2019

Role of cytochrome P450 2B6 polymorphisms in unexpected methadone death

Taha Ahmad
taha.ahmad.nj@gmail.com

ROLE OF CYTOCHROME P450 2B6 POLYMORPHISMS IN UNEXPECTED METHADONE DEATH

A dissertation submitted to
the Graduate College of
Marshall University
In partial fulfillment of
the requirements for the degree of
Doctor of Philosophy
In
Biomedical Sciences
by
Taha Ahmad
Approved by
Dr. Gary O. Rankin, Committee Chairperson
Dr. Monica A. Valentovic
Dr. Donald A. Primerano
Dr. Travis B. Salisbury
Dr. Richard D. Egleton

Marshall University
May 2019

APPROVAL OF THESIS

We, the faculty supervising the work of Taha Ahmad, affirm that the dissertation, *Role of Cytochrome P450 2B6 Polymorphisms in Unexpected Methadone Death*, meets the high academic standards for original scholarship and creative work established by the Biomedical Science Program and the Graduate College of Marshall University. This work also conforms to the editorial standards of our discipline and the Graduate College of Marshall University. With our signatures, we approve the manuscript for publication.

Dr. Gary O. Rankin, Committee Chairperson Date 4/26/19
Department of Biomedical Sciences

Dr. Monica A. Valentovic, Committee Member Date 4-26-2019
Department of Biomedical Sciences

Dr. Donald A. Primerano, Committee Member Date 04/26/2019
Department of Biomedical Sciences

Dr. Travis B. Salisbury, Committee Member Date 4/26/2019
Department of Biomedical Sciences

Dr. Richard D. Egleton, Committee Member Date 4/26/19
Department of Biomedical Sciences

DEDICATION

I dedicate this work to all those students who have struggled throughout their journey and felt hopeless, defeated, and despair, and to those who suffer and have suffered from anxiety and depression. This road was not an easy one, but with patience, persistence, perseverance, and faith, all things are possible, even if it takes nine years.

ACKNOWLEDGMENTS

I would like to take this opportunity to extend special thanks to each and every one of my committee members for keeping their doors open to let me pick their brains, to talk out loud and bounce ideas off of them, and for their expertise and useful critiques, which has aided in my growth as a researcher, and provided support in overcoming the numerous obstacles and setbacks I have faced throughout my research.

My deepest appreciation goes out to my advisor, Dr. Gary O. Rankin for taking me on and allowing me to pursue a project that aligned with my passion in forensic science. His professional guidance, wisdom, and support have helped throughout my time as a graduate student. I have had the chance to get to know him and admire the person he is.

Dr. Monica A. Valentovic, thank you for teaching me how to use and troubleshoot the HPLC. Your vast intelligence is intimidating and something I aspire to have one day.

Dr. Donald A. Primerano, thank you for being you, for always getting back to me so promptly, for being candid and frank and yet so softhearted when I needed your guidance throughout the years.

Dr. Travis B. Salisbury, thank you for always having a positive attitude, which gave me hope when I had doubts. Any student would be fortunate to have you as a mentor.

Dr. Richard D. Egleton, thank you for joining my committee late in the game and allowing me to vent out my frustrations when I needed it the most.

I would also like to show my gratitude to Dr. Jung Han Kim, Dr. Emine Koc, and Dr. Wei Li for donating materials crucial for the project; and to Dr. Nalini Santanam, Dr. Piyali Dasgupta, and Dr. Lawrence Grover for allowing me to use equipment from their laboratories.

Getting through my dissertation required more than just academic support, and I have Dr. Kristeena Wright and Caroline Hunter to thank for their friendship, and their personal and professional support, especially during our lunch dates on the fourth floor lobby at the BBSC.

Finally, I would like to give a heartfelt thanks to my mom, Yasmin Ahmad; my sisters, Feryal and Rabia Ahmad; my fiancé, Travis Dangelo; my friends, Mandana Patimetha, Daniel Rizov, and Uncle Rick; and countless other family members and friends who have unequivocally supported and encouraged me throughout these challenging years. This journey was a roller coaster ride, and your unwavering belief in me, love, pep talks, and advice during the lowest of the lows was never taken for granted. I am eternally grateful to all of you. I must not forget my two beautiful bunnies, Hobbit and Puddles, who were there for emotional comfort and companionship as I took on the challenge of putting to paper eight years' worth of research.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Methadone is a synthetic, long-acting opioid prescribed as an analgesic for chronic pain. It has a single chiral center forming two enantiomers, (R)-methadone and (S)-methadone, each having specific pharmacological actions. Concentrations of (R)- and (S)-methadone above therapeutic levels have the ability to cause serious, life-threatening, and fatal side effects. Cardiotoxicity is caused by elevated (S)-methadone levels by prolonging the QT interval of the heart's electrical cycle. In 2014, methadone accounted for only 1% of all opioids prescribed for pain, but was responsible for 3,400 of the 14,838 individuals (~23%) who died in the United States from overdoses due to prescription opioids. West Virginia (WV) and Kentucky (KY) continue to have significant increases in overdose death rates involving prescription opioids. These overdoses could be due in part to the pharmacogenetics of an individual, encompassing the ability to influence the pharmacokinetic and pharmacodynamic properties of methadone, contributing to toxicity. The principle determinant of the large interindividual variability of methadone pharmacokinetics is metabolism, causing a discordance in the relationship between dose, plasma concentrations, and side effects. Single nucleotide polymorphisms (SNPs) within the genes of drug metabolizing enzymes, cytochrome P450 (CYP), may be important contributing factors in altering CYP activity, methadone metabolism and pharmacodynamics. (S)-Methadone is stereoselectively metabolized in the liver by CYP2B6. The purpose of this study was to determine if one or more SNPs located within the *CYP2B6* gene contributes to a poor metabolizer phenotype for unexpected methadone deaths. A study cohort was obtained from the WV and KY Offices of the Chief Medical Examiner consisting of 125 Caucasians who suffered methadone-only fatal overdoses. Genotypic frequency of three intronic SNPs (rs2279344, rs4803419, and rs8192719) and five exonic SNPs (rs3211371, rs3745274, rs8192709,

rs12721655, and rs35979566) were investigated. The differences between the observed genotypic frequencies were compared to the regional control group of 255 deceased Caucasian individuals. The frequencies of the exonic SNP rs3745274 and intronic SNP rs8192719 were enhanced in the methadone-only group. Higher blood methadone concentrations were observed in individuals who were genotyped homozygous for SNP rs3211371. The five exonic SNPs genotyped, and two others (rs2279343 and rs35773040), were individually expressed in COS-1 cells. The effect of these SNPs on the catalytic activity of the CYP2B6 enzyme was assessed by evaluating the luminescence generated from the metabolism of luminogenic luciferin-2B6 substrate. Additionally, inhibition studies were also conducted using clopidogrel to determine that the activity observed was from the CYP2B6 enzyme. Methadone was used to evaluate competitive inhibition with luciferin-2B6 substrate at the active site. The effect on CYP2B6 activity by introducing individual SNP variants resulted in the following decreasing order: rs2279343 > rs3745274 > wild type (*CYP2B6*1*), rs3211371 > rs8192709 > rs35773040, rs35979566. Relative to the wild type CYP2B6, SNPs rs8192709, rs35773040, and rs35979566 yielded 57%, 81%, and 94% decreased activity, respectively. Methadone inhibited the activity of the CYP2B6 enzyme by displacing the luciferin-2B6 substrate. Therefore, our investigations suggest that the differences in catalytic activity by *CYP2B6* allelic variants rs8192709, rs35773040, and rs35979566 result in poor metabolizing phenotypes which may be contributing factors in decreased metabolism of (S)-methadone. Because CYP2B6 only plays a partial role in the metabolism of (S)-methadone, additional knowledge on the combination of SNPs on *CYP2B6*, together with SNPs on other methadone metabolizing CYP genes, can improve the proper therapeutic dosing for methadone, patient outcome, and the development of individualized medicine.

CHAPTER 1

EFFECTS OF CYTOCHROME P450 SINGLE NUCLEOTIDE POLYMORPHISMS ON METHADONE METABOLISM AND PHARMACODYNAMICS

The information in this chapter has been published in *Biochemical Pharmacology*.

Ahmad, T., Valentovic, M. A., & Rankin, G. O. (2018). Effects of cytochrome P450 single nucleotide polymorphisms on methadone metabolism and pharmacodynamics. *Biochemical Pharmacology, 153,* 196-204. doi:10.1016/j.bcp.2018.02.020

Taha Ahmad, Monica A. Valentovic, and Gary O. Rankin*

Department of Biomedical Sciences, Toxicology Research Cluster, Joan C. Edwards School of Medicine, Marshall University, Huntington, WV 25755-9310, USA

*Corresponding author at: Department of Biomedical Sciences, Joan C. Edwards School of Medicine, BBSC-435F, Marshall University, 1700 Third Avenue, Huntington, WV 25755, USA.

E-mail address: rankin@marshall.edu (G.O. Rankin).

Abstract

Methadone is a synthetic, long-acting opioid with a single chiral center forming two enantiomers, (R)-methadone and (S)-methadone, each having specific pharmacological actions. Concentrations of (R)- and (S)-methadone above therapeutic levels have the ability to cause serious, life-threatening, and fatal side effects. This toxicity can be due in part to the pharmacogenetics of an individual, which influences the pharmacokinetic and pharmacodynamic properties of the drug. Methadone is primarily metabolized in the liver by cytochrome P450 (CYP) enzymes, predominately by CYP2B6, followed by CYP3A4, 2C19, 2D6, and to a lesser extent, CYP2C18, 3A7, 2C8, 2C9, 3A5, and 1A2. SNPs located within CYPs have the potential to play an important role in altering methadone metabolism and pharmacodynamics. Several SNPs in the *CYP2B6*, *3A4*, *2C19*, *2D6*, and *3A5* genes result in increases in methadone plasma concentrations, decreased N-demethylation, and decreased methadone clearance. In particular, carriers of *CYP2B6*6/*6* may have a greater risk for detrimental adverse effects, as methadone metabolism and clearance are diminished in these individuals. *CYP2B6*4*, on the other hand, has been observed to decrease plasma concentrations of methadone due to increased methadone clearance. The involvement, contribution, and understanding the role of SNPs in *CYP2B6*, and other CYP genes, in methadone metabolism can improve the therapeutic uses of methadone in patient outcome and the development of personalized medicine.

1.1 Methadone Pharmacology

Shortly after methadone was introduced in the United States, the Food and Drug Administration (FDA) approved its use as an analgesic and antitussive agent (Institute of Medicine, 1995). During the 1960s, evidence for the usefulness of methadone in the treatment of and maintenance therapy for narcotic addiction was growing (Dole & Nyswander, 1965; Joseph,

Stancliff, & Langrod, 2000). A decade later, the FDA expanded the approval for physicians and hospital pharmacies to dispense methadone for treating opiate addiction in treatment programs only (Institute of Medicine, 1995). Enrollment in a methadone maintenance treatment (MMT) program also has the potential to be a public health strategy for decreasing the transmission of infectious diseases (e.g. HIV/AIDS, hepatitis C, or other blood-borne pathogens) by reducing the number of opioid users reusing contaminated needles (Drucker, Lurie, Wodakt, & Alcabes, 1998; Joseph et al., 2000; Somogyi, Barratt, Ali, & Coller, 2014).

Methadone is a synthetic, long-acting opioid with a single chiral center that forms two enantiomers, R-(-)-methadone (levo-methadone) and S-(+)-methadone (dextro-methadone) (Anderson & Kearney, 2000; Lisberg & Scheinmann, 2013). Both enantiomers have specific pharmacodynamic properties and pharmacological actions. (R)-Methadone is a μ-opioid receptor (MOR) agonist that mimics the body's natural endogenous opioids, endorphins and enkephalins. Binding of (R)-methadone to the MOR mediates therapeutic effects with the release of other neurotransmitters, including acetylcholine, dopamine, norepinephrine, and substance P (Anderson & Kearney, 2000). The effects propagated by this interaction yields reduction and elimination of drug cravings and withdrawal signs and symptoms of narcotics, analgesia, sedation, nausea, vomiting, constipation, miosis, antitussive effects, hypotension, mild bradycardia, and tolerance and dependence (Langrod, Lowinson, & Ruiz, 1981; Reisine, Law, Blake, & Tallent, 1996; Li, Kantelip, Gerritsen-van Schieveen, & Davani, 2008; Mallinckrodt Pharmaceuticals, Inc., 2016).

In the United States, methadone is clinically available only as a racemic formulation comprised of both (R)- and (S)-methadone enantiomers (Gaertner, Voltz, & Ostgathe, 2008; Kapur, Hutson, Chibber, Luk, & Selby, 2011; McCance-Katz, 2011). Methadone is currently

used as: (1) an approved opioid substitution therapeutic agent to suppress opioid-abstinence syndrome in opiate abuse and addiction (Stotts, Dodrill, & Kosten, 2009; Bart, 2012; Kreek et al., 2012; Peles, Linzy, Kreek, & Adelson, 2013; McDonough, 2013); (2) a second-line opioid in alleviating cancer pain and chronic non-malignant pain, such as back, joint, or neuropathic pain and other forms of severe pain (Taylor et al., 2000; Toombs & Kral, 2005; Jackman, Purvis, & Mallett, 2008; Fredheim, Moksnes, Borchgrevink, Kaasa, & Dale, 2009); and (3) a first-line treatment in patients with both opioid dependence and chronic pain (Kahan, Srivastava, Wilson, Mailis-Gagnon, & Midmer, 2006).

Methadone has contributed to a significant portion of opioid-related overdose deaths. However, this outcome is not attributed to the use of methadone for opioid dependence, rather from prescriptions of methadone for pain management (Jones, Baldwin, Manocchio, White, & Mack, 2016). Reports from the Centers for Disease Control and Prevention (CDC) indicate a 15.6% increase in opioid death rates from 2014 to 2015. Conversely, methadone death rates decreased by 9.1% due to factors such as efforts to reduce methadone therapy for pain, warning labels, clinical guidelines, and limiting high dose formulations (Rudd, Seth, David, & Scholl, 2016). However, death rates continued to increase through 2014 resulting from methadone overdosing in individuals between the ages of 55 – 64 years (Jones et al., 2016).

Concentrations of (R)- and (S)-methadone above therapeutic levels have negative and detrimental side effects. Elevated (R)-methadone levels may depress ventilation, inducing respiratory depression, by acting on the MORs expressed on respiratory centers in the brainstem (Mitchell et al., 2004; Silverman, Nettleton, Spencer, Wallisch, & Olsen, 2009; van der Schier, Roozekrans, van Velzen, Dahan, & Niesters, 2014). Conversely, an increase in (S)-methadone disposition causes cardiotoxicity through the blockage of the voltage-gated potassium channel of

the human ether-a-*go-go* related gene (hERG), subsequently prolonging the QT interval of an electrocardiogram leading to torsades de pointes. (S)-Methadone is 3.5 times more potent than (R)-methadone in blocking the hERG channel, thus attributing the stereoselectivity of methadone to cardiotoxicity (Eap et al., 2007; Ansermot et al., 2010; Dobrinas et al., 2013; Csajka, Crettol, Guidi, & Eap, 2016).

Methadone is primarily eliminated via hepatic metabolism by cytochrome P450 (CYP) enzymes through oxidative biotransformation. Methadone undergoes stereoselective N-demethylation followed by spontaneous cyclization to form the principle, inactive metabolite 2-ethyl-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) (Figure 1) (Moody, Alburges, Parker, Collins, & Strong, 1997; Oda & Kharasch, 2001; Ferrari, Coccia, Bertolini, & Sternieri, 2004; Nanovskaya et al., 2004; Lehotay et al., 2005; Lugo, Satterfield, & Kern, 2005). Methadone has a broad half-life of elimination ranging from 5 – 130 hours, with an average of 22 hours (Eap, Buclin, & Baumann, 2002). This extreme inconsistency in the elimination half-life exemplifies the large variability methadone possesses in its pharmacokinetics between individuals. Because of the interindividual variation, it is difficult to predict an individual's peak plasma levels, which may result in some individuals having a higher than normal plasma level that approaches the toxic or even fatal drug concentration. Comedication, age, gender, health, weight, and ethnicities can all be factors that contribute to this effect.

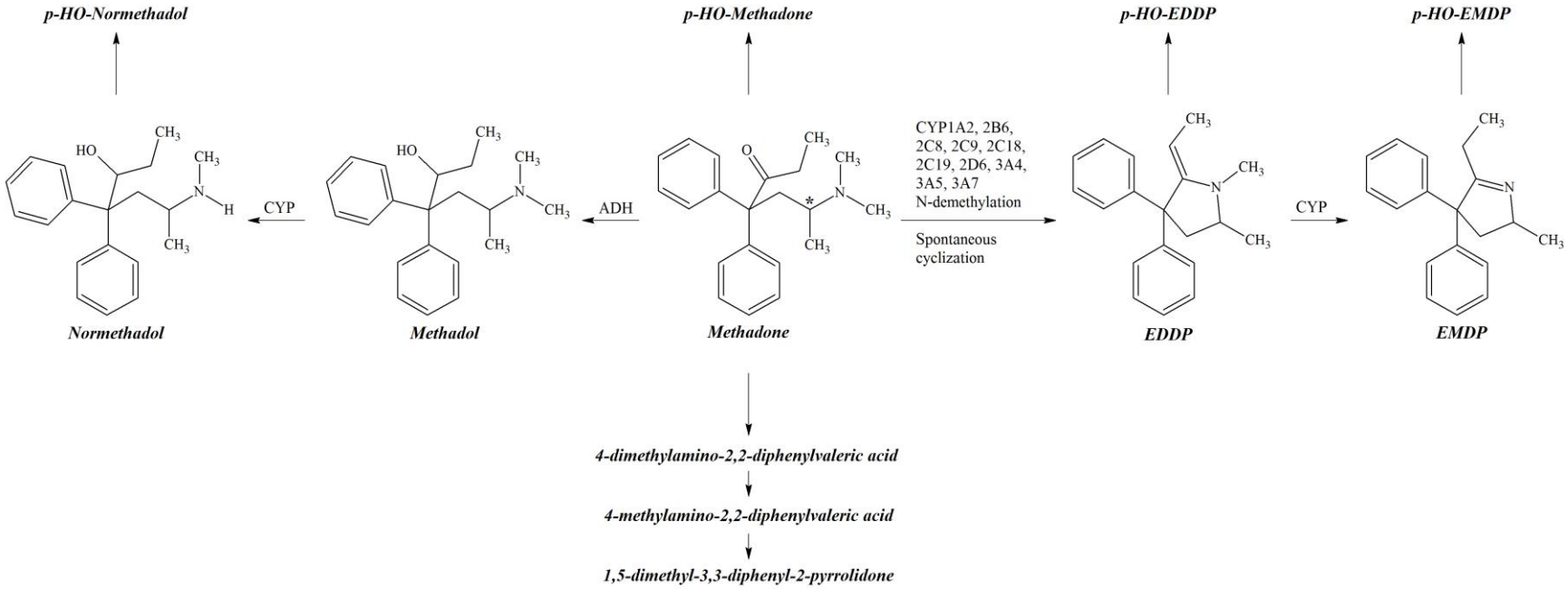

Figure 1: Eleven Methadone Metabolites Found in Human Excretion.
* Indicates chiral carbon atom. ADH, alcohol dehydrogenase; CYP, cytochrome P450; EDDP, 2-ethyl-1,5-dimethyl-3,3-diphenylpyrrolidine; EMDP, 2-ethyl-5-methyl-3,3-diphenyl-1-pyrroline; p-HO, para-hydroxy.

Genetic variation occurs within and among populations, leading to polymorphisms. SNPs are the most common type of genetic variation. A SNP is a variation of a single nucleotide at a specific position in the genome, some of which may contribute to changes in a gene, either in the coding (exons) or non-coding (introns) regions, or the regions between genes. A genetic variation within CYP genes responsible for the metabolism of methadone may be causal for altered function, which may result in methadone being rapidly eliminated from the body, thus preventing the drug from reaching therapeutic levels. Alternatively, the genetic variation may prolong elimination from the body, consequently, intensifying analgesic and detrimental adverse effects (Daly, 2004; Smith, 2009). The purpose of this review is to focus on the effects of SNPs of the various CYPs that metabolize methadone.

1.2 Methadone Metabolism

Eleven metabolites from methadone metabolism have been isolated and identified in urine and feces in humans (Dinis-Oliveira, 2016). Methadone predominantly forms the pharmacologically inactive pyrrolidine metabolites EDDP and 2-ethyl-5-methyl-3,3-diphenyl-1-pyrroline (EMDP). Methadol and normethadol are active analgesic metabolites of methadone, although this pathway is relatively minor (Sullivan & Due, 1973; Anggård et al., 1975). To a lesser extent, methadone, normethadol, EDDP, and EMDP are further hydrolyzed to form inactive *p*-hydroxy metabolites. Another minor pathway yields the inactive metabolites 4-dimethylamino-2,2-diphenylvaleric acid, 4-methylamino-2,2- diphenylvaleric acid, and 1,5-dimethyl-3,3-diphenyl-2-pyrrolidone (Figure 1) (Sullivan & Due, 1973; Anggård et al., 1975; Dinis-Oliveira, 2016).

The CYP enzymes involved in the formation of methadone metabolites in humans are CYP2B6, 3A4, 2C19, 2D6, and to a lesser extent, CYP2C18, 3A7, 2C8, 2C9, 3A5, and 1A2

(Chang, Fang, Lin, & Moody, 2011). These CYPs, however, metabolize methadone in a stereoselective manner, where CYP2C19, 3A7, and 2C8 preferentially metabolize (R)-methadone, CYP2B6, 2D6, and 2C18 primarily metabolize (S)-methadone, and CYP3A4 demonstrates no stereoselectivity in methadone metabolism (Chang et al., 2011; Lemke, Williams, Roche, & Zito, 2012). Methadone is N-demethylated to an intermediate compound, normethadone, which is then spontaneously cyclized to form EDDP. A second N-demethylation reaction occurs on EDDP to form EMDP (Sullivan & Due, 1973; Oda & Kharasch, 2001; Ferrari et al., 2004; Nanovskaya et al., 2004; DePriest, Puet, Holt, Roberts, & Cone, 2015). An alternate metabolic pathway in the formation of EMDP is a CYP mediated N-demethlyation of normethadone to form dinormethadone, which is then spontaneously cyclized to EMDP (Lemke et al., 2012). In a minor biotransformation pathway, the keto group of methadone is reduced by alcohol dehydrogenase to form methadol, which can be N-demethylated to form normethadol. The inactive (S)-methadone produces active metabolites of α-l-methadol and α-l-normethadol, which have analgesic properties comparable to (R)-methadone. (R)-Methadone, on the other-hand, is metabolized to biologically inactive metabolites of d-methadol and d-normethadol (Sullivan & Due, 1973; Anggård et al., 1975; Lemke et al., 2012).

Methadone's long ranging half-life can be partially attributed to the extreme interindividual pharmacokinetics and its stereoselective metabolism (Leshner, 1997; Boulton, Arnaud, & DeVane, 2001). (S)-Methadone exhibits a significantly shorter elimination half-life than (R)-methadone (Kristensen et al., 1996; DePriest et al., 2015). Inherited polymorphisms in CYPs have the potential to affect the metabolic rate of methadone in a stereoselective manner. The following sections will discuss the role of CYP SNPs in methadone metabolism and its consequences on biological activity.

1.3 Cytochrome P450 (CYP)

There are approximately 57 active human CYP genes grouped into 18 families and 44 subfamilies. Only 12 CYP enzymes, which belong to the CYP1, CYP2, or CYP3 families, are of major importance and responsible for the metabolism of more than 95% of therapeutic drugs (Wang & Tompkins, 2008; Wang, Li, & Zhou, 2009; Zanger & Schwab, 2013). The involvement and contribution of SNPs is inadequately understood in drug biotransformation, but are known to play a vital role in the differences in patient outcome and the development of personalized medicine (Solus et al., 2004). SNPs can cause a loss of function mutation, in which the splicing, expression, or transcription of the CYP gene is decreased or the protein structure is altered. Alternatively, gain of function mutations may also occur, resulting in an increased substrate turnover due to an increased number of functional gene copies, as well as variations in the promotor or amino acids (Johansson & Ingelman-Sundberg, 2008; Sadee et al., 2011; Zanger & Schwab, 2013). SNPs may be responsible for determining metabolic phenotype – ultra-rapid metabolizers (UMs), extensive metabolizers (EMs), intermediate metabolizers (IMs), and poor metabolizers (PMs) – and the binding affinity to CYPs, all while affecting the half-life, as well as the therapeutic and detrimental effects of methadone.

1.3.1 CYP1 Family

The CYP1 family consists of two subfamilies, encompassing three functional genes, *CYP1A1*, *1A2*, and *1B1*. CYP1A1 and CYP1B1 enzymes are mainly expressed extra-hepatically, whereas CYP1A2 accounts for 4-16% of the hepatic CYPs and is responsible for 8-10% of the drugs metabolized by CYP isoforms, including methadone (Daly, 2004; McGraw & Waller, 2012; Zanger & Schwab, 2013).

The interindividual variations in the mRNA and protein expression levels of CYP1A2 range from 15-40 fold, and a 40-130 fold interindividual difference in activity (Zhou, Liu, & Chowbay, 2009). To date, there are 192 genetic variants in *CYP1A2* (Fokkema et al., 2011; Fokkema & den Dunnen, 2017). An in silico study, by Wang et al. (2009), found 31 nonsynonymous SNPs, of which 24 were predicted to be deleterious by the SIFT and PolyPhen algorithms. (S)-Methadone is marginally metabolized by CYP1A2 (Zhou et al., 2009; Chang et al., 2011). However, results of an in vitro study, conducted by Wang and DeVane (2003), show neither the depletion of (R)- and (S)-methadone, nor the formation of EDDP by recombinant CYP1A2. Genetic variations in *CYP1A2* did not affect or had little influence on the plasma concentrations of methadone and EDDP (Fredheim et al., 2009; Zhou et al., 2009; Chang et al., 2011). Additionally, Crettol et al. (2006) observed *CYP1A2*1F* did not influence (R)-, (S)-, or (R,S)-methadone at the peak or trough levels in 245 MMT patients (96% white) (Table 1). Based on the current studies, *CYP1A2* SNPs have not been shown to impact the metabolism of methadone.

Alleles	dbSNP ID†	Key sequence change‡	Effect and key amino acid substitution (if any)	Effect on enzyme activity§
CYP1A2*1F	rs762551	intron 1, c.-9-154C>A		No influence on (R)-, (S)-, or (R,S)-MTD levels at trough or peak
CYP2B6*2	rs8192709	c.64C>T	R22C	↑ [(R,S)-MTD]/[EDDP] ratio
CYP2B6*4	rs2279343	c.785A>G	K262R	↑ (R,S)-MTD plasma levels ↓ (R,S)-MTD plasma levels ↑ MTD clearance ↑ (R)- and (S)-MTD N-demethylation
CYP2B6*5	rs3211371	c.1459C>T	R487C	↓ (S)-MTD plasma levels ↑ (S)-MTD clearance No effect on MTD clearance ↑ (R,S)-MTD plasma levels
CYP2B6*6	rs2279343 rs3745274	c.785A>G c.516G>T	K262R Q172H	↑ (S)- and (R,S)-MTD plasma levels ↑ MTD/Dose plasma ratio ↓ (R)- and (S)-MTD N-demethylation ↓ MTD clearance
CYP2B6*9	rs3745274	c.516G>T	Q172H	↑ (R,S)-MTD plasma levels ↑ [(S)-MTD/MTD Dose] plasma ratio ↓ (R)- and (S)-MTD N-demethylation ↓ (S)-MTD clearance
CYP2B6*11	rs35303484	c.136A>G	M46V	↑ (S)-MTD plasma levels ↓ (S)-MTD clearance
***CYP2B6* 3'UTR**	rs707265	c.*1355A>G		↑ [(S)-MTD/MTD Dose] plasma ratio ↓ (S)-MTD clearance
***CYP2B6* 3'UTR**	rs1038376	3'UTR, c.*1277A>T		↑ [(S)-MTD/MTD Dose] plasma ratio ↓ (S)-MTD clearance
***CYP2B6* intron 1**	rs10403955	c.172-468T>G		↑ [(S)-MTD/MTD Dose] plasma ratio ↓ (S)-MTD clearance
***CYP2B6* intron 5**	rs2279345	c.923-197T>C		↑ [(S)-MTD/MTD Dose] plasma ratio ↓ (S)-MTD clearance
CYP2C9*2	rs1799853	c.430C>T	R144C	No effect on MTD plasma levels

Alleles	dbSNP ID[†]	Key sequence change[‡]	Effect and key amino acid substitution (if any)	Effect on enzyme activity[§]
				↑ MTD/ Dose plasma ratio
CYP2C9*3	rs1057910	c.1075A>C	I359L	No effect on MTD plasma levels ↑ MTD/Dose plasma ratio
CYP2C19*2	rs4244285	c.681G>A	I331V; splicing defect	No effect on MTD plasma levels ↑ (R)-MTD plasma levels ↑ MTD/Dose plasma ratio ↑ EDDP plasma levels
CYP2C19*3	rs4986893	c.636G>A	W212X; stop codon	No effect on MTD plasma levels ↑ MTD/Dose plasma ratio
CYP3A4*1B	rs2740574	5'UTR, c.-392A>G		↑ (S)-MTD plasma levels
CYP3A5*3	rs776746	5'UTR, c.219-237A>G	Splicing defect	No effect on MTD plasma levels ↑ MTD metabolism (**1/*3* carrier) ↑ MTD/Dose plasma ratio (**3/*3*)

Table 1: Cytochrome P450 Single Nucleotide Polymorphisms Relevant to Methadone Metabolism

[†] National Center for Biotechnology Information (NCBI) dbSNP (NCBI Resource Coordinators, 2016)

[‡] Location of sequence change on mRNA for the respective CYP NM accession number (NCBI Resource Coordinators, 2016)

[§] (Crettol et al., 2005; Crettol et al., 2006; De Fazio et al., 2008; Fonseca et al., 2011; Wang et al., 2011; Dobrinas et al., 2013; Gadel et al., 2013; Kharasch & Stubbert, 2013; Lee et al., 2013; Levran et al., 2013; Wang et al., 2013; Carlquist et al., 2015; Gadel et al., 2015; Kharasch et al., 2015; Ahmad et al., 2017; Kringen et al., 2017)

More comprehensive information is available from the Human Cytochrome P450 Allele Nomenclature Committee (Zhou et al., 2017)

CYP, cytochrome P450; MTD, methadone; EDDP, 2-ethyl-1,5-dimethyl-3,3-diphenylpyrrolidine; UTR, untranslated region.

1.3.2 CYP2 Family

The CYP2 family represents about 30% of human CYP isoforms, making it the largest CYP family (Kim et al., 2015; Daly, 2016). The liver is comprised of five drug metabolizing CYP2 subfamilies (A-E), of which, the most pharmacologically important genes display the highest levels of polymorphism (Zanger & Schwab, 2013; Solus et al., 2004). Methadone is metabolized by several members of the CYP2 family: 2B6, 2C8, 2C9, 2C18, 2C19, and 2D6.

1.3.2.1 CYP2B

CYP2B6 has a 1-10% contribution to the total hepatic CYP pool, accounting for the metabolism of about 7-8% of pharmaceutical drugs (Zhou et al., 2009; Zanger & Schwab, 2013). Despite this enzyme's relatively low involvement in drug metabolism, CYP2B6 is the predominant determinant involved in the N-demethylation of methadone and clearance. CYP2B6 also displays stereoselectivity towards (S)-methadone (Chang et al., 2011; Kharasch & Stubbert, 2013). *CYP2B6* is one of the most polymorphic genes, having 74 allelic variants and 38 protein variants, which affect catalytic activity, transcriptional regulation, and splicing. The variants also affect mRNA and protein expression, exhibiting nearly 300 fold interindividual variability (Fokkema et al., 2011; Zanger & Klein, 2013; Zanger & Schwab, 2013; Ingelman-Sundberg, 2017; Zhou, Ingelman-Sundberg, & Lauschke, 2017; Fokkema & den Dunnen, 2018a).

CYP2B6 SNPs can alter the metabolic ratios of [methadone]/[EDDP]. A significant decrease in the metabolic ratio was observed in individuals carrying the *CYP2B6*2* allele, suggesting a role in an increased rate of metabolism. The data should be interpreted with caution because the subjects heterozygous for the variant only had a sample size of two (Ahmad, Sabet, Primerano, Richards-Waugh, & Rankin, 2017). A bioinformatics study also predicted *CYP2B6*2*

to alter the metabolic phenotype as determined by both the SIFT and PolyPhen algorithms (Wang et al., 2009).

Lee et al. (2013) studied the influence of *CYP2B6*4* on methadone plasma concentrations in 178 MMT patients in Taiwan. The study indicated an increase in (R,S)-methadone plasma levels in individuals homozygous for *CYP2B6*4*. The results were consistent with the Levran et al. (2013) study of 74 Israeli MMT patients; though neither study showed any significant differences in the increase in (R,S)-methadone plasma levels. On the other hand, Kharasch and colleagues (Kharasch, Regina, Blood, & Friedel, 2015) observed *CYP2B6*4* carriers had a significant decrease in (R)-, (S)-, and (R,S)- methadone plasma levels, with increased methadone metabolism and clearance. It is important to note that one of the limitations to this study was the small sample size (n=4). Gadel and colleagues (2015) demonstrated an increase in N-demethylation of methadone by *CYP2B6*4* in an in vitro study, supporting the findings of Kharasch et al. (2015).

*CYP2B6*5* shows contradicting data in its involvement in methadone clearance and plasma concentrations. In a genotyping study of methadone-only overdoses in 125 Caucasians, *CYP2B6*5* was linked to a significant increase in (R,S)-methadone plasma levels (Ahmad et al., 2017). In a study cohort consisting of 35 individuals with low (S)-methadone levels, *CYP2B6*5* was overrepresented, indicating an increased CYP2B6 activity (Dobrinas et al., 2013). The methadone plasma concentration of two individuals genotyped for homozygosity in *CYP2B6*5* appeared to remain unaltered when compared to that of the wild type *CYP2B6*1* (Kharasch et al., 2015).

*CYP2B6*6* is a haplotype consisting of two nonsynonymous variants, *CYP2B6*4* and *CYP2B6*9*. Numerous papers indicate an increase in (R,S)- and (S)-methadone plasma levels in

*CYP2B6*6/*6* individuals (Crettol et al., 2005; Crettol et al., 2006; Kharasch & Stubbert, 2013; Kharasch et al., 2015; Kringen et al., 2017). Gadel and colleagues (Gadel, Crafford, Regina, & Kharach, 2013) and Kharasch et al. (2015) have also demonstrated a decrease in methadone N-demethylation and decreased methadone clearance, respectively. An in vitro study found SNPs *CYP2B6*6* and *CYP2B6*9* to be catalytically deficient in EDDP formation (Gadel, Friedel, & Kharasch, 2015). Furthermore, increased (R,S)-methadone plasma levels were observed in 74 Israeli MMT patients homozygous for the *CYP2B6*9* SNP, though the increase was not statistically significant (Levran et al., 2013). A significant increase in plasma concentrations and a decrease in (S)-methadone metabolism was observed in 366 Taiwanese MMT patients encompassing the *CYP2B6*9* SNP (Wang et al., 2011). The studies observing the SNPs of the haplotype *CYP2B6*6* suggest that the diminishing metabolic activity of *CYP2B6*6/*6* is credited to the c.516G>T variant of *CYP2B6*9*.

Dobrinas et al. (2013) observed an overrepresentation of *CYP2B6*11* polymorphism in a high (S)-methadone level group of MMT patients, indicating a decline in CYP2B6 activity. Wang et al. (2011) examined polymorphisms in the intronic and 3'-untranslated region (UTR) regions of *CYP2B6*, and demonstrated the SNPs rs707265, rs2279345, rs1038376, and rs10403955 all correspond to increased [(S)-methadone/methadone dose] plasma ratios, and decreased (S)-methadone clearance (Table 1). The SNPs investigated in this section demonstrate a compelling link to the importance of genetic variations in *CYP2B6* and altered metabolism of methadone.

1.3.2.2 CYP2C

The CYP2C subfamily consists of four genes, *CYP2C8*, *2C9*, *2C18*, and *2C19*. The CYPs from these genes all contribute to the metabolism of methadone (Iribarne et al., 1996; Crettol et

al., 2005; Chang et al., 2011; Fonseca et al., 2011). CYP2C9 is the highest expressed isozyme of the four in liver, while CYP2C8 and CYP2C19 are expressed at 2 and 10 fold lower levels than CYP2C9 (Zanger & Schwab, 2013). CYP2C18 is expressed primarily in the skin (Zhou et al., 2009). CYP2C19 comprises 16% of the CYP2C family and metabolizes methadone to a greater extent, as compared to the CYP2C8, 2C9, and 2C18 isozymes (Dinis-Oliveira, 2016). The CYP2C family demonstrates stereoselectivity in methadone metabolism, where CYP2C8 and 2C19 predominately metabolize (R)-methadone, while CYP2C18 primarily metabolizes (S)-methadone.

Currently, there are no polymorphic studies on *CYP2C8* and *2C18* and their role in methadone metabolism. This is not surprising for *CYP2C18*, where only 8 variants have been reported to date (Fokkema et al., 2011; Fokkema & den Dunnen, 2015a). There are 97 genetic variants observed in *CYP2C8* (Fokkema et al., 2011; Fokkema & den Dunnen, 2015b), which has a minor role in the metabolism of methadone, probably contributing to the dearth of studies in the corresponding SNPs.

Although *CYP2C9* exhibits 378 genetic variants and *2C19* has 236 reported genetic variants (Fokkema et al., 2011; Fokkema & den Dunnen, 2018b; Fokkema & den Dunnen, 2019), only **2* and **3* have been examined for their respective CYP isozymes. Results from three separate studies showed no effect on methadone plasma levels with *CYP2C9*2* and *CYP2C9*3* genotypes in Caucasians (Crettol et al., 2005; Crettol et al., 2006; Fonseca et al., 2011). A significant increase in methadone serum concentrations/dose ratio with heterozygous carriers of *CYP2C9*2* and *CYP2C9*3* SNPs was observed in a study in Norway. Similar results were seen for the homozygous *CYP2C9*2* and *CYP2C9*3* individuals, albeit, there were no significant differences in methadone serum concentrations/dose ratios (Kringen et al., 2017).

In studies by Crettol et al. (2005; 2006), *CYP2C19*2* and *CYP2C19*3* were also found to lack an effect on methadone plasma levels. A study of 366 Taiwanese MMT patients showed homozygous carriers of *CYP2C19*2* were associated with a significant increase in (R)-methadone levels. Patients homozygous for *CYP2C19*3* also tended to exhibit an increase in (R)-methadone levels, although the data was not significantly different (Wang et al., 2013). Carlquist et al. (2015) observed an increase in the plasma levels of EDDP with *CYP2C19*2* in 25 MMT patients (20 Caucasian, 4 Hispanic, and 1 African American), indicating an increase in methadone metabolism. Kringen et al. (2017) noticed, in a Norwegian population, individuals homozygous and heterozygous carriers for *CYP2C19*2* and *CYP2C19*3* had an increase in methadone serum concentration/dose ratio, with significant differences seen only in heterozygotes. The contradicting results in the *CYP2C19* variants between these studies could be attributed to the ethnicity of the patients, where the frequencies of both *CYP2C19*2* and *CYP2C19*3* are higher in Asians than Caucasians (Table 1).

1.3.2.3 CYP2D

The CYP2D subfamily consists of only one protein coding gene, *CYP2D6*, which accounts for 2-5% of the hepatic CYP composition and ~25% of pharmacological drug metabolism (Zanger & Schwab, 2013; NCBI Resource Coordinators, 2016). CYP2D6 metabolizes both enantiomers of methadone, with a slightly greater propensity towards (S)-methadone (Wang & DeVane, 2003; Chang et al., 2011). Unlike SNPs in other CYP genes, CYP2D6 phenotype is determined by allele combinations. PMs consist of two non-functional alleles, IMs are comprised of two decreased activity alleles, EMs have at least one functional allele, and UMs encompass more than one functional allele and/or an allele with a promoter mutation (Fonseca et al., 2011). The major and most common mutation alleles are *CYP2D6*3*,

**4*, **5*, and **6*, accounting for 93-97% of alleles among PM phenotypes (Eap et al., 2001; Trescot & Faynboym, 2014). *CYP2D6*1* and *CYP2D6*2* alleles represent normal functional activity.

In a 2001 study conducted by Eap et al. (2001), CYP2D6 phenotypes had a significant influence on the (R)-, (S)-, and (R,S)-methadone blood concentrations, where higher concentrations were observed in PMs (*CYP2D6*4/*4, *4/*3, *4/*6*) and lower concentrations were measured in UMs (*CYP2D6*1/*1*). Similarly, Crettol and colleagues (2006), observed a 0.5 fold decrease in (S)-methadone and 0.7 fold decrease in (R)-methadone plasma levels in UMs (*CYP2D6*1/*1xN*) when compared to the EM/IM (*CYP2D6*1/*3, *1/*4, *1/*5, *1/*6*) group. In another study, the PM, IM, and EM phenotypes, representing alleles *CYP2D6*1, *1xN, *2, *3, *4, *4xN, *5, *6, *9, *10, *16, *28, *33,* and **41*, did not impact the clearance of (R)-, (S)-, or (R,S)-methadone (Coller et al., 2007). *CYP2D6*3, *4, *5, *6, *7,* and **8* are non-functional variants, and *CYP2D6*9, *10,* and **41* have a decrease in function (Coller et al., 2007). Fonseca et al. (2011) observed EM patients received significantly lower doses of methadone as compared to UMs. It was also concluded that, contradictory to the phenotypes, UM (*CYP2D6*1xN, *2xN*) patients had significantly higher methadone plasma levels than EM (*CYP2D6*1, *2, *3, *6, *35*) and PM (*CYP2D6*4/*4*) patients. Further investigations on the *CYP2D6* alleles need to be conducted to get a clearer understanding of their significance. The relationship between *CYP2D6* allelic variants and phenotypic expression is depicted in Table 2.

Alleles	dbSNP ID[†]	Key sequence change[‡]	Effect and key amino acid substitution (if any)	Effect on enzyme activity[§]	Phenotype
CYP2D6*1				Wild type Duplication of gene	EM
CYP2D6*1xN				Multiduplication of gene *N* active genes ↑ activity	UM
CYP2D6*2	rs16947 rs1135840	c.886C>T c.1457G>C	R296C S486T	Normal activity Duplication of gene	EM
CYP2D6*2xN				Multiduplication of gene *N* active genes ↑ activity	UM
CYP2D6*3	rs3574686	c.775delA	259Frameshift	Defective allele	PM
CYP2D6*4	rs1065852 rs28371703 rs28371704 rs3892097 rs1135840	c.100C>T c.271C>A c.281A>G c.506-1G>A c.1457G>C	P34S L91M H94R Splicing defect S486T	Defective allele	PM
CYP2D6*4xN				Defective allele Multiduplication of gene	PM
CYP2D6*5				Deletion of entire CYP2D6 gene	PM
CYP2D6*6	rs5030655	c.454delT	118Frameshift	Defective allele	PM
CYP2D6*7	rs5030867	c.971A>C	H324P	Defective allele	PM
CYP2D6*8	rs5030865 rs16947 rs1135840	c.505G>A c.886C>T c.1457G>C	G169R R296C S486T	Defective allele	PM
CYP2D6*9	rs5030656	c.841_843delAAG	K281del	Impaired function	IM
CYP2D6*10	rs1065852 rs1135840	c.100C>T c.1457G>C	P34C S486T	Impaired function	IM
CYP2D6*16		CYP2D7/2D6 hybrid	Frameshift; switch region exon7-intron 8		PM

Alleles	dbSNP ID[†]	Key sequence change[‡]	Effect and key amino acid substitution (if any)	Effect on enzyme activity[§]	Phenotype
CYP2D6*17	rs28371706	c.320C>T	T107I	Impaired function	IM
	rs16947	c.886C>T	R296C		
	rs1135840	c.1457G>C	S486T		
CYP2D6*28		19G>A	V7M	Unknown	Unknown
	rs78482768	c.451C>G	Q151E		
	rs16947	c.886C>T	R296C		
	rs1135840	c.1457G>C	S486T		
CYP2D6*29	rs61736512	c.406G>A	V136I	Impaired function	IM
	rs16947	c.886C>T	R296C		
	rs59421388	c.1012G>A	V338M		
	rs1135840	c.1457G>C	S486T		
CYP2D6*33	rs28371717	c.709G>T	A237S	Normal activity	EM
CYP2D6*35	rs769258	c.31G>A	V11M	Normal activity	EM
	rs16947	c.886C>T	R296C	Duplication of gene	
	rs1135840	c.1457G>C	S486T		
CYP2D6*36	rs1065852	c.100C>T	P34S	Defective allele	PM
	rs1135840	c.1457G>C	S486T		
CYP2D6*41	rs16947	c.886C>T	R296C	Impaired function	IM
	rs28371725	c.985+39G>A	Splicing defect		
	rs1135840	c.1457G>C	S486T		
CYP2D6*43	rs28371696	c.77G>A	R26H	Normal activity	EM
				Duplication of gene	
CYP2D6*45	rs28371710	c.463G>A	E155K	Normal activity	EM
	rs16947	c.886C>T	R296C	Duplication of gene	
	rs1135840	c.1457G>C	S486T		

Table 2: Cytochrome P450 2D6 and Their Corresponding Activity and Phenotype

[†] National Center for Biotechnology Information (NCBI) dbSNP (NCBI Resource Coordinators, 2016)

[‡] Location of sequence change on mRNA for the respective CYP NM accession number (NCBI Resource Coordinators, 2016)

[§] (Eap et al., 2001; Crettol et al., 2006; Coller et al., 2007; Johansson & Ingelman-Sundberg, 2008; Fonseca et al., 2011)

More comprehensive information is available from the Human Cytochrome P450 Allele Nomenclature Committee (Zhou et al., 2017)

CYP, cytochrome P450; del, deletion; EM, extensive metabolizer; IM, intermediate metabolizer; PM, poor metabolizer; UM, ultra-rapid metabolizer.

1.3.3 CYP3 Family

The CYP3 family consists of only one subfamily, CYP3A, and four functional genes, *CYP3A4*, *3A5*, *3A7*, and *3A43*. These enzymes play a dominant role in drug metabolism pathways (~50%). CYP3A4 is the most expressed enzyme in adult liver, while CYP3A7 is predominantly expressed in fetal liver and is down-regulated after birth. CYP3A5 is polymorphically expressed in the liver, where alternative splicing results in multiple transcript variants, exhibiting high and low level proteins (Lamba, Lin, Schuetz, & Thummel, 2012; Zanger & Schwab, 2013; NCBI Resource Coordinators, 2016).

Methadone has been shown to be N-demethylated by CYP3A4, 3A5, and 3A7 in a nonstereoselective manner (Foster, Somogyi, & Bochner, 1999; Chang et al., 2011). *CYP3A4* and *CYP3A5* have relatively low numbers of genetic variants, suggesting minor clinical importance in the clearance of their respective substrates (Zhou et al., 2009; Levran et al., 2013). This observation would be in line with the lack of polymorphic studies found for CYP3A and their role in methadone metabolism. However, in one study, the allelic frequency of *CYP3A4*1B* in 245 MMT patients (96% white) was observed and correlated with an increase in methadone plasma levels (Crettol et al., 2006). In the same study, the study cohort was genotyped for the deficient allele *CYP3A5*3*. The plasma levels of methadone were not influenced by the *CYP3A5* genotype. It is also interesting to note that there was a strong linkage between *CYP3A4*1B* and *CYP3A5*3* genotypes (Crettol et al., 2006). Similar results were observed by Fonseca, et al. (2011), where the genetic polymorphism of *CYP3A5* did not influence methadone plasma levels. One case report of a 25-year old male in a MMT program showed extensive metabolism of methadone to EDDP. The subject was found to be heterozygous for *CYP3A5*1* (*CYP3A5*1/*3*) (De Fazio, Gallelli, De Siena, De Sarro, & Scordo, 2008). A large amount of CYP3A5 is

expressed in individuals carrying at least one *CYP3A5*1* allele (De Fazio et al., 2008). In a recent study of 155 serum samples and 62 patients, homozygous carriers of *CYP3A5*3* demonstrated a significant increase in methadone serum concentration/dose ratio, indicating a decrease in methadone clearance (Kringen et al., 2017) (Table 1).

1.4 Consequences of Altered Methadone Metabolism

Polymorphisms in genes encoding CYP enzymes have the potential to shift their metabolic capacity or change the substrate specificity of the enzyme, eliciting variable consequences in drug treatment (Jurica & Sulcova, 2012; Tamási & Falus, 2012). These changes can be crucial in the therapeutic potency of methadone. The main metabolic pathway of methadone involves the N-demethylation by hepatic CYPs into the primary metabolite, EDDP. Changes in the rate of EDDP formation could influence the pharmacodynamics of methadone.

*CYP2B6*4* was the only SNP that was found to increase the clearance of methadone by the N-demethylation of both (R)- and (S)-methadone (Gadel et al., 2015; Kharasch et al., 2015). In turn, there would be a decrease in the disposition of (R,S)-methadone, reducing the pharmacological effect of methadone and inducing withdrawal symptoms. Individuals with this SNP may require a higher dose of methadone.

Several SNPs were found to increase the methadone plasma concentration due to a decrease in the metabolic activity of the CYPs. Three independent studies collectively demonstrated elevated plasma levels of (R,S)- and (S)-methadone with diminishing N-demethylation of (R)- and (S)-methadone due to *CYP2B6*2, *9* and *11* (Wang et al., 2011; Dobrinas et al., 2013; Levran et al., 2013; Gadel et al., 2015; Ahmad et al., 2017). *CYP2B6*6* is a haplotype consisting of both *CYP2B6*4* and *CYP2B6*9* variants. Carriers of *CYP2B6*6/*6* had diminished methadone metabolism and clearance, hence, an increase in methadone levels.

This activity is credited to the *CYP2B6*9* SNP, where individuals with *CYP2B6*4/*6* haplotype did not observe the metabolic inhibition (Crettol et al., 2005; Crettol et al., 2006; Gadel et al., 2013; Kharasch & Stubbert, 2013; Gadel et al., 2015; Kharasch et al., 2015; Kringen et al., 2017). Mutations located in the UTR and intronic regions are also relevant in the biotransformation of methadone. Table 1 shows the four SNPs in the UTR and intronic regions linked to a decreased clearance and increased concentration of methadone (Wang et al., 2011). CYP2B6 influences the metabolism of (S)- methadone and, to a far lesser extent, (R)-methadone. Individuals with the genotypes mentioned above, particularly *CYP2B6*6/*6*, have a greater risk of suffering torsades de pointes and sudden death. A lower dose of methadone may be required for treatment for individuals with these variants.

Reduced metabolism by CYP2C19 can lead to increased plasma concentrations of methadone, specifically (R)-methadone, which can potentiate depressed ventilation manifesting as a potentially fatal respiratory depression. Both *CYP2C19*2* and *CYP2C19*3* were associated with elevated methadone plasma concentrations (Wang et al., 2013; Kringen et al., 2017). However, these results could be variable in different ethnic groups.

CYP2D6 and CYP3A4/5 metabolize both enantiomers of methadone, though CYP2D6 has a slightly greater preference towards (S)-methadone. Decreased metabolism by these CYPs may heighten either detrimental effect due to augmented levels of methadone. *CYP3A4*1B* has been shown to increase (S)-methadone plasma levels (Crettol et al., 2006), which can lead to cardiotoxic effects. In a study of 136 Caucasian individuals who died of methadone-only overdosing, an enrichment was observed in the *CYP3A4*1B* SNP (Richards-Waugh, Primerano, Dementieva, Kraner, & Rankin, 2014). *CYP3A5*3* is a common SNP with high frequency in the Caucasian population, where a splicing defect causes a loss of function of the *CYP3A5* enzyme

(McGraw & Waller, 2012). This loss of function is exemplified in a couple of studies where the methadone plasma levels are elevated in *CYP3A5*3/*3* individuals, while metabolism is increased and plasma levels are decreased in individuals carrying at least one copy of *CYP3A5*1* (De Fazio et al., 2008; Gadel et al., 2013). The effects of SNPs located on the *CYP2D6* gene is slightly more complicated. CYP2D6-dependent metabolism of methadone has been studied based on the phenotypic variability determined by a combination of SNPs on the *CYP2D6* gene. There have not been any clear implications on the relationship between *CYP2D6* SNPs and methadone metabolism and their role on the pharmacokinetics and pharmacodynamics of methadone.

Since methadone is metabolized by a number of CYPs, a combination of any of the SNPs, within or between genes, can have varying effects on the pharmacological response of methadone treatment. A genome-wide pharmacogenomic study showed two *CYP2B6* haplotypes of rs8100458, rs7250601, rs7250991, rs11882424, rs8192719, and rs10853744 (T-A-A-T-C-G and T-C-C-T-T-T) accounted for the variation in (S)-methadone plasma levels (Yang et al., 2016). In order to understand the complexity of the effects of CYP SNPs on methadone pharmacokinetics and pharmacodynamics, inclusion of additional gene variants and haplotype variants is required. Though SNPs influence only part of methadone pharmacology, the impact of genetic variations can be compounded by drug interactions, environmental factors, sex, health, and other nongenetic factors. Co-administration of methadone with respiratory depressants, such as other opioids, alcohol, or benzodiazepines, may lead to a greater detrimental effect. Likewise, individuals with reduced liver function (e.g. cirrhosis) could have a reduced capacity to metabolize methadone.

1.5 Conclusions

Methadone is currently used as a treatment and maintenance therapy for opioid addiction and as an analgesic for severe chronic pain. The wide-ranging half-life of methadone may result in some individuals having higher than normal therapeutic levels leading to negative, detrimental, and fatal side effects. Elevated (R)-methadone levels may lead to respiratory depression, while an increase in plasma levels of (S)-methadone may lead to severe cardiac arrhythmias. A significant portion of opioid-related deaths have been contributed to the use of prescription methadone for pain management. The pharmacogenetics of an individual possesses the ability to affect the pharmacokinetics and pharmacodynamics of methadone. SNPs located on CYP genes shift the metabolic capacity or change the substrate specificity of the CYP enzyme, affecting the metabolism of methadone. SNPs *CYP2B6*6, *9, *11*, *CYP2C19*2, *3*, *CYP3A4*1B*, and *CYP3A5*3* result in increased methadone plasma concentrations, decreased N-demethylation, and decreased methadone clearance. Since *CYP2B6* is the major determinant of methadone clearance, *CYP2B6*6/*6* is of particular interest. Homozygous carriers of *CYP2B6*6/*6* expressed diminished methadone metabolism and clearance, thus these individuals have a greater propensity for detrimental adverse effects. However, *CYP2B6*4* demonstrated an increase in N-demethylation and methadone clearance. A better understanding of the role of CYP SNPs in methadone metabolism can improve the proper therapeutic dosing for methadone, patient outcome, and the development of individualized medicine.

1.6 Conflict of Interest

The authors declare no conflict of interest.

CHAPTER 2

TELL-TALE SNPS: THE ROLE OF CYP2B6 IN METHADONE FATALITIES

The information in this chapter has been published in *Journal of Analytical Toxicology*.

Ahmad, T., Sabet, S., Primerano, D. A., Richards-Waugh, L. L., & Rankin, G. O. (2017, May). Tell-tale SNPs: The role of CYP2B6 in methadone fatalities. *Journal of Analytical Toxicology, 41*(4), 325-333. doi:10.1093/jat/bkw135

Taha Ahmad[1], Samie Sabet[1], Donald A. Primerano[1], Lauren L. Richards-Waugh[2], and Gary O. Rankin[1,*]

[1]Department of Biomedical Sciences, Joan C. Edwards School of Medicine, Marshall University, 1700 Third Avenue, Huntington, WV 25755, USA, and [2]Forensic Science Department, Marshall University, 1401 Forensic Science Drive, Huntington, WV 25701, USA

*Corresponding author at: Department of Biomedical Sciences, Joan C. Edwards School of Medicine, BBSC-435F, Marshall University, 1700 Third Avenue, Huntington, WV 25755, USA. E-mail address: rankin@marshall.edu (G.O. Rankin).

Abstract

Cytochrome P450 (CYP) enzyme 2B6 plays a significant role in the stereoselective metabolism of (S)- methadone to 2-ethyl-1,5-dimethyl-3,3-diphenylpyrrolidine, an inactive methadone metabolite. Elevated (S)-methadone can cause cardiotoxicity by prolonging the QT interval of the heart's electrical cycle. Large interindividual variability of methadone pharmacokinetics causes discordance in the relationship between dose, plasma concentrations and side effects. The purpose of this study was to determine if one or more single nucleotide polymorphisms (SNPs) located within the *CYP2B6* gene contributes to a poor metabolizer phenotype for methadone in these fatal cases. The genetic analysis was conducted on 125 Caucasian methadone-only fatalities obtained from the West Virginia and Kentucky Offices of the Chief Medical Examiner. The frequency of eight exonic and intronic SNPs (rs2279344, rs3211371, rs3745274, rs4803419, rs8192709, rs8192719, rs12721655 and rs35979566) was determined. The frequencies of SNPs rs3745274 (**9*, c.516G>T, Q172H), and rs8192719 (21563C>T) were enhanced in the methadone-only group. Higher blood methadone concentrations were observed in individuals who were genotyped homozygous for SNP rs3211371 (**5*, c.1459C>T, R487C). These results indicate that these three *CYP2B6* SNPs are associated with methadone fatalities.

2.1 Introduction

Initially discovered and developed in Germany during World War II, methadone is a synthetic MOR agonist that has been used as an analgesic alternative to morphine when it was difficult to obtain (Lugo et al., 2005; Lisberg & Scheinmann, 2013; Somogyi et al., 2014). In 1965, methadone was introduced as a treatment regimen of opioid drug dependence or withdrawal symptoms in patients (Somogyi et al., 2014). Nearly a decade later, in 1976,

physicians were given permission to prescribe methadone as an analgesic to maintain adequate pain control (Toombs & Kral, 2005), for malignant pain in cancer patients and non-malignant pain that continues beyond the normal course of a disease or injury (Taylor et al., 2000; Jackman et al., 2008).

Methadone is clinically available in the USA only as a racemic mixture containing equal amounts of the (R)- and (S)-methadone enantiomers (Gaertner et al., 2008; Kapur et al., 2011; McCance-Katz, 2011). Each enantiomer has specific pharmacodynamic properties. The analgesic properties of methadone are attributed to the (R)-enantiomer, which has a 10-fold greater affinity for the MOR (Kristensen, Christensen, & Christrup, 1995; Foster et al., 1999) and is 8 – 50 times more potent than (S)-methadone (Lugo et al., 2005). As concentrations of (R)-methadone increase above therapeutic levels, negative side effects on MORs in the brainstem respiratory centers can be potentiated, thus mediating respiratory depression (Mitchell et al., 2004; Silverman et al., 2009). On the other hand, elevated (S)-methadone causes cardiotoxicity by prolonging the QT interval and subsequently leads to torsade de pointes by blocking the voltage-gated potassium channel of the human ether-a-*go-go* related gene (hERG) (Eap et al., 2007; Dobrinas et al., 2013). This stereoselective cardiotoxicity is attributed to (S)-methadone being 3.5 times more potent than (R)- methadone in blocking the hERG channel (Eap et al., 2007; Ansermot et al., 2010; Dobrinas et al., 2013).

Methadone metabolism occurs primarily through the route of oxidative biotransformation. The principle metabolite, EDDP, is formed during metabolism through an N-demethylation reaction followed by spontaneous cyclization (Moody et al., 1997; Foster et al., 1999; Ferrari et al., 2004; Nanovskaya et al., 2004; Lehotay et al., 2005). EDDP is further metabolized to EMDP. Neither of these metabolites is pharmacologically active (Oda &

Kharasch, 2001; Ferrari et al., 2004; Nanovskaya et al., 2004). CYP enzymes in the liver are responsible for the metabolism of methadone, which undergoes stereoselective N-demethylation (Lugo et al., 2005). CYP3A4, 2B6 and 2C19 account for majority of the conversion of methadone to EDDP and other inactive metabolites, with minor contributions from CYP2C8, 2D6, 2D8, 2C18 and 3A7 (Wang & DeVane, 2003; Chang et al., 2011; Kapur et al., 2011). (R)-Methadone is preferentially metabolized by CYP2C8 and CYP2C19, whereas (S)-methadone is primarily metabolized by CYP2B6 and CYP2D6. CYP3A4 has no stereoselectivity, metabolizing both enantiomers (Chang et al., 2011; Kapur et al., 2011).

Methadone is a difficult medication to safely prescribe due to extreme variability in interindividual pharmacokinetics, making the relationship between dose, plasma concentrations and effects difficult to define (Leshner, 1997; Boulton et al., 2001). This methadone plasma variability increases the risk of an unexpected death. According to the most recent CDC report (2012), methadone accounts for >30% of prescription opioid deaths, while it only represents 2% of all opioid prescriptions. Much of the interindividual variability in methadone pharmacokinetics may be due to genetic variations within the genes encoding CYP enzymes responsible for the metabolism of methadone (Li et al., 2008).

SNPs are the most common form of genetic variation in the CYP genes (Risch, 2000; Daly, 2004). *CYP2B6* is a highly polymorphic gene with 74 allelic variants equating to 38 protein variants and a series of subvariants, designated **1B* to **38* (Zhou et al., 2009; McGraw & Waller, 2012; Ingelman-Sundberg, 2017; Fokkema & den Dunnen, 2018a). These SNPs are thought to play a role in interindividual and interethnic differences in the response of drug metabolism by CYP2B6 (Zhou et al., 2009). It is known that CYP2B6 plays a major role in the biotransformation of many clinically important drugs, such as selegiline (Watanabe et al., 2010),

bupropion (Lang et al., 2004), efavirenz (Holzinger et al., 2012), artemether (Honda et al., 2011) and methadone (Eap et al., 2007; Ansermot et al., 2010; Dobrinas et al., 2013). These studies demonstrated that some allelic variants (e.g., rs2279344, rs3211371, rs3745274, rs4803419, rs8192709, rs8192719, rs12721655 and rs35979566) lead to a decreased expression or decreased enzyme activity of CYP2B6.

Several pharmacogenetic studies have been previously conducted involving methadone-related deaths or patients in a MMT program. However, these studies did not include cases where only methadone was detected; rather, those studies used mixed drug cases and/or had small sample sizes (Crettol et al., 2005; Eap et al., 2007; Dobrinas et al., 2013). The present work is unique because we genotyped and analyzed *CYP2B6* variants in 125 Caucasians involved with methadone-only fatalities. Eight key SNP variants present in the Caucasian population were genotyped to test for enrichment. The analyses performed in this study were aimed to provide insight into the association of *CYP2B6* SNPs in individuals who succumb to methadone intoxication in the methadone-only cases. Because exonic variants cause amino acid substitutions, which can affect the enzymatic activity and intronic regions, are responsible for splicing, frameshifting and binding microRNAs, SNPs in both of these regions were examined to determine their association with methadone toxicity in the study population.

2.2 Material and Methods

2.2.1 Chemicals and Enzymes

QIAamp DNA Micro extraction kits were purchased from Qiagen (Valencia, CA, USA). Absolute ethanol was purchased from Pharmco-Aaper (Shelbyville, KY, USA). MicroAmp Optical 96-well Reaction Plates, 96-well Fast PCR Plates, MicroAmp Optical Adhesive Film, TaqMan Universal PCR Master Mix No AmpErase UNG and TaqMan SNP Genotyping Assays

were purchased from Life Technologies™ (Foster City, CA, USA). Tris base, ethylenediaminetetraacetic acid (EDTA) and all other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2.2 Sample Collection

The characteristics of all of the samples used and studied in the paper were described in a previous study on methadone pharmacogenetics (Richards-Waugh et al., 2014). Briefly, the cases for this study were derived from fatalities due to methadone occurring from 2003 to 2008 in the Offices of the Chief Medical Examiner (OCMEs) of West Virginia (WV) and Kentucky (KY). Based on the toxicity criteria, 125 Caucasian cases (78 from WV and 47 from KY) related to methadone-only fatalities were selected. A control group of 255 cases was obtained from the WV OCME, in which toxicology immunoassays were negative for amphetamines, barbiturates, benzodiazepines, buprenorphine, cocaine, methadone, propoxyphene, opiates, oxycodone and tricyclic antidepressants. Demographic data for the WV cases, along with the number of KY cases are outlined in Table 3.

	Methadone-Only Overdoses	**Controls**
WV Cases	78	255
KY Cases*	47	0
Total Cases	125	255
Age Ranges (years)	1.75 – 58	2 – 88
Mean Age	34.07 ± 11.40	45.35 ± 18.70
Males (%)	58 (74.4%)	194 (76.1%)
Females (%)	20 (25.6%)	61 (23.9%)

Table 3: Demographic of Study Groups
*Age and gender were not available for the KY Cases

2.2.3 DNA Extraction

Following the manufacturer's protocol for dried blood spots, QIAamp DNA Micro extraction kits were used for the purification of genomic DNA from blood stain cards prepared by the WV and KY OCMEs. Extractions were performed in triplicate in order to increase overall

genomic DNA yields; the three DNA solutions were pooled and then concentrated by passage over a MiniElute column. Extracted DNA was quantitated by absorbance spectrophotometry on a NanoDrop 1000. Samples were diluted to a concentration of 1.78 ng/µL with Tris-EDTA buffer to a final volume of 100 µL for use in the SNP genotyping assays. All genomic DNA samples, initial extractions and 1.78 ng/µL working solutions, were stored at 4°C.

2.2.4 SNP Selection

Eight different SNPs within the *CYP2B6* gene were genotyped. Five exonic SNPs, rs3211371 (**5*, c.1459C>T, R487C), rs3745274 (**9*, c.516G>T, Q172H), rs8192709 (**2*, c.64C>T, R22C), rs12721655 (**8*, c.415A>G, K139E) and rs35979566 (**15*, c.1172T>A, I391N), were selected because they were considered polymorphic in the Caucasian population, and known to encode non-synonymous amino acid substitutions (Figure 2). The remaining three SNPs, rs2279344 (18273G>A), rs4803419 (15582C>T) and rs8192719 (21563C>T), were intronic and had Minor Allele Frequencies (MAFs) of at least 5% (Figure 2).

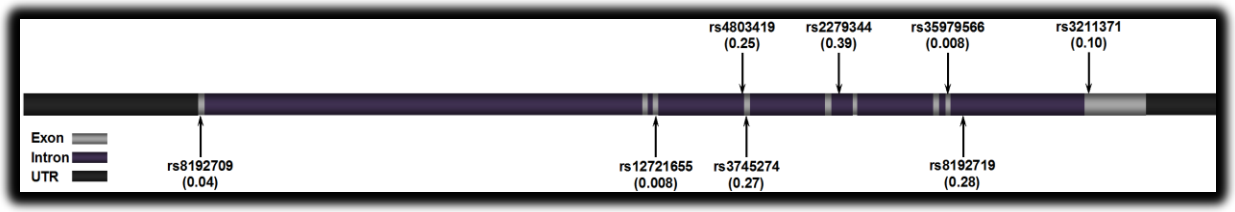

Figure 2: Location of Exonic and Intronic SNPs within the *CYP2B6* Gene
Minor allele frequency in parenthesis.

2.2.5 SNP Genotyping

The following Life Technologies™ TaqMan SNP Genotyping kits were used to determine *CYP2B6* genotypes: (i) C__26823974_30 (rs2279344), (ii) C__30634242_40 (rs3211371), (iii) C___7817765_60 (rs3745274), (iv) C___7817764_10 (rs4803419), (v) C___2818162_20 (rs8192709), (vi) C__22275631_10 (rs8192719), (vii) C__30634236_20

(rs12721655) and (viii) C__33845840_20 (rs35979566). Real-time polymerase chain reaction (RT-PCR) and allelic discrimination were carried out in a total volume of a 25 µL reaction, containing 20 ng of genomic DNA, TaqMan Universal PCR Master Mix No AmpErase UNG (1X) and a TaqMan SNP Genotyping Assay (1X). PCR parameters were set for polymerase activation at 95°C for 10 min followed by 50 cycles (rs2279344, rs3211371, rs3745274, rs4803419 and rs8192709) or 55 cycles (rs8192719) of denaturation at 92°C for 30 s and annealing/extension for 90 s at 60°C for each SNP on an ABI7000 Sequence Detection System. SNP rs12721655 and rs35979566 were genotyped on an ABI StepOnePlus™ Instrument using the RT-PCR parameters of 95°C for 10 min followed by 65 cycles of 92°C for 15 s and 60°C for 90 s. All SNP genotypes were determined using both the auto and manual allele call options on their respective instrument software.

2.2.6 Statistical Analysis

Allele frequencies for the control and methadone-only groups were calculated based on the allelic calls for all SNPs in each group. A χ^2 test with two degrees of freedom was used to assess the differences between the MAFs in the control group and the MAFs reported in the National Center for Biotechnology Information (NCBI) dbSNP database for the Caucasian population and between the control study group and the methadone-only fatality study group. P-values < 0.05 were considered to statistically significant. We also assessed whether genotypic frequencies for each SNP were in Hardy–Weinberg equilibrium (HWE) using a χ^2 test with two degrees of freedom. When performing the statistical analysis on the blood methadone concentrations and [methadone]/[EDDP] ratio, the methadone-only group was further divided into two groups. All 125 fatalities due to methadone were used for the analysis of blood methadone concentrations, while only the cases obtained from the WV OCME were used for the

analysis of [methadone]/[EDDP] ratios (the KY OCME did not determine EDDP concentrations, hence the [methadone]/[EDDP] ratio data were not available). The blood methadone concentration measurements were conducted by the OCMEs during the toxicological testing, and therefore, represent both the (R)- and (S)- methadone concentrations combined. The mean, standard deviation, standard error of the mean (SEM) and unpaired t-test with Welch's correction or one-way analysis of variance (ANOVA) with Tukey test correction was used to compare blood methadone concentrations (mg/L) between homozygotes for the major allele (ancestral), heterozygotes and homozygotes for the minor allele (variant) in WV/KY methadone-only cases. The same tests were also performed to compare [methadone]/[EDDP] ratio between ancestral, heterozygotes and variant alleles in the WV methadone-only cases. One-way ANOVA analyses were performed only on groups that had a minimum number of two cases for each genotype; an unpaired t-test with Welch's correction was performed in all other cases. Statistical analyses were carried out using GraphPad Prism 6 software version 6.02.

2.2.7 VEP of Non-synonymous SNP Variants

The consequences of the exonic SNPs were predicted using the online Variant Effect Predictor (VEP) tool on EnsemlGenomes (http://useast.ensembl.org/Tools/VEP). SIFT and PolyPhen scores were obtained by inputting and running the variant identifiers for Human (Homo sapiens) on VEP.

2.3 Results

Using the data from the allelic discrimination analysis, genotypic and MAFs were calculated for the eight SNPs in all groups (Table 4-11). Two SNPs, rs12721655 (MAF 0.8%) and rs35979566 (MAF 0.8%), were not included in the comparison between blood methadone

concentration and [methadone]/[EDDP] ratio because the variant allele was not observed in the methadone-only group.

rs2279344 (MAF = 39.4%)*	**Controls**	**Methadone-Only Overdoses (All)**	**WV Methadone-Only Overdoses (Male)**	**WV Methadone-Only Overdoses (Female)**
Calculated MAF	37.5%	38.4%	39.3%	25.0%
Homozygous Major Allele				
Expected	92.9	47.2	21.8	7.8
Observed	98	45	18	11
Heterozygotes				
Expected	120.8	56.7	26.3	9.4
Observed	120	59	32	8
Homozygous Minor Allele				
Expected	39.3	17.1	7.9	2.8
Observed	35	17	6	1
HWE				
χ^2	3.2E-02	0.11	2.19	8.9E-02
***P*-value**	0.98	0.95	0.33	0.96
Obs vs. Exp *P*-value	0.69	0.91	0.30	0.26

Table 4: Minor Allele Frequency, Observed, and Expected Genotypic Frequencies for rs227344

* The expected NCBI MAF is in parentheses beside the db SNP id.

rs3211371 (MAF = 10.4%)*	Controls	Methadone-Only Overdoses	WV Methadone-Only Overdoses (Male)	WV Methadone-Only Overdoses (Female)
Calculated MAF	10.3%	12.4%	13.8%	5.0%
Homozygous Major Allele				
Expected	203.1	100.6	46.7	16.1
Observed	205	97	43	18
Heterozygotes				
Expected	47.2	23.1	10.7	3.7
Observed	44	25	14	2
Homozygous Minor Allele				
Expected	2.7	1.3	0.6	0.2
Observed	4	3	1	0
HWE				
χ^2	0.82	0.79	1.3E-02	5.5E-02
***P*-value**	0.66	0.67	0.99	0.97
Obs vs. Exp *P*-value	0.67	0.30	0.46	0.55

Table 5: Minor Allele Frequency, Observed, and Expected Genotypic Frequencies for rs3211371

* The expected NCBI MAF is in parentheses beside the db SNP id.

rs3745274 (MAF = 27%)*	Controls	Methadone-Only Overdoses	WV Methadone-Only Overdoses (Male)	WV Methadone-Only Overdoses (Female)
Calculated MAF	22.4%	31.9%	35.1%	37.5%
Homozygous Major Allele				
Expected	133.2	74.7	34.3	12.0
Observed	156	58	25	6
Heterozygotes				
Expected	98.6	43.1	19.8	7.0
Observed	76	53	24	13
Homozygous Minor Allele				
Expected	18.2	6.2	2.9	1.0
Observed	18	13	8	1
HWE				
χ^2	3.94	3.0E -02	0.33	3.0
***P*-value**	0.14	0.99	0.85	0.22
Obs vs. Exp *P*-value	1.1E-02†	1.2E-03¶	1.8E-03¶	1.6E-02‖

Table 6: Minor Allele Frequency, Observed, and Expected Genotypic Frequencies for rs3745274

* The expected NCBI MAF is in parentheses beside the db SNP id.

Statistically significant in allele frequency as compared to the NCBI Caucasian population. †P < 0.01.

Statistically significant in allele frequency as compared to the control group, ‖P < 0.05, ¶P < 0.005.

rs4803419 (MAF = 25.1%)*	Controls	Methadone-Only Overdoses	WV Methadone-Only Overdoses (Male)	WV Methadone-Only Overdoses (Female)
Calculated MAF	31.1%	24.6%	25.0%	28.9%
Homozygous Major Allele				
Expected	142.5	57.9	27.5	9.0
Observed	126	67	31	8
Heterozygotes				
Expected	95.5	52.3	24.9	8.1
Observed	98	50	25	11
Homozygous Minor Allele				
Expected	16.0	11.8	5.6	1.8
Observed	30	5	2	0
HWE				
χ^2	2.53	1.35	1.30	3.15
***P*-value**	0.28	0.51	0.52	0.21
Obs vs. Exp *P*-value	8.2E-04‡	6.6E -02	0.25	0.23

Table 7: Minor Allele Frequency, Observed, and Expected Genotypic Frequencies for rs4803419

* The expected NCBI MAF is in parentheses beside the db SNP id.

Statistically significant in allele frequency as compared to the NCBI Caucasian population. ‡P < 0.001.

rs8192709 (MAF = 3.6%)*	Controls	Methadone-Only Overdoses	WV Methadone-Only Overdoses (Male)	WV Methadone-Only Overdoses (Female)
Calculated MAF	5.4%	2.8%	0.9%	2.5%
Homozygous Major Allele				
Expected	233.3	111.9	51.9	17.9
Observed	227	118	57	19
Heterozygotes				
Expected	17.4	12.7	5.9	2.0
Observed	21	7	1	1
Homozygous Minor Allele				
Expected	0.3	0.4	0.2	0.1
Observed	3	0	0	0
HWE				
χ^2	7.95	0.10	4.4E-03	1.3E-02
***P*-value**	1.9E-02§	0.95	1.00	0.99
Obs vs. Exp *P*-value	1.1E-05‡	0.20	9.3E -02	0.72

Table 8: Minor Allele Frequency, Observed, and Expected Genotypic Frequencies for rs8192709

* The expected NCBI MAF is in parentheses beside the db SNP id.

Statistically significant in allele frequency as compared to the NCBI Caucasian population. ‡P < 0.001.

§ Allele is out of Hardy-Weinberg Equilibrium, P < 0.05.

rs8192719 (MAF = 27.9%)*	Controls	Methadone-Only Overdoses	WV Methadone-Only Overdoses (Male)	WV Methadone-Only Overdoses (Female)
Calculated MAF	23.0%	33.6%	35.8%	39.5%
Homozygous Major Allele				
Expected	121.1	63.5	31.5	11.3
Observed	144	48	23	6
Heterozygotes				
Expected	93.7	37.9	18.8	6.7
Observed	71	46	22	11
Homozygous Minor Allele				
Expected	18.1	5.6	2.8	1.0
Observed	18	13	8	2
HWE				
χ^2	4.48	0.15	0.50	0.85
***P*-value**	0.11	0.93	0.77	0.65
Obs vs. Exp *P*-value	7.3E-03†	5.2E-04Δ	1.9E-03¶	4.5E-02‖

Table 9: Minor Allele Frequency, Observed, and Expected Genotypic Frequencies for rs8192719

* The expected NCBI MAF is in parentheses beside the db SNP id.

Statistically significant in allele frequency as compared to the NCBI Caucasian population. † $P < 0.01$.

Statistically significant in allele frequency as compared to the control group, ‖ $P < 0.05$, ¶ $P < 0.005$, Δ $P < 0.001$.

rs12721655 (MAF = 0.8%)*	Controls	Methadone-Only Overdoses	WV Methadone-Only Overdoses (Male)	WV Methadone-Only Overdoses (Female)
Calculated MAF	0.2%	0.0%	0.0%	0.0%
Homozygous Major Allele				
Expected	211.6	100.5	47.8	17.9
Observed	214	101	48	18
Heterozygotes				
Expected	3.4	0.5	0.2	0.1
Observed	1	0	0	0
Homozygous Minor Allele				
Expected	0	0	0	0
Observed	0	0	0	0
HWE				
χ^2	1.2E-03	ND	ND	ND
***P*-value**	1.00	ND	ND	ND
Obs vs. Exp *P*-value	0.42	0.79	0.89	0.96

Table 10: Minor Allele Frequency, Observed, and Expected Genotypic Frequencies for rs12721655

* The expected NCBI MAF is in parentheses beside the db SNP id.

ND: minor allele was not detected.

rs35979566 (MAF = 0.8%)*	Controls	Methadone-Only Overdoses	WV Methadone-Only Overdoses (Male)	WV Methadone-Only Overdoses (Female)
Calculated MAF	1.3%	0.0%	0.0%	0.0%
Homozygous Major Allele				
Expected	192.9	90.6	56.5	16.6
Observed	191	93	58	17
Heterozygotes				
Expected	3.1	2.3	1.5	0.4
Observed	5	0	0	0
Homozygous Minor Allele				
Expected	0	0	0	0
Observed	0	0	0	0
HWE				
χ^2	0.03	ND	ND	ND
***P*-value**	0.98	ND	ND	ND
Obs vs. Exp *P*-value	0.55	0.30	0.47	0.80

Table 11: Minor Allele Frequency, Observed, and Expected Genotypic Frequencies for rs35979566

* The expected NCBI MAF is in parentheses beside the db SNP id.

ND: minor allele was not detected.

2.3.1 HWE Analysis

Genotype frequencies within a group were considered to be in HWE if the resulting χ^2 value was < 5.99 (corresponding to a P-value > 0.05). SNP rs8192709 in the control group was out of HWE. The remaining five SNPs were in HWE for the control group. The methadone-only group was within HWE for all six SNPs.

2.3.2 Comparison of Observed and Expected Genotypic Frequencies

To assess for significant differences between the genotypic frequencies in the control groups and the NCBI frequencies for the Caucasian population, a χ^2 test with two degrees of freedom was used. We observed that the genotypic frequency of four SNPs (rs3745274, rs4803419, rs8192709 and rs8192719) was significantly different between our control group and that of the frequency in the NCBI population. SNPs rs3745274 and rs8192719 in the control

group had lower than expected MAFs compared to NCBI reported values, 22.4% vs 27.0% and 23.0% vs 27.9%, respectively, while SNPs rs4803419 and rs8192709 observed higher MAFs, 31.1% vs 25.1% and 5.4% vs 3.6%, respectively.

Since many of the control group SNP frequencies were discordant with the NCBI frequencies, we relied on genotype frequencies of the local control group to identify SNPs that could contribute to methadone toxicity. SNPs rs3745274 and rs8192719 exhibited a significant difference in the methadone-only group compared to the control group. For these two SNPs, the MAF in the methadone-only cases was greater than that of the control group, 31.9% vs 22.4% and 33.6% vs 23.0%, respectively. When assessing the observed and expected genotypic frequencies for the male and female groups, the differences in frequencies were preserved in the both groups. These results are delineated in Table 4.

2.3.3 Analysis of Blood Methadone Concentrations and [Methadone]/[EDDP] Ratios

We tested for associations between SNP genotypes and blood methadone concentrations using a one-way ANOVA, in conjunction with the Tukey test. For SNP rs3211371, these analyzes showed a significant increase in the mean blood methadone concentrations (mg/L) in participants carrying two copies of the variant allele (1.67 ± 0.85) as compared to either the heterozygote (0.52 ± 0.08) or homozygous ancestral genotype (0.59 ± 0.05). Figure 3 depicts the mean blood methadone concentrations, with SEM, of each of the genotypes for the SNPs tested. The metabolic ratio of [methadone]/[EDDP] was significantly lower in heterozygotes carrying the rs8192709 variant vs the homozygous ancestral genotype (Figure 4). No significant differences in ratios were observed for the remaining SNPs.

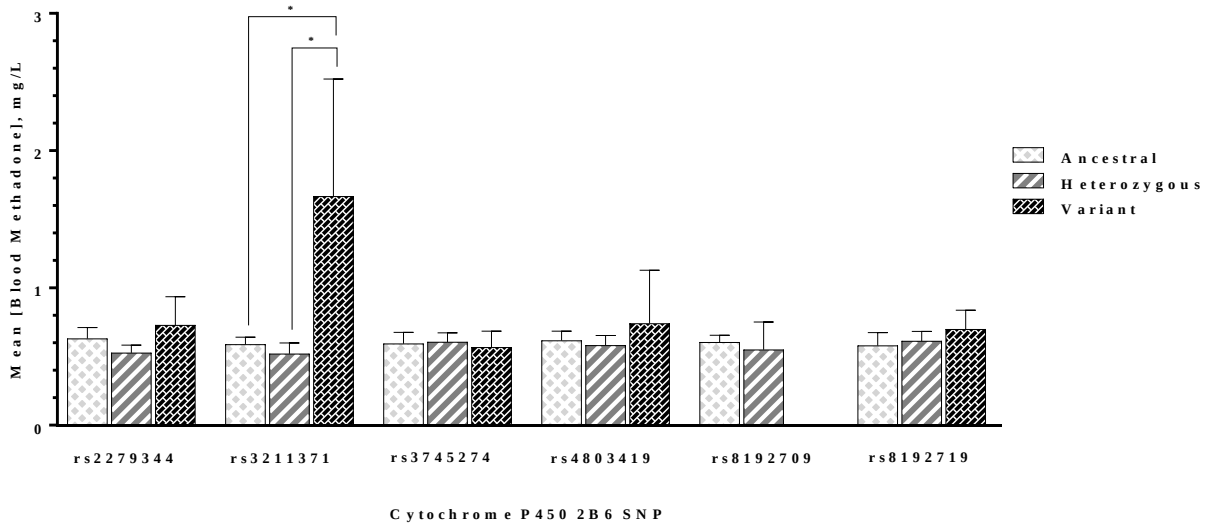

Figure 3: Comparison for Various *CYP2B6* SNPs with SEM of Mean Blood Methadone Concentrations

* Statistically significant difference, $P < 0.01$.

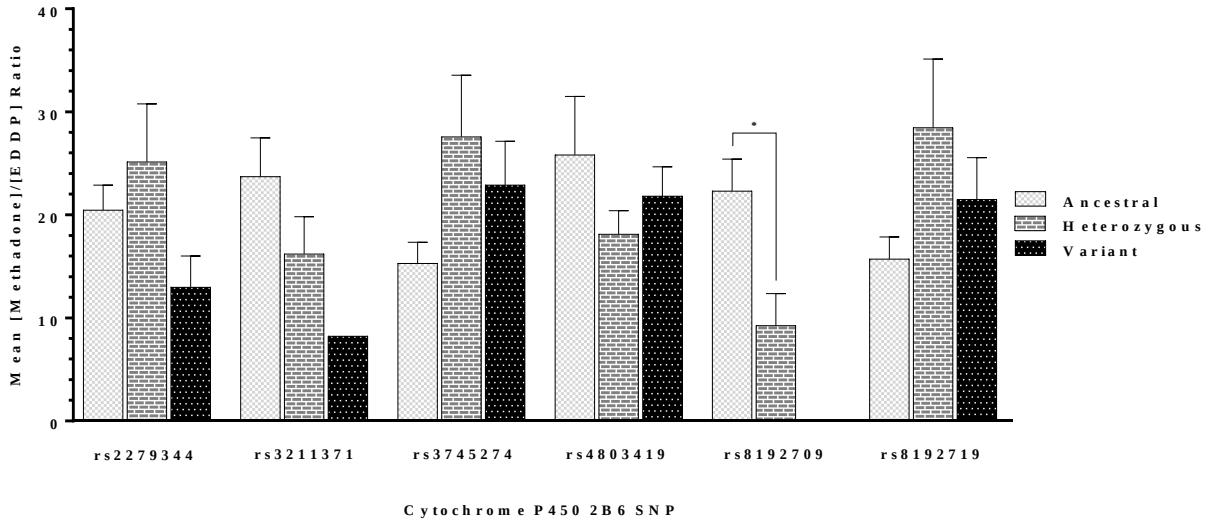

Figure 4: Comparison for Various *CYP2B6* SNPs with SEM of Mean [Methadone]/[EDDP] Ratios

* Statistically significant difference, $P < 0.05$.

2.3.4 Predicted Effects of SNPs on Protein Function

Amino acid substitution (AAS) at the corresponding positions for each of the SNPs were predicted for their effect on protein function. VEP reports the possible impact of the SNPs using the SIFT and PolyPhen algorithms. Both algorithms were consistent for rs3211371 and rs3745274, indicating that the AASs were tolerated and benign, while rs12721655 was predicted to affect the protein function in a deleterious and possibly damaging manner. Conflicting results were observed for rs8192709 and rs35979566, where the SIFT algorithm predicted the AASs to have a deleterious effect on the protein function, while PolyPhen predicted the substitutions to be benign.

2.4 Discussion

After oral administration, methadone can be detected in the blood within 15 – 45 min, and peak plasma concentrations can be attained at 2.5 – 4 h. The bioavailability of methadone is ~75%, with a wide range of 36 – 100% (Eap et al., 2002; Lugo et al., 2005; Kapur et al., 2011). About 86% of methadone binds to α1-acid glycoprotein (AGP), leaving 14% as free plasma methadone levels (Kapur et al., 2011). The methadone levels in the plasma decrease as the liver enzymes, CYPs, start to metabolize methadone to the inactive metabolites EDDP and EMDP. The elimination half-life of methadone ranges from 5 – 130 h, with a mean value of 22 h (Eap et al., 2002). The pharmacokinetics of methadone encompasses extreme interindividual variability attributed to factors such as, age, co-administered medications, disease, ethnicity (due to differing allelic frequency of genetic polymorphisms), gender and weight (Li et al., 2008; Kapur et al., 2011; Richards-Waugh et al., 2014). In a previous study, Richards-Waugh et al. (2014) demonstrated that an increasing number of SNPs present in *CYP3A4* was associated with an increasing blood level of methadone.

Due to the number of factors contributing to the pharmacokinetics of methadone, this study examined *CYP2B6* SNPs in 125 Caucasian individuals who suffered methadone-only fatalities. Our study eliminated the factors of co-medication and limited it to the allelic frequency of the Caucasian population in WV and KY. The methadone-only group was a non-random selection of individuals, chosen for the study based on a particular phenotype (fatalities due to methadone) of interest in the WV and KY region. In order to accurately determine any differences in the genotypic frequencies of the SNPs, the methadone-only group was compared to the regional control group. SNPs rs12721655 and rs35979566 did not exhibit any of the variant alleles in the methadone group or the control group. This observed genotypic frequency could be accredited to the low MAF of 0.8%. The study cohort may not have been large enough to reasonably observe the variant allele. The remaining alleles genotyped had a MAF of at least 2% as reported by the NCBI database. A significant enrichment of the minor alleles of rs3745274 and rs8192719 was observed in the methadone-only group compared to the control group, (32.2% vs 22.4% and 33.6% vs 23.0%), for the SNPs, respectively. The data suggests that these SNP may play a causative role in methadone fatalities. This finding is consistent with the observation that rs3745274 and rs8192719 are under-represented in a group of individuals with low (S)-methadone levels (Dobrinas et al., 2013) and suggesting their involvement with decreased CYP2B6 activity. The significant increase in the number of minor alleles was preserved in both the male and female cases, indicating that a particular gender does not have a predisposition to the mutation.

It is unclear if the enrichment of the minor alleles rs3745274 and rs8192719 leads to an enhancement of the blood level of (S)-methadone in this study, as enantiomer levels of (R)- and (S)-methadone were not determined at the time of autopsy. While total methadone blood levels

were not increased by the presence of these SNPs (Figure 3), the ratio of (R)- to (S)-methadone is unknown. If the ratio of (S)-methadone was increased due to these SNPs, then it is possible that an increased risk of death from cardiotoxicity would exist.

Parent-to-metabolite ratios are commonly used as an indicator of a recent intake of certain drugs and are not necessarily indicative of an acute poisoning. In some cases, the metabolic ratio may provide clues to indicate reduced metabolism of the drug due to a drug interaction or low metabolic capacity caused by genetics (Druid, Holmgren, Carlsson, & Ahlner, 1999). It was interesting to see a significant decrease ($P = 0.04$) in the metabolic ratio in individuals that were heterozygous for rs8192709. These data suggest that rs8192709 may have a role in metabolizing methadone at a faster rate. This analysis, however, should be interpreted with caution, as the number of individuals having a variant was minimal ($n = 2$), and neither were observed to be homozygous for the variant. A significant increase in the blood methadone concentration was observed for rs3211371 of homozygous variant alleles when compared to the ancestral alleles and the heterozygous genotypes ($P = 0.002$). This finding suggests that rs3211371 results in the reduced activity of CYP2B6. This finding is contrary to the findings of Dobrinas et al. (2013). In their study, rs3211371 was over-represented in low-level (S)-methadone groups, indicating an increased CYP2B6 activity. This study interprets the findings based on the various genotypes, whereas Dobrinas et al. (2013) interprets their findings on MAFs, which may be the reason of the contradictory postulations. Also, Dobrinas et al. (2013) measured enantiomeric levels of methadone in live MMT patients, while our results are from autopsy samples. It is also possible that the small sample size of the studies has resulted in these differences. Additional numbers of cases could be needed to resolve this difference in findings. A combination of the total number of variant alleles present for all of the SNPs tested for each

individual in the methadone-only group was determined. The blood methadone concentration and the [methadone]/[EDDP] ratio was compared to total number of minor alleles present, ranging from one to five copies of the variant alleles for *CYP2B6*. The data (not shown) did not support the assumption that multiple SNPs, rather than a single SNP, on the *CYP2B6* gene would increase the unexpected fatality from methadone.

SNP rs3211371 results in a conversion of arginine to cysteine at amino acid position 487, while glutamine is converted to histidine at amino acid position 172 as a result of rs3745274. Tools for annotating functional predictions of coding non-synonymous SNPs, such as SIFT and PolyPhen, suggested that both of these SNPs are tolerated or benign. On the other hand, rs8192709 encodes a conversion of arginine to cysteine at amino acid position 22, producing disagreeing predictions, where SIFT reports the variation to be deleterious, while PolyPhen predicts the mutation to be benign. *CYP2B6* has six substrate recognition sites (SRSs) along its sequence (Nguyen et al., 2008). SNPs rs3211371, rs3745274 and rs8192709 are not located within the known SRSs and confer minimal effects on methadone binding; however the SNPs may affect the hydrophobicity and conformation of the protein, resulting in alteration of the metabolic rate (Kamal, Lim, Tye, Ismail, & Choong, 2013). Furthermore, rs3745274 has been associated with increased levels of a hepatic splicing variant lacking exon 4 – 6 and decreased protein levels, caused by erroneous splicing, leading to decreased metabolism of substrates (Desta et al., 2007; Hofmann et al., 2008).

Physiologic differences occur between genders, which may affect the outcome of drug activity (Whitley & Linsdey, 2009). Gender influences the expression of CYPs and transporters, thus influencing pharmacokinetic parameters (Zanger & Schwab, 2013). Methadone accesses MOR site through the bloodstream, coming in contact with the plasma protein AGP. AGP is

capable of binding methadone and rendering it inactive (Behan, Cruickshank, Matthews-Smith, Bruce, & Smith, 2013). In general, females have a significantly greater binding capacity of AGP than males, suggesting that females could have a reduced level of free plasma drug levels (Kishino et al., 2002). In this study, there were no significant differences in the blood methadone concentration or the [methadone]/[EDDP] ratio between the male and female groups for any of the SNPs tested (data not shown).

It is important to note that since these cases were obtained from the WV and KY OCMEs, the medical and prescription history of the deceased individuals was not available. It is possible that drugs, prescription or natural supplements, or environmental compounds not detected by the normal toxicology screens could also have contributed to altered methadone metabolism. Additionally, it is unclear if the ratio of (R)- to (S)-methadone was changed in association with any *CYP2B6* SNPs examined. Research is currently underway to determine the effects of the exonic SNPs included in this study on the differential metabolism of (R)- and (S)-methadone.

2.5 Conclusions

The frequencies of two SNPs on the *CYP2B6* gene, rs3745274 (**9*, c.516G>T, Q172H) and rs8192719 (21563C>T) were significantly increased in the methadone-only group. SNP rs3211371 (**5*, c.1459C>T, R487C) exhibited a significant increase in the blood methadone concentration in the homozygous variant genotype, indicating that an individual carrying two copies of the minor allele for the SNP could have a poor methadone-metabolizer phenotype leading to an increased risk of fatal methadone intoxication. CYP2B6 only plays a partial role in the metabolism of (S)-methadone, and therefore the SNPs on this gene are only a part of the whole story. Richards-Waugh et al. (2014) studied the effects of SNPs on the *CYP3A4* gene,

which metabolizes both the (R)- and (S)- forms of methadone. It is possible that it is a combination of SNPs on both genes that leads to these unexpected fatalities.

2.6 Acknowledgments

The authors would like to thank the Marshall University Genomics Core Facility for use of shared instruments, and James Kraner, PhD and Mike Ward for acquisition of samples from the WV and KY OCMEs, respectively.

2.7 Funding

This project was supported in part by National Institutes of Health Grant P20RR016477 and P20GM103434 to the West Virginia IDeA Network for Biomedical Research Excellence. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

CHAPTER 3

EFFECTS OF CYTOCHROME P450 2B6 (CYP2B6) SINGLE NUCLEOTIDE POLYMORPHISMS ON CYP2B6 ACTIVITY: IMPLICATIONS FOR METHADONE METABOLISM

Abstract

Cytochrome P450 2B6 (CYP2B6) enzyme plays a significant role in the preferential stereoselective metabolism of (S)-methadone. Concentrations of (S)-methadone above therapeutic levels have the ability to cause serious, life-threatening, and fatal cardiac side effects. This toxicity could be due in part to the pharmacogenetics of an individual, which can alter the pharmacokinetic and pharmacodynamic properties of the drug and contributing to methadone overdose and death. One of the contributing factors for interindividual variability in biotransformation is genetic variation within the genes encoding CYP enzymes. Single nucleotide polymorphisms (SNPs), located within the *CYP2B6* gene were previously genotyped in 125 Caucasians involved in methadone-only overdose deaths to determine if these SNPs have the potential to play an important role in altering CYP2B6 activity. SNP mutations for rs2279344, rs3211371, rs3745274, rs8192709, rs12721655, rs35773040, and rs35979566 were introduced to wild type *CYP2B6* plasmid using Agilent QuikChange II Site-Directed Mutagenesis Kit. Sanger sequencing was performed for all genes to ensure each mutation was present. Each of the *CYP2B6* plasmids were individually expressed in COS-1 cell lines and the transfected cell lines were selectively grown. CYP2B6 microsomal protein was isolated from the COS-1 cells. The CYP2B6 activity in microsomal fractions was measured using a Promega P450-Glo CYP2B6 Assay Kit. Inhibition studies were conducted using clopidogrel to determine that the activity observed was from CYP2B6. Methadone was used to evaluate competition with luciferin-2B6 substrate at the active site. The effect of each individual SNP variant resulted in the

following decreasing order of CYP2B6 activity: rs2279343 > rs3745274 > wild type (*CYP2B6*1*), rs3211371 > rs8192709 > rs35773040, rs35979566. Relative to the wild type CYP2B6, SNPs rs8192709, rs35773040 and rs35979566 yielded 57%, 81%, and 94% decreased activity, respectively. The inhibition study with methadone indicated that the binding site is the same as luciferin-2B6 substrate. Therefore, our investigations suggest that rs8192709, rs35773040, and rs35979566 result in poor metabolizing phenotypes which may be contributing factors in decreased metabolism of (S)-methadone.

3.1 Introduction

The pharmacogenetics of an individual has the ability to alter the pharmacokinetic and pharmacodynamics properties of a drug. CYPs are heme-containing enzymes primarily found in the smooth endoplasmic reticulum of hepatic cells. These enzymes are responsible for catalyzing the oxidative biotransformation of most drugs. The principal determinant of the pharmacokinetic properties of xenobiotics is metabolism, thus affecting the resulting pharmacodynamic effects (Tanka, 1998; Turpeinen, 2006; Zanger & Schwab, 2013).

One of the contributing factors for interindividual variability in biotransformation is genetic variation within the genes encoding CYP enzymes. SNPs within a CYP gene may alter the enzymatic properties of the resulting protein, modify the metabolic response to a drug, and ultimately affect the drug's therapeutic and adverse effects (Ahmad et al., 2018).

Methadone has a wide-ranging half-life of elimination from 5 – 130 hours and a bioavailability ranging from 36 – 100%, making it a difficult medication to prescribe due to the extreme variability in the interindividual pharmacokinetics (Eap et al., 2002). This variation may be due to SNPs within the CYP genes responsible for the metabolism of methadone (Li et al., 2008). In most countries, methadone is administered as a racemic mixture of equal amounts of

(R)- and (S)-methadone. The CYP enzymes stereoselectively metabolize methadone, rendering distinct enantiomeric pharmacodynamics properties. While analgesic properties are ascribed to (R)-methadone, concentrations above therapeutic levels lead to respiratory depression by acting on the MOR controlling respiration in the brainstem (Mitchell et al., 2004; Silverman et al., 2009; van der Schier et al., 2014). Elevated (S)-methadone levels block the voltage-gated potassium channel of the hERG gene resulting in potentially fatal cardiotoxicity by prolonging the QT interval causing torsades de pointes (Eap et al., 2007; Ansermot et al., 2010; Dobrinas et al., 2013).

CYP2B6 enzyme plays a significant role in the preferential stereoselective metabolism of (S)-methadone. *CYP2B6* has 74 allelic variants and 38 haplotypes, rendering it one of the most polymorphic genes (Fokkema et al., 2011; Ingelman-Sundberg, 2017; Fokkema & den Dunnen, 2018a). The genetic variants affect catalytic activity, transcriptional regulation, splicing, and mRNA and protein expression. Therefore, SNPs in *CYP2B6*, and possibly other CYPs, could be contributing to the incidence of life-threatening and fatal cardiac side effects of (S)-methadone.

Our laboratory previously genotyped and analyzed numerous *CYP2B6* exonic and intronic SNP variants in 125 Caucasians involved in methadone-only fatalities (Ahmad et al., 2017). It was found that the frequencies of two SNPs on the *CYP2B6* gene, rs3745274 (**9*, c.516G>T, Q172H) and rs8192719 (21563C>T), were significantly increased in the methadone-only group. SNP rs3211371 (**5*, c.1459C>T, R487C) exhibited a significant increase in the blood methadone concentration, and rs8192709 (**2*, c.64C>T, R22C) may have a role in metabolizing methadone at a faster rate. The present work examined the effects of seven exonic *CYP2B6* SNPs, including the exonic SNPs genotyped in the previous study, in the metabolism of (S)-methadone.

3.2 Material and Methods

3.2.1 Material

Plasmid EX-NEG-M02, an empty control vector, was used as minus-P450 control. Plasmid EX-Q0553-M02 with the NCBI accession number: NM_000767.4 sequence, which cloned the human *CYP2B6* gene, was used as a mutagenesis template. All plasmids were obtained via GeneCopoeia (Rockville, MD). QuickChange II site-directed mutagenesis kit was purchased from Agilent Technologies (Santa Carla, CA). All primers designed to introduce the site-directed mutation were obtained from Sigma Life Sciences (The Woodlands, TX). DH5α *E. coli* cells, COS-1 cells, and clopidogrel bisulfate were generous gifts from Dr. Emine Koc, Dr. Jung Han Kim, and Dr. Wei Li, respectively (Marshall University, Huntington, WV, USA). QIAprep Spin Miniprep and DNeasy® Blood and Tissue kits were purchased from Qiagen (Germantown, MD). All microbiological and cell culture reagents were purchased from Fisher Scientific (Hampton, NH) and tissue culture plates were obtained from Midsci (Valley Park, MO). Pierce BCA protein assay kit was purchased from ThermoFisher Scientific (Waltham, MA). CYP2B6 (H-110) and goat anti-rabbit IgG-HRP antibodies were obtained from Santa Cruz Biotechnology (Dallas, TX). Phusion® High-Fidelity PCR Kit was purchased from New England Biolabs (Ipswich, MA). Microsome Isolation kit was purchased from BioVision (Milpitas, CA). Corning® Supersomes™ Human CYP2B6 + Oxidoreductase + b_5, and Corning® Gentest™ NADPH Regenerating System, Solutions A and B were purchased from Corning (Corning, NY). P450-Glo™ CYP2B6 Assay and Screening System and beetle luciferin, potassium sulfate were purchased from Promega (Madison, WI). Racemic methadone (6-dimethylamino-4,4-diphenylheptan-3-one) hydrochloride reference standard was obtained from Sigma-Aldrich (St. Louis, MO).

3.2.2 *CYP2B6* Plasmid Variants

3.2.2.1 Construction of *CYP2B6* Plasmid Variants

QuickChange II site-directed mutagenesis kit was used to generate various individual *CYP2B6* constructs from a plasmid carrying the human wild-type *CYP2B6* cDNA (*CYP2B6*1*): (i) rs2279343 (*CYP2B6*4*, c.785A>G, K262R); (ii) rs3211371 (*CYP2B6*5*, c.1459C>T, R487C); (iii) rs3745274 (*CYP2B6*9*, c.516G>T, Q172H); (iv) rs8192709 (*CYP2B6*2*, c.64C>T, R22C); (v) rs12721655 (*CYP2B6*8*, c.415A>G, K139E); (vi) rs35773040 (*CYP2B6*14*, c.419G>A, R140Q); and (vii) rs35979566 (*CYP2B6*15*, c.1172T>A, I391N). Following the manufacturer's conditions, the reactions were prepared in a final volume of 50 μL for each variant, containing 50 ng wild-type human *CYP2B6* plasmid, 125 ng each of the forward and reverse variant primer, 5 μL reaction buffer (10x), 1 μL dNTP mix, and ddH_2O. Before the start of each cycle, 1 μL of *PfuUltra* HF DNA polymerase was added to each reaction tube. The thermal cycling parameters were set for polymerase activation at 95°C for 30 s, followed by 12 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 1 min, and extension at 68°C for 8 min. Following the thermal cycling, the reactions were placed on ice for 2 min. The primers designed for the plasmid variants are listed in Table 12.

Target	Forward Primer (5' → 3')	Reverse Primer (5' → 3')
rs2279343 (*CYP2B6*4*)	CAGCGCCCCCAGGGACCTCATC	GATGAGGTCCCTGGGGGCGCTG
rs3211371 (*CYP2B6*5*)	CCCAACATACCAGATCTGCTTCCTGCCCCG	CGGGGCAGGAAGCAGATCTGGTATGTTGGG
rs3745274 (*CYP2B6*9*)	CCACCTTCCTCTTCCATTCCATTACCGCCAAC	GTTGGCGGTAATGGAATGGAAGAGGAAGGTGG
rs8192709 (*CYP2B6*2*)	GCTACTCCTGGTTCAGTGCCACCCTAACACC	GGTGTTAGGGTGGCACTGAACCAGGAGTAGC
rs12721655 (*CYP2B6*8*)	GGATGGGAGAGCGGAGTGTGGAG	CTCCACACTCCGCTCTCCCATCC
rs35773040 (*CYP2B6*14*)	GGATGGGAAAGCAGAGTGTGGAGGAGC	GCTCCTCCACACTCTGCTTTCCCATCC
rs35979566 (*CYP2B6*15*)	GAAGTATTTCTCAACCTGAGCACTGCTCTCC	GGAGAGCAGTGCTCAGGTTGAGAAATACTTC
Sanger Sequencing Primers	CAGCCTCCGGACTCTAGC CACTATGAGGGACTTCGGGA CGTCCTCCTCTTCCTTGCACTCC CAAGGACCTCATCGACACCTAC	TAATACGACTCACTATAGGG GATGGAGCAGATGATGTT
PCR Primers	ATAGAAGACACCGGGACCGA[*] CAGCCTCCGGACTCTAGC[†] CAAGGACCTCATCGACACCTAC[‡]	AGCAGTTTTCCCAGTCACGA[*] GATGGAGCAGATGATGTT[†] TAATACGACTCACTATAGGG[‡]

Table 12: Forward and Reverse Primers for Construction of CYP2B6 Plasmid Variants, Sanger Sequencing, and PCR Amplification

[*] Annealing temperature calculated from www.neb.com/TmCalculator is 65°C

[†] Annealing temperature calculated from www.neb.com/TmCalculator is 56°C; 1.5 µL of DMSO was added to the PCR reaction

[‡] Annealing temperature calculated from www.neb.com/TmCalculator is 63°C; 1.5 µL of DMSO was added to the PCR reaction

3.2.2.2 Transformation and Amplification of *CYP2B6* Plasmid Variant

Competent DH5α *E. coli* cells were incubated on ice for 30 min with 5 ng of the resulting PCR variants products. The cells were heat shocked at 42°C for 45 s and then placed back on ice to recover for 2 min. The transformed cells were allowed to grow overnight at 37°C on agar plates with 1x ampicillin for selection. An isolated colony from the agar plate, for each variant, was propagated in NZY+ broth with 1x ampicillin at 37°C overnight with shaking. Transformed DH5α *E. coli* cells were collected for plasmid extraction following the manufacturer's protocol for the QIAprep Spin Miniprep Kit. The purified plasmids were quantitated by absorbance spectrophotometry on a NanoDrop 1000. The *CYP2B6* variant plasmids were then given to the Marshall University Genomics Core Facility to conduct Sanger sequencing to verify the variants were present. Two forward primers and a reverse primer were used for sequencing all plasmids (listed in Table 12).

3.2.3 Transfection of COS-1 Cells

COS-1 cells were used for stable expression of the *CYP2B6* plasmid variants. For each individual variant, three wells in a 6-well tissue culture plate were seeded with 100,000 COS-1 cells in DMEM + 10% FBS. A lipid-DNA complex was formed using 300 ng of plasmid and 3 μL of Lipofectamine 2000 diluted to 200 μL with DMEM. The cells were washed with cold PBS and 800 μL of DMEM + 10% FBS was added to each plate along with 200 μL of their respective lipid-DNA complex (variant plasmid complexes for CYP2B6 expression or an empty control vector for negative control). The cells were grown in a humidified incubator at 37°C with 5% CO_2. The media was changed after 24 h to selective media (DMEM + 10% FBS + 1% P/S + 1mg/mL Geneticin) and incubated for an additional 24 h. The cells were recovered 48 h post-transfection from the three wells and diluted to one cell per well into 96-well tissue culture plates

to construct single clones. The remaining cells where stored under cryogenic conditions. Once the wells were about 80% confluent, the cells were gradually expanded to 48-well, 24-well, 12-well, 6-well, 60-mm, and 100-mm tissue culture plates, consecutively.

3.2.4 Determination of Protein Expression by Western Blot

The expression of CYP2B6 variant proteins in COS-1 single clone cell lines were assessed using Western blot analysis. Protein was extracted from cells grown to 80-90% confluency in a 60-mm tissue culture plate using 100 μL RIPA buffer with 1x protease inhibitor. The protein concentration for each sample was determined using the Pierce BCA protein assay kit, following the manufacturer's protocol. An aliquot of 10 μg of protein of each sample was denatured by boiling for 5 min. The proteins were separated on a 10% polyacrylamide gel for 1 h at 130V. The gel was then transferred to a nitrocellulose membrane for 1 h at 0.35A. The successful transfer and protein loading was verified using Ponceau S stain. The membrane was then blocked for nonspecific antibody binding using a 5% w/v non-fat milk/TBST solution (10 mM Tris-HCl, 150 mM NaCl, 0.1% Tween-20; pH 8.0) for 1 h. A rabbit polyclonal CYP2B6 (H-110) primary antibody (1:1000) was diluted in 5% non-fat milk/TBST and applied on the membrane for incubation at 4^{o}C overnight with continual rocking. The membrane was then washed three times with TBST for 7 min before the goat anti-rabbit HRP-linked secondary antibody (1:5000 dilution in 5% non-fat milk/TBST) was added for 1 h incubation. The membrane was again washed with TBST for 7 min, three times. The proteins were detected with the application of ECL and exposure to X-ray film. GAPDH was used as a loading control for all of the Western blots to confirm equal protein loading.

3.2.5 Verification of Plasmid Variant in Protein Expression

Once protein expression was determined via Western blotting, the *CYP2B6* gene was again sequenced to verify the protein was expressing the SNP variant. Each cell line was grown to 80-90% confluency in a 100-mm tissue culture plate. The DNA was purified from the cells following the manufacturer's protocol for the DNeasy® Blood and Tissue Kit. The extracted DNA was quantitated using the NanoDrop 1000. Phusion® High-Fidelity PCR Kit was used to amplify the *CYP2B6* gene in a PCR reaction. Following the manufacturer's conditions, the reactions were prepared in a final volume of 50 μL for each sample containing 150 ng of the purified template DNA, 10 μL of 5x Phusion HF, 2.5 μL each of 10 μM forward and reverse primer, 1 μL of 10 mM dNTPs, nuclease-free water to volume, and lastly 0.25 μL Phusion DNA polymerase. The thermocycling conditions were set on three stages: stage one for initial denaturation at 98ºC for 30 s, followed by stage two for 30 cycles of denaturation at 98ºC for 10 s, annealing at 51º, 56º, or 65ºC for 30 s, and extension at 72ºC for 26 s, and stage three for a final extension at 72ºC for 5 min. Following the cycling conditions, the samples were held at 4ºC. The primers designed for the PCR are listed in Table 12.

Positive PCR amplification was determined through Southern blot analysis on the PCR products using a 1% agarose gel with ethidium bromide at a final concentration of 0.5 μg/mL. The PCR products were then given to the Marshall University Genomics Core Facility to conduct Sanger sequencing to verify the variants were present. The sequencing primers used are listed in Table 12. DNA BASER Sequence Assembler v.4.36.0 was used to align the sequence reads to a reference sequence to form a single continuous contig for each sample. The resulting contig was subsequently aligned to the reference plasmid sequence using the multiple sequence alignment by CLUSTALW (https://www.genome.jp/tools-bin/clustalw).

3.2.6 Isolation and Quantitation of Microsomal Protein

After the confirmation of the presence of the *CYP2B6* SNP variants in the stable constructs of COS-1 cells, twenty 100-mm tissue culture plates of each cell line were grown to yield approximately 2 x 10^7 cells. The cells were collected for isolation of microsomal fractions by differential centrifugation following the manufacturer's protocol for BioVision's Microsome Isolation Kit. In short, all steps were performed on ice. The cells were homogenized via sonication and then centrifuged at 10,000 x g for 15 min at 4°C. The supernatant was then centrifuged at 25,000 x g for 20 min at 4°C. The resulting pellet was resuspended in storage buffer and stored at -80°C. The protein concentration for each microsomal extraction was determined using the Pierce BCA protein assay kit. An aliquot of 20 μg of microsomal protein for each sample was used for Western blot analysis as previously described. For each gel, 0.05, 0.1, 0.15, and 0.2 pmol of Corning® Supersome™ Human CYP2B6 was loaded as standards to produce a standard curve. Densitometry was measured with ImageJ PC-based software (National Institute of Health) to extrapolate the concentration of CYP2B6 in each COS-1 microsomal extract from the standard curve.

3.2.7 P450-Glo™ Assay

3.2.7.1 Measuring CYP2B6 SNP activity

The P450-Glo™ CYP2B6 assay was performed in accordance to the manufacturer's protocol in white opaque polystyrene nontreated flat-bottom 96-well plates. In short, 0.1 pmol of each CYP2B6 SNP variant microsomal protein were incubated with 25 μL of 2X substrate solution (final concentration of 3 μM luciferin-2B6) for 10 min at 37°C. CYP2B6 reactions were initiated with the addition of 25 μL 2X NADPH regeneration system and incubated for an additional 10 min at 37°C. An addition of 50 μL detection reagent was added to cease the

reaction and shaken on an orbital shaker at a frequency of 282 rpm for 10 s and incubated for 20 min at room temperature in the dark. Luminescence signals were measured using a Synergy HTX multi-mode microplate reader. The CYP2B6 activity was normalized to activity detected for the empty vector microsomal fractions (minus-P450 control). All reactions were performed in triplicate.

3.2.7.2 Inhibition of CYP2B6 activity

CYP2B6 was selectively inhibited with a final concentration of 0.5 μM and 1.0 μM clopidogrel to ensure the activity observed was from the CYP2B6 enzyme. Similarly, 0.1 pmol of CYP2B6*1 microsomal protein was incubated with 12.5 μL of 4X substrate solution (final concentration of 3 μM luciferin-2B6) and 12.5 μL of the appropriate clopidogrel stock solution for 10 min at 37ºC. The reaction was initiated with the addition of 25 μL 2X NADPH regeneration system and incubated for an additional 10 min at 37ºC. The reaction was terminated and luminescent signal was initiated with the addition of 50 μL detection reagent. The plate was shaken on an orbital shaker at a frequency of 282 rpm for 10 s and incubated for 20 min at room temperature in the dark. Luminescence signals were measured on a microplate reader as above. The CYP2B6 activity was normalized to activity detected for the untreated control at each incubation time point. All reactions were performed in triplicate.

Various concentrations of racemic methadone (3, 6, 12, 60, 120, and 240 μM) was also used to test for inhibition. The reactions were carried out and luminescence signals were measured and normalized in the same manner as the inhibition with clopidogrel. All reactions were performed in triplicate.

3.2.8 Statistical Analysis

Statistical analyses were carried out using GraphPad Prism software version 7.02. The CYP2B6 SNP activity was compared to wild type CYP2B6 activity using one-way ANOVA with Dunnett's multiple comparison test. *P*-values < 0.05 were considered to be statistically significant.

3.3 Results and Discussion

In order to test the effects of missense variants on human CYP2B6 enzymatic activity, we created a wild type CYP2B6 expression vector in plasmid EX-Q0553-M02 and a series of variant CYP2B6 expression vectors. We then expressed the resulting enzymes by stable transfection in COS-1 cells. The COS-1 cell line was selected because CYPs are not innately expressed by these cells. COS-1 cells also have ample oxidoreductase and cytochrome b_5 to support the CYP2B6 activity (Gonzalez & Korzekwa, 1995). The variant constructs included the following missense substitutions: (1) rs2279343 (*CYP2B6*4*, c.785A>G, K262R), (2) rs3211371 (*CYP2B6*5*, c.1459C>T, R487C), (3) rs3745274 (*CYP2B6*9*, c.516G>T, Q172H), (4) rs8192709 (*CYP2B6*2*, c.64C>T, R22C), (5) rs12721655 (*CYP2B6*8*, c.415A>G, K139E), (6) rs35773040 (*CYP2B6*14*, c.419G>A, R140Q), and (7) rs35979566 (*CYP2B6*15*, c.1172T>A, I391N) (Appendix B). We successfully transfected the wild type construct and all variant construct with the exception of the SNP rs12721655 variant plasmid. This observation may stem from lethal effects of the variant enzyme and is consistent to the predicted SIFT and PolyPhen algorithms of rs12721655 having a deleterious and possibly damaging protein function (Ahmad et al., 2017). As a result, no further studies were conducted on the SNP.

Expression of the wild type and variant proteins was confirmed by Western blot analysis on whole cell extracts with CYP2B6 (H-110) antibody (Figure 5); a 55kDa band was not

detected in the empty vector transfectant. After verification of protein expression, the presence of the *CYP2B6* genetic variants in COS-1cells was confirmed by amplification and sequencing of the *CYP2B6* gene (Appendix B).

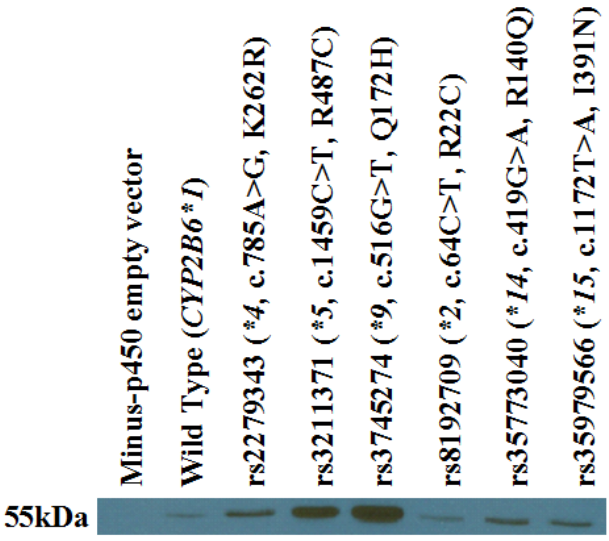

Figure 5: Western Blot Analysis of CYP2B6 Variant Protein Expression from Whole Cell Lysates

The activity of the wild type and variant enzymes was measured in COS-1 microsomal extracts using the Promega P450-Glo CYP2B6 Assay Kit. In this assay, CYP2B6 enzymes metabolized a luminogenic luciferin-2B6 substrate to produce d-luciferin that generates light proportional to the CYP2B6 activity. Since SNP rs8192709 produced a lower than expected microsomal extract, 0.086pmol of the variant proteins were used to compare SNP activity. The activity of the CYP2B6 protein variants was rs2279343 > rs3745274 > wild type (*CYP2B6*1*), rs3211371 > rs8192709 > rs35773040, rs35979566 (Figure 6). Similar results were seen using 0.1 pmol microsomal protein (data not shown). Compared to the wild type CYP2B6, SNPs rs2279343 and rs3745274 resulted in a 90% and 55% increase in CYP2B6 activity, respectively ($P < 0.0001$). In contrast, SNPs rs8192709, rs35773040, and rs35979566 yielded decreased

CYP2B6 activity by, 57%, 81%, and 94%, respectively (P < 0.0001). SNP rs3211371 had activity comparable to the wild type CYP2B6 enzyme (Figure 6).

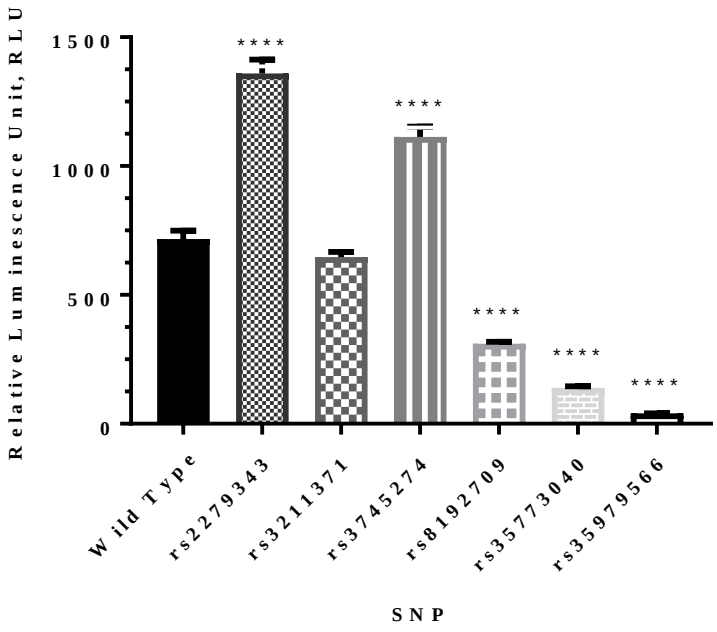

Figure 6: Effect of CYP2B6 SNPs on CYP2B6 Enzyme Activity.
Results are the mean with SEM of three P450-Glo reactions. 0.086 pmoles of 2B6 enzyme were loaded into assay.
**** Statistically significant difference with respect to wild type CYP2B6, $P < 0.0001$.

Clopidogrel is a selective inhibitor of CYP2B6, inactivating the cytochrome activity primarily through the destruction of the heme subunit. Clopidogrel is metabolically biotransformed to the active metabolite 2-oxo-clopidogrel by CYP2B6. Covalent modification of Cys475 of CYP2B6 by 2-oxo-clopidogrel may also lead to the loss of the catalytic activity of CYP2B6 (Zhang, Amunugama, Ney, Cooper, & Hollenberg, 2011). Using clopidogrel as an inhibition control confirmed that the induced activity observed was from the CYP2B6 enzyme. Addition of 0.5 μM clopidogrel and 1 μM clopidogrel to wild type CYP2B6 microsomal extracts resulted in reduction of CYP2B6 activity by 62% and 86%, respectively (P < 0.0001) (Figure 7).

The inhibitory concentration at which the CYP2B6 enzyme activity is reduced by 50% (IC_{50}) was determined to be 0.393 μM using the online source AAT Bioquest IC50 Calculator (Quest Graph™ IC50 Calculator, 2018).

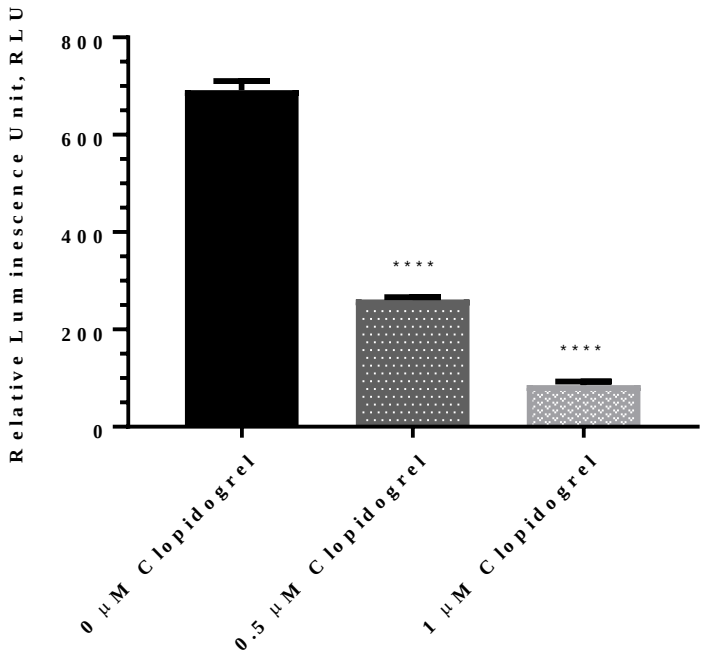

Figure 7: Wild Type CYP2B6 Enzyme Activity Inhibited by 0.5 μM and 1 μM of Clopidogrel
Results are the mean with SEM of three reactions. Statistically significant difference compared to untreated wild type CYP2B6 enzyme, $^{****}P < 0.0001$.

Racemic methadone was used as a test compound to determine the effect on the wild type CYP2B6 enzyme. Inhibition by clopidogrel determined the capacity of the system to detect inhibition by racemic methadone. Significant inhibition was not seen at racemic methadone concentrations of 3 μM, 6 μM, and 12 μM (data not shown). However, concentrations at 60 μM, 120 μM, and 240 μM, resulted in an inhibition of the wild type CYP2B6 enzyme with a significant decrease in activity by 24%, 34%, and 46%, respectively ($P < 0.005$, $P < 0.0005$, and

P < 0.0001, respectively) (Figure 8). The IC_{50} value was calculated to be 304 μM of racemic methadone, over 100 times the luciferin-2B6 concentration in the assays, indicating methadone is a weak competitive inhibitor for the substrate luciferin-2B6.

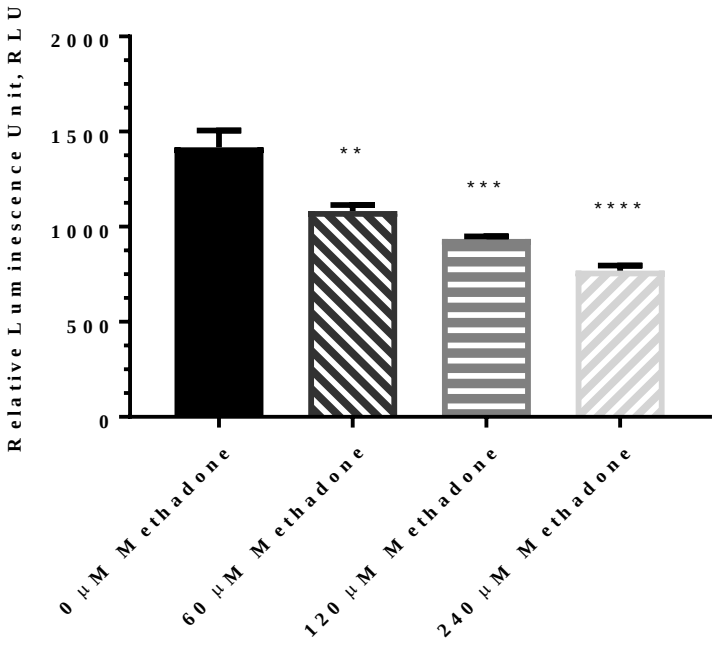

Figure 8: Wild Type CYP2B6 Enzyme Activity Inhibited by 60 μM, 120 μM, and 240 μM of Racemic Methadone
Results are the mean with SEM of three reactions. Statistically significant difference compared to untreated wild type CYP2B6 enzyme, $^{**}P < 0.005$, $^{***}P < 0.0005$, $^{****}P < 0.0001$.

SNP rs2279343 (K262R) also resulted in an increase in activity as compared to the wild type CYP2B6. In the CYP2B family, variant R262 is involved in a hydrogen-bonding network in the G and H helices, along with their associated loops, which includes H252, T255, D263, and D266. The ancestral K262 of CYP2B6 disrupts this hydrogen-bonding network, which may affect the G and I helices and alter the active site (Gay, Roberts, & Halpert, 2010). SNP rs2279343 reverts the lysine in position 262 to the CYP2B conserved arginine residue, which

may increase the plasticity of CYP2B6 to adopt different confirmations when binding to different substrates, thus resulting in an increase in the observed activity.

In previous studies, the frequency of the exonic SNP rs3745274 was observed to be significantly increased in 125 Caucasian individuals who suffered fatalities due to methadone, in the WV and KY regions (Ahmad et al., 2017), indicating SNPs rs3745274 may be linked to methadone toxicity overdosing. However, the activity of CYP2B6 SNP rs3745274 (Q172H) when compared to the wild type CYP2B6 yielded an increase in CYP2B6 activity ($P < 0.0001$), indicating the SNP results in an ultra-rapid metabolizer phenotype. These results were not in agreement to the findings by Gadel et al. (2015), who found that the *in vitro* intrinsic clearance of methadone to its principal metabolite, EDDP, was significantly less in the mutant than the wild type CYP2B6 from insect cells co-expressed with oxidoreductase and cytochrome b_5. Similarly, clinical genetic association studies of concentration-to-dosage ratios and methadone plasma concentrations were consistent with diminished methadone *N*-demethylation in the presence of the rs3745274 SNP (Wang et al., 2011; Levran et al., 2013).

One of the most common *CYP2B6* variant alleles is *CYP2B6*6*, a haplotype harboring both the rs2279343 (K262R) and rs3745274 (Q172H) non-synonymous coding SNPs. Individually, both of these SNPs increased the activity of CYP2B6 enzymes in our studies. Numerous *in vitro* and clinical studies reported *CYP2B6*6* genotype has been linked with an increase in methadone plasma levels, decreased methadone *N*-demethylation, and decreased methadone clearance (Gadel et al., 2015; Crettol et al., 2005; Crettol et al., 2006; Kharasch & Stubbert, 2013; Kharasch et al., 2015; Kringen et al., 2017). The *CYP2B6*6* allele has been associated with reduced protein levels, which may be correlated to rs3745274 involved in the erroneous hepatic splicing of exons 4, 5 and 6 yielding a truncated protein and concurrently

altering the catalytic activity of the enzyme. In silico analysis of the splice sites suggest an exonic splicing enhancer may be modulated by rs3745274 in exon 4. These observations were from the expression and analysis of minigene constructs created and expressed in COS-1 and Huh7 cell lines by Hofmann et al. containing all nine exons of *CYP2B6*, introns 2, 5, and 7, and the 5' and 3' parts of introns 3 and 6. The minigenes lacked introns 1, 4, and 8 and parts of intron 3 and 6 (Hofmann et al., 2008). However, linkage disequilibrium (LD) analysis in an Israeli population supported the inclusion of the intronic SNP rs8192719 (intron 8, g.21563C>T) in the *CYP2B6*6* haplotype (Levran et al., 2013). Although an LD analysis was not performed in our previous study, all subjects identified with the rs3745274 SNP also had the rs8192719 SNP (unpublished data). It is possible that decreased activity of *CYP2B6*6* may be causal from the intronic SNP, affecting splice-dependent quantitative and qualitative expression of CYP2B6, controlling the efficacy and toxicity of methadone.

The CYP2B6 activity of SNP rs3211371 was comparable to that of the wild type CYP2B6. In comparison to various clinical and decedent studies, these findings were not consistent. Individuals homozygous for SNP rs3211371 of methadone-only deceased cohort by Ahmad et al. (2017), had a significant increase in blood methadone concentrations, suggesting a PM phenotype. In another study, SNP rs3211371 was over-represented in individuals with low (S)-methadone levels, indicating an increase in CYP2B6 activity (Dobrinas et al., 2013). While, the IV and oral methadone plasma concentrations of SNP rs3211371 homozygote subjects resembled those seen in the cohort homozygous for the wild type CYP2B6 (Kharasch et al., 2015).

Reduced CYP2B6 activity was observed with SNP rs8192709, causing a non-synonymous amino acid change in position 22 from a large, positively charged arginine to a

medium, nonpolar nucleophilic cysteine. Protein folding may be interfered by cross bridges formed by cysteine residues, increasing the rigidity of the protein. SNP rs35773040 causes an amino acid change from a positively charged arginine to a polar, uncharged glutamine at position 140. This mutation is located at the end of the C-D loop (Nguyen et al., 2008), which may disrupt interactions with the D helix of CYP2B6, resulting in conformational modifications. Replacement of the hydrophobic Ile391 with a less bulky polar uncharged residue (asparagine), due to SNP rs35979566, diminished the CYP2B6 activity. This effect may be related to the position of this residue directly between the $\beta_{1\text{-}3}$ sheet and the K' helix (Nguyen et al., 2008).

CYP2B6 is only one enzyme involved in the metabolism of (S)-methadone. Cytochromes 2D6 and 2C18 also preferentially metabolize (S)-methadone, while CYP3A4 demonstrates no stereoselectivity, metabolizing both (R)- and (S)-methadone (Ahmad et al., 2018). The contradictory results seen in this and other studies with clinical data could be in part due to a combination of these CYPs and are not only that of CYP2B6 alone.

3.4 Conclusions

In summary, metabolically competent cell systems were generated by stable transfection with individual *CYP2B6* variant plasmids in COS-1 cells. The effect of introducing one exonic SNP resulted in the following decreasing order of CYP2B6 activity: rs2279343 > rs3745274 > wild type (*CYP2B6*1*), rs3211371 > rs8192709 > rs35773040, rs35979566. SNPs rs8192709, rs35773040 and rs35979566 yielded 57%, 81%, and 94% decreased activity relative to wild type CYP2B6, respectively. Methadone binds to the same active site and competes with the luciferin-2B6 substrate used to measure CYP2B6 activity. Therefore, our results suggest that these three SNPs could contribute to PM phenotypes and may influence the pharmacokinetics and pharmacodynamics of (S)-methadone.

3.5 Conflict of Interest

The authors declare no conflict of interest.

3.6 Funding

This project was supported in part by National Institutes of Health Grant P20GM103434 to the West Virginia IDeA Network for Biomedical Research Excellence, 2U54GM104942 to the West Virginia Clinical and Translational Science Institute, and GM121299-01A1 to the Appalachian Center for Cellular transport in Obesity Related Disorders. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

CHAPTER 4

SUMMARY, CONCLUSIONS, AND FUTURE DIRECTIONS

It is interesting to observe the history and trends of methadone use unfold over the last 20 years; once a miracle drug, methadone is now a killer cure. In the mid-1990s, methadone was increasingly prescribed as a treatment for chronic nonmalignant pain because of its long duration of analgesic action and relatively low cost (Paulozzi, Mack, & Jones, 2012). In 2003, West Virginia was one of the first states to start reporting increases in fatal overdoses associated with methadone (Lipman, 2008). By 2004, the methadone mortality rate in West Virginia increased 25-fold compared to 1999, while Kentucky showed the fourth largest increase at 15-fold (Fingerhut, 2006). The rate of methadone overdoses peaked in 2006, plateaued in 2007, and then declined 6.5%, on average, in each subsequent year (Jones et al., 2016; Faul, Bohm, & Alexander, 2017).

The main determinant of methadone mortality rates parallel the amount of methadone distributed for pain relief through prescriptions (Paulozzi et al., 2012). In 2006, the FDA issued a public health advisory for respiratory depression and cardiac arrhythmias leading to unintentional overdose deaths related to methadone used for pain, which was quickly followed with issuance of black box warnings by drug manufacturers (Christie, 2011). At the request of the Drug Enforcement Agency (DEA), manufacturers voluntarily limited the distribution of the 40 mg methadone formulation to MMT programs and hospitals at the beginning of 2008, since this dosage was not approved for treatment of pain. The steps taken by the FDA and DEA to improve the safety of methadone usage may have caused the resulting decline in methadone related mortality rate in consistence with the decrease in the national distribution of methadone (Paulozzi et al., 2012). However, the overdose death rate in people 55 – 64 years of age

continued to rise through 2014 and remains a drug that disproportionately contributes to fatal overdoses among opioid pain relievers (Paulozzi et al., 2012; Jones et al., 2016).

Methadone mortality rate can partially be attributed to the genetic makeup of an individual. SNPs in regions of the genome that express drug metabolizing enzymes, CYPs, may have consequences that lead to decreased metabolism of methadone leading to unintentional overdoses. The CYP2B6 enzyme is one of the predominant metabolizers of methadone, and a major determinant of stereoselective metabolism of (S)-methadone (Chang et al., 2011). Studying and understanding the pharmacogenetic factors in methadone metabolism can help mediate the proper dosing regimen for an individual for safe and effective therapeutic success in pain relief. Several pharmacogenetic studies on *CYP2B6* have been previously conducted involving patients in MMT programs (Crettol et al., 2005; Crettol et al., 2006; Eap et al., 2007; Wang et al., 2011; Dobrinas et al., 2013; Kharasch & Stubbert, 2013; Levran et al., 2013; Kharasch et al., 2015; Kringen et al., 2017). However, these studies had small sample sizes (Kharasch & Stubbert, 2013), included multidrug cases rather than single-drug methadone cases (Eap et al., 2007; Wang et al., 2011), or consisted of subject groups of mixed populations (Crettol et al., 2005; Crettol et al., 2006; Dobrinas et al., 2013; Levran et al., 2013; Kharasch et al., 2015). The work presented in this dissertation is from the largest methadone-only fatalities from a single population cohort. It is a unique pharmacogenetic study designed to gain knowledge in the prevalence and function of SNPs in *CYP2B6*.

The current study was designed to extract the genomic DNA of 125 Caucasian subjects from the Appalachian region (WV and KY) involved in methadone-only fatalities from blood stain cards, along with a control group consisting of 225 deceased Caucasian individuals from WV who screened negative for central nervous system drugs. Eight key exonic and intronic SNP

variants present in the Caucasian population were genotyped to test for enrichment in the subjects. The analyses performed in this study were aimed to provide insight into the association of *CYP2B6* SNPs in individuals who succumb to methadone intoxication in the methadone-only cases. Many of the control group SNP frequencies were significantly different from the MAF reported for the general Caucasian population in the NCBI dbSNP database (Tables 6 – 9). These differences indicated that the population in the Appalachia region has evolved, and as such, we relied on genotypic frequencies of the local control group to identify SNPs that could contribute to methadone toxicity. The frequencies of two SNPs on the *CYP2B6* gene, rs3745274 (**9*, c.516G>T, Q172H) and rs8192719 (21563C>T) were significantly increased in the methadone-only group compared to the control group (Tables 6 and 9). Associations were also analyzed between SNP genotypes and blood methadone concentrations. SNP rs3211371 exhibited a significant increase in the blood methadone concentration in the homozygous variant genotype as compared to either the heterozygous or homozygous ancestral genotype (Figure 3). This study suggested that these three SNPs may be associated as contributory factors to increased methadone mortality rates.

In addition to the pharmacogenetic association study, the effect of each individual exonic SNP on the activity of the CYP2B6 enzyme was determined. This current study was conducted by generating metabolically competent cell systems by stably transfecting individual *CYP2B6* exonic variant plasmids in COS-1 cell lines. The effect of each exonic SNP on enzyme activity was measured using a luminogenic CYP assay and compared to the wild type CYP2B6 enzyme. This study showed that the genetic variants of *CYP2B6* influences the enzymatic activity in the following decreasing order: rs2279343 > rs3745274 > wild type (*CYP2B6*1*), rs3211371 > rs8192709 > rs35773040, rs35979566. SNPs rs8192709, rs35773040, and rs35979566 yielded

57%, 81%, and 94% decreased activity relative to wild type CYP2B6, respectively. To further explore the effect of these SNPs on the metabolism of methadone, various methadone concentrations were added to the assay to competitively inhibit CYP2B6 activity. The data indicated that methadone also binds to the CYP2B6 active site and competes with the luminogenic substrate used to measure the activity. Therefore, our investigations suggest that these three SNPs may contribute to CYP2B6 PM phenotypes and may influence the pharmacokinetics and pharmacodynamics of (S)-methadone leading to unanticipated fatalities.

Since the time of the genotyping study, a TaqMan® SNP Genotyping kit for SNP rs35773040 (C__34816082_30) has become available. A custom designed assay for rs2279343 (AHBJ12M) can now also be obtained through ThermoFisher Scientific (Bloom et al., 2013). These kits should be used to genotype the methadone-only and control cases. With this additional information, the *CYP2B6*6* haplotype, which is made up by SNPs rs2279343 and rs3745274, can be identified in these individuals. Statistical analysis, conducted as described in Chapter 2, could reveal more SNPs that may be associated with methadone overdoses in the WV and KY populations.

The purpose of this study was to determine the role of *CYP2B6* SNPs in the unexpected death due to methadone overdoses. While it has unveiled SNPs enriched in those deceased individuals from the heart of the Appalachia region and SNPs that reduce the enzymatic activity of CYP2B6, there are several questions left unanswered. The data amongst the association and functionality studies are not complimentary. SNP rs3745274 was enhanced in methadone-only cases, however the SNP yielded an increase in CYP2B6 activity. Similarly, SNP rs3211371 was correlated with a significant increase in the blood methadone concentration, yet the effect of CYP2B6 activity was comparable to the wild type. While rs8192709 was associated with an

increase in methadone metabolism, the CYP2B6 activity was significantly decreased with the presence of this SNP. We determined the effect of individual SNPs on the activity of the CYP2B6 enzyme; however, the deceased individuals expressed multiple SNPs in their genetic profile for methadone metabolizing CYPs. The combined effect of SNPs on multiple CYPs may be the contributory factor to the discrepancies observed between the two studies. In order to understand the complexity of the effects of *CYP2B6* SNPs on methadone pharmacokinetics and pharmacodynamics, further functionality testing should be conducted with combinations of SNPs. For example, a plasmid constructed to contain the common haplotype *CYP2B6*6*, would be useful since clinical evidence suggests this genotype influences methadone disposition (Crettol et al., 2005; Crettol et al., 2006; Eap et al., 2007; Wang et al., 2011). Any number of *CYP2B6* SNP combinations would impart a greater understanding in the effects of the SNPs in CYP2B6 activity, be it a compound effect with multiple PM SNPs or a null effect with an extensive metabolizer SNP in combination with a PM SNP.

The CYP2B6 enzyme is only involved partially in the metabolism of (S)-methadone, and therefore, the SNPs on this gene are only a fraction of the whole story. Previous studies conducted by our laboratory determined the association of SNPs on the *CYP3A4* gene, which metabolizes both the (R)- and (S)- forms of methadone. It was found that SNPs rs2242480 and rs2740574 were enriched within the methadone-only group (Richards-Waugh et al., 2014). Studies on the effect of these *CYP3A4* SNPs should be conducted as outlined in Chapter 3. It is possible that it is a combination of SNPs on both the *CYP2B6* and *CYP3A4* genes leads to unintentional methadone overdoses. This additional research will help elucidate the role of the different CYPs and their polymorphisms in methadone pharmacokinetics and thus the therapeutic outcome.

REFERENCES

Ahmad, T., Sabet, S., Primerano, D. A., Richards-Waugh, L. L., & Rankin, G. O. (2017, May). Tell-tale SNPs: The role of CYP2B6 in methadone fatalities. *Journal of Analytical Toxicology, 41*(4), 325-333. doi:10.1093/jat/bkw135

Ahmad, T., Valentovic, M. A., & Rankin, G. O. (2018, July). Effects of cytochrome P450 single nucleotide polymorphisms on methadone metabolism and pharmacodynamics. *Biochemical Pharmacology, 153,* 196-204. doi:10.1016/j.bcp.2018.02.020

Anderson, I. B., & Kearney, T. E. (2000, January). Use of Methadone. *Western Journal of Medicine, 172,* 43-46. doi:10.1136/ewjm.172.1.43

Anggård, E., Gunne, L.-M., Holmstrand, J., McMahon, R. E., Sandberg, C.-G., & Sullivan, H. R. (1975). Disposition of methadone in methadone maintenance. *Clinical Pharmacology & Therapeutics, 17*(3), 258-266. doi:http://dx.doi.org/10.1002/cpt1975173258

Ansermot, N., Albayrak, Ö., Schläpfer, J., Crettol, S., Croquette-Krokar, M., Bourquin, M., . . . Eap, C. B. (2010, March 22). Substitution of (R,S)-Methadone by (R)-Methadone Impact on QTc Interval. *Archives of internal medicine, 170*(6), 529-536. doi:10.1001/archinternmed.2010.26

Bart, G. (2012, July). Maintenance Medication for Opiate Addiction: The Foundation of Recovery. *Journal of Addictive Diseases, 31*(3), 207-225. doi:10.1080/10550887.2012.694598

Behan, J. L., Cruickshank, Y. E., Matthews-Smith, G., Bruce, M., & Smith, K. D. (2013). The glycosylation of AGP and its associations with the binding to methadone. *BioMed Research International, 2013*(108902). doi:10.1155/2013/108902

Bloom, A. J., Martinez, M., Chen, L.-S., Bierut, L. J., Murphy, S. E., & Goate, A. (2013, November 15). CYP2B6 Non-Coding Variation Associated with Smoking Cessation Is Also Associated with Differences in Allelic Expression, Splicing, and Nicotine Metabolism Independent of Common Amino-Acid Changes. *PLoS One, 8*(11), e79700. doi:10.1371/journal.pone.0079700

Boulton, D. W., Arnaud, P., & DeVane, C. L. (2001). Pharmacokinetics and pharmacodynamics of methadone enantiomers after a single oral dose of racemate. *Clinical Pharmacology and Therapeutics, 70*(1), 48-57. doi:10.1067/mcp.2001.116793

Carlquist, J. F., Moody, D. E., Knight, S., Johnson, E. G., Fang, W. B., Huntinghouse, J. A., . . . Anderson, J. L. (2015). A Possible Mechanistic Link Between the CYP2C19 Genotype, the Methadone Metabolite Ethylidene-1,5-Dimethyl-3,3-Diphenylpyrrolidene (EDDP), and Methadone-Induced Corrected QT Interval Prolongation in a Pilot Study. *Molecular Diagnosis and Therapy, 19*(2), 131-138. doi:10.1007/s40291-015-0137-4

CDC. (2012, July 3). *Vital Signs: Prescription Painkiller Overdoses.* Retrieved August 2016, from Centers for Disease Control and Prevention: https://www.cdc.gov/vitalsigns/MethadoneOverdoses/index.html

Chang, Y., Fang, W. B., Lin, S.-N., & Moody, D. E. (2011, January). Stereo-Selective Metabolism of Methadone by Human Liver Microsomes and cDNA-Expressed Cytochrome P450s: A Reconciliation. *Basic & Clinical Pharmacology & Toxicology, 108*(1), 55-62. doi:10.1111/j.1742-7843.2010.00628.x

Christie, J. M. (2011, Spring-Summer). Opioid Prescribing: Methadone Risk Mitigation. *The Anesthesia Patient Safety Foundation Newsletter, 26*(1), pp. 1, 13-15. Retrieved April 6, 2019, from https://www.apsf.org/wp-content/uploads/newsletters/2011/spring/pdf/APSF201106.pdf

Coller, J. K., Joergensen, C., Foster, D. J., James, H., Gillis, D., Christrup, L., & Somogyi, A. A. (2007). Lack of influence of CYP2D6 genotype on the clearance of (R) - , (S) - and racemic-methadone. *International journal of clinical pharmacology and therapeutics, 45*(7), 410-417. doi:10.5414/CPP45410

Crettol, S., Déglon, J.-J., Besson, J., Croquette-Krokar, M., Gothuey, I., Hämmig, R., . . . Eap, C. B. (2005). Methadone enantiomer plasma levels, CYP2B6, CYP2C19, and CYP2C9 genotypes, and response to treatment. *Clinical Pharmacology and Therapeutics, 78*(6), 593-604. doi:10.1016/j.clpt.2005.08.011

Crettol, S., Déglon, J.-J., Besson, J., Croquette-Krokar, M., Hämmig, R., Gothuey, I., . . . Eap, C. B. (2006). ABCB1 and cytochrome P450 genotypes and phenotypes: Influence on methadone plasma levels and response to treatment. *Clinical Pharmacology and Therapeutics, 80*(6), 668-681. doi:10.1016/j.clpt.2006.09.012

Csajka, C., Crettol, S., Guidi, M., & Eap, C. B. (2016). Population Genetic-Based Pharmacokinetic Modeling of Methadone and its Relationship with the QTc Interval in Opioid-Dependent Patients. *Clinical Pharmacokinetics, 55*(12), 1521-1533. doi:10.1007/s40262-016-0415-2

Daly, A. K. (2004, December). Pharmacogenetics of the cytochromes P450. *Current Topics in Medicinal Chemistry, 4*(16), 1733-1744. doi:10.2174/1568026043387070

Daly, A. K. (2016, July 27). CYP2 Family. *The Biomedical & Life Sciences Collection*. Newcastle, Tyne and Wear, England: Henry Stewart Talks. Retrieved from https://hstalks.com/t/685/

De Fazio, S., Gallelli, L., De Siena, A., De Sarro, G., & Scordo, M. G. (2008). Role of CYP3A5 in Abnormal Clearance of Methadone Salvatore. *Annals of Pharmacotherapy, 42*(6), 893-897. doi:10.1345/aph.1K539

DePriest, A. Z., Puet, B. L., Holt, A. C., Roberts, A., & Cone, E. J. (2015, July). Metabolism and Disposition of Prescription Opioids: A Review. *Forensic Science Review, 27*(2), 115-145. Retrieved from

https://www.researchgate.net/profile/Edward_Cone/publication/280583461_Metabolism_and_Disposition_of_Prescription_Opioids_A_Review/links/55bf5fb708aed621de12fd90/Metabolism-and-Disposition-of-Prescription-Opioids-A-Review.pdf

Desta, Z., Saussele, T., Ward, B., Blievernicht, J., Li, L., Klein, K., . . . Zanger, U. M. (2007, June). Impact of CYP2B6 polymorphism on hepatic efavirenz metabolism in vitro. *PHARMACOGENOMICS, 8*(6), 547-558. doi:10.2217/14622416.8.6.547

Dinis-Oliveira, R. J. (2016). Metabolomics of methadone: clinical and forensic toxicological implications and variability of dose response. *Drug Metabolism Reviews, 48*(4), 568-576. doi:10.1080/03602532.2016.1192642

Dobrinas, M., Crettol, S., Oneda, B., Lahyani, R., Rotger, M., Choong, E., . . . Eap, C. B. (2013, February). Contribution of CYP2B6 alleles in explaining extreme (S)-methadone plasma levels A CYP2B6 gene resequencing study. *Pharmacogenetics and Genomics, 23*(2), 84-93. doi:10.1097/FPC.0b013e32835cb2e2

Dole, V. P., & Nyswander, M. (1965). A medical treatment for diacetylmorphine (heroin) addiction: A clinical trial with methadone hydrochloride. *JAMA, 193*(8), 646-650. doi:10.1001/jama.1965.03090080008002

Drucker, E., Lurie, P., Wodakt, A., & Alcabes, P. (1998). Measuring harm reduction: the effects of needle and syringe exchange programs and methadone maintenance on the ecology of HIV. *AIDS, 12* (Suppl A), S217-S230.

Druid, H., Holmgren, P., Carlsson, B., & Ahlner, J. (1999). Cytochrome P450 2D6 (CYP2D6) genotyping on postmortem blood as a supplementary tool for interpretation of forensic toxicological results. *Forensic Science International, 99*(1), 25-34. doi:10.1016/S0379-0738(98)00169-8

Eap, C. B., Broly, F., Mino, A., Haemmig, R., Déglon, J. J., Uehlinger, C., . . . Baumann, P. (2001). Cytochrome P450 2D6 genotype and methadone steady-state concentrations. *Journal of clinical psychopharmacology, 21*(2), 229-234. doi:10.1097/00004714-200104000-00016

Eap, C. B., Buclin, T., & Baumann, P. (2002, December). Interindividual variability of the clinical pharmacokinetics of methadone: implications for the treatment of opioid dependence. *Clinical Pharmacokinetics, 41*(14), 1153-1193. doi:10.2165/00003088-200241140-00003

Eap, C. B., Crettol, S., Rougier, J.-S., Schläpfer , J., Grilo, L. S., Déglon, J.-J., . . . Abriel, H. (2007, May). Stereoselective block of hERG channel by (S)-methadone and QT interval prolongation in CYP2B6 slow metabolizers. *Clinical Pharmacology and Therapeutics, 81*(5), 719-728. doi:10.1038/sj.clpt.6100120

Faul, M., Bohm, M., & Alexander, C. (2017, March 31). Methadone Prescribing and Overdose and the Association with Medicaid Preferred Drug List Policies — United States, 2007–

2014. *Morbidity and Mortality Weekly Report (MMWR), 66*(12), pp. 320-323. doi:http://dx.doi.org/10.15585/mmwr.mm6612a2

Ferrari, A., Coccia, C. P., Bertolini, A., & Sternieri, E. (2004). Methadone - Metabolism, pharmacokinetics and interactions. *Pharmacological Research, 50*(6), 551-559. doi:10.1016/j.phrs.2004.05.002

Fingerhut, L. A. (2006, December). Increases in Methadone-Related Deaths: 1999-2004. *National Center for Health Statistics*. Retrieved April 3, 2019, from https://www.cdc.gov/nchs/data/hestat/methadone1999-04/methadone1999-04.htm

Fokkema, I. F., & den Dunnen, J. T. (2015a, October 24). *CYP2C18 (cytochrome P450, family 2, subfamily C, polypeptide 18)*. Retrieved March 29, 2019, from LOVD v.3.0 - Leiden Open Variation Database Online gene-centered collection and display of DNA variants: https://www.lovd.nl/CYP2C18

Fokkema, I. F., & den Dunnen, J. T. (2015b, May 22). *CYP2C8 (cytochrome P450, family 2, subfamily C, polypeptide 8)*. Retrieved March 29, 2019, from LOVD v.3.0 - Leiden Open Variation Database Online gene-centered collection and display of DNA variants: http://www.LOVD.nl/CYP2C8

Fokkema, I. F., & den Dunnen, J. T. (2017, August 4). *CYP1A2 (cytochrome P450, family 1, subfamily A, polypeptide 2)*. Retrieved March 29, 2019, from LOVD v.3.0 - Leiden Open Variation Database Online gene-centered collection and display of DNA variants: https://www.LOVD.nl/CYP1A2

Fokkema, I. F., & den Dunnen, J. T. (2018a, September 14). *CYP2B6 (cytochrome P450, family 2, subfamily B, polypeptide 6)*. Retrieved March 29, 2019, from LOVD v.3.0 - Leiden Open Variation Database Online gene-centered collection and display of DNA variants: http://www.LOVD.nl/CYP2B6

Fokkema, I. F., & den Dunnen, J. T. (2018b, December 2018). *CYP2C19 (cytochrome P450, family 2, subfamily C, polypeptide 19)*. Retrieved March 29, 2019, from LOVD v.3.0 - Leiden Open Variation Database Online gene-centered collection and display of DNA variants: http://www.LOVD.nl/CYP2C19

Fokkema, I. F., & den Dunnen, J. T. (2019, February 26). *CYP2C9 (cytochrome P450, family 2, subfamily C, polypeptide 9*. Retrieved March 29, 2019, from LOVD v.3.0 - Leiden Open Variation Database Online gene-centered collection and display of DNA variants: http://www.LOVD.nl/CYP2C9

Fokkema, I. F., Taschne, P. E., Schaafsma, G. C., CellI, J., Laros, J. F., & den Dunnen, T. (2011). LOVD v.2.0: The next generation in gene variant databases. *Human Mutation, 32*(5), 557-563. doi:10.1002/humu.21438

Fonseca, F., de la Torre, R., Díaz, L., Pastor, A., Cuyàs, E., Pizarro, N., . . . Torrens, M. (2011, May). Contribution of cytochrome P450 and ABCB1 genetic variability on methadone

pharmacokinetics, dose requirements, and response. *PLoS ONE, 6*(5), e19527. doi:10.1371/journal.pone.0019527

Foster, D. J., Somogyi, A. A., & Bochner, F. (1999). Methadone N-demethylation in human liver microsomes: Lack of stereoselectivity and involvement of CYP3A4. *British Journal of Clinical Pharmacology, 47*(4), 403-412. doi:10.1046/j.1365-2125.1999.00921.x

Fredheim, O. M., Moksnes, K., Borchgrevink, P. C., Kaasa, S., & Dale, O. (2009, August). Clinical pharmacology of methadone for pain. *Acta Anaesthesiologica Scandinavica, 52*(7), 879-889. doi:10.1111/j.1399-6576.2008.01597.x

Gadel, S., Crafford, A., Regina, K., & Kharach, E. (2013). Methadone N-demethylation by the common CYP2B6 allelic variant CYP2B6.6. *Drug Metabolism and Disposition, 41*(4), 709-713. doi:10.1124/dmd.112.050625

Gadel, S., Friedel, C., & Kharasch, E. D. (2015). Differences in methadone metabolism by CYP2B6 variants. *Drug Metabolism and Disposition, 43*(7), 994-1001. doi:10.1124/dmd.115.064352

Gaertner, J., Voltz, R., & Ostgathe, C. (2008, August). Methadone: A Closer Look at the Controversy. *Journal of pain and symptom management, 36*(2), e4-7. doi:10.1016/j.jpainsymman.2008.04.007

Gay, S. C., Roberts, A. G., & Halpert, J. R. (2010). Structural Features of Cytochromes P450 and Ligands that Affect Drug Metabolism as Revealed by X-ray Crystallography and NMR. *Future Medicinal Chemistry, 2*(9), 1451-1468. doi:10.4155/fmc.10.229

Gonzalez, F. J., & Korzekwa, K. R. (1995). Cytochromes P450 Expression Systems. *Annual Review of Pharmacology and Toxicology, 35*(1), 369-390. doi:10.1146/annurev.pa.35.040195.002101

Hofmann, M. H., Blievernicht, J. K., Klein, K., Saussele, T., Schaeffeler, E., Schwab, M., & Zanger, U. M. (2008). Aberrant splicing caused by single nucleotide polymorphism c.516G>T , a marker of CYP2B6*6, is responsible for decreased expression and activity of CYP2B6 in Liver. *The Journal of Pharmacology and Experimental Therapeutics, 325*(1), 284-292. doi:10.1124/jpet.107.133306

Holzinger, E. R., Grady, B., Ritchie, M. D., Ribaudo, H. J., Acosta, E. P., Morse, G. D., . . . Haas, D. W. (2012). Genome-wide association study of plasma efavirenz pharmacokinetics in AIDS Clinical Trials Group protocols implicates several CYP2B6 variants. *Pharmacogenetics and Genomics, 22*(12), 858-867. doi:10.1097/FPC.0b013e32835a450b

Honda, M., Muroi, Y., Tamaki, Y., Saigusa, D., Suzuki, N., Tomioka, Y., . . . Hiratsuka, M. (2011). Functional characterization of CYP2B6 allelic variants in demethylation of antimalarial artemether. *Drug Metabolism and Disposition, 39*(10), 1860-1865. doi:10.1124/dmd.111.040352

Ingelman-Sundberg, M. (2017, February 9). The Human Cytochrome P450 (CYP) Allele Nomenclature Database Alle Nomenclature for Cytochrome P450 enzymes. (M. Ingelman-Sundberg , A. K. Daly, & D. W. Nebert, Eds.) Retrieved March 15, 2019, from https://www.pharmvar.org/htdocs/archive/index_original.htm

Institute of Medicine. (1995). *Federal Regulation of Methadone Treatment.* (R. A. Rettig & A. Yarmolinsky, Eds.) Washington, DC: The National Academies Press. doi:10.17226/4899

Iribarne, C., Berthou, F., Baird, S., Dréano, Y., Picart, D., Bail, J. P., . . . Ménez, J. F. (1996). Involvement of cytochrome P450 3A4 enzyme in the N-demethylation of methadone in human liver microsomes. *Chemical Research in Toxicology, 9*(2), 365-373. doi:10.1021/tx950116m

Jackman, R. P., Purvis, J. M., & Mallett, B. S. (2008, November 15). Chronic nonmalignant pain in primary care. *American Family Physician, 78*(10), 1155-1162. Retrieved from https://pdfs.semanticscholar.org/a0a8/8ebcc6d35229924ab9e5e6aa22947e0d59d2.pdf

Johansson, I., & Ingelman-Sundberg, M. (2008). CNVs of human genes and their implication in pharmacogenetics. *Cytogenetic and Genome Research, 123*, 195-204. doi:10.1159/000184709

Jones, C. M., Baldwin, G. T., Manocchio, T., White, J. O., & Mack, K. A. (2016, July 8). Trends in Methadone Distribution for Pain Treatment, Methadone Diversion, and Overdose Deaths — United States, 2002–2014. *Morbidity and Mortality Weekly Report (MMWR), 65*(26), pp. 667-671. doi:http://dx.doi.org/10.15585/mmwr.mm6526a2

Joseph, H., Stancliff, S., & Langrod, J. (2000, October/November). Methadone maintenance treatment (MMT): A Review of Historical and Clinical Issues. *The Mount Sinai Journal of Medicine, 67*(5 & 6), 347-364.

Jurica, J., & Sulcova, A. (2012). Determination of Cytochrome P450 Metabolic Activity Using Selective Markers. In J. Paxton (Ed.), *Topics on Drug Metabolism* (pp. 191-220). InTech. doi:10.5772/30236

Kahan, M., Srivastava, A., Wilson, L., Mailis-Gagnon, A., & Midmer, D. (2006, September 10). Opioids for managing chronic non-malignant pain: safe and effective prescribing. *Canadian Family Physician, 52*(9), 1091-1096. Retrieved from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1783736/

Kamal, N. N., Lim, T. S., Tye, G. J., Ismail, R., & Choong, Y. S. (2013). The effect of CYP2B6, CYP2D6, and CYP3A4 alleles on methadone binding: A molecular docking study. *Journal of Chemistry, 2013*(249642). doi:10.1155/2013/249642

Kapur, B. M., Hutson, J. R., Chibber, T., Luk, A., & Selby, P. (2011). Methadone: a review of drug-drug and pathophysiological interactions. *Critical Reviews in Clinical Laboratory Sciences, 48*(4), 171-195. doi:10.3109/10408363.2011.620601

Kharasch, E. D., & Stubbert, K. (2013). Role of cytochrome P4502B6 in methadone metabolism and clearance. *The Journal of Clinical Pharmacology, 53*(3), 305-313. doi:10.1002/jcph.1

Kharasch, E. D., Regina, K. J., Blood, J., & Friedel, C. (2015). Methadone Pharmacogenetics: CYP2B6 Polymorphisms Determine Plasma Concentrations, Clearance, and Metabolism. *Anesthesiology, 123*(5), 1142-1153. doi:10.1097/ALN.0000000000000867

Kim, J.-H., Cheong, H. S., Park, B. L., Kim, L. H., Shin, H. J., Na, H. S., . . . Shin, H. D. (2015). Direct sequencing and comprehensive screening of genetic polymorphisms on CYP2 family genes (CYP2A6, CYP2B6, CYP2C8, and CYP2E1) in five ethnic populations. *Archives of pharmacal research, 38*(1), 115-128. doi:10.1007/s12272-014-0348-y

Kishino, S., Nomura, A., Itoh, S., Nakagawa, T., Takekuma, Y., Sugawara, M., . . . Miyazaki, K. (2002). Age- and gender-related differences in carbohydrate concentrations of α1-acid glycoprotein variants and the effects of glycoforms on their drug-binding capacities. *European Journal of Clinical Pharmacology, 58*(9), 621-628. doi:10.1007/s00228-002-0530-x

Kreek, M. J., Levran, O., Reed, B., Schlussman, S. D., Zhou, Y., & Butelman, E. R. (2012, October). Opiate addiction and cocaine addiction: Underlying molecular neurobiology and genetics. *The Journal of Clinical Investigation, 122*(10), 3387-3391. doi:10.1172/JCI60390

Kringen, M. K., Chalabianloo, F., Bernard, J.-P., Bramness, J. G., Molden, E., & Høiseth, G. (2017, October). The combined effect of CYP2B6 genotype and other candidate genes on a steady-state serum concentration of methadone in opioid maintenance treatment. *Therapeutic Drug Monitoring, 39*(5), 550-555. doi:10.1097/FTD.0000000000000437

Kristensen, K., Blemmer, T., Angelo, H. R., Christrup, L. L., Drenck, N. E., Rasmussen, S. N., & Sjøgren, P. (1996). Stereoselective pharmacokinetics of methadone in chronic pain patients. *Therapeutic Drug Monitoring, 18*(3), 221-227. doi:10.1097/00007691-199606000-00001

Kristensen, K., Christensen, C. B., & Christrup, L. L. (1995, February). The mu1, mu2, delta, kappa opioid receptor binding profiles of methadone stereoisomers and morphine. *Life Sciences, 56*(2), PL45-PL50. doi:10.1016/0024-3205(94)00937-6

Lamba, J. K., Lin, Y. S., Schuetz, E. G., & Thummel, K. E. (2012). Genetic contribution to variable human CYP3A-mediated metabolism. *Advanced Drug Delivery Reviews, 54*(10), 256-569. doi:10.1016/j.addr.2012.09.017

Lang, T., Klein, K., Richter, T., Zibat, A., Kerb, R., Eichelbaum, M., . . . Zanger, U. M. (2004). Multiple novel nonsynonymous CYP2B6 gene polymorphisms in Caucasians: demonstration of phenotypic null alleles. *The Journal of pharmacology and experimental therapeutics, 311*(1), 34-43. doi:10.1124/jpet.104.068973

Langrod, J., Lowinson, J., & Ruiz, P. (1981). Methadone treatment and physical complaints: a clinical analysis. *The International Journal of the Addictions, 16*(5), 947-952. doi:10.3109/10826088109038903

Lee, H.-Y., Li, J.-H., Sheu, Y.-L., Tang, H.-P., Chang, W.-C., Tang, T.-C., . . . Liu, R.-H. (2013). Moving toward Personalized Medicine in the Methadone Maintenance Treatment Program: A Pilot Study on the Evaluation of Treatment Responses in Taiwan. *BioMed Research International, 2013*(741403). doi:10.1155/2013/741403

Lehotay, D. C., George, S., Etter, M. L., Graybiel, K., Eichhorst, J. C., Fern, B., . . . Kapur, B. (2005). Free and bound enantiomers of methadone and its metabolite, EDDP in methadone maintenance treatment: Relationship to dosage? *Clinical Biochemistry, 38*, 1088-1094. doi:10.1016/j.clinbiochem.2005.09.009

Lemke, T. L., Williams, D. A., Roche, V. F., & Zito, S. W. (2012). Central Analgesics. In T. L. Lemke, & D. A. Williams (Eds.), *Foye's principles of medicinal chemistry: Seventh edition* (7th ed., pp. 686-687). Wolters Kluwer Health Adis (ESP).

Leshner, A. I. (1997, August). Drug Abuse and Addiction Treatment Research. *Archives Of General Psychiatry, 54*(8), 691-694. doi:10.1001/archpsyc.1997.01830200015002

Levran, O., Peles, E., Hamon, S., Rand, M., Adelson, M., & Kreek, M. J. (2013, July). CYP2B6 SNPs are associated with methadone dose required for effective treatment of opioid addiction. *Addiction Biology, 18*(4), 709-716. doi:10.1111/j.1369-1600.2011.00349.x.

Li, Y., Kantelip, J.-P., Gerritsen-van Schieveen, P., & Davani, S. (2008). Interindividual variability of methadone response: impact of genetic polymorphism. *Molecular diagnosis & therapy, 12*(2), 109-124. doi:10.1007/BF03256276

Lipman, J. (2008). The Methadone Poisoning Epidemic. *The Forensic Examiner* (Summer 2008), pp. 38-46. Retrieved April 3, 2019, from https://www.deadiversion.usdoj.gov/mtgs/methadone_alert/forensic_examiner_17_38_2008.pdf#search=methadone

Lisberg, P., & Scheinmann, F. (2013). Is it Time to Consider Use of Levo-methadone (R-(-)-Methadone) to Replace Racemic Methadone? *Journal of Developing Drugs, 2*(2). doi:10.4172/2329-6631.1000109

Lugo, R. A., Satterfield, K. L., & Kern, S. E. (2005). Pharmacokinetics of Methadone. *Journal of Pain & Palliative Care Pharmacotherapy, 19*(4), 13-24. doi:10.1080/J354v19n04_05

Mallinckrodt Pharmaceuticals, Inc. (2016, December). Methadose™ Oral Concentrate (methadone hydrochloride oral concentrate USP) and Methadose™ Sugar-Free Oral Concentrate (methadone hydrochloride oral concentrate USP) dye-free, sugar-free, unflavored. *METHADOSE label*. Hazelwood, MO, USA. Retrieved November 3, 2017, from https://www.accessdata.fda.gov/drugsatfda_docs/label/2016/017116s029lbl.pdf

McCance-Katz, E. F. (2011, April). (R)-methadone versus racemic methadone: what is best for patient care? *Addiction, 106*(4), 687-688. doi:10.1111/j.1360-0443.2011.03374.x

McDonough, M. (2013, June). Opioid treatment of opioid addiction. *Australian Prescriber, 36*(3), 83-87. doi:10.18773/austprescr.2013.033

McGraw, J., & Waller, D. (2012). Cytochrome P450 variations in different ethnic populations. *Expert Opinion on Drug Metabolism & Toxicology, 8*(3), 371-382. doi:10.1517/17425255.2012.657626

Mitchell, T. B., Dyer, K. R., Newcombe, D., Salter, A., Somogyi, A. A., Bochner, F., & White, J. M. (2004). Subjective and physiological responses among racemic- methadone maintenance patients in relation to relative (S)- vs. (R)-methadone exposure. *British Journal of Clinical Pharmacology, 58*(6), 609-617. doi:10.1111/j.1365-2125.2004.02221.x British

Moody, D. E., Alburges, M. E., Parker, R. J., Collins, J. M., & Strong, J. M. (1997, December). The Involvement of Cytochrome P450 3A4 in theN-Demethylation ofl-α-Acetylmethadol (LAAM), norLAAM, and Methadone. *Drug Metabolism and Disposition, 25*(12), 1347-1353.

Nanovskaya, T. N., Deshmukh, S. V., Nekhayeva, I. A., Zharikova, O. L., Hankins, G. D., & Ahmed, M. S. (2004). Methadone metabolism by human placenta. *Biochemical Pharmacology, 68*, 583-591. doi:10.1016/j.bcp.2004.04.011

NCBI Resource Coordinators. (2016, January 4). Database resources of the National Center for Biotechnology Information. *Nucleic Acids Research, 44*(D1), D7-D19. doi:https://doi.org/10.1093/nar/gkv1290

Nguyen, T.-A., Tychopoulos, M., Bichat, F., Zimmermann, C., Flinois, J.-P., Diry, M., . . . de Waziers, I. (2008). Improvement of cyclophosphamide activation by CYP2B6 mutants: from in silico to ex vivo. *Molecular pharmacology, 73*(4), 1122-1133. doi:10.1124/mol.107.042861

Oda, Y., & Kharasch, E. D. (2001, September 1). Metabolism of Methadone and levo-alpha-Acetylmethadol (LAAM) by Human Intestinal Cytochrome P450 3A4 (CYP3A4): Potential Contribution of Intestinal Metabolism to Presystemic Clearance and Bioactivation. *The Journal of Pharmacology and Experimental Therapeutics, 298*(3), 1021-1032.

Paulozzi, L. J., Mack, K. A., & Jones, C. M. (2012, July 6). Vital Signs: Risk for Overdose from Methadone Used for Pain Relief — United States, 1999–2010. *Morbidity and Mortality Weekly Report (MMWR), 61*(26), pp. 493-497. Retrieved April 3, 2019, from *Morbidity and Mortality Weekly Report (MMWR)*: https://www.cdc.gov/mmwr/preview/mmwrhtml/mm6126a5.htm?s_cid=mm6126a5_w

Peles, E., Linzy, S., Kreek, M. J., & Adelson, M. (2013, November/December). Prospective study of QTc changes among former opiate addicts since admission to methadone

maintenance treatment: benzodiazepine risk. *Journal of addiction medicine, 7*(6), 428-434. doi:10.1097/ADM.0b013e3182a8a4f2

Quest Graph™ IC50 Calculator. (2018). (AAT Bioquest, Inc.) Retrieved March 20, 2019, from https://www.aatbio.com/tools/ic50-calculator

Reisine, T., Law, S. F., Blake, A., & Tallent, M. (1996). Molecular mechanisms of opiate receptor coupling to G proteins and effector systems. *Annals of the New York Academy of Sciences, 780*, 168-175. doi:10.1111/j.1749-6632.1996.tb15121.x

Richards-Waugh, L. L., Primerano, D. A., Dementieva, Y., Kraner, J. C., & Rankin, G. O. (2014). Fatal Methadone Toxicity: Potential Role of CYP3A4 Genetic Polymorphism. *Journal of Analytical Toxicology, 38*(8), 541-547. doi:10.1093/jat/bku091

Risch, N. J. (2000, June 15). Searching for genetic determinants in the new millennium. *Nature, 405*(6788), 847-856. doi:10.1038/35015718

Rudd, R. A., Seth, P., David, F., & Scholl, L. (2016). *Increases in drug and opioid-involved overdose deaths: United States, 2010–2015.* US Department of Health and Human Services. Atlanta: Centers for Disease Control and Prevention. doi:10.15585/mmwr.mm655051e1

Sadee, W., Wang, D., Papp, A. C., Pinsonneault, J. K., Smith, R. M., Moyer, R. A., & Johnson, A. D. (2011, February 2). Pharmacogenomics of the RNA world: structural RNA polymorphisms in drug therapy. *Clinical pharmacology and therapeutics, 89*(3), 355-365. doi:10.1038/clpt.2010.314

Silverman, D. A., Nettleton, R. T., Spencer, K. B., Wallisch, M., & Olsen, G. D. (2009, December 31). S-Methadone augments R-methadone induced respiratory depression in the neonatal guinea pig. *Respiratory Physiology & Neurobiology, 169*(3), 252-261. doi:10.1016/j.resp.2009.09.001

Smith, H. S. (2009, July). Opioid Metabolism. *Mayo Clinic Proceedings, 84*(7), 613-624. doi:10.4065/84.7.613

Solus, J. F., Arietta, B. J., Harris, J. R., Sexton, D. P., Steward, J. Q., McMunn, C., . . . Dawson, E. P. (2004). Genetic variation in eleven phase I drug metabolism genes in an ethnically diverse population. *Pharmacogenomics, 5*(7), 895-931. doi:10.1517/14622416.5.7.895

Somogyi, A. A., Barratt, D. T., Ali, R. L., & Coller, J. K. (2014). Pharmacogenomics of methadone maintenance treatment. *Pharmacogenomics, 15*(7), 1007-1027. doi:10.2217/pgs.14.56

Stotts, A. L., Dodrill, C. L., & Kosten, T. R. (2009, August). Opioid dependence treatment: options in pharmacotherapy. *Expert Opinion in Pharmacotherapy, 10*(11), 1727-1740. doi:10.1517/14656560903037168

Sullivan, H. R., & Due, S. L. (1973). Urinary metabolites of dl-methadone in maintenance subjects. *Journal of Medicinal Chemistry, 16*(8), 909-913. doi:10.1021/jm00266a009

Tamási, V., & Falus, A. (2012). Genetic and Epigenetic Factors Affecting Cytochrome P450 Phenotype and Their Clinical Relevance. In J. Paxton (Ed.), *Topics on Drug Metabolism* (pp. 87-110). InTech. doi:10.5772/36451

Tanka, E. (1998). Clinically important pharmacokinetic drug-drug interactions: role of cytochrome P450 enzymes. *Journal of Clinical Pharmacy and Therapeutics, 23*(6), 403-416. doi:10.1046/j.1365-2710.1998.00086.x

Taylor, W. F., Finkel, A. G., Robertson, K. R., Anderson, A. C., Toomey, T. C., Abashian, S. A., & Mann, J. D. (2000). Methadone in the Treatment of Chronic Nonmalignant Pain: A 2-Year Follow-up. *Pain Medicine, 1*(3), 254-259. doi:10.1046/j.1526-4637.2000.00027.x

Toombs, J. D., & Kral, L. A. (2005, April 1). Methadone treatment for pain states. *American Family Physician, 71*(7), 1353-1358. Retrieved from https://www.aafp.org/afp/2005/0401/p1353.html

Trescot, A. M., & Faynboym, S. (2014). A review of the role of genetic testing in pain medicine. *Pain Physician, 17*(5), 425-445.

Turpeinen, M. (2006). *Cytochrome P450 enzymes—in vitro, in vivo, and in silico studies.* (K. Nurkkala, Ed.) Oulu, Finland: Oulu University Press. Retrieved from http://jultika.oulu.fi/files/isbn9514282205.pdf

van der Schier, R., Roozekrans, M., van Velzen, M., Dahan, A., & Niesters, M. (2014, September 4). Opioid-induced respiratory depression: reversal by non-opioid drugs. *F1000Prime Reports*. doi:10.12703/P6-79

Wang, H., & Tompkins, L. M. (2008). CYP2B6: New Insights into a Historically Overlooked Cytochrome P450 Isozyme. *Current Drug Metabolism, 9*(7), 598-610. doi:10.2174/138920008785821710

Wang, J.-S., & DeVane, C. L. (2003). Involvement of CYP3A4, CYP2C8, and CYP2D6 in the metabolism of (R)- and (S)-methadone in vitro. *Drug Metabolism and Disposition, 31*(6), 742-747. doi:10.1124/dmd.31.6.742

Wang, L.-L., Li, Y., & Zhou, S.-F. (2009). A Bioinformatics Approach for the Phenotype Prediction of Nonsynonymous Single Nucleotide Polymorphisms in Human Cytochromes P450. *The American Society for Pharmacology and Experimental Therapeutics, 37*(5), 977-991. doi:10.1124/dmd.108.026047

Wang, S.-C., Ho, I.-K., Tsou, H.-H., Liu, S.-W., Hsiao, C.-F., Chen, C.-H., . . . Liu, Y.-L. (2013). Functional genetic polymorphisms in CYP2C19 gene in relation to cardiac side effects and treatment dose in a methadone maintenance cohort. *Omics: a journal of integrative biology, 17*(10), 519-526. doi:10.1089/omi.2012.0068

Wang, S.-C., Ho, I.-K., Tsou, H.-H., Tian, J.-N., Hsiao, C.-F., Chen, C.-H., . . . Liu, Y.-L. (2011). CYP2B6 polymorphisms influence the plasma concentration and clearance of the methadone S-enantiomer. *Journal of clinical psychopharmacology, 31*(4), 463-469. doi:10.1097/JCP.0b013e318222b5dd

Watanabe, T., Sakuyama, K., Sasaki, T., Ishii, Y., Ishikawa, M., Hirasawa, N., & Hiratsuka, M. (2010, July). Functional characterization of 26 CYP2B6 allelic variants (CYP2B6.2-CYP2B6.28, except CYP2B6.22). *Pharmacogenetics and Genomics, 20*(7), 459-462. doi:10.1097/FPC.0b013e32833bba0e

Whitley, H. P., & Linsdey, W. (2009). Sex-based differences in drug activity. *American Family Physician, 80*(11), 1254-1258.

Yang, H.-C., Chu, S.-K., Huang, C.-L., Kuo, H.-W., Wang, S.-C., Liu, S.-W., . . . Liu, Y.-L. (2016). Genome-Wide Pharmacogenomic Study on Methadone Maintenance Treatment Identifies SNP rs17180299 and Multiple Haplotypes on CYP2B6, SPON1, and GSG1L Associated with Plasma Concentrations of Methadone R- and S-enantiomers in Heroin-Dependent Patients. *PLoS Genetics, 12*(3), e1005910. doi:10.1371/journal.pgen.1005910

Zanger, U. M., & Klein, K. (2013, March). Pharmacogenetics of cytochrome P450 2B6 (CYP2B6): Advances on polymorphisms, mechanisms, and clinical relevance. *Frontiers in Genetics, 4*(24). doi:10.3389/fgene.2013.00024

Zanger, U. M., & Schwab, M. (2013). Cytochrome P450 enzymes in drug metabolism: Regulation of gene expression, enzyme activities, and impact of genetic variation. *Pharmacology and Therapeutics, 138*(1), 103-141. doi:10.1016/j.pharmthera.2012.12.007

Zhang, H., Amunugama, H., Ney, S., Cooper, N., & Hollenberg, P. F. (2011). Mechanism-based inactivation of human cytochrome P450 2B6 by clopidogrel: involvement of both covalent modification of cysteinyl residue 475 and loss of heme. *Molecular Pharmacology, 80*(5), 839-847. doi:10.1124/mol.111.073783

Zhou, S.-F. (2009). Polymorphism of human cytochrome P450 2D6 and its clinical significance: Part II. *Clinical pharmacokinetics, 48*(12), 761-804. doi:10.2165/11318070-000000000-00000

Zhou, S.-F., Liu, J.-P., & Chowbay, B. (2009, December). Polymorphism of human cytochrome P450 enzymes and its clinical impact. *Drug Metabolism Reviews, 41*(2), 89-295. doi:10.1080/03602530902843483

Zhou, Y., Ingelman-Sundberg, M., & Lauschke, V. M. (2017, October). Worldwide Distribution of Cytochrome P450 Alleles: A Meta-analysis of Population-scale Sequencing Projects. *Clinical Pharmacology & Therapeutics, 102*(4), 688-700. doi:10.1002/cpt.690

APPENDIX A: INSTITUTIONAL REVIEW BOARD APPROVAL

MARSHALL UNIVERSITY.
www.marshall.edu

Office of Research Integrity

April 25, 2019

Taha Ahmad
Department of Biomedical Sciences
Joan C. Edwards School of Medicine
Marshall University

Dear Ms. Ahmad:

This letter is in response to the submitted dissertation abstract entitled *"Role of Cytochrome P450 2B6 Polymorphisms in Unexpected Methadone Death."* After assessing the abstract it has been deemed not to be human subject research and therefore exempt from oversight of the Marshall University Institutional Review Board (IRB). The Code of Federal Regulations (45CFR46) has set forth the criteria utilized in making this determination. Since the study does not involve human subjects as defined in DHHS regulation 45 CFR §46.102(e) it is not considered human subject research. If there are any changes to the abstract you provided then you would need to resubmit that information to the Office of Research Integrity for review and determination.

I appreciate your willingness to submit the abstract for determination. Please feel free to contact the Office of Research Integrity if you have any questions regarding future protocols that may require IRB review.

Sincerely,

Bruce F. Day, ThD, CIP
Director

APPENDIX B: MULTIPLE SEQUENCE ALIGNMENT OF CYP2B6 SNP PLASMID VARIANTS[1] TO NCBI REFERENCE SEQUENCE PRE- AND POST-TRANSFECTION[2] OF COS-1 CELLS

CYP2B6 Plasmid (NM_000767.4)	ATGGAACTCA GCGTCCTCCT CTTCCTTGCA CTCCTCACAG GACTCTTGCT 50
Wild Type (*CYP2B6*1*)	ATGGAACTCA GCGTCCTCCT CTTCCTTGCA CTCCTCACAG GACTCTTGCT 50
rs2279343 (*CYP2B6*4*)	ATGGAACTCA GCGTCCTCCT CTTCCTTGCA CTCCTCACAG GACTCTTGCT 50
rs3211371 (*CYP2B6*5*)	ATGGAACTCA GCGTCCTCCT CTTCCTTGCA CTCCTCACAG GACTCTTGCT 50
rs3745274 (*CYP2B6*9*)	ATGGAACTCA GCGTCCTCCT CTTCCTTGCA CTCCTCACAG GACTCTTGCT 50
rs8192709 (*CYP2B6*2*)	ATGGAACTCA GCGTCCTCCT CTTCCTTGCA CTCCTCACAG GACTCTTGCT 50
rs12721655 (*8, c.415A>G)	ATGGAACTCA GCGTCCTCCT CTTCCTTGCA CTCCTCACAG GACTCTTGCT 50
rs35773040 (*CYP2B6*14*)	ATGGAACTCA GCGTCCTCCT CTTCCTTGCA CTCCTCACAG GACTCTTGCT 50
rs35979566 (*CYP2B6*15*)	ATGGAACTCA GCGTCCTCCT CTTCCTTGCA CTCCTCACAG GACTCTTGCT 50
CYP2B6 Plasmid (NM_000767.4)	ACTCCTGGTT CAGCGCCACC CTAACACCCA TGACCGCCTC CCACCAGGGC 100
Wild Type (*CYP2B6*1*)	ACTCCTGGTT CAGCGCCACC CTAACACCCA TGACCGCCTC CCACCAGGGC 100
rs2279343 (**4*, c.785A>G)	ACTCCTGGTT CAGCGCCACC CTAACACCCA TGACCGCCTC CCACCAGGGC 100
rs3211371 (*5, c.1459C>T)	ACTCCTGGTT CAGCGCCACC CTAACACCCA TGACCGCCTC CCACCAGGGC 100
rs3745274 (**9*, c.516G>T)	ACTCCTGGTT CAGCGCCACC CTAACACCCA TGACCGCCTC CCACCAGGGC 100
rs8192709 (**2*, c.64C>T)	ACTCCTGGTT CAGTGCCACC CTAACACCCA TGACCGCCTC CCACCAGGGC 100
rs12721655 (*8, c.415A>G)	ACTCCTGGTT CAGCGCCACC CTAACACCCA TGACCGCCTC CCACCAGGGC 100
rs35773040 (**14*, c.419G>A)	ACTCCTGGTT CAGCGCCACC CTAACACCCA TGACCGCCTC CCACCAGGGC 100
rs35979566 (**15*, c.1172T>A)	ACTCCTGGTT CAGCGCCACC CTAACACCCA TGACCGCCTC CCACCAGGGC 100

[1] Single nucleotide polymorphisms (SNPs) are highlighted in turquoise.

[2] Transfection of COS-1 cells with SNP rs12721655 variant plasmid was lethal and resulted in cell death. Therefore, Sanger sequencing on this SNP was performed only on the plasmid pre-transfection.

CYP2B6 Plasmid (NM_000767.4)	CCCGCCCTCT GCCCCTTTTG GGAAACCTTC TGCAGATGGA TAGAAGAGGC 150
Wild Type (*CYP2B6*1*)	CCCGCCCTCT GCCCCTTTTG GGAAACCTTC TGCAGATGGA TAGAAGAGGC 150
rs2279343 (**4*, c.785A>G)	CCCGCCCTCT GCCCCTTTTG GGAAACCTTC TGCAGATGGA TAGAAGAGGC 150
rs3211371 (**5*, c.1459C>T)	CCCGCCCTCT GCCCCTTTTG GGAAACCTTC TGCAGATGGA TAGAAGAGGC 150
rs3745274 (**9*, c.516G>T)	CCCGCCCTCT GCCCCTTTTG GGAAACCTTC TGCAGATGGA TAGAAGAGGC 150
rs8192709 (**2*, c.64C>T)	CCCGCCCTCT GCCCCTTTTG GGAAACCTTC TGCAGATGGA TAGAAGAGGC 150
rs12721655 (*8, c.415A>G)	CCCGCCCTCT GCCCCTTTTG GGAAACCTTC TGCAGATGGA TAGAAGAGGC 150
rs35773040 (**14*, c.419G>A)	CCCGCCCTCT GCCCCTTTTG GGAAACCTTC TGCAGATGGA TAGAAGAGGC 150
rs35979566 (**15*, c.1172T>A)	CCCGCCCTCT GCCCCTTTTG GGAAACCTTC TGCAGATGGA TAGAAGAGGC 150

CYP2B6 Plasmid (NM_000767.4)	CTACTCAAAT CCTTTCTGAG GTTCCGAGAG AAATATGGGG ACGTCTTCAC 200
Wild Type (*CYP2B6*1*)	CTACTCAAAT CCTTTCTGAG GTTCCGAGAG AAATATGGGG ACGTCTTCAC 200
rs2279343 (**4*, c.785A>G)	CTACTCAAAT CCTTTCTGAG GTTCCGAGAG AAATATGGGG ACGTCTTCAC 200
rs3211371 (**5*, c.1459C>T)	CTACTCAAAT CCTTTCTGAG GTTCCGAGAG AAATATGGGG ACGTCTTCAC 200
rs3745274 (**9*, c.516G>T)	CTACTCAAAT CCTTTCTGAG GTTCCGAGAG AAATATGGGG ACGTCTTCAC 200
rs8192709 (**2*, c.64C>T)	CTACTCAAAT CCTTTCTGAG GTTCCGAGAG AAATATGGGG ACGTCTTCAC 200
rs12721655 (*8, c.415A>G)	CTACTCAAAT CCTTTCTGAG GTTCCGAGAG AAATATGGGG ACGTCTTCAC 200
rs35773040 (**14*, c.419G>A)	CTACTCAAAT CCTTTCTGAG GTTCCGAGAG AAATATGGGG ACGTCTTCAC 200
rs35979566 (**15*, c.1172T>A)	CTACTCAAAT CCTTTCTGAG GTTCCGAGAG AAATATGGGG ACGTCTTCAC 200

CYP2B6 Plasmid (NM_000767.4)	GGTACACCTG GGACCGAGGC CCGTGGTCAT GCTGTGTGGA GTAGAGGCCA 250
Wild Type (*CYP2B6*1*)	GGTACACCTG GGACCGAGGC CCGTGGTCAT GCTGTGTGGA GTAGAGGCCA 250
rs2279343 (**4*, c.785A>G)	GGTACACCTG GGACCGAGGC CCGTGGTCAT GCTGTGTGGA GTAGAGGCCA 250
rs3211371 (**5*, c.1459C>T)	GGTACACCTG GGACCGAGGC CCGTGGTCAT GCTGTGTGGA GTAGAGGCCA 250
rs3745274 (**9*, c.516G>T)	GGTACACCTG GGACCGAGGC CCGTGGTCAT GCTGTGTGGA GTAGAGGCCA 250
rs8192709 (**2*, c.64C>T)	GGTACACCTG GGACCGAGGC CCGTGGTCAT GCTGTGTGGA GTAGAGGCCA 250
rs12721655 (*8, c.415A>G)	GGTACACCTG GGACCGAGGC CCGTGGTCAT GCTGTGTGGA GTAGAGGCCA 250
rs35773040 (**14*, c.419G>A)	GGTACACCTG GGACCGAGGC CCGTGGTCAT GCTGTGTGGA GTAGAGGCCA 250
rs35979566 (**15*, c.1172T>A)	GGTACACCTG GGACCGAGGC CCGTGGTCAT GCTGTGTGGA GTAGAGGCCA 250

CYP2B6 Plasmid (NM_000767.4)	TACGGGAGGC CCTTGTGGAC AAGGCTGAGG CCTTCTCTGG CCGGGGAAAA 300
Wild Type (*CYP2B6*1*)	TACGGGAGGC CCTTGTGGAC AAGGCTGAGG CCTTCTCTGG CCGGGGAAAA 300
rs2279343 (**4*, c.785A>G)	TACGGGAGGC CCTTGTGGAC AAGGCTGAGG CCTTCTCTGG CCGGGGAAAA 300
rs3211371 (**5*, c.1459C>T)	TACGGGAGGC CCTTGTGGAC AAGGCTGAGG CCTTCTCTGG CCGGGGAAAA 300
rs3745274 (**9*, c.516G>T)	TACGGGAGGC CCTTGTGGAC AAGGCTGAGG CCTTCTCTGG CCGGGGAAAA 300
rs8192709 (**2*, c.64C>T)	TACGGGAGGC CCTTGTGGAC AAGGCTGAGG CCTTCTCTGG CCGGGGAAAA 300
rs12721655 (*8, c.415A>G)	TACGGGAGGC CCTTGTGGAC AAGGCTGAGG CCTTCTCTGG CCGGGGAAAA 300
rs35773040 (**14*, c.419G>A)	TACGGGAGGC CCTTGTGGAC AAGGCTGAGG CCTTCTCTGG CCGGGGAAAA 300
rs35979566 (**15*, c.1172T>A)	TACGGGAGGC CCTTGTGGAC AAGGCTGAGG CCTTCTCTGG CCGGGGAAAA 300

CYP2B6 Plasmid (NM_000767.4)	ATCGCCATGG TCGACCCATT CTTCCGGGGA TATGGTGTGA TCTTTGCCAA 350
Wild Type (*CYP2B6*1*)	ATCGCCATGG TCGACCCATT CTTCCGGGGA TATGGTGTGA TCTTTGCCAA 350
rs2279343 (**4*, c.785A>G)	ATCGCCATGG TCGACCCATT CTTCCGGGGA TATGGTGTGA TCTTTGCCAA 350
rs3211371 (**5*, c.1459C>T)	ATCGCCATGG TCGACCCATT CTTCCGGGGA TATGGTGTGA TCTTTGCCAA 350
rs3745274 (**9*, c.516G>T)	ATCGCCATGG TCGACCCATT CTTCCGGGGA TATGGTGTGA TCTTTGCCAA 350
rs8192709 (**2*, c.64C>T)	ATCGCCATGG TCGACCCATT CTTCCGGGGA TATGGTGTGA TCTTTGCCAA 350
rs12721655 (*8, c.415A>G)	ATCGCCATGG TCGACCCATT CTTCCGGGGA TATGGTGTGA TCTTTGCCAA 350
rs35773040 (**14*, c.419G>A)	ATCGCCATGG TCGACCCATT CTTCCGGGGA TATGGTGTGA TCTTTGCCAA 350
rs35979566 (**15*, c.1172T>A)	ATCGCCATGG TCGACCCATT CTTCCGGGGA TATGGTGTGA TCTTTGCCAA 350

CYP2B6 Plasmid (NM_000767.4)	TGGAAACCGC TGGAAGGTGC TTCGGCGATT CTCTGTGACC ACTATGAGGG 400
Wild Type (*CYP2B6*1*)	TGGAAACCGC TGGAAGGTGC TTCGGCGATT CTCTGTGACC ACTATGAGGG 400
rs2279343 (**4*, c.785A>G)	TGGAAACCGC TGGAAGGTGC TTCGGCGATT CTCTGTGACC ACTATGAGGG 400
rs3211371 (**5*, c.1459C>T)	TGGAAACCGC TGGAAGGTGC TTCGGCGATT CTCTGTGACC ACTATGAGGG 400
rs3745274 (**9*, c.516G>T)	TGGAAACCGC TGGAAGGTGC TTCGGCGATT CTCTGTGACC ACTATGAGGG 400
rs8192709 (**2*, c.64C>T)	TGGAAACCGC TGGAAGGTGC TTCGGCGATT CTCTGTGACC ACTATGAGGG 400
rs12721655 (*8, c.415A>G)	TGGAAACCGC TGGAAGGTGC TTCGGCGATT CTCTGTGACC ACTATGAGGG 400
rs35773040 (**14*, c.419G>A)	TGGAAACCGC TGGAAGGTGC TTCGGCGATT CTCTGTGACC ACTATGAGGG 400
rs35979566 (**15*, c.1172T>A)	TGGAAACCGC TGGAAGGTGC TTCGGCGATT CTCTGTGACC ACTATGAGGG 400

CYP2B6 Plasmid (NM_000767.4)	ACTTCGGGAT GGGAAAGCGG AGTGTGGAGG AGCGGATTCA GGAGGAGGCT 450
Wild Type (*CYP2B6*1*)	ACTTCGGGAT GGGAAAGCGG AGTGTGGAGG AGCGGATTCA GGAGGAGGCT 450
rs2279343 (**4*, c.785A>G)	ACTTCGGGAT GGGAAAGCGG AGTGTGGAGG AGCGGATTCA GGAGGAGGCT 450
rs3211371 (**5*, c.1459C>T)	ACTTCGGGAT GGGAAAGCGG AGTGTGGAGG AGCGGATTCA GGAGGAGGCT 450
rs3745274 (**9*, c.516G>T)	ACTTCGGGAT GGGAAAGCGG AGTGTGGAGG AGCGGATTCA GGAGGAGGCT 450
rs8192709 (**2*, c.64C>T)	ACTTCGGGAT GGGAAAGCGG AGTGTGGAGG AGCGGATTCA GGAGGAGGCT 450
rs12721655 (*8, c.415A>G)	ACTTCGGGAT GGGAGAGCGG AGTGTGGAGG AGCGGATTCA GGAGGAGGCT 450
rs35773040 (**14*, c.419G>A)	ACTTCGGGAT GGGAAAGCAG AGTGTGGAGG AGCGGATTCA GGAGGAGGCT 450
rs35979566 (**15*, c.1172T>A)	ACTTCGGGAT GGGAAAGCGG AGTGTGGAGG AGCGGATTCA GGAGGAGGCT 450

CYP2B6 Plasmid (NM_000767.4)	CAGTGTCTGA TAGAGGAGCT TCGGAAATCC AAGGGGGCCC TCATGGACCC 500
Wild Type (*CYP2B6*1*)	CAGTGTCTGA TAGAGGAGCT TCGGAAATCC AAGGGGGCCC TCATGGACCC 500
rs2279343 (**4*, c.785A>G)	CAGTGTCTGA TAGAGGAGCT TCGGAAATCC AAGGGGGCCC TCATGGACCC 500
rs3211371 (**5*, c.1459C>T)	CAGTGTCTGA TAGAGGAGCT TCGGAAATCC AAGGGGGCCC TCATGGACCC 500
rs3745274 (**9*, c.516G>T)	CAGTGTCTGA TAGAGGAGCT TCGGAAATCC AAGGGGGCCC TCATGGACCC 500
rs8192709 (**2*, c.64C>T)	CAGTGTCTGA TAGAGGAGCT TCGGAAATCC AAGGGGGCCC TCATGGACCC 500
rs12721655 (*8, c.415A>G)	CAGTGTCTGA TAGAGGAGCT TCGGAAATCC AAGGGGGCCC TCATGGACCC 500
rs35773040 (**14*, c.419G>A)	CAGTGTCTGA TAGAGGAGCT TCGGAAATCC AAGGGGGCCC TCATGGACCC 500
rs35979566 (**15*, c.1172T>A)	CAGTGTCTGA TAGAGGAGCT TCGGAAATCC AAGGGGGCCC TCATGGACCC 500

CYP2B6 Plasmid (NM_000767.4)	CACCTTCCTC TTCCAGTCCA TTACCGCCAA CATCATCTGC TCCATCGTCT 550
Wild Type (*CYP2B6*1*)	CACCTTCCTC TTCCAGTCCA TTACCGCCAA CATCATCTGC TCCATCGTCT 550
rs2279343 (**4*, c.785A>G)	CACCTTCCTC TTCCAGTCCA TTACCGCCAA CATCATCTGC TCCATCGTCT 550
rs3211371 (**5*, c.1459C>T)	CACCTTCCTC TTCCAGTCCA TTACCGCCAA CATCATCTGC TCCATCGTCT 550
rs3745274 (**9*, c.516G>T)	CACCTTCCTC TTCCATTCCA TTACCGCCAA CATCATCTGC TCCATCGTCT 550
rs8192709 (**2*, c.64C>T)	CACCTTCCTC TTCCAGTCCA TTACCGCCAA CATCATCTGC TCCATCGTCT 550
rs12721655 (*8, c.415A>G)	CACCTTCCTC TTCCAGTCCA TTACCGCCAA CATCATCTGC TCCATCGTCT 550
rs35773040 (**14*, c.419G>A)	CACCTTCCTC TTCCAGTCCA TTACCGCCAA CATCATCTGC TCCATCGTCT 550
rs35979566 (**15*, c.1172T>A)	CACCTTCCTC TTCCAGTCCA TTACCGCCAA CATCATCTGC TCCATCGTCT 550

CYP2B6 Plasmid (NM_000767.4) TTGGAAAACG ATTCCACTAC CAAGATCAAG AGTTCCTGAA GATGCTGAAC 600
Wild Type (*CYP2B6*1*) TTGGAAAACG ATTCCACTAC CAAGATCAAG AGTTCCTGAA GATGCTGAAC 600
rs2279343 (**4*, c.785A>G) TTGGAAAACG ATTCCACTAC CAAGATCAAG AGTTCCTGAA GATGCTGAAC 600
rs3211371 (**5*, c.1459C>T) TTGGAAAACG ATTCCACTAC CAAGATCAAG AGTTCCTGAA GATGCTGAAC 600
rs3745274 (**9*, c.516G>T) TTGGAAAACG ATTCCACTAC CAAGATCAAG AGTTCCTGAA GATGCTGAAC 600
rs8192709 (**2*, c.64C>T) TTGGAAAACG ATTCCACTAC CAAGATCAAG AGTTCCTGAA GATGCTGAAC 600
rs12721655 (*8, c.415A>G) TTGGAAAACG ATTCCACTAC CAAGATCAAG AGTTCCTGAA GATGCTGAAC 600
rs35773040 (**14*, c.419G>A) TTGGAAAACG ATTCCACTAC CAAGATCAAG AGTTCCTGAA GATGCTGAAC 600
rs35979566 (**15*, c.1172T>A) TTGGAAAACG ATTCCACTAC CAAGATCAAG AGTTCCTGAA GATGCTGAAC 600

CYP2B6 Plasmid (NM_000767.4) TTGTTCTACC AGACTTTTTC ACTCATCAGC TCTGTATTCG GCCAGCTGTT 650
Wild Type (*CYP2B6*1*) TTGTTCTACC AGACTTTTTC ACTCATCAGC TCTGTATTCG GCCAGCTGTT 650
rs2279343 (**4*, c.785A>G) TTGTTCTACC AGACTTTTTC ACTCATCAGC TCTGTATTCG GCCAGCTGTT 650
rs3211371 (**5*, c.1459C>T) TTGTTCTACC AGACTTTTTC ACTCATCAGC TCTGTATTCG GCCAGCTGTT 650
rs3745274 (**9*, c.516G>T) TTGTTCTACC AGACTTTTTC ACTCATCAGC TCTGTATTCG GCCAGCTGTT 650
rs8192709 (**2*, c.64C>T) TTGTTCTACC AGACTTTTTC ACTCATCAGC TCTGTATTCG GCCAGCTGTT 650
rs12721655 (*8, c.415A>G) TTGTTCTACC AGACTTTTTC ACTCATCAGC TCTGTATTCG GCCAGCTGTT 650
rs35773040 (**14*, c.419G>A) TTGTTCTACC AGACTTTTTC ACTCATCAGC TCTGTATTCG GCCAGCTGTT 650
rs35979566 (**15*, c.1172T>A) TTGTTCTACC AGACTTTTTC ACTCATCAGC TCTGTATTCG GCCAGCTGTT 650

CYP2B6 Plasmid (NM_000767.4) TGAGCTCTTC TCTGGCTTCT TGAAATACTT TCCTGGGGCA CACAGGCAAG 700
Wild Type (*CYP2B6*1*) TGAGCTCTTC TCTGGCTTCT TGAAATACTT TCCTGGGGCA CACAGGCAAG 700
rs2279343 (**4*, c.785A>G) TGAGCTCTTC TCTGGCTTCT TGAAATACTT TCCTGGGGCA CACAGGCAAG 700
rs3211371 (**5*, c.1459C>T) TGAGCTCTTC TCTGGCTTCT TGAAATACTT TCCTGGGGCA CACAGGCAAG 700
rs3745274 (**9*, c.516G>T) TGAGCTCTTC TCTGGCTTCT TGAAATACTT TCCTGGGGCA CACAGGCAAG 700
rs8192709 (**2*, c.64C>T) TGAGCTCTTC TCTGGCTTCT TGAAATACTT TCCTGGGGCA CACAGGCAAG 700
rs12721655 (*8, c.415A>G) TGAGCTCTTC TCTGGCTTCT TGAAATACTT TCCTGGGGCA CACAGGCAAG 700
rs35773040 (**14*, c.419G>A) TGAGCTCTTC TCTGGCTTCT TGAAATACTT TCCTGGGGCA CACAGGCAAG 700
rs35979566 (**15*, c.1172T>A) TGAGCTCTTC TCTGGCTTCT TGAAATACTT TCCTGGGGCA CACAGGCAAG 700

CYP2B6 Plasmid (NM_000767.4)	TTTACAAAAA CCTGCAGGAA ATCAATGCTT ACATTGGCCA CAGTGTGGAG 750
Wild Type (*CYP2B6*1*)	TTTACAAAAA CCTGCAGGAA ATCAATGCTT ACATTGGCCA CAGTGTGGAG 750
rs2279343 (**4*, c.785A>G)	TTTACAAAAA CCTGCAGGAA ATCAATGCTT ACATTGGCCA CAGTGTGGAG 750
rs3211371 (**5*, c.1459C>T)	TTTACAAAAA CCTGCAGGAA ATCAATGCTT ACATTGGCCA CAGTGTGGAG 750
rs3745274 (**9*, c.516G>T)	TTTACAAAAA CCTGCAGGAA ATCAATGCTT ACATTGGCCA CAGTGTGGAG 750
rs8192709 (**2*, c.64C>T)	TTTACAAAAA CCTGCAGGAA ATCAATGCTT ACATTGGCCA CAGTGTGGAG 750
rs12721655 (*8, c.415A>G)	TTTACAAAAA CCTGCAGGAA ATCAATGCTT ACATTGGCCA CAGTGTGGAG 750
rs35773040 (**14*, c.419G>A)	TTTACAAAAA CCTGCAGGAA ATCAATGCTT ACATTGGCCA CAGTGTGGAG 750
rs35979566 (**15*, c.1172T>A)	TTTACAAAAA CCTGCAGGAA ATCAATGCTT ACATTGGCCA CAGTGTGGAG 750

CYP2B6 Plasmid (NM_000767.4)	AAGCACCGTG AAACCCTGGA CCCCAGCGCC CCCAAGGACC TCATCGACAC 800
Wild Type (*CYP2B6*1*)	AAGCACCGTG AAACCCTGGA CCCCAGCGCC CCCAAGGACC TCATCGACAC 800
rs2279343 (**4*, c.785A>G)	AAGCACCGTG AAACCCTGGA CCCCAGCGCC CCCAGGGACC TCATCGACAC 800
rs3211371 (**5*, c.1459C>T)	AAGCACCGTG AAACCCTGGA CCCCAGCGCC CCCAAGGACC TCATCGACAC 800
rs3745274 (**9*, c.516G>T)	AAGCACCGTG AAACCCTGGA CCCCAGCGCC CCCAAGGACC TCATCGACAC 800
rs8192709 (**2*, c.64C>T)	AAGCACCGTG AAACCCTGGA CCCCAGCGCC CCCAAGGACC TCATCGACAC 800
rs12721655 (*8, c.415A>G)	AAGCACCGTG AAACCCTGGA CCCCAGCGCC CCCAAGGACC TCATCGACAC 800
rs35773040 (**14*, c.419G>A)	AAGCACCGTG AAACCCTGGA CCCCAGCGCC CCCAAGGACC TCATCGACAC 800
rs35979566 (**15*, c.1172T>A)	AAGCACCGTG AAACCCTGGA CCCCAGCGCC CCCAAGGACC TCATCGACAC 800

CYP2B6 Plasmid (NM_000767.4)	CTACCTGCTC CACATGGAAA AAGAGAAATC CAACGCACAC AGTGAATTCA 850
Wild Type (*CYP2B6*1*)	CTACCTGCTC CACATGGAAA AAGAGAAATC CAACGCACAC AGTGAATTCA 850
rs2279343 (**4*, c.785A>G)	CTACCTGCTC CACATGGAAA AAGAGAAATC CAACGCACAC AGTGAATTCA 850
rs3211371 (**5*, c.1459C>T)	CTACCTGCTC CACATGGAAA AAGAGAAATC CAACGCACAC AGTGAATTCA 850
rs3745274 (**9*, c.516G>T)	CTACCTGCTC CACATGGAAA AAGAGAAATC CAACGCACAC AGTGAATTCA 850
rs8192709 (**2*, c.64C>T)	CTACCTGCTC CACATGGAAA AAGAGAAATC CAACGCACAC AGTGAATTCA 850
rs12721655 (*8, c.415A>G)	CTACCTGCTC CACATGGAAA AAGAGAAATC CAACGCACAC AGTGAATTCA 850
rs35773040 (**14*, c.419G>A)	CTACCTGCTC CACATGGAAA AAGAGAAATC CAACGCACAC AGTGAATTCA 850
rs35979566 (**15*, c.1172T>A)	CTACCTGCTC CACATGGAAA AAGAGAAATC CAACGCACAC AGTGAATTCA 850

CYP2B6 Plasmid (NM_000767.4)	GCCACCAGAA CCTCAACCTC AACACGCTCT CGCTCTTCTT TGCTGGCACT 900
Wild Type (*CYP2B6*1*)	GCCACCAGAA CCTCAACCTC AACACGCTCT CGCTCTTCTT TGCTGGCACT 900
rs2279343 (**4*, c.785A>G)	GCCACCAGAA CCTCAACCTC AACACGCTCT CGCTCTTCTT TGCTGGCACT 900
rs3211371 (**5*, c.1459C>T)	GCCACCAGAA CCTCAACCTC AACACGCTCT CGCTCTTCTT TGCTGGCACT 900
rs3745274 (**9*, c.516G>T)	GCCACCAGAA CCTCAACCTC AACACGCTCT CGCTCTTCTT TGCTGGCACT 900
rs8192709 (**2*, c.64C>T)	GCCACCAGAA CCTCAACCTC AACACGCTCT CGCTCTTCTT TGCTGGCACT 900
rs12721655 (*8, c.415A>G)	GCCACCAGAA CCTCAACCTC AACACGCTCT CGCTCTTCTT TGCTGGCACT 900
rs35773040 (**14*, c.419G>A)	GCCACCAGAA CCTCAACCTC AACACGCTCT CGCTCTTCTT TGCTGGCACT 900
rs35979566 (**15*, c.1172T>A)	GCCACCAGAA CCTCAACCTC AACACGCTCT CGCTCTTCTT TGCTGGCACT 900

CYP2B6 Plasmid (NM_000767.4)	GAGACCACCA GCACCACTCT CCGCTACGGC TTCCTGCTCA TGCTCAAATA 950
Wild Type (*CYP2B6*1*)	GAGACCACCA GCACCACTCT CCGCTACGGC TTCCTGCTCA TGCTCAAATA 950
rs2279343 (**4*, c.785A>G)	GAGACCACCA GCACCACTCT CCGCTACGGC TTCCTGCTCA TGCTCAAATA 950
rs3211371 (**5*, c.1459C>T)	GAGACCACCA GCACCACTCT CCGCTACGGC TTCCTGCTCA TGCTCAAATA 950
rs3745274 (**9*, c.516G>T)	GAGACCACCA GCACCACTCT CCGCTACGGC TTCCTGCTCA TGCTCAAATA 950
rs8192709 (**2*, c.64C>T)	GAGACCACCA GCACCACTCT CCGCTACGGC TTCCTGCTCA TGCTCAAATA 950
rs12721655 (*8, c.415A>G)	GAGACCACCA GCACCACTCT CCGCTACGGC TTCCTGCTCA TGCTCAAATA 950
rs35773040 (**14*, c.419G>A)	GAGACCACCA GCACCACTCT CCGCTACGGC TTCCTGCTCA TGCTCAAATA 950
rs35979566 (**15*, c.1172T>A)	GAGACCACCA GCACCACTCT CCGCTACGGC TTCCTGCTCA TGCTCAAATA 950

CYP2B6 Plasmid (NM_000767.4)	CCCTCATGTT GCAGAGAGAG TCTACAGGGA GATTGAACAG GTGATTGGCC 1000
Wild Type (*CYP2B6*1*)	CCCTCATGTT GCAGAGAGAG TCTACAGGGA GATTGAACAG GTGATTGGCC 1000
rs2279343 (**4*, c.785A>G)	CCCTCATGTT GCAGAGAGAG TCTACAGGGA GATTGAACAG GTGATTGGCC 1000
rs3211371 (**5*, c.1459C>T)	CCCTCATGTT GCAGAGAGAG TCTACAGGGA GATTGAACAG GTGATTGGCC 1000
rs3745274 (**9*, c.516G>T)	CCCTCATGTT GCAGAGAGAG TCTACAGGGA GATTGAACAG GTGATTGGCC 1000
rs8192709 (**2*, c.64C>T)	CCCTCATGTT GCAGAGAGAG TCTACAGGGA GATTGAACAG GTGATTGGCC 1000
rs12721655 (*8, c.415A>G)	CCCTCATGTT GCAGAGAGAG TCTACAGGGA GATTGAACAG GTGATTGGCC 1000
rs35773040 (**14*, c.419G>A)	CCCTCATGTT GCAGAGAGAG TCTACAGGGA GATTGAACAG GTGATTGGCC 1000
rs35979566 (**15*, c.1172T>A)	CCCTCATGTT GCAGAGAGAG TCTACAGGGA GATTGAACAG GTGATTGGCC 1000

CYP2B6 Plasmid (NM_000767.4)	CACATCGCCC TCCAGAGCTT CATGACCGAG CCAAAATGCC ATACACAGAG 1050
Wild Type (*CYP2B6*1*)	CACATCGCCC TCCAGAGCTT CATGACCGAG CCAAAATGCC ATACACAGAG 1050
rs2279343 (**4*, c.785A>G)	CACATCGCCC TCCAGAGCTT CATGACCGAG CCAAAATGCC ATACACAGAG 1050
rs3211371 (**5*, c.1459C>T)	CACATCGCCC TCCAGAGCTT CATGACCGAG CCAAAATGCC ATACACAGAG 1050
rs3745274 (**9*, c.516G>T)	CACATCGCCC TCCAGAGCTT CATGACCGAG CCAAAATGCC ATACACAGAG 1050
rs8192709 (**2*, c.64C>T)	CACATCGCCC TCCAGAGCTT CATGACCGAG CCAAAATGCC ATACACAGAG 1050
rs12721655 (*8, c.415A>G)	CACATCGCCC TCCAGAGCTT CATGACCGAG CCAAAATGCC ATACACAGAG 1050
rs35773040 (**14*, c.419G>A)	CACATCGCCC TCCAGAGCTT CATGACCGAG CCAAAATGCC ATACACAGAG 1050
rs35979566 (**15*, c.1172T>A)	CACATCGCCC TCCAGAGCTT CATGACCGAG CCAAAATGCC ATACACAGAG 1050
CYP2B6 Plasmid (NM_000767.4)	GCAGTCATCT ATGAGATTCA GAGATTTTCC GACCTTCTCC CCATGGGTGT 1100
Wild Type (*CYP2B6*1*)	GCAGTCATCT ATGAGATTCA GAGATTTTCC GACCTTCTCC CCATGGGTGT 1100
rs2279343 (**4*, c.785A>G)	GCAGTCATCT ATGAGATTCA GAGATTTTCC GACCTTCTCC CCATGGGTGT 1100
rs3211371 (**5*, c.1459C>T)	GCAGTCATCT ATGAGATTCA GAGATTTTCC GACCTTCTCC CCATGGGTGT 1100
rs3745274 (**9*, c.516G>T)	GCAGTCATCT ATGAGATTCA GAGATTTTCC GACCTTCTCC CCATGGGTGT 1100
rs8192709 (**2*, c.64C>T)	GCAGTCATCT ATGAGATTCA GAGATTTTCC GACCTTCTCC CCATGGGTGT 1100
rs12721655 (*8, c.415A>G)	GCAGTCATCT ATGAGATTCA GAGATTTTCC GACCTTCTCC CCATGGGTGT 1100
rs35773040 (**14*, c.419G>A)	GCAGTCATCT ATGAGATTCA GAGATTTTCC GACCTTCTCC CCATGGGTGT 1100
rs35979566 (**15*, c.1172T>A)	GCAGTCATCT ATGAGATTCA GAGATTTTCC GACCTTCTCC CCATGGGTGT 1100
CYP2B6 Plasmid (NM_000767.4)	GCCCCACATT GTCACCCAAC ACACCAGCTT CCGAGGGTAC ATCATCCCCA 1150
Wild Type (*CYP2B6*1*)	GCCCCACATT GTCACCCAAC ACACCAGCTT CCGAGGGTAC ATCATCCCCA 1150
rs2279343 (**4*, c.785A>G)	GCCCCACATT GTCACCCAAC ACACCAGCTT CCGAGGGTAC ATCATCCCCA 1150
rs3211371 (**5*, c.1459C>T)	GCCCCACATT GTCACCCAAC ACACCAGCTT CCGAGGGTAC ATCATCCCCA 1150
rs3745274 (**9*, c.516G>T)	GCCCCACATT GTCACCCAAC ACACCAGCTT CCGAGGGTAC ATCATCCCCA 1150
rs8192709 (**2*, c.64C>T)	GCCCCACATT GTCACCCAAC ACACCAGCTT CCGAGGGTAC ATCATCCCCA 1150
rs12721655 (*8, c.415A>G)	GCCCCACATT GTCACCCAAC ACACCAGCTT CCGAGGGTAC ATCATCCCCA 1150
rs35773040 (**14*, c.419G>A)	GCCCCACATT GTCACCCAAC ACACCAGCTT CCGAGGGTAC ATCATCCCCA 1150
rs35979566 (**15*, c.1172T>A)	GCCCCACATT GTCACCCAAC ACACCAGCTT CCGAGGGTAC ATCATCCCCA 1150

CYP2B6 Plasmid (NM_000767.4)	AGGACACAGA AGTATTTCTC ATCCTGAGCA CTGCTCTCCA TGACCCACAC 1200
Wild Type (*CYP2B6*1*)	AGGACACAGA AGTATTTCTC ATCCTGAGCA CTGCTCTCCA TGACCCACAC 1200
rs2279343 (**4*, c.785A>G)	AGGACACAGA AGTATTTCTC ATCCTGAGCA CTGCTCTCCA TGACCCACAC 1200
rs3211371 (**5*, c.1459C>T)	AGGACACAGA AGTATTTCTC ATCCTGAGCA CTGCTCTCCA TGACCCACAC 1200
rs3745274 (**9*, c.516G>T)	AGGACACAGA AGTATTTCTC ATCCTGAGCA CTGCTCTCCA TGACCCACAC 1200
rs8192709 (**2*, c.64C>T)	AGGACACAGA AGTATTTCTC ATCCTGAGCA CTGCTCTCCA TGACCCACAC 1200
rs12721655 (*8, c.415A>G)	AGGACACAGA AGTATTTCTC ATCCTGAGCA CTGCTCTCCA TGACCCACAC 1200
rs35773040 (**14*, c.419G>A)	AGGACACAGA AGTATTTCTC ATCCTGAGCA CTGCTCTCCA TGACCCACAC 1200
rs35979566 (**15*, c.1172T>A)	AGGACACAGA AGTATTTCTC AACCTGAGCA CTGCTCTCCA TGACCCACAC 1200

CYP2B6 Plasmid (NM_000767.4)	TACTTTGAAA AACCAGACGC CTTCAATCCT GACCACTTTC TGGATGCCAA 1250
Wild Type (*CYP2B6*1*)	TACTTTGAAA AACCAGACGC CTTCAATCCT GACCACTTTC TGGATGCCAA 1250
rs2279343 (**4*, c.785A>G)	TACTTTGAAA AACCAGACGC CTTCAATCCT GACCACTTTC TGGATGCCAA 1250
rs3211371 (**5*, c.1459C>T)	TACTTTGAAA AACCAGACGC CTTCAATCCT GACCACTTTC TGGATGCCAA 1250
rs3745274 (**9*, c.516G>T)	TACTTTGAAA AACCAGACGC CTTCAATCCT GACCACTTTC TGGATGCCAA 1250
rs8192709 (**2*, c.64C>T)	TACTTTGAAA AACCAGACGC CTTCAATCCT GACCACTTTC TGGATGCCAA 1250
rs12721655 (*8, c.415A>G)	TACTTTGAAA AACCAGACGC CTTCAATCCT GACCACTTTC TGGATGCCAA 1250
rs35773040 (**14*, c.419G>A)	TACTTTGAAA AACCAGACGC CTTCAATCCT GACCACTTTC TGGATGCCAA 1250
rs35979566 (**15*, c.1172T>A)	TACTTTGAAA AACCAGACGC CTTCAATCCT GACCACTTTC TGGATGCCAA 1250

CYP2B6 Plasmid (NM_000767.4)	TGGGGCACTG AAAAAGACTG AAGCTTTTAT CCCCTTCTCC TTAGGGAAGC 1300
Wild Type (*CYP2B6*1*)	TGGGGCACTG AAAAAGACTG AAGCTTTTAT CCCCTTCTCC TTAGGGAAGC 1300
rs2279343 (**4*, c.785A>G)	TGGGGCACTG AAAAAGACTG AAGCTTTTAT CCCCTTCTCC TTAGGGAAGC 1300
rs3211371 (**5*, c.1459C>T)	TGGGGCACTG AAAAAGACTG AAGCTTTTAT CCCCTTCTCC TTAGGGAAGC 1300
rs3745274 (**9*, c.516G>T)	TGGGGCACTG AAAAAGACTG AAGCTTTTAT CCCCTTCTCC TTAGGGAAGC 1300
rs8192709 (**2*, c.64C>T)	TGGGGCACTG AAAAAGACTG AAGCTTTTAT CCCCTTCTCC TTAGGGAAGC 1300
rs12721655 (*8, c.415A>G)	TGGGGCACTG AAAAAGACTG AAGCTTTTAT CCCCTTCTCC TTAGGGAAGC 1300
rs35773040 (**14*, c.419G>A)	TGGGGCACTG AAAAAGACTG AAGCTTTTAT CCCCTTCTCC TTAGGGAAGC 1300
rs35979566 (**15*, c.1172T>A)	TGGGGCACTG AAAAAGACTG AAGCTTTTAT CCCCTTCTCC TTAGGGAAGC 1300

CYP2B6 Plasmid (NM_000767.4)	GGATTTGTCT TGGTGAAGGC ATCGCCCGTG CGGAATTGTT CCTCTTCTTC 1350
Wild Type (*CYP2B6*1*)	GGATTTGTCT TGGTGAAGGC ATCGCCCGTG CGGAATTGTT CCTCTTCTTC 1350
rs2279343 (**4*, c.785A>G)	GGATTTGTCT TGGTGAAGGC ATCGCCCGTG CGGAATTGTT CCTCTTCTTC 1350
rs3211371 (**5*, c.1459C>T)	GGATTTGTCT TGGTGAAGGC ATCGCCCGTG CGGAATTGTT CCTCTTCTTC 1350
rs3745274 (**9*, c.516G>T)	GGATTTGTCT TGGTGAAGGC ATCGCCCGTG CGGAATTGTT CCTCTTCTTC 1350
rs8192709 (**2*, c.64C>T)	GGATTTGTCT TGGTGAAGGC ATCGCCCGTG CGGAATTGTT CCTCTTCTTC 1350
rs12721655 (*8, c.415A>G)	GGATTTGTCT TGGTGAAGGC ATCGCCCGTG CGGAATTGTT CCTCTTCTTC 1350
rs35773040 (**14*, c.419G>A)	GGATTTGTCT TGGTGAAGGC ATCGCCCGTG CGGAATTGTT CCTCTTCTTC 1350
rs35979566 (**15*, c.1172T>A)	GGATTTGTCT TGGTGAAGGC ATCGCCCGTG CGGAATTGTT CCTCTTCTTC 1350
CYP2B6 Plasmid (NM_000767.4)	ACCACCATCC TCCAGAACTT CTCCATGGCC AGCCCCGTGG CCCCAGAAGA 1400
Wild Type (*CYP2B6*1*)	ACCACCATCC TCCAGAACTT CTCCATGGCC AGCCCCGTGG CCCCAGAAGA 1400
rs2279343 (**4*, c.785A>G)	ACCACCATCC TCCAGAACTT CTCCATGGCC AGCCCCGTGG CCCCAGAAGA 1400
rs3211371 (**5*, c.1459C>T)	ACCACCATCC TCCAGAACTT CTCCATGGCC AGCCCCGTGG CCCCAGAAGA 1400
rs3745274 (**9*, c.516G>T)	ACCACCATCC TCCAGAACTT CTCCATGGCC AGCCCCGTGG CCCCAGAAGA 1400
rs8192709 (**2*, c.64C>T)	ACCACCATCC TCCAGAACTT CTCCATGGCC AGCCCCGTGG CCCCAGAAGA 1400
rs12721655 (*8, c.415A>G)	ACCACCATCC TCCAGAACTT CTCCATGGCC AGCCCCGTGG CCCCAGAAGA 1400
rs35773040 (**14*, c.419G>A)	ACCACCATCC TCCAGAACTT CTCCATGGCC AGCCCCGTGG CCCCAGAAGA 1400
rs35979566 (**15*, c.1172T>A)	ACCACCATCC TCCAGAACTT CTCCATGGCC AGCCCCGTGG CCCCAGAAGA 1400
CYP2B6 Plasmid (NM_000767.4)	CATCGATCTG ACACCCCAGG AGTGTGGTGT GGGCAAAATA CCCCCAACAT 1450
Wild Type (*CYP2B6*1*)	CATCGATCTG ACACCCCAGG AGTGTGGTGT GGGCAAAATA CCCCCAACAT 1450
rs2279343 (**4*, c.785A>G)	CATCGATCTG ACACCCCAGG AGTGTGGTGT GGGCAAAATA CCCCCAACAT 1450
rs3211371 (**5*, c.1459C>T)	CATCGATCTG ACACCCCAGG AGTGTGGTGT GGGCAAAATA CCCCCAACAT 1450
rs3745274 (**9*, c.516G>T)	CATCGATCTG ACACCCCAGG AGTGTGGTGT GGGCAAAATA CCCCCAACAT 1450
rs8192709 (**2*, c.64C>T)	CATCGATCTG ACACCCCAGG AGTGTGGTGT GGGCAAAATA CCCCCAACAT 1450
rs12721655 (*8, c.415A>G)	CATCGATCTG ACACCCCAGG AGTGTGGTGT GGGCAAAATA CCCCCAACAT 1450
rs35773040 (**14*, c.419G>A)	CATCGATCTG ACACCCCAGG AGTGTGGTGT GGGCAAAATA CCCCCAACAT 1450
rs35979566 (**15*, c.1172T>A)	CATCGATCTG ACACCCCAGG AGTGTGGTGT GGGCAAAATA CCCCCAACAT 1450

CYP2B6 Plasmid (NM_000767.4)	ACCAGATCCG CTTCCTGCCC CGCTAG 1476
Wild Type (*CYP2B6*1*)	ACCAGATCCG CTTCCTGCCC CGCTAG 1476
rs2279343 (**4*, c.785A>G)	ACCAGATCCG CTTCCTGCCC CGCTAG 1476
rs3211371 (**5*, c.1459C>T)	ACCAGATCTG CTTCCTGCCC CGCTAG 1476
rs3745274 (**9*, c.516G>T)	ACCAGATCCG CTTCCTGCCC CGCTAG 1476
rs8192709 (**2*, c.64C>T)	ACCAGATCCG CTTCCTGCCC CGCTAG 1476
rs12721655 (*8, c.415A>G)	ACCAGATCCG CTTCCTGCCC CGCTAG 1476
rs35773040 (**14*, c.419G>A)	ACCAGATCCG CTTCCTGCCC CGCTAG 1476
rs35979566 (**15*, c.1172T>A)	ACCAGATCCG CTTCCTGCCC CGCTAG 1476

APPENDIX C: LIST OF ABBREVIATIONS

AAS… amino acid substitution

ADH… alcohol dehydrogenase

AGP… α1-acid glycoprotein

ANOVA… analysis of variance

CDC… Centers for Disease Control and Prevention

CYP… cytochrome P450

DEA… Drug Enforcement Agency

del… deletion

DNA… deoxyribonucleic acid

EDDP… 2-ethyl-1,5-dimethyl-3,3-diphenylpyrrolidine

EM… extensive metabolizer

EMDP… 2-ethyl-5-methyl-3,3-diphenyl-1-pyrroline

FDA… Food and Drug Administration

hERG… human ether-a-*go-go* related gene

HWE… Hardy-Weinberg equilibrium

IM… intermediate metabolizer

KY… Kentucky

LD… linkage disequilibrium

MAF… minor allele frequency

MMT… methadone maintenance treatment

MOR… μ-opioid receptor

MTD… methadone

NADPH… nicotinamide adenine dinucleotide phosphate

NCBI… National Center for Biotechnology Information

OCME… Offices of the Chief Medical Examiner

p-HO… para-hydroxy

PolyPhen… polymorphism phenotyping

PM… poor metabolizer

RT-PCR… real-time polymerase chain reaction

SEM… standard error of the mean

SIFT… scale-invariant feature transform

SNP… single nucleotide polymorphism

SRS… substrate recognition site

UM… ultra-rapid metabolizer

UTR… untranslated region

VEP… variant effect predictor

WV… West Virginia

APPENDIX D: CURRICULUM VITAE

TAHA AHMAD, PHD

Phone: (201) 705-7815
Taha.Ahmad.NJ@gmail.com

1443 15th Street
Huntington, WV 25701

EDUCATION

PhD Marshall University, Biomedical Sciences May 2019
Emphases: Pharmacology, Physiology and Toxicology
Dissertation: "Role of Cytochrome P450 2B6 Polymorphisms in Unexpected Methadone Death"
Committee: Dr. Gary O. Rankin, Dr. Monica A. Valentovic, Dr. Donald A. Primerano, Dr. Travis B. Salisbury, Dr. Richard D. Egleton

MSFS Marshall University, Forensic Science May 2009
Emphases: DNA Analysis, Crime Scene Investigation, and Computer Forensics

BS New Jersey City University, Biology and Chemistry May 2005
Minored in Mathematics

LABORATORY SKILLS

Experimental Techniques

- Gel electrophoresis, PCR, Light Microscope, Spectrophotometer, HPLC/UV-Vis, UPLC/MS/MS
- Extract genomic DNA from blood stain cards via QIAamp DNA Micro DNA Kit
- Quantitate extracted DNA using NanoDrop 1000
- Genotype CYP2B6 and CYP2C19 SNPs using TaqMan® SNP Genotyping Assay on the ABI 7000 Sequence Detection and StepOnePlus instrument and software
- Determine relationship and correlation of SNPs in CYP2B6 and CYP2C19 genes to decreased metabolism of methadone leading to fatal concentrations
- Perform mutations using a QuikChange II Site-Directed Mutagenesis Kit on CYP2B6 plasmids, transformation of DH5α and XL-1 Blue cells for amplification of the plasmids, purification and sequencing of the plasmids to ensure presence of mutations
- Perform routine cell culture, cell maintenance and storage, transfections, and selection of COS-1 cells
- Isolate microsomal protein and quantitate protein using Pierce BCA protein assay
- Measure CYP2B6 activity using P450-Glo™ Assay
- Develop a method for an HPLC/UV-Vis to separate enantiomers of methadone and its metabolite EDDP

Software

- Microsoft Office (Word, Excel, Access, PowerPoint, Outlook), Chromas Lite, DNA Baser Assembler, ImageJ, GraphPad Prism, ChemSketch

PUBLICATIONS

Journal Publications

Ahmad, T., Valentovic, M. A., and Rankin, G. O., "Effects of Cytochrome P450 Single Nucleotide Polymorphisms on Methadone Metabolism and Pharmacodynamics," Biochemical Pharmacology, 153, 2018, pp. 196-204.

Ahmad, T., Sabet, S., Primerano, D.A., Richards-Waugh, L.L., and Rankin, G.O., "Tell-tale SNPs: The Role of CYP2B6 in Methadone Fatalities," J Anal Toxicol. 41(4), 2017, pp. 325-333.

Lee, S.B., Clabaugh, K.C., Silva, B., Odigie, K.O., Coble, M.D., Loreille, O., Scheible, M., Fourney, R.M., Stevens, J., Carmody, G.R., Parsons, T.J., Pozder, A., Eisenberg, A.J., Budowle, B., **Ahmad, T.**, Miller, R.W., and Crouse, C.A., "Assessing a Novel Room Temperature DNA Storage Medium for Forensic Biological Samples," Forensic Sci Int Genet. 6(1), 2012, pp. 31-40.

PRESENTATIONS

Selected for Oral Presentation

Ahmad, T., "The Significance of CYP2B6 Genetic Polymorphisms in Unexpected Fatalities of Methadone Users in Caucasians of WV and KY Appalachia Region," Health Science Center 28th Annual Research Day, Marshall University, Huntington, West Virginia, March 2016.

Oral Presentations

Ahmad, T., "Cell Culture and Transfection," Lecture, Marshall University, Huntington, West Virginia, November 2016.

Ahmad, T., "S-Methadone: The Evil Twin," Spring Seminar Series, Marshall University, Huntington, West Virginia, April 2016.

Ahmad, T., "Sex, Drugs, & Rock 'N' Roll: The Science Behind Addiction," Fall Seminar Series, Marshall University, Huntington, West Virginia, November 2014.

Ahmad, T., "Methadone: Killing More Than Just Pain," Fall Seminar Series, Marshall University, Huntington, West Virginia, October 2014.

Ahmad, T., "Whole Exome Gene Sequencing," Lecture, Marshall University, Huntington, West Virginia, February 2013.

Ahmad, T., "What's in YOUR Genes? A Look at Methadone Overdose," Spring Seminar Series, Marshall University, Huntington, West Virginia, April 2011.

Ahmad, T., "Quantifiler® DUO vs Plexor® HY on the Stratagene Mx3005P™," Fall Seminar Series, Marshall University Forensic Science Center, Huntington, West Virginia, October 2009.

Ahmad, T., "Biomatrica DNA SampleMatrix® – A New Prospect for Forensic DNA Sample Storage," Marshall University Forensic Science Center, Huntington, West Virginia, October 2009.

Ahmad, T., "The Purpose and Importance of Using Kits," Lecture, Marshall University Forensic Science Center, Huntington, West Virginia, October 2009.

Ahmad, T., "Cases Using Microscopy: Yes, There is a Use to Microscopy," Lecture, Marshall University Forensic Science Center, Huntington, West Virginia, October 2009.

Ahmad, T., "The Application of GIS in Forensic Science Investigations," Spring Seminar Series, Marshall University Forensic Science Center, Huntington, West Virginia, April 2009.

Poster Presentations

Ahmad, T., Salisbury, T.B., Valentovic, M.A., Primerano, D.A., and Rankin, G.O., "Effects of Cytochrome P450 2B6 (CYP2B6) Single Nucleotide Polymorphisms on CYP2B6 Activity: Implications for Methadone Metabolism," Health Science Center 31st Annual Research Day, Marshall University, Huntington, West Virginia, March 2019.

Ahmad, T., Richards-Waugh, L.L., Sabet, S., and Rankin, G.O., "The Significance of CYP2B6 Genetic Polymorphisms in Unexpected Fatalities of Methadone Users in Caucasians of WV and KY Appalachia Region," Appalachian Regional Cell Conference, Huntington, West Virginia, November 2015.

Ahmad, T., Sabet, S., Richards-Waugh, L.L., and Rankin, G.O., "Role of CYP2B6 Genetic Polymorphisms in Unexpected Methadone Fatality," American Academy of Forensic Sciences, Orlando, Florida, February 2015.

Ahmad, T., Richards-Waugh, L.L., Sabet, S., and Rankin, G.O., "Role of CYP2B6 Genetic Polymorphisms in Unexpected Fatalities of Caucasian Methadone Users," Appalachian Regional Cell Conference, Huntington, West Virginia, November 2014.

Ahmad, T., Sabet, S., Richards-Waugh, L.L., and Rankin, G.O., "Contribution of CYP2B6 Genetic Polymorphisms in Unexpected Fatalities of Methadone Users," Ohio Valley Regional Chapter of the Society of Toxicology, Dayton, Ohio, September 2014.

Ahmad, T., Richards-Waugh, L.L., Sabet, S., and Rankin, G.O., "Contribution of CYP2B6 Alleles in Sudden Death of Methadone Users: A CYP2B6 Genetic Polymorphism Study," Society of Toxicology. Phoenix, Arizona, March 2014.

Ahmad, T., Sabet, S., Richards-Waugh, L.L., and Rankin, G.O., "Genetic Polymorphism in CYP2B6 and Sudden Death of Methadone Users in West Virginia and Kentucky," Health Science Center 25th Annual Research Day, Marshall University, Huntington, West Virginia, March 2013.

Sabet, S., **Ahmad, T.**, Rankin, G.O., and Richards-Waugh, L.L., "Genetic Polymorphism in CYP2B6 and Sudden Death of Methadone Users in WV and KY," WV-INBRE Summer Research Symposium, Morgantown, West Virginia, July 2012.

Ahmad, T., Miller, R.W., McGuckian, A.B., Conover-Sikorsky, J., Crouse, C.A., and Staton, P.J., "Biomatrica DNA SampleMatrix® – A New Prospect for Forensic DNA Sample Storage," Marshall University Forensic Science Research Day, Huntington, West Virginia, April 2009.

Ahmad, T., Miller, R.W., McGuckian, A.B., Conover-Sikorsky, J., Crouse, C.A., and Staton, P.J., "Biomatrica DNA SampleMatrix® – A New Prospect for Forensic DNA Sample Storage," American Academy of Forensic Sciences. Denver, Colorado, February 2009.

HONORS AND AWARDS

Oral Basic Science 1st Place 2016
Health science center 28th annual research day at Marshall University oral presentation winner for "The Significance of CYP2B6 Genetic Polymorphisms in Unexpected Fatalities of Methadone Users in Caucasians of the WV and KY Appalachia Region."

Graduate Student Organization Scholarship 2015
Recognizes individuals who have demonstrated outstanding performance in their program, as well as to the local community. Scholarship amount of $500.

Who's Who Among Students in American Universities and Colleges 2015
Recognizes students with high academic standing and have demonstrated leadership competencies.

LEADERSHIP EXPERIENCES

Marshall University School of Medicine Graduate Student Organization (MUSOM GSO)
President, 2014 – 2015
Member, 2010 – Present

The Sherab Education Project
Volunteer, 2012

Marshall University Forensic Identification Association (MUFIA) Student Organization
Member, 2007 – 2009