycodone are excreted via the kidneys, therefore in individuals with renal impairment drug accumulation can occur causing opioid sensitivity (Murphy 2005; Johnson 2007; Neerkin *et al.* 2012). The extent of renal impairment influences the choice of opioid as well as the recommended dose. King *et al.* (2011) undertook a systematic review of the literature and concluded that opioids such as fentanyl, afentanil and methadone are the least likely to cause harm whereas morphine causes toxicity in renal impairment (King *et al.* 2011).

2.1.4.3 Choice, dose and route of opioid administration

The choice of opioid, dose and route of administration alters the rate and duration of analgesic/euphoric effect and influences the occurrence and severity of side effects (Benyamin *et al.* 2008; Wolff *et al.* 2012; Mori *et al.* 2013; Daoust *et al.* 2015). For example between 0.3-1.5 mg of IV administered hydromorphone, oxymorphone and buprenorphine are required to achieve the same analgesic effect as 10mg of IV morphine, whereas higher doses of IV meperidine (75mg) are needed (Vallejo *et al.*

2011). The route of drug administration influences the peak effect of the drug; the peak effect of IM/SC morphine occurs 45-90 minutes following administration whereas the effects of IV morphine are experienced more rapidly (15-30 minutes) and orally administered morphine effects have a slower onset of action (2-4 hours) (Lugo and Kern 2002).

2.1.4.4 Drug-drug interactions

Drug-drug interactions (DDIs) can alter the intended pharmacokinetic (absorption, distribution, metabolism and elimination) and pharmacodynamic (desired effects and side effects) properties of a drug (Gaertner *et al.* 2012). The DDIs can either enhance or decrease opioid effect, primarily by modulating opioid metabolism and therefore influencing their elimination rate (Maurer and Bartkowski 1993; Overholser and Foster 2011). DDIs are more frequent amongst opioids that are metabolised by phase I CYP450 enzymes as CYP enzymes are also involved in the metabolism of ~50% of drugs (Overholser and Foster 2011). As such DDIs are more frequent with methadone, oxycodone and codeine use than for morphine which is metabolised by phase II conjugation enzymes.

Opioid response can be enhanced by CYP inhibitors such as telithromycin, itraconazole, ketoconazole and ritonavir that prevent the metabolism of oxycodone and methadone. The decreased metabolism results in increased opioid exposure causing greater opioid effect (Hagelberg *et al.* 2009; Nieminen *et al.* 2010a; Samer *et al.* 2010; Gronlund *et al.* 2010; 2011a, 2011b; Kummer *et al.* 2011). Alternatively CYP450 inducers (rifampin, St John's wort) can increase the rate of opioid metabolism by stimulating transcription factors that upregulate the expression of CYP enzymes. The increased CYP presence accelerates the rate of opioid clearance (Ferrari

et al. 2004; Nieminen *et al.* 2009, 2010b; McCance-Katz *et al.* 2010; Rowland and Tozer 2010).

2.1.4.5 Prior opioid experience

Opioid dependant individuals require higher and more frequent doses of analgesics to obtain adequate pain relief than opioid naive populations (Alford *et al.* 2013). For example, methadone maintained patients required higher than normal doses of morphine to obtain analgesia (Doverty *et al.* 2001; Athanasos *et al.* 2006) and showed significant tolerance to remifantanil (Hay *et al.* 2008). The increased opioid dose is thought, in part, to be a result of an increased sensitivity to pain (Hay *et al.* 2009; Wachholtz *et al.* 2015). Additionally ineffective analgesia may also be obtained from the development of complete cross-tolerance between different opioids (Athanasos *et al.* 2006; Compton *et al.* 2012; Wachholtz *et al.* 2015).

2.1.4.6 Genetic variations

Understanding the implications of genetic variations has the potential to enhance clinical outcomes (Argoff 2010). For example, genetic and clinical data can already be used to accurately predict an individual's response to irinotecan, ababcavir and warfarin (Higashi *et al.* 2002; Innocenti *et al.* 2004; Limdi *et al.* 2008; Mallal *et al.* 2008; Schwarz *et al.* 2008). This use of genetic data to prescribe medication is termed "personalised medicine" as it allows the drug with the best efficacy and side effect profile for that individual to be prescribed first time, rather than prescribed by traditional methods such as clinicians experience or trial-by-error (Argoff 2010). This facilitates prospective decision making regarding choice of the correct dose of the correct drug for a given patient and reduces subsequent side-effects. Thus in a number of different areas of medicine including oncology and haematology, pharmacogenomics has revolutionised drug prescribing.

2.1.4.6.1 OPRM1 polymorphisms

Despite there being >100 polymorphisms, the most studied SNP in *OPRM1* is the 118A>G that has a 10-15% minor allele frequency within the Caucasian population (Stamer and Stuber 2007) and a much higher frequency of 47% in Asian populations (Ono *et al.* 2009). The 118A>G polymorphism causes an amino acid exchange at position 40 of the μ-opioid receptor, from asparagine (Asn; N) to aspartic acid (Asp; D) (N40D) deleting a putative N-glycosylation site in the extracellular receptor region (Huang *et al.* 2012). The loss of the N-glycosylation site has been implicated in addiction vulnerability (Kroslak *et al.* 2007; Nagaya *et al.* 2012), clinical drug overdose vulnerability (Manini *et al.* 2013) and conflicting evidence exists concerning the effect of 118A>G on response to opioids.

Morphine consumption in a population of Taiwanese patients (n = 120) following knee replacement surgery was greater in 118GG patients (40.4 ± 22 mg) than 118AA patients (25.3 ± 15.5 mg) (Chou *et al.* 2006a). The observation of greater morphine consumption in 118GG patients post-surgery was replicated in female populations of Taiwanese (n = 80) and Chinese (n = 588) patients (Chou *et al.* 2006b; Sia *et al.* 2008). However ethnicity is postulated to influence morphine requirements as Malay (n = 241) and Indian (n = 137) populations showed no difference in morphine consumption in a study undertaken by Tan *et al.* (2009b) whereas the Chinese subgroup (n = 620) did. Similarly a lack of association between the 118A>G polymorphism and post-surgery morphine consumption in a population of Black and White subjects was reported by Coulbault *et al.* (2006).

Conflicting evidence for opioid response in acute and chronic pain in association with the 118A>G polymorphism have also been reported. Ross *et al.* (2005) reported no

relationship between 118A>G and morphine response in a Caucasian cancer population (n = 162). However patients homozygous for the variant allele (118GG) have been associated with poorer response to morphine and requirement of increased morphine to achieve adequate pain control compared to 118AA counterparts (Klepstad *et al.* 2004; Campa *et al.* 2008). Conversely Janicki *et al.* (2006) found that the minor G allele was less common in chronic opioid patients requiring higher doses of opioid analgesics than homozygous wild type.

A meta-analysis published in 2009 (Walter and Lotsch 2009) and further summary published in 2013 (Walter *et al.* 2013) discourages the use of 118A>G as a solitary marker of clinical relevance but emphasises its importance as part of a complex system that underlies response to analgesics. The effect of a combination of polymorphisms was illustrated by Hayashida *et al.* (2008) who observed that individuals carrying a 118G allele-containing haplotype required more opioids to obtain the same analgesia as carriers of other haplotypes. The association of the 118G allele-containing haplotype was more significantly associated with opioid requirements than analysing 118A>G polymorphism by itself (Hayashida *et al.* 2008).

2.1.4.6.2 CYP450 and COMT polymorphisms

CYP2D6 is a highly polymorphic gene, the genetic variations of which can be categorised into different phenotypes; poor metabolisers (PMs), extensive metabolisers (EMs) and ultra-rapid metabolisers (UMs). PMs have two non-functional alleles (e.g. *CYP2D6*4/*4* or **4/*6*) that cannot metabolise substrates resulting in the risk of adverse events, whereas UMs have three or more functional alleles (e.g. *CYP2D6*1XN*) that rapidly metabolise substrates leading to poor drug response (de Leon *et al.* 2006). Post-surgery patients with *CYP2D6* PM status treated with codeine

and tramadol had decreased analgesia (Persson *et al.* 1995; Stamer *et al.* 2003). Both codeine and tramadol undertake their pharmacological action via their metabolites and as such individuals with non-functioning *CYP2D6* alleles obtain less analgesia. At the other extreme, individuals carrying *CYP2D6* UM status have high plasma morphine concentrations following codeine administration and therefore experience greater sedation (Kirchheiner *et al.* 2007). Oxycodone is also a substrate for *CYP2D6* however polymorphisms in *CYP2D6* have not been associated with pharmacodynamic changes in oxycodone response despite *CYP2D6* polymorphisms causing pharmacokinetic alterations (Andreassen *et al.* 2012). Inhibition of both *CYP2D6* and *CYP3A4* however not only increased oxycodone plasma concentrations but also increased drowsiness and deterioration of performance of healthy subjects compared to healthy subjects taking a placebo (Gronlund *et al.* 2010) suggesting that altered oxycodone response is dependent upon both *CYP2D6* and *CYP3A4*.

Like *CYP2D6, CYP2B6* is highly polymorphic (Zanger and Klein 2013) and the polymorphisms influence the expression of the metabolic enzymes. For example *CYP2B6*5* (1459C>T, R487C), **6* (516G>T, Q172H; 785A>G, K262R) and **18* (983T>C, I328T) cause reduced expression of the CYP2B6 enzyme compared to the wild type that influence opioid activity (Turpeinen and Zanger 2012; Zanger and Klein 2013). Of particular interest is *CYP2B6*6* that has been shown to be associated with higher steady state plasma methadone concentrations (Crettol *et al.* 2005, 2006; Eap *et al.* 2007; Wang *et al.* 2011), requirement for lower methadone doses (Hung *et al.* 2011; Levran *et al.* 2013a) and longer clearance rate of methadone (Kharasch *et al.* 2014).

Catechol-*O*-methyltransferase (encoded by the *COMT* gene) is responsible for conjugating endogenous opioids such as dopamine and norepinephrine. These

endogenous opioids are instrumental in pain relief and genetic variations in *COMT* have been associated with pain perception, opioid response, dependence and addiction (Diatchenko *et al.* 2005, 2006; Dai *et al.* 2010; Lee and Lee 2011; de Gregori *et al.* 2013). In particular, the SNP 472A>G (rs4680) that codes for a Val158Met substitution is responsible for reducing *COMT* activity by decreasing its thermostability (Lotta *et al.* 1995; Rakvag *et al.* 2005; Oosterhuis *et al.* 2008). Patients that carry the Met/Met genotype have been reported to require less morphine than Val carriers following surgery and when used to alleviate cancer related pain (Rakvag *et al.* 2005; Reyes-Gibby *et al.* 2007; de Gregori *et al.* 2013).

Alongside the 472A>G polymorphism, three SNPs create haplotypes that have been associated with increased median doses of morphine and patients' having greater pain sensitivity, -98A>G, 408C>G and 186C>T of *COMT* (Diatchenko *et al.* 2005; Rakvag *et al.* 2008). The side effects of morphine have also been reported to be less in carriers of variant alleles within *COMT*. For example, nausea was decreased in 158Met carriers following surgery (Kolesnikov *et al.* 2011) and drowsiness and confusion was decreased in cancer patients with the variant allele at -4873A>G (rs740603) (Ross *et al.* 2008).

2.1.4.6.3 Transport protein polymorphisms

The metabolites as well as parent drug rely on transport proteins such as P-glycoproteins (P-gp) to reach their sites of action. Variations within the P-gp encoded by the *ABCB1/MDR1* gene has shown to significantly influence the bioavailability of many opioids (Coller *et al.* 2006; Campa *et al.* 2008). According to the National Centre for Biotechnology Information (NCBI) there are more than 50 SNPs in the coding region of the human transport gene *ABCB1/MDR1* (Fung and Gottesman 2009). The

majority of the SNPs are located within the intracellular regions, with only 3 SNPs located in the extracellular loop, 4 SNPs within 2 of the transmembrane domains (T9 and T12) and of those SNPs none change the glycosylation or phosphorylation sites, or the ATP binding domains (Fung and Gottesman 2009). Genetic variations within in *ABCB1/MDR1*, specifically 1236C>T, 2677G>T and 3435C>T have been associated with not only variable analgesic efficacy to morphine and oxycodone but also variable degrees of adverse effects (Campa *et al.* 2008; Ross *et al.* 2008; Fujita *et al.* 2010; Zwisler *et al.* 2010).

2.2 DNA methylation

DNA methylation is an important gene regulatory epigenetic mechanism essential for mammalian development and genome stability (Jaenisch and Bird 2003; Jones and Liang 2009; Smith and Meissner 2013). It is classified as an epigenetic mechanism because unlike genetic variations such as single nucleotide polymorphisms, repeats, insertions or deletes, the sequence of DNA bases remains unaltered as illustrated in Figure 2-13. Instead methyl groups are covalently bound to the DNA which influences whether or not DNA is transcribed and subsequently translated into proteins or non-coding/ribosomal/transfer RNA. The epigenetic patterns in the genome are referred to as the epigenome and unlike the genome, the epigenome is variable and dynamic, responding to environmental influences and cellular stress (Hammoud *et al.* 2013).

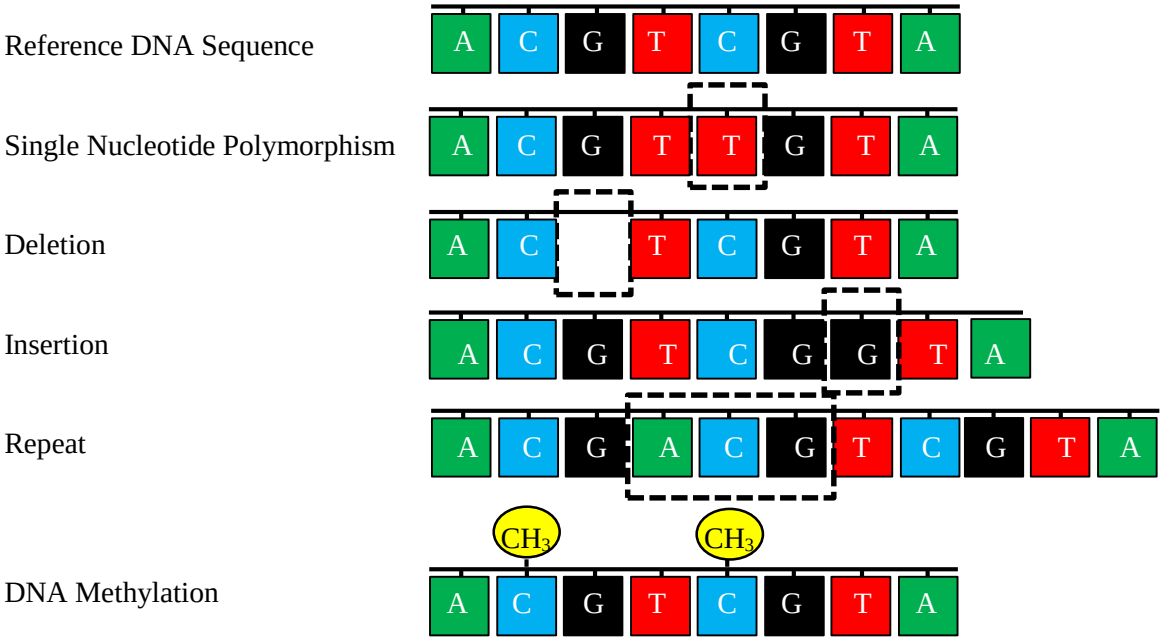

Figure 2-13. Genetic variations and DNA methylation
Genetic variations alter the underlying DNA sequence (variant highlighted by dashed box); single nucleotide polymorphism (SNP) = a base change from the reference sequence, deletion = deleted base, insertion = additional base, and repeat = repeated sequence. Epigenetic mechanisms such as DNA methylation do not alter the underlying DNA sequence; instead methyl groups (CH_3) are added to the DNA. A = adenosine; C = cytosine; G = guanine; T = thymine.

Methylation occurs at the 5 carbon of the DNA base cytosine (Figure 2-14). This process is undertaken by a group of enzymes called DNA methyltransferases (DNMTs). The DNMTs achieve methylation by enveloping a cytosine base that has been flipped out from the DNA helix. A covalent bond forms between the enzyme and substrate base which causes an increased negative charge at position 5- of the cytosine. To neutralise the charge, a methyl group donated from *S*-adenosyl-methionine (SAM) is added to C5 which in turn causes the cleavage of the covalent bond between the enzyme and the substrate base freeing the DNMT to methylate additional cytosine bases (Jeltsch 2002; Goll and Bestor 2005).

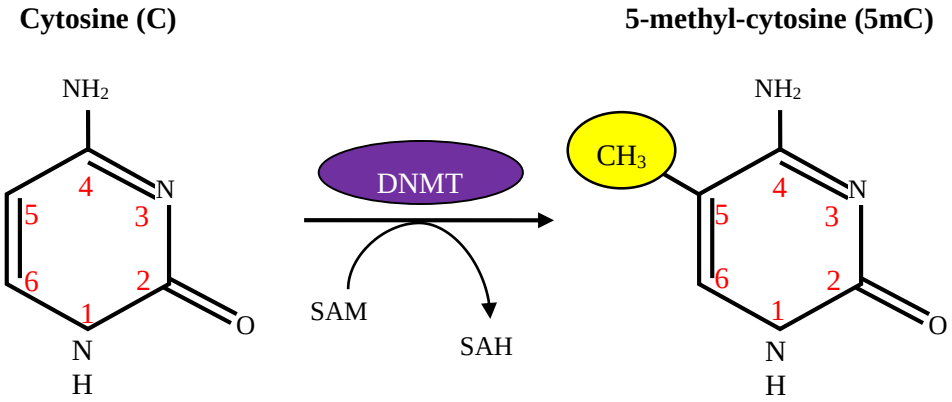

Figure 2-14. Positioning of methyl group on a cytosine base
A methyl group (CH_3) donated from *S*-adenosyl-methionine (SAM) is covalently bonded to the 5 carbon on the cytosine base; a process facilitated by DNA methyltransferase (DNMT). SAH = *S*-adenosyl-homocysteine.

In mammalian genomes, methylation predominately occurs when cytosine and guanine bases fall in succession within the DNA sequence; referred to as CpG dinucleotides, CpGs (Kim *et al.* 2010a). Methylation can also occur at CpH dinucleotides (where H = adenosine [A] / cytosine [C] / thymine [T]) however CpH methylation has only been observed in mammalian oocytes, pluripotent embryonic stem cells and mature neurons (Lister *et al.* 2009; Xie *et al.* 2012; Lister *et al.* 2013; Shirane *et al.* 2013) and is absent in somatic tissue (Ramsahoye *et al.* 2000; Ziller *et*

al. 2011). Unlike CpGs which demonstrate cell-type-specific methylation (Lister *et al.* 2009), CpH methylation has great inter-individual variability and are therefore not thought to have a gene regulatory function (Medvedeva *et al.* 2014). As the function of CpH methylation is unknown the focus of this study will be CpG methylation.

In the adult human genome the majority, ~60-90%, of CpG sites are methylated (Puig & Agrelo 2012; Smith and Meissner 2013), however the distribution of CpGs, and their methylation status is uneven within the genome (Figure 2-15). Approximately 99% of the human genome has a CpG deficit, in intronic and intergenic regions for example, and the CpGs in these regions are mostly methylated. However ~1% of the human genome has clusters of CpG sites, particularly in promoter regions near transcription start sites, and these CpG sites are largely unmethylated (Deaton & Bird 2011). The lack of CpG homogeneity throughout the genome is as a result of the instability of methylated-cytosine that deaminates to thymine (Weber *et al.* 2007; Cohen *et al.* 2011).

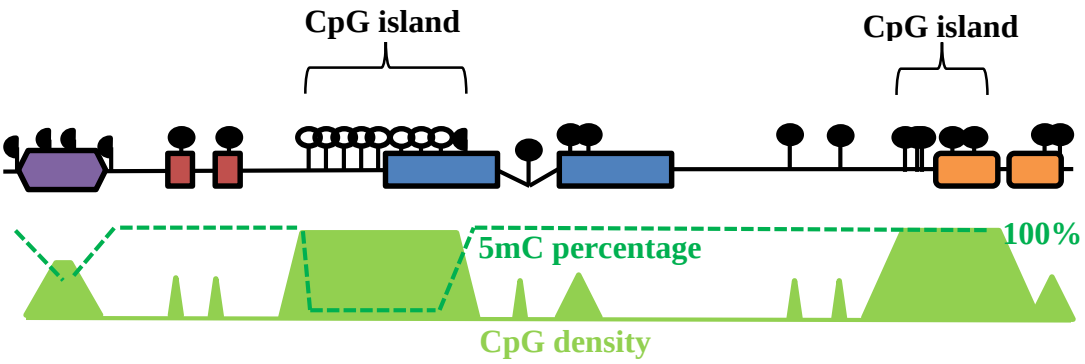

Figure 2-15. Distribution of methylated and unmethylated CpGs within the mammalian genome
Unmethylated CpGs (empty lollipops) and hemi-methylated CpGs (half lollipops) cluster within promoter regions of coding regions (blue rectangles), termed CpG islands, and near enhancer sequences (purple hexagon). If the island is methylated then transcription factors can bind and the gene can be expressed. However, if the CpG island is methylated (filled lollipop), e.g. islands associated with imprinted genes (orange rectangles), then transcription factors cannot bind and the gene is silenced. Throughout the rest of the genome CpGs are sparse and generally methylated silencing repeat elements within the DNA (red squares). Figure adapted from Baubec and Schubeler 2014.

2.2.1 Functions of DNA methylation

The arrangement and methylation status of CpG sites within the genome is important since DNA methylation influences whether DNA is transcribed or repressed. For example, intergenic and intronic regions that were thought to contain "junk" DNA actually contain potentially harmful transposable / repetitive elements such as SINE, LINE, DNA transposons and simple repeats that can act as functional genome reshapers (Han & Boeke 2005; Beauregard *et al.* 2008; Goodier & Kazazian 2008; Su *et al.* 2012). If the CpGs repressing these repetitive elements become hypomethylated it can cause genomic instability leading to genetic disorders and cancer (Kato *et al.* 2007; Daskalos *et al.* 2009; Choi *et al.* 2009; Baccarelli *et al.* 2010; Xie *et al.* 2010). One such disorder is immunodeficiency, centromeric instability, and facial anomaly (ICF) syndrome that occurs when pericentromeric repeats are not repressed. Lack of repression is a result of missense mutations in the DNA methyltransferase, DNMT3b. As the pericentromeric repeats are not repressed they cause rearrangements near the centromeres leading to the affected individuals being susceptible to infectious diseases and having facial abnormalities (Gopalakrishnan *et al.* 2009).

Methylation is also fundamental for long term repression and silencing of genes on the inactive X chromosome (Splinter *et al.* 2011). During the embryonic stages of female mammalian development, one X chromosome undergoes region wide inactivation by a *cis*-acting non-coding RNA, *Xist*. This inactivation is shortly followed by recruitment of proteins and a change in chromatin structure brought about by DNA methylation that inhibits transcription factor access to the DNA (Payer and Lee 2008). If both of the X chromosomes were active then twice the number of genes

associated with the X chromosome would be produced compared to males resulting in metabolic imbalance (Gartler & Goldman 2001).

As well as maintaining the inactivation of the X chromosome, methylation is important for establishing and maintaining imprinted genes (Miranda & Jones 2007; Bartolomei 2009; Ferguson-Smith 2011). The rule for the majority of genes is that both alleles, the paternal allele and the maternal allele, are expressed equally. However, when genes are imprinted only one of the parental alleles are expressed. For example, the insulin-like growth factor II (IGF2) is only expressed from the paternal allele (Pedone *et al.* 1999) as the maternal allele is repressed by DNA methylation. Failure to maintain gene imprinting can lead to numerous developmental defects such as Prader-Willi syndrome (Gillessen-Kaesbach *et al.* 1995), Beckwith-Widemann syndrome (Kubota *et al.* 1994) and Angelman syndrome (Das *et al.* 1997).

Although other epigenetic modifications such as histone modifications can alter chromatin structure and gene expression, they are reversible so long-term gene inactivation is not maintained (Shi *et al.* 2004; Takeuchi *et al.* 2006). McGarvey *et al.* (2006) demonstrated that artificially reversing histone modifications did not alter gene expression if the promoter region was highly methylated. However, active *in vitro* demethylation of promoter regions does activate a gene (Kaminskas *et al.* 2005) despite the presence of other chromatin / gene altering modifications suggesting methylation is the predominant controlling factor.

DNA methylation is also fundamental in cellular differentiation that enables organisms to exist. During embryogenesis essential methylation programming occurs that determine which genes are expressed, as well as determining cell fate (Christensen *et al.* 2009; Huang & Fan 2010). To understand the influence of DNA methylation on

cellular differentiation, Laurent *et al.* (2010) compared the methylation profile of different cells: an undifferentiated cell; human embryonic stem cell, and differentiated cells; a fibroblastic derivative of the human embryonic stem cell, neonatal fibroblasts and a fully differentiated adult monocyte. Their study found that embryonic stem cells had higher global methylation in comparison to fibroblasts and monocytes, however genes associated with pluripotency and development had less methylation in embryonic cells than differentiated cells. For example, the *POU5F1* / *OCT4* gene that encodes for a transcription factor important for embryonic development has less methylation within the transcription start site in embryonic cells than the differentiated cells. This lack of methylation within the transcription start site allows the necessary transcription of the gene for embryogenesis, but becomes increasingly methylated during differentiation when the transcription factor is not as functionally important for cell development.

Similarly, HOX genes that are responsible for the orientation and basic structure of an organism are less methylated during embryogenesis allowing appropriate anatomical arrangement and are more methylated in differentiated cells. Different methylation patterns are also seen in different tissue samples. For example, CpG sites in adult and infant blood are typically more methylated than CpG sites in tissues such as placenta, brain and kidney (Christensen *et al.* 2009). A study conducted by Byun *et al.* (2009) discovered that one particular locus, *MST1R*, was heavily methylated in the brain compared to other tissues. This particular gene encodes for a receptor that is involved with the development of epithelial tissue and bone that is less in demand in brain tissue than other tissues (Byun *et al.* 2009).

2.2.2 DNA methyltransferases (DNMTs)

There are four members in the DNMT family, three enzymes that actively undertake DNA methylation, DNMT1, DNMT3A and DNMT3B (Goll and Bestor 2005), and DNMT3L, a protein that shares homology with DNMT3A and DNMT3B but lacks catalytic activity (Gowher *et al.* 2006; Kareta *et al.* 2006). The domains of the DNMTs cause a preference for either *de novo* methylation (establishment of new methylation patterns, during germ development for example) or for maintenance methylation (maintaining methylation patterns following DNA replication). DNMT1, DNMT3A and DNMT3B contain a large N-terminal catalytic domain and smaller C-terminal domain. The localisation of the DNMT in the nucleus and interaction with other proteins, DNA and chromatin is regulated by domains within the N-terminal. The C-terminal is the enzymatically active domain responsible for the transfer of the methyl group to the 5 carbon of cytosine (Jeltsch 2002).

DNMT3L, although not catalytically active is also required for DNA methylation. DNMT3L is unable to bind SAM and has very weak affinity for DNA (Gowher *et al.* 2006; Kareta *et al.* 2006) however has been shown to co-localise and stimulate the activity of DNMT3A and DNMT3B *in vivo* (Chedin *et al.* 2002; Chen *et al.* 2005a; Jia *et al.* 2007) and *in vitro* (Suetake *et al.* 2004; Gowher *et al.* 2006; Kareta *et al.* 2006). DNMT3L-knockout mouse studies have shown that DNMT3L is important for establishing methylation on imprinted genes. Without DNMT3L, de novo methylation does not occur in the germline resulting in mouse sterility (Bourc'his and Bestor 2004; O'Doherty *et al.* 2011).

2.2.2.1 *De novo* methylation and maintenance methylation

The different DNMTs methylate cytosines at various periods in cell development. DNMT3A and DNMT3B are more prevalent during germ cell development when new

methylation patterns are established, *de novo* methylation (Figure 2-16a). Whereas DNMT1 is the primary DNMT for maintaining methylation patterns following DNA replication (Kim *et al.* 2009) as illustrated in figure 2-16b. However all of the DNMTs are involved in both de novo and maintenance methylation to some extent (Riggs and Xiong 2004).

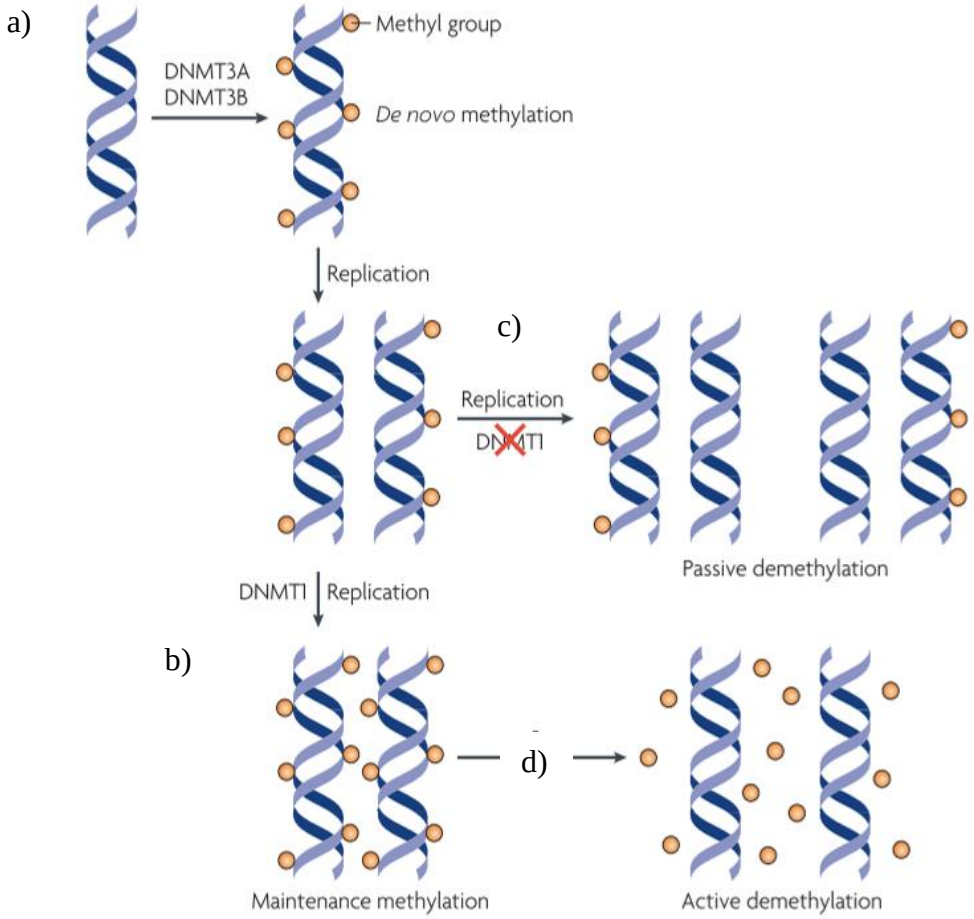

Figure 2-16. Functions of DNMTs
a) DNMT3A and DNMT3B are prevalent during cell development to induce de novo methylation patterns. b) During cell replication, DNMT1 is more prevalent as it has a preference for hemi-methylated DNA; c) without the presence of DNMT1 the DNA passively demethylates causing cell abnormalities; d) active demethylation by enzymes such as TET (figure taken from Wu & Zhang 2010).

Once the methylation patterns have been established and the DNA undergoes replication, DNMT1 quickly methylates the newly synthesised DNA strand preserving the methylation pattern of the parent strand (Kim *et al.* 2009) (Figure 2-16b). DNMT1

is considered to be the major maintenance methyltransferase as it has a preference for hemi-methylated DNA (Pradhan *et al.* 1999). Transcription of DNMT1 occurs mostly during the S-phase of the cell cycle (Robertson *et al.* 2000) when it is most needed to methylate newly generated hemi-methylated DNA. DNMT1 achieves this by occupying a position at the replication fork from which it can methylate the CpG dinucleotides before the chromatin reassembles (Jurkowska *et al.* 2011). DNMT1 has domains that can interact with the DNA polymerase processivity factor, proliferating cell nuclear antigen (PCNA)- interacting binding partner, NP95 (Chuang *et al.* 1997) which would ensure its localisation to the replication fork (Arand *et al.* 2012). NP95 specifically attracts DNMT1 to the parental methylated strand orientating the enzyme and its activity to the newly synthesised, unmethylated strand (Bostick *et al.* 2007; Sharif *et al.* 2007). Disruption of DNMT1-PCNA interaction only causes a small change in methylation (Egger *et al.* 2006; Schermelleh *et al.* 2007). The ubiquitin-like PHD and RING finger domain containing protein (UHRF1) could also fulfil a similar function (Bostick *et al.* 2007; Arita *et al.* 2008; Avvakumov *et al.* 2008). DNMT1 may be recruited by UHRF1 to hemi-methylated sites in DNA even after replication (Bostick *et al.* 2007; Arita *et al.* 2008; Avvakumov *et al.* 2008; Jones and Liang 2009).

DNMT1 however is not efficient so *de novo* methylation by DNMT3a and DNMT3b helps re-establish the precursor methylation pattern (Jeong *et al.* 2009; Starkweather & Pair 2011). DNMT3A and 3B are anchored to nucleosomes, presumably to methylated regions, and do not read the methylation but are proposed to methylate sites missed by DNMT1 during DNA replication (Jeong *et al.* 2009). Mice lacking DNMT3A and DNMT3B have up to 30% of CpG sites hemimethylated in repeat regions, and embryonic cells gradually lose DNA methylation with each cell division

(Liang *et al.* 2002; Chen *et al.* 2003). The passive demethylation results in abnormal cell development (Wu & Zhang 2010).

2.2.3 Mechanism of gene regulation by DNA methylation

The exact mechanism by which CpG methylation inhibits gene transcription is unclear; but possible suggested mechanisms include:

1. **Direct blocking of transcription factor binding sites (TFBS).**

The addition of methyl groups to the CpG dinucleotides, in or surrounding, transcription factor binding sites can interrupt the recognition sequence to which transcription factors bind (Klose and Bird 2006; Maunakea *et al.* 2010). There are ~1400 transcription factors in the mammalian genome (Vaquerizas *et al.* 2009) however the sensitivity of these transcription factors to DNA methylation have only been tested in a minority (Medvedeva *et al.* 2014). The transcription factors listed in Table 2-1 have CpG dinucleotides within their recognition motifs and the majority of transcription factors are adversely affected when these CpGs are methylated.

Table 2-1. Impact of methylated DNA on transcription factor binding site recognition

Transcription Factor	Effect of methylation on transcriptional activity	Reference
Aryl hydrocarbon receptor (AhR)	Inhibitory	Shen and Whitlock 1989 Sudheer *et al.* 2010
Activator protein 1 (AP-1)	Inhibitory	Comb and Goodman 1990 Ng *et al.* 2013
cAMP response element binding protein (CREB)	Inhibitory	Iguchi and Schaffner 1989 Kim *et al.* 2007 Wen *et al.* 2010
Specificity protein 1 (Sp1)	Inhibitory No effect	Macleod *et al.* 1994 Lal *et al.* 2009 Wang *et al.* 2010 Harrington *et al.* 1988; Holler *et al.* 1988
E2F	E2F1-5 and E2F2 = Inhibitory; E2F2-5 = no effect	Campanero *et al.* 2000
Cdc2	Inhibitory	Campanero *et al.* 2000
YY1	Inhibitory	Kim *et al.* 2003
n-Myc	Inhibitory	Perini *et al.* 2005
NGFI-A	Inhibitory	Weaver *et al.* 2007
c-myc	Inhibitory	Prendergast *et al.* 1991
CTFC	Inhibitory	Bell and Felsenfeld 2000 Renaud *et al.* 2007 Wang *et al.* 2012
P120(ctn_-binding partner Kaiso	No effect; however greater affinity for unmethylated binding sites.	Daniel *et al.* 2002
USF1	No effect	Prendergast *et al.* 1991
TFE3	No effect	Prendergast *et al.* 1991

For example, the transcription factor n-Myc is prevented from binding to the promoters of the epidermal growth factor receptor (EGFR) and caspase-8 protein (CASP8) if the E-box (CACGTG) is methylated (Perini *et al.* 2005). In vitro experiments have shown that the binding activity of Yin Yang 1 (YY1) is repressed if the YY1-binding site (CCAT / ATGG) in the paternally-expressed gene 3 protein (PEG3) is methylated (Kim *et al.* 2003). However the effect of methylation on Sp1 transcription factor binding is variable. Some authors have found that SP1 is not affected by DNA methylation (Harrington *et al.* 1988; Holler *et al.* 1988) whereas others have demonstrated that Sp1 binding may be affected by methylation of specific CpGs within the Sp1 binding motif (Clark *et al.* 1997) or affected by hypermethylation surrounding the Sp1 binding site (Zhu *et al.* 2003).

The positioning of transcription factor (TF) motifs effects the level of methylation as TF motifs <1000bp from transcription start sites had significantly less methylation than TF motifs >1000bp from transcription start sites (Eckhardt *et al.* 2006). TF motifs near transcription start sites of development and housekeeping genes are hypomethylated to enable normal growth. When these CpG islands become methylated the transcription of the respective gene is inhibited resulting in gene silencing (Jones & Baylin 2002). Experiments have been undertaken that illustrate the repression of gene expression by artificial methylation (reviewed in Jurkowska & Jeltsch 2010), as well as the restoration of gene expression by demethylating endogenously methylated DNA regions using DNA methyltransferase inhibitors (Baylin 2005; reviewed in Kelly *et al.* 2010). This is not the case for all genes, for example, genes with high CpG density are hypomethylated and repressed if the CpG island becomes hypermethylated (Weber *et al.* 2007; Meissner *et al.* 2008), however genes with intermediate CpG density acquire differentiation-dependant hypermethylation and are repressed with hypermethylation (Maatouk *et al.* 2006; Weber *et al.* 2007; Farthing *et al.* 2008; Meissner *et al.* 2008; Borgel *et al.* 2010), whereas low CpG density promoters which are generally hypermethylated remain transcriptionally active regardless of methylation state (Weber *et al.* 2007; Meissner *et al.* 2008).

2. Indirect blocking of transcription factor binding sites.

a. Methyl-CpG binding protein complexes block transcription factor binding sites.

Methylated CpGs recruit proteins that contain a methyl-CpG binding domain (MBD), methyl-CpG binding zinc finger domains, and SET and RING Associated (SRA) domains (Figure 2-17). Proteins with MBD that bind to methylated DNA include

MeCP2, MBD1, MBD2 and MBD4 (Klose and Bird 2006). Kaiso, ZBTB4 and ZBTB38 contain zinc finger domains and UHRF1 and UHFR2 have SRA domains. When these proteins bind they, and their associated co-repressor complexes, may block transcription factor binding (Nan *et al.* 1998; Jones *et al.* 1998; Sharif *et al.* 2007; Quenneville *et al.* 2011).

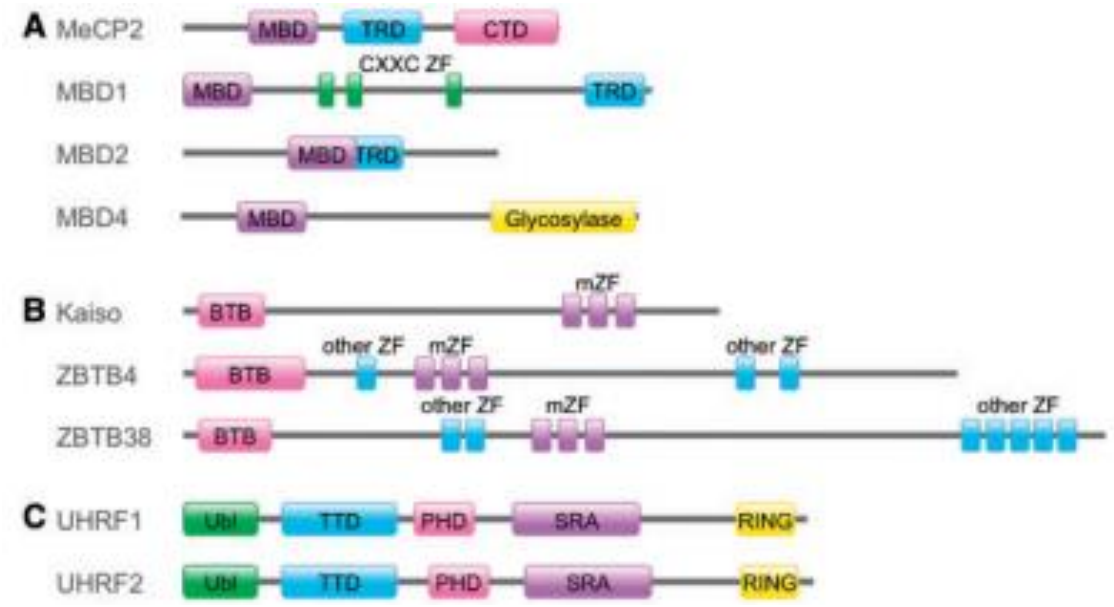

Figure 2-17. Proteins recruited to methylated CpGs
BTB = Broad complex, Tramtrack, Bric and brac; CTD = chromatin compaction; CXXC ZF = cysteine rich unmethylated-CpG-binding zinc finger; glycolase = DNA glycolase activity; MBD = methyl binding domain; mZF = methyl-CpG-binding zinc fingers; PHD = plant homeodomain finger domain; SRA = SET and RING associated domain; RING = really interesting new gene finger domain; TRD = transcriptional repression domain; TTD = tandem tudor domain; Ubl = ubiquitin-like domain. Image taken from Fournier *et al.* 2012.

b. Alteration of chromatin / nucleosome configuration

The proteins complexes formed by MeCP2, MBD1, MBD2 and MBD4 can also silence gene expression by altering the formation of the chromatin (Tate and Bird 1993). The MBD proteins recruit histone deacetylases (HDAC) and / or DNA methyltransferases (DNMTs) that remove an acetyl group and / or add a methyl group to H3 and H4 histone tails, respectively. Acetyl groups on histone tails is associated

with open chromatin, euchromatin that allows access to the transcription machinery. The removal of acetyl groups and addition of methyl groups creates an inaccessible chromatin configuration, heterochromatin, that inhibits transcriptional activity (Kimura and Shiota 2003; Fuks *et al.* 2003; Klose and Bird 2006; Bogdanovic and Veenstra 2009). Furthermore, CpG methylation can contribute to gene transcription repression by forming a more rigid and compact nucleosome conformation, wrapping the DNA tighter around the histone and changing the topology of nucleosomal DNA limiting access of the underlying DNA to transcription factors (Choy *et al.* 2010; Lee and Lee 2011).

2.2.4 Factors that influence DNA methylation

2.2.4.1 Diet

Epigenetic mechanisms, although inherently stable, can be affected by nutrition, exposure to drugs / chemicals and age (Barres & Zierath 2011). The most widely used example to illustrate the effect of nutrition on DNA methylation is the *Apis mellifera* (Honeybee). Worker and queen bees begin life as genetically identical larvae, however, the larvae that are fed royal jelly become queen bees and those that are not fed royal jelly become worker bees (Kucharski *et al.* 2008). Specifically, the protein royalactin has been proposed as the factor that induces queen / worker bee differentiation, as bees fed with purified royalactin had an increased body size and ovary development (Kamakura 2011).

In human populations, deficiencies in nutrients essential for DNA methylation cause aberrant methylation profiles (Sharp *et al.* 2008). These nutrients include folate, choline, vitamin B_6, vitamin B_{12} and methionine that are modulated by one-carbon metabolism (Figure 2-18). Folate, methionine and choline are methyl group donors,

and vitamin B_6 and vitamin B_{12} facilitate the methyl group donation. From methionine, S-adenosylmethionine (SAM) is synthesised which donates its methyl group to cytosine, facilitated by DNMTs. The loss of the methyl group reduces SAM to S-adenosylhomocysteine (SAH). An accumulation of SAH results in methylation inhibition as the synthesis of SAH from homocysteine is favourable over the hydrolysation of SAH to homocysteine. Therefore there is no homocysteine to accept a methyl group from folate or choline to form methionine, and no SAM is synthesised resulting in no DNA methylation (Chmurzynska 2010).

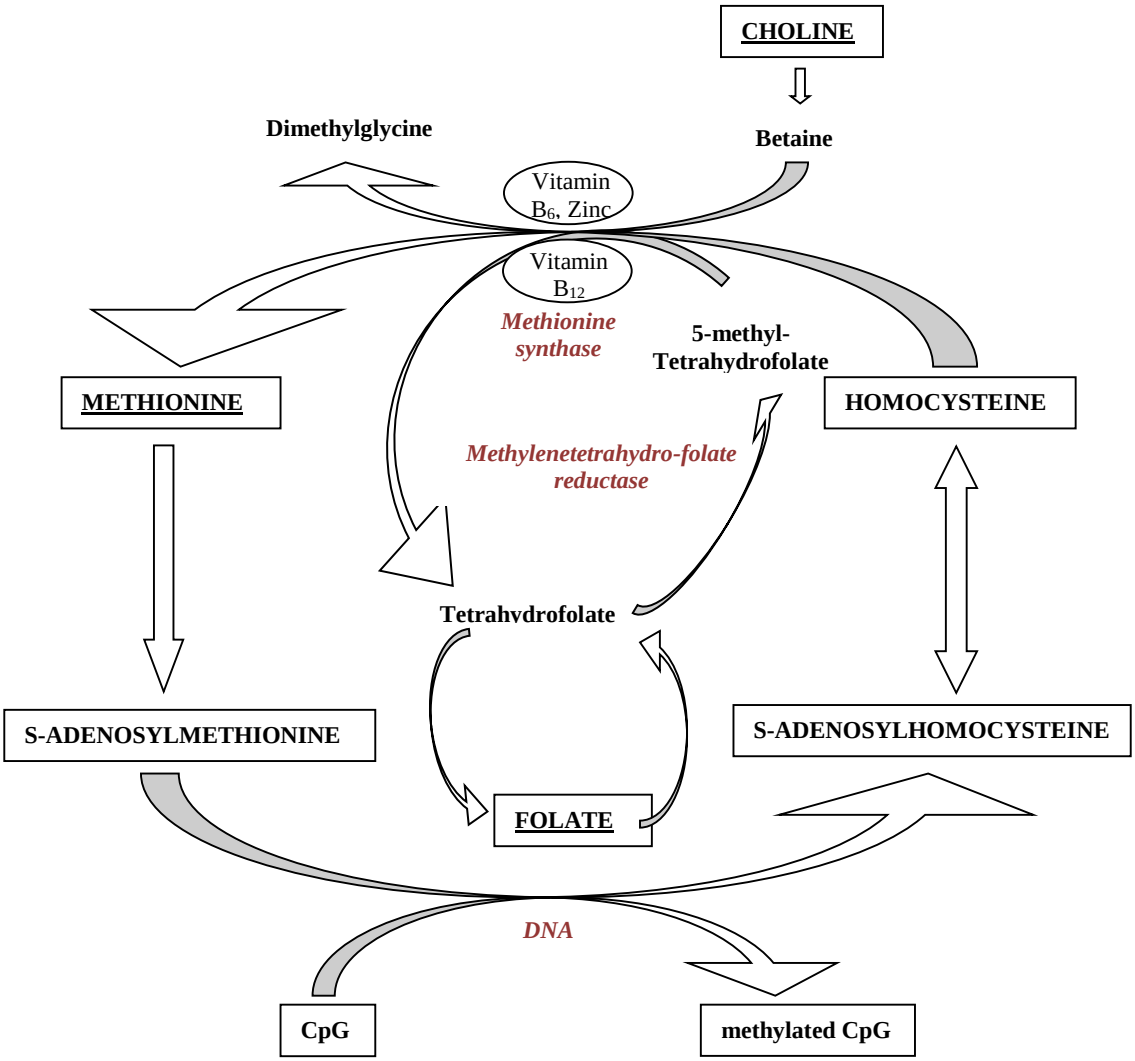

Figure 2-18. Simplified version of one-carbon metabolism showing some major metabolic intermediates, cofactors and dietary sources of methyl groups
Methionine, choline and folate are the major donors of methyl groups for DNA methylation however a number of enzymes and vitamins are necessary for successful methyl group donation; enzymes are written in red and italics, essential vitamins are inside ellipses. Information to create figure obtained from Mason (2003) and Chmurzynska (2010).

Without the donation of methyl groups from these factors, or the cofactors that facilitate methyl group donation, methylation cannot take place (Sharp *et al.* 2008). As normal DNA methylation is not maintained, repetitive elements within intronic and intergenic regions, as well as oncogenes can become available for transcription (Choi & Mason 2000; Wilson *et al.* 2007) and genes critical for tumour repression and growth regulatory genes become repressed promoting and progressing carcinogenesis (Sharp *et al.* 2008). Studies carried out using mice and rats on various methyl donor group deficient diets demonstrate the impact on genome methylation status (Powell *et al.* 2005; Pogribny *et al.* 2006; Huang *et al.* 2008; Pogribny *et al.* 2009). A global loss of DNA methylation was reported as well as hypermethylation of promoter regions.

These abnormal methylation patterns were reversible if the rodents were returned to methyl-sufficient diets within a relatively short time period, but the longer the rodents were methyl-deficient, the more likely the abnormal methylation would remain contributing to the progression of the cancer (Pogribny *et al.* 2009). A review by Kim (2003) concluded that the dose of folate and the stage of the disease in which folate is taken are important as folate supplementation during the initial stages of cancer can be beneficial, but later in cancer development can act as a cancer catalyst. It is commonly known that human cancer patients experience a loss of appetite during the advanced stages of their disease (Halliday *et al.* 2012) so may not intake the necessary nutrients for DNA methylation maintenance. However, the addition of nutrient supplements to cancer patients' treatment does not always change the outcome of the cancer for the better (Andreeva *et al.* 2012) and may even increase the chance of prostate cancer in men (review by Wien *et al.* 2012).

2.2.4.2 Age

Age also has an impact on DNA methylation status (Christensen *et al.* 2009). Maintenance of DNA methylation profiles deteriorate with age (Li *et al.* 2010) so DNA regions typically hypermethylated become hypomethylated, and hypomethylated promoter regions become hypermethylated. This results in the expression of genes that are normally silenced and the repression of previously expressed genes (Richardson 2003). Mechanisms that are thought to cause these aberrant methylation profiles include; altered expression of DNMTs, increased plasma concentrations of homocysteine (de Bree *et al.* 2005) and less efficient DNA repair (Gorbunova *et al.* 2007).

2.2.4.3 Environmental and drug exposures

Exposure to trace metals within the environment have also been reported to alter normal methylation patterns. These metals can alter the DNA methylation by interfering with metabolism of methinonine / folate (Rowling *et al.* 2002); by competing for the intracellular methyl group inhibiting its use for DNA methylation (Sutherland & Costa 2003); or by directly inhibiting the methyltransferases (Cox 1985; Takiguchi *et al.* 2003).

For arsenic to be metabolised, a methyl group must be donated which directly competes with the DNA methylation process for the SAM molecule. When arsenic was introduced into the diet of rats, DNA from liver tissue was hypomethylated (Davis *et al.* 2000), this outcome was supported by Chen *et al.* (2004) and Reichard *et al.* (2007), however they also reported gene-specific hypermethylation. Exposure to nickel can trigger *de novo* methylation of tumor suppressor genes making it a potent environmental carcinogen (Cangul *et al.* 2002). When Lee *et al.* (1995) exposed *Escherichia coli gtp* gene to nickel the transfected gene was inactivated. Cadmium

also causes genome-wide demethylation, and one method in which it achieves this is by inhibiting methyltransferases (Takiguchi *et al.* 2003). It has been speculated that these metals alter DNA methylation by metal induced oxidative stress.

The reactive oxygen species (ROS) produced by the metals interfere with the DNMT:DNA interaction resulting in no methylation (Baccarelli and Bollati 2009). In response to ROS, the body produces the antioxidant glutathione (GSH) (Schwartz *et al.* 2005; Chahine *et al.* 2007). GSH is produced from homocysteine that is fundamental in the one-carbon metabolism pathway. The redirection of homocysteine for GSH production causes a decreased availability of methyl donors and therefore DNA hypomethylation (Hitchler and Domann 2009). In addition, ROS can directly damage the DNA reducing the binding affinity of methyl-CpG binding proteins (MBPs) that recruit DNA methyltransferases (DNMTs) to methylate the DNA (Valinluck *et al.* 2004; Baccarelli and Bollati 2009).

A study by Bollati *et al.* (2007) reported that prolonged exposure to airborne benzene can alter methylation patterns. They found that the benzene exposed individuals had a global loss of DNA methylation and hypermethylation of gene-specific methylation. Exposure to benzene has been associated with an increased risk of acute myelogenus leukaemia (Bird *et al.* 2005).

Alongside accidental exposures to trace metals and air pollutants, substances we choose to consume can affect DNA methylation. Alcohol has been found to alter the DNA methylation patterns in several tissues including liver, oesophagus, colon and uterus tissue. These alterations lead to genetic and phenotypic changes (Zhou *et al.* 2011b). Alcohol achieves these genetic changes via two methods; directly affecting the methyl donors and inhibiting enzymes (methionine synthase and methionine

adenosyltransferase) important for one-carbon metabolism (Mason and Choi 2005). Christensen *et al.* (2010) suggest that alcohol's disruption of the one-carbon metabolism is the mechanism that makes it carcinogenic.

Drugs such as cannabis and opioids are also thought to alter methylation by stimulating DNMT activity through G-protein-coupled receptor-mediated cascade pathways (Paradisi *et al.* 2008; Doehring *et al.* 2013). The effect of drug exposure on the epigenome, specifically gene alterations made in the reward, motivations and pleasure centres of the brain are postulated to play a role in the development of addiction, dependence and tolerance (Kalivas *et al.* 2005; Hyman *et al.* 2006; Koob and Kreek 2007).

Acute cocaine exposure was observed to increase the expression of DNMT3a and 3b in the nucleus accumbens (NAc) and stimulate MeCP2 binding causing hypermethylation of the protein phosphatase-1 catalytic subunit (*PP1c*) promoter. Pharmacological inhibition of DNMT activity resulted in attenuated down regulation of PP1c in the NAc and delayed the development of cocaine induced behavioural sensitization (Anier *et al.* 2010). Therefore, alterations to *OPRM1* methylation may influence the analgesic effects of opioids and could play a role in the development of tolerance.

2.2.4.4 Smoking

Tobacco exposure has been associated with mortalities through causing numerous cancers, cardiovascular and metabolic diseases and respiratory diseases (Centres for Disease Control and Prevention 2011). These diseases are instigated by the chemicals within tobacco smoke such as carcinogens, nicotine and carbon monoxide that can

damage DNA and alter epigenetic mechanisms such as DNA methylation (Lee and Pausova 2013).

Exposure to cigarette smoke is considered one of the most influential factors for modifying DNA methylation patterns (Breitling *et al.* 2011; Lee and Pausova 2013; Shenker *et al.* 2013; Zeilinger *et al.* 2013). Possible mechanisms for cigarette smoke mediated DNA methylation alterations are shown in Figure 2-19, as summarised by Lee and Pausova (2013). Carcinogen induced double-stranded DNA breakage results in the recruitment of DNMT1 that has been suggested to alter the normal methylation profile (Mortusewicz *et al.* 2005). The recruited DNMT1 methylates CpG sites adjacent to the DNA repair site regardless of the CpG site's previous methylation status (Cuozzo *et al.* 2007).

Secondly, activation of the nicotine acetylcholine receptor increases intracellular calcium; the calcium increase leads to downstream stimulation of the transcription factor cAMP response element-binding protein that can alter gene expression (Shen and Yakel 2009). Via this pathway it has been suggested that DNMT1 expression is down-regulated in cortical and hippocampal GABAergic neurons resulting in hypomethylation and subsequent reduced expression of genes such as GAD67 (Satta *et al.* 2008).

Thirdly, cigarette smoke exposure can modulate the expression and activity of DNA-binding proteins, e.g. Sp1 and PEA3 (Mercer *et al.* 2009; Di *et al.* 2012; Wallace *et al.* 2012; Shin *et al.* 2015). Cigarette smoke exposure has been observed to increase Sp1 protein expression in lung epithelial cells resulting in increased transcription of the *MUC5AC* gene that is the major inducible mucus gene in the airway (Di *et al.* 2012).

Fourthly, the carbon monoxide within cigarette smoke decreases cellular oxygen content (Jensen *et al.* 1991), to which gene regulatory hypoxia-inducible factors (HIFs) respond (Majmundar *et al.* 2010). These transcription factors regulate enzymes that facilitate the synthesis of methyl donors thereby altering DNA methylation (Avila *et al.* 1998; Liu *et al.* 2011). Hypoxia has also been observed to alter the expression of DNMT1 and DNMT3a (Liu *et al.* 2011).

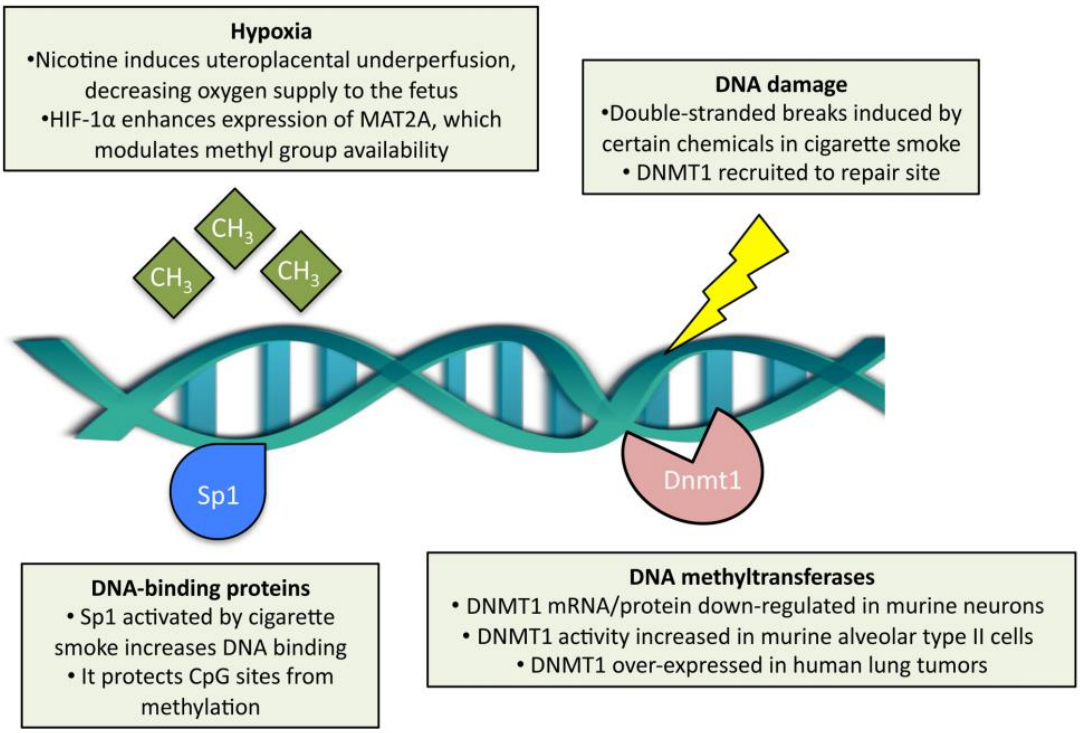

Figure 2-19. Mechanisms by which smoking effects DNA methylation
Cigarette smoke has been shown to modulate DNA methyltransferase 1 (DNMT1) content, both at the transcript and protein level, and enzymatic activity separately in different cell types. Double stranded DNA breaks may be induced by cigarette smoke, which subsequently recruits DNMT1 adjacent to the repair site. DNA binding proteins, such as Sp1, are activated by cigarette smoke and protect CpG sites from de novo methylation. In the context of prenatal exposure, cigarette smoke induces hypoxia in the embryo, which in turn modulates methyl group availability. Image obtained from Lee and Pausova (2013).

In vivo and *in vitro* experiments have shown that smoke exposure can alter DNA methylation. For example, repetitive elements within the genome such as LINE and AluYb8 are hypomethylated in diseased tissues of smokers (current or former) compared to non-smokers (Smith *et al.* 2007; Furniss *et al.* 2008). Global hypomethylation was also reported in cord serum DNA and buccal swabs of infants

exposed to prenatal smoke exposure (Breton *et al.* 2009; Guerrero-Preston *et al.* 2010). Hypomethylation of repetitive elements is associated with genomic instability leading to genetic mutations, insertions and deletions (Cordaux and Batzer 2009).

The genomic instability and resulting genomic mutations have been implicated in disease development. For example, infants exposed to prenatal smoke exposure have a greater susceptibility of developing diseases such as asthma (Wang and Pinkerton 2008), cancer (Filippini *et al.* 2000), obesity (Toschke *et al.* 2003; Sharma *et al.* 2008), type II diabetes (Montgomery and Ekbom 2002).

In addition to global hypomethylation, the extent of methylation at specific CpG loci varies between smoke and non-smoke exposed individuals. CpGs within genes associated with tumour suppression (*APC, CDH13, p16, RAR-beta, RASSF1A*), genomic stability (*MGMT*), or xenobiotic metabolism (*GSTP1*) or transportation (*ABCB1/MDR1*) are hypermethylated in smokers compared to non-smokers suggesting less gene expression (Zöchbauer - Müller *et al.* 2003; Enokida *et al.* 2006; Liu *et al.* 2010). However, genes associated with coagulation (F2RL3) and inflammation (*GPR15*) are hypomethylated in smokers (Breitling *et al.* 2011; Wan *et al.* 2012). The varying methylation of different genes suggests that tobacco smoke targets specific genes for hypermethylation and hypomethylation (Vaissiere *et al.* 2009).

2.2.5 DNA methylation and opioid genes

Although there are currently no direct links between methylation status of opioid related genes and opioid response, studies have been carried out that research the methylation status of opioid genes in relation to stage of disease, contribution to

disease progression, susceptibility to drug addiction and use of the methylation level as a biomarker for drug resistance (not opioids).

The expression of *OPRM1* is affected by DNA methylation. Embryonic P19 cells do not express the μ-opioid receptor as the promoter region of the *OPRM1* gene is heavily methylated. Once cells become differentiated, the promoter region is hypomethylated allowing expression of the gene. To further illustrate that DNA methylation is a prominent factor for *OPRM1* expression, the P19 cells were artificially demethylated and *OPRM1* was expressed (Hwang *et al.* 2007; Hwang *et al.* 2010). The majority of authors who have analysed *OPRM1* have focused on the promoter region of the gene. Within this region there are reported to be a number of transcription factors (Sp1, AP-1, NFkB, Stat6, IL-4) and 3 nucleosomes (Hwang *et al.* 2007; Hwang *et al.* 2010). In vitro experiments infecting hybrid T and B cells with Simian immunodeficiency virus (SIV) showed that SIV induced methylation of Sp1 transcription binding sites inhibited the expression of *OPRM1* (Liu *et al.* 2009). Therefore, methylation of CpG sites within the regions that contain the motifs for the transcription factors and nucleosomes are of importance. Variable *OPRM1* methylation levels have been reported between different ethnicities (Nielsen *et al.* 2010), former drug users and non-drug users (Nielsen *et al.* 2009) and ischemic cells and non-ischemic cells (Formisano *et al.* 2007) in human studies, and social (Hao *et al.* 2011) and diet (Vucetic *et al.* 2010) factors in rats.

The majority of studies that analyse the P-glycoprotein opioid transport protein (*ABCB1*/*MDR1* gene) are concerned with the methylation level at different stages of cancers such as colon and breast cancer. As cancer progresses, the *ABCB1*/*MDR1* gene becomes more methylated (Enokida *et al.* 2004; Muggerud *et al.* 2010). However, Dejeux *et al.* (2010) found that hypomethylation within the promoter region of the

gene was correlated with progressive disease and that the level of methylation could indicate the patient's drug response. This opinion was supported by Boettcher *et al.* (2010) who found that the *ABCB1*/*MDR1* was hypomethylated in breast and ovarian carcinoma and the resulting increase in gene expression could be an indicator of resistance to doxorubicin, a cancer chemotherapy drug.

With regards to studies involving opioid metabolising enzymes, there are few studies relating to the phase I metabolising enzymes important for oxycodone and methadone metabolism. However, the importance of studying these enzymes has been noted. Ingelman-Sundberg *et al.* (2007) located CpG islands within the genes and speculated that methylated CpG sites may play a role in gene expression. Kim *et al.* (2010b) found methylated sites within the *CYP2D6* gene but this methylation was not associated with drug response to tamoxifen.

2.2.6 DNA Methylation analysis

Numerous methods have been employed to analyse the methylation profile of a DNA sample (summarised in Laird *et al.* 2010). The initial step of methylation analysis is to distinguish between methylated cytosines and unmethylated cytosines (Johansson 2008). To achieve this, the DNA can be enzyme digested, bisulfite converted or undergo affinity enrichment pre-treatment. The literature has shown (Nielsen *et al.* 2009, Chrobov *et al.* 2010) that methylation at specific CpG loci correlate with drug response. Therefore, affinity enrichment pre-treatments would not be appropriate for this study as they determine the average methylation status of a defined region. Bisulfite conversion and restriction enzymes allow the methylation status of particular loci to be determined.

Bisulfite conversion was the first method developed to distinguish between the two cytosine states (Frommer *et al.* 1992). The unmethylated cytosine is converted to uracil by addition of bisulfite across cytosine's 5-6 double bond. Hydrolytic deamination of the unmethylated cytosine produces a bisulphate uracil derivative that undergoes alkali treatment to remove the sulfonate group resulting in uracil (Clark *et al.* 1994) (Figure 2-20). Following bisulfite pre-treatment the uracil bases are converted to thymine during PCR amplification whereas the methylated cytosines remain as cytosines. The DNA sequence can then be determined by gel separation following direct sequencing (also known as Sanger bisulfite sequencing) or capillary electrophoresis (bisulfite conversion sequencing). Alternatively the bisulfite converted sample can be sequenced by pyrosequencing.

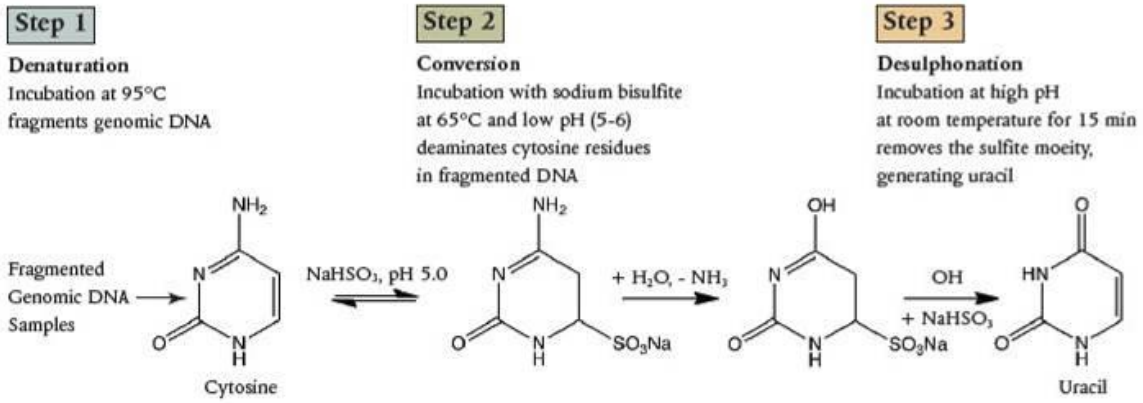

Figure 2-20. Process of bisulfite conversion
Figure taken from EpiMark Bisulfite Conversion Kit technical reference, available at http://www.neb.com/nebecomm/tech_reference/epigenetics/Bisulfite_Conversion.asp#.UJAy228mQo8

During pyrosequencing nucleotide bases are added to the single stranded DNA one at a time. Once a nucleotide is incorporated into the complimentary DNA strand pyrophosphate (PPi) is released. ATP sulfurylase converts PPi to ATP which in turn converts luciferase to oxyluciferase. Oxyluciferase produces light proportional to the amount of ATP produced which represented in a pyrogram as a peak. Any unincorporated bases are degraded by apyrase before the next nucleotide is added and

the process begins again (Figure 2-21). Methylated cytosines are indicated as cytosine, and unmethylated cytosines are indicated by a thymine. Fundamentally, the methylated sites are detected the way in which a C/T SNP would be identified. Pyrosequencing is not the cheapest method of analysis but the sample preparation is relatively quick and easy compared to direct sequencing.

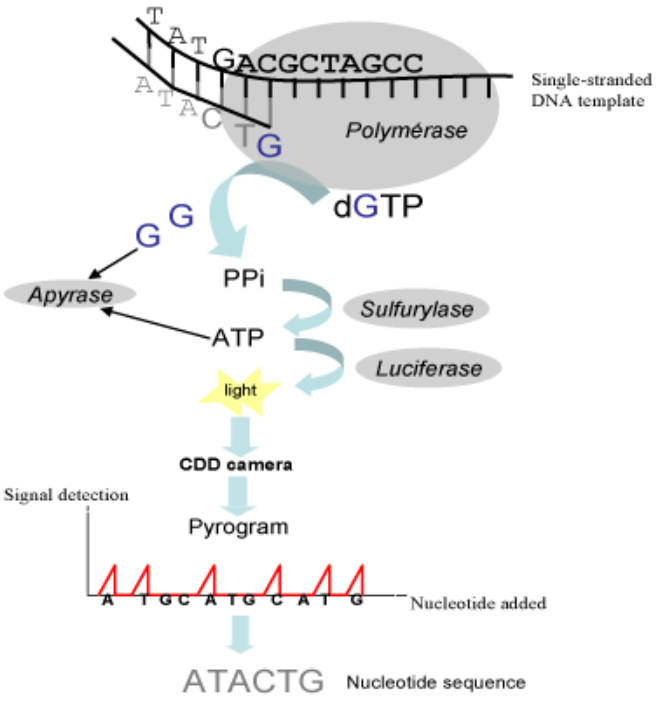

Figure 2-21. Mechanism of pyrosequencing
Sequencing primers anneal to single stranded DNA and aided by polymerase complimentary nucleotides are added to synthesised DNA. Nucleotides are added to the single stranded DNA sequentially; if that nucleotide is next in the sequence then it is incorporated into the DNA which releases pyrophosphatase. Via ATP sulfyrase, pyrophosphatase is converted to ATP that converts luciferase to oxyluciferase resulting in the production of light. This light is captured by a CCD camera and the intensity of the light is directly proportional to the number of nucleotides incorporated into the newly synthesised DNA sequence. Any unused nucleotides are broken down by apyrase and the next nucleotide is added to the DNA mix (image available at http://www.omicsonline.com/ArchiveJCSB/2009/February/03/Images/JCSB2.74Figure1.htm).

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3.0 GENERIC METHODS

To determine the effect of *ABCB1/MDR1*, *CYP2D6* and *OPRM1* gene DNA methylation, in conjunction with commonly investigated genetic variations, on response to opioid analgesics a population of cancer patients requiring morphine or oxycodone for pain relief were recruited. First though the influence of smoking and opioid exposure on gene DNA methylation was established in a population of smokers and non-smokers, and in a population of former-heroin dependant women and their babies. Gene DNA methylation has also been shown to be tissue dependant so methylation of *ABCB1/MDR1*, *CYP2D6* and *OPRM1* in various tissues were investigated.

3.1 Ethical considerations and patient recruitment

As the populations for each of the exploratory studies consisted of human volunteers, ethical approval was obtained to ensure that the outcomes of the project outweighed any adverse effects on the participants. The ethical processes for each population and recruitment process are detailed below.

3.1.1 Does smoking affect buccal DNA methylation?

DNA methylation is known to be affected by age, lifestyle, environmental exposures, smoking and diet. To determine the effect of smoking on the DNA methylation on *CYP2D6* and *OPRM1* in buccal cells a population of Caucasian smokers and non-smokers were recruited by the PhD candidate. A protocol was written (Appendix A 1.0) by the PhD candidate and Ethical approval sought and obtained from Bournemouth University Science, Technology and Health Research Ethics panel. Women on Bournemouth University campus grounds were approached and were

asked whether they would like to participate in a study concerning the influence of smoking on DNA. The women were eligible to participate in the study if they were between the ages of 18 and 50 and were not exposed to regular opioid use. If the women met the criteria they were asked to read a participant information sheet (Appendix A 1.1) and sign a consent form (Appendix A 1.2). The women were asked to provide their age (as methylation has been shown to alter with age), smoking status and whether or not they took opioids on a regular basis (Appendix A 1.3). Following completion of the consent form and questionnaire oral swabs were collected by the PhD candidate as described in section *3.2.1.1 Oral swabs*.

3.1.2 Methadone-prescribed opioid-dependant mothers and their new-born babies

To determine the effect of *in utero* drug exposure on the methylation of targeted opioid genes, buccal swabs from a population of pregnant former-heroin addicts on methadone maintenance treatment programmes and their babies were analysed. The protocol, ethics submission and collection of buccal DNA were undertaken by Dr Helen Mactier and Dr Cheryl Gillis at Glasgow Royal Infirmary. All methadone-presribed opioid-dependent (MPOD) mothers delivering after 36 completed weeks' gestation in a single large inner-city maternity unit in Glasgow were eligible to participate in this study. Potential subjects were identified within the first few days after delivery. The mothers had been managed within an established multidisciplinary service for women with substance misuse problems; antenatal care included ongoing methadone maintenance provided in collaboration with social work and addiction services and tailored to symptoms. Sufficient methadone was prescribed to eliminate physical withdrawals, with the aim of reducing towards the lowest acceptable dose of

methadone prior to delivery. Following written, informed consent demographic data included maternal age and prescribed dose of methadone at delivery, as well as infant gestation, birth weight and duration of hospital stay. All babies were nursed in the postnatal ward with their mothers and NAS was managed according to protocol with twice daily scoring using a local version of the Lipsitz scoring system (Lipsitz 1975). Those infants scoring 5 or more on two consecutive occasions despite consoling and/or with poor feeding or ongoing weight loss after 5 days were commenced on oral morphine at 60 µg/kg six times daily. This dose could be escalated to 80 µg/kg six times daily if the baby remained symptomatic, otherwise oral morphine was weaned daily by 10 µg/kg/dose. If NAS symptoms were not controlled by oral morphine, phenobarbititone was added to the treatment regime.

The control population of non-opioid-dependant mothers comprised 17 smoking mother-baby dyads from similar postcodes to the MPOD mothers, and 15 non-smoking mother-baby dyads from more affluent areas of Glasgow as assessed by the DEPCAT scale. The DEPCAT scale is derived from the Carstairs and Morris index of deprivation that amalgamates areas into four groups (DEPCAT 1-2 = affluent, 3-4 = intermediate, 5 = deprived, 6-7 = very deprived) based on level of overcrowding, male unemployment, car ownership and the proportion of people in households in social class 4 or 5 (NHS Lothian).

Oral swabs were obtained from the mothers and babies within 24-48 hours of delivery as described in *3.2.2.1 Oral swabs*. Plasma samples were obtained from the MPOD mothers as detailed in *3.2.2.2 Plasma samples* within 24 hours of delivery.

3.1.3 Tissue specific DNA methylation

3.1.3.1 Dundee blood and tissue samples

To help ascertain the effect that frequent opioid use has on DNA methylation of opioid important genes, post-mortem samples from deaths associated with heroin overdose, as decided by the resident pathologists, were obtained from Sir James Black Mortuary, Dundee. Tissue samples were collected as described in section *3.2.3*.

For comparison to the opioid exposed population, blood samples from a control non-drug using Dundonian population were obtained. Opioid naivety was determined by toxicological screening and drug / medical history. Ethical approval was not required as the results of the genetic analysis formed part of the medico-legal examiners investigation. Following genetic analysis, reports were written and incorporated into the pathologists report.

3.1.3.2 Edinburgh tissue samples

Non-opioid exposed tissue samples were required to ascertain the "normal" methylation of opioid related genes in liver, psoas muscle and thalamus. To achieve this the Edinburgh Tissue and Brain Bank was approached to provide liver, psoas muscle and thalamus from non-opioid exposed individuals. To obtain the samples a "tissue user request form" was completed and the management group of the tissue bank decided whether or not the proposed project was worthy of using their samples. To obtain the tissue samples from Edinburgh tissue bank a MTA was set up.

3.1.4 Pilot trial: Personalising opioid therapy for cancer pain relief

3.1.4.1 Royal Bournemouth, Christchurch and Poole Hospitals

To determine the effect of methylation and SNPs on opioid response, an opioid naïve population requiring either morphine or oxycodone were recruited from Royal Bournemouth Hospital, the MacMillan Unit, Forest Holme and Poole Hospital. To gain ethical approval, the PhD candidate wrote a protocol and supporting documents (Appendix B 1.0) and submitted them to the South Berkshire B Ethics Committee via the Integrated Research Application Service.

Eligibility for study participation was determined by lead clinician of each patient following the criteria detailed in the inclusion / exclusion document (Appendix B 1.1). The reasons for why patients could not be recruited into the study were recorded so that recruitment rate could be increased in future studies. Those patients that were eligible to participate in the study were provided with a patient information sheet and consent form (Appendix B 1.2 and B 1.3) and were approached by the PhD candidate. The patients were also asked if they would be willing to allow a member of the research team to visit them at their homes to discuss the study; if yes the patient was asked to sign a home visit permission form (Appendix B 1.4). The patient was given at least 24 hours to consider participating in the study before being asked to sign the consent form. If the patient chose not to participate, they were asked to provide a reason to help with future recruitment rate; but were under no obligation to give a reason if they chose not to.

When morphine or oxycodone use was imminent, baseline date was collected. This information included a demographic questionnaire (Appendix B 1.5), pain

questionnaire (Appendix B 1.7), side effect questionnaire (Appendix B 1.8), constipation assessment (Appendix B 1.9), and opioid history record (Appendix B 1.10).

Within 24 hours of the first opioid dose the patient was asked to complete a pain questionnaire and side effect questionnaire; these questionnaires were repeated on alternate days from initial dose. Once the patient was considered a morphine / oxycodone responder or non-responder they were asked to complete the pain, side effect and constipation questionnaire and provide blood and oral fluid samples. One blood sample and oral swab were obtained for genetic analyses and one blood sample and oral fluid sample were obtained for metabolite analysis. If the patient did not respond to their first-line opioid and they were switched to an alternative opioid the reason for the opioid switch was recorded in Appendix B 1.11 and biological samples were collected. If the patients were switched to either morphine or oxycodone then the patients were asked the same questions at the same time intervals as their first-line opioid.

Patients were considered stable on an opioid when their average pain over the last 24 hours was 4 or less for at least 2 days, and side effects experiences were either “none at all” or “a little”. Responses of >4 on the pain questionnaire, and “quite a bit” and “very much” responses on the side effect questionnaire constituted an opioid non-responder. If the initial opioid was ineffective, and the patients were switched to the alternative study opioid (either morphine or oxycodone) the patient continued to participate in the study. Once the patient reached a stable or switch time point blood samples were taken for toxicological analysis, and methylation and SNP analyses. In

addition, buccal swabs were obtained from a subset of cancer patients to determine the suitability of buccal swabs over blood for genetic and epigenetic analysis. Buccal swab DNA collection is less invasive and has a gentler collection procedure so would be more comfortable for the cancer patients who undergo innumerable tests and blood collections.

From the participating Bournemouth and Christchurch, and Poole NHS Foundation trusts 21 patients diagnosed with cancer requiring either morphine or oxycodone gave informed consent to participate in the study. Of the 21 patients, 18 patients saw the study through to completion providing pain and side effect questionnaire responses at the designated intervals and blood samples at the end of the study. Buccal swabs were obtained from 17 of the 18 patients who completed the study as the buccal collection devices were not ready when the first patient was recruited. The three patients that agreed to participate but did not complete the study withdrew for a number of reasons; 1) did not complete the questionnaire responses at the designated time points, 2) failed to provide blood and buccal swabs in a timely manner and, 3) felt daunted by the number of healthcare professionals contacting them throughout their treatment so decided against study participation.

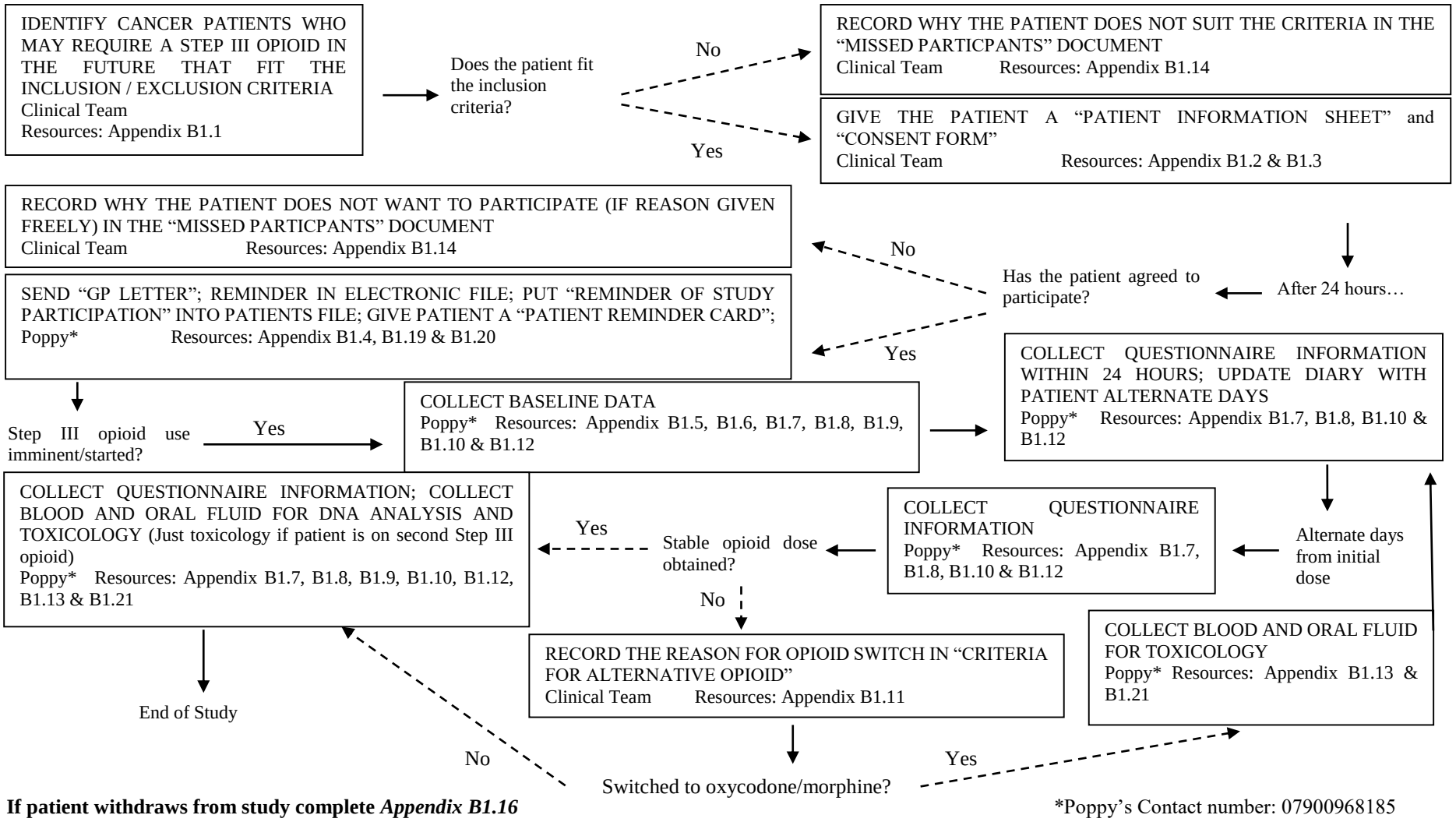

Figure 3-1. Flow diagram of pharmacogenetic study

3.1.4.2 Royal Marsden Hospital

A collaboration was formed between Royal Marsden Hospital and Bournemouth University to increase the study population size. Plasma samples and pain and side effect responses were obtained from 129 patients by Dr Joy Ross and her team at Royal Marsden Hospital.

To analyse the plasma samples a material transfer agreement (MTA) was arranged. This document allowed the samples to be transferred from the donor site (Royal Marsden Hospital), to the site of analysis (Randox Laboratories, N. Ireland for toxicology analysis and Royal Bournemouth Hospital for methylation analysis by the PhD candidate, and LCG Genomics for SNP analysis).

3.2 Sample collection

The tissue type selected for genetic, epigenetic and toxicological analysis varied between the different populations studied because of sample availability, ease of collection as well as ethical limitations (range of tissues obtained are listed in Table 3-1). For example, only buccal cells were obtained from drug and non-drug exposed babies as the use of buccal swabs is a relatively non-invasive and pain free DNA collection method suitable for babies. DNA from each population was analysed for SNPs and DNA methylation in all samples collected.

In addition to genetic analyses, samples from five of the populations underwent toxicological analysis (relevant populations emphasised in italics in Table 3-1). The concentrations of opioids were determined in blood or plasma samples.

Table 3-1. Tissue samples collected from each study population for genetic, epigenetic and toxicological analyses

Study Population	Buccal swab	Whole blood	Plasma	Liver	Thalamus	Psoas Muscle
Mothers: *Methadone* / non-drug exposed	Yes		Yes*			
Babies: Methadone / non-drug exposed	Yes					
Cancer patients: RBH, PH	Yes	Yes				
Cancer patients: RMH			Yes			
***Methadone* / non-opioid deaths**		Yes				
Heroin deaths		Yes		Yes	Yes	Yes
Non-drug exposed tissues				Yes	Yes	Yes
Smokers / non-smokers	Yes					
Breast cancer survivors		Yes				

Key: * = Plasma samples only obtained from the methadone exposed mothers; RBH = Royal Bournemouth Hospital; RMH = Royal Marsden Hospital; PH = Poole Hospital; *italics* = populations whose blood or plasma samples were also analysed for drug concentrations.

3.2.1 Does smoking affect buccal DNA methylation?

3.2.1.1 Oral swabs

Buccal DNA from smokers and non-smokers were collected by the PhD student using Catch-All™ sample collection swabs (Cambio Ltd, Cambridge). The Catch-All™ swabs consist of a cotton bud-like collector encapsulated within a hard case for ease of storage. Using this swab, epithelial cells from the inner cheeks are collected from which genomic DNA can be extracted.

Prior to collection of the epithelial cells from buccal tissues, subjects were requested to rinse their mouths twice with water. Both inner cheeks of the subject were swabbed by the researcher, firmly rolling the collector up to 20 times over the entire cheek. Swabs were allowed to air dry for 15-20 minutes and then stored at -20°C in the original packaging. For longer term storage the swabs were stored at -70°C.

3.2.2 Methadone-prescribed opioid-dependant mothers and their new-born babies

3.2.2.1 Oral swabs

Mother and baby buccal DNA samples were collected by the Glasgow Royal Infirmary Team. Samples were stored as described in section *3.2.1.1* and when batches of 20 DNA samples were obtained the samples were posted to Bournemouth University on dry ice to be analysed by the PhD candidate.

3.2.2.2 Plasma samples

The Glasgow Royal Infirmary Team obtained ~3mL venous blood samples from each mother, spun down the sample and collected the plasma portion. Plasma samples were stored at -70°C before being sent to Imperial College, London, on dry ice for drug analysis.

3.2.3 Tissue specific DNA methylation

3.2.3.1 Blood samples

Post-mortem blood was collected from the ligated femoral vein by pathologists at Sir James Black Mortuary in Dundee. For drug analysis blood was collected in tubes containing sodium fluoride and potassium oxalate preservative (BD Vacutainer®, New Jersey). Samples were stored at -70°C before undergoing toxicological screening by the staff at the Centre of Forensic and Legal Medicine, Dundee. Blood samples from 43 post-mortems associated with heroin toxicity were obtained for toxicological analyses as were 41 blood samples from non-drug related post-mortems.

3.2.3.2 Tissue samples

Tissue samples were collected by pathologists at Sir James Black Mortuary (Dundee) from heroin associated deaths as part of the routine post-mortem procedure. The primary purpose of the tissue samples was to ascertain the distribution of opioids in different tissues post-mortem therefore the right lobe of the liver sample was collected, the point furthest from the central cavity and stomach where drug accumulation may occur. A small sample of psoas major muscle was collected from near the spine once the cadaver's organs were eviscerated. In addition to the liver and muscle a small sample of thalamus was obtained. Complete tissue sets (blood, liver, psoas muscle and thalamus) were obtained from 30 heroin associated post-mortems.

Liver, psoas muscle and thalamus from opioid naïve individuals, as determined by drug screening and case history, were collected and stored by the Edinburgh Brain and Tissue Bank. The frozen samples were transported to Royal Bournemouth Hospital on dry ice where they were analysed by the PhD candidate.

3.2.4 Pilot trial: Personalising opioid therapy for cancer pain relief

3.2.4.1 Oral swabs

The PhD candidate collected the oral swabs from cancer patients at Royal Bournemouth Hospital and Poole Hospital as described in *3.2.1.1*.

3.2.4.2 Blood samples

Peripheral blood samples were obtained by phlebotomists and clinicians at Royal Bournemouth Hospital, Poole Hospital and Royal Marsden Hospital. Blood intended for genetic analysis was collected in purple topped tubes containing EDTA. For drug analysis blood was collected in tubes containing sodium fluoride and potassium oxalate preservative (BD Vacutainer®, New Jersey). Samples were stored at -70°C before undergoing opioid analysis at Randox laboratories (analysis undertaken by PhD candidate), methylation analysis at Royal Bournemouth hospital (analysis undertaken by PhD candidate) or SNP analysis (analysis undertaken by LGC Genomics).

3.3 Toxicological analysis

3.3.1 Methadone-prescribed opioid-dependant mothers

Plasma samples from 18 of the methadone-prescribed opioid-dependant mothers were analysed for methadone and its metabolite 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) by the PhD candidate at the Toxicology Unit, Imperial College, London. Drugs were isolated from 1mL of plasma using liquid-liquid extraction. Deionised water (3mL), 1mL of internal standard ($Clomipramine_{d3}$), 0.15mL 0.880 ammonia solution and 6mL of diethyl ether was added to the plasma samples. The samples were capped, agitated (10 minutes) and centrifuged (5 minutes, 500g). The organic layer was removed and added to a glass tube containing 5mL 0.1M hydrochloric acid. Tubes were agitated (10 minutes), centrifuged (5 minutes, 500 x g) and the organic layer discarded. 0.15mL 0.880 ammonia solution and 5mL of diethyl ether were added to the glass tube and agitated (10 minutes) and centrifuged (5 minutes, 500 x g) for a final time. The organic layer was carefully pipetted into a glass tube and left to evaporate overnight.

For detection by gas chromatography mass spectrometry analysis the extracted drugs were reconstituted in 0.035mL acetonitrile. A Hewlett Packard 6890 gas chromatograph linked to a 5973 mass spectrometer was used to detect the analytes of interest. The oven temperature started at 80°C and was ramped to 310°C at a rate of 16°C/minute with a total run time of 30 minutes per sample. A splitless injector at a temperature of 280°C was used with helium as the carrier gas. Compounds of interest were separated using a Restek Rxi-5ms capillary column (30m x 5mm, 0.25µm) at a carrier gas flow rate of 1mL/minute.

3.3.2 Tissue specific DNA methylation

3.3.2.1 Determination of methadone – opioid overdoses

Blood samples from deaths associated with methadone toxicity were analysed by the toxicologists at the Forensic and Legal Department at Dundee University. The methadone was extracted from the blood sample using liquid-liquid extraction. To 0.5mL of blood sample 0.5mL internal standard was added (desipramine) and vortex mixed. Chlorobutane (5mL) was added to each sample, agitated and centrifuged for 5 minutes at 1500 x g. The chlorobutane layer was pipetted carefully into a clean tube and 0.1mL of 0.05M sulphuric acid was added to each tube. The sample was agitated and centrifuged again for 5 minutes at 1500 x g. Following centrifugation the chlorobutane layer was aspirated carefully and the remaining sulphuric acid layer containing any drug was transferred into HPLC vials for analysis.

The 43 blood samples obtained from opioid associated deaths were analysed for a range of drugs by the Forensic and Legal Medicine department, Dundee. The drugs screened for included alprazolam, amitriptyline and metabolite, amphetamine, citalopram, diazepam, fluoxetine and metabolite, gabapentin, methadone and metabolite, mirtazapine, morphine and metabolites, naloxone, oxazepam, oxycodone, paracetamol, phenazepam, procyclidine, quetiapine and metabolite, quinine, temazepam and tramadol.

3.3.2.2 Determination of morphine – opioid overdoses

Blood samples (150 µL) were diluted with 150 µL equine plasma and spiked with 50 µL of $Morphine_{d3}$ and 50 µL of $M3G_{d3}$ (IS). After vortexing, 1mL of 0.5 M ammonium carbonate solution was added to each sample, and extracted according to the procedure in Taylor and Elliott (2009). Briefly solid phase extraction columns (Bond Elut C18,

6 mL, Varian) were conditioned using 2 mL methanol followed by 2 mL water and then 1 mL 0.5 M ammonium carbonate buffer. Diluted and spiked samples were loaded onto the column and allowed to elute at a rate of ~1 mL/min. The columns were washed with 5 mL of 0.005 M ammonium carbonate buffer and air dried under vacuum for 5 minutes. Drugs were eluted using 1 mL 70:30 acetonitrile:water. The evaporated eluent was reconstituted with 100 µL of freshly made LC-MS mobile phase (96% phase A: 4% phase B), transferred into a HPLC vial and analysed.

A 1200 Agilent HPLC system interfaced with an Applied Biosystem 3200 Q TRAP triple-quadrupole linear ion trap mass spectrometer (AB Applied Biosystem, Foster City, CA, USA) equipped with an electrospray ionization source (ESI) was used to analyse all the samples. Separation of all analytes was accomplished using a Phenomenex Synergi 4µ Polar-RP 80A Column (150 mm × 2 mm × 4µm) protected by a Phenomenex Security Guard column (Macclesfield, UK). Mobile Phase A consisted of a 1mM ammonium formate and 0.1% formic acid solution (around pH 2.7). Mobile Phase B was a 50% acetonitrile solution with 1mM ammonium formate and 0.1% formic acid (around pH 3.8).

3.3.3 Pilot trial: Personalising opioid therapy for cancer pain relief

To determine the concentration of morphine / oxycodone and their metabolites in blood and plasma samples a solid phase extraction followed by HPLC-MS/MS method developed by Randox Laboratories was used. Cancer patient blood or plasma samples were transported to County Antrim and analysed using the method described below.

Samples were prepared for HPLC-MS/MS analysis by adding 1.5mL 2% phosphoric acid in phosphate buffer to 500µL of plasma/blood. Blood samples were centrifuged for 3 minutes at 1000 x g. SPE columns (Isolute®, Biotage) were conditioned using

2mL methanol and 2mL distilled water. The sample (0.5mL) was loaded onto the SPE column and allowed to flow through at a rate of 1mL/min. Columns were subsequently washed using 2mL of 0.1M hydrochloric acid followed by 2mL of methanol. The columns were air dried for 10 minutes before elution using 2mL 5% ammonia in methanol. Eluates were dried down under nitrogen and reconstituted in 200µL deionised water.

To separate the analytes of interest a 1200 Series HPLC coupled with an Ion Trap 6320 Mass Spectrometer (Agilent Technologies) was used. Separation of the compounds was achieved using a Phenomenex column (150 x 3mm; 5µm) kept at 50°C. Drugs were eluted using a gradient elution of 100% mobile phase A (0.1% formic acid v/v in water) to 100% mobile phase B (0.1% formic acid in methanol) at a flow rate of 0.75mL/minute.

3.4 SNP and gene duplication/deletion analysis

3.4.1 DNA extraction

3.4.1.1 DNA from buccal swabs

DNA was extracted from the oral swabs by immersing the swab in an Eppendorf containing 500µL of QuickExtract DNA solution (Cambio Ltd., Cambridge). The swab was pressed firmly against the side of the Eppendorf tube at least 5 times ensuring the majority of the liquid containing the buccal cells remained in the tube. Each Eppendorf tube was vortex mixed for 10 seconds and incubated for 1 minute at 65°C. Following incubation the samples were vortex mixed for 15 seconds and incubated again at 98°C for 2 minutes. Finally the extracted DNA was vortexed for 15 seconds and stored at -70°C until further analysis.

3.4.1.2 DNA from blood and tissue

DNA was extracted from blood and tissue samples using the Qiagen DNeasy® Blood & Tissue kit. The kit contains prepared reagents that are added to samples in sequence. The reagents include proteinase K and four buffers; AL, AW1, AW2 and AE. Proteinase K enables cell lysis to release the DNA; buffers AL, AW1 and AW2 are used to purify the DNA and buffer AE is an elution buffer.

To extract DNA from blood samples, cells from 100µL of blood were first lysed using 20µL proteinase K and volume made up to 220µL using phosphate buffer solution. 200µL of buffer AL was added and samples were thoroughly vortex mixed ensuring a homogeneous solution and incubated at 56°C for 10 minutes. Ethanol (200µL) was added to each sample and vortex mixed. The entire sample was transferred to a DNeasy mini spin column that was placed inside a 2mL collection tube. Following centrifugation (1 minute at 300 x g) the flow-through collected was discarded and

500µL of buffer AW1 was added to each tube. The DNeasy mini spin columns were centrifuged again and waste discarded. 500µL of buffer AW2 was added and centrifuged for 3 minutes at 6000 x g to dry and alleviate any interfering residual ethanol from the DNeasy membrane. The DNeasy mini spin columns were transferred to clean Eppendorf tubes and 50µL of buffer AE was added directly onto the DNeasy membrane. The samples were incubated for 15 minutes at room temperature and centrifuged for 1 minute at 300 x g to elute the DNA.

To extract DNA from the tissue samples an additional sample preparation method was necessary. Frozen tissue samples (25mg) were cut into small sections and 180µL of ATL buffer and 20µL of proteinase K was added to each tube. The samples were then left to incubate overnight at 56°C. Once the samples were completely lysed 200µL buffer AL was added and extracted following the same method as the blood samples.

3.4.1.3 DNA from plasma

DNA was extracted from plasma using the QIAmp DNA mini kit (Qiagen). The extraction method was similar to that employed for extracting DNA from blood and tissue samples (*section 3.4.1.2*); the only differences being that instead of 100µL of sample being added, 200µL of plasma was mixed with the proteinase K and no phosphate buffer was added.

3.4.2 DNA quantification

Each extracted DNA sample was quantified using the NanoDrop 1000 Spectrophotometer. A 1µL sample was aliquotted from the stock solution onto the NanoDrop 1000 pedestal that contains the end of a fibre optic cable (the receiving fibre). The sampling arm that contains the source fibre optic cable was then placed over the sample and light passed from the source fibre to the receiving fibre. The

amount of light of absorbed by the sample was compared to the light absorbance of a water control sample and the quantity of DNA was recorded.

3.4.3 DNA preparation and packaging for LGC

To determine the genotypes of individuals at 13 different SNP sites extracted genomic DNA was analysed by LGC genomics (SNPs detailed in Table 3-2 and their locations illustrated in Figures 3-2 to 3-6). The SNPs chosen for analysis were based upon previous research, summarised in section *2.1.4.6*. In addition, methadone, heroin and oxycodone samples were analysed for *CYP2D6* deletions and duplications (Table 3-3) using a Hy-beacon assay. An external company rather than in-house analysis was used is it was more cost effective. As the SNP aspect of this research project was not novel, it was felt that the use of an external company was justifiable.

Table 3-2. Table of SNPs analysed by LGC genomics

SNP ID	Sequence	NCBI Frequency (Caucasian)	Synonymous / Non-synonymous
ABCB1 **1236C>T** **rs1128503**	TCCTGGTAGATCTTGAAGGG [C/T] CTGAACCTGAAGGTGCAGA	C = 0.55 T = 0.45	Synonymous Glycine
ABCB1 **2677G>T** **rs2032582**	TAAGAAAGAACTAGAAGGT [G/T] CTGGGAAGGTGAGTCAAAC	G = 0.53 T = 0.47	Non-synonymous Serine>Threonine
ABCB1 **3435C>T** **rs1045642**	TGGTGTCACAGGAAGAGAT [C/T] GTGAGGGCAGCAAAGGAGG	C = 0.43 T = 0.57	Synonymous Isoleucine
COMT **-98A>G** **rs6269**	TCTGAACCTTGCCCCTCTGC [A/G] AACACAAGGGGGCGATGGT	A = 0.51 G = 0.49	
COMT **186C>T** **rs4633**	AGCAGCGCATCCTGAACCA [C/T] GTGCTGCAGCATGCGGAGC	C = 0.48 T = 0.52	Synonymous Histidine
COMT **408C>G** **rs4818**	TGTCACCAGGGGCGAGGCT [C/G] ATCACCATCGAGATCAACC	C = 0.56 G = 0.44	Synonymous Leucine
COMT **472A>G** **rs4680**	GATGGTGGATTTCGCTGGC [A/G] TGAAGGACAAGGTGTGCAT	A = 0.52 G = 0.48	Non-synonymous Valine>Methionine
CYP2B6 **516G>T** **rs3745274**	GGACCCCACCTTCCTCTTCCA [G/T] TCCATTACCGCCAACATCATC	G = 0.73 T = 0.27	Non-Synonymous Glutamine>Histidine
CYP2B6 **785A>G** **rs2279343**	CCTGGACCCCAGCGCCCCCA [A/G] GGACCTCATCGACACCTACC	A = 0.79 G = 0.21	Non-Synonymous Lysine>Arginine
CYP2D6 **1707T>delT** **rs5030655**	CAAGAAGTCGCTGGAGCAG [T/-] GGGTGACCGAGGAGGCCGC	T = 0.99 deleted T = 0.01	Non-Synonymous Tryptophan>Glycine
CYP2D6 **1845G>A** **rs3892097**	ACCCGCATCTCCCACCCCCA [G/A] GACGCCCCTTTCGCCCCAAC	G = 0.76 A = 0.24	
CYP2D6 **2459A/delA** **rs35742686**	TGAGCTGCTAACTGAGCAC [A/-] GGATGACCTGGGACCCAGC	A = 0.99 deleted A = 0.01	Non-Synonymous Arginine>Glycine
OPRM1 **118A>G** **rs1799971**	ACTTGTCCCACTTAGATGGC [A/G] ACCTGTCCGACCCATGCGGT	A = 0.85 G = 0.15	Non-Synonymous Asparagine>Aspartic Acid

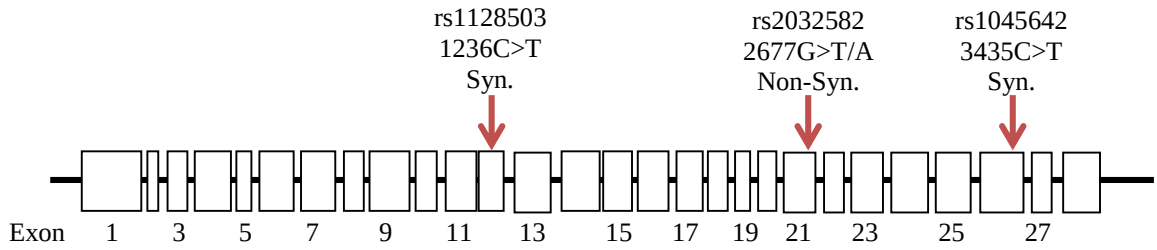

Figure 3-2. Location of 1236C>T, 2677G>T/A and 3435C>T SNPs in relation to the 28 exons that code for the ABCB1/MDR1 transport protein
The polymorphisms are labelled by their reference SNP number (rs), position in the gene in relation to the ATG start codon, the nucleotide base change (A = adenine, C = cytosine, G = guanine, T = thymine) and whether or not the polymorphism results in an amino acid substitution; Syn. = synonymous – amino acid unchanged, Non-Syn. = non-synonymous – amino acid substitution. Boxes represent coding exons, black line indicates intronic regions (image adapted from Meletiadis et al. 2006).

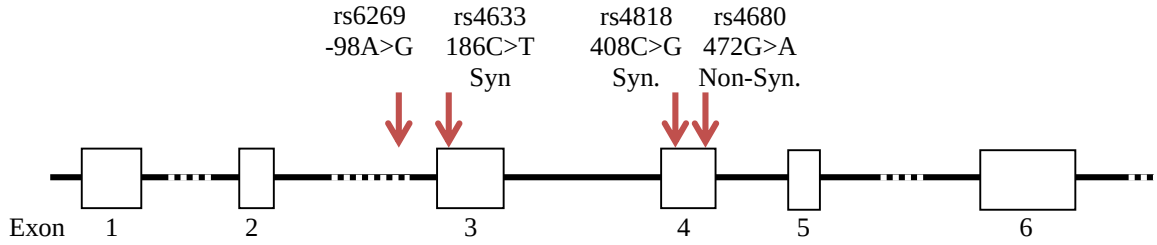

Figure 3-3. Location of -98A>G, 186C>T, 408C>G and 472G>A SNPs in relation to the 6 exons that code for the COMT metabolising enzyme
The polymorphisms are labelled by their reference SNP number (rs), position in the gene in relation to the ATG start codon, the nucleotide base change (A = adenine, C = cytosine, G = guanine, T = thymine) and whether or not the polymorphism results in an amino acid substitution; Syn. = synonymous – amino acid unchanged, Non-Syn. = non-synonymous – amino acid substitution. Boxes represent coding exons, black line indicates intronic regions (image adapted from Rakvåg et al. 2008).

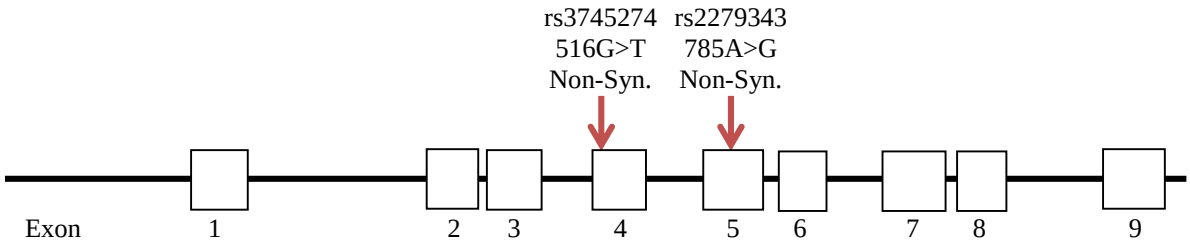

Figure 3-4. Location of 516G>T and 785A>G SNPs in relation to the 9 exons that code for the CYP2B6 metabolising enzyme
The polymorphisms are labelled by their reference SNP number (rs), position in the gene in relation to the ATG start codon, the nucleotide base change (A = adenine, C = cytosine, G = guanine, T = thymine) and whether or not the polymorphism results in an amino acid substitution; Non-Syn. = non-synonymous – amino acid substitution. Boxes represent coding exons, black line indicates intronic regions (image adapted from Jacob *et al.* 2004).

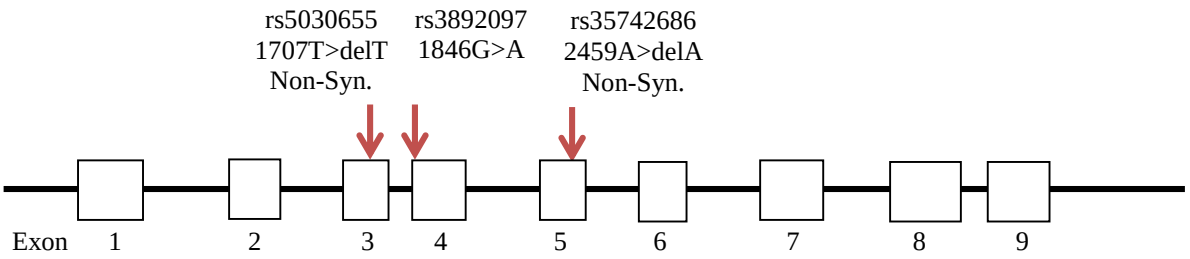

Figure 3-5. Location of 1707T>delT, 1846G>A and 2459A>delA SNPs in relation to the 9 exons that code for the CYP2D6 metabolising enzyme
The polymorphisms are labelled by their reference SNP number (rs), position in the gene in relation to the ATG start codon, the nucleotide base change (A = adenine, C = cytosine, G = guanine, T = thymine) and whether or not the polymorphism results in an amino acid substitution; Non-Syn. = non-synonymous – amino acid substitution. Boxes represent coding exons, black line indicates intronic regions (image adapted from Sistonen *et al.* 2007).

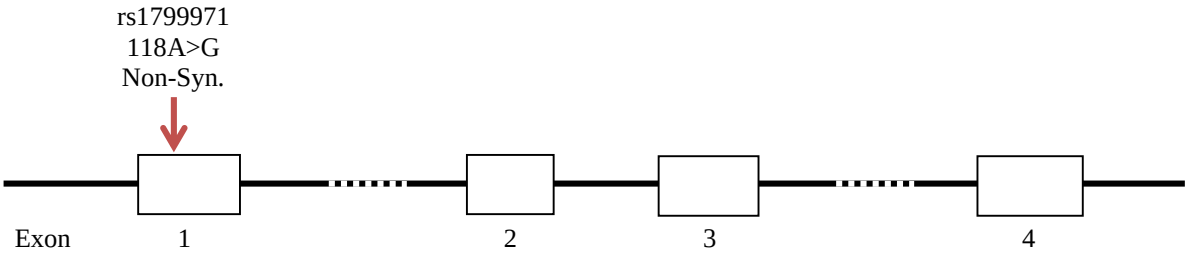

Figure 3-6. Location of 118A>G SNP in relation to the 4 exons that code for the μ-opioid receptor
The polymorphism is labelled by its reference SNP number (rs), position in the gene in relation to the ATG start codon, the nucleotide base change (A = adenine, G = guanine) and whether or not the polymorphism results in an amino acid substitution. Non-Syn. = non-synonymous – amino acid substitution. Boxes represent coding exons, black line indicates intronic regions (image adapted from Zhang *et al.* 2006).

Table 3-3. Table of *CYP2D6* variants analysed using Hy-Beacons assay

***CYP2D6* variant**	**Number of copies**	**Function**
***1/*1**	Two copies	Normal
***1/*5**	One copy	
***1xN**	More than two copies	Increased
***5/*5**	*CYP2D6* deleted	Decreased

3.4.3.1 DNA quantity and volume

DNA samples were standardised to approximately 5 ng/μL using a Nanodrop 1000 spectrophotometer. For analysis of the 13 SNPs and *CYP2D6* deletions and

duplications a minimum volume of 40 µL was required. The samples, volumes and quantities of DNA samples sent to LGC are listed in Appendix C1.0.

3.4.3.2 Packaging, transportation

The 380 samples (MPOD mothers and their babies, n = 40; opioid naïve mothers and their babies, n = 64; heroin associated deaths, n = 43; methadone associated deaths, n = 77; RBH, FH and MU cancer patients, n = 18; RMH cancer patients, n =128; and 10 control samples) were transported to LGC in four 96 well plates, each plate with an empty well as a negative template control. To ensure that no samples leaked during transportation the 96 well plates were sealed using foil seals (purchased from Fisher Scientific) and frozen at -70˚C. The frozen samples were transported on dry ice to LGC genomics using Davies International courier service.

3.5 DNA methylation analysis

3.5.1 Pyrosequencing method - trial

Pyrosequencing was undertaken by the PhD candidate at Royal Bournemouth hospital, Molecular Pathology department using their Pyromark Q24 pyrosequencer (Qiagen).

The gene chosen for preliminary investigation was *OPRM1*. This gene was chosen as it contains a high density of CpG dinucleotides within the promoter region and exon 1 (Figure 3-7) and has been shown to have variable methylation between different ethnicities (Nielsen *et al.* 2010) and individuals' exposure to drugs (Nielsen *et al.* 2009; Chorbov *et al.* 2010).

TCCTTGGATCGCTTTGCGCAAAATCCACCCCTTTTCCCTCCTCCCTCCCTTCCAGCCTCCGAATCCCGCATGGCCCACGCTCCCCTCCTGCAGC
GGTGCGGGGCAGGTGATGAGCCTCTGTGAACTACTAAGGTGGGAGGGGGCTATACGCAGAGGAGAATGTCAGATGCTCAGCTCGGTCCC
CTCCGCCTGACGCTCCTCTCTGTCTCAGCCAGGACTGGTTTCTGTAAGAAACAGCAGGAGCTGTGGCAGCGGCGAAAGGAAGCGGCTGAG
GCGCTTGGAACCCGAAAAGTCTCGGTGCTCCTGGCTACCTCGCACAGCGGTGCCCGCCCGGCCGTCAGTACCATGGACAGCAGCGCTGC
CCCCACGAACGCCAGCAATTGCACTGATGCCTTGGCGTACTCAAGTTGCTCCCCAGCACCCAGCCCCGGTTCCTGGGTCAACTTGTCCCACT
TAGATGGCAACCTGTCCGACCCATGCGGTCCGAACCGCACCGACCTGGGCGGGAGAGACAGCCTGTGCCCTCCGACCGGCAGTCCCTCCA
TGATCACG

Figure 3-7. CpG sites located in the promoter region and exon 1 of *OPRM1*
CG represent potential variable methylation sites, CG show sites that have been previously researched and have shown variation between populations (information gathered from Nielsen *et al.* 2008, Nielsen *et al.* 2010 and Chorbov *et al.* 2011). Yellow highlight indicates the *OPRM1* start codon.

3.5.1.1 Primer design

Eight CpG dinucleotides were selected from the promoter region and exon 1 of *OPRM1* and their analysis was divided into two assays; 5 CpGs within the promoter region of *OPRM1* and 3 CpGs in exon 1 (Figure 3-8).

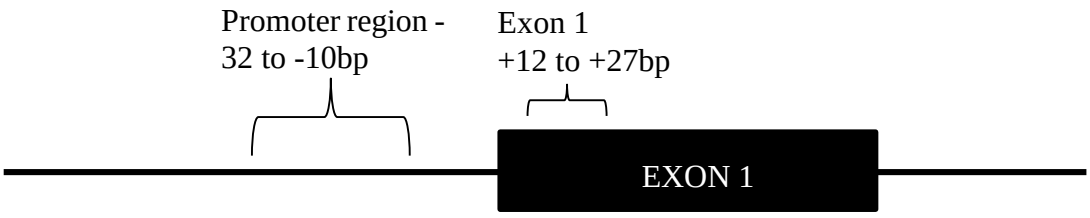

Figure 3-8. Promoter region and exon 1 of *OPRM1* with CG regions of interest indicated
Promoter region -32, -25, -18-, 14, -10; Exon 1 +12, +23, +27; bp: base pairs

To design the primers for bisulfite treated DNA, PSQ Assay Design software (Biotage) and MethPrimer (available online) were used. The DNA sequence was obtained from UCSC genome browser (http://genome.ucsc.edu/). To emulate the effect of bisulfite treatment the DNA sequence was pasted into a Microsoft Word document and all CpG dinucleotides within the sequence were changed to YG; all remaining "C" bases converted to "T". The region of interest was then pasted into the primer design software and suitable primers were suggested. Initially primers without biotin tags were ordered and tested to verify that the correct DNA sequence was amplified. Once suitable PCR products were obtained the biotin labelled primers were ordered (Table 3-4).

Primers were obtained from Eurofins mwg|operon and reconstituted with water using the concentrations listed in the table below to make a final primer concentration of 100µmol/µL (Table).

Table 3-4. *OPRM1* primers; reconstitution and optimum annealing temperature

Primer Name	Sequence	H_2O (µL)	Optimum annealing temp. (°C)
OPRMpy -30 to -7 F	GGATTGGTTTTTGTAAGAAATAGTA	253	53.5
OPRMpy -30 to -7 R	CTAAAAACAACCCTACTATCCATAATAC	226	59.3
OPRMpy +12 to +27 F	GGATTGGTTTTTGTAAGAAATAGTA	252	54.8
OPRMpy +12 to +27 R	CTAAAAACAACCCTACTATCCATAATAC	211	53.8
OPRMpy -30 -7 R BIO	Bio-CTAAAAACAACCCTACTATCCATAATA	499	59.3
OPRMpy +12 +27 BIO	Bio-GGATTGGTTTTTGTAAGAAATAGTA	512	54.8
OPRMpy -30 -7 Seq	AGTTTAGGTGTTTTTGGTTA	361	Na
OPRMpy +12 +27 Seq	CCAAAACATCAATACAATTA	510	Na

3.5.1.2 Bisulfite conversion

DNA was bisulfite converted using EZ-96 DNA Methylation-Gold™ Kit (Zymo Research). CT conversion reagent (130µL) was added to the supplied 96 well

conversion plate. To each well a 20µL aliquot of DNA (~25ng/µL) was added and incubated at 98°C for 10 minutes followed by 64°C for 2.5 hours. Binding buffer (600µL) was pipetted into the wells of the Zymo-Spin^{TM} I-96 binding plate and DNA was transferred from the conversion plate to the binding plate. Samples were centrifuged for 5 minutes at 3000 x g and the waste was discarded. The M-wash buffer (400µL) was then added and plate centrifuged for 5 minutes at 3000 x g. M-desulphonation buffer (200µL) was added to each well and left to stand for 20 minutes at room temperature. Following centrifugation, two wash steps using the M-wash buffer (400 µL) were undertaken, one with a centrifuge time of 5 minutes and the second with a centrifuge time of 10 minutes (3000 x g). The binding plate was placed on top of the elution plate and 15µL of M-Elution Buffer was added directly to each membrane. The plate was left to stand for 5 minutes before the DNA was eluted by centrifuging the plates for 3 minutes at 3000 x g.

3.5.1.3 PCR optimisation

To amplify the regions of interest 1µL of bisulfite converted DNA was amplified following the protocol in Table 3-5. The success of the PCR amplification was determined by gel electrophoresis. A 1.5% gel was made using agarose and 1 x Tris-acetate-EDTA buffer (TAE; Thermo Scientific). PCR products were visualised using 1µL loading buffer (Gel Stain Red, Biotium).

Table 3-5. Sample preparation of bisulfite converted DNA for PCR amplification

	Volume (µL)
PCR H_20	19.1
Buffer x10	2.5
dNTPs	2.0
Bisulfite converted DNA	1.0
Forward primer	0.1
Reverse primer	0.1
GoTaq polymerase	0.2

To obtain the best PCR product each primer set underwent PCR at 6 different temperatures using a Veriti Thermocycler. The temperature ranged from 50°C to 62°C. If faint bands were obtained the number of cycles was increased from 35 to 40 cycles. The temperature program is summarised in Figure 3-9.

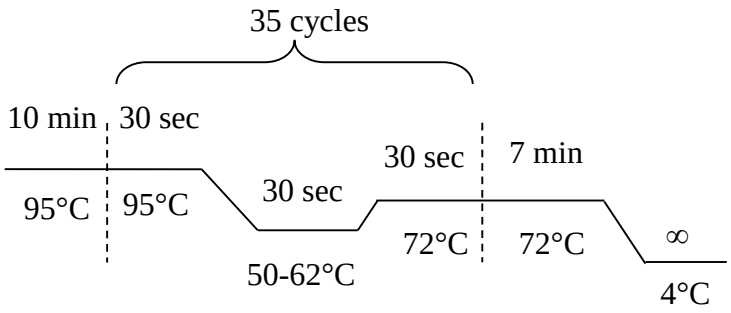

Figure 3-9. Temperature program for amplification of samples for sequencing

3.5.1.3.1 Examination of PCR reaction by gel electrophoresis

The success of the PCR amplification was determined by gel electrophoresis. A 1.5% gel was made using agarose and 1xTAE buffer. PCR products were visualised using 1µL loading buffer (Gel Stain Red). Figure 3-10 illustrates that 62°C was too hot for both primer sets as the presence of PCR product was faint and inconsistent in the 5 DNA samples amplified (Figure 3-10). 50°C was not hot enough for the +12 primer set as there was evidence of non-specific products. The optimum temperature for the

+12 primer set was 58°C as a vivid PCR product with no smear was produced. The optimal temperature for the -30 primer set was 50°C, however at all of the temperatures the -30 primer set produced a secondary product.

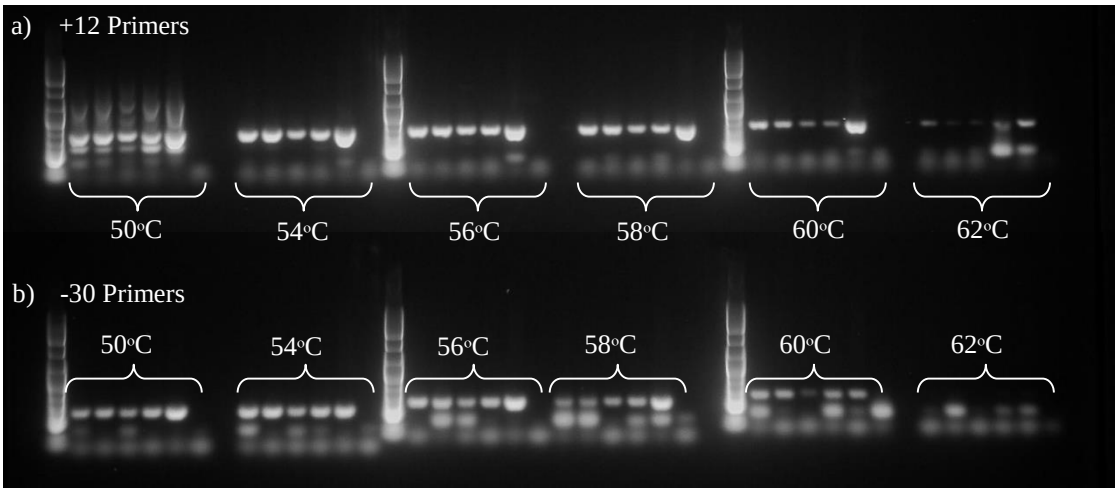

Figure 3-10. *OPRM1* +12 and -30 PCR optimisation
Five bisulfite converted genomic DNA samples and a PCR blank were amplified for two regions of the *OPRM1* gene at a range of temperatures (50°C to 62°C). a) Gel obtained for the +12 primer set, b) gel obtained for the -30 primer set. Although the DNA ladder was smeared as a result of overloading, the amplicon sizes were as predicted.

The secondary product obtained using the -30 primer set was alleviated when the PCR product was cleaned using SureClean implying that there was an excess of primers within the PCR reaction (Figure 3-11).

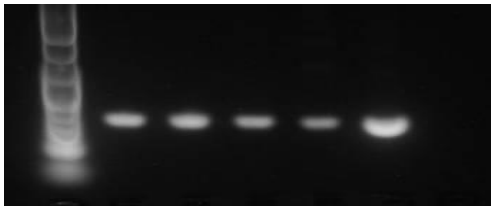

Figure 3-11. SureClean purification of OPRM1 -30 primer set

3.5.1.3.2 Primer concentration optimisation

To relieve the -30 primer set of excess primers 16 replicates were carried out, each of which had varying F and/or R primer concentrations. Much less PCR product was obtained when the lowest concentration (0.05µM) of each primer was utilised as

designated by bands that were very faint (Figure 3-12, a). As the primer concentration increased more PCR product was replicated; however, there was also greater formation of secondary product. In general less secondary product was produced when 0.05 or 0.1µM of F primer was added suggesting that excess F primer causes the secondary product (Figure 3-12, a, b, e, f, i, j, m, n). Of the 16 different combinations of F and R primer concentrations 0.05µM of F primer and 0.2µM of R primer gave the cleanest and brightest PCR product and the faintest secondary product (Figure 3-12, i).

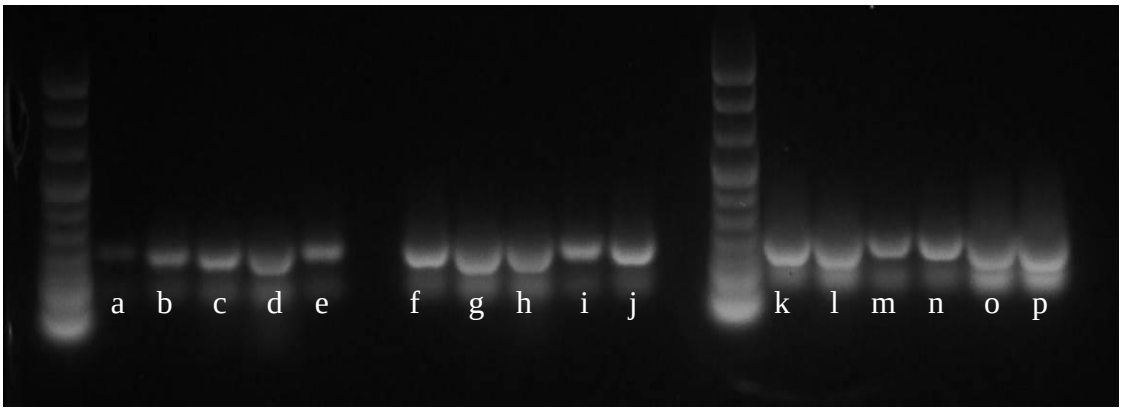

Figure 3-12. -30 primer concentration optimisation
A genomic DNA sample was amplified using the following concentrations of forward (F) and reverse (R) primers to amplify the -30 to -7bp of *OPRM1*: a) F:0.05µM, R:0.05µM; b) F: 0.1µM, R: 0.05µM; c) F: 0.2µM, R: 0.05µM; d) F: 0.4µM, R: 0.05µM; e) F: 0.05µM, R: 0.1µM; f) F: 0.1µM, R: 0.1µM; g) F: 0.2µM, R: 0.1µM; h) F: 0.4µM, R: 0.1µM; i) F: 0.05µM, R: 0.2µM; j) F: 0.1µM, R: 0.2µM; k) F: 0.2µM, R: 0.2µM; l) F: 0.4µM, R: 0.2µM; m) F: 0.05µM, R: 0.4µM; n) F: 0.1µM, R: 0.4µM; o) F: 0.2µM, R: 0.4µM; p) F: 0.4µM, R: 0.4µM.

3.5.1.4 ABI310 genetic analyser

To ensure that the correct PCR product was amplified the replicated DNA was purified and subjected to sequencing using the ABI310 (Life Technologies). The PCR product was alleviated of excess PCR products using SureClean (Bioline). SureClean (15µL) was added to 15µL of PCR product. The samples were shaken for 1 minute and left to stand for 10 minutes. Following centrifugation the supernatant was carefully decanted leaving the DNA pellet in the bottom of the tube. To the pellet was added 100µL of

70% ethanol. Samples were shaken for 1 minute using a vortex mixer and centrifuged for 10 minutes. The supernatant was decanted and remaining pellet left to air dry. The DNA was resuspended in 10µL of water.

F and R primers were diluted to make a stock solution of 3.3µM. Either the F or R primer (1µL) was added to a 0.5µL cleaned PCR product. BigDye (2µL; Life Technologies), Sequencing buffer (4µL; Life Technologies) and 12.5µL of water were also added to each sample. All samples were then placed in the Thermocycler and underwent the following temperature program (Figure 3-13):

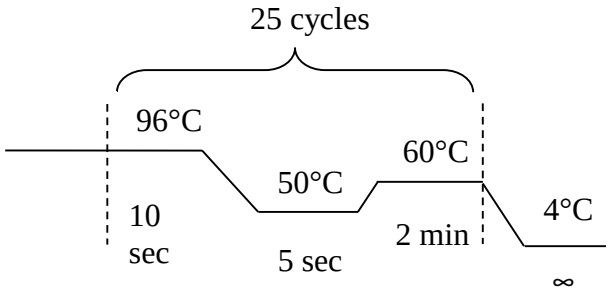

Figure 3-13. Incubation temperature program
Samples were incubated 96°C for 10 seconds, 50°C for 5 seconds, and 60°C for 2 minutes for 25 cycles, the temperature was then lowered to 4°C.

An aliquot of the sample, 20µL, was added to 50µL 100% ethanol and 2µL sodium acetate buffer solution. Following a 1 minute vortex the samples were centrifuged for 30 minutes. During this 30 minute window the sequencer was prepared for analysis. Fresh buffer, water and POP polymer (Life Technologies) were prepared if necessary and the sample list created. After centrifugation the supernatant was decanted and 100µL 70% ethanol was added to the residue and centrifuged for 10 minutes. The ethanol was decanted and the residue left to air dry before 20µL deionised formamide was added and transferred to sequencing tubes. Denaturation was carried out by incubating the tubes for 2 minutes at 94°C and then placing them in an ice bucket for ~1 minute before loading onto the sequencer.

3.5.1.5 Pyrosequencing

Once it was known that the correct PCR product was being obtained, pyrosequencing was undertaken. PCR product (10µL) was added to 2µL sepherose beads (Sigma Aldrich), 40µL pyrosequencing binding buffer (Qiagen) and 28µL water. A commercial wash station under vacuum (Qiagen) was used. Initially the probes were washed with water and dried under vacuum before placing them in the sampling plate. Successive washes were undertaken using 70% ethanol (5 seconds), Pyromark denaturation solution (5 seconds; Qiagen) and pyrosequencing wash buffer (10 seconds; Qiagen). The vacuum was turned off and the probes placed inside a plate containing annealing buffer (Qiagen) that interrupts the bonds between the DNA and probes. The annealing buffer also contained 1µL of sequencing primer at a concentration of 0.3µM (sequencing primer obtained from Eurofins). Appropriate volumes of ddNTPs, enzyme and substrate were aliquotted into the pyrosequencer cartridge (as determined by the pyrosequencer software) and samples were analysed following the designed assay.

3.5.1.5.1 Assay on OPRM1 -32bp to -7bp assay

The majority of tests for the -32 to 7bp assay initially failed because of the following errors: the sequence detected did not match the reference sequence, there was a low signal-to-noise ratio and peak height was too low for quantification. These errors occurred as there was not enough DNA present to give a strong signal (Figure 3-14 i). This low quantity of DNA caused irregular peak heights, for example Figure 3-14 ii peaks 5-9 should all be the same height as the expected sequence dictates that there is one G base followed by one T base, one A, one T, one A. The T bases are giving greater responses than the other bases suggesting that there are more T bases in the sample than there are in the reference sequence (Figure 3-14 b). Also, additional peaks

were detected in the sample (Figure 3-14 iii). As a result of these issues a second set of samples were amplified using a PCR programme with 40 PCR cycles instead of 35 cycles to increase the quantity of product. The increased PCR cycles enhanced the pyrosequencing response marginally as the peak response increased from 15 to 40 but the peaks were still too low to be quantified (Figure 3-14 c). Although the signal response was still too low for methylation level quantification the increased quantity of DNA did improve the consistency of peak height and alleviated the sample of spurious peaks. The temperature of the heating block used during the annealing phase was increased which enhanced peak height, alleviated the pyrogram of interfering spurious peaks completely and produced consistent peak heights.

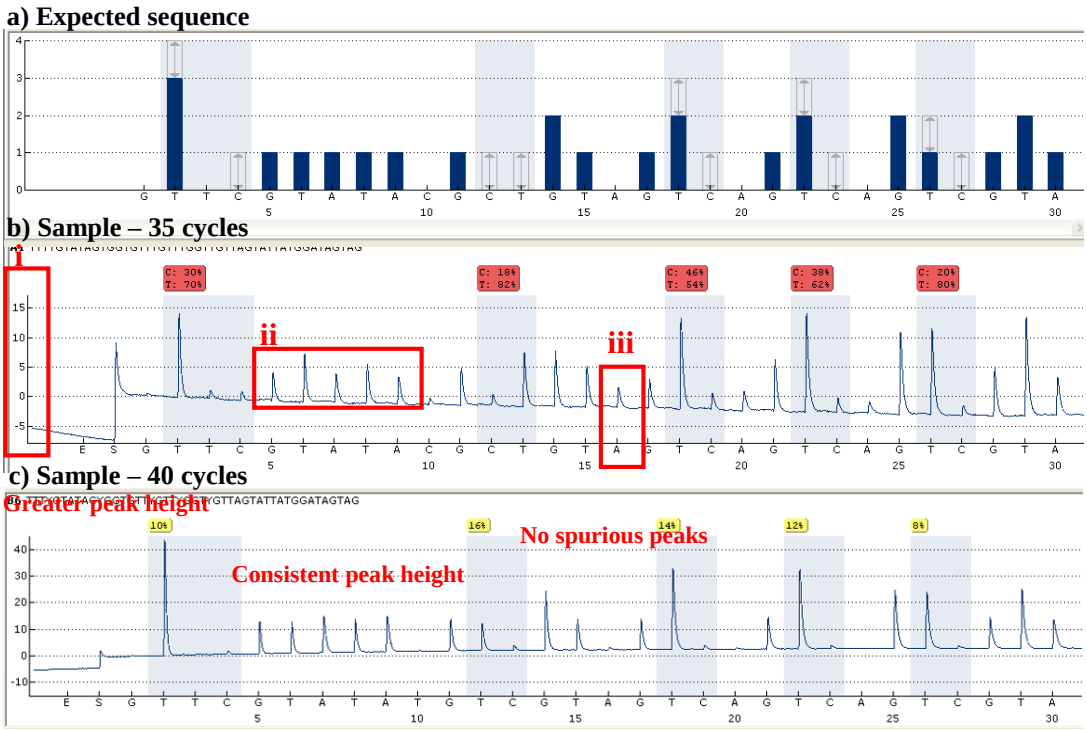

Figure 3-14. Pyrograms of the *OPRM1* promoter region from DNA samples that underwent either 35 or 40 PCR amplification cycles
a) Expected DNA sequence, b) sequence obtained from sample that underwent 35 PCR cycles, c) sequence obtained from sample that underwent 40 PCR cycles. i) low scale, ii) inconsistent peaks, iii) spurious peaks.

3.5.1.5.2 Assay on OPRM1 +12bp to +27bp assay

3 CpG sites were investigated using the +12bp to +27bp primers. Similarly to the promoter region pyrosequencing results, a low signal response was reported. Following the increase of temperature during the annealing phase all 3 CpG sites of interest could be analysed.

3.5.2 Designing methylation assays for *ABCB1/MDR1* and *CYP2D6*

Promoter region CpG sites in *ABCB1/MDR1* (Figure 3-15; n = 11) were selected based on research undertaken by Dejeux *et al*. (2010). Dejeux *et al*. (2010) suggested that methylation of *ABCB1/MDR1* could predict the response to, and efficacy of doxorubicin treatment. Differential methylation of *ABCB1/MDR1* promoter region methylation was also observed in positive and negative tumours (Muggerud *et al*. 2010). Therefore *ABCB1/MDR1* was considered an appropriate candidate for variable response to opioids.

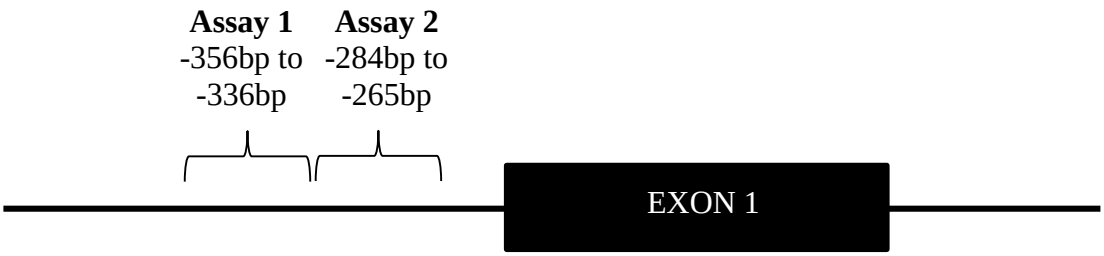

Figure 3-15. Promoter region and exon 1 of *ABCB1/MDR1* with CG regions of interest

The final gene for methylation analysis was *CYP2D6*. In 2007, Ingelman-Sundberg *et al.* (2007) published an article reviewing epigenetic mechanisms in cytochrome P450 enzymes. Methylation on *CYP1A1* (Okino *et al.* 2006), *CYP1B1* (Tokizane *et al.* 2005), and *CYP2W1* (Karlgren *et al.* 2006; Gomez *et al.* 2007) was shown to affect gene expression. Although no research had been undertaken on *CYP2D6* methylation, Ingelman-Sundberg *et al.* (2007) illustrated that *CYP2D6* contains a high density of

CpG dinucleotides and postulated that *CYP2D6* expression may be regulated by epigenetic mechanisms. To the best of our knowledge, the only research to be undertaken on *CYP2D6* methylation was published by Kim *et al.* (2010b). Kim *et al.* (2010b) reported no differences in *CYP2D6* promoter region methylation between tamoxifen-resistant and control tissues. To date, the influence of *CYP2D6* methylation on opioid response has not been investigated. As genetic variants within *CYP2D6* have been shown to influence drug response it was considered an important gene to investigate. Therefore, 3 CpG sites were chosen for exploration (boxed in Figure 3-16).

GTGACTACATTAGGGTGTATGAGCCTAGCTGGGAGGTGGATGGCCGGGTCCACTGAAACCCTGGTTATCCCAGAAGGCTTTGCAGGCTTC
AGGAGCTTGGAGTGGGGAGAGGGGGTGACTTCTCCGACCAGGCCCCTCCACCGGCCTACCCTGGGTAAGGGCCTGGAGCAGGAAGCAGG
GGCAAGAACCTCTGGAGCAGCCCATACCCGCCCTGGCCTGACTCTGCCACTGGCAGCACAGTCAACACAGCAGGTTCACTCACAGCAGAG
GGCAAAGGCCATCATCAGCTCCCTTTATAAGGGAAGGGTCACGCGCGCTCGGTGTGCTGAGAGTGTCCTGCCTGGTCCTCTGTGCCTGGTGG
GGTGGGGGTGCCAGGTGTGTCCAGAGGAGCCCATTTGGTAGTGAGGCAGGTATGGGGCTAGAAGCACTGGTGCCCCTGGCCGTGATAGT
GGCCATCTTCCTGCTCCTGGTGGACCTGATGCACCGGCGCCAACGCTGGGCTGCACGCTACCCACCAGGCCCCCTGCCACTGCCCGGGCTG
GGCAACCTGCTGCATGTGGACTTCCAGAACACACCATACTGCTTCGACCAGGTGAGGGAGGAGGTCCTGGAGGGCGGCAGAGGTCCTGA
GGATGCCCCACCACCAGCAAACATGGGTGGTGGGTGAAACCACAGGCTGGACCAGAAGCCAGGCTGAGAAGGGGAAGCAGGTTTGGGG
GACTTCCTGGAGAAGGGCATTTATACATGGCATGAAGGACTGGATTTTCCAAAGGCCAAGGAAGAGTAGG

Figure 3-16. CpG sites located in the promoter region and exon 1 of *CYP2D6*
CG represents potential variable methylation sites, CG show the CpG loci to be investigated.

3.5.2.1 Primers for *ABCB1/MDR1* and *CYP2D6*

Primers were designed following the method described in *3.5.1.1 Primer design* with the exception of the primers for *ABCB1/MDR1*. Primers for *ABCB1/MDR1* were those utilised by Dejeux *et al*. (2010). Primers were ordered from Eurofins mwg|operon and reconstituted in water to make a primer concentration of 100µmol/µL (Table 3-6).

Table 3-6. Primers for PCR and pyrosequencing

Primer Name	Sequence	H_2O (µL)	Optimum annealing temp. (°C)
B1 and B2 F	AAAACAAAATTAAAAATCTAACAAC	682	56.4
B1 and B2 R BIO	TTAGATTTAGGAGTTTTTGGAGTAG	721	49.9
B1 seq	TGGTATTGGATTATGTTGTT	460	na
B2 seq	TGGGTGGGAGGAAGT	279	na
CYP2D6 F BIO	TGGAGTAGGAAGTAGGGGTAAGAAT	412	61.3
CYP2D6 R	AACACAAAAAACCAAACAAAACAC	410	54.2
CYP2D6 seq	AAACACTCTCAACACACC	243	na

3.5.2.2 Bisulfite conversion

The methodology for bisulfite conversion has been described previously described (see section *3.5.1.2 Bisulfite conversion*).

3.5.2.3 PCR amplification

For the PCR reaction, 1-4µL of bisulfite converted DNA was used (Table 3-7) dependent upon the quality and quantity of DNA being analysed (for example, 4µL of plasma DNA, 1µL of DNA from whole blood). The volume of the PCR reaction was maintained at 25µL by adjusting the volume of water.

Table 3.7. Master mix for amplification of *OPRM1*, *ABCB1* and *CYP2D6*

	Volume (µL)
PCR H_20	19.1-16.1
Buffer x10	2.5
dNTPs	2.0
Bisulfite converted DNA	1.0-4.0
Forward primer	0.1
Reverse primer	0.1
GoTaq polymerase	0.2

The optimal temperature for each set of primers was established following the same method as ‘4.2.3 PCR optimisation’. The optimum temperatures and number of PCR cycles for each assay is shown in Figure 3-17.

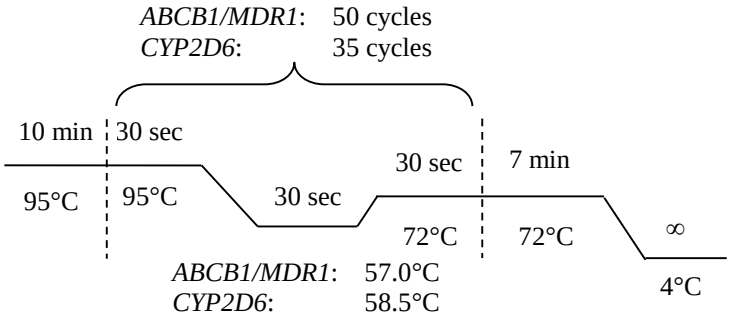

Figure 3-27. Temperature program for amplification of samples for sequencing

3.6 Statistical analysis

All statistical analyses were undertaken using SPSS v.20 (IBM). Normality was verified by Kolmogorov-Smirnov or Shapiro-Wilks tests (P = >0.05, data normally distributed). Data that was non-normally distributed was transformed using square root or LG10 transformations and Kolmogorov-Smirnov or Shapiro-Wilks tests undertaken again.

Relationships between two or more sets of categorical variables were assessed by Chi-squared tests utilising the Fisher exact test.

3.6.1 Normally distributed data

Student two-tailed t-tests were used when continuous variables with normal distribution were to be compared between two categorical/nominal independent datasets. If more than two independent datasets were used then one-way ANOVA was used to determine their relationship with continuous variables. The relationship between two continuous variables was assessed using Pearson's correlation.

3.6.2 Non-normally distributed data

If data was non-normally distributed and could not be corrected by transformations the Mann-Whitney U test was employed for testing relationships of continuous variables between two categorical/nominal independent datasets. If more than two independent datasets were used then Kruskal Wallis H test was used to determine their relationship with continuous variables. The relationship between two continuous variables was assessed using Spearman's rho correlation.

4.0 IMPACT OF SMOKING ON BUCCAL DNA METHYLATION OF OPIOID IMPORTANT GENES

4.1 Introduction

As smoke exposure has been shown to effect the methylation of a variety of genes (see section *2.2.4.4*), the influence of tobacco smoke on opioid related genes was of interest. Variable response to opioids as a result of genetic variations have been reported within a clinical setting (see section *2.1.4.*6), therefore smoke-induced genetic alterations may play a role in opioid response. The influence of smoke exposure on opioid related genes is of importance when studying subjects with addiction to drugs as many drug users are also smokers. Therefore a population of non-opioid exposed current smokers, former smokers and non-smokers were recruited and the extent of methylation on the promoter region of *OPRM1* and *CYP2D6* was determined.

4.2 Materials and methods

4.2.1 Sample collection

Participants were recruited as described in *3.1.1*. Buccal swabs were collected using Catch-All^{TM} brushes following the protocol in *3.2.1.1*. The study was approved by the Bournemouth University Science, Technology and Health Research Ethics Panel.

4.2.2 Methylation analysis

CYP2D6 and *OPRM1* gene DNA methylation was determined as described in *3.5*.

4.3 Results

Participants (n = 93) were classified into five groups: never-smokers (n = 43); ex-smokers (n = 16), last smoked 1-24 years prior to study participation; smoke <15 cigarettes per day (n = 23); smoke >15 cigarettes per day (n = 6); and social smokers (n = 5) (Table 4-1). A cut-off of 15 cigarettes per day indicating low / high consumption was chosen based on other studies (e.g. Bjartveit and Tvardel 2005).

The median age of the study population was 29 with a range of 19-52. Of the 93 participants 25 were undergraduate students, 34 were postgraduate students (Masters or PhD) and 34 were teaching staff. The age ranges of each smoking or non-smoking group are shown in Table 4-1. Using the Kruskal Wallis H test no difference was found in age between the women who have never smoked (median 26) and those who smoked previously (median 24). However the ex-smokers tended to be older than the social smokers (P = 0.018) and older than those who smoke <15 cigarettes a day (P = 0.004).

Table 4-1. Age of smoking and non-smoking study participants

	n	Age range	Median age	*CYP2D6* methylation % SD (n)	*OPRM1* methylation % SD (n)
Never smoked	43	20 - 50	26	90.8 ±1.7 (43)	11.7 ±5.0 (24)
Ever smoked	50	19 - 50	24	91.0 ±1.8 (43)	10.5 ±3.1 (43)
Ex-smoker	16	21 - 50	33	91.1 ±2.5 (12)	10.1 ±2.7 (13)
<15 cig./day	23	19 - 48	22	91.0 ±1.4 (21)	10.7 ±3.0 (19)
>15 cig./day	6	19 - 40	24	90.8 ±2.6 (5)	10.8 ±5.2 (6)
Occasional*	5	20 -32	20	90.9 ±1.1 (5)	10.9 ±1.5 (5)

*"Social smokers", smoke less than 3 cigarettes per week in a drinking environment.

Methylation data for *CYP2D6* and *OPRM1* was obtained in 92% and 72% of cases respectively. *OPRM1* methylation data from an additional 23 individuals was obtained

from the smoker and non-smoker buccal swabs, however during data transfer from the pyrosequencer USB to the computer the information was lost as a result of operator error. The cases could not be reanalysed within the timeframe of the PhD.

No relationships were observed following Spearman's rho correlation between age and methylation of either *CYP2D6* (correlation coefficient .099, $P = 0.363$) or *OPRM1* (correlation coefficient .088, $P = 0.481$). In addition, no methylation differences were observed in *CYP2D6* or *OPRM1* between the different smoking statuses ($P = 0.991$, figure 4-1; $P = 0.822$, figure 4-2, respectively).

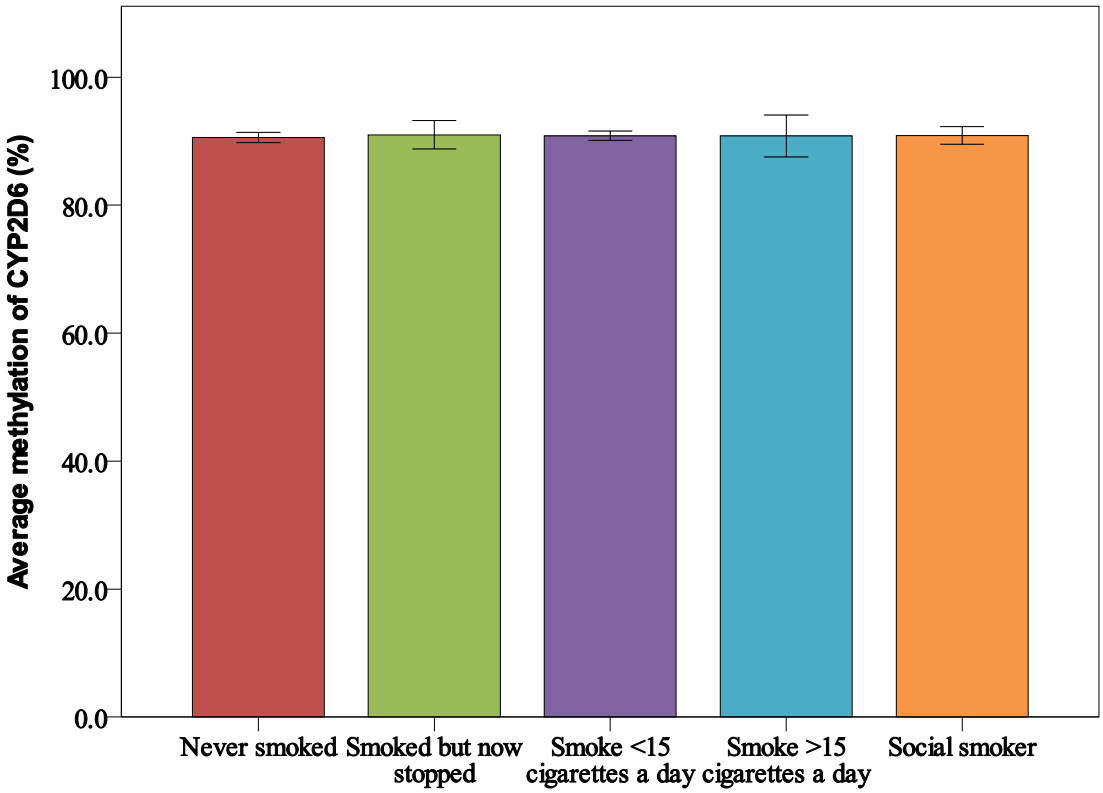

Figure 4-1. *CYP2D6* methylation in non-smokers, ex-smokers, frequent smokers (<15 or >15 cigarettes/day) and social smokers

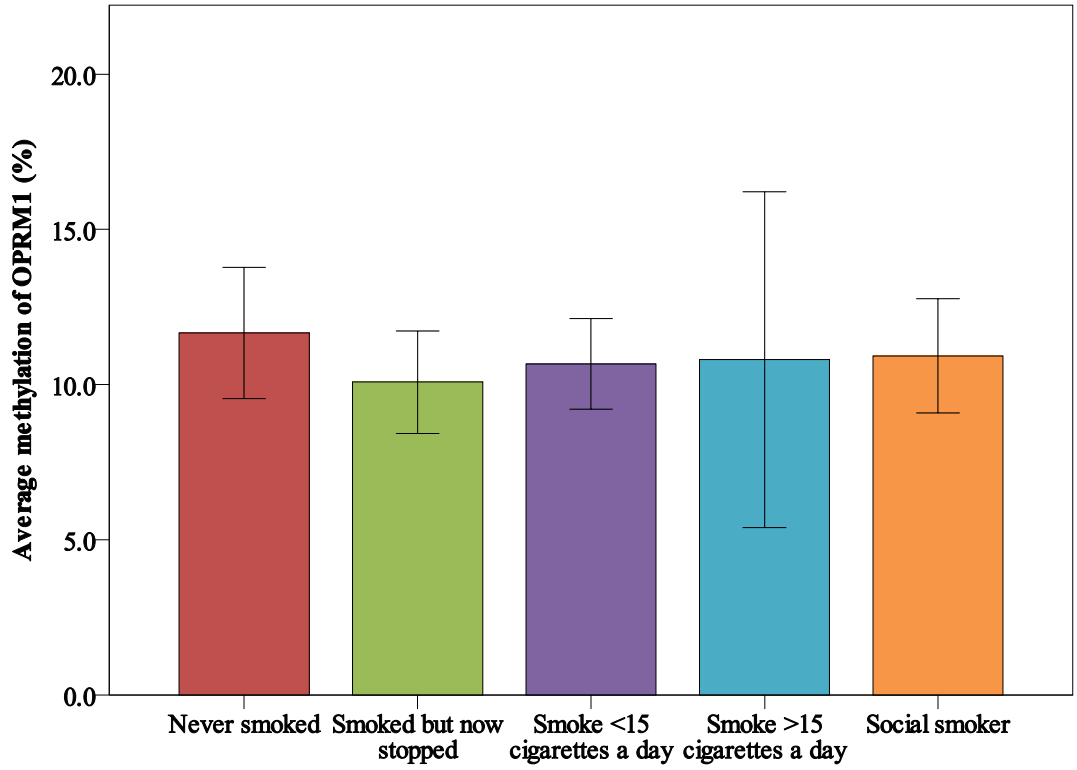

Figure 4-2. *OPRM1* methylation in non-smokers, ex-smokers, frequent smokers (<15 or >15/day cigarettes) and social smokers

4.4 Discussion

Regardless of an individual's smoking exposure the average methylation of 3 CpG sites in *CYP2D6* and 8 CpG sites in *OPRM1* were unchanged. Previous studies have shown that smoke exposure can cause methylation alterations, however these alterations were observed at repetitive elements within diseased tissues (Smith *et al.* 2007; Furniss *et al.* 2008) and promoter regions, or individual CpG loci of specific genes (Zöchbauer-Müller *et al.* 2003; Enokida *et al.* 2006; Marsit *et al.* 2007; Kupfer *et al.* 2010; Wan *et al.* 2014). Promoter region CpG sites within *OPRM1* were investigated in this study as previous research reported higher methylation in lymphocyte and sperm DNA of opioid exposed individuals compared to controls (Nielsen *et al.* 2009; Chorbov *et al.* 2010). The increased methylation was postulated to be as a result of opioid exposure; however the effect of smoking was not taken into consideration. The data obtained from this study suggests that the CpGs investigated in *CYP2D6* and *OPRM1* of buccal swab DNA are not targets for smoke induced methylation changes.

The lack of variable methylation between smokers and non-smokers in this study may in part be explained by tissue choice. Epithelium cells of heavy smokers from four different aerodigestive tract tissues were compared and tissue specific methylation was identified (Zöchbauer-Müller *et al.* 2003). Similarly, Chien (2011) compared methylation in blood and buccal swabs and observed variable methylation. The methylation differences between paired blood and buccal swabs being greater in samples obtained from smokers than non-smokers (Chien 2011). Smoke induced methylation differences were observed on CpG sites within the gene body of *OPRM1* in peripheral blood samples of adolescents exposed to maternal smoking (Lee *et al.*

2014). The study by Lee *et al.* (2014) showed that methylation levels were lower in adolescents who were exposed to nicotine prenatally compared to non-nicotine prenatally exposed adolescents. The differences in *OPRM1* gene body methylation are thought to play a role in regulating the alternative splice sites (Shukla *et al.* 2011; Li-Byarlay *et al.* 2013; Gelfman and Ast 2013) and may also regulate *OPRM1* by inhibiting alternative promoters (Maunakea *et al.* 2010).

The results from this pilot population suggest that DNA methylation of *CYP2D6* and *OPRM1* is not influenced by smoking therefore methylation differences observed in later populations may represent the influence of other factors.

4.4.1 Study limitations

Limited information was collected regarding the type of cigarettes smoked, i.e. branded or own rolled. This may have an influence on DNA methylation as nicotine (the DNA altering chemical) percentage content of different cigarettes may vary. The smoking groups were also quite generalised but groupings were necessary to obtain substantial groups for comparison. Each individual's style of smoking may also alter the risk of DNA methylation as the volume of smoke inhaled and the length of time the smoke was held in the oral cavity will differ. As diet has been shown to change gene DNA methylation it would have been of interest to investigate nutritional intake, specifically folate and other methyl group donors. Comparison of gene DNA methylation with length of time smoked would also have been of interest rather than just current smoking status. However this study was undertaken as a pilot population to ascertain whether any gene DNA methylation differences were observed between smokers and non-smokers and to encourage participation the questionnaires were kept minimal.

5.0 METHADONE-PRESCRIBED OPIOID-DEPENDANT MOTHERS AND THEIR NEW-BORN BABIES: Babies Born to Methadone-Prescribed Opioid-Dependant Mothers have Elevated DNA Methylation on *ABCB1/MDR1*, *CYP2D6* and *OPRM1*.

5.1 Introduction

Since the early 1900s, methadone has been used for opioid-dependant women during pregnancy as it has fewer risks than illicit opioid use (National Institutes of Health Consensus Development Panel, 1998; Fisher and Fisher 2000). Despite the popularity of methadone there are still significant risks associated with its use for the developing foetus. Infants of methadone-prescribed mothers tend to have shorter gestation periods and therefore lower birth weights, heights, and smaller head circumferences, and are at risk of developing neonatal abstinence syndrome (NAS) (Shaw and McIvor 1994; Dashe *et al.* 2002; Arlettaz *et al.* 2005; Fajemirokun-Odudeyi *et al.* 2006; Kakko *et al.* 2008; Dryden *et al.* 2009; Wouldes and Woodward 2010; Cleary *et al.* 2011).

The negative effects of intrauterine methadone have been shown to continue into early childhood influencing post-natal weight gain, head circumference, height and trends towards poorer cognitive, psychomotor and behavioural performance compared to non-opioid exposed infants / children (Bauman and Levine 1986; Soepatmi 1994; Darke *et al.* 2000; Pirastu *et al.* 2006; Whitham *et al.* 2010; Konijnenberg and Melinder 2013; Baldacchino *et al.* 2014).

With regards to methadone, Nielsen *et al.* (2008) reported significantly increased DNA methylation of *OPRM1* in lymphocytes of former heroin addicts compared to

controls. These observations were later replicated by Chorbov *et al.* (2010) and Doehring *et al.* (2013) who concluded that opioid misuse may alter *OPRM1* methylation. To the best of our knowledge, the influence of intrauterine methadone on the developing foetal DNA methylation has not yet been reported. Wachman *et al.* (2014) compared methylation levels in *OPRM1* of methadone exposed infants requiring NAS treatment, and those that did not require NAS. However they did not have a control population to determine the methylation profile of a non-opioid exposed population.

The aim of this study was to examine the different methylation status in selected opioid-related genes (*ABCB1*, *CYP2D6* and *OPRM1*) between, a) babies born to MPOD mothers and babies of non-opioid exposed mothers, b) MPOD mothers and non-opioid mothers, c) MPOD mothers and their babies, and d) non-opioid exposed mothers and their babies.

5.2 Materials and methods

5.2.1 Sample collection

Mother and baby dyads were recruited as described in *3.1.2* and samples collected as detailed in *3.2.2*.

5.2.2 Toxicological analysis

Plasma samples from 18 of the MPOD mothers were analysed for methadone and its metabolite 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) at the Toxicology Unit, Imperial College London as previously described in *3.3.1*.

5.2.3 DNA methylation analysis

DNA was extracted from the Catch-All™ oral swabs as described in *3.4.1.1* and methylation profile determined using the pyrosequencing method detailed in *3.5*.

5.3 Results

MPOD mothers were prescribed a mean methadone dose of 59 ±27.6 mg. Methadone plasma concentrations were determined in 18 of the 21 samples. A median methadone concentration of 245 ng/mL was observed with a range of 60-720 ng/mL. In addition to the prescribed methadone, during pregnancy all the mothers had smoked cigarettes and consumed other prescription / illicit drugs e.g. benzodiazepines. The majority of opioid-dependant mothers lived in the more deprived areas of Glasgow, as ascertained by their DEPCAT score (86%). Of the 21 babies born to opioid-dependant mothers, 11 required treatment for NAS using either oramorph (73%) or phenobarbititone (27%). The MPOD mothers and mothers in the control group were not only matched by postcode, but also by age (32 ±4.2 vs. 30 ±5.3 years, P = 0.268, *df* 51), gestation period (272 ±9.4 vs. 277 ±9.2 days, P = 0.122, *df* 51) and smoking status. Baby birth weight was significantly lower in the opioid-dependant group than the non-opioid exposed control group (2815 ±352.5 vs. 3386 ±533.7 grams, P = <0.001, *df* 51).

5.3.1 Methylation differences between methadone exposed and non-opioid exposed mothers and babies

Using independent t-tests, mean methylation of *ABCB1/MDR1*, *CYP2D6* and *OPRM1* was shown to be greater in babies born to MPOD mothers than babies in the control population (P = <0.001, *df* 47, figure 5-1). The most prominent difference was observed in *ABCB1/MDR1* (17.8% ±0.5 vs. 2.6% ±0.4), followed by *OPRM1* (8.5% ±0.3 vs. 3.5% ±1.6), then *CYP2D6* (92.1% ±1.2 vs. 89.3% ±2.4). As observed in the baby comparison, the greatest methylation difference between the MPOD mothers and control mothers was seen in *ABCB1/MDR1* (18.4% vs 3.0% respectively, P = <0.001,

df 18, figure 5-2). A statistically significant difference was also observed in *CYP2D6* (92.6% vs. 90.5%, *P* = 0.001, *df* 45) but not *OPRM1* (8.7% ±0.4 vs. 8.1% ±2.1, *P* = 0.147, *df* 31).

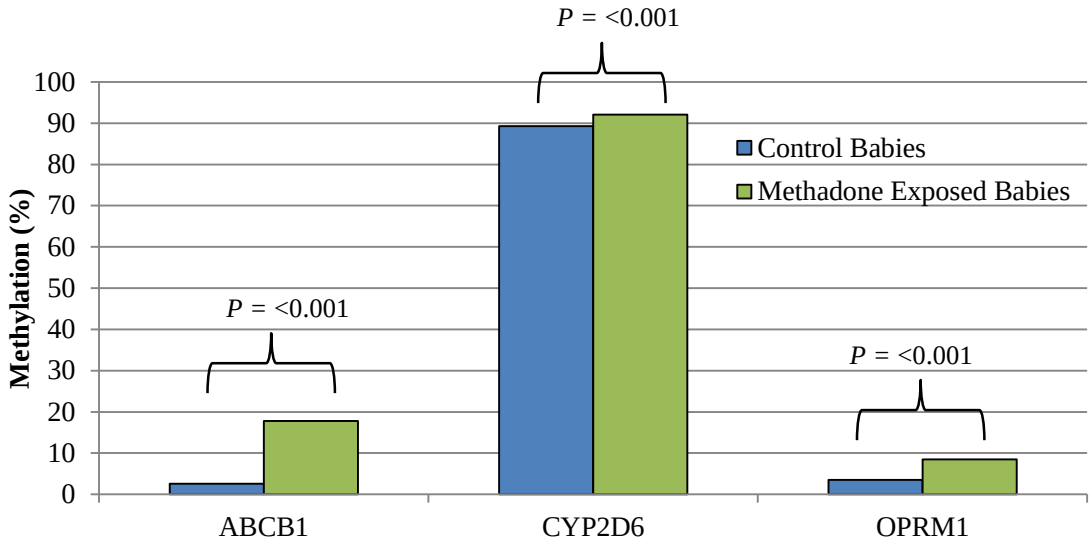

Figure 5-1. Methylation differences between methadone exposed babies and controls in *ABCB1/MDR1, CYP2D6* and *OPRM1*
Control babies (left columns in blue) were significantly less methylated on *ABCB1/MDR1*, *CYP2D6* and *OPRM1* than babies exposed to methadone *in utero* (right columns in green).

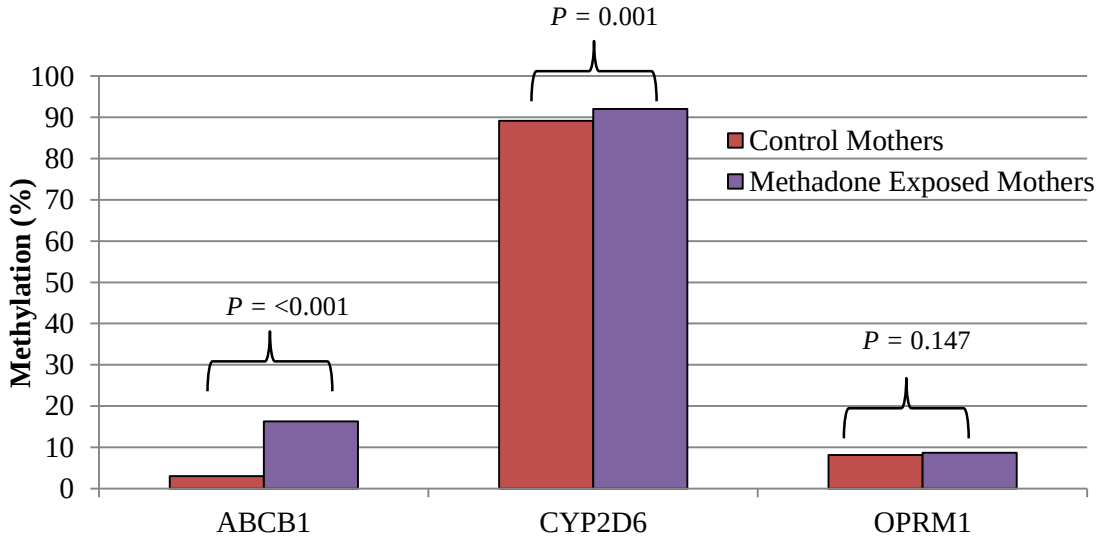

Figure 5-2. Methylation differences between methadone exposed mothers and controls in *ABCB1/MDR1, CYP2D6* and *OPRM1*
Control mothers (left columns in red) were significantly less methylated on *ABCB1/MDR1* and *CYP2D6* than methadone exposed mothers (right columns in purple), however no difference was observed on *OPRM1*.

In the control population the mothers' methylation levels in *OPRM1* and *ABCB1/MDR1* were greater than that of their babies (*P* = <0.001, *df* 53 and *P* = 0.018, *df* 27 respectively) but no difference in methylation was observed in *CYP2D6* (*P* = 0.852, *df* 62). Between the MPOD mothers and their babies no differences were observed in any of the genes investigated (Figure 5-3).

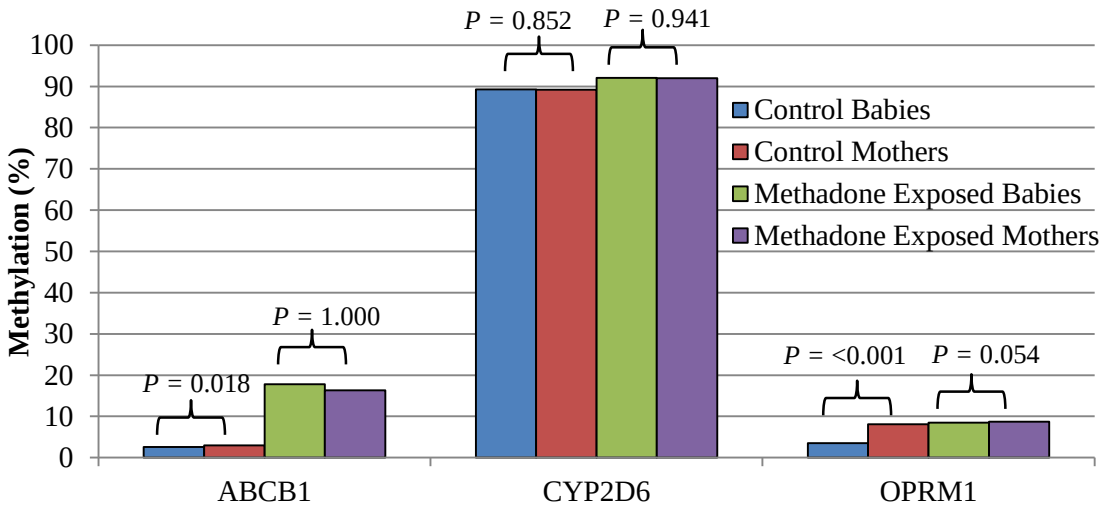

Figure 5-3. Methylation differences between methadone exposed mothers – baby dyads and control mother – baby dyads in *ABCB1/MDR1, CYP2D6* and *OPRM1*
Opioid exposed mother and baby dyads (green and purple columns) had similar methylation on the three genes investigated. Non-opioid exposed babies (blue column) had less methylation on *ABCB1/MDR1* and *OPRM1* than their mothers (red column), however no difference was observed on *CYP2D6*.

5.3.2 Relationship between methylation and smoking / residential status

Exposure to the chemicals within cigarettes, and residence within deprived or affluent regions of Glasgow did not impact the methylation in any of the genes investigated of either the mothers or their babies (Table 5-1).

Table 5-1. No relationship was observed between smoking exposure or residential status and *ABCB1/MDR1, CYP2D6* and *OPRM1* gene DNA methylation

	Smoking Exposure		Residential Status	
	P (2-tailed)	*df*	*P* (2-tailed)	*df*
ABCB1/MDR1				
Control Babies	0.083	18	0.083	18
Control Mothers	0.176	7	0.176	7
Methadone Exposed Babies	nd	nd	0.722	17
Methadone Exposed Mothers	nd	nd	0.746	17
CYP2D6				
Control Babies	0.213	30	0.350	30
Control Mothers	0.317	21	0.274	30
Methadone Exposed Babies	nd	nd	0.323	17
Methadone Exposed Mothers	nd	nd	0.662	16
OPRM1				
Control Babies	0.112	28	0.112	28
Control Mothers	0.059	27	0.135	27
Methadone Exposed Babies	nd	nd	0.599	18
Methadone Exposed Mothers	nd	nd	0.992	17

Independent sample t-tests were undertaken on control mother and baby, and methadone exposed mother and baby samples. *P* values of <0.05 were considered significant. nd = no data: all of the methadone exposed mothers were smokers so no comparison between smokers and non-smokers could be made for the methadone exposed population.

5.3.3 Relationship between methylation and NAS development

There were no methylation differences between the babies of MPOD mothers that required NAS treatment or did not require NAS treatment in any of the genes analysed (Table 5-2).

Table 5-2. No relationship was observed between the development of NAS and *ABCB1/MDR1, CYP2D6* and *OPRM1* gene DNA methylation

	NAS Development	
	P (2-tailed)	*df*
ABCB1/MDR1	0.343	17
CYP2D6	0.992	17
OPRM1	0.992	18

Independent sample t-tests were undertaken on control mother and baby, and methadone exposed mother and baby samples. *P* values of <0.05 were considered significant.

5.3.4 Methylation differences between paired plasma and buccal DNA samples

The methylation percentages observed in the buccal swabs were statistically different from the methylation found in paired plasma samples provided by the MPOD mothers. This statistical difference was observed in each of the three genes analysed (Figure 5-4). Methylation was higher in buccal DNA than plasma DNA at the CpG sites investigated in *ABCB1/MDR1* and *OPRM1*; however plasma had higher methylation in *CYP2D6* than buccal DNA. Bivariate Spearman's rho correlation showed no relationship between buccal and plasma *ABCB1/MDR1* (.362, P = 0.140, n = 18) *CYP2D6* (-.007, P = 0.980, n = 15) or *OPRM1* (.178, P = 0.543, n = 14) DNA methylation levels.

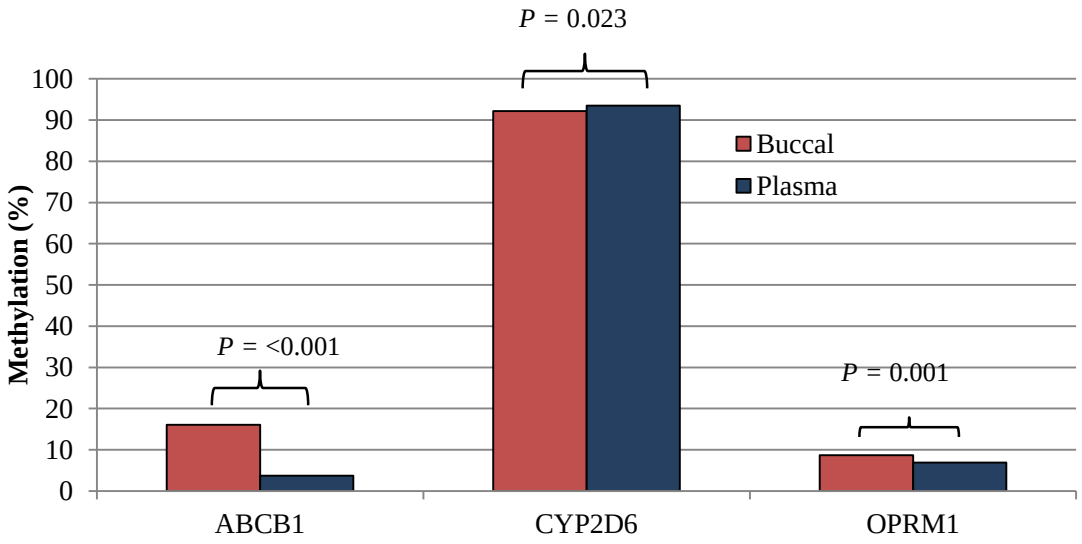

Figure 5-4. MPOD mother buccal and plasma methylation
The difference between buccal and plasma gene DNA methylation was assessed using the related-samples Wilcoxon signed rank test as the data for plasma DNA methylation was non-normally distributed and could not be normalised.

5.4 Discussion

The aim of this study was to determine the effect of *in utero* methadone exposure on baby methylation in *ABCB1/MDR1*, *CYP2D6* and *OPRM1*. The results suggest that *in utero* exposure to methadone could increase DNA methylation of babies born to MPOD mothers; alternatively, or as a co-factor, the methadone associated lifestyle could influence DNA methylation patterns.

The opioid exposed babies had significantly higher methylation of each of the genes investigated compared to the non-opioid exposed babies. Unlike previous studies, the methylation differences observed between the opioid exposed and non-opioid exposed babies are much more prominent, especially the CpG sites investigated in *ABCB1/MDR1*. In studies comparing opioid exposed adults to non-opioid exposed adults, significant methylation differences of only a few percent have been reported (Nielsen *et al.* 2009; Chorbov *et al.* 2010; Doehring *et al.* 2013) e.g. at CpG -18 of *OPRM1* average methylation in methadone exposed individuals was 25.4% compared to 21.4% in opioid naïve individuals. However, the opioid exposed babies in this study had ≥ double the methylation of the control babies. The reported methylation levels by Chorbov *et al*. and Doehring *et al*. are not comparable as adult populations were used, not babies and methylation has been shown to be age dependant (Heyn and Estellar 2012). Also, methylation has been shown to be tissue-specific (Zhang *et al.* 2013) so DNA from lymphocytes, whole blood, sperm and brain tissue are not be comparable to the buccal DNA collected for this study.

To our knowledge the only study that has investigated CpG sites in saliva samples from neonates was published by Wachman *et al.* (2014) who reported methylation levels in *OPRM1* that were lower than found in our opioid exposed population (~5.1%

v 8.9%) but greater than the methylation levels observed in our control population (~5.1% v 3.8%). Wachman *et al.* (2014) did not have a non-opioid exposed cohort for comparison. They did, however find a methylation difference between babies that required NAS treatment and those that did not (at CpG -10 in *OPRM1*); and between babies that required more than 2 treatments to alleviate NAS compared to those that had 0-1 treatments (CpG -10, -14 and +84). The results of this study do not support the report that relationship between CpG methylation and requirement of NAS treatment as observed by Wachman *et al.* (2014). The proposition that this could be a sample size effect should not be excluded.

The gene methylation disparity between the opioid exposed babies and non-opioid exposed babies could be explained by the influence of methadone exposure during fertilisation and embryonic development. This period is essential for correct development and is a sensitive period of epigenetic reprogramming. Epigenetic marks are made during foetal development persist to adulthood (McKay *et al.* 2012), therefore exposure to methadone / methadone-lifestyle during this sensitive period could adversely affect the babies DNA methylation with possible consequences in later life. Intrauterine exposures have been associated with increased risk of developing diseases later in life (Jirtle and Skinner 2007; Reamon-Buettner and Borlak 2007).

Potential mechanisms (as discussed by Doehring *et al.* 2013) by which methadone could increase DNA methylation is by G-protein-coupled receptor-mediated increase of DNA methyltransferase activity. Stimulation of the CB1R cannabinoid receptor, a G-protein-coupled receptor, is thought to increase global methylation status by triggering the activation of p38 and, to a lesser extent, the p42/44 mitogen-activated

protein kinase-dependant (MAPK) pathways that stimulate transcription factors (Paradisi *et al.* 2008). Methadone binds to the mu-opioid receptor, which is a G-protein-coupled receptor like CB1R and therefore also triggers the MAPK pathways (Morse *et al.* 2011).

The increased methylation observed in babies of MPOD mothers however may or may not exclusively be a result of methadone exposure. Research into the lifestyle of heroin addicts indicates that these individuals experience chaotic, stressful, high risk lifestyles (Hulse *et al.* 1998; Gyarmarthy *et al.* 2009), with risk of developing depression and anxiety (Sordo *et al.* 2012; Chahua *et al.* 2013). These factors have been associated with variable methylation (McGowan *et al.* 2009; de Rooij *et al.* 2012). Methadone-maintenance treatment improves opioid-dependant individuals' quality of life (Xiao *et al.* 2010; Wang *et al.* 2012), however stresses are still experienced (Barry *et al.* 2011). Alongside stressful lifestyles, opioid dependant individuals tend to have a poor diet (Himmelgreen *et al.* 1998; Nazrul Islam *et al.* 2002; Saeland *et al.* 2009; Neale *et al.* 2012) and be poly-drug users (SAMHSA 2007; Brecht *et al.* 2008; Jansson *et al.* 2012). The combination of these factors is thought to contribute to the negative impact on the developing foetus (Bell and Lau 1995; Hulse *et al.* 1998) and therefore may alter their DNA methylation profiles (Hogg *et al.* 2012).

The results showed that the methadone exposed babies had similar methylation levels to that of their MPOD mothers in each of the genes investigated. Less methylation was expected in the methadone exposed baby population compared to the MPOD mothers as previous studied have shown that methylation of CpG rich regions can be age associated (Gopisetty *et al.* 2006; Heyn and Esteller 2012). However, age related

methylation changes can be gene specific and to the best of our knowledge there is little age-methylation related data on the CpG sites investigated as part of this study. The data obtained from the control (non-opioid exposed) baby and mother population however illustrated that methylation of CpG sites within the promoter regions of *ABCB1/MDR1* and *OPRM1* differ, but not in *CYP2D6*. As the paired mother-baby dyads were exposed to the same diet, stresses and chemical exposures, the increased methylation differences observed on *ABCB1/MDR1* and *OPRM1* of the control mothers is thought to be due to the acquisition of methylation over time, before pregnancy.

When comparing the methylation in MPOD mothers and controls it is apparent that MPOD mothers have increased methylation on *ABCB1/MDR1* and *CYP2D6* genes. The methylation differences observed were not attributable to the mothers' age, whether they smoked or not, or the areas they reside in. As such, the increased methylation in MPOD mothers is thought to be as a result of regular opioid exposure, or the associated lifestyle. There are no studies to date that have explored the relationship between DNA methylation on *ABCB1/MDR1* and *CYP2D6* and methadone exposure. However there have been previous studies investigating the methylation of CpG sites within *OPRM1*. These studies observed CpG point-wise differences between the opioid exposed and control populations in *OPRM1* (Nielsen *et al.* 2009; Chorbov *et al.* 2010; Doehring *et al.* 2013) although, those findings were not replicated in our data.

To maintain DNA methylation patterns essential methyl group donors such as folate, choline and methionine are required. These nutrients are obtained from foods such as fruits, vegetables, beans and nuts (La Brosse and Albrecht 2012) and diets that

deficient of these nutrients have been shown to cause global hypomethylation (Jacob *et al.* 1998; Rampersaud *et al.* 2000; Pufulete *et al.* 2005; Niculescu *et al.* 2006). High fat and sugary maternal diets have also shown promoter hypomethylation of rat/mice studies in brain (Vucetic *et al.* 2010), but the changes can be sex-specific (Gallou-Kabani *et al.* 2010). A limitation of the present study was that the nutritional intake of mothers was not obtained. The MPOD mothers were considered nutrient and vitamin deficient. If so, our work suggests that diet is not the factor causing the increased methylation observed in *OPRM1*, *ABCB1/MDR1* and *CYP2D6* of babies born to MPOD mothers.

Alcohol has been reported to impact DNA methylation (Christensen *et al.* 2010; Lambert *et al.* 2011) and although there is no literature on benzodiazepines and methylation, the behavioural and neurobehavioral problems of new-borns exposed to prescribed and illicit psychoactive substances has been reported (Jansson *et al.* 2012). In addition the majority of MPOD mothers were prescribed or admitted illicit benzodiazepine use, and had an unknown amount of alcohol consumption.

The methadone as well as the stresses and diet that babies born to MPOD mothers are exposed to may alter the DNA methylation of important opioid related genes. These alterations established during a sensitive period of epigenetic programming are then maintained during cell replication which may impact the babies' behaviour and susceptibility to disease and drug use later in life. Our findings suggest that the methylation changes on different genes are not diet related.

5.4.1 Study limitations

Numbers for this pilot population were small for the number of variables investigated. A bigger population and undertaking a multiple regression would have been beneficial. Additional information from the methadone exposed and opioid naïve populations would also have been useful. For example, the maternal diet, measurement of stress indicators and a record of confounding substrates (e.g. alcohol and prescribed and illicit drug use).

6.0 METHADONE-PRESCRIBED OPIOID-DEPENDANT MOTHERS AND THEIR NEW-BORN BABIES: The *CYP2B6*6* polymorphism protects babies exposed to methadone *in utero* from neonatal abstinence syndrome development.

6.1 Introduction

Opioid use during pregnancy has increased over the last two decades, as has the number of women of child bearing age that are opioid dependant (Unger *et al.* 2012; Brogly *et al.* 2014; Goettler and Tschudin 2014). This is of concern as unplanned pregnancies amongst opioid users are common because of irregular / absent menstrual cycles as well as ineffective use of contraceptive drugs (Kreek *et al.* 1999; Herrmann *et al.* 2014). Infants born to opioid addicted mothers can suffer congenital abnormalities, foetal growth restriction, preterm birth, impaired neurodevelopment and may experience opioid withdrawal, neonatal abstinence syndrome (NAS) (Bandstra *et al.* 2010).

Of the infants exposed to intrauterine methadone, ~60-80% exhibit symptoms of NAS (Jones *et al.* 2010; Welle-Strand *et al.* 2013; Gawronski *et al.* 2014). NAS manifests as tremors, high pitched crying, unrest, yawning, sneezing, vomiting, diarrhoea, fever and poor sucking. The onset of these withdrawal symptoms can occur anytime from 48 hours to 10 days following birth (Dryden *et al.* 2009; Smirk *et al.* 2014). A study by Dryden *et al.* (2009) showed that of 450 infants exposed to methadone *in utero* almost 50% required pharmacological treatment for NAS and as such were admitted to the neonatal care unit. The length of stay within the neonatal unit ranged from 1 – 108 days (median 13 days). Although infants born to opioid-dependant women only

represented 2.9% of all hospital births, the infants occupied 18.2% of the neonatal cots reflecting their increased medical needs. The costs are not only restricted to infant monitoring, but also spent on providing breastfeeding and parenting support to the mothers as well as social work assessment and support in the community (Dryden *et al.* 2009). The subsequent postnatal care of the opioid exposed infants draws heavily upon healthcare resources (Dryden *et al.* 2009; Patrick *et al.* 2012; Smirk *et al.* 2014); therefore identification of factors predicting NAS development may enable reduced observation or early discharge of less susceptible infants resulting in large financial savings.

A number of factors have been associated with development NAS, including dose of methadone prescribed, exposure to tobacco and whether or not the infant is breast fed (Kaltenback *et al.* 2012). Recent research by Wachman *et al.* (2013, 2014) also suggested the use of genetic and epigenetic variations for predicting the development of NAS. They found that polymorphisms within the μ-opioid receptor, encoded by *OPRM1*, and the metabolising enzyme for endogenous opioids, *COMT*, were associated with requirement for NAS treatment and extended stay at hospital following birth. In addition, Wachman *et al.* (2014) found that DNA methylation was greater within the promoter region of *OPRM1* of the infants who required two or more treatments for NAS. Identification of genetic variations that may aid NAS prediction early among infants at risk is useful so as to allow appropriate monitoring. The NAS predictors could also negate prolonged hospital stays for neonates who are less likely to develop withdrawal, reducing the healthcare costs associated with NAS monitoring e.g. cot occupancy or staff undertaking NAS assessments every 4 hours.

6.2 Materials and methods

6.2.1 Sample collection

Participant selection and requirements described in *3.1.2*. Oral swab and plasma samples were collected as detailed in *3.2.2.1* and *3.2.2.2*, respectively.

6.2.2 Toxicological analysis

Plasma samples from 18 of the MPOD mothers were analysed for methadone and its metabolite 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) at the Toxicology Unit, Imperial College London as previously described in *3.3.1*. Plasma samples were not collected from two MPOD mothers.

6.2.3 SNP and gene duplication / deletion analysis

DNA was extracted from buccal swabs following the protocol detailed in *3.4.1.1* and sent to LCG genomics for SNP analysis (following the preparation detailed in *3.4.3*).

6.3 Results

The mothers (n=20) had an average age of 32 ±4 and were prescribed methadone doses of 59 ±28 mg; alongside the methadone the mothers all smoked. Their babies had a mean gestation period of 272 ±9 days and at birth weighed 2815.4 ±353.5 g. After birth the babies had a median length of stay at hospital of 11 days (range 5 – 42 days). Of the 20 methadone exposed babies recruited, 10 required treatment for NAS. Between the babies requiring and not requiring NAS treatment there was no difference in length of gestation, birth weight, maternal methadone dose, or maternal plasma methadone concentration (Table 6-1) as assessed by independent t-tests. Fisher's exact test showed that there was no relationship between infants that suffered from NAS and their mothers' DEPCAT score, and also no association between NAS development and likelihood of baby remaining in the mother's care. Babies of older mothers had an increased likelihood of requiring NAS treatment ($P = 0.025$, df 18), and babies that were treated for NAS required a longer stay at hospital than the babies not requiring NAS treatment ($P = 0.004$, df 18).

Table 6-1. Summary of demographic and methylation data of methadone exposed babies (n=20) treated or not treated for Neonatal Abstinence Syndrome

Variables	Babies requiring NAS treatment (n=10)	Babies not requiring NAS treatment (n=10)	*P* value
Gestation Days (SD)	274 (±9)	270 (±10)	0.408, *df* 18
Birth Weight Grams (SD)	2731 (±315)	2876 (±398)	0.378, *df* 18
Maternal methadone dose mg (SD)	66 (±24)	54 (±32)	0.351, *df* 18
Length of hospital stay Days (range)	20 (±11)	7 (±2)	0.004, *df* 18
Maternal age Years (SD)	34 (±4)	30 (±4)	0.025, *df* 18
Discharged to maternal care	5	6	0.367, *df* 18
DEPCAT score >4 Total	8	7	1.000, *df* 18
Maternal methadone concentration dose corrected (SD)*	4.6 (±2)	5.3 (±9)	0.591, *df* 18
Maternal smoking Total	10	10	1.000, *df* 18

* dose corrected = methadone plasma concentration / prescribed methadone dose.

6.3.1 Associations between *ABCB1/MDR1, COMT, CYP2B6, CYP2D6* and *OPRM1* genes and NAS development

Buccal DNA was obtained from all 20 babies, however insufficient sample resulted in some samples (as detailed in Table 6-2) not being analysed for the SNPs of interest in *ABCB1/MDR1*, *COMT*, *CYP2B6*, *CYP2D6* and *OPRM1*.

Table 6-2. Baby genotype frequencies (n=20)

Single nucleotide polymorphism	No.	Genotype Frequencies	Allele Frequencies
ABCB1/MDR1 **1236C>T** **rs1128503**	19	CC = 0.21	C = 0.475 T = 0.525
		CT = 0.53	
		TT = 0.26	
ABCB1/MDR1 **2677G>T** **rs2032582**	20	GG = 0.15	G = 0.450 T = 0.550
		GT = 0.60	
		TT = 0.25	
ABCB1/MDR1 **3435C>T** **rs1045642**	19	CC = 0.21	C = 0.420 T = 0.580
		CT = 0.42	
		TT = 0.37	
COMT **186C>T** **Rs4633**	18	CC = 0.11	C = 0.390 T = 0.610
		CT = 0.56	
		TT = 0.33	
COMT **158A>G** **rs4680**	20	GG = 0.15	G = 0.450 A = 0.550
		GA = 0.60	
		AA = 0.25	
COMT **408C>G** **rs4818**	18	CC = 0.39	C = 0.610 G = 0.390
		CG = 0.44	
		GG = 0.17	
COMT **-98A>G** **rs6269**	20	GG = 0.15	G = 0.375 A = 0.625
		GA = 0.45	
		AA = 0.40	
CYP2B6 **516G>T** **rs3745274**	18	GG = 0.50	G = 0.750 T = 0.250
		GT = 0.50	
		TT = 0.00	
CYP2B6 **785A>G** **rs2279343**	19	AA = 0.50	A = 0.750 G = 0.250
		AG = 0.50	
		GG = 0.00	
CYP2D6	20	1 func. allele = 0.10	
		2 func. alleles = 0.75	
		>2 func. alleles = 0.15	
CYP2D6 **1707T>delT** **rs5030655**	20	TT = 0.95	T = 0.975 - = 0.025
		T- = 0.05	
		- - = 0.00	
OPRM1 **118A>G** **rs1799971**	19	AA = 0.89	A = 0.945 G = 0.055
		AG = 0.11	
		GG = 0.00	

NB *CYP2D6* 1845 G>A (rs3892097) and *CYP2D6* 2459 A>delA (rs35742686) are not included in the table as all the infants carried the same genotypes at these polymorphisms.

Of the 13 SNPs analysed, all the babies carried the same genotype at two *CYP2D6* SNPs (rs3892097 and rs35742686) so are not represented in Table 6-2. In relation to the development of NAS, *CYP2B6* 516G>T and 785A>G were observed to be associated (Table 6-3; $P = 0.015$, df 18 and 0.023, df 18, respectively). Babies carrying the homozygous wild type genotype were more likely develop NAS and require treatment that heterozygous babies. No relationship between NAS development and

any other SNP was observed. There was also no relationship between the number of copies of *CYP2D6* and NAS development.

Table 6-3. Association between baby genotype and NAS development as determined by Fisher's exact test

	ABCB1/MDR1					
	1236		2677		3435	
	CC	CT/TT	GG	GT/TT	CC	CT/TT
NAS treated	2	7	2	8	2	7
Untreated	2	8	1	9	2	8
***P* value**	1.000		1.000		1.000	

	COMT							
	62		158		408		-98	
	CC	CT/TT	GG	GA/AA	GG	GC/CC	GG	GA/AA
NAS treated	1	9	1	9	1	7	1	9
Untreated	1	7	2	8	2	8	2	8
***P* value**	1.000		1.000		1.000		1.000	

	CYP2B6			
	516		785	
	GG	GT/TT	AA	AG/GG
NAS treated	8	2	7	2
Untreated	1	7	2	8
***P* value**	0.015		0.023	

	CYP2D6			
	Number of copies		1707	
	1	≥2	TT	T-
NAS treated	0	10	9	1
Untreated	2	8	10	0
***P* value**	0.474		1.000	

	OPRM1	
	118	
	AA	AG/GG
NAS treated	8	1
Untreated	9	1
***P* value**	1.000	

The maternal genotype of the SNPs investigated did not predict likelihood of their babies requiring NAS treatment (Table 6-4).

Table 6-4. Association between maternal genotype and baby NAS development as determined by Fisher's exact test

	ABCB1/MDR1					
	1236		2677		3435	
	CC	CT/TT	GG	GT/TT	CC	CT/TT
NAS treated	2	7	2	8	2	7
Untreated	2	5	4	6	2	6
***P* value**	1.000		0.628		1.000	
	COMT							
	62		158		408		-98	
	CC	CT/TT	GG	GA/AA	GG	GC/CC	GG	GA/AA
NAS treated	1	8	1	8	1	7	2	8
Untreated	2	5	2	5	2	6	1	6
***P* value**	0.550		0.550		1.000		1.000	
	CYP2B6			
	516		785	
	GG	GT/TT	AA	AG/GG
NAS treated	5	2	7	3
Untreated	1	5	1	5
***P* value**	0.103		0.119	
	CYP2D6	
	Number of copies	
	1	≥2
NAS treated	0	8
Untreated	2	5
***P* value**	0.200	
	OPRM1	
	118	
	AA	AG/GG
NAS treated	8	1
Untreated	7	0
***P* value**	1.000	

6.3.2 Genetic variations and methadone dose and plasma concentrations

Independent t-tests were undertaken to ascertain whether there was a relationship between maternal methadone dose and plasma methadone concentrations, and genotypes. A relationship between maternal dose of methadone and copies of *CYP2D6*

was observed but no other genes (Table 6-5). Mothers with only one functioning allele of *CYP2D6* required less methadone prescribed than mothers with two or more functioning alleles (19 ±23 mg v 64 ±21 mg; $P = 0.016$, df 13).

Table 6-5. Association between maternal genotype and baby NAS development as determined by Fisher's exact test

	Maternal methadone dose mg (SD)		Maternal methadone plasma concentration, ng/mL (SD)	
	Homozygous wild-type	Heterozygous/ homozygous mutant	Homozygous wild-type	Heterozygous/ homozygous mutant
ABCB1				
1236C>T	65 (30)	59 (28)	260 (269)	275 (188)
2677G>T	57 (35)	61 (26)	242 (220)	308 (216)
3435C>T	46 (37)	61 (26)	255 (273)	307 (226)
COMT				
62C>T	70 (25)	61 (30)	447 (276)	281 (207)
158G>A	70 (25)	56 (26)	447 (276)	268 (219)
408G>C	70 (25)	55 (26)	447 (276)	267 (210)
-98G>A	72 (28)	56 (29)	327 (156)	214 (166)
CYP2B6				
516G>T	62 (28)	53 (29)	266 (233)	324 (281)
785A>G	65 (28)	55 (28)	289 (194)	343 (265)
OPRM1				
118A>G	54 (28)	65 (-)	296 (246)	320 (-)

NB SNPs are not included in the table if all the mothers carried the same genotypes at these polymorphisms. No range is given for OPRM1 as only one mother had the heterozygous/homozygous mutant genotype.

6.4 Discussion

The results obtained indicate that babies carrying a mutated allele at *CYP2B6* 516G>T and 785A>G are less likely to require NAS treatment than babies homozygous wild type. *CYP2B6* is the predominant CYP isoform responsible for inactivating methadone to the metabolite 2-ethyl-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) modulating methadone plasma concentrations and clearance (Kharasch *et al.* 2008; 2013). The combination of the 516G>T SNP and 785A>G SNP forms the *CYP2B6**6 haplotype that has been associated with higher steady-state plasma methadone concentrations (Crettol *et al.* 2005; Eap *et al.* 2007; Wang *et al.* 2011) and requirement of lower methadone doses (Hung *et al.* 2011; Levran *et al.* 2013). *CYP2B6**6 haplotype causes higher plasma methadone concentrations and lower methadone doses as it is catalytically deficient compared to the wild type (Gadel *et al.* 2013) resulting in poorer methadone metabolism compared to the wild-type. Therefore, babies with *CYP2B6**6 are less likely to require NAS treatment because the babies' plasma methadone concentration has a slower rate of decline so withdrawal is less abrupt. Following birth, babies carrying the *CYP2B6* wild-type efficiently and rapidly metabolise the maternally transferred methadone and suffer opioid withdrawal symptoms.

The relationships observed by Wachman *et al.* (2013) between the *OPRM1* SNP 118A>G and *COMT* 158A>G with length of hospital stay and medical treatment of NAS were not supported by this study. Babies with the AA genotype at both SNPs required longer hospital stays and were more likely to require NAS treatment. However the lack of corroboration between our data and that of Wachman *et al.* (2013) could be a sample size effect in addition to a low frequency of mutated alleles in our study population.

This is the first study to report a relationship between the *CYP2B6*6* genotype, associated with poor metaboliser status, and a requirement for NAS treatment. These findings indicate that babies carrying the *CYP2B6* wild type are more susceptible to developing NAS.

6.4.1 Study limitations

Further studies with a larger population of exposed babies is required before definitive conclusions can be drawn. The influence of other *in utero* exposures must also be considered in future studies. All of the babies in this study were not only exposed to methadone but nicotine and various other prescription and illicit drugs. A detailed record of the mother's drug / alcohol / smoking consumption should be obtained.

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7.0 TISSUE SPECIFIC DNA METHYLATION: DNA methylation of *ABCB1/MDR1*, *CYP2D6* and *OPRM1* in individuals whose deaths were attributed to heroin toxicity.

7.1 Introduction

Heroin is a highly addictive semi-synthetic opioid that rapidly crosses the blood-brain barrier affecting the central nervous (Tamrazi and Almast 2012; Ruha and Levine 2014), cardiovascular (Dettmeyer *et al.* 2009; Darke *et al.* 2010), respiratory (Pattinson 2008; van der Schier *et al.* 2014), gastrointestinal (Holzer 2014) and endocrine systems (Elliott *et al.* 2010); as well as the skin (Fink *et al.* 2011; Onesti *et al.* 2014). The effects of intravenously injected heroin usually occur within 30 seconds and include an initial warm rush of sedation and reduction in blood pressure and heart rate creating a sense of well-being and euphoria (Fernandez and Libby 2013). The sedative effects last between 2-4 hours and in cases of addiction may be followed by the onset of withdrawal symptoms of restlessness, diarrhoea, vomiting and aches and pains (Handelsman *et al.* 1987; Cowan *et al.* 2005). Heroin users develop dependence for the drug, craving the effects of heroin and negating the withdrawal effects through continued heroin use. Over time however the addict can develop tolerance to heroin and subsequently require increased doses of the drug to obtain the desired euphoria (Hoffer *et al.* 2012; Craft and Lustyk 2013).

At a molecular level, opioid exposure has been observed to alter DNA methylation. When investigating gene methylation however it is important to be aware of tissue specific methylation. Genome wide analysis has shown that methylation between different tissues is generally well conserved, with tissues of similar function having

the highest correlation tissue (Rakyan *et al.* 2008; Lokk *et al.* 2014). However tissue specific differentially methylated regions occur in promoters of genes associated with tissue specific function (Davies *et al.* 2012; Sleiker *et al.* 2013; Lokk *et al.* 2014). For example, genes involved with blood vessel development and morphogenesis are hypomethylated in arteries, and immune response and leukocyte activation genes are hypomethylated in tonsil tissue (Lokk *et al.* 2014). Therefore, when investigating the effect of gene methylation on protein action it is important to obtain DNA from the relevant tissue.

In a bid to increase our understanding of methylation in drug addicted individuals, the specific DNA methylation on *ABCB1/MDR1*, *CYP2D6* and *OPRM1* in blood, liver, psoas muscle and thalamus was examined in deaths attributed to heroin toxicity. The extent of methylation in liver, psoas muscle and thalamus was compared against samples obtained from non-drug related deaths via the Edinburgh Brain and Tissue Bank. Methylation of *ABCB1/MDR1*, *CYP2D6* and *OPRM1* in blood was compared against a geographically similar post-mortem population of non-drug related deaths. Identification of functional changes of opioid exposure may lead to the development of a powerful therapeutic tool (Kovatsi *et al.* 2011; Kouidou *et al.* 2010; Fragou *et al.* 2011) that could ease the financial impact of opioid dependence on society and assist with the interpretation of post-mortem toxicology results.

7.2 Materials and methods

7.2.1 Sample collection

Samples were obtained as described in *3.1.3* and *3.2.3*.

7.2.2 Toxicological analysis

Concentrations of morphine and its metabolites, morphine-3-glucuronide (M3G) and morphine-6-glucuronide (M6G) were determined by LC-MS as described in Chapter *3.3.2*.

7.2.3 SNP and gene duplication / deletion analysis

SNP and gene duplication were ascertained following the protocols in *3.4*.

7.2.4 DNA methylation analysis

Gene DNA methylation was determined as described in *3.5*.

7.3 Results

The opioid exposed individuals (n = 43) ranged between 21 – 65 years old with a mean age of 35; the majority of whom were male (79%). Both of the control cohorts, the Dundonian population from which non-opioid exposed blood was obtained and the Edinburgh tissue bank liver, psoas muscle and thalamus donors, were older than the opioid exposed population (P = <0.001, Table 7-1). There was no gender distribution difference between the opioid exposed and control populations (Table 7-1).

Table 7-1. Age, gender and gene average DNA methylation of opioid exposed and control populations

		Opioid exposed (n = 43)	Control – blood (n = 41)	Control – liver, muscle and thalamus (n = 10)
Age (mean, range)		35, 21-65	56, 24-84	55, 44-76
Gender (% male)		79	85	70
ABCB1/MDR1* median methylation	**Blood**	1.9 (1.2-3.2)	2.2 (1.5-5.7)	na
	Liver	1.7 (1.1-3.4)	na	na
	Muscle	1.7 (1.1-2.5)	na	na
	Thalamus	2.3 (1.9-2.8)	na	na
***CYP2D6* average methylation**	**Blood**	92.6 (88.1-94.1)	92.8 (80.3-94.9)	na
	Liver	20.3 (9.2-77.9)	na	84.0 (14.8-87.8)
	Muscle	84.5 (81.4-87.9)	na	84.1 (20.8-89.4)
	Thalamus	88.5 (79.1-91.6)	na	86.4 (32.3-89.7)
***OPRM1* average methylation**	**Blood**	8.2 (4.6-13.1)	9.1 (4.5-19.7)	na
	Liver	7.7 (5.3-10.9)	na	5.6 (3.4-8.1)
	Muscle	3.5 (3.0-4.1)	na	4.4 (3.6-8.0)
	Thalamus	3.7 (2.9-6.2)	na	6.1 (3.6-9.2)

**ABCB1MDR1* methylation data had non-normal distribution, therefore the median methylation and range were provided rather than the mean.

The methylation status of *ABCB1/MDR1*, *CYP2D6* and *OPRM1* was determined in blood, liver psoas muscle and thalamus samples from opioid associated deaths (Table 7-1). Control methylation data was obtained for *CYP2D6* and *OPRM1* from blood, liver, muscle and thalamus but *ABCB1/MDR1* methylation was only determined in

non-opioid exposed blood samples (Table 7-1). Not all of the samples were successfully analysed for methylation for a number of reasons, detailed below (the number of samples successfully analysed using each assay is shown in Figures 7-3 to 7-7):

1) poor quality DNA was extracted as a result of tissues being subject to repeated freeze-thaw cycles;
2) bisulfite conversion of DNA was undertaken using plates with only 96 wells and as such bisulfite conversions were restricted to converting 90 samples per run (with the remaining six spaces used for negatives, methylated and unmethylated controls);
3) complete consumption of the 15µL of bisulfite converted DNA sample as a result of preparation or instrumental error;
4) insufficient time and budget in the PhD timescale to reanalyse samples with missing methylation results.

7.3.1 Age and methylation

7.3.1.1 ABCB1/MDR1 DNA methylation and age

No relationships were observed between age and *ABCB1/MDR1* gene DNA methylation in any of the tissues investigated in opioid exposed and opioid naïve individuals (Table 7-2).

Table 7-2. Correlation between *ABCB1/MDR1* gene DNA methylation and age in opioid exposed and opioid naïve individuals

	Opioid exposed				Opioid naïve			
	Blood	**Liver**	**Psoas**	**Thalamus**	**Blood**	**Liver**	**Psoas**	**Thalamus**
Pearson r	-.081	-.082	.212	.236	.038	nd	nd	nd
Sig. (2-tailed)	.619	.697	.279	.267	.827	nd	nd	nd
N	40	25	28	24	25	nd	nd	nd

7.3.1.2 CYP2D6 DNA methylation and age

Pearson correlation showed no relationship between age and opioid exposed *CYP2D6* gene methylation in any of the tissues investigated. Non-opioid exposed liver, muscle and thalamus sample *CYP2D6* gene methylation however were positively correlated with age according to Spearman's Rank correlation (Table 7-3; Figure 7-1).

Table 7-3. Correlation between *CYP2D6* gene DNA methylation and age in opioid exposed and opioid naïve individuals

	Opioid exposed				Opioid naïve			
	Blood	**Liver**	**Psoas**	**Thalamus**	**Blood**	**Liver**	**Psoas**	**Thalamus**
Pearson r	-.027	.025	.006	-.096	-.267	.636	.867	.830
Sig. (2-tailed)	.865	.902	.975	.641	.100	.048	.002	.003
N	42	27	27	26	39	10	9	10

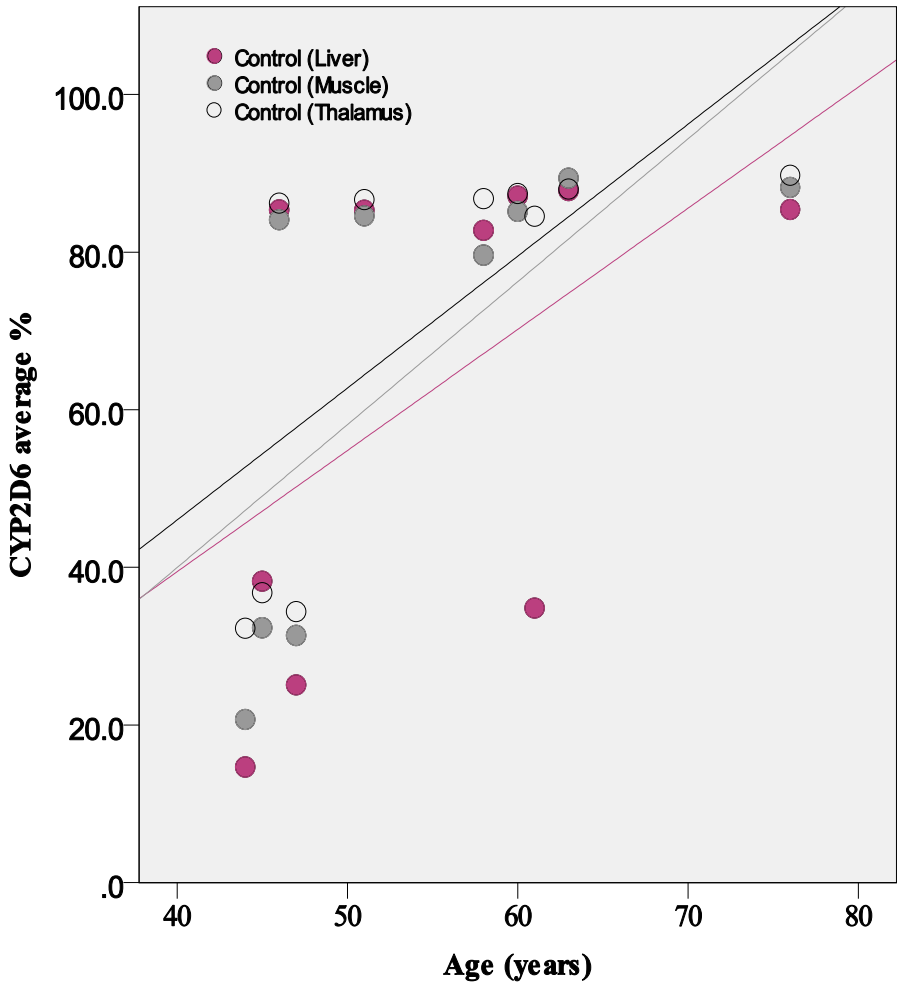

Figure 7-1. Relationship between *CYP2D6* methylation and liver, muscle and thalamus from non-opioid exposed samples
The average methylation (%) of the 3 CpG sites investigated in CYP2D6 of liver (pink filled circle), muscle (grey filled circle) and thalamus (white filled circle) samples were shown to be positively correlated with age, P = 0.048, 0.002 and 0.003 respectively.

7.3.1.3 OPRM1 DNA methylation and age

Utilising bivariate correlation age was compared to the methylation status of each gene (Table 7-4). Pearson's correlation showed that the average DNA methylation of *OPRM1* was positively correlated with age in thalamus of opioid exposed individuals (Figure 7-2). No other relationships were observed between age and *OPRM1* methylation in any other opioid exposed tissues. No relationship between age and *OPRM1* methylation in opioid naïve tissue samples were observed.

Table 7-4. Pearson correlation between *OPRM1* gene DNA methylation and age in opioid exposed and opioid naïve individuals

	Opioid exposed				Opioid naïve			
	Blood	**Liver**	**Psoas**	**Thalamus**	**Blood**	**Liver**	**Psoas**	**Thalamus**
Pearson r	.013	.324	.249	.588	.182	-,445	-.642	.674
Sig. (2-tailed)	.935	.141	.220	.003	.364	.230	.062	.056
N	39	22	26	24	27	9	9	9

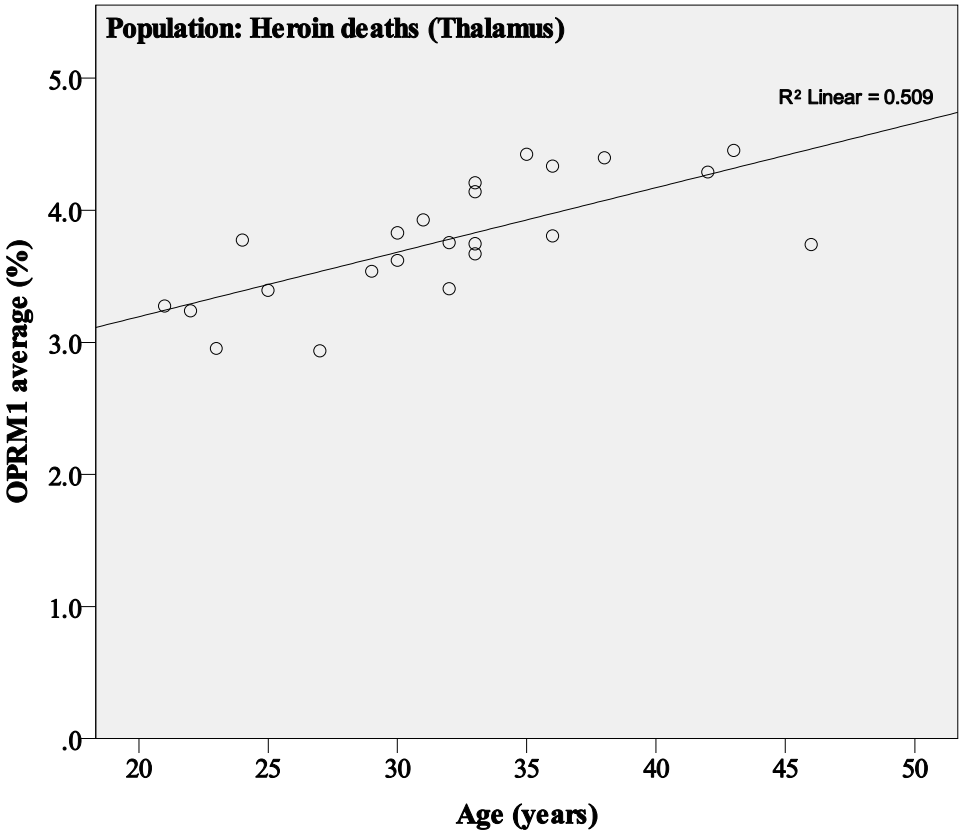

Figure 7-2. Relationship between age and *OPRM1* DNA methylation in thalamus samples of opioid exposed individuals
Each data point represents the age and average methylation of the 8 CpG sites investigated in *OPRM1* (%) of thalamus samples from individuals exposed to opioids (n=24). The regression line shows a positive correlation between age and *OPRM1* methylation (P = <0.003).

7.3.2 Gender and methylation

Independent-samples Mann-Whitney U tests were undertaken on the data represented in Table 7-5 and no relationship between gender and extent of methylation in *ABCB1/MDR1*, *CYP2D6* or *OPRM1* in any tissue investigated was found.

Table 7-5. Gender differences in *ABCB1/MDR1*, *CYP2D6* and *OPRM1* gene methylation in opioid exposed and opioid naïve populations

			Opioid exposed (n = 43)	Control – blood (n = 41)	Control – liver, muscle and thalamus (n = 10)
***ABCB1* median methylation**	**Blood**	**Male**	1.9 (1.2-3.2)	2.1 (1.5-5.7)	nd
		Female	2.0 (1.6-2.1)	3.1 (2.9-3.3)	nd
	Liver	**Male**	1.7 (1.1-3.4)	nd	nd
		Female	1.6 (1.2-2.7)	nd	nd
	Muscle	**Male**	1.7 (1.1-2.2)	nd	nd
		Female	1.8 (1.5-2.5)	nd	nd
	Thalamus	**Male**	2.3 (1.9-2.8)	nd	nd
		Female	2.1 (1.9-2.5)	nd	nd
***CYP2D6* average methylation**	**Blood**	**Male**	92.2 (89.6-94.1)	92.9 (85.7-94.9)	nd
		Female	93.0 (88.1-94.0)	92.0 (80.3-93.9)	nd
	Liver	**Male**	27.4 (9.2-77.9)	nd	85.3 (25.1-87.8)
		Female	22.6 (13.7-35.1)	nd	38.2 (14.7-85.4)
	Muscle	**Male**	84.8 (81.4-87.9)	nd	84.9 (31.4-89.4)
		Female	84.5 (83.0-85.7)	nd	32.3 (20.7-84.1)
	Thalamus	**Male**	87.9 (79.1-90.7)	nd	86.8 (34.4-89.7)
		Female	88.5 (83.7-91.6)	nd	36.8 (32.3-86.2)
***OPRM1* average methylation**	**Blood**	**Male**	8.0 (4.6-13.1)	9.9 (4.5-17.5)	nd
		Female	8.0 (6.3-9.3)	10.1 (8.6-19.7)	nd
	Liver	**Male**	7.8 (5.3-10.9)	nd	4.6 (3.4-8.1)
		Female	7.4 (5.9-9.9)	nd	7.2 (6.7-7.6)
	Muscle	**Male**	3.5 (3.0-4.0)	nd	4.1 (3.6-8.0)
		Female	3.6 (3.0-4.1)	nd	6.4 (5.2-6.6)
	Thalamus	**Male**	3.8 (2.9-4.5)	nd	5.6 (3.6-8.2)
		Female	3.8 (3.4-4.3)	nd	8.1 (3.6-5.6)

7.3.3 Intra-individual gene methylation tissue differences in opioid associated deaths

Blood, liver, muscle and thalamus obtained from individuals whose deaths were associated with opioid toxicity had tissue methylation differences for each gene investigated (Figure 7-3 to 7-7, paired t-tests undertaken). CpG sites within the promoter region of *ABCB1/MDR1* were statistically significantly more methylated in thalamus (2.3%) than liver (1.7%, $P = 0.004$) and muscle (1.7%, $P = <0.001$) (Figure 7-3).

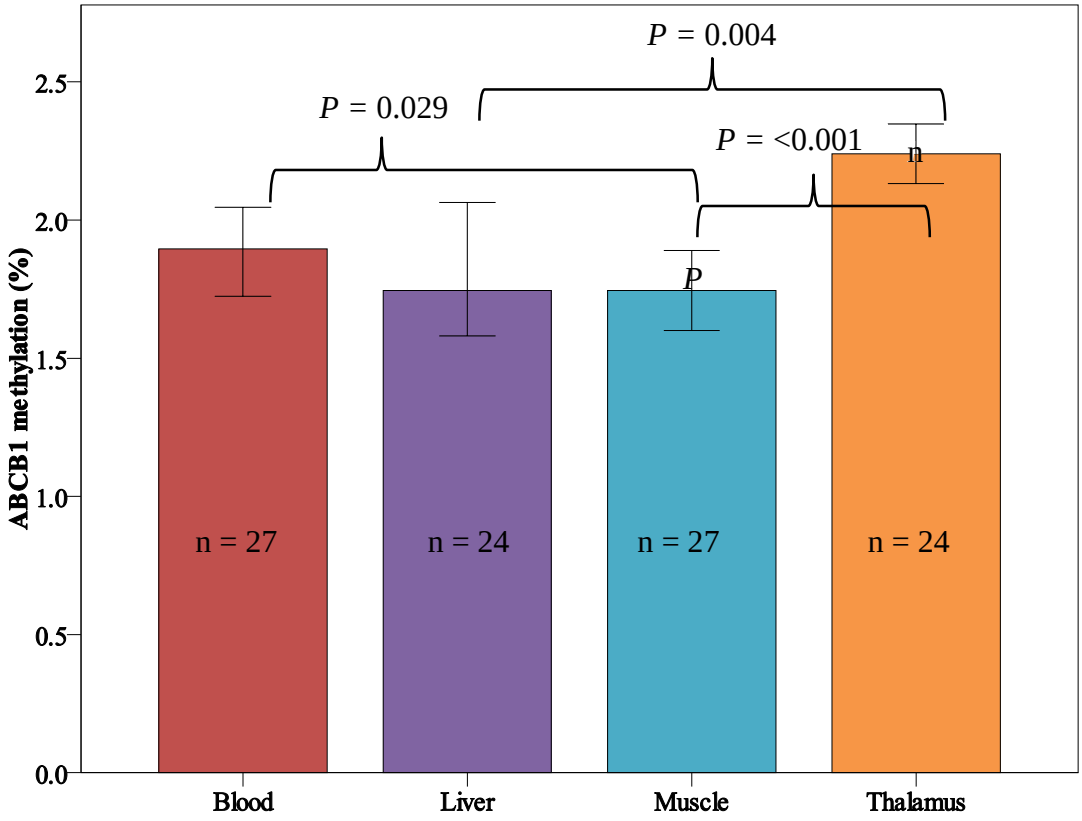

Figure 7-3. Median *ABCB1/MDR1* methylation (%) in blood, liver, muscle and thalamus obtained from opioid associated deaths
Median methylation scores and range of data points (error bars) were plotted as data had non-normal distribution. The brackets illustrate statistically significant methylation differences between paired tissue samples, e.g. the greatest *ABCB1/MDR1* methylation difference was observed between thalamus and blood samples, $P = <0.001$.

The methylation differences observed at the 3 CpG sites investigated in *CYP2D6* were significantly more emphasised (Figure 7-4). The median methylation of *CYP2D6* was 20% in the liver samples of opioid associated deaths which was statistically significantly lower than blood (93%), muscle (85%) and thalamus (88%), *P* = <0.001. In addition blood samples showed higher *CYP2D6* methylation (*P* = <0.001) whereas similar *CYP2D6* methylation was observed in muscle and thalamus obtained from the same individual.

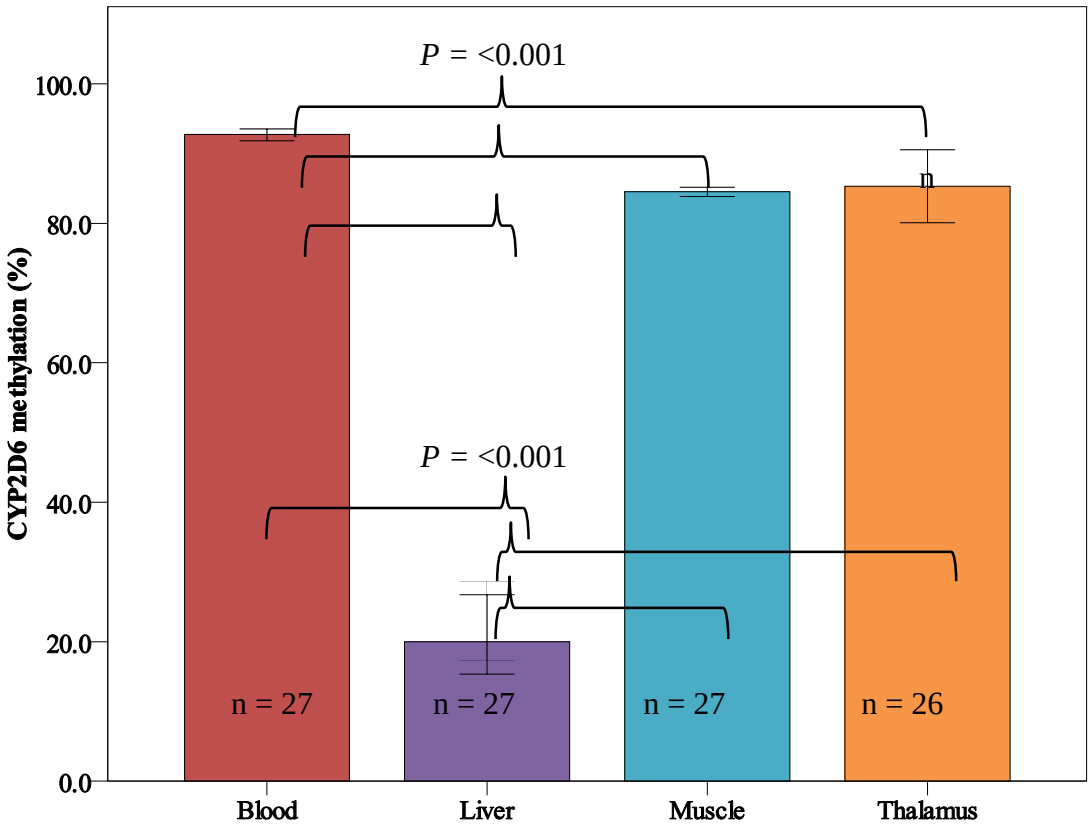

Figure 7-4. Average *CYP2D6* methylation (%) in blood, liver, muscle and thalamus obtained from opioid associated deaths
Bars represent the average methylation (%) of 3 CpG sites in *CYP2D6* in blood (red), liver (purple), muscle (blue) and thalamus (orange) samples. The error bars represent the 95%confidence interval. The brackets illustrate statistically significant methylation differences between paired tissue samples, e.g. the liver had less methylation than related blood, muscle and thalamus samples, *P* = <0.001.

Tissue methylation differences were also observed in *OPRM1*. Blood (8.2%) and liver (7.7%) samples taken from the same individual had similar methylation (Figure 7-5). The methylation observed in the blood and liver was statistically significantly higher than the methylation in muscle (3.5%) and thalamus (3.7%), P = <0.001.

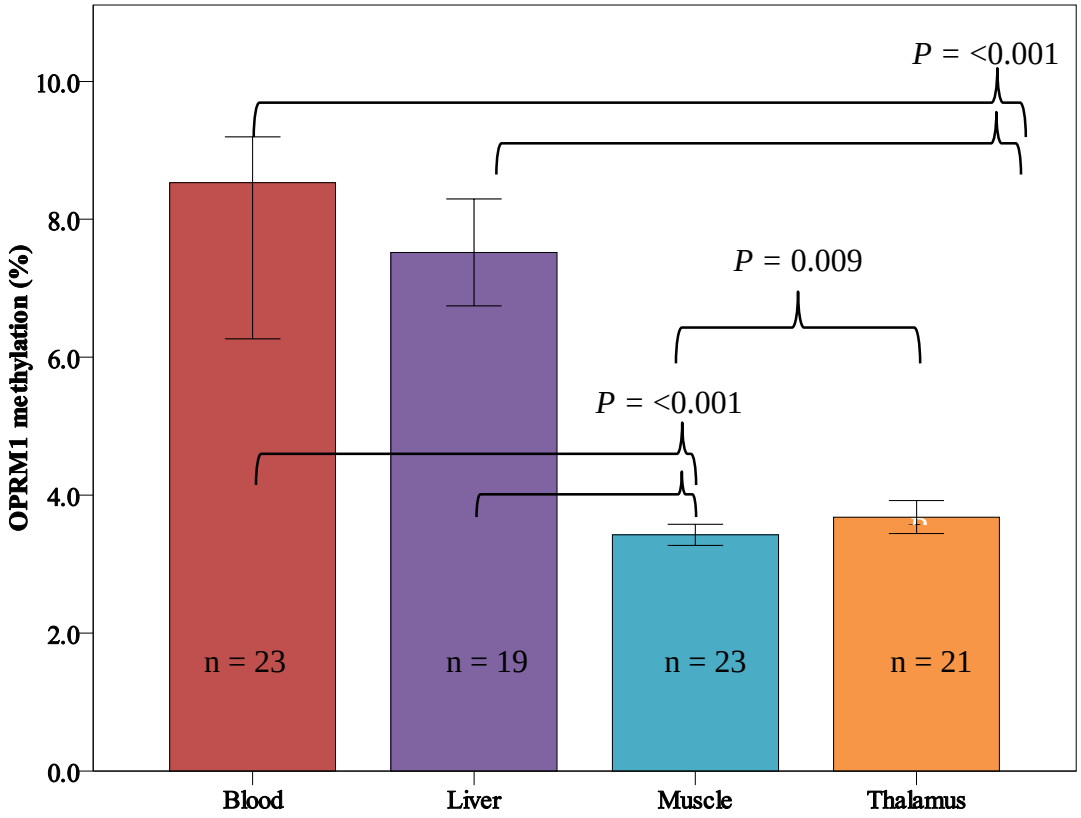

Figure 7-5. Average *OPRM1* methylation in blood, liver, muscle and thalamus obtained from opioid associated deaths
Bars represent the average methylation (%) of 8 CpG sites in *OPRM1* in blood (red), liver (purple), muscle (blue) and thalamus (orange) samples. The error bars represent the 95%confidence interval. The brackets illustrate statistically significant methylation differences between paired tissue samples, e.g. blood and liver samples had higher methylation than related muscle and thalamus samples, P = <0.001.

7.3.4 Intra-individual gene methylation tissue differences in controls

Unlike the tissues obtained from deaths associated with opioid toxicity, there was not a statistically significant difference with respect to *CYP2D6* methylation between liver, muscle or thalamus samples obtained from non-opioid associated deaths (Figure

7-6). There was also no tissue methylation differences observed in *OPRM1* (Figure 7-7).

As a consequence of time and cost restrictions, and availability of space on bisulfite conversion plates, methylation analysis of *ABCB1/MDR1* was not undertaken in the control tissues. As the methylation differences in opioid exposed samples were more prominent in *CYP2D6* and *OPRM1*, determining the methylation profile in these genes was prioritised over *ABCB1/MDR1* methylation.

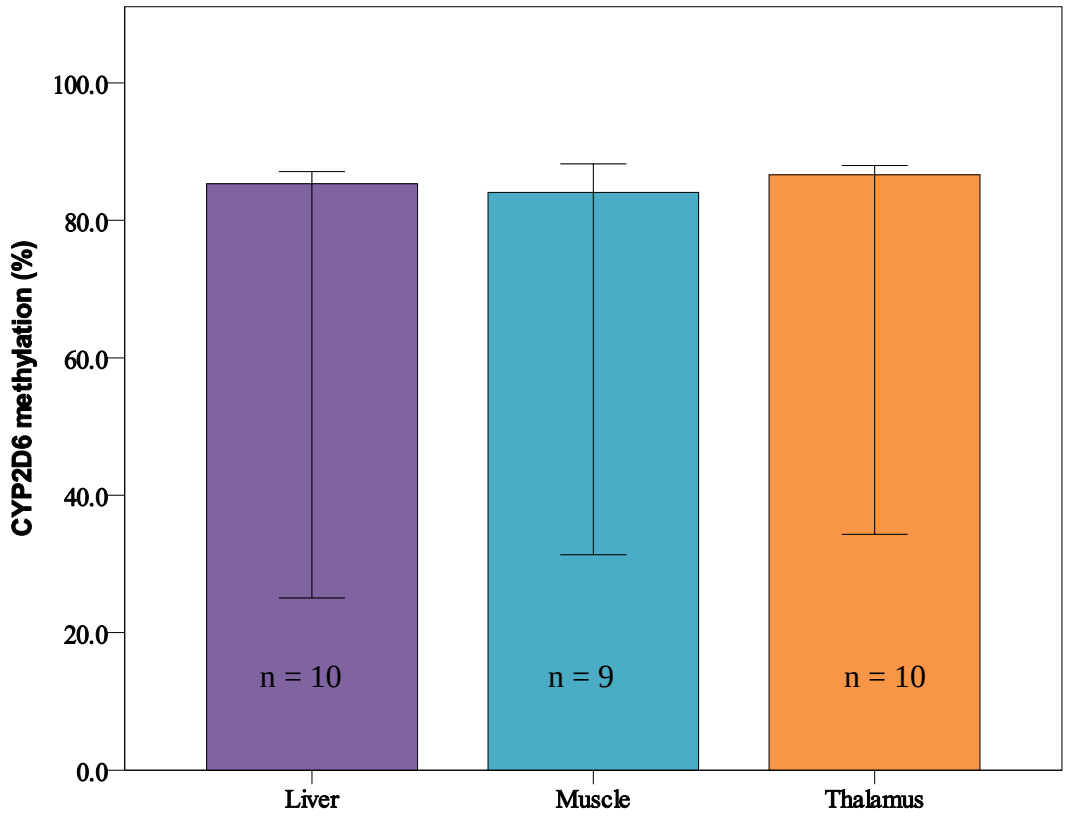

Figure 7-6. Median *CYP2D6* methylation (%) in liver, muscle and thalamus obtained from non-opioid associated deaths
Median methylation scores and range of data points (error bars) were plotted as data had non-normal distribution.

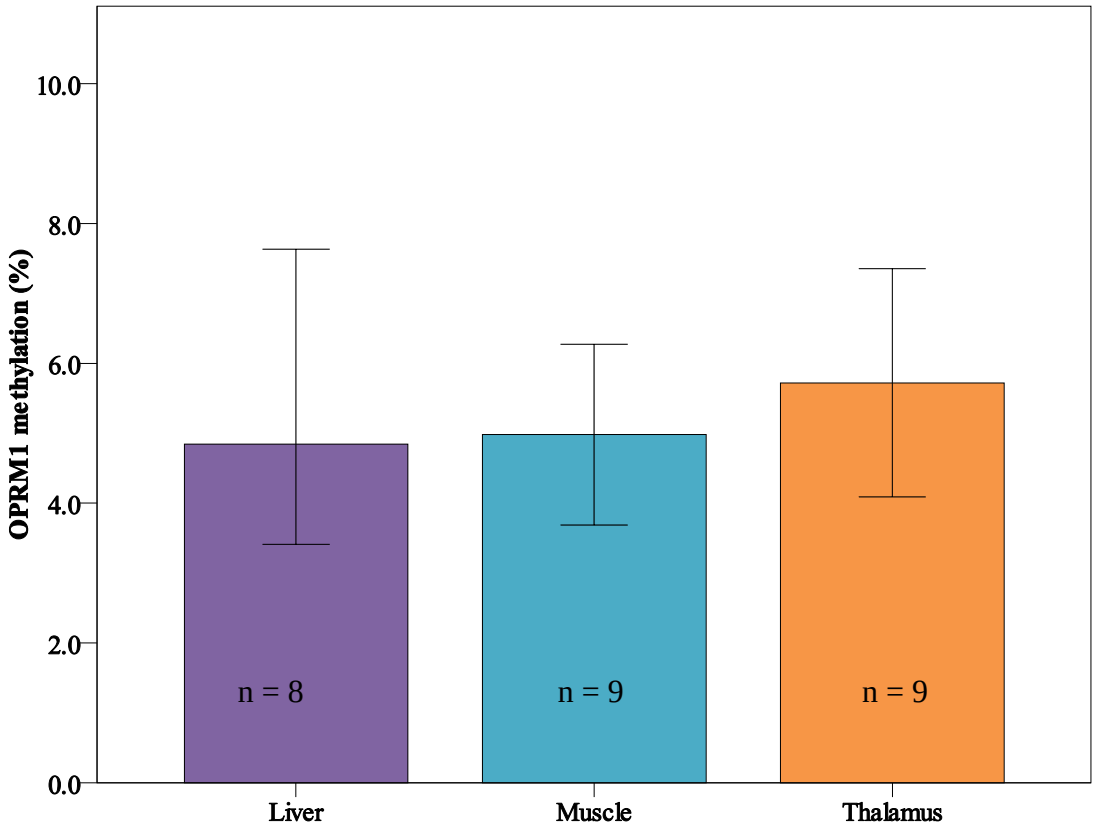

Figure 7-7. Average *OPRM1* methylation (%) in liver, muscle and thalamus obtained from non-opioid associated deaths
Bars represent the average methylation (%) of 8 CpG sites in *OPRM1* in liver (purple), muscle (blue) and thalamus (orange) samples. The error bars represent the 95%confidence interval.

7.3.5 Opioid exposed tissues v non-opioid exposed tissues

The degree of *CYP2D6* methylation in liver samples of opioid exposed individuals was statistically significantly lower than in liver samples of individuals whose deaths were non-opioid related (Figure 7-8, $P = 0.043$). Comparison of opioid and non-opioid exposed muscle and thalamus revealed no difference in *CYP2D6* methylation.

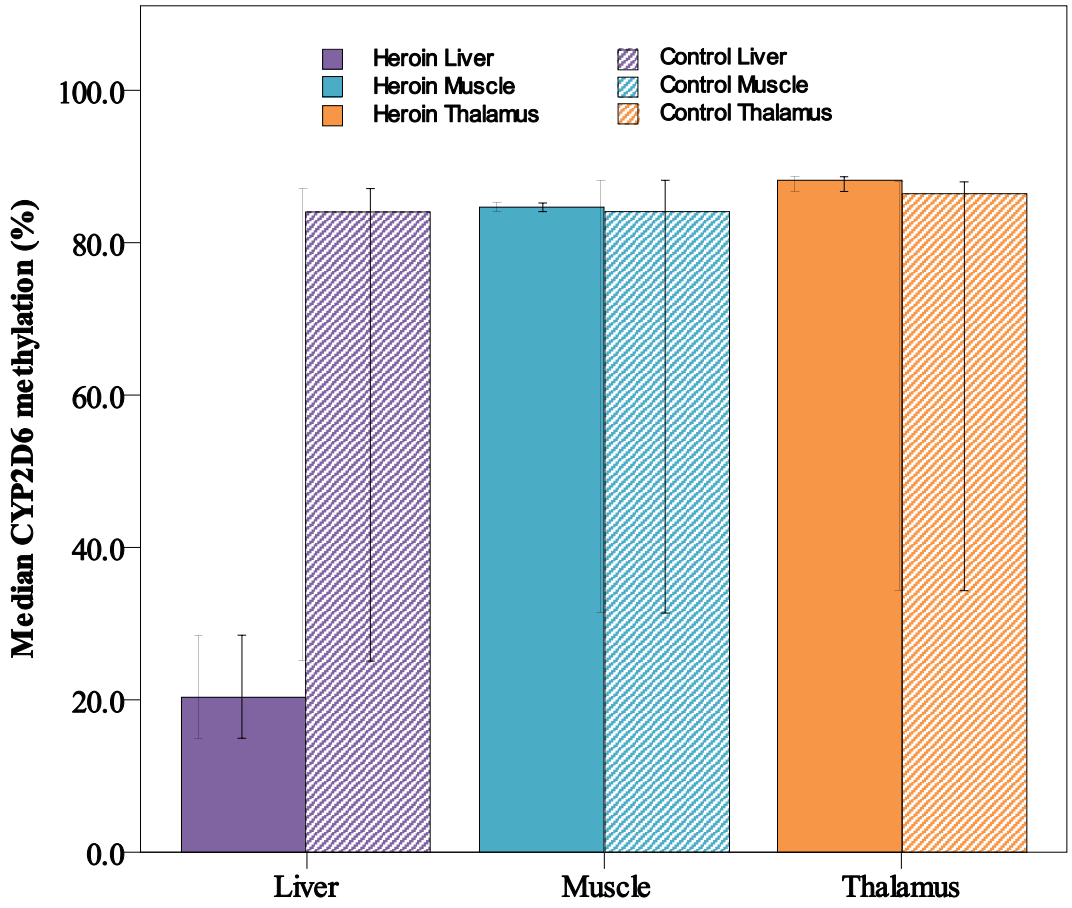

Figure 7-8. *CYP2D6* methylation (%) in liver, muscle and thalamus from opioid and non-opioid associated deaths
Median methylation scores and range of data points (error bars) were plotted as data had non-normal distribution. Opioid exposed liver samples (solid purple) were statistically significantly less methylated than opioid naïve liver samples (striped purple), $P = 0.043$.

Statistically significant *OPRM1* methylation differences were observed in each tissue between the opioid exposed population and non-opioid exposed population (Figure 7-9). Methylation was higher in opioid exposed liver samples compared to controls (7.6% v 5.6%, $P = 0.002$). The converse was seen for muscle and thalamus, the average methylation of *OPRM1* was lower in opioid exposed muscle (3.5% v 5.1%, $P = 0.012$) and thalamus (3.9% v 6.1%, $P = 0.015$) than the controls.

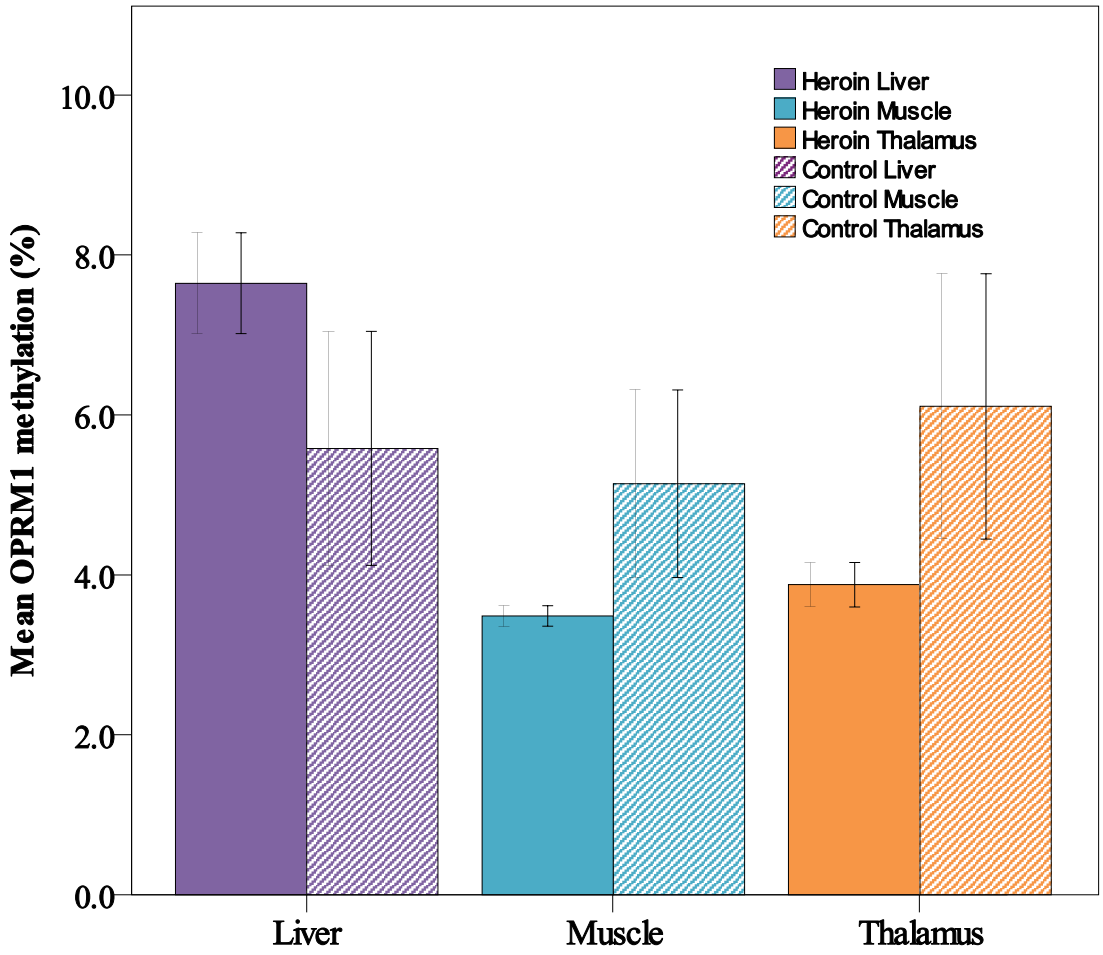

Figure 7-9. *OPRM1* methylation in liver, muscle and thalamus from opioid and non-opioid associated deaths
Bars represent the average methylation (%) of 8 CpG sites in *OPRM1* in opioid exposed (solid colour) and opioid naïve (striped) liver (purple), muscle (blue) and thalamus (orange) samples. The error bars represent the 95%confidence interval.

Methylation of *ABCB1/MDR1* and *OPRM1* in blood obtained from non-opioid exposed deaths was greater than *ABCB1/MDR1* and *OPRM1* methylation in opioid exposed blood samples (P = 0.009 and P = 0.009 respectively), Figure 7-10. There was no statistical difference in methylation of *CYP2D6* between opioid exposed individuals or non-opioid exposed blood samples.

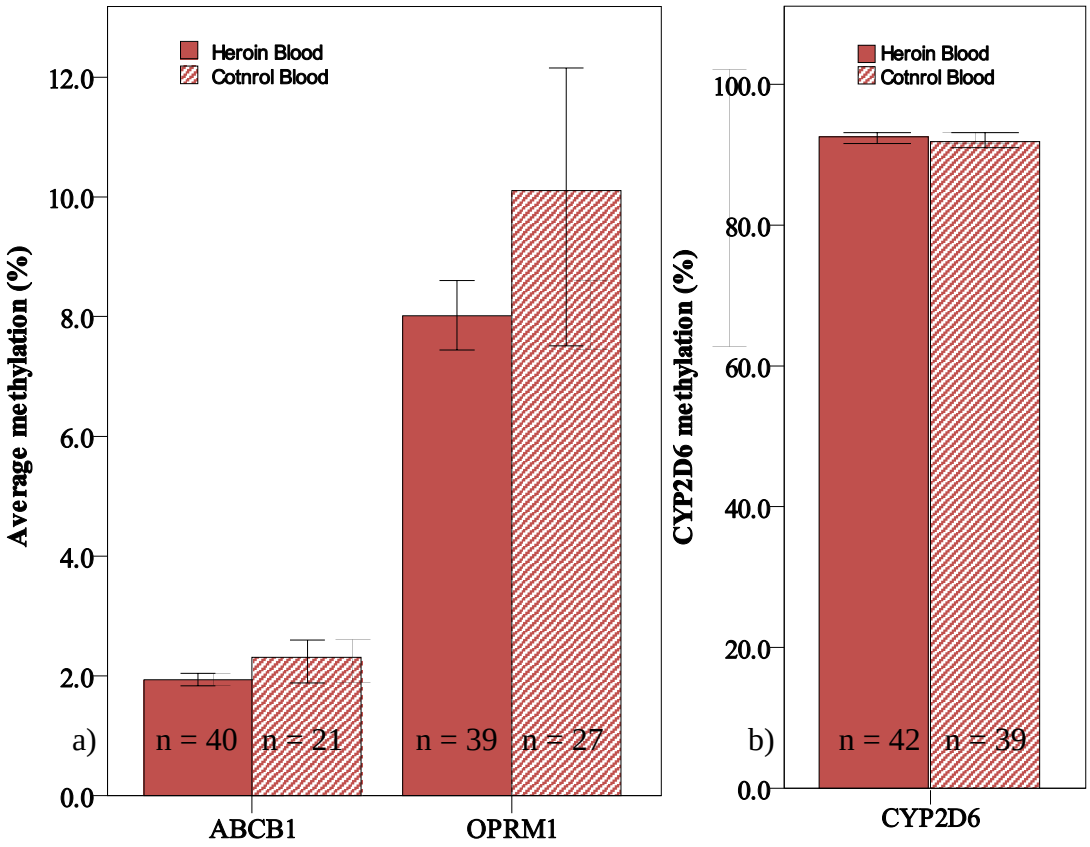

Figure 7-10. Average methylation (%) of a) *ABCB1*, *OPRM1* and b) *CYP2D6* in blood obtained from opioid exposed and non-opioid exposed donors
Bars represent the average methylation (%) of 11 CpG sites in *ABCB1*, 8 CpG sites in *OPRM1* and 3 CpG sites in *CYP2D6* in opioid exposed (solid red) and opioid naïve (striped red) blood samples. The error bars represent the 95% confidence interval.

7.3.6 Genetic variations v blood drug concentrations

Morphine was detected in 39 of the 43 heroin attributed deaths at concentrations ranging from 10 – 1350 ng/mL (median 160 ng/mL). Analysis for metabolites of morphine was also undertaken. Morphine-3-glucuronide (M3G) was detected at concentrations of 10 – 1120 ng/mL (median 140 ng/mL) in 42 of the blood samples, and morphine-3-glucuronide (M6G) concentrations ranged from 10 – 660 ng/mL (median 40 ng/mL) in 32 blood samples. Independent samples t-test showed that the only difference between morphine / metabolite concentrations and genotype was observed in the *ABCB1/MDR1* transport protein (Table 7-6). Individuals carrying the

TT genotype at rs1128503 and rs1128503 had lower morphine:M3G (transformed using LG10 to obtain normal distribution) than individuals with either CC/CT (P = 0.004) and GG/GT (P = 0.001), respectively.

Table 7-6. Average morphine and metabolite concentrations in different genotypes

			MOR	M3G	M6G	MOR:M3G	MOR:M6G	M3G:M6G
ABCB1	rs1128503	TT	177.3	250.0	59.0	0.6*	3.4	5.67
		CC/CT	240.0	249.7	80.5	2.5*	6.1	4.8
	rs2032582	TT	138.0	229.1	55.6	0.5*	3.0	5.7
		GG/GT	251.4	257.3	80.9	2.5*	6.1	4.8
	rs1045642	TT	229.2	209.3	56.4	1.0	5.2	5.3
		CC/CT	218.8	270.7	82.9	2.4	5.3	4.9
COMT	rs4633 TT	TT	222.5	312.2	141.4	1.3	2.7	5.1
		CC/CT	222.3	232.2	54.8	2.1	6.0	5.0
	rs4680	AA	222.5	312.2	141.4	1.3	2.7	5.1
		GG/GA	222.3	232.2	54.8	2.1	6.0	5.0
	rs4818	CC	213.3	273.1	108.2	2.5	4.3	4.5
		GG/GC	226.3	238.9	55.7	1.7	5.8	5.4
	rs6269	AA	213.3	273.1	108.2	2.5	4.3	4.5
		GG/GA	226.3	238.9	55.7	1.7	5.8	5.4
CYP2B6	rs3745274	GG	251.8	246.7	95.6	1.5	4.1	5.1
		TT/TG	177.3	252.7	55.7	2.3	5.8	4.4
	rs2279343	AA	238.1	208.7	54.0	1.9	5.4	5.9
		GG/GA	203.9	302.2	91.2	2.0	5.1	4.3
CYP2D6	<2 functioning alleles		396.7	443.3	243.3	1.2	3.2	2.8
	≥2 functioning alleles		207.8	234.5	56.2	2.0	5.5	5.3

*Statistical differences observed (Independent t-test, P = <0 .05). MOR = morphine; M3G = morphine-3-glucuronide; M6G = morphine-6-glucuronide; MOR:M3G, MOR:M6G, M3G:M6G = drug concentration ratio between MOR and M3G, MOR and M6G, and M3G and M6G, respectively.

The relationships between drug concentrations and extent of *ABCB1/MDR1*, *CYP2D6* and *OPRM1* methylation in each tissue is shown in Table 7-7. The methylation of *OPRM1* positively correlated with MOR:M3G ratio (following Log10 transformation as a result of non-normal distribution), P = 0.030 .437 significant at the 0.01 level (2-tailed)**. *CYP2D6* methylation positively correlated with M3G concentration (P = 0.021), and negatively correlated with MOR:M3G (P = 0.030, -.454*) and M3G:M6G

(*P* = 0.021, -.479*). *OPRM1* methylation negatively correlated with M3G concentration (*P* = 0.035), and positively correlated with MOR:M3G (*P* = 0.015, .510) and M3G:M6G (*P* = 0.015, .510*).

Table 7-7. Morphine and metabolite concentrations vs tissue specific methylation

		MOR+	M3G+	M6G++	MOR:M3G++	MOR:M6G+	M3G:M6G
***ABCB1* methylation**	Blood+	.122 .477 36	-.182 .266 39	.021 .912 29	.301 .079 35	.143 .460 29	.294 .087 35
	Liver++	-.184 .413 22	-.042 .846 24	-.097 .711 17	-.138 .522 21	-.352 .166 17	-.040 .862 21
	Muscle	-.205 .325 25	-.369 .058 27	-.181 .459 19	.109 .613 24	.210 .388 19	-.028 .898 24
	Thalamus	.035 .877 22	.098 .655 23	-.298 .246 17	-.256 .262 21	.093 .724 17	-.293 .197 21
***CYP2D6* methylation**	Blood+-	.054 .750 37	-.051 .753 40	.288 .123 30	.132 .441 36	-.058 .763 30	.012 .945 36
	Liver++	.120 .586 23	.179 .381 26	.405 .095 18	-.276 .213 22	-.279 .262 18	-.193 .390 22
	Muscle	.159 .457 24	.451* .021 26	.318 .198 18	-.454* .030 23	-.075 .769 18	-.479* .021 23
	Thalamus	-.140 .534 22	-.045 .836 24	-.318 .214 17	-.116 .617 21	.207 .425 17	-.224 .329 21
***OPRM1* methylation**	Blood	.234 .169 36	-.262 .112 38	.103 .587 30	.437** .009 35	.330 .075 30	.267 .109 35
	Liver	-.077 .755 19	-.263 .249 21	.339 .236 14	.016 .949 18	-.229 .431 14	.160 .525 18
	Muscle	.046 .835 23	-.423* .035 25	.062 .808 18	.510* .015 22	.254 .309 18	.510* .015 22
	Thalamus	-.087 .715 20	-.297 .179 22	-.204 .466 15	.120 .623 19	.242 .385 15	-.139 .571 19

+SQRT transformations; +-negative SQRT; ++LG10; *significant at the 0.05 level (2-tailed); **significant at the .01 level (2-tailed). MOR = morphine; M3G = morphine-3-glucuronide; M6G = morphine-6-glucuronide; MOR:M3G, MOR:M6G, M3G:M6G = drug concentration ratio between MOR and M3G, MOR and M6G, and M3G and M6G, respectively.

7.4 Discussion

Intra-individual tissue specific differences were observed in *ABCB1/MDR1*, *CYP2D6* and *OPRM1* of tissue samples obtained from opioid exposed individuals. The most prominent tissue methylation difference was observed on the *CYP2D6* gene. *CYP2D6,* which is an important metabolising enzyme, was statistically significantly less methylated in the liver samples of opioid exposed individuals than related blood, muscle or thalamus samples (P = <0.001). The lower *CYP2D6* methylation in muscle and thalamus was expected as the liver is the primary organ for drug detoxification so protein expression is required for drug metabolism. The explanation for this difference may be as follows; tissue specific differentially methylated regions have been observed in genome wide analyses (Zhang *et al.* 2013; Lokk *et al.* 2014).

Promoter regions of genes associated with tissue specific function are hypomethylated, for example genes involved with blood vessel development and morphogenesis were hypomethylated in arteries, and immune response and leukocyte activation genes were hypomethylated in tonsil tissue (Lokk *et al.* 2014). Liver samples from controls did not have lower *CYP2D6* methylation than related muscle and thalamus samples, but were significantly more methylated than drug exposed liver samples (P = 0.002). The lower *CYP2D6* methylation in drug exposed individuals may be the body's response to adapt to drug exposures. *CYP2D6* accounts for 2% of the hepatic cytochrome P450 content (Shimada *et al.* 1994), and is fundamental in the metabolism of a wide variety of drugs and endogenous compounds (Wang *et al.* 2013; Ravindranath 2014).

The individuals whose deaths were attributed to opioid toxicity did not only have morphine and metabolites in their systems, but also a whole host of xenobiotic substances that are metabolised by *CYP2D6*. The lower methylation of *CYP2D6* may

result in increased expression of metabolising enzymes to enable the metabolism of a range of xenobiotic substances that the opioid dependant individuals consumed. Alternatively the lower methylation of *CYP2D6* in the opioid exposed population could be a result of age. The control population had a median age of 55, whereas the population of opioid exposed individuals had a median age of 35 (P = 0.001). A positive relationship was observed between control liver, muscle and thalamus *CYP2D6* methylation and age (P = 0.048, 0.002 and 0.003 respectively). However, the control group of consisted of only 10 individuals spanning an age range of ~30 years so a larger population is required to corroborate the *CYP2D6* methylation-age relationship.

Despite the age disparity and differences in drug exposures *CYP2D6* methylation in blood of controls and opioid users was similar. *CYP2D6* was hypermethylated and as drug metabolism by *CYP2D6* does not occur in the blood higher gene methylation was expected compared to liver methylation. Previous research has shown that *CYP2D6* expression is not only apparent in the liver but also in the brain in a region- and cell-specific manner, the extent of methylation is induced by a variety of drugs and toxins (Miksys *et al.* 2002; Miksys and Tyndale 2004; Mann *et al.* 2008; Ferguson and Tyndale 2011). The variable expression of *CYP2D6* in the brain has been suggested to be a cause for inter-individual variation in response to centrally acting drugs (Zhou *et al.* 2013). *CYP2D6* expression has been observed in the frontal cortex, cerebellum, hippocampus, hypothalamus and thalamus (Miksys *et al.* 2002; Kircheiner *et al.* 2011; Mann *et al.* 2012). In this study, the three CpG sites investigated in *CYP2D6* in thalamus were hypermethylated in opioid exposed samples. The hypermethylation of *CYP2D6* in opioid exposed thalamus samples suggest that expression is inhibited reducing the functional role of *CYP2D6* within the brain.

7.4.1 Methylation of *ABCB1/MDR1* gene

The methylation difference observed in *ABCB1/MDR1* between the different tissues was not as prominent as *CYP2D6* methylation. Thalamus exhibited statistically significantly higher methylation than liver and muscle (P = 0.004 and <0.001 respectively); however the eleven CpG sites investigated had an average methylation of only 2%. The hypomethylation suggests that the *ABCB1/MDR1* would be expressed in each of the tissues investigated therefore transporting xenobiotics away from tissues back into the blood (Choudhuri and Klaassen 2006).

ABCB1/MDR1 transcription site hypomethylation has been observed in normal (non-diseased) breast, brain and pancreatic tissue (Dejeux *et al.* 2010; Muggerud *et al.* 2010; Chen *et al.* 2012; Oberstadt *et al.* 2013). In diseased tissue *ABCB1/MDR1* methylation of up to 85% has been reported and this increased methylation has been postulated to be a cause of drug resistance development (Andersson *et al.* 2003; Lu and Shervington 2008; Dejeux *et al.* 2010; Muggerud *et al.* 2010; Oberstadt *et al.* 2013). *ABCB1/MDR1* expression can influence drug resistance as the transporter alters the delivery of drugs to target tissues therefore affecting the bioavailability, distribution and excretion of drugs (Choudhuri and Klaassen 2006). Absence or inhibition of the *ABCB1/MDR1* transporter results in drug accumulation within tissues (Lankas *et al.* 1997; Borst and Elferink 2002). Therefore, the *ABCB1/MDR1* hypomethylation in the liver, muscle and thalamus of opioid exposed population suggests that the *ABCB1/MDR1* transporter is expressed and drugs are transported back into the bloodstream protecting vital organs.

Control *ABCB1/MDR1* methylation data was obtained from blood samples. *ABCB1/MDR1* methylation in blood samples from controls was significantly higher than in the opioid exposed samples (P = 0.009). As the control population were ~20

years older than the opioid exposed population (mean age 56 v 35 years old, P = <0.001) the increased methylation is postulated to be an artefact of aging.

7.4.2 Methylation of *OPRM1* gene

The methylation on *OPRM1* of opioid exposed samples was significantly greater in blood and liver samples than muscle and thalamus (P = <0.001). As opioids exert their effects on the central nervous system hypomethylation of *OPRM1* that may enable expression of the mu-opioid receptor in the thalamus samples was not unexpected. In comparison to the control thalamus samples, the opioid exposed thalamus were less methylated on *OPRM1* (3.9% v 6.1%, P = 0.015) that may result in greater expression of the mu-opioid receptor. Our data suggests that the lower methylation of the receptor within the opioid exposed thalamus samples compared to the controls may be a result of age. *OPRM1* methylation was positively correlated with age in the opioid exposed thalamus samples (P = <0.0005, Figure 7-2).

No age relationship was observed in the control thalamus samples but this could be a population size affect as only 10 control individuals were analysed. The control population had a median age 20 years older than the opioid exposed population *OPRM1* and age (P = <0.001). Therefore the lower *OPRM1* methylation in opioid exposed thalamus may be the influence of age rather than an adaptive response to opioid exposure. The relationship between age and *OPRM1* methylation in thalamus has not yet been reported. CpG site specific methylation increases in frontal cortex, temporal cortex, pons and cerebellum have been reported with age (Hernandez *et al.* 2011) as has the relationship between age and methylation markers within blood (Hannum *et al.* 2013). Increased methylation was observed in genes associated with DNA-binding factors and transcription factors (Hernandez *et al.* 2011) suggesting that

age related methylation changes are associated with maintenance of transcriptional programs.

7.5 Conclusion

Tissue specific methylation differences of *ABCB1/MDR1*, *CYP2D6* and *OPRM1* were observed within the blood, liver, muscle and thalamus of opioid exposed individuals. The gene methylation differences appear to be related to the function of the tissue investigated, e.g. the metabolising enzyme *CYP2D6* was less methylated in the liver, the primary site of drug detoxification. The same methylation differences were not observed for *CYP2D6* and *OPRM1* in liver, muscle and thalamus from the ten control individuals. As a result of the small population and wide age range of the control population the tissue specific methylation differences that were observed in the opioid exposed population may have been masked. Additionally, although the control population were known not to be chronic opioid users a record of drug history was not obtained.

The *ABCB1/MDR1*, *CYP2D6* and *OPRM1* methylation differences observed between the control and opioid exposed populations are likely to be explained by age rather than drug exposure. Previous studies investigating the methylation differences of *OPRM1* in sperm and lymphocytes of opioid exposed and controls found that methylation was higher in the opioid exposed individuals. For this study to be comparable an age matched control population of similar size should be recruited.

To further the work, mRNA expression studies should be undertaken to determine the functional consequences of the methylation differences observed between the different tissues as well as between the opioid exposed and control populations.

8.0 PILOT TRIAL: PERSONALISING OPIOID THERAPY FOR CANCER PAIN RELIEF. The influence of DNA methylation and SNPs on opioid response

8.1 Introduction

At present cancer pain management involves careful titration of an initial opioid (usually morphine) according to response (analgesia and side-effects), opioid switching if necessary, and further titration of the alternative opioid. There is currently no way of prospectively predicting an individual's response to opioids for cancer pain. In many instances patients experience significant pain and / or side-effects during analgesic stabilisation which is a process based largely on trial and error.

In terms of cancer pain, the development of a model incorporating clinical, genetic, epigenetic and metabolite data may potentially allow prospective prediction of response to different opioid drugs. Personalised prescribing involving choosing the correct dose of the correct opioid for each individual patient would result in more rapid pain management, reduced side-effects and potentially a better quality of life. The studies published to date have not provided any data that can be used in such modelling. Therefore we hypothesised that the epigenetic mechanism DNA methylation, in addition to genetic polymorphisms in opioid related genes, influence response to morphine and oxycodone.

8.2 Materials and methods

8.2.1 Sample collection

Participants were recruited as described in *3.1.4*. Plasma and blood samples were collected as detailed in *3.2.4*.

8.2.2 Toxicological analysis

Blood samples were analysed for morphine, M3G, M6G, oxycodone, noroxycodone and oxymorphone following the method described in Chapter *3.3.3*.

8.2.3 SNP and gene duplication / deletion analysis

SNPs and *CYP2D6* deletions and duplications were determined following the protocol detailed in *3.4*.

8.2.4 DNA methylation analysis

Analysis of promoter region methylation of *ABCB1/MDR1*, *CYP2D6* and *OPRM1* was undertaken as described in *3.5*.

8.3 Results

In total, 147 patients were recruited into the study (Figure 8-1). The recruited population were largely Caucasian (93%), had an average age of 61 years (±13 years) and consisted of 69 males and 78 females. The smoking status of the Royal Marsden patients was unknown; of the remaining 18 patients 13 had never smoked, two were former smokers and three participants smoked >20 cigarettes per week.

The participants were diagnosed with cancers including breast, upper and lower gastrointestinal, sarcoma, lung, gynaecological, urinary tract, pancreas and hepatobiliary, haematological, prostate, malignant melanoma, and head and neck. Opioids were prescribed most frequently to alleviate somatic bony pain (41%), defined as a dull ache or tender hot spot, followed by somatic pain (37%), sharp pain, burning or prickling on the body surface or deep tissue; and finally visceral pain (22%), pressure-like or squeezing pain, poorly localised.

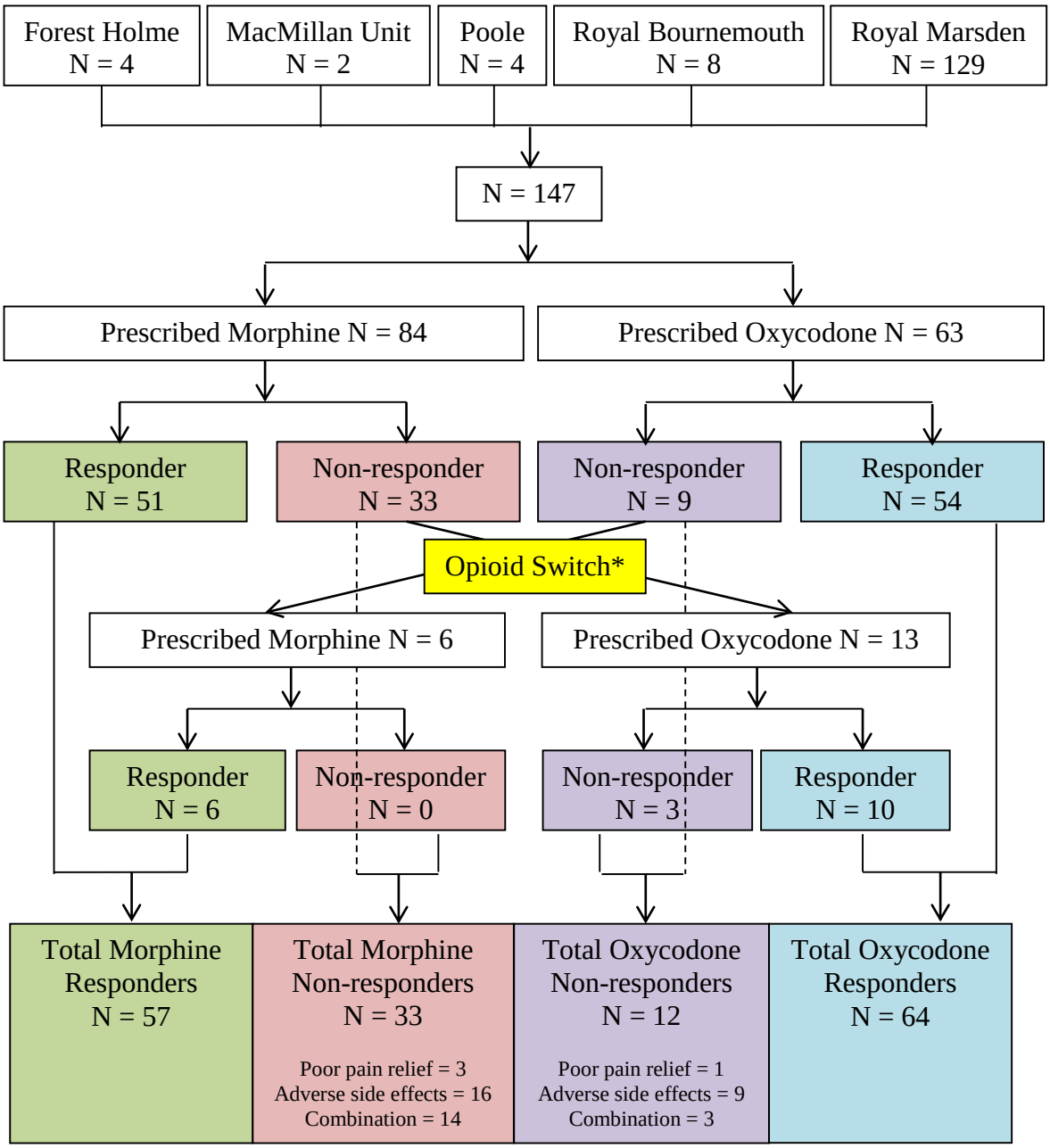

Figure 8-1. Responders and non-responders to morphine and oxycodone in an opioid naïve cancer population

*Non-responders switched to alternative opioid, be it the alternative study opioid or a non-study opioid.

Morphine was the first-line opioid for 84 participants (Figure 8-1); 51 responded well to the morphine reporting pain scores of ≤4 and no / mild side effects (e.g. RB5 Figure 8-2 and 8-3), however 33 were deemed to be non-responders based upon their questionnaire responses. For example RB1 obtained inadequate pain relief (Figure 8-2), MU1 obtained inadequate pain relief and adverse side effects (Figure 8-2 and 8-3

respectively), and RB4 obtained adequate analgesia but suffered from intolerable drowsiness (Figure 8-2 and 8-3 respectively). Oxycodone was prescribed to 13 of the morphine non-responders and 10 obtained adequate analgesia with no / acceptable side effects. The remaining morphine non-responders were switched to alternative non-study opioids.

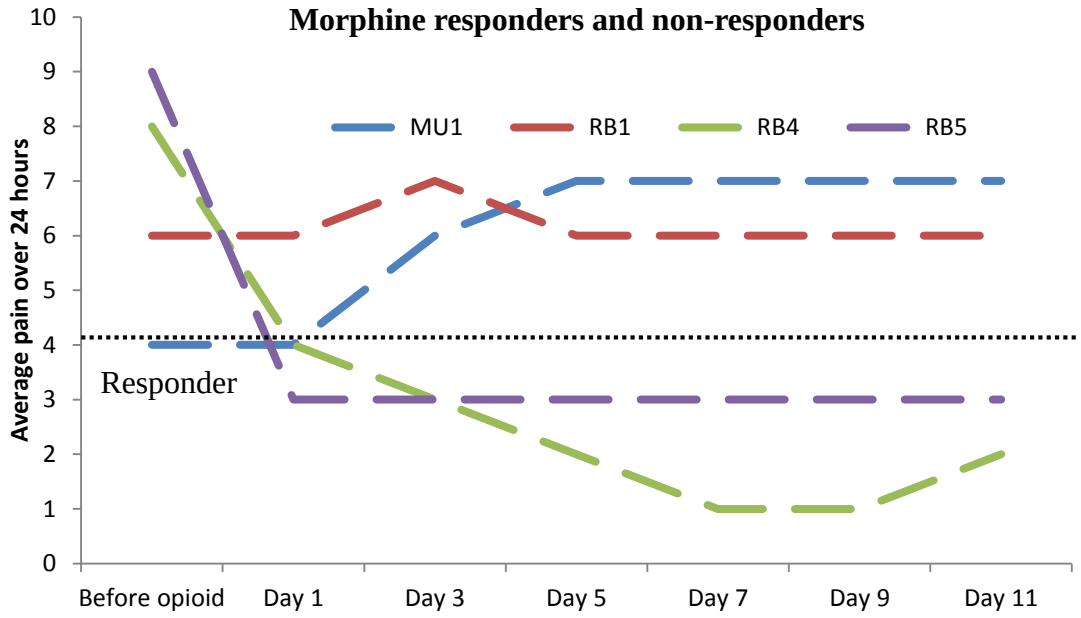

Figure 8-2. Average pain score / 24 hours over an 11 day period of morphine responders and non-responders
RB4 and RB5 were considered non-responders to morphine as their average pain score was below 4 on the 11 point likert scale.

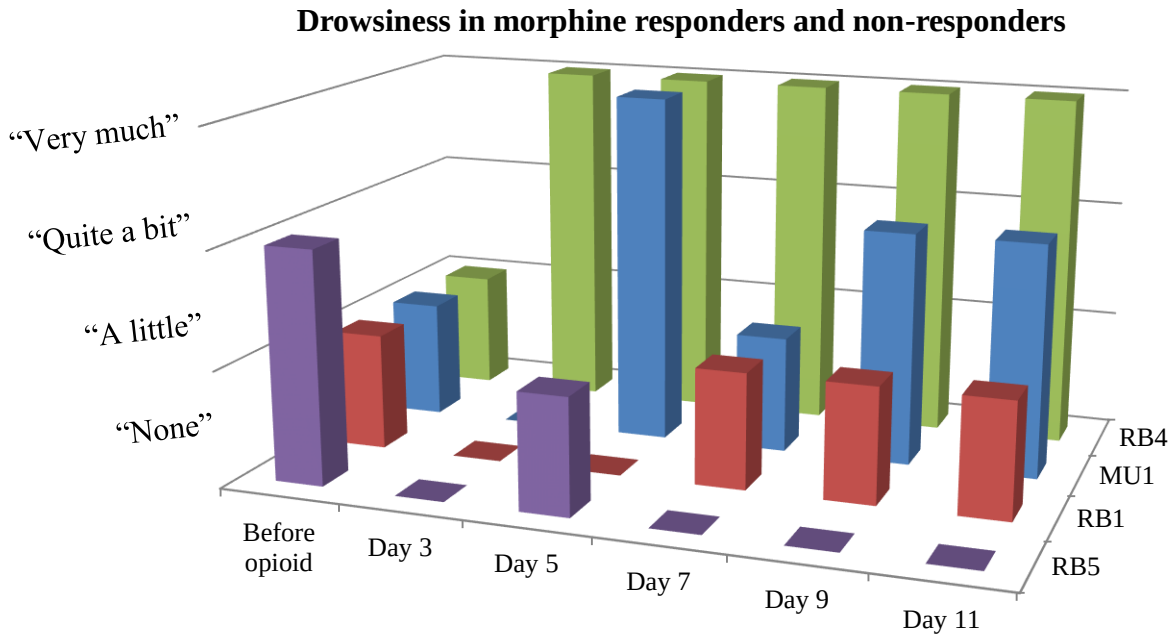

Figure 8-3. Drowsiness in morphine responders and non-responders over study period
MU1 and RB4 were considered morphine non-responders as they reported drowsiness values of "quite a bit" and "very much".

Oxycodone was prescribed as the first-line opioid to 63 patients and 54 obtained adequate pain relief and tolerable side effects, RB7 for example (Figure 8-4 and 8-5 respectively). The nine oxycodone non-responders were classified as non-responders as a result of inadequate pain relief (e.g. FH2 Figure 8-4), inadequate pain relief and intolerable side effects (e.g. RB8 Figure 8-4 and 8-5 respectively) or adequate pain relief but intolerable side effects (e.g. MU2 Figure 8-4 and 8-5 respectively). Six of the oxycodone non-responders were switched to morphine and obtained suitable analgesia and no / minimal side effects. The remaining 3 oxycodone non-responders were switched to non-study opioids.

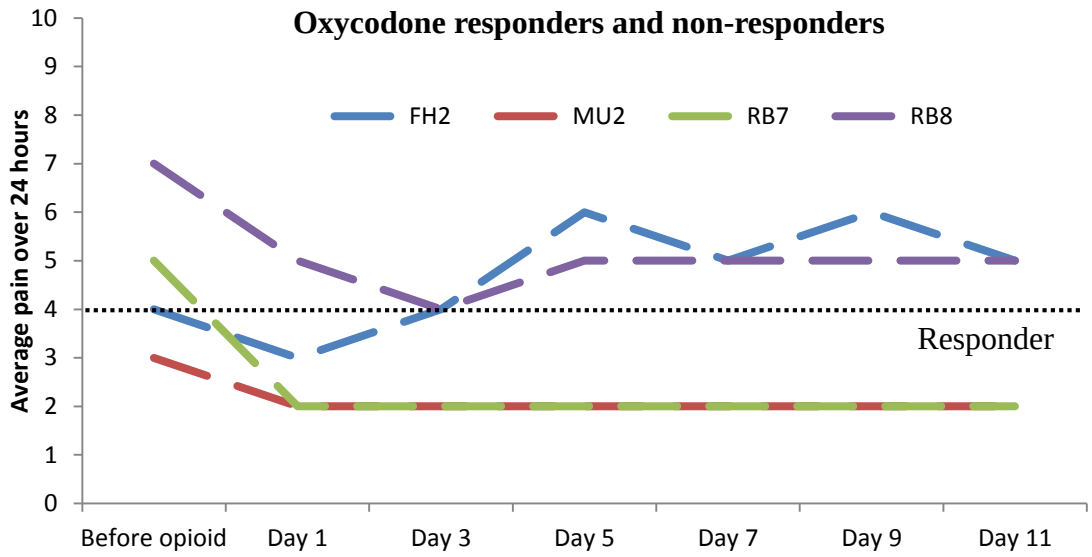

Figure 8-4. Average pain score / 24 hours over an 11 day period of oxycodone responders and non-responders
RB7 and MU2 were considered non-responders to oxycodone as their average pain score was below 4 on the 11 point likert scale.

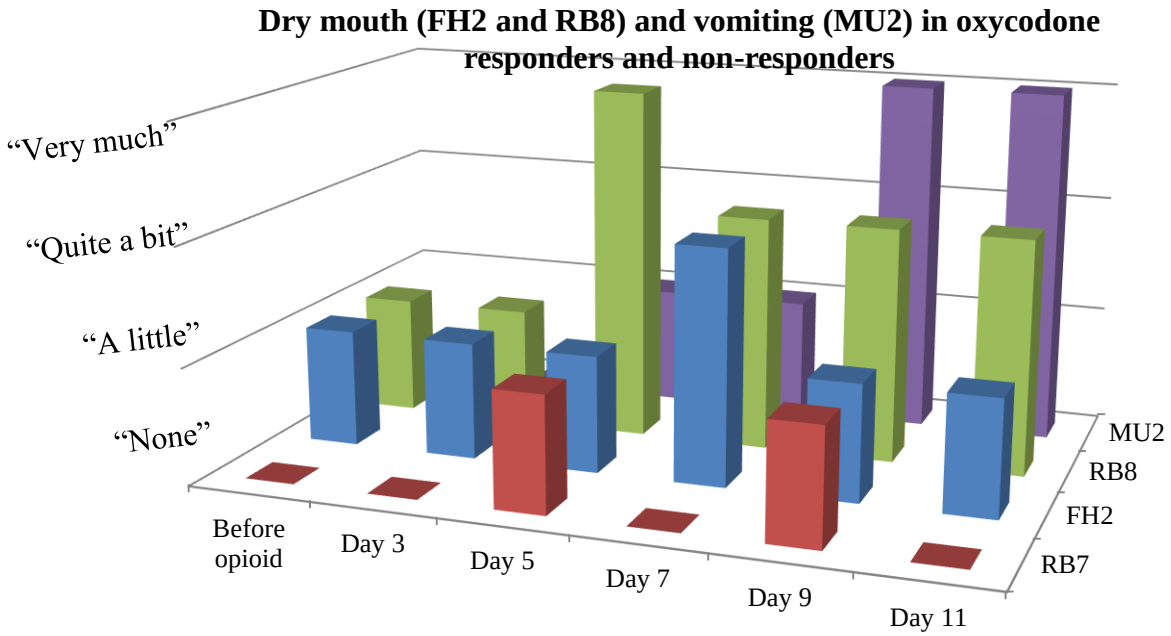

Figure 8-5. Dry mouth and vomiting in oxycodone responders and non-responders over study period
MU2 and RB8 were considered morphine non-responders as they reported dry mouth values of "quite a bit" and "very much".

8.3.1 Methylation

8.3.1.1 Paired tissue gene DNA methylation comparison

Paired blood and buccal samples were obtained from 17 cancer patients obtained from Forest Holme, Poole Hospital, MacMillan Unit and Royal Bournemouth hospital (Figure 8-1) (1 patient from this group of hospitals did not provide an oral swab). The paired samples t-test showed that blood and buccal swab DNA had statistically significantly different methylation in *ABCB1/MDR1* and *OPRM1* (Table 8-1, *P* = <0.001 and 0.013, respectively) whereas no difference was observed in *CYP2D6* (*P* = 0.351). There was no correlation between blood and buccal swab methylation obtained from the same individual in any of the genes investigated.

Table 8-1. Relationship between gene DNA methylation in paired blood and buccal samples

		N	Mean SD	*P (2-tailed)*	Correlation
ABCB1/MDR1	**Blood**	15	2.4 ±.35	*<0.001*	-.206
	Buccal		12.8 ±5.0		
CYP2D6	**Blood**	17	92.1 ±1.03	*0.351*	.118
	Buccal		91.6 ±1.76		
OPRM1	**Blood**	11	2.5 ±.33	*0.013*	-.400
	Buccal		3.2 ±.58		

8.3.1.2 Gene DNA methylation and effect of age in morphine / oxycodone responders and non-responders

Methylation data was not obtained from all recruited cancer patients because, a) plasma samples were completely consumed during toxicology analyses, b) low concentrations of DNA in plasma samples, and c) DNA was absent in plasma sample as a result of repeat freeze-thaw cycling. As such 46 patient blood or plasma samples were analysed for *ABCB1/MDR1* methylation, 44 for *CYP2D6* and 27 for *OPRM1*

methylation (Table 8-2). *OPRM1* methylation in blood was positively correlated with age (.515**, P = 0.006) as determined by Spearman's rho. The strength of this relationship was much more evident in the morphine responder group and non-responder group (Table 8-2, Figure 8-6) than oxycodone exposed patients. No relationships between age and *ABCB1/MDR1* and *CYP2D6* methylation were observed.

Of the 17 buccal swabs obtained, *CYP2D6* methylation was obtained from all 17 participants, 16 for *OPRM1* and 11 for *ABCB1/MDR1* (Table 8-2). With the exception of the negative relationship between *CYP2D6* methylation in buccal swabs of morphine responders and age (Table 8-2) there were no correlations between age and buccal gene DNA methylation.

Table 8-2. Relationship between age and gene blood and buccal DNA methylation in opioid responders and non-responders

		ABCB1/MDR1	***CYP2D6***	***OPRM1***
Blood	**Pearson r**	-.015	-.064	.515**
	***P* (2-tailed)**	.921	.678	.006
	N	46	44	27
Morphine responder	**Pearson r**	-.045	.041	.745**
	***P* (2-tailed)**	.855	.881	.008
	N	19	16	11
Morphine non-responder	**Pearson r**	-.167	.160	.704**
	***P* (2-tailed)**	.569	.602	.007
	N	14	13	13
Oxycodone responder	**Pearson r**	.030	.054	-.085
	***P* (2-tailed)**	.912	.848	.893
	N	16	15	5
Oxycodone non-responder	**Pearson r**	.130	-.265	.792
	***P* (2-tailed)**	.836	.612	.060
	N	5	6	6
Buccal	**Pearson r**	.113	-.248	.047
	***P* (2-tailed)**	.741	.337	.862
	N	11	17	16
Morphine responder	**Pearson r**	.510	-.788*	.120
	***P* (2-tailed)**	.490	.035	.797
	N	4	7	7
Morphine non-responder	**Pearson r**	-.091	.402	.122
	***P* (2-tailed)**	.865	.323	.794
	N	6	8	7

* Correlation is significant at the 0.05 level (2-tailed); ** Correlation is significant at the 0.01 level (2-tailed)

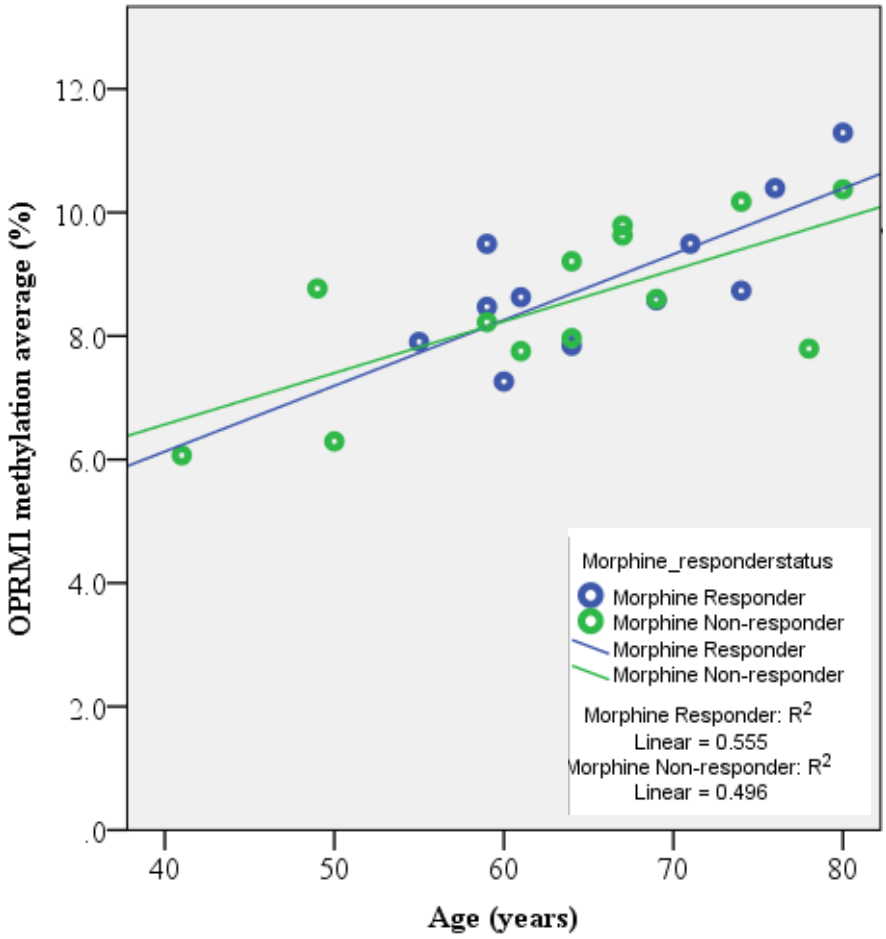

Figure 8-6. Relationship between age and *OPRM1* methylation in morphine responders and non-responders

8.3.2 SNP and gene duplication / deletion analysis

8.3.2.1 Morphine responders v morphine non-responders

Age, dose, drug concentrations and methylation of *ABCB1/MDR1*, *CYP2D6* and *OPRM1* did not differ between morphine responders and non-responders (Table 8-3). A statistically significant difference was observed between *OPRM1* 118AA and 118AG/GG and morphine responder status (Figure 8-7). Morphine responders were more likely to carry a variant allele at *OPRM1* 118A>G than non-responders (P = 0.048).

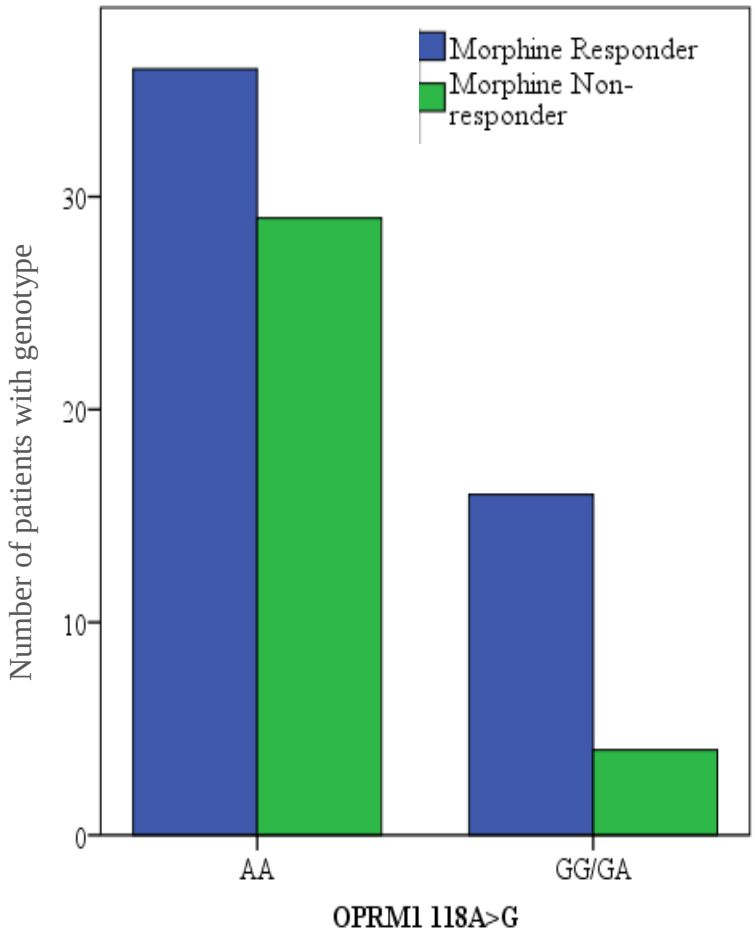

Figure 8-6. *OPRM1* 118AA v AG/GG genotype in morphine responders and non-responders

Table 8-2. Gender, age, dose, drug concentration, genotype and methylation differences between morphine responders and morphine non-responders

	Responders	Non-responders	*P*
Gender, male / female	27 / 30	18 / 15	*0.512*
Age, years	60 ±11	62 ±12	*0.396*
Dose, median in mg (range)	86 (15-240)	88 (20-360)	*0.525*
MOR*	0.41 ±0.14 (n=35)	0.46 ±0.17 (n=15)	*0.237*
M3G*	3.10 ±0.85 (n=41)	3.07 ±1.69 (n=21)	*0.935*
M6G*	1.15 ±0.34 (n=40)	1.16 ±0.64 (n=20)	*0.939*
MOR:M3G*	0.13 ±0.04 (n=35)	0.13 ±0.05 (n=15)	*0.806*
MOR:M6G**	-0.94 ±0.36 (n=35)	-0.93 ±0.34 (n=15)	*0.929*
M3G:M6G, median (range)	7.35 (4.58-33.13) (n=40)	8.64 (4.38-30.59) (n=20)	*0.442*
***ABCB1/MDR1* 1236 CC, CT/TT**	16, 32	12, 19	*0.626*
***ABCB1/MDR1* 2677 GG, GT/TT**	16, 33	13, 20	*0.531*
***ABCB1/MDR1* 3435 CC, CT/TT**	11, 41	7, 26	*0.995*
***COMT* 62 CC, CT/TT**	14, 35	9, 21	*0.892*
***COMT* 158 GG, GA/AA**	13, 34	8, 21	*0.994*
***COMT* rs4818 GG, GC/CC**	9, 42	7, 24	*0.585*
***COMT* rs6269 GG, GA/AA**	9, 42	6, 24	*0.792*
***CYP2B6* 516 GG, GT/TT**	26, 20	18, 12	*0.764*
***CYP2B6* 785 AA, AG/GG**	20, 25	15, 15	*0.637*
***CYP2D6* 1846 GG/GA, AA**	18, 0	11, 2	*0.085*
***OPRM1* 118 AA, AG/GG**	36, 16	29, 4	*0.048*
***CYP2D6* *1/*1, *1XN**	4, 3	3, 1	*0.554*
Blood *ABCB1/MDR1 methylation %, SD (n)**	1.8 ±0.6 (n=19)	1.7 ±0.5 (n=14)	*0.481*
Blood *CYP2D6* methylation %, SD (n)	88.1 ±4.4 (n=16)	90.9 ±2.8 (n=13)	*0.057*
Blood *OPRM1* methylation %, SD (n)	8.9 ±1.2 (n=11)	8.5 ±1.4 (n=13)	*0.446*
Buccal ABCB1*/MDR1* methylation	3.5 ±0.23 (n=4)	3.1 ±0 .72 (n=6)	*0.610*
Buccal *CYP2D6* methylation %, SD (n)	90.9 ±2.31 (n=7)	92.1 ±1.21 (n=8)	*0.281*
Buccal *OPRM1* methylation %, SD (n)	11.9 ±5.25 (n=7)	12.6 ±4.93 (n=7)	*0.805*

* = SQRT transformation; ** = LG10 transformation; t-tests were undertaken for normally distributed data; Mann-Whitney U for non-normal distribution; Pearson chi square for comparing ordinal data. MOR = morphine; M3G = morphine-3-glucuronide; M6G = morphine-6-glucuronide; MOR:M3G, MOR:M6G, M3G:M6G = drug concentration ratio between MOR and M3G, MOR and M6G, and M3G and M6G, respectively.

The frequency of *ABCB1/MDR1*, *COMT*, *CYP2B6* and *CYP2D6* haplotypes in morphine responders and non-responders did not statistically significantly differ (Table 8-4).

Table 8-3. Frequency of haplotypes in morphine responders and non-responders

		Responders	**Non-responders**	***P***
CYP2D6	Decreased *4/*4; *4/*4XN; *4/*6	0	2	.172
	Normal *1/*1; *1/*3; *1/*4; *1/*5; *1/*6	48	29	
	Increased *1/*1XN; *1XN/*4	3	1	
ABCB1/MDR1	Homozygous wild type 1236CC, 2677GG, 3435CC	10	5	.482
	Variant 1236CT/TT, 2677GT/TT, 3435CT/TT	34	26	
CYP2B6	Wild type 516GG, 785AA	20	14	.882
	Variant 516GT/TT, 785AG/GG	20	13	
COMT	Wild type 472AA, 408CC, -98AA	14	9	.895
	Variant 472AG/GG, 408CG/GG, -98AG/GG	30	18	

8.3.2.2 Oxycodone responders vs oxycodone non-responders

Age, dose, drug concentrations and methylation of *ABCB1/MDR1*, *CYP2D6* and *OPRM1* did not differ between oxycodone responders and non-responders (Table 8-5). Statistically significant differences were observed between three SNPs and oxycodone responder status, *ABCB1/MDR1* 1236C>T and 3435C>T, and *CYP2B6* 516G>T (Figure 8-8). Oxycodone responders were more likely to carry a variant allele at *ABCB1/MDR1* 1236C>T and 3435C>T than non-responders, whereas the variant allele at *CYP2B6* 516G>T was more prominent in the non-responder group ($P = 0.037$, 0.013 and 0.003 respectively).

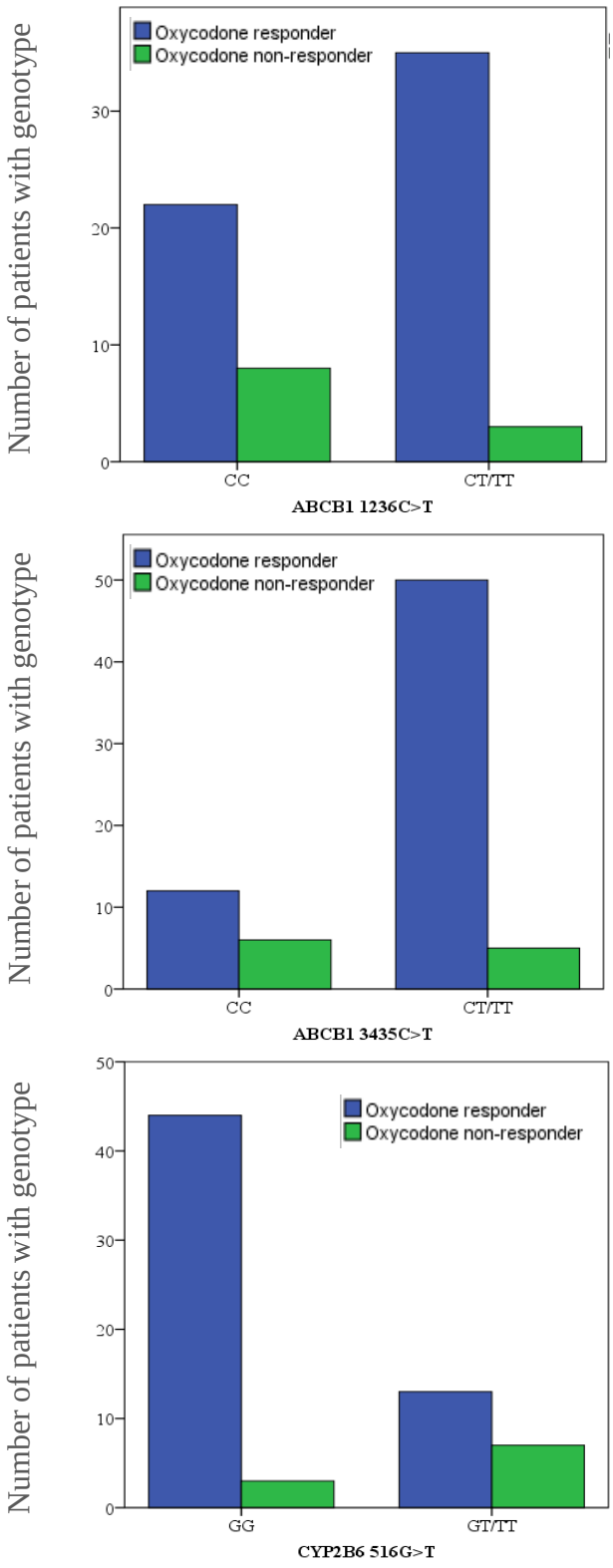

Figure 8-7. a) *ABCB1/MDR1* 1236 CC v CT/TT, b) *ABCB1/MDR1* 3435 CC v CT/TT and c) *CYP2B6* GG v GT/TT genotype in oxycodone responders and non-responders

Table 8-4. Gender, age, dose, drug concentration, genotype and methylation differences between oxycodone responders and oxycodone non-responders

	Responders	Non-responders	*P*
Gender, male / female	29, 35	7, 5	*0.407*
Age, years	62 ±13	61 ±12	*0.856*
Dose*	1.7 ±0.28	1.9 ±.043	*0.128*
Oxy	0.73 ±0.38 (n=45)	0.65 ±.60 (n=6)	*0.640*
nOxy	0.60 ±0.37 (n=45)	0.47 ±0.41 (n=6)	*0.429*
OxyMor*	-1.66 ±0.21 (n=7)	-1.91 (n=1)	*0.312*
Oxy:nOxy*	0.11 ±0.25 (n=45)	0.09 ±.21	*0.744*
Oxy:OxyMor	36.55 ±20.44 (n=7)	50.76	*0.540*
nOxy:OxyMor	27.71 ±20.80 (n=7)	21.05	*0.775*
***ABCB1/MDR1* 1236 CC, CT/TT**	22, 35	8, 3	*0.037*
***ABCB1/MDR1* 2677 GG, GT/TT**	23, 39	7, 4	*0.099*
***ABCB1/MDR1* 3435 CC, CT/TT**	12, 50	6, 5	*0.013*
***COMT* 62 CC, CT/TT**	16, 40	3, 8	*0.930*
***COMT* 158 GG, GA/AA**	12, 39	2, 8	*0.808*
***COMT* rs4818 GG, GC/CC**	10, 46	2, 10	*0.922*
***COMT* rs6269 GG, GA/AA**	9, 50	1, 10	*0.592*
***CYP2B6* 516 GG, GT/TT**	44, 13	3, 7	*0.003*
***CYP2B6* 785 AA, AG/GG**	30, 19	3, 7	*0.070*
***OPRM1* 118 AA, AG/GG**	42, 20	7, 4	*0.789*
***CYP2D6* *1/*1, *1XN**	44, 10	7, 3	*0.407*
Blood *ABCB1/MDR1 methylation %, SD (n)**	3.2 ±1.3 (n=16)	4.5 ±4.1 (n=4)	*0.578*
Blood *CYP2D6* methylation %, SD (n)	87.6 ±4.8 (n=15)	84.7 ±11.1 (n=5)	*0.592*
Blood *OPRM1* methylation %, SD (n)	10.4 ±2.6 (n=5)	8.4 ±1.7 (n=5)	*0.259*
Buccal ABCB1/*MDR1* methylation	2.4 ±0.38 (n=2)	2.8 ±0.47 (n=2)	*0.700*
Buccal *CYP2D6* methylation %, SD (n)	92.3 ±0.52 (n=3)	92.4 ±0.56 (n=3)	*1.000*
Buccal *OPRM1* methylation %, SD (n)	16.2 ±0.94 (n=3)	9.2 ±0.05 (n=2)	*0.200*

***** lg10 transformation; t-tests were undertaken for normally distributed data; Mann Whitney U for non-normal distribution; pearson chi square for comparing ordinal data; Oxy = oxycodone; nOxy = noroxycodone; OxyMor = oxymorphone.

The statistically significant difference between oxycodone responders and non-responders observed in *ABCB1/MDR1* at a single SNP level remained significant during haplotype analysis (P = 0.010, Table 8-6). However, there was no significant difference between homozygous wild type *CYP2B6* 516GG and 785AA haplotype versus 516GT/TT and 785AG/GG haplotype in oxycodone responders and non-responders (Table 8-6).

Table 8-5. Frequency of haplotypes in oxycodone responders and non-responders

		Responders	**Non-responders**	***P***
CYP2D6	Decreased *4/*4; *4/*4XN; *4/*6	0	0	0.530
	Normal *1/*1; *1/*3; *1/*4; *1/*5; *1/*6	53	10	
	Increased *1/*1XN; *1XN/*4	10	3	
ABCB1/MDR1	Homozygous wild type 1236CC, 2677GG, 3435CC	10	6	0.010
	Variant 1236CT/TT, 2677GT/TT, 3435CT/TT	45	5	
CYP2B6	Wild type 516GG, 785AA	29	4	0.207
	Variant 516GT/TT, 785AG/GG	18	6	
COMT	Wild type 472AA, 408CC, -98AA	15	3	0.700
	Variant 472AG/GG, 408CG/GG, -98AG/GG	30	8	

8.4 Discussion

No differences were found in promoter region methylation of *ABCB1/MDR1*, *CYP2D6* and *OPRM1* in blood of morphine / oxycodone responders or non-responders. This is the first study to investigate methylation of opioid related genes in relation to response to either morphine or oxycodone in a cancer population. Although there were no methylation differences between opioid responders and non-responders, the 118A>G polymorphism in *OPRM1* was found to be associated with morphine responder status; and polymorphisms in *ABCB1/MDR1* (1236C>T and 3435C>T) and *CYP2B6* (516G>T) were associated with oxycodone responder status. The statistical differences observed in *ABCB1/MDR1* of oxycodone responders and non-responders remained significant when comparing homozygous wild type at 1236C>T and 2677G>T and 3435C>T to carriers of the variant allele haplotype ($p = .010$).

The data indicates that the variant allele *OPRM1* 118G was more prevalent in morphine responders than non-responders ($P = 0.048$) which is contrary to the existing research. Previous studies have shown that carriers of a mutated allele at 118A>G obtain less pain relief and require higher doses of morphine to achieve analgesia than homozygous wild type counterparts (Klepstad *et al.* 2004; Reyes-Gibby *et al.* 2007; Campa *et al.* 2008; Tan *et al.* 2009; Sia *et al.* 2008, 2013). Carriers of the mutated allele are postulated to have reduced sensitivity to opioids as a result of, 1) the loss of a putative glycosylation site, and 2) the creation of an additional CpG site susceptible to DNA methylation. The loss of a glycosylation site and possible methylation within exon 1 are thought to reduce receptor signalling efficacy and receptor expression (Mestek *et al.* 1995; Kroslak 2007; Oertel 2009, 2012; Mura 2013). However, the reduced response to opioids was not replicated by other chronic pain studies (Janicki *et al.* 2006; Lotsch *et al.* 2009; Klepstad *et al.* 2011) and was not apparent in our study

population. The lack of reproducibility between studies may be a result of sample size as well as confounding factors such as gene-gene and gene-environment interactions, differences in pain perception, environmental influences, multiple comorbidities and polydrug use (Chou *et al.* 2006a; Likar *et al.* 2008).

Heterozygous or homozygous carriers of the polymorphism at *ABCB1/MDR1* 1236C>T, 2677G>T and 3435C>T were more likely to respond to oxycodone than homozygous wild type. Variable doses and responses to opioids in conjunction with these polymorphisms have been previously reported. Doses of methadone and morphine were higher in carriers of 1236CC, 2677GG and / or 3435CC (Coller *et al.* 2006; Lotsch *et al.* 2009; Barratt *et al.* 2012; Bastami *et al.* 2014). The *ABCB1/MDR1* wild type genotypes have been also associated with adverse side effects following morphine consumption compared to carriers of heterozygous or homozygous mutations (Coulbault *et al.* 2006; Fujita *et al.* 2010).

In relation to analgesic response, individuals with 3435TT genotype obtained an increased analgesic effect to morphine (Campa *et al.* 2008). This effect was not evident in our morphine responder / non-responder population and was also not replicated by Sia *et al.* (2010) who found no association between *ABCB1/MDR1* polymorphisms and morphine consumption, pain scores or side effects. In addition Zwisler *et al.* (2009; 2012) reported no association between *ABCB1/MDR1* 2677G>T and 3435C>T polymorphism and response to oxycodone in a population of postoperative patients. However in a population of 33 healthy patients exposed to experimental pain, better antinociception and less adverse side effects were experienced in individuals carrying a variant allele at 2677G>T and 3435C>T (Zwisler *et al.* 2010).

The studies to date concerning the influence of *ABCB1/MDR1* polymorphisms on expression and function remain inconclusive as a result of conflicting findings (Fung and Gottesman 2009). For example, Hoffmeyer *et al.* (2000) and Kim *et al.* (2001) observed decreased expression of *ABCB1/MDR1* in individuals carrying the T allele at 3435C>T and 2677G>T, respectively. However the expression of *ABCB1/MDR1* was found to be increased in carriers of the T allele in a Japanese and Caucasian population (Sakaeda *et al.* 2002; Siegmund *et al.* 2002). Research undertaken by Wang *et al.* (2005b) concluded that 3435T was associated with mRNA stability as transcripts carrying the T allele degraded faster than wild type transcripts. But this finding was not supported by other researchers (Kimchi-Sarfaty *et al.* 2007; Gow *et al.* 2008; Hung *et al.* 2008).

The 1236C>T and 3435C>T SNPs are synonymous so do not cause an amino acid substitution, however synonymous SNPs in other genes have functional consequences (Chamary *et al.* 2006; Sauna *et al.* 2007) and therefore 1236C>T and 34335C>T could modify *ABCB1/MDR1* function. A combination of mutations, i.e. haplotypes, are postulated to alter the shape of *ABCB1/MDR1* by causing different folding that changes substrate specificity compared to the wild type (Kimchi-Sarfaty *et al.* 2007; Hung *et al.* 2008; Fung and Gottesman 2009). Therefore the greater frequency of the heterozygous or homozygous mutations of 1236C>T, 2677G>T and 3435C>T in the oxycodone responder group may be an *ABCB1/MDR1* modification that is advantageous in this cancer population.

Oxycodone is primarily metabolised by *CYP3A4* to noroxycodone, and to a lesser extent *CYP2D6* to the active metabolite oxymorphone. Genetic variability in *CYP2D6* has been estimated to cause reduced enzyme activity in 5-10% of Caucasians and rapid metabolism in 1-7% (Evans *et al.* 1980; Bertilsson *et al.* 1992; Lovlie *et al.* 1996;

Bathum *et al.* 1998; Heiskanen *et al.* 1998). The influence of *CYP2D6* was not observed in this study which may be a result of small sample size. However the 516G>T variant in *CYP2B6* was observed to be more prevalent in the oxycodone non-responder group compared to oxycodone responders.

CYP2B6 polymorphisms (750T>C, 516G>T, 785A>G) have been associated with decreased *CYP2B6* expression resulting in increased substrate blood concentrations, e.g. methadone, postulated to increase toxicity susceptibility (Lang *et al.* 2001; Crettol *et al.* 2007; Eap *et al.* 2007; Bunten *et al.* 2010). Although *CYP2B6* does not metabolise oxycodone, adverse drug reactions as a result of drug-drug and genetic-drug interactions in addition to nutrition, disease and environmental influences have been documented (Stamer and Stuber 2007; Gudin 2012). For example, inhibition of CYP3A or *CYP2D6* by ketoconazole or quinine respectively, can alter the efficacy of oxycodone as an analgesic (Hagelberg *et al.* 2009; Samer *et al.* 2010). The inhibition of one enzyme has been observed to increase the metabolic rate of the alternative enzyme. In the case of CYP3A inhibition, *CYP2D6* activity increases the metabolism of oxycodone into the active metabolite oxymorphone. The increased concentration of oxymorphone results in increased analgesia, but also increased toxicity (Samer *et al.* 2010). The effect is further enhanced if the individual has an ultrarapid *CYP2D6* metaboliser status in addition to an inhibited CYP3A enzyme (Samer *et al.* 2010). As the *CYP2B6* 516G>T polymorphism is associated with reduced expression, concomitant medication metabolised by *CYP2B6* may be in competition with the oxycodone for alternative metabolising enzymes (*CYP2D6*, *CYP3A4*) resulting in slower detoxification of oxycodone, causing toxicity or enhanced effect.

The results of this research show that the average methylation of 11 CpG sites investigated in *ABCB1/MDR1*, 3 CpG sites in *CYP2D6* and 8 CpG sites in *ORPM1*

are not indicators of response to morphine or oxycodone. The lack of relationship does not mean that these genes or methylation is not associated with responder status.

The promoter region of *ABCB1/MDR1*, *CYP2D6* and *OPRM1* were explored in this study as that is where CpG density was greatest and potential transcription factor binding sites are located. However, variable methylation in regions less densely populated by CpG sites and within the gene body have been associated with altered gene function and disease development (Shann *et al.* 2008; Ball *et al.* 2009; Irizarry *et al.* 2009; Illingworth *et al.* 2010; Maunakea *et al.* 2010; Deaton *et al.* 2011; Kinoshita *et al.* 2013). Therefore, other CpG sites further upstream from the transcription start site or surrounding alternative promoters in the gene body of *ABCB1/MDR1*, *CYP2D6* or *OPRM1* may influence opioid responder status. Alternatively, no relationship was observed between the genes' methylation profiles and morphine or oxycodone response as a result of the tissue investigated.

Blood was analysed as previous research detected variable methylation in blood samples of *OPRM1* between different populations (Nielsen *et al.* 2009, 2010). Buccal swabs were investigated to determine its usefulness as a pain free, easy and relatively non-invasive alternative to blood for genetic analysis. However the *ABCB1/MDR1* transport protein is located at blood-tissue membranes, *CYP2D6* is primarily expressed in the liver and the μ-opioid receptor in the central nervous system. Therefore, the methylation profile of these genes within their associated tissue may reflect response to opioids. The variable methylation of *ABCB1/MDR1*, *CYP2D6* and *OPRM1* of opioid dependant and opioid naïve populations has been discussed in *Chapters 5* and *7*.

The variable response to morphine and oxycodone is likely to be a complex phenomenon contributed to by a number of interacting clinical and genetic factors (Diatchenko *et al.* 2005). Studies to date have focused on single genetic variants, or at most the interaction of two variants. More complex modelling is required to explore the concept of epistasis i.e. gene-gene and gene-environment interactions (Moore 2003).

The choice of gene investigated also needs to be widened as focus is mainly on *OPRM1*, *COMT* and *ABCB1/MDR1*. There has been only one genome-wide association study in cancer patients taking opioids, and this data has not been replicated (Galvan *et al.* 2011). To take into account the wide range of variables that have been postulated to influence opioid response, a large population is required to provide statistically significant findings.

9.0 THESIS DISCUSSION

The aim of this thesis was to explore the influence of opioid-related gene DNA methylation on analgesic response to morphine and oxycodone. Humans obtain different responses to morphine and oxycodone as a result of a numerous factors, including the formulation of the opioid and the route of administration, body composition and organ dysfunction, the use of concomitant medication or illicit drugs, and genetic variations within genes involved in opioid pharmacokinetics and pharmacodynamics. Although relationships between each of these factors and opioid response have been obtained, they are not fully understood. Other factors must be contributing to produce the variable response to opioids. Therefore it was decided to investigate the epigenetic gene regulatory mechanism, DNA methylation, on the relevant genes governing metabolism, membrane transport and efficacy in relation to pain response.

Patients that require either morphine or oxycodone to alleviate pain are already in considerable discomfort, therefore relatively non-intrusive and pain free method for the epigenetic analysis is a favourable approach. Obtaining genomic DNA using buccal swabs is a clearly a better method than from blood. Therefore analysis of DNA methylation status of those aforementioned genes was largely undertaken using buccal DNA, but paired blood samples were obtained for comparison in the final studied pilot population.

Previous research has shown that DNA methylation, unlike genetic polymorphisms, is a dynamic mechanism influenced by environmental factors. Two such factors that have been shown to alter 'normal' DNA methylation patterns are smoking and chronic opioid use. As such a population of smokers and non-smokers were recruited and the DNA methylation profiles of opioid related genes (*ABCB1/MDR1*, *CYP2D6* and

OPRM1) were investigated between the two sample groups. It was hoped that DNA polymorphisms and methylation of these opioid related genes could be a set of useful biomarkers for revealing opioid response in a readily accessible and non-intrusive way.

Does smoking influence buccal DNA methylation?

Despite the fact that 50 female smokers and 43 female non-smokers between the ages of 18-50 were recruited for the study, no difference of methylation on any of the above mentioned genes were observed. Previous studies investigating the response of smoke exposure on buccal DNA methylation found that widespread changes occur. However, the smoke exposure induced methylation changes were observed at repetitive elements (Smith *et al.* 2007; Breton *et al.* 2009) or mapped to genes involved in the detoxification of the carcinogenic agents in tobacco smoke, i.e. polycyclic hydrocarbons (PAH), such as the aryl hydrocarbon receptor repressor (*AHRR*), *CYP1A1* and *CYP1B1* (Zeilinger *et al.* 2013; Teschendorff *et al.* 2015). *CYP2D6* is not one of the genes significantly associated with PAH metabolism (Shimada *et al.* 2001) which could explain why no *CYP2D6* methylation differences were observed between smokers and non-smokers in this study.

OPRM1 methylation however was reported to be associated with smoke exposure (Lee *et al.* 2014). Nonetheless, this study is not comparable since Lee *et al.* (2014) explored DNA methylation of blood samples (rather than buccal swabs) from adolescents who were prenatally exposed to maternal cigarette smoking. In addition, although Lee *et al.* (2014) analysed CpG sites within the introns and exons of the *OPRM1* gene, they did not report DNA methylation in the promoter region CpGs. It

is intriguing if DNA methylation in the promoter region or in the intron/exon region could affect gene expression in the same way.

It is also questionable if the DNA methylation states revealed from blood samples could truly reflect the same DNA methylation states in the brain tissue where the OPRM1 receptor is expressed. The importance of tissue specific methylation has already been highlighted in numerous studies when investigating the influence of smoke exposure on DNA methylation of genes (Zöchbauer_Müller *et al.* 2003; Chien 2011; Teschendorff *et al.* 2015). However, for this study, ascertaining the effect of smoke exposure on the methylation of opioid related genes within buccal cells was the aim so that any differences found could be taken into account when investigating and comparing other populations. Analysis of buccal samples of smokers and non-smokers demonstrated that there is no DNA methylation differences on the promoters of *CYP2D6* and *OPRM1* genes. Therefore, those methylation differences observed in later populations were considered as results of other variables, not the influence of smoking.

Methadone-prescribed opioid-dependant mothers and their new-born babies: Babies Born to Methadone-Prescribed Opioid-Dependant Mothers have Elevated DNA Methylation on *ABCB1/MDR1*, *CYP2D6* and *OPRM1*.

To assess the effect of chronic opioid exposure on DNA methylation, woman undergoing methadone maintenance treatment programmes and their new-born babies, and opioid naïve women-baby dyads were recruited and the same genes were analysed as the smoking population. Previous studies showed that chronic opioid exposure altered DNA methylation (Nielsen *et al.* 2009; Chorbov *et al.* 2010). However, the focus of the studies by Nielsen *et al.* (2009) and Chorbov *et al.* (2010) was on the μ-opioid receptor in lymphocytes and sperm, respectively. As such it was

necessary to investigate the methylation of *ABCB1/MDR1*, *CYP2D6* and *OPRM1* in other tissues, namely buccal cells, blood, liver, muscle and thalamus, of opioid exposed individuals compared to non-opioid exposed individuals.

The opioid-dependant mothers had been prescribed methadone during pregnancy. Non-opioid exposed mother-baby dyads were recruited as controls and matched by age and postcode to negate the effects of aging and potentially deprived living standards on gene DNA methylation. In spite of the relatively small populations of mother-baby dyads (opioid exposed n = 21; non-opioid exposed n = 31), this research showed that methylation was statistically different between the opioid exposed adults and opioid naïve adults (*ABCB1/MDR1* and *CYP2D6*, but not *OPRM1*); and between the opioid exposed and opioid naïve babies (in all genes investigated, $P = <0.001$). The increased methylation of the opioid exposed populations compared to the control populations could not be explained by age, smoking status or area of residence and as such was attributed to the effect of regular opioid exposure or lifestyle associated with opioid dependency.

To date, the influence of chronic opioid exposure on DNA methylation of *ABCB1/MDR1*, *CYP2D6* and *OPRM1* genes from adult buccal tissue has not been published. However increased methylation within the opioid exposed populations was expected as opioid exposure, and the stressful lifestyle and poor diet associated with opioid dependency has been previously reported to cause variable DNA methylation in tissues other than buccal DNA (McGowan *et al.* 2009; Nielsen *et al.* 2009; Chorbov *et al.* 2010; de Rooij *et al.* 2012; Doehring *et al.* 2013). Conversely to the existing literature (Nielsen *et al.* 2009; Chorbov *et al.* 2010; Doehring *et al.* 2013), no *OPRM1*

DNA methylation differences were observed between the opioid exposed and opioid naïve mothers. The disparity between previous studies and this study may be a result of tissue specific methylation.

With regards to baby DNA methylation, one recent study by Wachman *et al.* (2014) investigated methylation of *OPRM1* in saliva samples of opioid exposed babies. Unfortunately, they did not recruit an opioid naïve population of babies for comparison. The methylation percentages that Wachman *et al.* (2014) reported for the opioid exposed babies were lower than those found in our opioid exposed babies, but greater methylation levels were observed than that of our opioid naïve population. This suggests that opioid exposure, and / or the lifestyle associated with opioid dependency increases the methylation of *OPRM1*. The extent of DNA methylation may be influenced by maternal dose of methadone as the mothers recruited by Wachman *et al.* (2014) were receiving lower doses of methadone compared to the mothers recruited as part of this study.

The purpose of the work undertaken by Wachman *et al.* (2014) was not to explore the effects of opioid exposure on DNA methylation, but to ascertain whether DNA methylation of *OPRM1* influences the development of NAS. They found that the -10 CpG site was hypermethylated in babies requiring NAS treatment compared to those that did not, but this difference did not retain significance after multiple testing correction. The lack of relationship between *OPRM1* DNA methylation and likelihood of developing NAS observed by Wachman *et al.* (2014) was corroborated by this study suggesting that DNA methylation is not a suitable biomarker for the likelihood of developing NAS.

Methadone-prescribed opioid-dependant mothers and their new-born babies: The *CYP2B6*6* polymorphism protects babies exposed to methadone *in utero* from neonatal abstinence syndrome development.

Although no relationship between DNA methylation and NAS development was observed, a relationship that has not been previously reported was found between *CYP2B6* polymorphisms and NAS development. Babies carrying a variant allele at *CYP2B6* 516G>T and 785A>G (*CYP2B6*6* genotype) were statistically less likely to require NAS treatment than babies with the *CYP2B6*1* genotype. *CYP2B6* is the predominant metabolising enzyme of methadone and the *CYP2B6*6* genotype has been reported to catalytically deficient compared to the wild-type (Gadel *et al.* 2013).

The reduced metabolic activity of *CYP2B6*6*, associated with the poor metaboliser phenotype, is postulated to have protected babies carrying this genotype from NAS development by prolonging the presence of maternally transferred methadone; whereas babies with the wild-type would efficiently and rapidly metabolise methadone resulting in the opioid withdrawal symptoms signifying NAS. Increased methadone plasma concentrations following methadone administration and lower requirement of methadone dose of individuals with *CYP2B6*6* compared to wild-type has been reported in adult populations (Crettol *et al.* 2005; Eap *et al.* 2007; Bunten *et al.* 2011; Hung *et al.* 2011; Wang *et al.* 2011; Levran *et al.* 2013) but there is no literature with regards to baby NAS development and *CYP2B6* polymorphisms.

Other genes associated with opioid pharmacokinetics and pharmacodynamics have been associated with the development of NAS. A study undertaken by Wachman *et al.* (2013) found that babies heterozygous or homozygous for the variant allele at *OPRM1* 118A>G were less likely to require treatment for NAS that those babies with homozygous wild-type. This finding was not observed in our population of

methadone exposed babies, as reported in *Chapter 6*. The lack of relationship between the *OPRM1* 118A>G SNP and NAS development may be a result of limited sample size as well as low occurrence of the mutated allele in our studied human subjects.

Tissue specific DNA methylation: DNA methylation of *ABCB1/MDR1*, *CYP2D6* and *OPRM1* in individuals whose deaths were attributed to heroin toxicity.

The second opioid exposed population investigated were heroin associated fatalities undergoing autopsy at Sir James Black Mortuary, Dundee. Tissue specific DNA methylation was investigated using these post-mortem blood, liver, psoas muscle and thalamus samples. Comparison of methylation within opioid exposed intra-individual tissue samples showed that *ABCB1/MDR1* had higher methylation in thalamus compared to liver and muscle; *CYP2D6* was less methylated in liver tissue compared to blood, muscle and thalamus tissue; and *OPRM1* was less methylated in thalamus and muscle compared to DNA obtained from blood and liver (detailed in *Chapter 7*).

The variable gene methylation between the tissues appeared to be related to tissue function. The most prominent example being the hypomethylation of *CYP2D6* in liver compared to its hypermethylation in other tissues investigated. *CYP2D6* is an important hepatic metabolising enzyme, fundamental in the metabolism of a wide variety of drugs and endogenous compounds (Wang *et al.* 2013; Ravindranath 2014). The hypomethylated *CYP2D6* gene in the liver samples is speculated to enable the protein to be expressed more readily in the liver so as to enable detoxification of not only the opioids consumed but also the wide variety of drugs detected in the postmortem blood samples (drugs listed in *3.3.2*).

Clearly further research is required to determine the effect of variable gene methylation on expression of proteins within various tissues. Tissue-specific differentially methylated regions have been observed in genome wide analyses (Zhang *et al.* 2013; Lokk *et al.* 2014). For example, the receptor involved with the development of epithelial tissue and bone, *MST1R*, is less in demand in the brain tissue and is therefore heavily methylated in comparison to other tissues (Byun *et al.* 2009). Analysis of the post-mortem population has shown that methylation of the opioid related genes is tissue specific. In addition the post-mortem population illustrated that there was no correlation between methylation of related samples. This study highlights that gene methylation in peripheral tissues does not necessarily suggest that methylation is not influencing gene expression in the tissue of gene function, thereby altering opioid response.

Pilot trial: Personalising opioid therapy for cancer pain relief. The influence of DNA methylation and SNPs on opioid response.

Unlike smoking, opioid exposure influenced DNA methylation on opioid related genes. Therefore, an opioid naïve population of patients requiring either morphine or oxycodone to alleviate cancer related pain were recruited to determine the influence of DNA methylation on opioid responder status. In addition to DNA methylation analysis, genetic variations in *ABCB1/MDR1*, *COMT*, *CYP2B6*, *CYP2D6* and *OPRM1* that have been associated with opioid response by previous studies (summarised in

2.2.5) were investigated. This study is the first to show that buccal and plasma *ABCB1/MDR1*, *CYP2D6* and *OPRM1* DNA methylation is not indicative of how an opioid naïve population would respond to morphine or oxycodone analgesics.

Although DNA methylation was not associated with opioid response the *OPRM1* 118A>G, *ABCB1/MDR1* 1236C>T, *ABCB1/MDR1* 3435C>T, and *CYP2B6* 516G>T polymorphisms were statistically significantly different between opioid responders and non-responders. Individuals carrying the variant allele at *OPRM1* 118A>G were more likely to be respond well to morphine analgesia which is inconsistent with previous studies. Previous research has indicated that carriers of 118G allele obtain less pain relief and require higher doses of morphine to achieve analgesia (Klepstad *et al.* 2004; Campa *et al.* 2008; Tan *et al.* 2009; Sia *et al.* 2008, 2013).

However the lack of a relationship between the 118A>G polymorphism and opioid response has also been reported (Janicki *et al.* 2006; Klepstad *et al.* 2011). Three of the studies that observed a relationship between 118A>G and morphine response were undertaken using Asian populations administered morphine during childbirth or hysterectomy surgery (Sia *et al.* 2008; Tan *et al.* 2009; Sia *et al.* 2013). As reported by Tan *et al.* (2009), ethnicity, independent of the *OPRM1* 118A>G polymorphism, is a significant contributor to opioid response. Therefore the Caucasian population recruited for this study is not comparable to studies of Asian populations.

Also the studies by Sia *et al.* (2008, 2013) and Tan *et al.* (2009) detail the use of intravenous morphine, and as discussed in *2.1.3* the route of opioid administration alters drug efficacy and duration as a result of differing bioavailability and rate at which the opioid reaches the target site. Intravenous administration of morphine results in a more rapid analgesic response as it by-passes first-pass metabolism and therefore reduces the influence of genes such as *ABCB1/MDR1* which plays a role in blood-tissue opioid transportation. As such, the study undertaken by Klepstad *et al.* (2004) is the most similar to this study as a Caucasian population was recruited and were prescribed oral morphine for cancer related pain. In addition, similar population

sizes were recruited by Klepstad *et al.* (2004) and this study. Possible explanations, then, for the contradictory *OPRM1* 118A>G polymorphism and opioid response outcomes include (as postulated by Chou *et al.* 2006; Likar *et al.* 2008) confounding factors such as gene-gene and gene-environment interactions, environmental influences, multiple comorbidities and drug-drug interactions. To explore the effect of all these factors a large population of patients would need to be recruited.

Carriers of the variant alleles at *ABCB1/MDR1* 1236C>T and 3435C>T, and *CYP2B6* 516G>T were more likely to respond to oxycodone than those cancer patients with the wild-type genotype. The statistical difference observed in the *ABCB1/MDR1* polymorphisms of oxycodone responders and non-responders was retained when the wild-type haplotype was compared to the variant haplotype (P = 0.010). The occurrence of better opioid response (with regards to pain as well as side effects) in patients with a variant allele at *ABCB1/MDR1* 1236C>T and 3435C>T has been previously reported (Coulbault *et al.* 2006; Campa *et al.* 2008; Fujita *et al.* 2010; Zwisler *et al.*2010). The polymorphisms are associated with a transport protein with reduced expression and / or efficacy (Hassan *et al.* 2009) and as a result the pharmacological effects of opioids are prolonged through increased absorption and CNS accumulation. Further, larger studies should be undertaken to corroborate these findings.

The largest population of patients to be recruited to date is by the European Pharmacogenetic Opioid Study (EPOS) group (n = 2294). The EPOS found no relationship between any polymorphism and opioid response (Klepstad *et al.* 2011); however their cancer patient population lacked homogeneity. Patients experiencing neuropathic pain were merged with those suffering somatic and visceral pain which operate through different pain signalling pathways (Woolf 2011). Not only that, but a

wide range of opioids with different cell signalling transduction pathways were prescribed (morphine, oxycodone, fentanyl in addition to other opioids) via different routes of administration.

As part of this study, the choice of opioid was limited to oral administrated morphine or oxycodone and patients suffering from neuropathic pain were considered not eligible for the study. It should be noted that the target population of cancer patients to be recruited was 200. However, a number of delays were encountered whilst designing and writing the protocol as well as obtaining ethical approval that resulted in a shortened period for patient recruitment (full details of delays encountered attached as Appendix D1.0). Although >18 months were consumed by the administration aspects, a considerable amount knowledge was learnt from having to choose appropriate wording for writing the protocol and supporting appendices, liaising with clinicians and corresponding with the ethics committee.

Conclusion

In conclusion, DNA methylation on the *ABCB1/MDR1*, *CYP2D6* and *OPRM1* gene promoter regions of blood and buccal samples cannot be used to predict individual response to morphine and oxycodone. However this research showed that DNA methylation of the genes is tissue-specific. Therefore it is plausible that differences in DNA methylation on the above genes between opioid responders and non-responders may reflect the functional state (on or off expression) of the genes in specific tissues of relevance. As such future research should investigate the DNA methylation states of *ABCB1/MDR1*, *CYP2D6* and *OPRM1* genes in cells obtained from blood-tissue barriers, liver and central nervous system, respectively, in relation to opioid response outcome. *Chapter 5* and *7* illustrated that chronic opioid use, or the lifestyle associated

with opioid dependency are important factors to be considered when exploring DNA methylation of opioid related genes, whereas smoking did not influence their DNA methylation (*Chapter 4*).

Although DNA methylation was not associated with responder status, relationships were observed with polymorphisms in *ABCB1/MDR1* and *OPRM1* genes. These findings clearly need to be confirmed in future studies with larger population sample sizes. The identification of SNPs associated with opioid response is potentially very valuable for clinicians prescribing opioids to patients in need of Step III opioids to alleviate pain.

Additionally this study has identified genetic polymorphisms that could act as a diagnostic tool for predicting the susceptibility of babies exposed to methadone *in utero* to develop NAS. The *CYP2B6*6* genotype is more frequent in babies who do not require treatment for opioid withdrawal symptoms. Indicators of NAS development enables appropriate monitoring of babies who are more susceptible to developing NAS, which could negate unnecessary long hospital stays for babies protected from developing NAS. The reduced hospital stay of babies less susceptible to NAS development would reduce the healthcare costs associated with NAS monitoring.

In conclusion this study has;

1. suggested that smoke exposure does not influence buccal DNA methylation of *CYP2D6* or *OPRM1* therefore smoking was not considered as a methylation altering factor for these genes.

2. illustrated that opioid exposure, or the opioid associated lifestyle increases gene DNA methylation so an opioid naïve population was necessary to investigate opioid response in relation to gene methylation.
3. identified genetic polymorphisms that could act as a diagnostic tool for predicting the susceptibility of babies exposed to methadone *in utero* to develop NAS. The *CYP2B6*6* genotype is more frequent in babies who do not require treatment for opioid withdrawal symptoms.
4. confirmed previous studies that DNA methylation is tissue specific therefore methylation of relevant genes may be of more interest than DNA analysis of genes in peripheral tissues.
5. determined that methylation of *ABCB1/MDR1*, *CYP2D6* and *OPRM1* did not influence the response to morphine or oxycodone in an opioid naïve population, however polymorphisms in *ABCB1/MDR1* and *OPRM1* may.

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Does smoking affect buccal DNA methylation?

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1. Background

Chemicals within in tobacco smoke can damage DNA. This damage can be in the form of mutations which alters the DNA sequence or epigenetic mechanisms that add or remove compounds to the DNA sequence. One such epigenetic mechanism is called DNA methylation and involves the addition of methyl groups onto particular sequences of DNA; specifically when cytosine and guanine bases fall in succession (CpG). DNA methylation is an essential mechanism for gene regulation. If a gene is heavily methylated then that gene is effectively switched off as transcription factors cannot bind and translate the DNA sequence into a protein.

Recently we undertook a study involving mothers on maintenance treatment programmes whilst pregnant. From both the mother and newborn baby buccal swabs were obtained. The methylation of CpG sites in the mu-opioid receptor (OPRM1), transport protein (ABCB1) and 3 CpG sites in CYP2D6, a phase one metabolising enzyme were determined in the buccal DNA. The aim of this study was to establish the effect of prolonged opioid exposure on the mother's buccal DNA methylation of opioid important genes, and to determine the effect of methadone exposure in utero on their neonate. Unfortunately all of the mothers who were taking methadone were also smokers. Smoking has been shown to alter buccal DNA methylation, causing

variable methylation of certain genes compared to non-smokers (Breitling et al. 2011; Chien et al 2011). Similar smoking-related DNA methylation gene specific and genome wide alterations have been observed in neonates exposed to smoking in utero (Breton et al 2009; Flom et al. 2011). However to date, the effect of smoking on methylation of the mu-opioid receptor (OPRM1), transport protein (ABCB1) and phase I metabolising enzyme, CYP2D6, has not been reported. Therefore it is not known whether the methylation profile observed in the methadone exposed mother and baby population is as a result of the methadone exposure, tobacco smoke or is a "normal" methylation profile.

Hypothesis: Smoking causes increased DNA methylation in buccal DNA compared to non-smoker buccal DNA methylation.

2. Study Design

We plan to undertake a prospective non-interventional fully anonymised genetic study. Study subjects will only be asked to complete a short questionnaire and provide a buccal swab.

2.1 Study Population

The aim is to enrol 100 females between the ages of 18 and 50; 50 smokers and 50 non-smokers.

2.2 Population Recruitment and Requirements

2.2.1 Bournemouth University staff, PhD researchers and students will be approached and asked to read a patient information sheet that details the background and purpose of the study.

2.2.2 The eligible volunteers must sign an informed consent form after having read the patient information sheet to participate in the study.

2.2.3 On introduction to the study the volunteer will be assigned a unique study number. This unique study number will be matched to the buccal swab provided. There will be no record linking a volunteer to their study number.

2.2.4 The volunteer's age, smoking history and opioid use will be recorded.

2.2.5 Following obtaining consent and completing the questionnaire volunteers will be asked to provide a buccal swab. Buccal swabs will be collected using Catch-All™ brushes for DNA analysis.

2.3 Sample Collection, Storage and Analysis

2.3.1 To collect buccal DNA volunteers will be asked to swill their mouth with water a couple of times to remove any external contaminants.

2.3.2 The cotton bud-like buccal swab will be rubbed gently on the inner side of both cheeks 10-20 times each.

2.3.3 The buccal swab will be air dried in a hard plastic case until sample is dry (about 20 minutes).

2.3.4 Samples will be stored at -20 until extraction at Royal Bournemouth Hospital.

2.3.5 Methylation analysis will be undertaken at Royal Bournemouth Hospital

Molecular Pathology Department using previously developed pyrosequencing assays.

3. Statistical Analysis

Methylation profile of each gene will be compared between smokers and non-smokers using t-tests if the data is normally distributed and there are no outliers. Alternatively independent nonparametric tests will be undertaken.

The methylation profile of the smokers and non-smokers will also be compared to the methylation profile of methadone exposed mothers to ascertain whether the mothers' methylation is impacted by opioid exposure.

4. Costs of the project

Analysis will be undertaken by Poppy McLaughlin at the Centre for Forensic and Biological Sciences, Bournemouth University, and Molecular Pathology department, Royal Bournemouth hospital under the direct supervision of Prof. David Osselton and Dr Tamas Hickish. The cost of all analyses will be borne by the Centre for Forensic and Biological Sciences, Bournemouth University (buccal swabs - already purchased) and Royal Bournemouth hospital charitable funds (for pyrosequencing analysis – already purchased).

5. Timeline

It is anticipated that 50 smokers and 50 non-smokers can be recruited within a four week period.

6. Summary and likely outcomes from this project

The results from this study will illustrate the impact of smoking on buccal DNA methylation. If there is no difference in methylation between smokers and non-

 4 smokers then the methylation profile found in the methadone mothers is more likely to be due to their methadone use than smoking. This will show that methadone has an adverse effect of buccal DNA and may alter drug response.

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APPENDIX A1.1

Does smoking affect buccal DNA?

Participant Information Sheet

You are being invited to participate in a research study. Before you decide, it is important for you to understand why the research is being done and what it will involve. Please read the following information carefully and discuss it with others if you wish. Ask us if anything is unclear or if you would like more information.

What is the purpose of the study?

Buccal DNA (genetic material collected from the mouth cavity) has been obtained from mothers taking methadone to alleviate their craving for other drugs, specifically heroin. These buccal samples have been analysed for a genetic mechanism that plays a role in gene expression. This genetic mechanism is called DNA methylation. DNA methylation is known to be affected by age, lifestyle, environmental exposures, smoking and diet. We decided to research what affect continued methadone exposure has on buccal DNA methylation however all the mothers analysed were also smokers. Therefore we do not know whether the DNA methylation identified is as a result of methadone use, a result of smoking, or could be a combination of the two factors.

In order to determine the effect of smoking on buccal DNA methylation we would like to collect and analyse buccal samples from female smokers and non-smokers.

Why have I been chosen?

We are asking a sample of female staff and students at Bournemouth University if they will take part in this study.

Do we need to take part?

No. It is entirely up to you. If you do decide that you will take part you will be given this information sheet to keep and be asked to sign a consent form. You will be free to withdraw from the study at any time and you won't have to give a reason.

What will happen to me if I take part?

After signing the consent form we will collect a mouth swab from you to check your genes (DNA). The mouth swab is painless and will only take a few seconds. The DNA samples will be stored and analysed at the University of Bournemouth. We will keep a note of your smoking status and whether or not you take drugs, but otherwise the samples provided will be completely anonymised.

APPENDIX A1.1

What are the possible risks of taking part?
There are no risks from taking part in this study.

What are the possible benefits of taking part in this study?
There is not expected to be any direct benefit to yourself from taking part in this study.

Suggestions and complaints.
If taking part in this research project harms you there are no special compensation arrangements. If you wish to complain about any aspect of the way you have been treated during the course of this study a complaint in writing can be made to Prof David Osselton, C237, Talbot Campus, Bournemouth University, BH12 5BB.

Will our taking part in this study be kept confidential?
All information collected about you during the course of this research will be kept strictly confidential.

What will happen to the results of the research study?
Information gathered from this study will be analysed and the results submitted for publication in a medical journal. Information may also be presented at scientific meetings. You will not be identified in any presentation or written document.

Who is funding this research?
The mouth swab tests will be carried out at the University of Bournemouth as part of a PhD.

Who has reviewed this study?
This study has been approved by Bournemouth University Ethics Committee.

Contact for further information
Poppy McLaughlin, PhD researcher, can be contacted at any time on 01202 961831.

Thank you for taking time to consider this research study.

Yours sincerely,

Poppy McLaughlin

APPENDIX A1.2

Bournemouth University

CONSENT FORM

Study: Does smoking affect buccal DNA?

Name of Researcher: Poppy McLaughlin

Study Number:

Please initial each box

1. I confirm that I have read and understand the information sheet dated March 2014 (version 1) for the above study and have had the opportunity to ask questions. ☐

2. I understand that my participation is voluntary, and that I am free to withdraw consent at any time without giving any reason. ☐

APPENDIX A1.3

Bournemouth University

Study: Does smoking affect buccal DNA?

Questionnaire

Study No:

1. How old are you? _____ years old

2. Please choose one of the following statements and complete as necessary:
   a. I have never smoked
   b. I used to smoke but stopped in _____ (year)
   c. I smoke less than 15 cigarettes a day
   d. I smoke more than 15 cigarettes a day

3. Do you take regular opioids, e.g. morphine, methadone, heroin, oxycodone?
   a. No
   b. Yes, I take _____

Thank you for your participation!

Pilot Trial - Pharmacogenetic determinants of inter-individual response to opioid analgesia: a feasibility and explorative study to support a large multicentre study.

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Contents

Protocol
Version 1.4
24/01/2013

Contents 1
1 Study Summary 2
2 Background 4
2.1 Opioids for cancer pain 4
2.2 Inter-individual variation in response to opioids for cancer pain 4
2.3 Pharmacogenetics of opioid response 7
2.4 Previously published data in this area 8
2.5 Opioid blood concentrations 13
3 Rationale for Study 13
4 Aims and Objectives 14
4.1 Primary Objectives 14
4.2 Secondary Objectives 14
5 Study Design 15
5.1 Centres 16
5.2 Study Population 16
5.3 Patient Eligibility Criteria 16
5.4 Patient Exclusion Criteria 16
5.5 Patient Enrolment 17
5.6 Consent and Confidentiality 17
5.7 Initial Patient Assessment 18
5.8 Additional Patient Assessments 19
5.9 Sample Collection 20
5.9.1 Sample Collection 20
5.10 Sample Analysis Location and Storage 21
5.11 Study Endpoints 22
6 Sample Analyses 23
6.1 Genetic Analysis 23

6.2 Opioid Quantification 24
7 Statistical Considerations 24
7.1 Hypotheses 25
7.2 Statistician 26
8 Safety Reporting 26
8.1 Protocol Violations 27
9 Quality Control and Assurance 28
9.1 Trial monitoring committee 28
9.2 Committee for clinical research 28
9.3 Ethical approval 28
10 Financial and Insurance Matters 29
10.1 Study funding 29
10.2 Trial sponsor 29
11 Publication Policy 29
11.1 Informing subjects of results 29
11.2 Dissemination of results 29
11.3 Peer review 29
12 National/Local Implications for Future Practice, Education and Research 30
12.1 Change to clinical practice 30
12.2 Predictive model 30
13 References 30

1 Study Summary

Morphine is the initial analgesic opioid of choice for relieving chronic cancer pain that cannot be controlled by non-opioid analgesics, however patient responses are variable. Whilst many patients achieve satisfactory pain relief from morphine, 1030% of patients obtain inadequate or no pain relief and may suffer intolerable adverse side effects such as nausea and constipation (Cherny *et al*. 2001; Ross *et al*.

2005; Oertel *et al*. 2006). The dose of analgesic is titrated gradually until affective analgesia is obtained with minimal side effects but this process can be slow, as well as painful for the patients. In the event that patients do not obtain the desired pain relief from morphine or have unacceptable side effects, alternative opioids such as oxycodone are prescribed but these analgesics also produce variable inter-individual responses. This "trial-by-error" method for drug administration has a detrimental effect on patients' quality of life as well as being expensive for health care organisations.

The literature has shown that genetic variations can lead to varying analgesic affects of opioids. As of yet no study has created an algorithm that takes into account the various polymorphisms as well as lifestyle choices to calculate the most appropriate opioid and the correct dose on first administration rather on a trial-by-error basis. Initially a pilot study will be carried out to gauge participant recruitment rate, compliance with the study, participant drop-out rate and to explore genetic and toxicological information. Patients with cancer who require a Step III analgesic such as morphine or oxycodone to relieve cancer related pain will be asked to participate in this observational, non-invasive study. Treatment of patient's cancer pain will not be affected by participating in this study, however patients will be asked to complete questionnaires and provide blood and oral fluid samples. Each patient will be asked to rate the efficacy of their pain relief by completing pain and side effect questionnaires at regular intervals until they are on a stable dose of either morphine or oxycodone (usually within 7-10 days). If the initial opioid is ineffective and the patients are switched to the alternative study opioid (either morphine or oxycodone), the patient may continue to participate in the pilot trial. The participants' blood and oral fluid samples would be analysed to quantify opioid concentrations and to identify potential genetic variants which may affect drug response. The opioid concentrations detected in the blood and oral fluid samples would be compared to ascertain the suitability of using oral fluid in place of blood samples for monitoring opioid levels. Oral fluid collection is a less invasive and has a gentler collection procedure so would be more comfortable for the cancer patients who undergo innumerable tests and blood collections. Previous research (Hansen *et al.* 2007) has shown that patients prefer to provide oral fluid samples over blood samples.

2 Background

2.1 Opioids for cancer pain

The World Health Organisation (WHO) defined a pain relief step ladder in 1986 (Burton and Cleeland 2001) that directs the pharmacological management of cancer pain. This is a step-wise approach, starting with the use of non opioids (e.g. paracetamol and aspirin) for mild pain (Step I), adding weak opioids (e.g. codeine) for moderate pain (Step II), and using regular strong opioids such as morphine for moderate to severe pain (Step III).

There are a number of different strong (Step III) opioids available, including morphine, oxycodone, fentanyl, alfentanil and methadone. Some opioids differ in their mode of metabolism and therefore some are recommended as being safer in certain circumstances than others e.g. alfentanil in renal failure. There is little evidence however to suggest superiority of one opioid over another in terms of efficacy (Hanks *et al.* 2001). There have been very few randomized controlled trials comparing different opioids and those that have been carried out have tended to be small.

It is standard clinical practice in the UK to commence patients with moderate to severe cancer pain on a regular (usually 4 hourly) low dose of a short-acting immediate release preparation of an opioid (usually morphine). The opioid dose is then adjusted over a number of days depending on the level of pain relief achieved and whether or not the patient is experiencing opioid related side-effects. When / if the patient reaches a state where the pain is well controlled without intolerable opioid side-effects, he / she is generally converted to a modified release longer acting opioid which just needs to be administered once or twice a day.

2.2 Inter-individual variation in response to opioids for cancer pain

Clinical experience and a growing number of studies have demonstrated marked variability in individual patient's response in terms of the level of pain relief

achieved, the side-effects experienced and the daily dose of opioid required (Argoff 2010). Up to 30% of cancer patients on oral morphine for pain are known as "morphine non-responders" (Cherny *et al.* 2001; Ross *et al*. 2005; Oertel *et al*. 2006). These "morphine non-responders" present in a number of different ways (Mercadante *et al.* 2006):

- Patients who achieve good analgesia but with intolerable side-effects.
- Patients who do not achieve good analgesia because of dose-limiting sideeffects.
    - It is not uncommon for opioid side-effects such as nausea, vomiting, sedation, confusion, hallucinations and myoclonus to become apparent as the dose is increased. These toxicities may often be managed with other medications e.g. antiemetics / antipsychotics but sometimes they persist or become intolerable and thus dose-limiting. In this case, patients (morphine non-responders) are switched from morphine to an alternative opioid.
- Patients who do not achieve good analgesia but do not experience sideeffects either, despite escalating morphine doses.

The analgesic variability of morphine has been reported as early as the 1980's when Austin *et al.* (1980) reported dramatic pain responses with minimal changes in morphine serum concentrations. Recommendations for individualised doses have been suggested (Tamsen *et al.* 1979) with administrations of morphine varying 1000-fold between patients (EAPC 1996). Although most work in this area had been carried out in patients on morphine, inter-individual variation in response to other strong opioids for cancer pain also exists. Emerging evidence suggests that there may be two broad groups of opioid non-responders:

- Patients who do not achieve an adequate clinical response to the initial opioid shortly after initiation, when the opioid dose is relatively low, reflecting that not all drugs are efficacious in all patients. "Heterogeneity of treatment effects" is seen with most pharmaceutical medications and may be explained in part by individual pharmacokinetic or pharmacodynamic factors.

- Patients who appear to become non-responsive to the initial opioid at either higher doses or after chronic opioid therapy. This may be due to development of physical tolerance to the initial opioid (Slatkin 2009).

Recognition of this substantial inter-individual variation in response to morphine for cancer pain has resulted in the emergence of "opioid switching" as a clinical manoeuvre to redress the balance between analgesia and side-effects. If patients are considered to be "non-responders" to the initial opioid, it is common clinical practice to switch them to an alternative strong opioid (Quigley 2003; Dale *et al.* 2011). For example, if a patient is a non-responder to morphine, he / she may be switched to oxycodone.

The theoretical basis supporting this practice is that although all strong opioids act on opioid receptors, there are pharmacokinetic and pharmacodynamic differences which may explain why some opioids are more or less effective in different patients. Morphine exerts its action by binding to the μ-opioid receptor which is expressed by the *OPRM1* gene. Morphine is metabolised by UDP-Glucuronosyltransferase-2B7 (UGT2B7) in the liver, principally to morphine-3-glucuronide (M3G) and to a lesser extent, morphine-6-glucuronide (M6G). M3G does not have any analgesic affect, on the contrary M6G has been found to be pharmacologically active; the extent of potency depends on the route of administration. M6G also carries out its pharmacological effects on the μ-opioid receptor (Smith and South 2001). Oxycodone on the other hand, is a semi-synthetic opioid that has agonist activity on the μ, δ and κ-opioid receptors. Oxycodone metabolism is carried out by cytochrome P450s. CYP2D6 *O*-demethylates oxycodone to a potent narcotic analgesic, oxymorphone and CYP3A4 and CYP3A5 *N*-demethylate oxycodone to noroxycodone, an inactive metabolite (Baselt 2002). Oxymorphone is a more potent analgesic than oxycodone and has a higher binding affinity to the μ-opioid receptor than oxycodone. Oxymorphone is further metabolised in the liver by CYP2D6 and CYP3A4 (Childers *et al*. 1979).

The evidence supporting the efficacy of opioid switching is based on observational studies and clinical anecdote, mainly because large randomised controlled trials in

this area have not been carried out. However most studies demonstrate a reduction in pain and side-effect scores with the alternative opioid (Dale *et al.* 2011).

2.3 Pharmacogenetics of opioid response

Inter-individual variation in response to opioids for cancer pain may be a result of factors such as age, gender, disease and lifestyle that affect the pharmacodynamic and pharmacokinetic properties of a drug. A recent study of over 2000 patients suggested that psychological issues and sleep deprivation may also play a role (Knudsen *et al.* 2011). To date no clinical factors (with the exception of renal impairment) have been identified which can be used prospectively to predict opioid response in this cohort. Therefore there is a growing interest in the possibility that an individual's genetic makeup may influence opioid response. Pharmacogenetics is the study of genetic variations that lead to differing drug responses. The word itself is derived from "pharmacology", the study of drugs activity on the body and "genetics", the study of inherited traits (Bukaveckas 2004). In many areas of medicine, pharmacogenetics has yielded some very exciting results. An individual's response to drugs such as irinotecan, abacavir and warfarin can now be accurately predicted through integration of genetic and clinical data. This facilitates prospective decision making regarding choice of the correct dose of the correct drug for a given patient and reduces subsequent side-effects. Thus in a number of different areas of medicine including oncology and haematology, pharmacogenetics has revolutionised drug prescribing.

One of the best characterised pharmacogenetic phenomena involves codeine. Codeine, a weak opioid, is metabolised to morphine via the genetically heterogeneous enzyme CYP2D6 (Dayer *et al.* 1988; Sindrup *et al.* 1990). Up to 10% of Caucasians (and varying proportions of other populations) lack CYP2D6 activity. These are known as "poor metabolisers" and thus experience little analgesia from codeine, as compared to "extensive metabolisers". CYP2D6 gene duplication on the other hand is associated with ultra-rapid metabolism of codeine to morphine, found in 3% of Caucasians. There is a case report of fatal neonatal opioid toxicity in a child who was being breastfed by a CYP2D6 ultra-rapid metabolising mother (Koren *et al.* 2006). Variation in at least 16 alleles has been shown to influence

CYP2D6 activity (Sachse *et al.* 1997). Surprisingly, the impact of genetic variation in CYP2D6 on codeine response has not been extensively studied in cancer patients.

2.4 Previously published data in this area

There have been a number of clinical studies examining the role of pharmacogenetics on response to opioids for cancer pain. Overall the results of these genetic association studies have provided limited and often conflicting results. The reasons for this include:

1. Small study size. Until the recent European Pharmacogenetics on Opioids study (EPOS) (Klepstad *et al.* 2011), the largest genetic association studies in this field included less than 300 subjects (Rakvag *et al.* 2005; Ross *et al.* 2005). The recent EPOS was a multi-centre study in which 2294 cancer patients from 17 centres in 11 European countries were included. Although the clinical applicability of the genetic association results from this study is somewhat inconclusive, this study supports a multi-centre study design to achieve adequate study numbers in a field where, due to the poorly nature of many of the patients, recruitment is often challenging.

2. Lack of consensus on study outcomes. There is no consensus in research in this field as to the best clinical phenotype of opioid response. Some studies have examined daily opioid dose requirements (regardless of the level of pain control achieved), some have explored a single side-effect. Similarly different studies use different pain measures.

3. Only a limited numbers of genes and genetic variants have been assessed. The opioid pharmacogenetic studies in cancer patients which have shown some positive results have primarily focused attention on three genes; OPRM, COMT and MDR1. The main findings from these studies are summarised in Table 1. There has been only one genome-wide association study in cancer patients taking opioids, and this data has not been replicated (Galvan *et al.* 2011).

4. Response to opioids for cancer pain is likely to be a complex phenomenon, contributed to by a number of interacting clinical and genetic factors (Diatchenko *et al.* 2005). Studies to date have focused on single genetic variants, or at most the interaction of two variants. More complex modelling is required to explore the concept of epistasis i.e. gene-gene and gene-environment interactions (Moore 2003).

A patient's response to either morphine or oxycodone may depend on a number of factors including their body's ability to carry out drug absorption, distribution, metabolism and elimination. Resultant drug concentration at the target site and the number and morphology of target receptors, together with variation in multiple downstream events after receptor / ligand binding will also influence individual response. Thus, genetic variation of multiple genes may influence an individual's response to opioids (Ross *et al*. 2005).

Single nucleotide polymorphisms (SNPs) are the most common type of genetic variant at a DNA level. A SNP represents a change in a single nucleotide at a particular position along the DNA strand. All published genetic association studies of response to opioids for cancer pain have examined SNPs. DNA deacetylation, phosphorylation and methylation can also affect drug response as these variations can alter the expression of a gene (Doehring *et al*. 2011). Former heroin addicts have a higher level of methylation in the promoter region of the OPRM1 gene compared to control populations (Nielsen *et al*. 2010). Associations between degree of methylation and alcohol dependence and nicotine addiction have also been found (Bleich *et al*. 2006; Philibert *et al*. 2008). Due to these epigenetic changes, the expression of the μ-opioid receptor may be altered therefore altering efficacy (Nielson *et al*. 2010). Similarly, links between methylation of the COMT gene and nicotine dependence have been found (Xu *et al*. 2010).

Table 1: Summary of the published pharmacogenetic studies of response to opioids for cancer pain*

Author	N	Genes examined	Opioid	Clinical outcome	Type of study
Klepstad	99	OPRM1	Morphine	Daily morphine dose	Prospective observational study. Single time point
Rakvag	197, 207	COMT			
Reyes-Gibby	207	OPRM1 / COMT			
Campa	145	OPRM1	Morphine	Change in pain scores between baseline and week 1	Prospective observational study. Scores measured 1 week apart
Holthe	77, 175	UGT2B7	Morphine	Morphine, M-3-G and M-6-G concentrations	Prospective observational study. Single time point
Ross	186	OPRM1 Barrestin2 Stat6 UGT2B7	Morphine	"Morphine responders" versus "non-responders". Morphine responders were taking morphine for at least 1 month with good pain control and minimal side-effects. Morphine non-responders had inadequate pain control and/or intolerable side-effects	Prospective observational study. Single time point
	228	COMT MDR1		Central side-effects of morphine (drowsiness, confusion, hallucinations)	

Reyes-Gibby	140	IL-6 TNF-α	Any strong opioid including morphine, oxycodone, fentanyl, methadone	Morphine equivalent daily dose Pain severity Change in pain severity	Prospective observational study. Scores measured at baseline and 30 days post first assessment at the Supportive Care Centre for pain management
Klepstad 2011	2294	OPRM1, OPRD1, OPRK1, ARRB2, GNAZ, HINT1, Stat6, ABCB1, COMT, HRH1, ADRA2A, MC1R, TACR1, GCH1, DRD2, DRD3, HTR3A, HTR3B, HTR2A, HTR3C, HTR3D, HTR3E, HTR1, CNR1	Any strong opioid including morphine, oxycodone, fentanyl, methadone	Daily morphine (or morphine equivalent) dose Nausea and vomiting Extremes of pain relief**	Prospective observational study. Single time point

*modified from Droney *et al.* 2011

** A genome wide association study was carried out on a subset of patients (n=1008) using a pooled DNA approach

2.5 Opioid blood concentrations

Opioid concentrations in the blood vary depending on the route of drug administration, dose and frequency of dose, disease, liver and kidney function as well as a person's ethnicity, age and lifestyle. As a result these variables can have a considerable impact on the interpretation of drug concentrations found in various samples.

No study to date has been able to find a definitive correlation between opioid blood concentrations and clinical response. Therapeutic concentrations of morphine and oxycodone range from 0.01 – 0.07 mg/L and 0.015 – 0.062 mg/L in plasma samples, respectively (Moffat *et al*. 2011). Peak plasma concentrations after intramuscular administration of oxycodone were 0.034 mg/L whereas slightly higher peak plasma concentrations of 0.038 mg/L were found in the same patients when given an oral dose of oxycodone (Pöyhiä *et al*. 1993). However, cancer patients receiving daily oxycodone doses may have steady-state plasma concentrations at much higher concentrations than normal therapeutic doses. Hardy *et al*. (2011) reported plasma concentrations of 0.001 to 0.256 mg/L from cancer patients receiving 10 – 600 mg of sustained released oxycodone a day. Similarly concentrations equal to or above the therapeutic range were found in cancer patients receiving morphine sulphate tablets and morphine sulphate solution for an extended period of time (Neumann *et al*. 1982).

3 Rationale for Study

At present cancer pain management involves careful titration of an initial opioid (usually morphine) according to response (analgesia and side-effects), opioid switching if necessary, and further titration of the alternative opioid. There is currently no way of prospectively predicting an individual's response to opioids for cancer pain. In many instances patients experience significant pain and / or sideeffects during analgesic stabilisation which is a process based largely on trial and error. In terms of cancer pain, the development of a model incorporating clinical, genetic and metabolite data may potentially allow prospective prediction of response Pilot Trial: Personalising Opioid Therapy for Cancer Pain Relief

to different opioid drugs. Personalised prescribing involving choosing the correct dose of the correct opioid for each individual patient would result in more rapid pain management, reduced side-effects and potentially a better quality of life. The studies published to date have not provided any data that can be used in such modelling.

This pilot study has been designed to ascertain the feasibility and logistics of recruiting cancer patients, and explore genetic variations which may affect an individual's response to opioid analgesics. The investigators have considerable experience in carrying out similar studies in palliative care patient populations (Ross et al. 2005; Riley et al. 2006; Ross et al. 2006; Ross et al. 2008) so we are confident that the patient requirements for participation in this study are not too burdensome or lengthy. The information gathered from this pilot study will be taken forward to a large, multi-centre study which will have the aim of obtaining and integrating robust, meaningful clinical and laboratory data into a model to explore inter-individual variability in patient response to opioids.

4 Aims and Objectives

4.1 Primary Objectives

- Feasibility:
    - Evaluate the success rate of patient recruitment
    - Analgesic outcome (focussed observation over 7-10 days on commencement of step III opioid morphine/oxycodone).
    - Evaluate any variation between sites in palliative care procedures that might impact a larger multicentre trial.
- Explore the logistics of patient sample collection and analysis. 4.2 Secondary Objectives Exploration of polymorphisms within DNA sequences that may enhance or decrease opioid response in patient studied (analgesia and / or side-effect profile);

15 ○ Identify SNPs and the degree of methylation occurring on receptor, metabolising and transportation genes and determine its influence on opioid response.

- Establish whether oral and parenteral sample collection provides comparable information (DNA extraction; Opioid concentrations).

5 Study Design

This is a prospective non-interventional observational study. This is not a clinical trial of an investigative medicinal product. Study subjects will receive their normal clinical care. Involvement in this study will only require volunteers to complete a number of observational questionnaires and to provide blood and oral fluid samples (illustrated in Figure 1).

5.1 Centres

Three NHS Foundation Trusts will participate in this pilot study; Royal Bournemouth and Christchurch Hospitals, Poole Hospital, and the Royal Marsden Hospital (Fulham site, London, and Sutton site, Surrey).

5.2 Study Population

The aim is to enrol 200 patients with advanced cancer who are commencing use of Step III opioid analgesics.

5.3 Patient Eligibility Criteria

A patient will be eligible for inclusion in this study if all of the following criteria apply:

5.3.1 A cancer diagnosis

5.3.2 Requiring regular morphine or oxycodone, via any route, to relieve cancer related pain.

5.3.3 Age 18 years or above.

5.3.4 Able to give written informed consent.

5.3.5 Able to understand and complete questionnaires in English.

5.4 Patient Exclusion Criteria

A patient will not be eligible for the trial if any of the following criteria apply:

5.4.1 Does not consent to participate with the study.

5.4.2 Patients lack capacity to fully understand their requirements.

5.4.3 Patients who are pregnant.

5.4.4 Patients with predominately neuropathic pain.

5.4.5 Creatinine levels 1.5 x upper limit of normal

5.5 Patient Enrolment

5.5.1 All patients who are being started on a Step III opioid or being converted from another Step III analgesic (e.g. fentanyl) to morphine or oxycodone, will be considered for entry to the study and will be screened for inclusion according to the eligibility criteria above.

5.5.2 Inpatients and outpatients will be eligible for inclusion in this study.

5.5.3 Patients currently on Step II analgesia will be given a Patient Information Sheet. This would serve to educate the patient regarding the trial and empower them to contact the research team directly or indirectly to participate if they were to require Step III analgesia in the future.

5.5.4 Patients can discuss the objectives, risks, benefits, inconveniences, and their right to withdraw from the study with a member of the clinical team before deciding whether or not to participate in the study.

5.5.5 Patients will have at least 24 hours to read the Patient Information Sheet and discuss with family / friends / members of the clinical team before deciding whether or not to participate in the study.

5.5.6 Patients who do not enter the trial either because of personal preference or were missed by the recruitment team will be noted along with reasons for lack of inclusion (if patients volunteer a reason).

5.6 Consent and Confidentiality

5.6.1 Eligible patients will be approached by a member of the clinical research team.

5.6.2 Consent may be obtained from the patient during routine hospital visits; alternatively the patient will be visited by a member of the research team at their home address subject to the patient’s consent. Visits to patients’ homes will only occur if the patient has given permission for home visits

to take place. The patient's agreement to allow home visits will be recorded in the patient's notes.

5.6.3 Patients must sign and date an Informed Consent form after having read the Patient Information Sheet. Special considerations will be given to explain the implications of genetic testing and permission will be gained for collecting and storing samples for future analyses. No less than 24 hours will given from having being invited to taking consent for the study.

5.6.4 Three copies of the consent form will be made, one copy retained by the patient, one copy to be stored in the patient's medical record and the remaining copy to be stored in the study file.

5.6.5 On introduction to the study, patients would be assigned a unique study number and data collated will be anonymised. Only designated members of the team would have access to patient identifiers.

5.6.6 All data will be stored in line with principle of GCP, the Data Protection Act and trust policies regarding information governance.

5.7 Initial Patient Assessment

5.7.1 Information regarding patient's cancer type, prescribed medication for treating the cancer, concomitant medication (including other analgesic agents, laxatives and saliva substitutes), biochemistry and haematology results and liver and kidney function tests will be collated by a member of the research team.

5.7.2 Patient demographic data will also be collected, including age, gender, smoking history and ethnicity.

5.7.3 Before initial morphine or oxycodone dose or within 24-48 hours of initial dose, patients will be asked about pain severity using a validated modified Brief Pain Inventory questionnaire. This tool tests five dimensions of pain severity using an 11-point numerical rating scale.

5.7.4 Opioid-specific side effects will be assessed using a 4 point adjectival Likert scale (None, a little, quite a bit, and very much) which has been used in a number of studies and is adapted from the European Organisation for Research and Treatment of Cancer (EORTC) questionnaire.

5.7.5 On entry to the study, patients will be asked to fill in a constipation questionnaire that has been used in a number of previous studies.

5.7.6 On entry to the study oral health will be scored according to NCI CTC (common toxicity criteria) version 3.

5.8 Additional Patient Assessments

5.8.1 Patients will be asked to fill in a modified Brief Pain Inventory and opioid side-effect assessment within 24 hours of initial opioid dose and every other day until they are stabilised on an opioid or switched to an alternative opioid.

5.8.2 A constipation questionnaire will be completed when a stable opioid dose is reached or when the patient's opioid is switched to an alternative opioid.

a. The definition of whether a patient is a responder or non-responder to their opioid will be determined by both objective assessment of the clinical team and also whether or not they achieve adequate pain control. A patient will be considered a responder to the opioid and having reached a stable opioid dose when:

- the patient requires ≤ 2 breakthrough pain doses / per day,
- average pain score ≤4,
- minimal / acceptable side-effects (≤ "a little" on the likert scale).

There may be circumstances where the patient may be considered to have reached a stable opioid dose and therefore would be considered a "responder" but who do not fulfil these criteria (e.g. if the patient is

unable to carry out pain and side-effect assessments or if the patient is experiencing incident pain). The clinical reasons for deviation from these factors will be documented.

5.8.3 In the event a stable dose is not obtained because the patient does not achieve adequate pain control despite escalating opioid dose and / or experiences dose-limiting opioid side-effects, then he / she will be considered a non-responder to that opioid.

- the patient requires > 2 breakthrough pain doses / per day,
- average pain score > 4 for a period up to 10 days,
- unacceptable side-effects ("very much" on the likert scale).

5.8.4 Patients who do not respond to the initial study opioid will be switched to an alternative opioid. If the patient's alternative opioid is either morphine or oxycodone the patient will remain in the study and asked to complete the following questionnaires at the time intervals stated below:

- Modified brief pain inventory and side effect assessment within 24 hours of first opioid dose and every other day until they are stabilised on an opioid / switched to an alternative opioid
- Constipation questionnaire once patient is stable on the opioid / switched to an alternative opioid.

5.8.5 NCI CTC version 3 assessment of oral health will be scored whenever oral fluid is collected (see 5.9.1.2)

5.9 Sample Collection

5.9.1 Sample Collection

5.9.1.1 Once consent has been obtained, blood and oral fluid samples will be collected.

a. 2 mL whole blood will be collected in tubes containing EDTA for DNA analysis.

b. Buccal swabs will be collected using Catch-All™ Brushes for DNA analysis.

5.9.1.2 Following patient stabilisation on an opioid, a blood and oral fluid sample for opioid concentration analysis will be collected or when switched to an alternative analgesic.

a. 10 mL blood samples will be collected in sodium fluoride and potassium oxalate tubes for parent drug and metabolite analysis.

b. 2 mL oral fluid will collected using Concateno Certus oral fluid collection devices for parent drug and metabolite analysis.

5.9.1.3 In the event a stable dose is not obtained and the patients are switched to either oxycodone or morphine as the alternative opioid, the following blood and oral fluid samples will be collected:

a. 10 mL blood samples will be collected in sodium fluoride and potassium oxalate tubes for parent drug and metabolite analysis.

b. 2 mL oral fluid will collected using Concateno Certus oral fluid collection devices for parent drug and metabolite analysis.

5.10 Sample Analysis Location and Storage

5.10.1 Samples will initially be stored at their respective sites in freezers.

a. Whole blood preserved with EDTA for DNA analysis stored at -80°C.

b. Whole blood preserved with sodium fluoride and potassium oxalate for toxicological analysis stored at -80°C.

c. Catch-All™ buccal swabs for DNA analysis stored at -80°C.

d. Oral fluid samples in a pH 6 buffer will be stored at -80°C.

5.10.2 Tissue trackers will be in place at all sites in line with GCP.

5.10.3 Parent drug and drug metabolite analyses will be carried out at Bournemouth University.

a. Material transfer agreements (MTA) will be in place so that blood and oral fluid samples can be transported in batches to Bournemouth University, Applied Sciences department for toxicological analysis by P. McLaughlin.

5.10.4 Genetic analyses will be undertaken at Bournemouth University**,** Royal Bournemouth hospital Molecular Pathology department and Imperial College London. Relevant MTA will be in place to support this. External agencies may be recruited to design and run the genetic assays for this study.

5.10.5 Long term storage of;

a. DNA samples at Imperial College, London and Royal Bournemouth Hospital.

b. Blood and oral fluid samples used for drug analysis at Royal Bournemouth Hospital.

5.10.6 In the event of a participant withdrawing from the study, the subject has the option to request that their blood and oral fluid samples and their data are destroyed (explained in patient information sheet and consent process). If the subject does not request this then their samples and data will be used in this study.

a. Samples that have been requested to be destroyed will be incinerated at their respective hospital

5.11 Study Endpoints

5.11.1 Patients will have completed the study when they have:

a. Completed the modified Brief Pain Inventory and side effect and constipation assessments when stabilised on a study opioid / switched to a non study opioid.

b. Provided blood and oral fluid samples for parent drug and metabolite analysis when stabilised on a study opioid / switched to a non study opioid.

5.11.2 Patients will terminate the study prematurely if;

a. Non-response to opioid morphine / oxycodone and switched to an alternative opioid treatment.
b. Patient dies.
c. Patient stops taking Step III opioids on clinical grounds.
d. Patient clinically deteriorates or loses capacity and is unable to complete study requirements.

6 Sample Analyses

6.1 Genetic Analysis

6.1.1 Blood and oral fluid samples would be used to sequence the individuals DNA to help identify regions which affect their response to opioid analgesia. Sequences for genes involved in opioid metabolism, transportation and receptor genes will be obtained from online databases such as NCBI to help identify SNPs, methylated regions and design primers.

6.1.2 Candidate genetic variants which effect the metabolism, transportation and receptor sites for morphine and oxycodone, such as, but not limited to, μ-opioid receptor, β-arrestin2, stat6, UGT2B7, HNF1-α, OCT-1, MDR-1, COMT, CYP3A4, CYP3A5, □-opioid receptor will be examined and explored. Potential candidate genes and SNPs for which assays have already been validated in our laboratory are included in appendix 17. Samples will be genotyped using SSP-PCR.

6.1.3 Oral swabs will be collected using CatchAll™ Brushes and DNA quantified using Human Quantifiler® kit and the 48 well StepOne™ real-time PCR instrument (Applied Biosystems). DNA would be extracted from blood using the Qiagen DNeasy Blood and Tissue Kit and quantified in the same manner as the oral swab.

6.1.4 To identify new SNPs and areas of methylation, the Applied Biosystems Genetic Analyzer 310 will be used which has the ability to sequence regions up to 500 bp.

6.2 Opioid Quantification

6.2.1 Whole blood: Monitor opioid concentrations by quantifying morphine, oxycodone and their metabolites using Gas Chromatography – tandem Mass Spectrometry (GC-MS/MS) and/or High Pressure Liquid Chromatography – tandem Mass Spectrometry (HPLC-MS/MS)

6.2.2 Oral fluid: The oral fluid collected will be diluted with a buffer and analysed using GC-MS/MS or HPLC-MS/MS as per blood analysis.

7 Statistical Considerations

A study population of 200 has been chosen to ensure that meaningful data can be collected to explore the secondary objectives of this pilot trial. The primary objective, ascertaining the feasibility and logistics of carrying out a study of this nature, could be carried out with a smaller population however there are many variables within this study which will affect the statistical significance of the results we obtain. Patients will be taking either morphine or oxycodone, they will be of various ages, gender and ethnicity so a larger population is needed to determine genetic variants which affect patient response to morphine / oxycodone analgesics.

Initially the data will be explored using morphine responder versus non-responder as the primary clinical outcome. This study will focus on the early stage of morphine response versus non-response, which is likely to be due to pharmacokinetic and pharmacodynamic differences rather than later changes in response which are likely to be related to the development of tolerance. The clinical data will also be explored to define the clinical phenotype of opioid response using more detailed pain and side-effect scores. This study is powered to 200 based on the primary clinical phenotype of morphine responder versus non-responder. Up to 30% of cancer patients are “morphine non-responders”. Therefore it is expected that approximately 140 morphine responders and 60 morphine non-responders will be recruited to this

study. These numbers are comparable to studies previously carried out in this field (Rakvag *et al.* 2005; Ross *et al.* 2005). These numbers are sufficient to allow preliminary exploration of approaches for clinical phenotype definition incorporating pain and side-effect scores and to build preliminary multi-variate models of genegene and gene-environment interactions.

The patient samples will undergo genetic screening in addition to opioid quantification. Exploratory relationships found between DNA polymorphisms and analgesic effect will be noted and carried forward to a multi-centre study where statistical power will be obtained by using a large study population and sample size can be more reliably defined using the initial estimates. The indicative information provided by this study can aid the choice of genetic variations analysed in the larger collaborative study. This study requires multivariate analysis due to the volume of data and variable data to be collected. SPSS software will be used to find statistically significant associations and trends. Preliminary modelling will be carried out to explore gene-gene and gene environment interactions.

7.1 Hypotheses

Null hypothesis 1: There is no relationship between pain control and opioid:

a) Receptor phenotypes;
b) Transporter phenotypes;
c) Metabolising phenotypes.

Null hypothesis 2: There is no relationship between side effects and opioid:

a) Receptor phenotypes;
b) Transporter phenotypes;
c) Metabolising phenotypes.

Null hypothesis 3: There is no methylation in the:

a) Receptor;
b) Transporter;
c) Metabolising

genes that will affect the patients response to opioid analgesia.

Null hypothesis 4: There is no association between opioid concentrations, parent drug or metabolites, and opioid:

a) Receptor phenotypes;
b) Transporter phenotypes;
c) Metabolising phenotypes.

Null hypothesis 5: Oral swabs and blood samples will be equal in enabling quality DNA extraction.

Null hypothesis 6: Oral fluid and blood samples will be equally sensitive for opioid concentration evaluation.

7.2 Statistician

Professor Peter Thomas, Professor of Health Care Statistics and Epidemiology, Bournemouth University, Dorset.

8 Safety Reporting

This is an observational non-interventional study. This trial is designed to identify patients who are not responders to either morphine or oxycodone i.e. they may experience inadequate analgesia or they may experience side-effects from morphine or oxycodone. The clinical and research team may be alerted to the fact that the patient is not responding to their current opioid by the patient presenting with "opioid toxicity". In some instances these side-effects or lack of adequate analgesia may necessitate hospitalisation. The presence of opioid-specific side-effects or hospitalisation in this instance is not considered an adverse event and will be recorded on the relevant CRF in the relevant section.

Many, if not most, of the patients who are entered onto this trial will have advanced metastatic cancer. These patients have a limited prognosis and withdrawal of some patients due to death or disease progression is expected and will be recorded. These are expected outcomes in this study population and do not constitute adverse events. The clinical team will be informed of response to the opioid as documented in the study files.

The following trial-specific situations will be recorded in the CRF and documented as adverse events (AE):

- Death due to opioid toxicity (i.e. respiratory depression that is deemed by medical judgement to be due to opioid medication).
- Opioid toxicity that is life-threatening (i.e. respiratory depression that is deemed by medical judgement to be due to opioid medication).

All members of the research team who observe an AE are responsible for reporting it to the Consultant in Charge immediately.

8.1 Protocol Violations

8.1.1 Protocol violations are any unplanned or unintended changes or deviations from the approved study protocol, consent document or recruitment process. Protocol violations may be major or minor in severity.

8.1.2 Record of both major and minor protocol violations will be kept prospectively by the research team using the trial protocol violation log. The log pertaining to each study subject will be kept in their CRF.

8.1.3 A major protocol violation is a deviation that has an impact on subject safety, may substantially alter risks to subjects, may have an effect on the integrity of the study data, or may affect the subject's willingness to participate in the study. Major protocol violations must be reported to the Study Sponsor within 24 hours, and CCR and REC must be notified in a timely fashion. The PI has ultimate responsibility for reporting protocol violations to the Study Sponsor. The Study Sponsor must report the major violation to MHRA within 5 working days. Examples of major violations include;

- Failure to obtain informed consent from the subject.
- Enrolling a subject who does not meet the inclusion and exclusion criteria.

8.1.4 A minor protocol violation is one that does not impact subject safety, compromise the integrity of the study data or affect the subject's willingness to participate in the study. Minor protocol violations do not need to be reported immediately. They are documented and submitted to

R&D department at annual intervals. Examples of minor violations include;

- Use of unapproved recruitment procedures or materials (slightly altered).
- Inappropriate consent process documentation (dated by someone other than the subject, missing signature of person obtaining consent, incorrect date on consent).
- Use of expired or outdated consent or CRF documentation.
- Due to the poorly nature of many of the study subjects, it is likely that study visits or completion of questionnaires may occur outside the protocol-prescribed window (for example if the subject was on holiday, unable to be contacted, or was ill and was late for assessment). Similarly some patients may decline or postpone blood or oral fluid sampling. These will not be considered protocol violations as part of this study but the dates of the assessments etc will be recorded in the CRF. These data will be important in the feasibility analysis.

9 Quality Control and Assurance

9.1 Trial monitoring committee

The Royal Bournemouth Hospital Trial Management Group will monitor the conduct and progress of the trial.

9.2 Committee for clinical research

Approval has been granted by the R & D department at the Royal Bournemouth Hospital.

9.3 Ethical approval

Ethical approval has been sought from the South Central Berkshire B Ethics Committee.

10 Financial and Insurance Matters

10.1 Study funding

The trial will be funded by Royal Bournemouth Hospital and Bournemouth University.

10.2 Trial sponsor

Royal Bournemouth Hospital has accepted the role of sponsor for this study.

11 Publication Policy

11.1 Informing subjects of results

Subjects are asked if they would like to be informed of the results of this study during the process of consent and their answer is recorded on the consent form. If they agree to being informed, results will be disseminated by post when the results are available. Results will be presented to the academic staff at Bournemouth University as well as the staff at The Royal Bournemouth Hospital, Poole Hospital and The Royal Marsden at hospital meetings.

11.2 Dissemination of results

Information from this study will be disseminated nationally and internationally.

11.3 Peer review

Results of the trial will be collated, analysed and presented for publication in peer review scientific journals.

12 National/Local Implications for Future Practice, Education and Research

12.1 Change to clinical practice

The information obtained from this pilot trial regarding patient recruitment rate, drop-out rate, compliance with study requirements and potential genetic variations involved with opioid response, will enable a large multi-centre study to be carried out. A substantial palliative care population will then be recruited so as to ascertain links between genetic variations and opioid response.

If genetic variants can be linked with opioid response it will allow physicians to administer an appropriate opioid and an effective dose rather than prescribing opioids on a trial-by-error basis. This will not only reduce discomfort of the cancer patient but also reduce costs to the health care systems as analgesics would be used effectively and the need to administer drugs for side effects would also be reduced.

12.2 Predictive model

A predictive model of an individual's likely tolerance to a particular opioid will enable appropriate, evidenced-based prescribing of Step III analgesics. A model would minimise the development or avoidance of intolerable side effects and, overall, improve in quality of life for each patient.

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APPENDIX B1.1

INCLUSION AND EXCLUSION CRITERIA

Pilot Trial: Personalising Opioid Therapy for Cancer Pain Relief

Centre: …… **Date:** …… (dd/mm/yyyy)

Patient Identification Number: ……

INCLUSION

*The answer must be **YES** to all the questions below to be eligible for entry into the study:*

Have a cancer diagnosis.	Yes	No
Requiring regular morphine or oxycodone, via any route, for cancer related pain.		No
Age 18 years or above.	Yes	No
Able to give written informed consent.	Yes	No
Able to understand and complete questionnaires in English.	Yes	No

EXCLUSION

*The answer must be **NO** to all the questions below to be eligible for entry in to the study:*

Does not consent to participate with the study. No		Yes
Patient lacks capacity to fully understand their requirements. No		Yes
Patient is pregnant. No		Yes
Patient has predominately neuropathic pain. No		Yes
Creatinine clearance 1.5 x upper limit of normal No		Yes

ELIGIBILITY

The patient is suitable to be included in the study?	Yes	No

If Yes;

Date Patient Information Sheet and Consent Form received: ……..

(dd/mm/yyyy)

The Royal Bournemouth and Christchurch Hospitals NHS Foundation Trust

APPENDIX B1.2

PATIENT

INFORMATION SHEET

Pilot Trial: Personalising Opioid Therapy for Cancer Pain Relief

'We would like to tell you about a research study that you might like to consider participating in. It is important for you to understand why the research is being undertaken and what it will involve. Please take the time to read the following information carefully and discuss it with friends, relatives and your doctor if you wish. Ask your doctor or nurse if there is anything that is not clear or if you would like more information. Thank you for reading this.' **What is the purpose of the study?**

A number of studies have been carried out that found links between a person's genetic make-up and how they respond to pain killers. However, these studies have either only a small number of participants which means that their findings may not represent the wider population or they have not collected adequate information about the effectiveness and side effects of the pain killers. We would like to carry out a study to evaluate the difficulties associated with enrolling patients into studies and monitor how well participant requirements are fulfilled. This study will also help establish the costs and time restraints involved in exploring patient samples for genetic make-up variations and pain killer concentrations. The information gained from this pilot study will be carried forward to a large national study in which we hope to identify links between genetic make-up variations and pain relief response. The ultimate goal is to be able to give patients the right pain killer at the right dose first time so effective and fast pain relief can be obtained.

What are we researching and why?

A number of pain killers can be prescribed for the treatment of cancer pain, the two most widely used being morphine and oxycodone. The majority of people who receive morphine obtain successful pain relief, however a small number of people find that morphine is not effective in relieving pain and that it causes side effects. In the event that patients do not receive adequate pain relief from morphine, oxycodone has been recommended as an alternative pain killer. Nonetheless, ineffective pain relief and side effects have been reported with the use of this drug also.

At the moment, the most effective pain relief drug for cancer patients cannot be predicted, nor can the drug which will give the least side effects. It is possible that because of individual differences in our genetic make-up, some people will achieve better pain control and fewer side effects by taking morphine whilst others will find greater benefit with oxycodone. We are interested to know if by identifying differences in individual genes we

will be able to predict, in the future, which pain killers will work better for individual patients with fewer or no side effects.

Why would I be invited to take part?

Your clinician may decide to start you on a new medication for your pain. If your clinician chooses either morphine or oxycodone to relieve your cancer related pain we would like to record the effectiveness and possible side effect profile of these medications.

Do I have to take part in the study?

It is up to you to decide whether or not to take part. If you decide to take part you are still free to change your mind or withdraw at any time and without giving a reason. This will not affect the standard of treatment or care you receive.

What will happen to me if I take part?

You will be asked to complete questionnaires about your pain and any side effects that you experience at the start of the study and every other day until you are stable on a drug. You will also be asked detailed questions about your bowel function at the start of the study and again once you are stable on either morphine or oxycodone. Constipation is a common side-effect of morphine and one which may be improved with laxatives. The clinical team will adjust the dose of your laxatives on each occasion in order to minimise constipation in conjunction with the NHS Hospital Palliative Care symptom control guidelines.

You will be asked to provide blood and oral fluid samples before you start your new medication and once you are stable on a pain killer. In the event that you do not respond to the initial pain killer, a blood and oral fluid sample will be taken and you will be switched to an alternative drug. If this drug is either morphine or oxycodone we will ask you to provide additional blood and oral fluid samples until you are stable on the second pain killer. The use of any drugs other than morphine and oxycodone to manage your cancer pain means you can no longer participate in the study.

Participation in this study will not affect or delay your pain relief treatment.

What will happen to my samples?

The samples collected will be used to analyse the genes involved in response to and breakdown of morphine and oxycodone. If you choose to withdraw from the study we will retain your samples unless you request that your samples be destroyed.

We would like to store your blood and oral fluid samples for possible future tests and if you consent to take part, this includes permission for us to do so. We will only do research related to pain and side effect profiles on your samples. We will not seek further permission from you for doing any

additional research, but approval will be obtained from the local Ethics Committee for any further research using your donation.

What are the possible side effects?

Common side effects of morphine and oxycodone include drowsiness, sickness, constipation, confusion, hallucinations and itchiness. Most of these side effects tend to occur at the beginning of treatment and usually wear off after several days. If symptoms persist it may be necessary to either reduce the dose of drug or change to an alternative medication.

What are the possible benefits of taking part?

This study will not directly benefit you however the results from this research may enable us to find out more about why some people react differently to painkillers and this will benefit future patients by allowing us to improve our pain management protocols.

What happens when the research study stops?

If your pain has improved, you will continue the treatment that you have been allocated for as long as you need it.

Will my taking part in this study be kept confidential?

If you consent to take part in the research any of your medical records may be looked at by a member of the research team. Your name, however, will not be disclosed outside the hospital. You will not be identified in any report or publication that arises as a result of the study. With your permission, your GP and community team will be informed about the study and that you are participating in it.

What will happen to the results of the research study?

Results of this study are likely to be available in 2014/2015. They will be presented in a range of publications.

Who is organising and funding the research?

The research will be funded by Royal Bournemouth Hospital, Bournemouth.

Who has reviewed the study?

South Central Berkshire B Ethics Committee has approved this study.

What now?

If you decide to voluntarily take part in this study, then you'll be asked to sign a consent form. You will be given not less than 24 hours to make your decision as to whether you wish to take part. With your permission you may be visited at your home by a member of the research team who will go through the study process thoroughly with you and obtain consent. Ideally we would like to see you before you start the new pain killers as we would

like to gather some baseline information. However, if you agree to participate and the initial questionnaires have been taken within 48 hours of your first dose of morphine or oxycodone you will still be able to participate in the study.

What if I have any concerns?

If you have any concerns or other questions about this study or the way it has been carried out, you should contact your study doctor (see below) on telephone number (see below), or you may contact the hospital complaints department.

Any complaints will be assessed on a case by case basis and will be dealt with or forwarded on to the relevant regulatory bodies as required. We recommend that you obtain a copy of your hospitals complaints procedure or policy if you intend to make a complaint.

Contact for Further Information

Thank you for reading this information sheet. If you require any further information then please contact the following: **Dr. Tamas Hickish on 01202 704789 (Consultant)**

He can discuss any aspect of the study with you and change your treatment if necessary. There will always be someone to talk to you at any time of the day or night.

The Royal Bournemouth and Christchurch Hospitals NHS Foundation Trust

APPENDIX B1.3

CONSENT FORM

Pilot Trial: Personalising Opioid Therapy for Cancer Pain Relief

Centre: …… **Date:** …… (dd/mm/yyyy)

Patient Identification Number: ……

Name of Chief Investigator: **Dr. Tamas Hickish**

	Please INITIAL boxes
1. I confirm that I have read and understand the information sheet dated……..... (Version……) for the above study and that I have had an opportunity to ask questions which have been answered to my satisfaction.	☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving a reason, and my medical care and legal rights will not be affected.	☐
3. I agree to give samples of blood and oral fluid for research in this project. I understand how the sample will be collected and that giving a sample for this research is voluntary.	☐
4. I understand that should I withdraw from this study I can request that my data and blood and oral fluid samples will be destroyed.	☐
5. I understand that I will be asked to complete questionnaires regarding the pain I experience as well as side effects felt at intervals throughout my treatment.	☐
6. I am willing to allow access to my medical records to check that the study is being carried out correctly. I have been assured that strict confidentiality will be maintained.	☐
7. I understand that my doctor and / or I, (as appropriate) will be informed if any of the results of the medical tests done as a part of the research are important for my health.	☐
8. I understand that I will not benefit financially if this research leads to the development of a new treatment or medical test.	☐

9. I agree that my GP may be notified about my participation in this study.

10. I agree to give a sample of blood and oral fluid for research in this project. I understand that my blood and oral fluid samples will be stored and I agree that samples may be sent to Bournemouth University and Imperial College for analysis.

I agree that my samples may be used for possible use in future projects related to pain, response to opioids and side effect profiles. I understand that these projects will be carried out by researchers from the Department of Palliative Medicine at the Royal Marsden Hospital and Bournemouth University. The investigators will not seek further permission from me for doing any additional research, but approval will be obtained from the local Ethics Committee for any further research using my donation. ☐

11. I understand that this study will include genetic research aimed at understanding more about genetic differences and individual response to morphine and oxycodone. ☐

12. I understand that the results of these investigations are unlikely to have any implications for me personally. ☐

I would like to be informed of the results of this study. Yes / No ☐

.....	.....	.....
Name of Patient (BLOCK CAPITALS)	Date	Signature
.....	.....	.....
Name of Person obtaining consent (If different from Principal Investigator)	Date	Signature
.....	.....	.....
Principal Investigator	Date	Signature

The Royal Bournemouth and Christchurch Hospitals NHS Foundation Trust

APPENDIX B1.4

Poole Hospital
Longfleet Road
Poole
Dorset
B

H15 2JB Date: Dear Dr.

RE:

I am writing to inform you that this patient has kindly consented to take part in the following study which has been approved by South Central Berkshire B ethics committee:

Pilot Trial: Personalising Opioid Therapy for Cancer Pain Relief

Patients whom require the transition from a Step II opioid to a Step III opioid, either morphine or oxycodone, are being approached to participate in the study. A blood and oral fluid sample will be taken to determine their genetic phenotype and opioid metabolite levels. The patients will also be asked to complete pain and side effect questionnaires.

The patients will be asked to provide samples and complete the questionnaires:

A: At time of entry to the study.
B: Alternate days from initial dose (pain and side effect questionnaires only).
C / D: Stabilised on first line opioid / Before opioid switch if non-response to first line opioid. **E:** Alternate days from initial dose of second Step III opioid (pain and side effect questionnaires only).
F / G: Stabilised on second line opioid / Before opioid switch if non-response to second line opioid.

It is anticipated that stability on an opioid takes between 7-10 days. However, if the patient does not respond to the initial analgesic the maximum duration for a patient to be involved in the study is up to 28 days.

The pain questionnaire will involve the patients rating their pain on a scale of “0” (no pain) to “10” (worst imaginable pain). The side effect questionnaire will ask which side effects they experience and at what severity. The constipation assessment will ask for descriptions of their stool movement over the period of a week.

I enclose the patient information sheet. If you require any further information about the study, please do not hesitate to contact me.

Yours sincerely,

Dr. Tamas Hickish

01202 704789

DEMOGRAPHIC INFORMATION

Pilot Trial: Personalising Opioid Therapy for Cancer Pain Relief

Centre: …… **Date:** ……
(dd/mm/yyyy)

Patient Identification Number: ……

Gender Male / Female **Date of Birth** .....
(dd/mm/yyyy)

Cancer Diagnosis
.....

Height .....(cms) **Weight** .....(Kgs)

Is your Natural Hair Colour Red? Yes / No

Ethnicity

White;
British ☐
Irish ☐
Other, please specify ☐ .....

Black;
Caribbean ☐
African ☐
Other, please specify ☐ .....

Asian;
Indian ☐
Pakistani ☐
Bangladeshi ☐
Other, please specify ☐ .....

Mixed;
White and Black Caribbean ☐
White and Black African ☐
White and Asian ☐
Other, please specify White and ☐ .....

Jewish; ☐
Sephardic
Ashkenazi

Chinese ☐

Other Ethnic Background, please specify
……

Quantity Smoked, on average, per week;

None ☐
1-10 ☐
11-20 ☐
21-30 ☐
>20 ☐

Number of hours spent excercising, per week;

None ☐
1-10 ☐
11-20 ☐
21-30 ☐
>20 ☐

MODIFIED BRIEF PAIN INVENTORY

Pilot Trial: Personalising Opioid Therapy for Cancer Pain Relief

Centre: …… **Date:** ……
(dd/mm/yyyy)

Patient Identification Number:

……

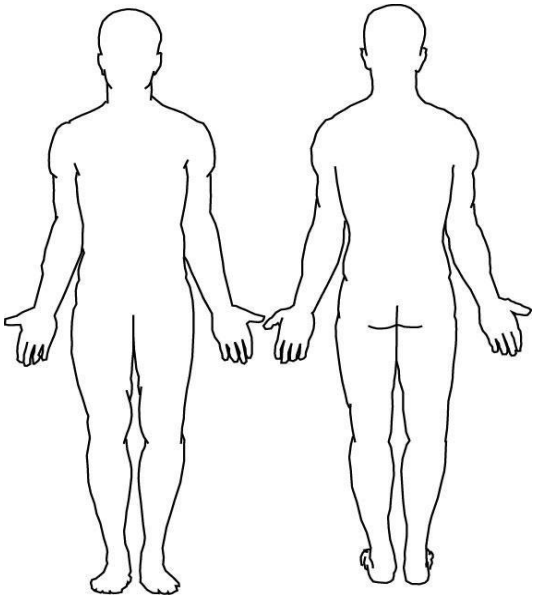
Time points: (please circle)

A: At time of entry to the study.
B: Alternate day from initial dose (initial opioid).
C: Stabilised on first line opioid (approx one week).
D: Before second alternative opioid, in the event of non-response to first opioid.
E: Alternate day from initial dose (alternative opioid).
F: Stabilised on second line opioid (approx one week post-switch).
G: Before third alternative opioid, in the event of non-response to first opioid.

1. **Throughout our lives, most of us have had pain from time to time (such as minor headaches, sprains and toothaches). Have you had pain other than these everyday kinds of pain today?**

   1. Yes 2. No

2. **On the diagram, shade in the areas where you feel pain.**



3. What type of pain is the participant describing? (please circle)

Somatic	Body surface (usually sharper and may have a burning or pricking quality)or deep tissues (i.e. musculoskeletal)
Somatic (bony)	dull ache within bones or tender hot spot
Visceral	Pain due to infiltration, compression, extension or stretching of the thoracic, abdominal or pelvic viscera (internal organs within a cavity). Visceral pain is usually not well localized and described pressure-like/squeezing.
Neuropathic	Due to injury to the nervous system, usually burning or tingling.

PAIN SCORES

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

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

2. **Please rate your pain by circling the one number that best describes your pain at its ***least*** in the last 24 hours.**

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

3. **Please rate your pain by circling the one number that best describes your pain on ***average*.**

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

4. **Please rate your pain by circling the one number that describes how much pain you have ***right now*.**

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

5. **In the last 24 hours, how much ***relief*** have pain treatments or medications provided for your pain? Please circle the one percentage that most shows how much relief you have received?**

   0% 10% 20% 30% 40% 50% 60% 70% 80% 90% 100%
   No relief *Complete relief*

Investigators Signature: Date:

B1.8 SIDE EFFECT SCORES

Pilot Trial: Personalising Opioid Therapy for Cancer Pain Relief

Centre: …… **Date:** ……
(dd/mm/yyyy)

Patient Identification Number: ……

Time points: (please circle)

A: At time of entry to the study.
B: Alternate day from initial dose (initial opioid).
C: Stabilised on first line opioid (approx one week).
D: Before second alternative opioid, in the event of non-response to first opioid.
E: Alternate day from initial dose (alternative opioid).
F: Stabilised on second line opioid (approx one week post-switch).
G: Before third alternative opioid, in the event of non-response to first opioid.

During the Past Week, have you:

	Not at All	A Little	Quite a Bit	Very Much
Felt Nauseous?	0	1	2	3
Vomited?	0	1	2	3
Had a Dry Mouth	0	1	2	3
Had Constipation?	0	1	2	3
Had Diarrhoea?	0	1	2	3
Been Drowsy?	0	1	2	3
Felt Confused, Disorientated or had Hallucinations?	0	1	2	3
Had Bad Dreams?	0	1	2	3

Please record any other adverse effects you have experienced with either morphine or oxycodone, and score appropriately as above:

Symptom	**Not at All**	**A Little**	**Quite a Bit**	**Very Much**

……	0	1	2	3
……	0	1	2	3

B1.9

CONSTIPATION ASSESSMENT

Pilot Trial: Personalising Opioid Therapy for Cancer Pain Relief

Centre: …… **Date:** ……
(dd/mm/yyyy)

Patient Identification Number:
……

Time points: (please circle)

A: At time of entry to the study.
C: Stabilised on first line opioid (approx one week).
D: Before second alternative opioid, in the event of non-response to first opioid.
F: Stabilised on second line opioid (approx one week post-switch).
G: Before third alternative opioid, in the event of non-response to first opioid.

Thank you for taking part in this study. Many of these questions appear similar. Please answer them individually.

Are you satisfied with how your bowel has functioned during the PAST WEEK? Please Circle

YES NO

In the PAST WEEK have you been constipated? Please Circle

Not at All A Little Quite a Bit Very Much

In the PAST WEEK please rate how constipated you have been?

Please Circle

0 1 2 3 4 5 6 7 8 9 10

Not at All

Worst Imaginable

What does being constipated mean to you?

…….

……

Constipation Assessment

1

During the PAST WEEK has your bowel activity been;

More constipated — Usual for you ☐ — Less constipated

a) A little more constipated ☐ — a) A little less constipated ☐
b) Quite a bit more constipated ☐ — b) Quite a bit less constipated ☐
c) A lot more constipated ☐ — c) A lot less constipated ☐

During the PAST WEEK did you open your bowels;

More frequently — Usual amount ☐ — Less frequently

a) A little more frequently ☐ — a) A little less frequently ☐
b) Quite a bit more frequently ☐ — b) Quite a bit less frequently ☐
c) A lot more frequently ☐ — c) A lot less frequently ☐

During the PAST WEEK how many times did you open your bowels?

More than once a day ☐ _____ times a week ☐ None at all ☐

Everyone's USUAL bowel activity is different. How many times a week do you USUALLY open your bowels?

_____

During the PAST WEEK what has your stool consistency been like in general? (Please tick)

Type 1		Separate hard lumps, like nuts (hard to pass) ☐
Type 2		Sausage-shaped but lumpy ☐
Type 3		Like a sausage but with cracks on the surface ☐
Type 4		Like a sausage or snake, smooth and soft ☐
Type 5		Soft blobs with clear-cut edges ☐
Type 6		Fluffy pieces with ragged edges, a mushy stool ☐
Type 7		Watery, no solid pieces. **Entirely Liquid** ☐

Constipation Assessment

2 3
B1.9

During the PAST WEEK has your stool been HARD? (Please circle)

Never Rarely Occasionally Nearly always

During the PAST WEEK did you have to STRAIN to open your bowels? (Please circle)

Not at all A little Quite a bit Very much

Laxatives taken during the PAST WEEK:			
Laxative Name	Dose and how often taken a day?	How many days was the medication taken?	Any side effects with this medication?

During the PAST WEEK how active have you been?

☐ Fully active, able to carry out all activities without restriction

☐ Fully mobile, able to carry out light work. Unable to carry out physically strenuous activity

☐ Mobile, capable of self-care, unable to carry out any work. Up and about more than 50% of waking hours

☐ Unable to carry out self-care. Totally confined to bed or chair

During the PAST WEEK what has your food intake been like? (Please circle)

As usual Less than usual More than usual

During the PAST WEEK what has your fibre intake been like? (Please circle)

As usual Less than usual More than usual

During the PAST WEEK what has your fluid intake been like? (Please circle)

As usual Less than usual More than usual

Investigators Signature: **Date:**

APPENDIX B1.10

OPIOID HISTORY AND TITRATION

Pilot Trial: Personalising Opioid Therapy for Cancer Pain Relief

Centre: …… **Date:** ……(dd/mm/yyyy)

Patient Identification Number: ……

Time points:

A: At time of entry to the study.
B: Alternate day from initial dose (initial opioid).
C: Stabilised on first line opioid (approx one week).
D: Before second alternative opioid, in the event of non-response to first opioid.
E: Alternate day from initial dose (alternative opioid).
F: Stabilised on second line opioid (approx one week post-switch).
G: Before third alternative opioid, in the event of non-response to morphine / oxycodone.

PREVIOUS STEP 2 ANALGESIC	**APPROX. START DATE**	**APPROX. FINISH DATE**
Name:		
Dose:		
Time Point A: STEP 3 ANALGESIC (PLEASE CIRCLE)		
Morphine	Oxycodone	
Dose:	**Frequency of Dose**:	
TITRATION		**TIME/DATE**
Time Point B		
Dose:		
Frequency of Dose:		

Breakthrough Opioid: Dose: Frequency of dose:	
Time Point B	
Dose:	
Frequency of Dose:	
Breakthrough Opioid: Dose: Frequency of dose:	

Opioid history and titration

TITRATION (Cont.)	**TIME/DATE**
Time Point B	
Dose:	
Frequency of Dose:	
Breakthrough Opioid: Dose: Frequency of dose:	
Time Point C	
Dose:	
Frequency of Dose:	
Breakthrough Opioid: Dose: Frequency of dose:	
Time Point D	
Dose:	
Frequency of Dose:	
Breakthrough Opioid: Dose: Frequency of dose:	
Time Point E*	
Morphine Oxycodone	
Dose:	
Frequency of Dose:	
Time Point E	
Dose:	
Frequency of Dose:	
Breakthrough Opioid: Dose: Frequency of dose:	

Time Point E	
Dose:	
Frequency of Dose:	

APPENDIX B1.10

TITRATION (cont.)	**DATE**
Breakthrough Opioid: Dose: Frequency of dose:	
Time Point F	
Dose:	
Frequency of Dose:	
Breakthrough Opioid: Dose: Frequency of dose:	
Time Point G	
Opioid:	
Dose:	
Frequency of Dose:	

*In the event that the patient is not stabilised on either morphine or oxycodone and is switched to a non-study opioid complete Time Point G.

ADDITIONAL TIME POINTS	**DATE**
Time Point ___	
Dose:	
Frequency of dose:	
Breakthrough Opioid: Dose: Frequency of dose:	
Time Point ___	
Dose:	
Frequency of dose:	
Breakthrough Opioid: Dose: Frequency of dose:	
Time Point ___	
Dose:	
Frequency of dose:	
Breakthrough Opioid: Dose: Frequency of dose:	
Time Point ___	
Dose:	
Frequency of dose:	
Breakthrough Opioid: Dose: Frequency of dose:	

APPENDIX B1.11

Criteria for changing to an alternative opioid

Pilot Trial: Personalising Opioid Therapy for Cancer Pain Relief

Centre: …… **Date:** ……
(dd/mm/yyyy)

Patient Identification Number: ……

Please give reason for changing to an alternative opioid

1) **Uncontrolled Pain** ☐

2) **Opioid Toxicity**

Side effects which are intractable despite appropriate intervention and at a level unacceptable to the patient)

- Nausea ☐
- Vomiting ☐
- Constipation ☐
- Drowsiness ☐
- Hallucinations ☐
- Nightmares ☐
- Pruritis ☐
- Myoclonus ☐
- Any other symptom

Describe…….

Was patient already on medications to control this symptom? Yes / No If so, what (Medication and dose)?

.....

Table 1 Concentrations and volumes of DNA samples sent to LGC Genomics for SNP analysis

Name	Type	DNA conc. (ng/µL)	Vol (µL)	Name	Type	DNA conc. (ng/µL)	Vol (µL)	Name	Type	DNA conc. (ng/µL)	Vol (µL)	Name	Type	DNA conc. (ng/µL)	Vol (µL)
M01	Plasma	4	50	**B10**	Buccal	17	40	**424_12**	Liver	28	40	**DC06**	Blood	6	30
M02	Buccal	23	40	**B11**	Buccal	23	40	**433_11**	Psoas	20	40	**DC07**	Blood	5	25
M03	Buccal	14	35	**B12**	Buccal	15	40	**448_12**	Psoas	21	40	**DC08**	Blood	5	20
M04	Buccal	21	40	**B13**	Buccal	19	40	**480_11**	Blood	22	40	**DC09**	Blood	15	20
M05	Buccal	16	35	**B14**	Buccal	13	40	**482_11**	Blood	12	30	**DC10**	Blood	8	30
M06	Buccal	27	40	**B15**	Buccal	26	40	**494_12**	Blood	6	40	**DC11**	Blood	7	35
M07	Buccal	18	35	**B16**	Buccal	16	40	**500_11**	Blood	18	40	**DC12**	Blood	26	40
M08	Buccal	21	35	**B17**	Buccal	22	40	**535_11**	Blood	18	40	**DC13**	Blood	23	20
M09	Buccal	19	35	**B18**	Buccal	16	40	**585_11**	Blood	22	40	**DC14**	Blood	9	40
M10	Plasma	3	50	**B19**	Buccal	17	40	**576_11**	Psoas	19	40	**DC15**	Blood	21	40
M11	Buccal	17	40	**B20**	Buccal	19	40	**582_11**	Blood	13	30	**DC16**	Blood	7	20
M12	Buccal	19	40	**055_12**	Blood	15	35	**604_10**	Liver	21	40	**DC17**	Blood	13	40
M13	Buccal	24	40	**089_12**	Liver	26	40	**618_10**	Liver	30	40	**DC18**	Blood	19	15
M14	Buccal	14	40	**147_11**	Blood	22	40	**619_10**	Blood	5	40	**DC19**	Blood	12	35
M15	Buccal	24	40	**163_11**	Blood	16	40	**636_12**	Blood	9	40	**DC20**	Blood	8	30
M16	Buccal	26	40	**182_12**	Liver	17	40	**649_12**	Psoas	16	40	**DC21**	Blood	18	30
M17	Buccal	26	40	**207_12**	Blood	19	40	**652_10**	Blood	17	40	**DC23**	Blood	11	40
M18	Buccal	20	40	**217_12**	Blood	14	40	**704_10**	Liver	25	40	**DC24**	Blood	15	25
M19	Buccal	20	40	**245_12**	Blood	22	40	**737_10**	Liver	18	40	**DC25**	Blood	15	20
M20	Buccal	19	40	**288_11**	Psoas	8	40	**779_11**	Blood	21	40	**DC26**	Blood	16	40
B01	Buccal	15	40	**336_12**	Blood	18	40	**781_10**	Liver	16	40	**DC27**	Blood	18	40
B02	Buccal	20	40	**345_12**	Blood	10	40	**801_10**	Blood	15	35	**DC29**	Blood	11	30
B03	Buccal	11	30	**346_12**	Blood	16	35	**818_10**	Blood	22	40	**DC31**	Blood	17	40

Table 4 continued: Concentrations and volumes of DNA samples sent to LGC Genomics for SNP analysis

Name	Type	DNA conc. (ng/µL)	Vol (µL)	Name	Type	DNA conc. (ng/µL)	Vol (µL)	Name	Type	DNA conc. (ng/µL)	Vol (µL)	Name	Type	DNA conc. (ng/µL)	Vol (µL)
B04	Buccal	20	40	**380_12**	Blood	14	40	**863_11**	Blood	16	40	**DC32**	Blood	17	40
B05	Buccal	19	35	**383_11**	Liver	23	40	**873_11**	Blood	16	40	**DC33**	Blood	13	40
B06	Buccal	21	40	**383_12**	Blood	23	40	**DC01**	Blood	7	35	**DC34**	Blood	14	40
B07	Buccal	16	40	**388_12**	Liver	24	40	**DC02**	Blood	10	30	**DC35**	Blood	6	40
B08	Buccal	17	40	**396_11**	Liver	23	40	**DC04**	Blood	6	30	**DC36**	Blood	12	30
B09	Buccal	21	40	**398_11**	Blood	7	40	**DC05**	Blood	5	20	**DC37**	Blood	16	30
DC38	Blood	24	40	**DC70**	Blood	16	25	**FH4**	Buccal	21	40	**RMH36**	Plasma	3	50
DC39	Blood	17	40	**DC71**	Blood	8	35	**FH5**	Buccal	16	40	**RMH37**	Plasma	3	50
DC40	Blood	14	30	**DC72**	Blood	14	40	**MU1**	Buccal	12	40	**RMH38**	Plasma	3	50
DC41	Blood	15	30	**DC73**	Blood	15	40	**MU2**	Buccal	16	40	**RMH39**	Plasma	4	50
DC44	Blood	14	40	**DC74**	Blood	17	40	**RMH02**	Plasma	3	50	**RMH40**	Plasma	1	50
DC45	Blood	8	30	**DC75**	Blood	22	40	**RMH03**	Plasma	3	50	**RMH43**	Plasma	4	50
DC46	Blood	17	40	**DC76**	Blood	25	40	**RMH04**	Plasma	5	50	**RMH44**	Plasma	2	50
DC47	Blood	5	30	**DC77**	Blood	17	40	**RMH05**	Plasma	2	50	**RMH45**	Plasma	2	50
DC48	Blood	8	30	**DC78**	Blood	17	40	**RMH07**	Plasma	3	50	**RMH47**	Plasma	3	50
DC49	Blood	18	40	**DC79**	Blood	10	40	**RMH08**	Plasma	3	50	**RMH48**	Plasma	4	50
DC50	Blood	8	40	**DC80**	Blood	23	40	**RMH10**	Plasma	6	50	**RMH49**	Plasma	3	50
DC51	Blood	9	25	**DC81**	Blood	21	20	**RMH11**	Plasma	3	50	**RMH52**	Plasma	4	50
DC52	Blood	9	40	**DC82**	Blood	9	40	**RMH12**	Plasma	4	50	**RMH55**	Plasma	4	50
DC53	Blood	25	40	**DC83**	Blood	14	40	**RMH13**	Plasma	3	50	**RMH56**	Plasma	4	50
DC54	Blood	23	15	**DC84**	Blood	10	25	**RMH15**	Plasma	2	50	**RMH57**	Plasma	3	50
DC55	Blood	15	35	**RB1**	Blood	19	40	**RMH16**	Plasma	2	50	**RMH58**	Plasma	5	50
DC56	Blood	14	25	**RB2**	Buccal	16	38	**RMH17**	Plasma	3	50	**RMH59**	Plasma	7	50

Table 4 continued: Concentrations and volumes of DNA samples sent to LGC Genomics for SNP analysis

Name	Type	DNA conc. (ng/µL)	Vol (µL)	Name	Type	DNA conc. (ng/µL)	Vol (µL)	Name	Type	DNA conc. (ng/µL)	Vol (µL)	Name	Type	DNA conc. (ng/µL)	Vol (µL)
DC57	Blood	23	40	**RB3**	Buccal	19	40	**RMH19**	Plasma	3	50	**RMH60**	Plasma	3	50
DC58	Blood	15	25	**RB4**	Buccal	22	40	**RMH20**	Plasma	2	50	**RMH61**	Plasma	6	50
DC59	Blood	11	30	**RB5**	Buccal	14	40	**RMH21**	Plasma	6	50	**RMH62**	Plasma	5	50
DC60	Blood	15	25	**RB6**	Buccal	12	40	**RMH22**	Plasma	2	50	**RMH63**	Plasma	3	50
DC61	Blood	15	35	**RB7**	Buccal	18	40	**RMH25**	Plasma	2	50	**RMH64**	Plasma	2	50
DC62	Blood	22	40	**RB8**	Buccal	18	40	**RMH26**	Plasma	4	50	**RMH66**	Plasma	2	50
DC63	Blood	12	35	**PH1**	Buccal	24	40	**RMH28**	Plasma	3	50	**RMH68**	Plasma	4	50
DC65	Blood	19	10	**PH2**	Buccal	23	38	**RMH30**	Plasma	3	50	**RMH70**	Plasma	2	50
DC66	Blood	10	40	**PH3**	Buccal	21	40	**RMH31**	Plasma	3	50	**RMH74**	Plasma	5	50
DC67	Blood	10	30	**PH4**	Buccal	27	40	**RMH32**	Plasma	2	50	**RMH77**	Plasma	4	50
DC68	Blood	14	35	**FH2**	Buccal	14	40	**RMH33**	Plasma	2	50	**RMH79**	Plasma	6	50
DC69	Blood	14	30	**FH3**	Buccal	14	40	**RMH35**	Plasma	3	50	**RMH81**	Plasma	3	50
RMH82	Plasma	4	50	**RMH125**	Plasma	3	50	**RMH170**	Plasma	4	50	**BC14**	Buccal	22	45
RMH83	Plasma	12	50	**RMH126**	Plasma	5	50	**RMH171**	Plasma	4	50	**BC15**	Buccal	18	45
RMH84	Plasma	2	50	**RMH127**	Plasma	23	50	**RMH172**	Plasma	2	50	**BC16**	Buccal	10	45
RMH85	Plasma	2	50	**RMH129**	Plasma	2	50	**RMH174**	Plasma	2	50	**BC17**	Buccal	9	45
RMH86	Plasma	6	50	**RMH130**	Plasma	4	50	**RMH175**	Plasma	3	50	**BC18**	Buccal	17	45
RMH87	Plasma	2	50	**RMH132**	Plasma	4	50	**RMH176**	Plasma	3	50	**BC19**	Buccal	11	45
RMH89	Plasma	2	50	**RMH133**	Plasma	2	50	**RMH179**	Plasma	2	50	**BC20**	Buccal	10	45
RMH90	Plasma	3	50	**RMH136**	Plasma	5	50	**RMH182**	Plasma	2	50	**BC21**	Buccal	16	45
RMH91	Plasma	3	50	**RMH137**	Plasma	4	50	**RMH185**	Plasma	4	50	**BC22**	Buccal	17	45
RMH92	Plasma	2	50	**RMH138**	Plasma	5	50	**RMH187**	Plasma	4	50	**BC23**	Buccal	13	45
RMH93	Plasma	2	50	**RMH141**	Plasma	5	50	**RMH191**	Plasma	4	50	**BC24**	Buccal	14	45

Table 4 continued: Concentrations and volumes of DNA samples sent to LGC Genomics for SNP analysis

APPENDIX C1.0

Name	Type	DNA conc. (ng/µL)	Vol (µL)	Name	Type	DNA conc. (ng/µL)	Vol (µL)	Name	Type	DNA conc. (ng/µL)	Vol (µL)	Name	Type	DNA conc. (ng/µL)	Vol (µL)
RMH94	Plasma	5	50	**RMH142**	Plasma	2	50	**RMH192**	Plasma	6	50	**BC25**	Buccal	13	45
RMH95	Plasma	4	50	**RMH144**	Plasma	4	50	**RMH193**	Plasma	5	50	**BC26**	Buccal	13	45
RMH97	Plasma	1	50	**RMH143**	Plasma	4	50	**RMH194**	Plasma	3	50	**BC27**	Buccal	16	45
RMH98	Plasma	2	50	**RMH145**	Plasma	2	50	**RMH198**	Plasma	6	50	**BC28**	Buccal	8	45
RMH99	Plasma	3	50	**RMH146**	Plasma	3	50	**RMH199**	Plasma	3	50	**BC29**	Buccal	15	45
RMH100	Plasma	3	50	**RMH147**	Plasma	3	50	**BC01**	Buccal	12	45	**BC30**	Buccal	6	45
RMH101	Plasma	2	50	**RMH148**	Plasma	3	50	**BC02**	Buccal	20	45	**BC31**	Buccal	12	45
RMH103	Plasma	7	50	**RMH149**	Plasma	2	50	**BC03**	Buccal	17	45	**BC32**	Buccal	20	45
RMH104	Plasma	3	50	**RMH152**	Plasma	6	50	**BC04**	Buccal	16	45	**MC01**	Buccal	12	45
RMH106	Plasma	3	50	**RMH153**	Plasma	3	50	**BC05**	Buccal	21	45	**MC02**	Buccal	14	45
RMH107	Plasma	3	50	**RMH155**	Plasma	3	50	**BC06**	Buccal	17	45	**MC03**	Buccal	16	45
RMH113	Plasma	2	50	**RMH157**	Plasma	2	50	**BC07**	Buccal	14	45	**MC04**	Buccal	18	45
RMH116	Plasma	2	50	**RMH158**	Plasma	1	50	**BC08**	Buccal	12	45	**MC05**	Buccal	20	45
RMH119	Plasma	6	50	**RMH161**	Plasma	3	50	**BC09**	Buccal	11	45	**MC06**	Buccal	19	45
RMH121	Plasma	3	50	**RMH163**	Plasma	4	50	**BC10**	Buccal	16	45	**MC07**	Buccal	15	45
RMH122	Plasma	2	50	**RMH165**	Plasma	3	50	**BC11**	Buccal	12	45	**MC08**	Buccal	15	45
RMH123	Plasma	2	50	**RMH166**	Plasma	2	50	**BC12**	Buccal	12	45	**MC09**	Buccal	13	45
RMH124	Plasma	2	50	**RMH168**	Plasma	7	50	**BC13**	Buccal	16	45	**MC10**	Buccal	13	45
MC11	Buccal	14	45	**MC17**	Buccal	14	45	**MC23**	Buccal	15	45	**MC28**	Buccal	16	45
MC12	Buccal	19	45	**MC18**	Buccal	12	45	**MC24**	Buccal	17	45	**MC29**	Buccal	18	45
MC13	Buccal	15	45	**MC19**	Buccal	11	45	**MC25**	Buccal	21	45	**MC30**	Buccal	17	45
MC14	Buccal	17	45	**MC20**	Buccal	17	45	**MC26**	Buccal	13	45	**MC31**	Buccal	12	45
MC15	Buccal	19	45	**MC21**	Buccal	13	45	**MC27**	Buccal	17	45	**MC32**	Buccal	18	45
MC16	Buccal	17	45	**MC22**	Buccal	13	45								

DIFFICULTIES FACED OBTAINING STUDY SAMPLES FROM CANCER PATIENTS

1.0 Delays encountered whilst writing the protocol

Getting to the stage to be able to recruit patients into the pharmacogenetic study was much slower than anticipated. The initial hurdle was writing the protocol. The protocol was written in collaboration with clinicians at Bournemouth and London who had experience with numerous clinical trials, but due to their workload and the distance between Bournemouth and London finding time to discuss the study proved to be difficult. Despite the difficulties the collaboration with the Royal Marsden Hospital was a necessity as the study would not have been possible without their support and guidance. A few of the difficulties encountered when writing the protocol are detailed below:

1.1 Incorporation of patients prescribed morphine and oxycodone.

At the Royal Bournemouth Hospital the practice is to treat cancer pain using morphine. The Royal Marsden Hospital and MacMillan unit however use oxycodone as an alternative to morphine. If the study was to solely include patients prescribed morphine only a small study population would be obtained that would have poor statistical power. The study was altered to include patients prescribed oxycodone, but only oxycodone as the number of variables introduced had to be kept to a minimum.

1.2 Patient inclusion / exclusion criteria.

A number of amendments were made to the inclusion / exclusion criteria as each hospital had different definitions and time points as to when patients' pain relief dose should be escalated to Step III analgesics. Also, the use of morphine to relieve cancer related pain is in part determined by the patients liver function but both hospitals measure liver function using different scales therefore a common unit had to be discussed and agreed upon.

1.3 Transportation of samples.

All samples from the cancer patients needed to be transported to the Royal Bournemouth Hospital or Bournemouth University for drug / genetic analysis. For this to happen Material Transfer Agreements (MTAs) were set up.

1.4 Sample storage.

It was thought the samples would be analysed and stored at Bournemouth University however, the university does not have a human tissue license so the samples had to be stored at Royal Bournemouth Hospital and logged into their Tissue Auditor system.

1.5 Determining the study endpoint / when a patient was stable on an opioid.

Different authors have different opinions on what classifies as a stable response to an opioid. For example, if a patient has a 4-point improvement change on the 11-point pain scale from before taking the opioid compared to a week later the patient

may be considered an opioid responder. However, the patient could still have pain e.g. pain score of 6 which is over the acceptable level of pain. As such, a patient was considered stable once their pain was ≤4 on the 11-point pain scale for 3 continuous days.

1.6 Funding of the study, e.g. for research nurses and sample analysis.

Many discussions arose concerning the funding of the pilot study. As the research was part-funded by Randox and Bournemouth University the necessary drug standards and reagents for toxicological and genetic analysis were funded by them. One of the major concerns was who would collect the data from patients, including their demographic details and responses to questionnaires. It was proposed that at the Bournemouth and Poole sites existing research nurses would take on this project in addition to any other work without any additional pay. However for ease, all the data collection was the responsibility of the PhD researcher.

1.7 Statistician

The Royal Marsden group are very experienced in carrying out pharmacogenetic studies so the initial thought was to use their statistician to help interpret the data. However the statistician had to be associated with the study sponsor so Professor Peter Thomas was approached instead.

2.0 Delays encountered obtaining approval from the ethical committee Ethic delays

The second hurdle arose trying to complete the online ethics submission as this was a time consuming endeavour. Careful attention had to be made to the phrasing of each answer to ensure clarity. Despite having assistance from staff at Bournemouth Hospital who were experienced with ethics applications and the time taken to complete the protocol and ethics application the South Berkshire Ethics Committee expressed a number of concerns with the original proposal. The first ethics application was submitted, and was heard on 10th October 2011 but obtained an unfavourable opinion.

The concerns of South Berkshire B Ethics Committee are detailed below.

2.1 Aims and objectives of the study.

There was a lot of confusion as to what the study was supposed to achieve. The PhD proposal was to create an algorithm to allow clinicians to calculate which opioid to use and at what dose based on a patients phenotype and personal data. However, previous research demonstrated that large populations were needed to obtain associations between pain relief response and genetic variations. To obtain a statistically meaningful population a number of hospitals would have to be involved which would lead to issues including who would fund the research nurses and how would the large volume of samples be analysed. As such, it was decided that this project would be a pilot study so the main aim became to determine the feasibility of carrying out a pharmacogenetic study and secondary aims would be to find genetic variations indicative of affecting patient pain relief response.

2.2 Population size.

A small patient population could be used to achieve the primary aim as the recruitment rate and compliance with the study could easily be calculated and multiplied up to determine the number of patients that need to be approached in the larger study to obtain meaningful samples and data. However, to find genetic associations to pain relief response a larger population was needed to obtain significant results as there are many variables that may affect the patient response. These variables include the patient's age, ethnicity, gender, cancer type, concomitant medication and lifestyle. A larger population would allow the patients to be separated into more comparable categories but a sample size of 200 would give an indication of genetic variations that may be of interest.

2.3 Number of samples to be collected and what samples.

At the beginning of this PhD samples were going to be collected from the cancer patients' post-mortem. However, the Royal Marsden group looked unfavourably upon collecting samples post-mortem and this opinion was later mirrored by the Ethics Committee despite a Patient Participant Panel approving of the post-mortem sample collection. Eventually it was agreed by the participating researchers that only antemortem samples would be collected. Blood and oral fluid samples were to be collected once patients were stable on an opioid or switched to an alternative opioid. Ideally a blood and oral fluid sample would have also been collected following the first dose of Step III opioid. However the collection of these samples within a 24 hour time frame would have proved difficult so this sample collection was removed.

2.4 Patient information sheet

Similarly to the protocol, a number of amendments were made to the protocol support documents. The document with the most revisions was the patient information sheet as the Ethics Committee felt it did not portray the main aim of the study effectively and was not written in a sensitive enough manner for this sick study population. The Ethics Committee also required additional information to be added to the patient information sheet, the newly added information is shown in Figure 1-1.

"**What will happen to the results of the research study?** Results of this study are likely to be available in 2014 / 2015. They will be presented in a range of publications."

"What if I have any concerns? If you have any concerns or other questions about this study or the way it has been carried out, you should contact your study doctor (see below) on telephone number (see below), or you may contact the hospital complaints department. Any complaints will be assessed on a case by case basis and will be dealt with or forwarded on to the relevant regulatory bodies as required. We

Figure 1-1. Additional information added to the Patient Information Sheet

Once the changes had been made, the ethics application was resubmitted and heard on

17th January 2012. A favourable ethical opinion subject to changes was obtained. The Ethics Committee however wanted considerable changes to the protocol, either emphasising the primary objective relating to feasibility of monitoring patients over

a 28 day period, or changing the aim of the protocol completely. The primary objective was emphasised in the protocol along with other minor amendments by Dr. Tamas Hickish (chief investigator and PhD supervisor) and favourable ethical opinion was given on 17th February 2012.

2.5 Minor and major amendments

Following favourable ethical opinion to undertake a study no changes can be made to the protocol and supporting documents without the approval of either the main Ethics Committee or the study sponsor. Minor amendments only have to be reviewed by the study sponsor. Within 2 months of obtaining ethical approval to undertake the study minor amendments were made to the existing documents and additional supporting documents were created. As these changes did not impact the study protocol or influence the patients the proposed changes were sent to, and approved by, the Royal Bournemouth Hospital acting as study sponsor (Table 1-1).

Table 1-1. Changes made to existing protocol documents or newly created supporting documents

Document	Changes
Patient Information Sheet	Contact details for Dr Hickish updated
GP Letter	Contact details for Dr Hickish updated
Information for Staff	Contact details for Dr Hickish updated and document renamed "Information for staff" from "Nurse Information".
Initial Assessment	Biochemistry information collated reduced down to test results that may have an impact on study outcome.
Reminder Card	Newly created
Patient Reminder Cards	Newly created
Patient Sample Stickers	Newly created

The main Ethics Committee has to approve any substantial protocol amendments; these include changes to the study methodology or significant changes to study documentation. Throughout the initial months of undertaking the study it was

noticed that cancer outpatients requiring either morphine or oxycodone were being missed during the recruitment process. The outpatients could be given a patient information sheet during their visit with the oncologist but a 24 hour period was required between providing the patient information sheet and signing the consent form. Therefore it was necessary for the patient to return to the hospital the following day to provide consent which is an inconvenience to the patient and would make their participation less likely. As such a major amendment was made to the protocol that allowed for a member of the research team to visit the patient at their home, with the patient's consent. During the patient's appointment with the oncologist, the patient would be provided with a brief overview of the study and a patient information sheet. If the patient was interested in the study and did not object to a member of the research team visiting their home they were asked to sign a "Home visit permission form". The oncologist would then contact the PhD researcher who would contact and visit the patient 24 hours after the consent form was provided. Signing the home visit permission form did not mean the patient was obliged to participate in the study. The change to the protocol is underlined and in bold in Figure 1-2.

5.6.1 Consent may be obtained from the patient during routine hospital visits; alternatively the patient will be visited by a member of the research team at their home address subject to the patient's consent. Visits to patients' homes will only occur if the patient has given verbal permission for home visits to take place. The patient's verbal agreement to allow home visits will be recorded in the patient's

Figure 1-2. Additions made to the protocol

An additional sentence was added to the patient information sheet so patients would be aware that a member of the research team may visit them at their home (Figure 13).

What now?

If you decide to voluntarily take part in this study, then you'll be asked to sign a consent form. You will be given not less than 24 hours to make your decision as to whether you wish to take part. **With your permission you may be visited at your home by a member of the research team who will go through the study process thoroughly with you and obtain consent.** Ideally we would like to see you before you start the new pain killers as we would like to gather some baseline information. However, if you agree to participate and the initial …

questionnaires

Figure 1-3. Additional changes made to the Patient Information Sheet (bold text)

The amended protocol, patient information sheet and home visit permission form were given a favourable opinion by the main Ethics Committee on 11th February 2013 and resulted in a marked increase in patient recruitment (Figure 1-4).

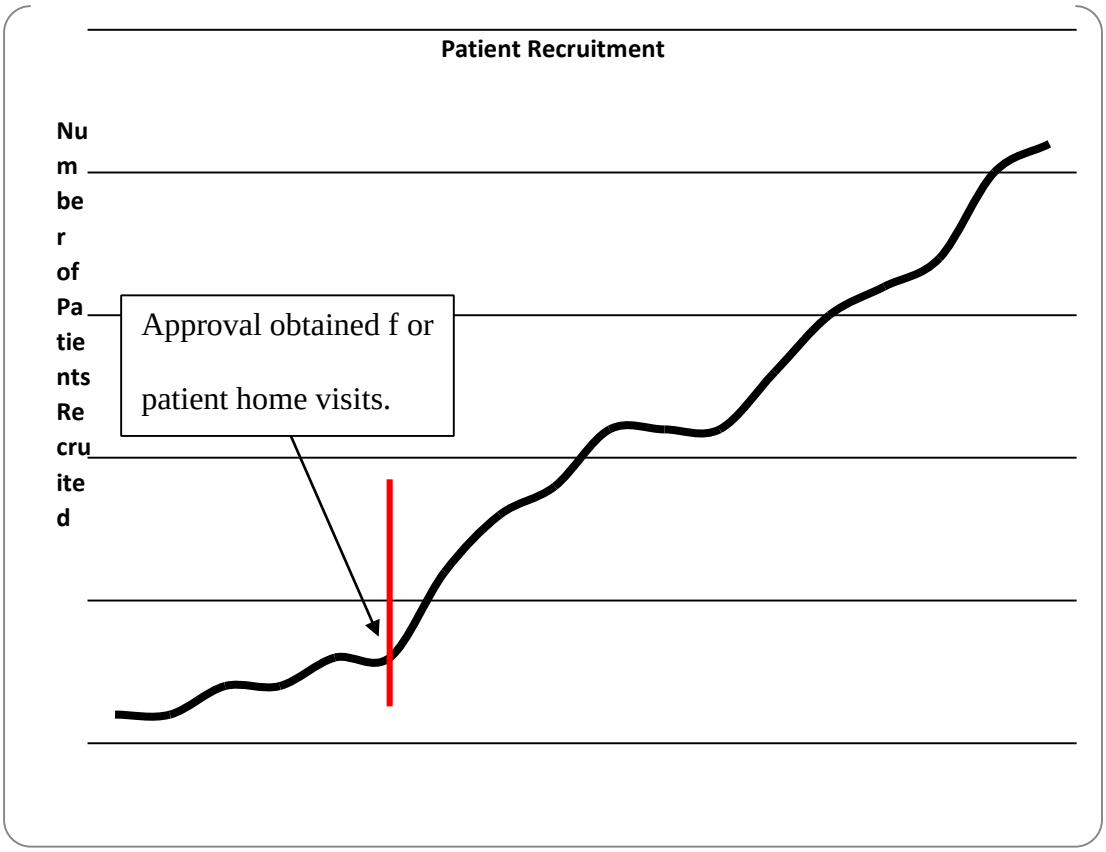


Figure 1-4. Recruitment rate of cancer patients into pharmacogenetic study

2.6 Extension of study period and annual reviews

The PhD commenced in October 2010 due to be completed by September 2013; therefore the original ethical approval to undertake the pharmacogenetic study expired on 2nd October 2013. However as ethical approval was not obtained until February

2012 due to difficulties writing the protocol and ethics application, Bournemouth University granted a one year extension onto the PhD. To enable a greater patient population to be recruited an extension to ethics was applied for. Approval to continue the study until October 2014 was sought with the aim of recruiting a more

realistic patient group of 50. South Berkshire B approved the ethics application extension.

From the date ethical approval was obtained to undertake the study, annual reports had to be completed. These forms check the progress of the study and ask for details on any protocol changes or breaches. Once completed the forms are sent to the R&D departments of each participating site as well as the main Ethics Committee.

2.7 Patient recruitment issues

Patient recruitment was much slower than anticipated considering the number of cancer patients diagnosed each week that may require an opioid analgesic to alleviate cancer pain. Initially the recruitment rate was slow as for the first 6 months only one oncologist was recruiting patients so that any flaws in the recruitment process could be ironed out before additional personnel were involved. In those 6 months three patients were successfully recruited but obtaining consent in a timeframe convenient for the patient proved to be difficult. Following approval from South Berkshire B Ethics Committee to visit patients at their homes the study was re-presented to clinicians and nurses at each participating NHS site. The study was received with enthusiasm by the oncology teams with a good intake of patients over the first 4 months (Figure 1-4). A lull in patient recruitment occurred over summer 2013 as participating clinicians took annual leave and covering clinicians were unaware of whether the patient had or had not been informed of the study.

To encourage patient recruitment following the summer period Multidisciplinary Team meetings (MDTs) were attended at each participating NHS site by the PhD researcher. The presence of the PhD researcher at the meetings acted as a visual

reminder of the study being undertaken and also meant that any patients being discussed at the meetings could be flagged by the researcher as a potential participant.

Attendance at these meetings did help identify patients, however the meetings were held on Tuesday afternoon in Poole and Thursday mornings in Christchurch, each lasting up to 3 hours on a weekly basis. With attendance to the meetings as well as the time spent travelling to and from the MDT meetings, valuable experiment time was being lost. Therefore, meetings were attended on a fortnightly basis when possible. Unfortunately often within the two week period potential participants were prescribed either morphine or oxycodone but not considered for the trial (Figure 1-5). If less than 48 hours had passed since the initial opioid dose and the clinician thought the patient had the capacity to provide retrospective answers to the questionnaires then the patient could be recruited into the study. More often than not the time period was longer than 48 hours.

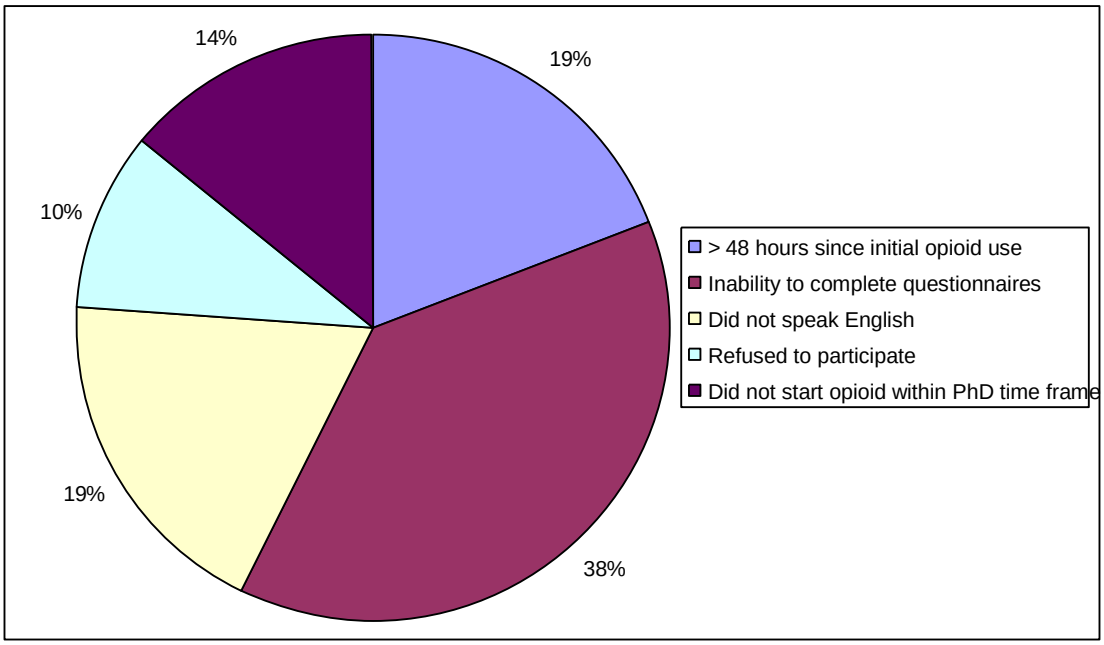


Figure 1-5. Pie chart showing the number of, and reason for, patients not being included in the pharmacogenetic study over a six week period.

The second most common reason for patients not being approached to participate in the study was their lack of capacity to complete the questionnaires, as assessed by their clinicians. Patients who were thought to lack capacity had diseases such as dementia and Alzheimer's or suffered from confusion and short term memory loss that would result in inaccurate answers. In addition, patients who were hard of hearing were not approached to participate in the study as it would be difficult for the researcher to conduct the necessary questionnaires over the phone.

Alongside missed participants, some patients could not participate in the study as they did not comply with the eligibility criteria. For example, patients who could not speak or understand written English were not included in the study.

The majority of patients who were approached to participate were happy to contribute to the study. However, two people did not want to participate. Although it

was not necessary for them to provide a reason for refusing the study they both volunteered their opinions. The first patient to reject the offer to participate in the study did not want anything extra to think about on top of their terminal cancer diagnosis. The second patient said they did not want to “feel like a guinea pig”.

Three patients who agreed to participate in the study, signed the consent forms and completed the initial questionnaires did not see the study through to completion as they did not require the escalation to morphine or oxycodone for pain relief in the time frame of the PhD.