COMPARISON OF POLYMORPHIC CYP2D6, CYP2C19 AND CYP2A6 IN CANADIAN NATIVE INDIAN, CAUCASIAN AND CHINESE POPULATIONS

by

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Abstract

A number of human cytochrome P450 enzymes are polymorphically expressed. This results in inter-individual differences in the ability to metabolize substrate drugs. More importantly, they exhibit significant inter-ethnic differences in the frequencies of mutant alleles and frequencies of the poor metabolizer genotype and phenotype. Although P450 enzymes have been studied extensively in Caucasian and Asian populations, very little is known about their genetic variation in Canadian Native Indians.

Mitochondrial DNA studies have shown that Canadian Native Indians are descendants of north Asian populations which crossed the Bering Strait between 13,000 and 30,000 years ago. This agrees with previous evidence gathered from linguistic, dental and morphological studies. We have therefore hypothesized that Canadian Native Indians will resemble Asians, and differ from Caucasians, with respect to the frequencies of CYP2D6, CYP2C19 and CYP2A6 mutant alleles.

We have studied the screened 159 Canadian Native Indian subjects for CYP2C19 (CYP2C19*2 and CYP2C19*3) and CYP2A6 (CYP2A6*2 and CYP2A6*3). These data have been analyzed and compared to Caucasian and Asian data previously reported in literature and data from our own laboratory. Canadian Native Indians were significantly different from both Caucasian and Asian populations with respect to the genotype patterns of both CYP2C19 and CYP2A6.

This study was unique in that it investigated both the CYP2C19 and CYP2A6 polymorphisms in one population, thereby allowing us to determine whether there was any association between the CYP2C19 and CYP2A6 genotypes. Analysis of Canadian Native Indians suggests that there is an association between the presence of the *CYP2C19* and *CYP2A6* mutant alleles such that the co-occurrence of these two alleles is higher than would be predicted based on their frequencies in this population.

These results suggest that Canadian Native Indians do not resemble either Caucasians or Asians with respect to the variant allele frequencies of these polymorphic cytochromes P450. This is most likely due to 1) selective pressures which are unique to North America and 2) Canadian Native Indians' unique evolutionary history.

Acknowledgments

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Introduction

Cytochrome P450 Superfamily

The cytochromes P450 (P450) are a superfamily of enzymes which are involved in the oxidative metabolism of a wide variety of endogenous and exogenous compounds such as drugs, steroids, prostaglandins and pheromones (Nebert and Gonzalez, 1985). These enzymes are present in both eukaryotic and prokaryotic species. The number of P450 genes in any given mammalian species has been estimated to range between 60 and 200 (Nelson *et al.*, 1996).

P450 Protein and Gene Nomenclature

P450 nomenclature is based on amino acid sequence homology. P450 genes with derived amino acid sequence homology of >40% are grouped into the same family, whereas P450 genes with derived amino acid sequence homology of >55% are grouped into the same subfamily (Nelson *et al.*, 1996). To date, 14 mammalian P450 gene families and 26 P450 gene subfamilies have been identified (Nelson *et al.*, 1996).

Cytochrome P450 gene nomenclature consists of: 1) the italicized root "CYP" denoting cytochrome P450, 2) an Arabic numeral representing the P450 family, 3) a letter representing the P450 subfamily and 4) an Arabic numeral representing the actual gene (Nelson *et al.*, 1996). For instance, *CYP2D6* is P450 gene 6 belonging to the D subfamily of family 2.

Human Genome Nomenclature for *CYP2D6* Mutant Alleles

Initially, CYP2D6 mutant alleles were named using an arbitrary system in which a letter following the gene name was used (i.e., *CYP2D6*A). Some alleles were assigned in alphabetical sequence according to the order in which they were discovered. For instance, the first mutant alleles

identified in Caucasian populations were denoted "*CYP2D6*A" and "*CYP2D6*B" (Heim & Meyer, 1990). Others were assigned names based on the functional consequence of the allele. The *CYP2D6*D allele, for example, is responsible for the deletion of the entire CYP2D6 gene (Steen *et al.*, 1995). Finally, a number of CYP2D6 mutant alleles were named according to the populations in which they were discovered. For example *CYP2D6*Ch_1 and *CYP2D6J* were initially detected in Chinese and Japanese populations, while *CYP2D6Z* was found in a Zimbabwean population (Masimirembwa *et al.*, 1995).

The discovery of CYP2D6 variant alleles has progressed at such a rapid rate in recent years that the development of a standardized nomenclature was required. In 1996, Daly *et al.*, proposed such a system which has since been adopted by many researchers in the field. Daly proposed the following:

> "All alleles are termed *CYP2D6* followed by an asterisk. Specific alleles are named by Arabic numerals or a combination of Arabic numerals followed by a capitalized Latin letter. There are no spaces between gene, asterisk and allele and the entire gene-allele symbol is italicized (e.g., *CYP2D6*1*). Since a number of alleles share common key mutations but differ with respect to other base changes, these should be given the same Arabic number (denoting an allele group) and distinguished by capitalized Latin letters (denoting allele subgroups)."

In addition, in order for new alleles to be classified, they should contain mutations which have functional consequences on transcription, splicing, translation, post-transcriptional/ post-translational modifications, or amino acid sequence (Daly *et al.*, 1996). The trivial nomenclature, proposed nomenclature, mutations, effects and enzyme activity for different CYP2D6 alleles are listed in Table 1.1 (modified from Daly *et al.*, 1996).

Table 1.1 ***CYP2D6* Allele Nomenclature.**

Allele	Trivial Name	Causative Mutation	Effect	Activity *in vivo*	Activity *in vitro*
*CYP2D6**1	wild-type	none		normal	normal
*CYP2D6**2	*CYP2D6*L		R296C, S486T	decreased	decreased
*CYP2D6**2XN	*CYP2D6*L2		R296C, S486T	increased	
*CYP2D6**3	*CYP2D6*A	A2637 deletion	frameshift	none	none
*CYP2D6**4A	*CYP2D6*B	G1934A	splicing defect	none	none
*CYP2D6**4B	*CYP2D6*B	G1934A	splicing defect	none	none
*CYP2D6**4C		G1934A	splicing defect		
*CYP2D6**4D		G1934A	splicing defect		
*CYP2D6**5	*CYP2D6*D	*CYP2D6* deleted	*CYP2D6* deleted	none	
*CYP2D6**6A	*CYP2D6*T	T1795 deleted	frameshift	none	
*CYP2D6**6B		T1795 deleted	frameshift	none	
*CYP2D6**7	*CYP2D6*E	A3023C	H324P	none	
*CYP2D6**8	*CYP2D6*G	G1846T	stop codon	none	
*CYP2D6**9	*CYP2D6*C	A2701-3 or G2702-4 deleted	K281 deleted	decreased	decreased
*CYP2D6**10A	*CYP2D6*J	C188T	P34S, S486T	decreased	
*CYP2D6**10B	*CYP2D6*Ch$_1$	C188T	P34S, S486T	decreased	decreased
*CYP2D6**10C	*CYP2D6*Ch$_2$	C188T	P34S, S486T	decreased	decreased
*CYP2D6**11	*CYP2D6*F	G971C	splicing defect	none	
*CYP2D6**12		G212A	G42R, R296C S486T	none	
*CYP2D6**13		2D6/2D7	frameshift	none	
*CYP2D6**14		G1846A	P34S, G169R, R296C, S486T	none	
*CYP2D6**15		T226 insertion		none	
*CYP2D6**16	*CYP2D6*D2	2D6/2D7	frameshift	none	
*CYP2D6**17	*CYP2D6*Z		T107I, R296S, S486T	decreased	decreased

The new nomenclature system for CYP2D6 alleles has also been extended to CYP2C19 and CYP2A6 alleles. The new nomenclature for CYP2A6 and CYP2C19 mutant alleles is listed in Table 1.2 (modified from Daly *et al.*, 1996).

Table 1.2 Mutant Allele Nomenclature for CYP2C19 and CYP2A6.

Allele	Trivial Name	Causative Mutation	Effect	Activity *in vivo*	Activity *in vitro*
*CYP2C19*1*	wild-type	none		normal	normal
*CYP2C19*2*	CYP2C19m$_1$	$G_{681}A$ Exon 5	aberrant splicing	none	none
*CYP2C19*3*	CYP2C19m$_2$	$G_{636}A$	premature stop codon	none	none
*CYP2A6*1*	wild-type	none		normal	normal
*CYP2A6*2*	CYP2A6v$_1$	$L_{160}H$	possible decreased stability	normal or absent	none
*CYP2A6*3*	CYP2A6v$_2$	AA substitutions in exon 3, 6, 8	unknown	none	unknown

Function of P450 Enzymes

One of P450 enzymes' functions is the metabolism of xenobiotics. Many lipophilic compounds, including drugs and environmental pollutants, are metabolized by P450 enzymes whose main role is to make these compounds more hydrophilic, thereby increasing their elimination from the body. This occurs via Phase I or functionalization reactions which involve the addition of a hydrophilic functional group, such as -OH, to lipophilic compounds, thereby increasing their water solubility and increasing their renal elimination (Gonzalez and Nebert, 1990 & 1995).

P450 Enzyme Multiplicity and Overlapping Substrate Specificity

Overlapping substrate specificity is a key feature which makes P450 enzymes highly effective detoxifiers of foreign compounds, a single P450 enzyme can metabolize a number of structurally unrelated compounds while any one chemical can be metabolized by a number of different P450 enzymes. For instance, CYP2D6 is involved in the oxidative metabolism of over 40 commonly used drugs including beta-blockers, tricyclic antidepressants and MAO inhibitors. At the same time, dextromethorphan, which is used as a CYP2D6 probe substrate, can be metabolized by both CYP2D6 and CYP3A. While the V_{max} for dextromethorphan metabolism by both enzymes is comparable, the K_m values differ significantly. K_m for O-demethylation (CYP2D6) was 7 μM while the K_m for N-demethylation (CYP3A) was 650 μM suggesting that CYP3A plays only a minor role in dextromethorphan metabolism in humans (Jacqz-Aigrain *et al.*, 1993). In summary, enzyme multiplicity and overlapping substrate specificity enable the P450 enzyme system to detoxify a large variety of xenobiotics.

Factors Affecting P450 Enzyme Activity

The metabolic activity of P450 proteins may be regulated at various levels (Table 1.3). Deletions, insertion, or substitutions in the coding sequence of the gene may affect the amount of active enzyme present or enzyme function. Mutations in regulatory sequences, defects in transcription or RNA processing may result in variable amounts or quality of mRNA produced. In addition, mRNA stability may have an impact on the amount of enzyme produced. Finally, enzyme induction or inhibition may alter the V_{max} thereby altering the enzyme's ability to metabolize substrates (Meyer *et al.*, 1992).

Table 1.3 Possible Mechanisms Causing a Genetic Deficiency of Drug-metabolizing Enzymes (Meyer *et al.*, 1992).

I.	**DNA/RNA level**
	a. Deletion, insertion, or rearrangement mutation of gene
	b. Defect in transcription, RNA processing, or RNA stability
II.	**At enzyme protein level**
	a. Decreased intracellular concentration or absence of enzyme protein
	• Diminished rate or lack of synthesis
	• Accelerated degradation of labile enzyme variant
	b. Normal intracellular concentration of mutant enzyme protein
III.	**At level of enzyme function**
	a. Decreased affinity for substrates (increased Km)
	b. Decreased maximal velocity (decreased Vmax)
	c. Combination of a. and b.
	d. Change in the stereo-selectivity of the reaction

Role in Animal-plant Warfare

Cytochromes P450 are believed to play an important role in "animal-plant warfare". It has been suggested that as animals began ingesting plants approximately 1200 million years ago, plants responded by evolving to produce chemical defence mechanisms in the form of phytoalexins in order to make themselves less palatable and/or digestible to animals. In turn, animals have developed a battery of xenobiotic metabolizing enzymes to counteract the effects of these plant-derived xenobiotics. Consequences of such co-evolution and plant-animal warfare include polymorphisms of drug metabolizing enzymes (Gonzalez and Nebert, 1990).

P450 Polymorphisms

Although many cytochrome P450 proteins may be induced and/or inhibited by xenobiotics, the P450 enzymes discussed here, CYP2D6, CYP2C19, and CYP2A6, are regulated primarily at the genetic level. Most of the inter-individual and inter-ethnic variation in human CYP2D6, CYP2C19 and perhaps CYP2A6 activities can be accounted for by genetic polymorphisms. According to Vogel and Motusky (1986), a polymorphism is: "a Mendelian or monogenic trait that exists in the population in at least two phenotypes, neither of which are rare - that is neither of which occurs with a frequency of less than 1-2%". Consequently, populations can be divided phenotypically into poor metabolizers (PMs) and extensive metabolizers (EMs) with respect to CYP2D6, CYP2C19 and CYP2A6 based on the ability to metabolize selective P450 substrate drugs (i.e., CYP2D6: dextromethorphan; CYP2A6: coumarin, etc.).

CYP2D6 Polymorphism

The CYP2D6 polymorphism is the most studied P450 human polymorphism. It has been studied at the DNA, mRNA and protein level (Meyer, 1990). Metabolism of CYP2D6 substrates exhibits significant inter-individual variation, ranging from deficient to ultra-rapid (Sachse *et al.*, 1997). Approximately 5-10% of Caucasians are homozygous for defective CYP2D6 alleles. These individuals express an inactive form of the CYP2D6 enzyme or lack the CYP2D6 protein altogether. As a result, their ability to metabolize CYP2D6 substrate drugs such as dextromethorphan or debrisoquine is significantly compromised relative to Caucasian EM individuals. In contrast, some individuals have multiple copies of the CYP2D6 gene which results in ultra-rapid CYP2D6 activity (Akilillu *et al.*, 1996; Johansson *et al.*, 1993).

Inter-ethnic Differences in CYP2D6 Activity

In addition to variations within ethnic groups, CYP2D6 also exhibits significant inter-ethnic differences in populations' mean metabolic activity. Significant inter-ethnic differences exist in CYP2D6 PM frequencies and in mean metabolic activity of CYP2D6 EM individuals. For example, whereas Caucasian populations exhibit PM frequencies in the range of 5 to 10%, Asian and African populations exhibit PM frequencies of approximately 1% (Bertilsson *et al.*, 1992; Lou *et al.*, 1987; Iyun *et al.*, 1986; Lennard *et al.*, 1992; Marinac *et al.*, 1995; Masimirembawa *et al.*, 1993; Simooya *et al.*, 1993; Sommers *et al.*, 1991; Spina *et al.*, 1994; Xu *et al.*, 1990; Yao *et al.*, 1990). Moreover, Asian EM individuals exhibit a lower mean CYP2D6 metabolic activity than do Caucasian EM subjects. This difference in activity appears as a right-ward shift in the frequency distribution of Chinese subjects (Figure 1.1).

Molecular/Genetic Basis of Variation in CYP2D6 Activity

The human CYP2D6 gene is located on the long arm of chromosome 22 in region q13.1 (Gonzalez *et al.*, 1988, Gough *et al*, 1992). CYP2D6 is located in close proximity to two CYP2D pseudogenes, CYP2D7P and CY2D8P, which share a high sequence homology (>90%) with the functional CYP2D6 gene (Heim and Meyer, 1990). These pseudogenes also contain a number of deleterious mutations, some of which are also found in CYP2D6 mutant alleles. Consequently, special care must be taken when genotyping for CYP2D6 alleles not to amplify the CYP2D7P and CYP2D8P pseudogenes in order to avoid inaccurate genotype results.

Inter-individual differences in CYP2D6 activity are caused by one or more mutations in the CYP2D6 gene. CYP2D6 PM individuals lack CYP2D6 activity due to deleterious mutations in both CYP2D6 alleles. Family studies have shown that CYP2D6 PM subjects are homozygous for these

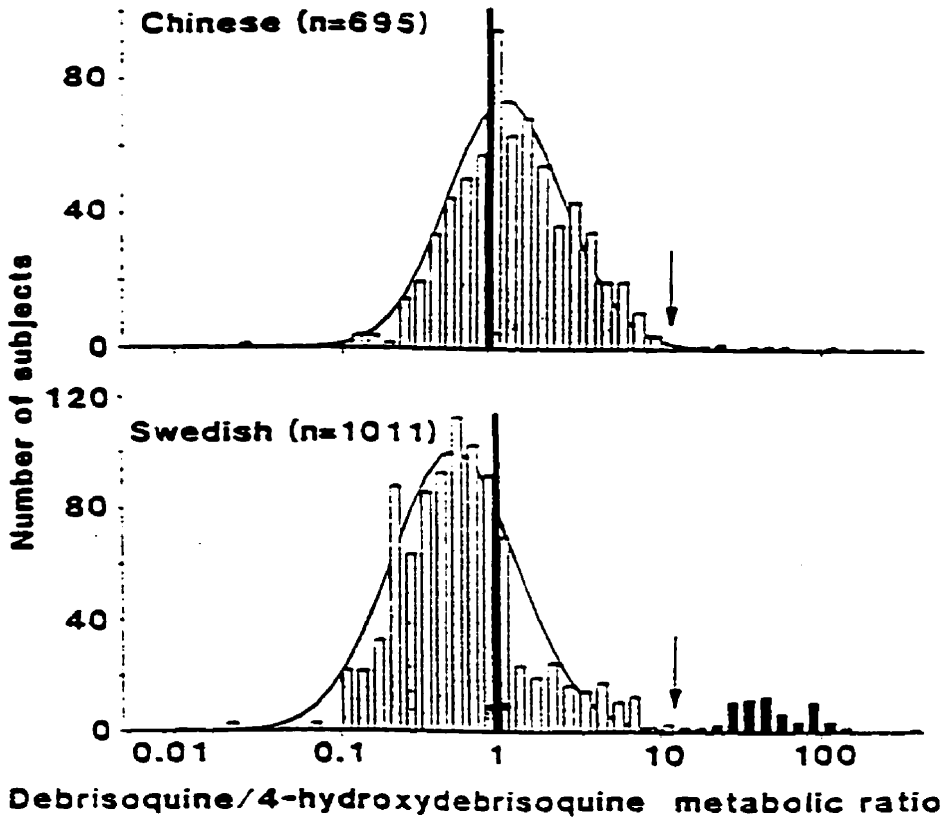

Figure 1.1 **Frequency distributions of the debrisoquine hydroxylation index in Swedish and Chinese populations**. This figure demonstrates the bimodal nature of the frequency distribution. The antimode, which is indicated by the arrow, may be used to classify individuals into CYP2D6 extensive metabolizers (EM) and poor metabolizers (PM).The solid line symbolized a metabolic ratio of 1. Most Chinese subjects have MR ratios greater than 1 while most Caucasian subjects have MR ratios below 1 (Kalow & Bertilsson, 1994).

deleterious mutations whereas EM subjects can be either homozygous or heterozygous for the wild-type allele (Mura *et al.*, 1993; Evans *et al.*, 1980, Steiner *et al.*, 1985). In addition, several CYP2D6 alleles have been shown to be responsible for a CYP2D6 protein with decreased catalytic activity *in vitro* and *in vivo* (Tyndale *et al.*, 1991; Broly & Meyer 1993; Johansson *et al.*, 1994).

*CYP2D6*3* and *CYP2D6*4* Null Mutant Alleles

To date, at least 17 CYP2D6 variant alleles have been identified, 12 of which code for deficient CYP2D6 activity and are therefore termed null mutant alleles (Daly *et al.*, 1996). *CYP2D6*4* (*CYP2D6B*) is by far the most common CYP2D6 null mutant allele in Caucasian populations (20-23%), accounting for approximately 82% of all Caucasian mutant alleles (Broly *et al.*, 1991; Gonzalez and Idle, 1994; Sachse *et al.*, 1997). *CYP2D6*3*, which has an allele frequency of 2%, accounts for roughly 8% of Caucasian *CYP2D6* mutant alleles. Combined, the *CYP2D6*3* and *CYP2D6*4* mutant alleles account for approximately 90% of CYP2D6 poor metabolizers. (Sachse *et al.*, 1997).

*CYP2D6*10* Mutant Allele

In addition to null mutant alleles which, when present in a homozygous state, result in a PM phenotype, there exist CYP2D6 mutant alleles which code for a CYP2D6 protein with decreased metabolic activity (Johansson *et al.*, 1994). The most common of these alleles is *CYP2D6*10* which was previously known as *CYP2D6*Ch_1. The *CYP2D6*Ch_2 and *CYP2D6J* mutant alleles are similar to *CYP2D6*Ch_1 with the exception of exon 9, where they contain additional silent nucleotide changes. The new nomenclature proposed by Daly (1996) classifies these alleles as *CYP2D6**10A, B and C, with the letters denoting silent mutations which are believed to have no further functional

consequences.

While *CYP2D6*4* is the most common mutant allele in Caucasian populations, *CYP2D6*10* is the most common CYP2D6 mutant allele (47-70%) in populations of Asian origin (Lee and Jeyaseelan, 1994; Wang *et al.*, 1993; Armstrong *et al.*, 1994; Dahl *et al.*, 1995). Expression of *CYP2D6*10* genes in COS-1 cells has shown that the causative mutation in this allele is a C to T substitution at position 188 in exon 1. This substitution results in a proline to serine amino acid substitution in a region which is highly conserved among cytochrome P450 gene families 1 and 2 (Ibeanu *et al.*, 1996). The result is a CYP2D6 protein which exhibits lower catalytic activity than the wild-type protein (Johansson, 1994). This decrease in activity has been shown *in vivo*. Figure 1.2 demonstrates the effect of the *CYP2D6*10* (Ch_1) genotype on Japanese subjects' ability to 4-hydroxylate debrisoquine (Lee & Jeyaseelan, 1994).

Inter-ethnic Variation in CYP2D6 Mutant Allele Frequencies

CYP2D6 mutant allele frequencies exhibit significant inter-ethnic variations (Table 1.4). For example, *CYP2D6*4* is the most common mutant allele in Caucasian populations, but is absent in populations of Asian descent. In contrast, *CYP2D6*10*, which is present in Asian populations at frequencies of 47% to 71%, is present in only 5% of Caucasians (Armstrong *et al.*, 1994). As might be expected, these variations in CYP2D6 mutant allele frequencies result in significant inter-ethnic differences in frequency distributions of CYP2D6 activity.

The absence of *CYP2D6*4* in populations of Asian descent is responsible for the relatively low (<1%) prevalence of the CYP2D6 PM phenotype in these populations. However, due to a relatively high *CYP2D6*10* allele frequency, Asian CYP2D6 EM individuals exhibit significantly higher metabolic ratio values for dextromethorphan and debrisoquine metabolites than do Caucasian

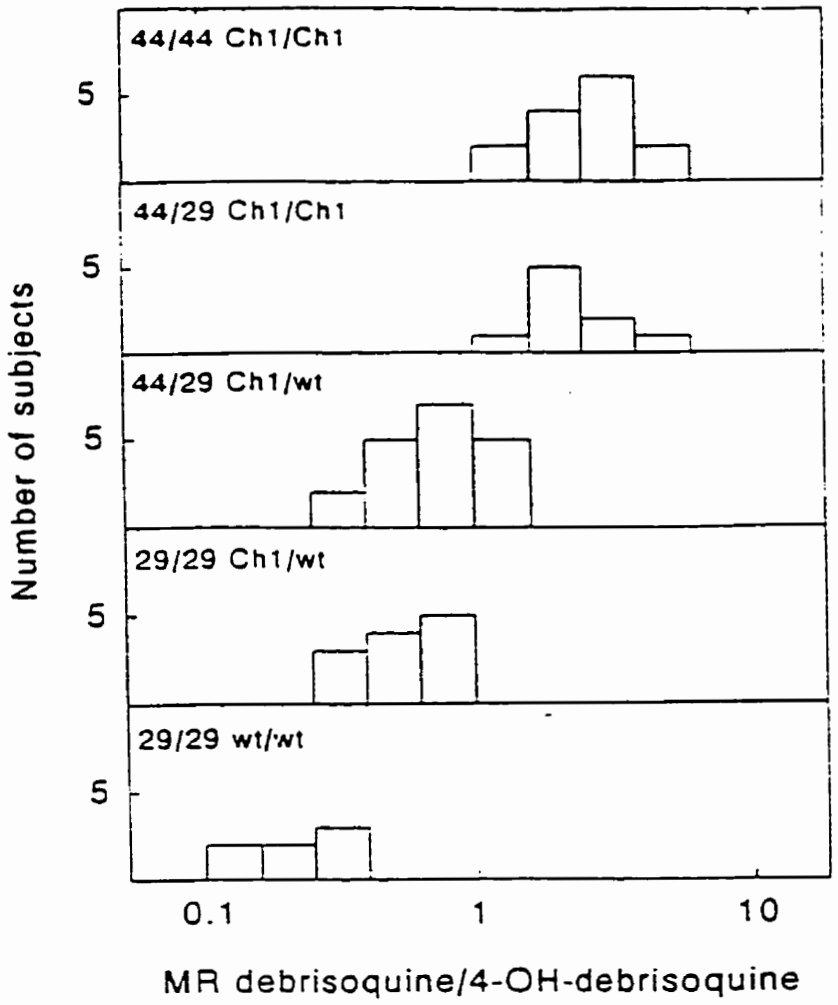

Figure 1.2 **Frequency distributions of debrisoquine metabolic ratio as a function of *CYP2D6*1* (wt) and *CYP2D6*10* (Ch1) alleles**. Presence of the CYP2D6*10 allele results in decreased CYP2D6 mean metabolic activity when compared to subjects carrying the CYP2D6*1 (wt) allele. Homozygous *CYP2D6*10* (Ch1) subjects exhibit higher metabolic ratios than heterozygous subjects, which in turn exhibit higher metabolic ratios values than *CYP2D6*1* (wt) homozygous subjects (Johansson *et al.*, 1994)

EM subjects (Lee and Jeyaseelan, 1994; Wang *et al.*, 1993; Dahl *et al.*, 1995; Armstrong *et al.*, 1994). In contrast, high *CYP2D6*4* allele frequencies (20-23%) in Caucasians result in a Caucasian PM frequency of 7-10%, while low *CYP2D6*10* frequencies result in EM individuals which exhibit lower MR values than do Asian EM subjects.

Although it is the *CYP2D6*4* and *CYP2D6*10* mutant alleles which exhibit the greatest differences in allele frequencies between Caucasian and Asian population, other *CYP2D6* mutant alleles also vary in these and other ethnic groups (Table 1.4.).

Table 1.4 **Inter-ethnic differences in select CYP2D6, CYP2C19 and CYP2A6 mutant allele frequencies (%).**

Enzyme	Mutant Allele	Caucasian	Chinese	Japanese	African
CYP2D6	*CYP2D6*3*	2	0	0	0
	*CYP2D6*4*	20-23	0	2	2.5
	*CYP2D6*5*	5	2	10	4
	*CYP2D6*9*	1	0		0
	*CYP2D6*10*	1.5-5	47-70	43	6
	PM %	**5-10**	**<1**	**2**	**3-6**
CYP2C19	*CYP2C19*2*	13	25-32	23-29	16-25
	*CYP2C19*3*	<1	5-6	10-13	0
	PM %	**1-6**	**14-17**	**14-18**	**2-5**
CYP2A6	*CYP2A6*2*	2-17	0-11	20	0
	*CYP2A6*3*	0-7	6-12	28	2.5
	Allele Derived PM %	**<1-6**	**1-3**	**23**	**<1**

Clinical Consequences of CYP2D6 Polymorphism: Drug Metabolism

CYP2D6 is involved in the oxidative metabolism of over 40 commonly used drugs, which include tricyclic antidepressants, hypnotics, beta-blockers, amphetamines and opioids (Figure 1.3). With such a wide array of CYP2D6 substrate drugs, it is not surprising that differences in CYP2D6 activity have been implicated in a number of potentially dangerous drug responses (Kroemer and Eichelbaum, 1995). The most common complications involving *CYP2D6*-substrate drugs are concentration-dependent toxicity reactions. Such adverse drug reactions (ADRs) are usually observed in CYP2D6 PM patients due to their inability to biotransform the active drug to an inactive metabolite.

Such complications have been observed with a number of clinically used drugs which are CYP2D6 substrates. For example, the anti-arrhythmic propafenone has been shown to reach plasma levels of 6000 ng/ml in CYP2D6 poor metabolizers. This is eight to ten times above the expected plasma levels and is caused by poor metabolizers' inability to carry out CYP2D6-mediated propafenone 5-hydroxylation (Lee *et al.*, 1990; Botsch *et al.*, 1993; Kroemer and Eichelbaum, 1995) This may ultimately result in serious toxicity-related ADRs which include cardiogenic shock, pulmonary edema and arrhythmias (Kroemer and Eichelbaum, 1995).

While certain drugs can reach toxic levels in CYP2D6 PM patients, others may not reach therapeutic concentrations. This occurs in situations where an inactive parent drug is bioactivated by CYP2D6 to an active compound. For example, codeine, which is bioactivated to more active morphine via CYP2D6, provides negligible analgesic benefit to CYP2D6 PM patients who are unable to carry out this CYP2D6-mediated bioactivation (Caraco *et al.*, 1996).

A number of studies have reported CYP2D6-dependent disposition of other clinically used drugs. Among those studied are: maprotiline (Firkunsy & Gleiter, 1994), tramadolol (Pouslen *et al.*,

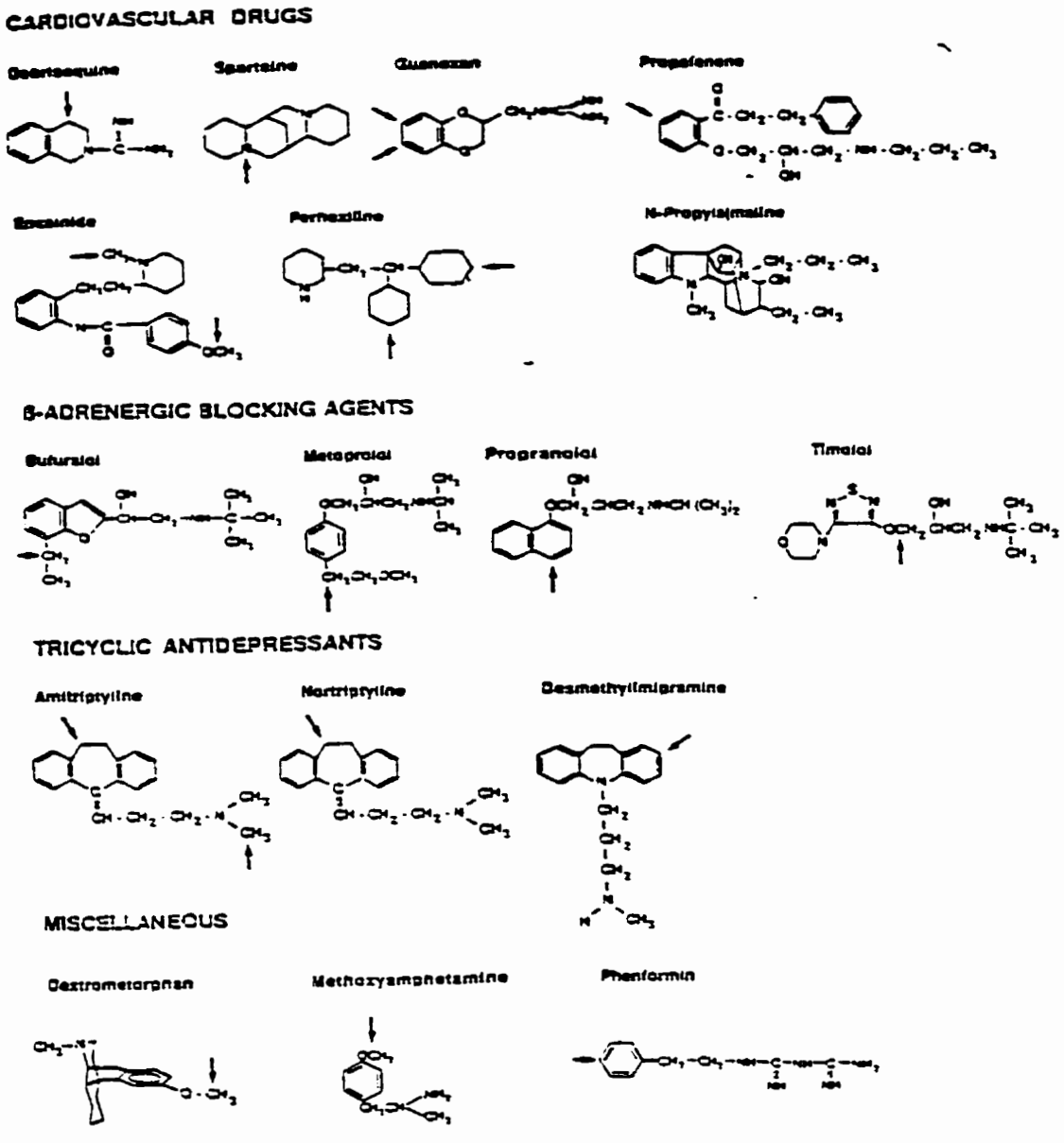

Figure 1.3 **Chemical structures of drugs with deficient metabolism in poor metabolizers of debrisoquine.** The arrow designates the site of oxidation (Meyer *et al.*, 1992).

1994), encainide (Funck-Brentano *et al.*, 1989), propranolol (Lai *et al.*, 1995), hydrocodone (Otton *et al.*, 1993), dihydrocodeine (Fromm *et al.*, 1995), nefazodone (Barbhaiya and Greene, 1996), fluvoxamine (Carrillo *et al.*, 1996), fluoxetine (Hamelin *et al.*, 1996), haloperidol (Llerena *et al*, 1992), metoprolol (Lennard *et al.*, 1982) and desipramine (Table 1.5) (Bertilsson and Aberg-Wistedt, 1982).

Finally, the CYP2D6 polymorphism has also been implicated in the metabolism of drugs of abuse. These include opioids, cocaine and amphetamines (Tyndale *et al.*, in press; Sellers *et al.*, 1994, Wu *et al.*, 1994, Sproule *et al.*, 1994).

Table 1.5 Selected substrates, inhibitors and inducers of CYP2D6.

Substrates		Inhibitors	Inducers
Amitriptyline	Hydrocodone	Ajmalicine	None Known
Citalopram	Imipramine	Chinidin	
Clonipramine	Methoxyamphetamine	Corynanthine	
Clozapine	Methoxyphenamine	Fluoxetine	
Codeine	Metoprolol	Lobelin	
Desipramine	Mexiletene	Propidin	
Dextromethorphan	Nortriptyline	Quinidine	
Encainide	Paroxetine	Trifluperidol	
Flecainine	Perhexilene	Yohimbine	
Fluoxetine	Perphenazine		
Flunarizine	Propafenone		
Haloperidol	Propranolol		

Drug Interactions

Yet another clinical complication which may arise is a drug interaction. This occurs when

patients are given two drugs, both of which are CYP2D6 substrates. The drug with higher affinity for CYP2D6 occupies the enzyme, thereby significantly decreasing or attenuating biotransformation of the second drug. This results in drug accumulation and toxicity-related ADRs (Belpaire and Bogaert, 1996). Alternatively, if both drugs have similar affinities for CYP2D6, patients may experience toxicity symptoms of both drugs (Figure 1.4; Nielsen *et al.*, 1990)

In effect, CYP2D6 EM patients who are taking two CYP2D6-substrate drugs may be prone to the same problems as CYP2D6 PM patients, namely toxicity symptoms, because they may resemble PM patients with respect to CYP2D6 phenotype as a result of competitive inhibition. This phenomenon is referred to as phenocopying (Figure 1.4) (Nielsen *et al.*, 1990, from Caraco, 1996).

CYP2D6 Activity as a Factor in Disease Susceptibility

In recent years, several studies have associated CYP2D6 phenotype and/or genotype status with disease (Table 1.6). These include Parkinson's disease (PD) (Akhmedova *et al.*, 1995; Tsuneoka *et al.*, 1993; Smith *et al.*, 1992; Armstrong *et al.*, 1992; Iwahashi, 1994; Kondo & Kanazawa, 1993; Kurth & Kurth, 1993; Benitez *et al.*, 1990; Barbeau *et al.*, 1985; Poirier *et al.*, 1987; Fonne-Pfister *et al.*, 1988), Alzheimer's disease (AD) (Chen *et al.*, 1995), various forms of cancer (Ayesh *et al.*, 1984, Ayesh & Idle, 1985, Caesar *et al*, 1987, Roots *et al.*, 1988; Hirvonen *et al.*, 1993; Agundez *et al.*, 1996; Legrand *et al.*, 1996; Speirs *et al.*, 1990), epilepsy (Borlak *et al.*, 1994), systemic lupus erythematosus (Baer *et al.*, 1986), and Balkan nephropathy (Ritchie *et al.*, 1983; Nikolov *et al*, 1991). Whether CYP2D6 plays a functional role, such as bioactivation of procarcinogens to carcinogens, or whether it is in genetic linkage with a proto-oncogene is still unknown (Meyer *et al.*, 1992). As an example, the PM phenotype has been associated with an increased susceptibility to PD. Recently, a mechanism by which this may occur has been proposed. Laboratory results have shown

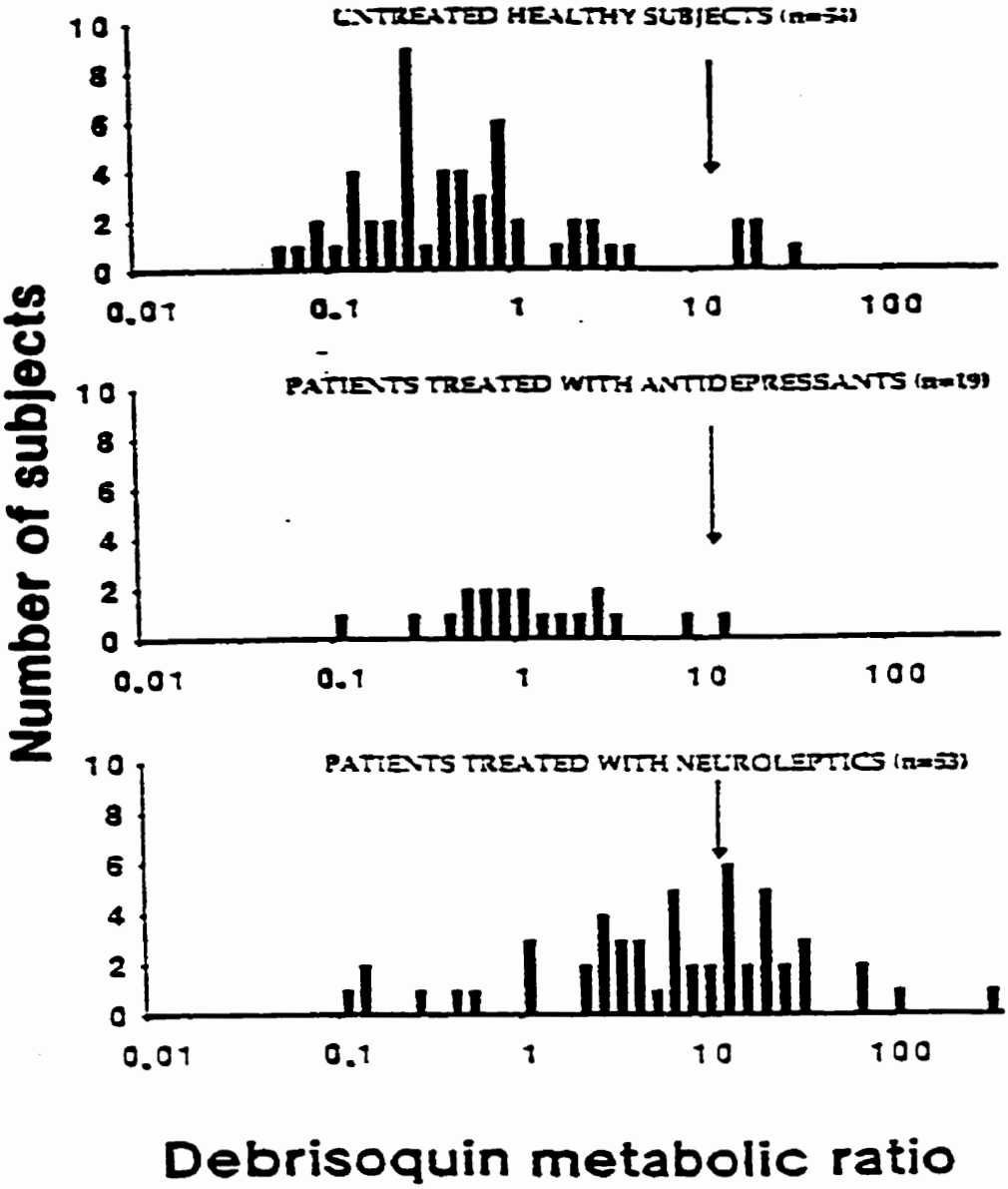

Figure 1.4 **Comparison of frequency distributions of debrisoquine metabolic ratio between untreated patients and patients treated with antidepressants or neuroleptics.** The frequency distributions of the patients treated with antidepressants and neuroleptics is shifted to the right relative to the untreated patients suggesting that CYP2D6 is inhibited by these drugs. In the case of neuroleptics, a large proportion of patients have been phenotyped as CYP2D6 poor metabolizers due to competitive inhibition of the CYP2D6 protein. The arrow indicates a debrisoquine metabolic ratio of 12.6 which is used as the antimode to distinguish between extensive and poor metabolizers (Nielsen *et al.*, 1990).

Table 1.6 ***CYP2D6*-associated Disease Susceptibility.**

Phenotype	Condition	Risk	References
CYP2D6 PM	Systemic lupus erythmatosus	increased	Baer *et al.*, 1986
	Epilepsy	increased	Brolak *et al.*, 1994
	Alzheimer's Disease	increased no effect	Chen *et al.*, 1995; Benitez *et al.*, 1993; Liu *et al*, 1992
	Parkinson's Disease	increased	Barbeau *et al.*, 1988 Akhmedova *et al.*, 1995; Tsuneoka *et al.*, 1993; Smith *et al.*, 1992; Armstrong *et al.*, 1992; Iwahashi, 1994; Kondo & Kanazawa, 1993; Kurth & Kurth, 1993; Benitez *et al.*, 1990; Barbeau *et al.*, 1985; Poirier *et al.*, 1987;
		no effect	Plante-Bordeneuve *et al.*, 1994; Gudjonsson *et al.*, 1990; Steiger *et al.*, 1991; Liu *et al.*, 1992
CYP2D6 EM	Lung Cancer	increased	Hirvonen *et al.*, 1993; Roots *et al.*, 1988
	Bladder Cancer	increased	Romkes *et al.*, 1996
	Hepatocellular Carcinoma	increased	Agundez *et al.*, 1996 Speirs *et al.*, 1990
		no effect	Legrand *et al.*, 1996
	Colon Cancer	increased	
	Balkan nephropathy	increased	Nikolov *et al.*, 1991 Ritchie *et al.*, 1983

that a PD-like syndrome can be caused by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). MPTP is bioactivated by monoamine oxdiase B to a neurotoxic metabolite, 1-methyl-4-phenylpyridine (MPP^+). CYP2D6 activity is believed to provide some neuroprotection against this toxicity as it is involved in the N-demethylation of MPP+. This reaction inactivates the neurotoxin and facilitates its' removal from the CNS (Coleman *et al.*, 1996). Since CYP2D6 has been implicated in the detoxification pathway of MPP+, CYP2D6 PM subjects are believed to have an increased risk of developing Parkinson's due to a deficiency in the detoxification pathway (Plante-Bordeneuve *et al.*, 1994; Steiger *et al.*, 1992). At the same time, CYP2D6 PM subjects are believed to have a decreased risk of developing certain types of cancer. A number of studies have shown a significantly lower prevalence of the CYP2D6 PM phenotype in cancer patients than in control subjects (Agundez *et al.*, 1996; Roots *et al.*, 1988; Hirvonen *et al.*, 1993). This suggests that the PM phenotype may be a protective factor against some forms of cancer due to the fact that PM subjects do not activate procarcinogens to the same extent that EM subjects do (Caporaso *et al.*, 1995).

CYP2C19 Polymorphism

As with CYP2D6, human populations can be phenotypically divided into CYP2C19 EM and PM subjects based on the ability to metabolize CYP2C19-substrate drugs such as mephenytoin (Kupfer and Preisig, 1984). Mephenytoin is an anticonvulsant drug which is given in a racemic mixture. The R-enantiomer is N-demethylated, while the S-enantiomer is rapidly 4-hydroxylated. The CYP2C19 polymorphism was originally identified when inter-individual differences were observed in the mephenytoin S/R ratio (Kupfer, 1981). This was eventually attributed to a difference in the 4-hydroxylation of S-mephenytoin which is mediated by CYP2C19.

Inter-ethnic differences in CYP2C19 Activity

CYP2C19 has been studied in populations of varying ethnic backgrounds. As with CYP2D6, there are significant inter-ethnic differences in CYP2C19 activity. Approximately 1% to 5% of Caucasians are poor metabolizers of S-mephenytoin (Bertilsson 1992; Drohse, 1989; Inaba, 1984; Jacqz, 1989; Nakamura, 1985; Pollock, 1991; Riviriego, 1993; Wedlund, 1989). In contrast, Asian populations exhibit PM frequencies between 15% and 23% (Xie *et al*, 1996; Bertilsson 1992, Horai, 1989; Kubota, 1996; Nakamura, 1985; Sohn, 1992; Takakubo, 1996).

CYP2C19 Genotype: *CYP2C19*2* and CYP219*3 Mutant Alleles

The CYP2C19 PM phenotype is inherited as an autosomal recessive trait (Kupfer & Preisig, 1984; Inaba *et al.*, 1986). CYP2C19 PM individuals are homozygous for two mutant alleles which result in no active protein being expressed, while CYP2C19 EM individuals are either homozygous or heterozygous for the *CYP2C19*1* (wild-type) allele. Two CYP2C19 mutant alleles, which are responsible for the PM phenotype, have been described (deMorais *et al.*, 1994a; 1994b). Additional null mutant alleles, which are present at very low frequencies, have been identified, however, these data have not yet been published.

*CYP2C19*2* contains a $G_{681}A$ substitution in exon 5 of the CYP2C19 gene. This nucleotide substitution results in the creation of an aberrant splice site which shifts the reading frame of the mRNA and produces a premature stop codon 20 amino acids downstream of the mutation. The result is a truncated, non-functional 234 amino acid CYP2C19 protein which lacks the heme-binding region (de Morais *et al.*, 1994a). The *CYP2C19*2* mutant allele accounts for 83% of Caucasian CYP2C19 PMs and 75% of Japanese CYP2C19 PM alleles (deMorais *et al.*, 1994a). This suggests that additional CYP2C19 null mutant alleles exist.

A second CYP2C19 mutant allele, called *CYP2C19*3*, contains a $G_{636}A$ substitution mutation in exon 4 of the CYP2C19 gene. This mutation creates a premature stop codon which results in the formation of a truncated and inactive protein (deMorais *et al.*, 1994b). Although *CYP2C19*3* accounts for the remaining 25% of Japanese CYP2C19 PM alleles not accounted for by *CYP2C19*2*, it has not been found in Caucasian populations (de Morais *et al.*, 1994b). This evidence suggests, that there exist additional CYP2C19 mutant alleles which account for the remaining 17% of Caucasian CYP2C19 PM alleles which are not explained by *CYP2C19*2* or *CYP2C19*3*. Presumably, some of these individuals can be explained by the recently identified CYP2C19 null mutant alleles.

Clinical Consequences of CYP2C19 Polymorphism

CYP2C19 is involved in the metabolism of a wide variety of drugs including diazepam (Caraco *et al.*, 1995), omeprazole (Chang *et al.*, 1995: Caraco *et al.*, 1995; Caraco *et al.*, 1996), hexobarbital (Kato *et al.*, 1992) and proguanil (Ward *et al.*, 1991) (Figure 1.5, Table 1.7). For example, significant inter-individual and inter-ethnic differences exist in the clearance of diazepam. CYP2C19 EM individuals exhibit diazepam plasma clearance twice that of CYP2C19 PM individuals (Bertilsson *et al.*, 1989).

In addition, due to the higher proportion of heterozygous individual in Asian populations, significant differences in diazepam clearance have been observed between EM individuals of Caucasian and Asian origin (Ghoneim *et al.*, 1981). In fact, many Hong Kong physicians have been reported to prescribe lower doses of diazepam for Chinese than for Caucasian patients (Kumana *et al.*, 1987).

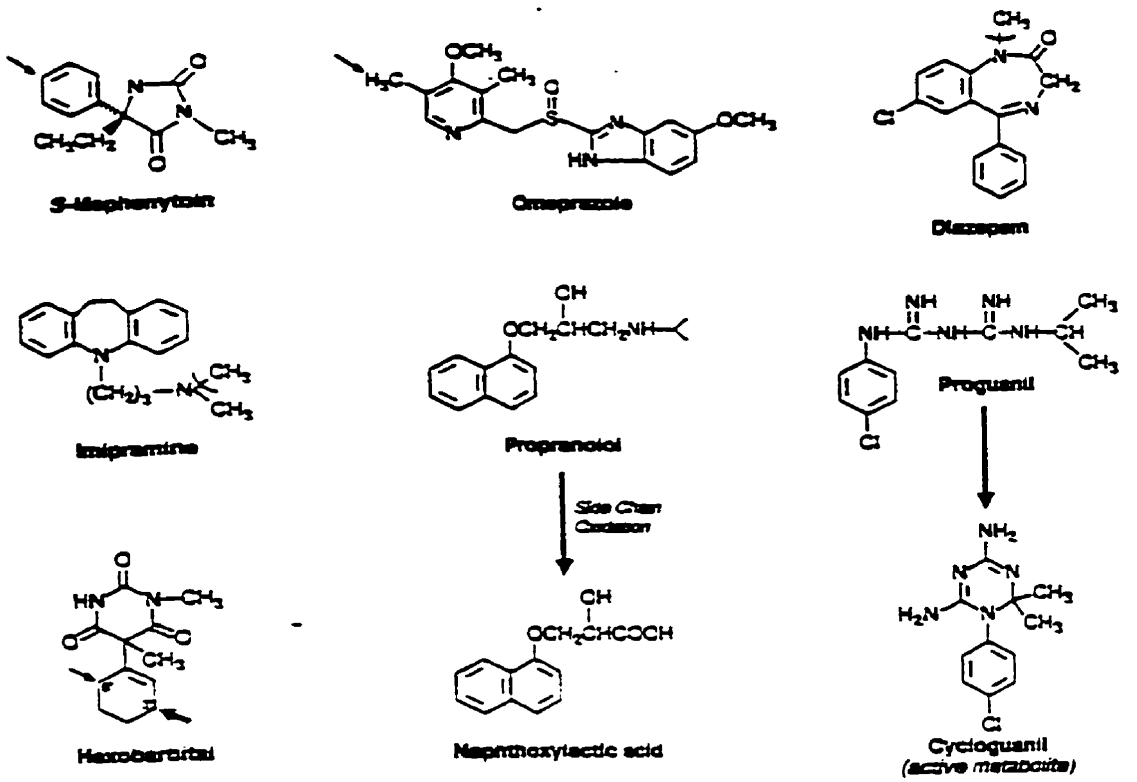

Figure 1.5 **Structures of substrates metabolized by CYP2C19**. Sites of metabolism are indicated with arrows (hydroxylation) or curved lines (demethylation) (Goldstein and de Morais, 1994).

Table 1.7 **Drugs Metabolized by CYP2C19** (Daniel and Edeki, 1996).

Hydroxylation Pathway	N-Demethylation Pathway
(S)-mephenytoin	(R)-mephenytoin
desmethyldiazepam	diazepam
(R)-mephobarbital	amitriptyline
(-)-hexobarbital	imipramine
nirvanol	clomipramine
moclobemide	citalopram

In addition, proguanil, which is bioactivated to cycloguanil is less effective as an antimalarial drug in PM individuals than in EM individuals due to decreased bioactivation via CYP2C19 (Ward *et al.*, 1991). It has been suggested that this may mean that the drug is less effective in Asians than in Caucasians due to the higher proportion of heterozygotes and thus, lower enzyme activity in the former population (Bertilsson, 1995).

CYP2C19 Inhibition and Induction

Non-genetic factors, such as concomitant use of certain drugs, may be responsible for altering CYP2C19 phenotype. Presence of CYP2C19 substrate drugs, such as omeprazole, may affect phenotyping results due to competitive inhibition of the CYP2C19 protein. In addition, certain disease states, such as acute hepatitis and cirrhosis, have been reported to decrease CYP2C19 enzymatic activity (Breimer *et al.*, 1975; Zilly *et al.*, 1978) Finally, there have been reports of age being a factor in CYP2C19 activity, with older patients exhibiting decreased activity (Wan *et al.*, 1997).

CYP2A6 Polymorphism

Recently, inter-individual variations in CYP2A6 activity have been observed (Rautio *et al.*, 1992). Work on CYP2A6 began in the mid-1980's when Pelkonen reported the presence of a new phenobarbital-inducible enzyme in human, mouse, rabbit and guinea pig liver microsomes. This enzyme, which was responsible for the hydroxylation of coumarin (1,2-benzopyrone), was termed coumarin hydroxylase (COH) (Pelkonen *et al.*, 1985; Raunio *et al.*, 1988).

At about the same time, a new human P450 was isolated and sequenced using a rat CYP2A1 cDNA probe. This new P450, which was termed CYP2A3 (now CYP2A6), exhibited high deduced amino acid sequence homology with rat CYP2A3 and mouse CYP2A3 (Yamano, 1989). Within a year, the CYP2A3 protein was expressed in Hep G2 cells, and it was reported that the enzyme was able to carry out coumarin 7-hydroxylation.

Studies of CYP2A in human liver microsomes identified a 50 kDa protein which showed a strong correlation between the amount of protein and coumarin 7-hydroxylation activity and between the amount of CYP2A3 mRNA and coumarin 7-hydroxylation (Yamano, 1990). In addition, the rat CYP2A antibody completely abolished coumarin 7-hydroxylation activity in human liver microsomes. These findings, combined with further kinetic investigation, established that CYP2A3 was the primary enzyme responsible for coumarin 7-hydroxylation in humans and that CYP2A3 and COH were in fact one and the same enzyme (Yamano et al., 1990).

While screening a liver bank for CYP2A3 activity, Yamano reported up to a 40-fold variation in enzyme activity and significant differences in mRNA levels between liver samples. Most importantly, three specimens were found to have no detectable CYP2A3 mRNA. Maurice (1991)

quantified the new CYP2A protein, now termed CYP2A6, in human liver samples and found that the concentration of CYP2A6 in human liver samples varied up to 144 fold and accounted for approximately 10% of total P450 content (Maurice *et al.*, 1991).

Two *in vivo* studies, using coumarin as a CYP2A6 substrate drug, have shown that frequency distributions of 7-hydroxycoumarin urinary excretion do not exhibit a bimodal distribution, which is indicative of a genetic polymorphism (Rautio *et al*, 1992; Iscan *et al.*, 1994). However, a small number of individuals in both studies exhibited urinary 7-hydroxycoumarin excretion well below the mean suggesting that these individuals may be heterozygous for a null or decreased activity CYP2A6 allele (Figure 1.6).

Genetic Basis for Variation in CYP2A6 Activity

To investigate the possibility of a genetic polymorphism, the CYP2A6 gene locus was characterized (Yamano *et al.*, 1990; Bale *et al*, 1990; Hoffman *et al.*, 1995). CYP2A6 is localized on human chromosome 19q13.2 in a tight 350kb cluster containing members of the CYP2A, CYP2B and CYP2F families. CYP2A6 is in close proximity to CYP2A7 and CYP2A13 which are highly homologous to CYP2A6 with respect to nucleotide sequence.

CYP2A6 Mutant Alleles

To date, there are two CYP2A6 mutant alleles which are believed to be responsible for decreased CYP2A6 activity. *CYP2A6*2* was the first CYP2A6 mutant allele identified (Yamano *et al.*, 1990). It consists of a single amino acid substitution which results in a $L_{160}H$ amino acid substitution. Expression of this protein in Hep G2 cells resulted in a protein lacking coumarin 7-hydroxylation activity. A population study by Cholerton and Idle revealed a *CYP2A6*2* mutant allele

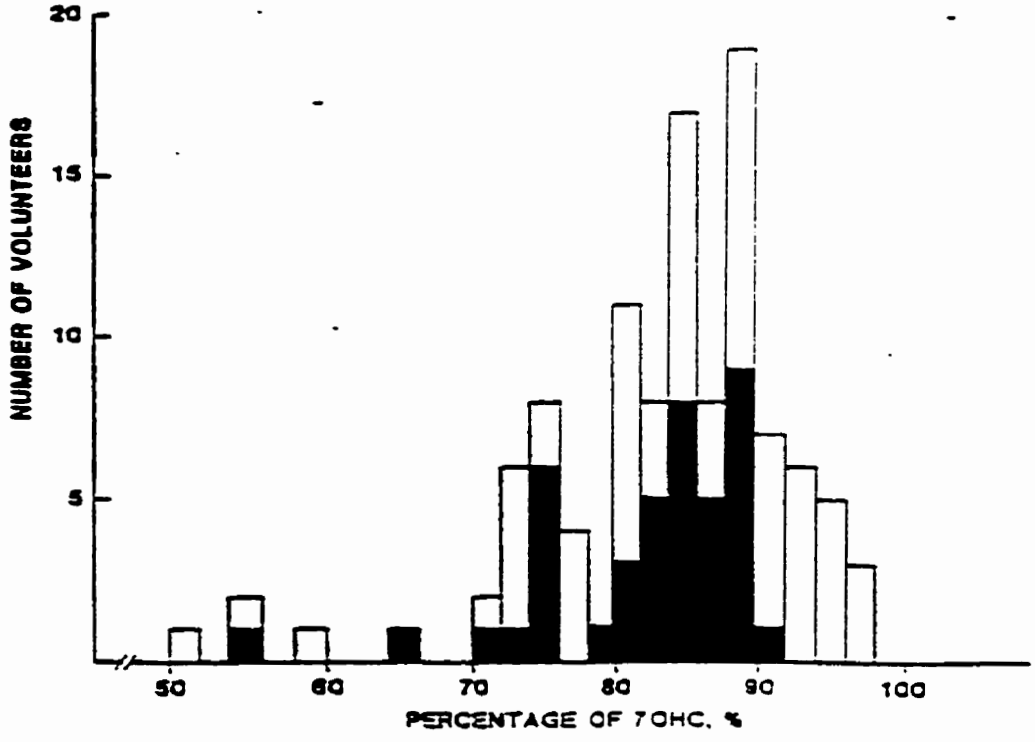

Figure 1.6 **Frequency distribution of percentage of coumarin dose excreted as 7-hydroxycoumarin metabolite**. Although statistical analysis revealed no antimode, which would be indicative of a bimodal distribution, several individuals display excretion of 7-hydroxycoumarin which is well below the mean. The black and white bars denote males and females, respectively (Cholerton *et al.*, 1992).

frequency of 1% (Fernandez-Salguero and Gonzalez, 1995). However, the *CYP2A6*2* mutant allele did not explain all low activity individuals. A subsequent study reported *CYP2A6*2* allele frequencies ranging from 0% in African-American to 20% in Japanese populations (Fernandez-Salguero *et al.*, 1995).

A second CYP2A6 mutant allele, *CYP2A6*3*, which is characterized by a mutation in exons 3, 6 and 8 was also identified (Fernandez-Salguero *et al.*, 1995). This allele was found at frequencies ranging from 0% to 28% in various ethnic groups (Table 1.4).

Following phenotyping for CYP2A6 activity and genotyping for *CYP2A6*2* and *CYP2A6*3*, Fernandez-Salguero (1995) found that there was a lack of concordance between phenotype and genotype. Although three subjects who were deficient in coumarin 7-hydroxylation were genotyped as *CYP2D6**2 homozygotes, there were also three *CYP2D6**2/*CYP2D6**2 individuals who exhibited coumarin metabolism similar to that of *CYP2D6**1/*CYP2D6**2 individuals. To date, the role of the *CYP2A6*2* and *CYP2A6*3* mutant alleles is not clearly understood. Despite earlier results, it is still not clear whether a CYP2A6*2/CYP2A6*2 genotype results in a CYP2A6 PM phenotype. Reliable phenotype and genotype data on CYP2A6 are still lacking as only small populations have been studied. Although initial studies do show significant inter-individual and inter-ethnic differences in both CYP2A6 mutant allele frequencies and enzyme activity, larger populations need to be studied to rule out or confirm the presence of a genetic CYP2A6 polymorphism (Table 1.4; Fernandez-Salguero *et al.*, 1995; Shimada *et al.*, 1994; Shimada *et al.*, 1996).

CYP2A6 Plays a Role in Nicotine Metabolism in Humans

P450 enzymes were initially implicated in nicotine metabolism in the early 1980s (Nakayama *et al.*, 1982). These findings have been confirmed in more recent studies with the help of cDNA

expressed human P450s. Of the 12 P450 enzymes expressed *in vitro*, CYP2B6 exhibited the highest rate of nicotine metabolism (Flammang *et al.*, 1992). However, since CYP2B6 accounts for less than 1% of total P450 content in the liver, it is not believed to play a major role in nicotine metabolism *in vivo*. Subsequent studies using human liver microsomes have shown that CYP2A6 protein content strongly correlates with nicotine to cotinine biotransformation (Berkman *et al.*, 1995; Nakajima *et al.*, 1996; Messina *et al.*, in press). The nicotine to cotinine pathway accounts for approximately 70-80% of nicotine metabolism in humans (Nakajima *et al.*, 1996). Given that nicotine is the reinforcing stimulus in tobacco, it is possible that CYP2A6 activity may play an important role in regulating tobacco smoking behaviour (Gfroerer, 1995). Individuals with low CYP2A6 activity may not be able to eliminate nicotine from the body as quickly as individuals with high CYP2A6 activity. As a result, individuals with low CYP2A6 activity may experience more negative effects following their first cigarette and may be less likely to continue smoking. In addition, smokers who exhibit low CYP2A6 activity may smoke fewer cigarettes per day, or smoke cigarettes with lower nicotine content due to decreased nicotine to cotinine biotransformation thereby lowering their exposure to toxic chemicals present in cigarette smoke.

Clinical Consequences of CYP2A6 Activity

CYP2A6 has also been implicated in the bioactivation of a number of carcinogens, suggesting that inter-individual differences in CYP2A6 activity may be associated with cancer risk (Fernadez-Salguero, 1995, Liu *et al.*, 1996; Yamazaki *et al.*, 1992: Pelkonen & Raunio, 1995). One group of compounds which has been shown to be bioactivated to carcinogens are nitrosamines. Nitrosamines are bioactivated to reactive alkyldiazohydroxides via the CYP2A6 pathway (Yamazaki *et al.*, 1992). Given that nitrosamines are present in tobacco smoke, it is possible that smokers with high CYP2A6

activity may be at significantly increased risk of developing tobacco-related cancers compared to smokers with decreased or normal CYP2A6 activity. This may be due to their increased ability to bioactivate nitrosamines to genotoxic products and due to increased exposure to tobacco which may be a consequence of faster nicotine metabolism. In effect, these two mechanisms may have an additive or synergistic effect in determining cancer risk in smokers with high CYP2A6 activity.

Non-Genetic Factors Affect CYP2A6 Activity

CYP2A6 activity, as determined by 7-hydroxylation of coumarin, has been shown to be significantly decreased in volunteers drinking grapefruit juice (Merkel *et al.*, 1993). Although the mechanism of inhibition is poorly understood, it is believed that two flavonoids, naringin and aglycon naringenin, are responsible for the decrease in coumarin 7-hydroxylation (Merkel *et al.*, 1993). Decreased CYP2A6 activity has also been associated with liver disease, whereas increased CYP2A6 activity has been associated with lignocaine, an anti-epileptic agent (Sotaniemi *et al.*, 1995). To date, no mechanism for this has been postulated.

Evolutionary Origins of Canadian Native Indians

The evolutionary origins of modern North American Indian populations have been the focus of numerous debates in recent years. It is currently believed that today's North American Indian peoples are descendants of Asian populations which migrated to North America across the Bering Strait between Siberia and Alaska (Meltzer, 1993). This migration occurred between 13,000 and 30,000 years ago (Schanfield, 1992; Zegura, 1987; Weiss, 1994; Torroni *et al.*, 1994; Cann, 1994).

Today, native North American peoples are divided into three linguistic groups which consist of the Amerindians of North, Central, and South America, the Na-dene of northern North America

and the Aleut-Eskimo of the far north (Wallace & Torroni, 1992). Most human geneticists attribute the linguistics differences to independent migrations. This is in agreement with the Greenberg theory which states that the Amerindian, the Na-dene and the Aleut-Eskimo arrived in North America in three separate migratory waves (Figure 1.7). The three groups exhibited significant variations in language, morphology and genetic markers prior to the migrations, giving rise to the heterogenous groups of today (Greenberg, 1986).

Greenberg's hypothesis is supported by evidence gathered in various research disciplines including anthropology, archaeology, linguistics, taxonomy, and most recently, genetics. Early genetic research has focused on studies of blood groups markers. However, most recently, mitochondrial DNA (mtDNA) analysis has emerged as an ideal tool for such studies.

Mitochondrial DNA is well suited to determining ethnic and geographic origins of populations for a number of reasons. First, mtDNA mutations accumulate along radiating maternal lineages. Second, since mtDNA is inherited in the maternal line, mutation is the only source of variation. Third, all human mtDNAs are components of a single mtDNA lineage, indicating a single origin. Finally, mtDNA variation correlates highly with the ethnic and geographic origins of the samples studied.

In summary, although recent genetic studies have challenged Greenberg's theory, Greenberg's explanation for Amerindian heterogeneity is still the most accepted among human geneticists. For the purposes of this discussion, the recent controversy is academic as the most important point is that mtDNA and morphological, linguistic and dental data suggest that Canadian Native Indians are descendants of ancient Asian populations.

Figure 1.7 **Hypothesized waves of migration of original North American populations.** According to Greenberg's hypothesis, modern Na-Dene, Amerind and Eskimo-Aleut are descendants of Asian population which crossed the Bering Strait in three separate migrations (Science 274:31-32, 1996).

Hypotheses

There is ample evidence suggesting that the Canadian Native Indian peoples are unique descendants of North Asian populations. Consequently, we hypothesize that the Canadian Native Indian population will resemble Asian populations and differ from Caucasian populations with respect to the CYP2D6, CYP2C19 and CYP2A6 polymorphisms. The specific hypotheses are as follows:

CYP2D6 Genotype

i. The frequencies of the *CYP2D6*3* and *CYP2D6*4* mutant alleles are significantly lower in the Canadian Native Indians than in Caucasians and not significantly different from Asians.

ii. The frequency of the *CYP2D6*10* mutant allele, which results in decreased CYP2D6 activity, is significantly higher among Canadian Native Indians than among Caucasians but is not significantly different from Asians.

CYP2D6 Phenotype

i. The frequency of the poor metabolizer phenotype is significantly lower in Canadian Native Indians than in Caucasians and not significantly different from Asians. This is due to the relatively low frequencies of the *CYP2D6*3* and *CYP2D6*4* mutant alleles in the Canadian Native Indian population.

ii. The frequency distribution of the Canadian Native Indian O-demethylation ratio

(ODMR) for dextromethorphan is shifted to the right relative to the Caucasian ODMR frequency distribution, thereby resembling Asian populations. This is due to the relatively high prevalence of the *CYP2D6*10* mutant allele which encodes a CYP2D6 protein with decreased catalytic activity.

CYP2C19 Genotype

i. The frequency of the *CYP2C19*2* mutant allele is significantly higher in Canadian Native Indians than in previously studied Caucasians and not significantly different from Asians.

ii. The *CYP2C19*3* mutant allele in Canadian Native Indians is present at frequencies similar to those found in Asian populations and significantly higher than observed in previously studied Caucasian populations.

CYP2A6 Genotype

i. The frequency of the *CYP2A6*2* mutant allele in Canadian Native Indian subjects is not significantly different from Caucasian or Asian populations.

ii. The frequency of the *CYP2A6*3* mutant allele in Canadian Native Indians is significantly different from Caucasians but not significantly different from Asians.

Materials and Methods

Subjects

Ethics Approval

All study protocols were approved by the Addiction Research Foundation Ethics Review Committee.

Sample Size Determination

Before subjects were recruited, it was important to calculate the sample size required to detect a significant difference between Canadian Native Indian, Caucasian, and Chinese mutant allele frequencies. The sample size calculations for this study were determined using Easystat software which performs sample size calculations based on; estimated rate of characteristic in control group, expected change of characteristic in study group, size of control group, desired power of the test, desired significance level, etc.

Sample size determination was based on the prevalence of the CYP2D6 PM phenotype and the allelic frequencies of the *CYP2D6*3*, *CYP2D6*4* and *CYP2D6*10* mutant alleles in previously studied Caucasian populations and the expected frequencies in the Canadian Native Indian population.

At a significance level of α=0.05, power of 1-β =0.8, it was determined that samples of approximately 180 Canadian Native Indian subjects were required to perform a statistical comparison between CYP2D6 PM phenotype frequencies and the allelic frequencies of CYP2D6 mutant alleles; *CYP2D6*3*, *CYP2D6*4* and *CYP2D6*10*. The hypothesized allele frequencies in Canadian Native Indians were based on previously studied Asian populations were 0%, 0% and 70% for the *CYP2D6*3*, *4 and *10 mutant alleles, respectively.

Caucasian (n=355) and Chinese (n=85) subjects, which were recruited for previous studies in our laboratory were used as control groups for the CYP2D6 phenotyping part of this study. All subjects had been previously classified into CYP2D6 PMs and CYP2D6 EMs according to their dextromethorphan ODMR values. Genotype results were not available for these subjects as blood samples were not collected at the time of the previous recruitments. However, blood samples and DNA were available for 70 recently recruited Chinese subjects who were genotyped for CYP2D6, CYP2C19, and CYP2A6 mutant alleles. In addition, Caucasian subjects were genotyped for CYP2A6 mutant alleles (n=270) and the *CYP2D6*3* and *CYP2D6*4* mutant alleles (n=276).

Subject Recruitment

Subjects were recruited in the Metropolitan Toronto area through the Mental Health Unit of the Addiction Research Foundation (A.R.F.). A.R.F. Ethics Review Committee-approved posters and newspaper advertisements were utilized in the recruitment of Canadian Native Indian participants (Appendix 1). A significant number of study participants were also recruited through word of mouth at Native community centres in the Toronto area.

Inclusion Criteria

All subjects were required to meet the following criteria before being accepted into the study:

i. Must have at least one grandparent of Canadian Native Indian descent.

ii. Healthy male or female.

iii. 16 to 70 years of age.

iv. Willingness to sign the Consent Form and abide by the rules of the study.

Exclusion Criteria

Subjects were excluded from the study if they had:

i. Known sensitivity to dextromethorphan.

ii. Medical or psychiatric reasons that require further investigation or treatment and would preclude participation (eg. patients who were taking medications which are known CYP2D6 substrates, ie. fluoxetine).

iii. No Canadian Native Indian ancestry.

Interview

All study participants took part in a brief interview prior to blood and urine sample collection. Most subjects were interviewed at the Mental Health Unit of the Addiction Research Foundation. Additional subjects were interviewed on-site at local Native community centres.

Interview Training

Interviews were carried out by trained nurses and/or students. Interviewers were trained at the Mental Health Unit of the Addiction Research Foundation by B. Sproule, PharmD. Training involved two seminar sessions. Following instruction sessions, all trainees were required to conduct mock interviews with Addiction Research Foundation employees who posed as study subjects. Mock interviews were audio recorded and played back to the interviewers at the conclusion of each interview at which time the recorded data were evaluated for accuracy.

Written Consent

All study participants were required to read a description of the study protocol and a consent

form after which they had ample opportunity to ask any questions regarding the study (Appendix 2). All subjects were required to sign the consent form in the presence of a witness before blood or urine samples could be collected. All subjects in this study were volunteer participants, who had the option of withdrawing at any time during the study.

Race Determination

The study protocol required that all subjects be of Canadian Native Indian descent. According the Canadian Government, the current definition of "Canadian Indian" has both legal and cultural connotations (Indian Act, 1978). Since this definition omits the issue of race, it is inadequate for a pharmacogenetic study such as this as it does not tell us anything about the ancestry or genetic make up of the individuals in question. According to the Government of Canada's definition, certain individuals may be considered Indian (ie. status Indian) despite not having any Indian ancestry. On the other hand, "full-blooded" Canadian Indians may have no Indian status as determined by the Indian Act of 1978. To ensure that study subjects were Indian with respect to race, their Indian ancestry was determined as follows:

a. Subjects' own declaration as to whether or not he/she is a Canadian Indian.

b. A family history was provided by the subjects. This included the ethnic background and names of parents, grandparents, and great-grandparents.

c. Calculation of the Blood Quantum Factor (BQF) as determined by the subjects' family history.

The BQF is a racial quantitator which is often used by the United States Government to settle land claims and distinguish Indians from non-Indians (Frideres, 1993). In this study, the BQF was used to determine the proportion of Canadian Native Indian ancestry for each subject. The BQF ranges

from 0 to 100 depending on the individual's ancestry. For example, an individual with a father of full Canadian Native Indian ancestry and a mother of non-Canadian Native Indian ancestry would be assigned a BQF of 50. On the other hand, an individual who had three grandparents of full Canadian Native Indian ancestry and one Caucasian grandparent would be assigned a BQF of 75.

Subjects' ancestral data was recorded on the Genetics of Addiction Questionnaire (GAQ) (Appendix) and the Ethnic Background Form based on the above criteria.

Genetics of Addiction Questionnaire

The Genetics of Addiction Questionnaire (GAQ) was used to record information on subjects' age, sex, ethnic background, current medication use and patterns of substance use/abuse including alcohol, tobacco, opiates, amphetamines etc. The GAQ is a combination of a demographic questionnaire, the Wilkinson Psychoactive Drug Use History Questionnaire and the Diagnostic and Statistical Manual (DSM-IV) of the American Psychiatric Association. The GAQ was used to gather demographic data and to diagnose abuse and/or dependence on alcohol, amphetamines, cocaine, opiates and tobacco.

CYP2D6 Phenotyping

Dextromethorphan Administration and Urine Sample Collection

Oral dextromethorphan, in the form of a 30 mg tablet (SmithKline-Beecham Pharma, Oakville, Ontario), was used as a CYP2D6 probe drug. Subjects took the tablet at bedtime, and collected overnight urine first thing in the morning. In cases where the subjects needed to pass urine during the night, they were asked to do so in the sample bottle provided. Samples were returned to the laboratory on the morning of urine collection at which time urine volume and pH were measured.

Urine samples were then aliquoted to 12 ml vials and stored at -20° C.

High Performance Liquid Chromatography (HPLC)

Urine dextromethorphan (DEX) and three of its' metabolites, namely, dextrorphan (DOR), 3-methoxymorphinan (3-MM) and 3-hydroxymorphinan (3-HM) (Hoffmann-La Roche Ltd., Nutley, New Jersey, USA) were analysed using high performance liquid chromatograph (HPLC) (Chen *et al.*, 1990).

Frozen urine samples were thawed, vortexed and centrifuged for 5 seconds. One quarter of a millilitre (0.25 ml) of urine and an equal volume of 0.2 M acetate buffer pH 5.0 (J.T. Baker Canada, Phillipsburg, NJ, USA) containing B-glucoronidase (Sigma, St. Louis, MO, USA) in concentration of 4.8 mg/ml were pipetted into polypropylene conical tubes which were incubated overnight at 37°C.

On the following morning, the required amount of standards was added to blank urines in order to obtain a standard curve. Internal standard (100 μl Buspirone, Bristol Myers) and 100 μl of 1 M carbonate buffer pH 10 were added to each tube. In order to optimize the extraction procedure, each sample was adjusted to a pH of 11.3-11.6 using 0.5 N NaOH.

For the extraction step, 3 ml of exctractant (hexane:ether 4:1; v:v) (Fluka Chemika-Biochemika, Buchs, Switzerland), was added to each tube. Tubes were vortexed for 10 minutes and centrifuged at 3000 rpm for 5 minutes. The organic layer was back extracted into 200 μl of 0.01N HCl. Tubes were vortexed for 10 minutes, and centrifuged at 3000 rpm for 5 minutes once again. The organic top layer was aspirated and the inorganic layer was washed with 1 ml of ether under the fumehood. The samples were vortexed for 5 minutes and centrifuged for another 5 minutes at 3000 rpm. The organic top layer was aspirated once again. Finally, 30 μl of the 200 μl clean extract was

injected into the HPLC system.

The extracted samples were analysed on a phenyl, 5 μm, 15 x 0.46 cm chromatography column (Chromatography Sciences Company, Montreal, Quebec) on a Hewlett Packard HPLC System (Pump and Autosampler series 1050, Integrator 3396 series II, Hewlett Packard Co., Palo Alto, CA, USA). A mobile phase of buffer:acetonitrile - 80:20 (v:v) (Buffer=10 mM KH_2PO_4 containing 1 mM Heptanesulfonic acid, pH adjusted to 3.8 with orthophosphoric acid) was used for the injections at a flow rate of 1.5-1.7 ml/min. An ABI Spectroflow 980 UV detector was used.

CYP2D6 Phenotype Determination

CYP2D6 phenotype was assigned based on subjects' ability to convert dextromethorphan to its metabolites, dextrorphan, 3'-methoxymorphinan, and 3'-hydroxymorphinan (Figure 3.1). The O-demethylation ratio (ODMR) of dextromethorphan was used to determine CYP2D6 activity. The dextromethorphan ODMR is a ratio which is calculated using the amount of parent drug (DEX) and metabolites (DOR, 3-HM, 3-MM) detected in an 8 hour urine sample. The ODMR is calculated as follows:

ODMR = (DEXTROMETHORPHAN + 3-MM)/(DOR + 3-HM)

A larger Caucasian population (n=355) was used to determine the anti-mode of the bimodal dextromethorphan log ODMR frequency distribution. Probit plot analysis revealed that the anti-mode was located at approximately -0.3 (Figure 3.2). Consequently, subjects with log ODMR values above -0.3 were classified as PMs, whereas subjects with log ODMR below -0.3 were classified as EMs.

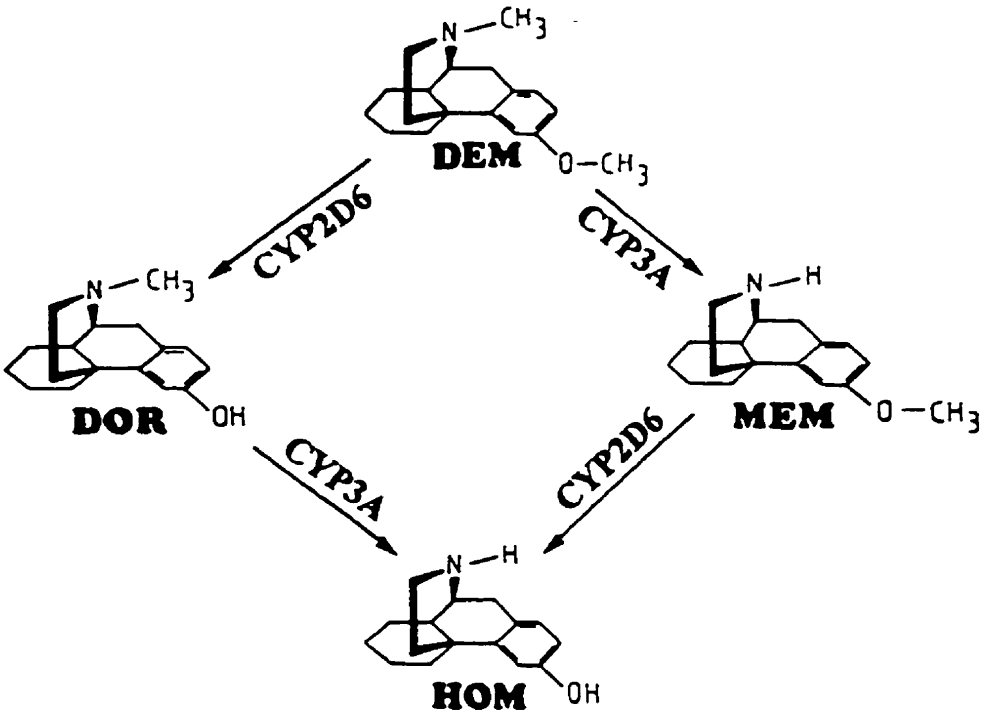

Figure 3.1 **Major routes of dextromethorphan metabolism in humans** (Jacqz-Aigrain *et al*, 1996).

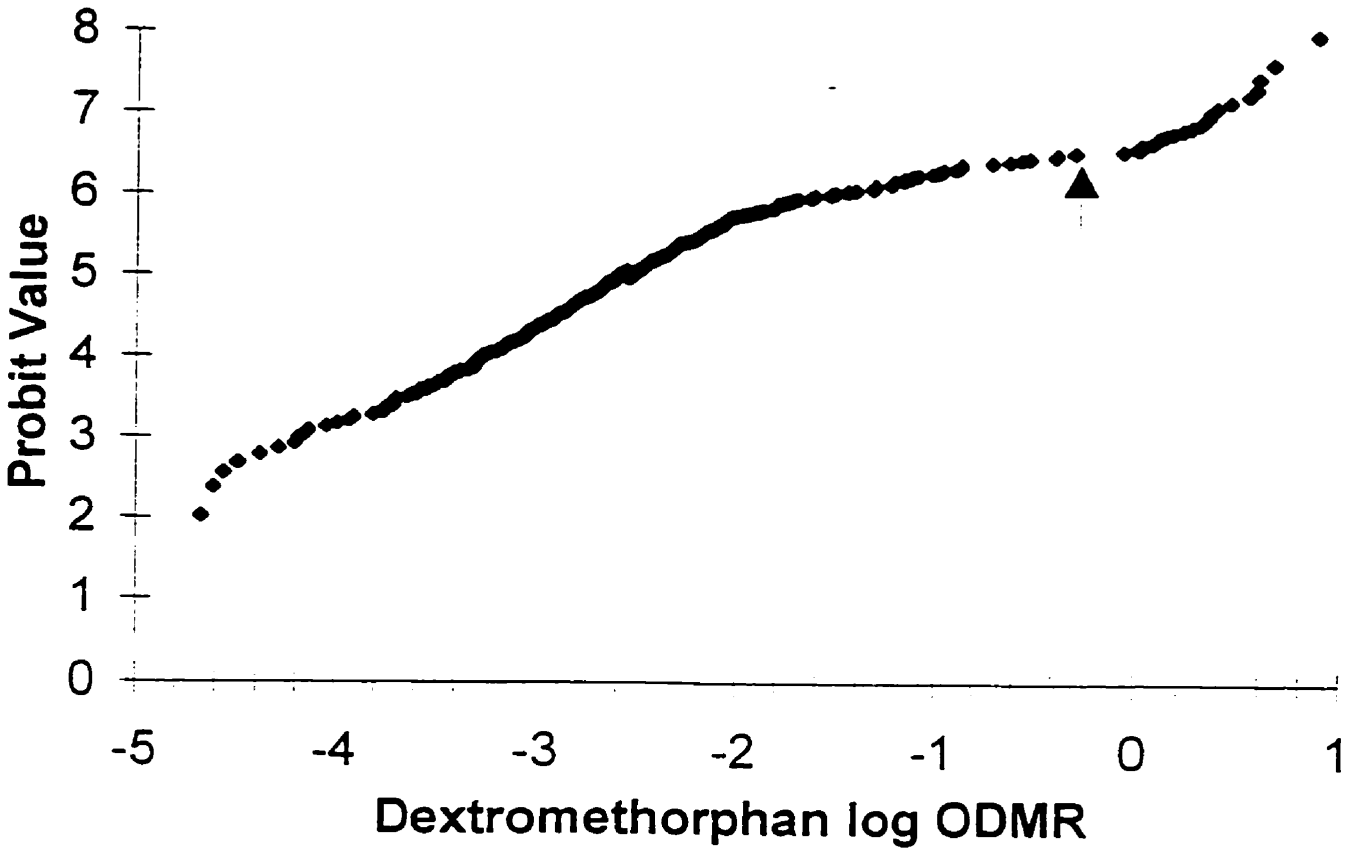

Figure 3.2 **Probit plot of 355 Caucasian subjects.** The arrow points to an inflection point at a log ODMR of -0.3. This point represents the antimode which can be used to distinguish between CYP2D6 PM and EM subjects.

Genotyping

Blood Sample Collection

Venous blood samples were collected from all study subjects. Twelve ml of whole blood was collected in Vacutainer Tubes (Becton Dickinson, Rutherford, New Jersey) containing ACD solution (citric acid, sodium citrate, glucose and distilled water). Shortly after collection, samples were transferred to scintillation vials and stored at 4°C. If DNA was not extracted within two weeks of collection, samples were transferred to a -20°C freezer. Genomic DNA was extracted using a variation of Sambrook's (1989) protocol. One ml of thawed blood and 2 ml of lysis buffer (10 mM Tris, pH=8.0, 10 mM NaCl, 4 mM DTT, 20 g/ml RNase, and 2% SDS) were incubated for 1 hour in a 37°C water bath. 100 μL of proteinase K (1 mg/50 ml) (Boehringer Mannheim, Laval, Quebec) was then added and incubated in a 56°C water bath. Following incubation, 3 ml of Tris-saturated (pH=8.0) phenol was added to the solution and tubes were placed on a shaker for 5 minutes. Tubes were centrifuged for 5 minutes at 5000 g. The aqueous phase was transferred to new tubes and the extraction process was repeated two more times using equal volumes of phenol:choloroform:isoamyl alcohol (25:25:1 v:v:v) (Sigma, Mississauga, Ontario). The extraction process was repeated one additional time with chloroform alone (Sigma, Mississauga, Ontario). Two times volume of 100% ethanol (Commercial Alcohols Inc., Brampton, Ontario) was used to precipitate the DNA at -20°C for 30 minutes. Upon removal from the freezer, DNA was washed with 70% ethanol. Tubes were then centrifuged for 10 minutes at 12,000 g. Ethanol was removed and the remaining pellet was dried at 65°C for 5 minutes. The dried pellet was resuspended in 40 μl of TE buffer (10 mM Tris HCl, pH=8.0, 1 mM EDTA). The absorbance of the DNA solution at 260 nm and 280 nm was measured using a Beckman DU-70 Spectrometer (Beckman, Mississauga, Ontario) The concentration of DNA was determined by calculation the OD_{260} (OD_{260} of 1 equals 50 μg/ml DNA).

Primer Preparation

Primers for all PCR assays were synthesized by the Hospital for Sick Children Biotech Service in Toronto. Primers were delivered in lyophilized form and were diluted with 500 μL of autoclaved water. The concentration of the primer solution was measured using OD260 calculations. Primers were aliquoted to small volumes of appropriate concentrations. Primer sequences were identical to those described in the literature (Heim and Meyer, 1990, Johansson *et al.*, 1994, deMorais *et al.*, 1994a and b, Fernandez-Salguero *et al.*, 1995). Primers are listed in Table 3.1.

CYP2D6

All Canadian Native Indian samples were analysed for the *CYP2D6*3*, *CYP2D6*4* and *CYP2D6*10* alleles.

I. *CYP2D6*3* and *CYP2D6*4*

The *CYP2D6*3* and *CYP2D6*4* mutant alleles were detected using a modified version of the polymerase chain reaction (PCR) described by Heim and Meyer (1990). *CYP2D6* is in close proximity to CYP2D7P and CYP2D8P, both of which exhibit high degree of nucleotide identity with the functional *CYP2D6* gene (Heim & Meyer, 1990). These pseudogenes also contain some of the mutations present in *CYP2D6*. Therefore, in order to increase specificity and decrease false priming to the pseudogene sequences, a double amplification, nested PCR approach was used (Figure 3.3).

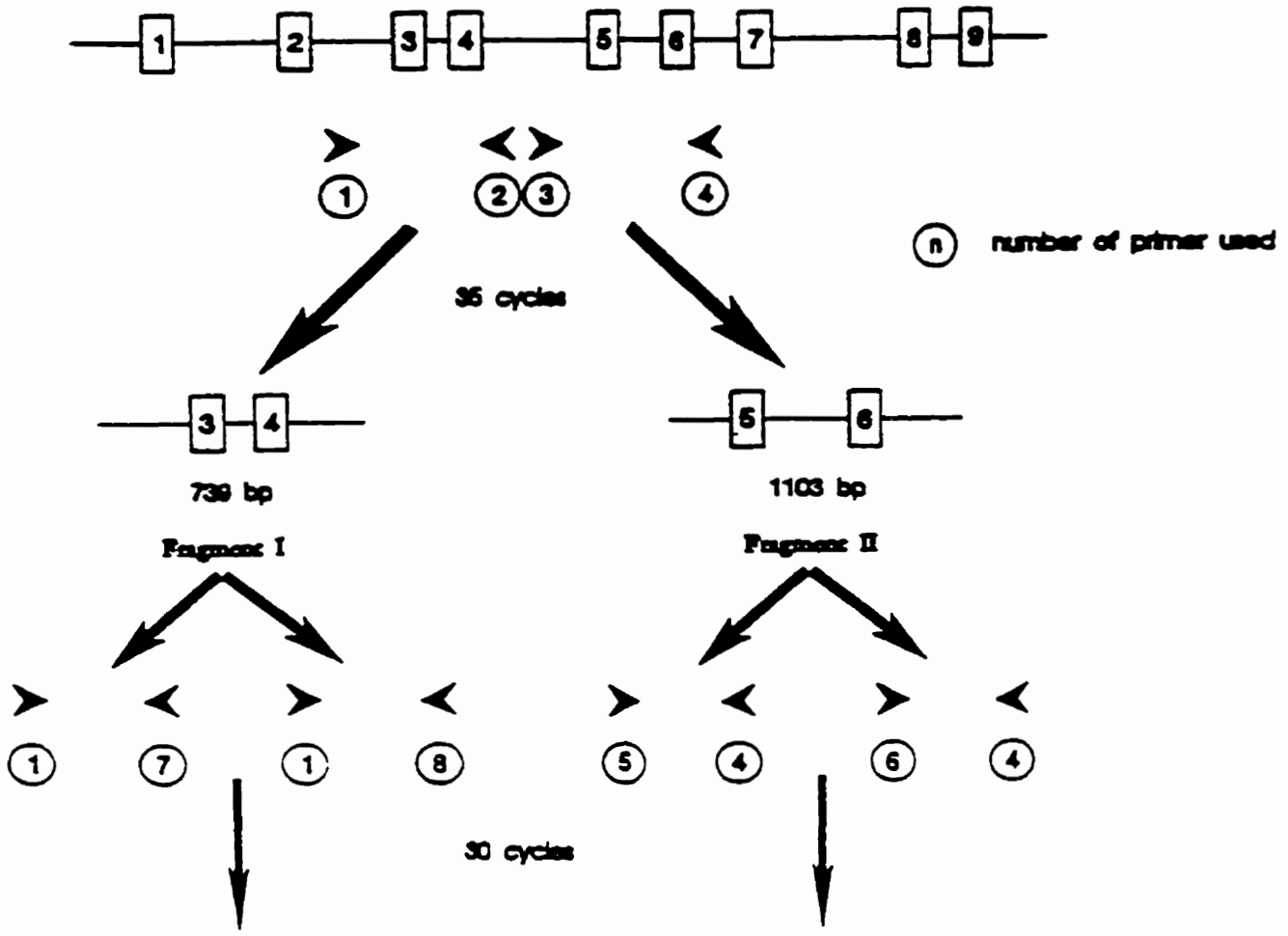

Figure 3.3 **Schematic diagram depicting allele-specific PCR approach used to detect the CYP2D6*3 and CYP2D6*4 null mutant alleles.** A nested-PCR strategy, which involved a CYP2D6-specific amplification to amplify the exons containing nucleotide changes, was used. Fragments I and II were then analyzed using allele-specific PCR to detect nucleotide changes present in the CYP2D6*3 and CYP2D*4 mutant alleles or the wild-type gene. (Heim & Meyer, 1990).

Table 3.1 Primers used for PCR genotyping assays.

Assay	Name	Sequence (5'-3')
*CYP2D6*3* (A)	P3	GCGGAGCGAGAGACCGAGGA
	P4	CCGGCCCTGACACTCCTTCT
	P5	GCTAACTGAGCACA
	P6	GCTAACTGAGCACG
*CYP2D6*4* (B)	P1	ATTTCCCAGCTGGAATCC
	P2	GAGACTCCTCGGTCTCTC
	P7	CGAAAGGGGCGTCC
	P8	CGAAAGGGGCGTCT
*CYP2D6*10* (Ch_1)	P9	ACCAGGCCCCTCCACCGG
	P10	TCTGGTAGGGGAGCCTCAGC
*CYP2C19*2* (m_1)	P40	AATTACAACCAGAGCTTGGC
	P41	TATCACTTTCCATAAAAGCAAG
*CYP2C19*3* (m_2)	P42	TATTATTATTGTTAACTAATATGA
	P43	ACTTCAGGGCTTGGTCAATA
*CYP2A6*2* (v_1) and *CYP2A6*3* (v_2)	F4	CCTCCCTTGCTGGCTGTGTCCCAAGCTTAGGC
	R4	CGCCCCTTCCTTTCCGCCATCCTGCCCCCAG
	E3F	GCGTGGTATTCAGCAACGGG
	E3R	TCGTGGGTGTTTTCCTTC

The first amplification uses a set of primers which are *CYP2D6* gene-specific. These primers amplify exons 3 through exon 6 of the *CYP2D6* gene without amplifying the corresponding CYP2D7P or CYP2D8P exons. The first amplification product undergoes subsequent second amplifications which are used to detect the point mutations in exon 4 (*CYP2D6*4*) and exon 5 (*CYP2D6*3*) (Figure 3.3).

Each genomic DNA sample undergoes two parallel first amplifications. One reaction, which amplifies exons 3 and 4, was performed with primers 1 and 2 (P1 and P2). The other first amplification reaction, which amplifies exons 5 and 6, was performed with primers 3 and 4 (P3 & P4).

The PCR conditions were modified from those described by Heim and Meyer in order to optimize the assay for our system. The most important changes were those in $MgCl_2$ and dNTP concentration and annealing temperature. The concentrations of both $MgCl_2$ and dNTP were increased due to the fact that the rate of false-positive results was unacceptable when using the original conditions described by Heim and Meyer. In addition, the annealing temperature of the first amplification was increased from 52°C to 56°C. These changes were made to ensure that second amplification only occurred when there was a match between primer and template. This was determined through the use of genotype control samples.

The first amplifications were performed in a reaction mixture of 30 μl containing 2.0 mM $MgCl_2$, 20 mM Tris HCL (pH=8.4), 50 mM KCl, 4% DMSO, 2.0 mM total dNTPs, 0.36 μM of each primer, 0.9 U Taq polymerase (Gibco BRL, Life Technologies, Burlington, Ontario). PCR conditions were as follows: denaturation step at 80°C for 2 min; 35 cycles of 94°C for 90 sec, 56°C for 90 sec, and 72°C for 90 sec. All PCR reactions were performed using a MJ DNA Engine (MJ Research, Inc., Watertown, Massachusetts). The P1&P2 first amplification yielded a PCR product of 739 bp This product served as a template for the second amplification reaction.

The P1&P2 (739 bp) first amplification product underwent two parallel second amplifications with P1&P7 (wildtype-specific) and P1&P8 (*CYP2D6**4-specific) to yield a 564 bp product. This reaction was performed in a PCR mixture of 20 μl containing 2.0 mM $MgCl_2$, 20 mM Tris HCl (pH=8.4), 50 mM KCl, 2.0 mM total dNTPs, 0.625 μM of each primer, 1.25 U of Taq polymerase and 1 μl of first amplification product as template. PCR was carried out at an initial denaturation of

80 °C for 2 min , 30 cycles of 60 sec at 94 °C, 60 sec at 50 °C and 60 sec at 72 °C. 15 μl of each sample was mixed with 2 μl ethidium bromide and run on a 1.2% agarose gel (Gibco BRL, Life Technologies).

The presence of the P1&P7 band (564 bp) was indicative of a wild-type sequence (G1934) in exon 4, while the presence of the P1&P8 (564 bp) band diagnosed the G1934A substitution, which is the causative mutation of the *CYP2D6*4* allele (Figure 3.4).

The CYP2D6*3 assay, which involved a first amplification with P3&P4 and a second amplification with P4&P5 and P4&P6 was carried out under the same conditions as the CYP2D6*4 assay with the exception of the primer sets. The first amplification yielded a product of 1123 bp which was used as a template for the second amplification reaction. The second reaction yielded a product 588 bp in length. The presence of the P4&P5 band was indicative of a wild-type sequence at A2637 of exon 5, while presence of the P4&P6 band indicated presence of the A2637 deletion, which is the causative mutation of the CY2D6*3 allele (Figure 3.4).

II. *CYP2D6*10* (Ch_1)

The $C_{188}T$ base pair substitution, which is the causative mutation in all three *CYP2D6*10* mutant alleles (*10A, *10B and *10C) was detected using PCR and RFLP PCR amplification was performed using primers 9 and 10 (P9 and P10, Table 3.1). The reaction conditions were determined empirically through the use of control samples, which consisted of individuals with the following genotypes: C188/C188, C188/T188 and T188/T188. The assay was optimized in order to ensure that no false-positive and/or false-negative results were obtained for the control samples. A mixture of 50 μl containing 20 mM Tris HCl (pH=8.4), 50 mM KCl, 2.0 mM dNTP, 4% DMSO, 1.5 mM $MgCl_2$, 0.35 μM of each primer, 3 U of Taq polymerase (Gibco BRL), and 2.5 μl of DNA template. The

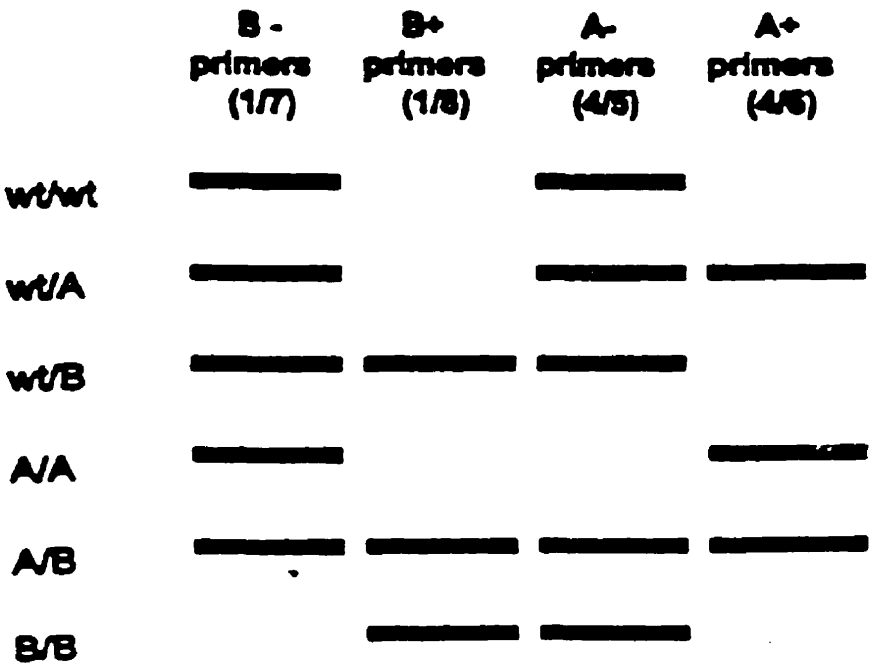

Figure 3.4 PCR banding patterns for CYP2D6*3 (A) and CYP2D6*4 (B) genotyping.

PCR reaction yielded a 517 bp DNA fragment.

Twenty μl of PCR product was incubated in a total volume of 25 μl of 1X NEBuffer 4 (50 mM potassium acetate, 20 mM Tris acetate, 10 mM magnesium acetate, 1 mM DTT, pH 7.9 @ 25°C) (New England Biolabs, Beverly Massachusetts, USA) containing 1 U of Hph I enzyme (New England Biolabs). Hph I digestion was carried out for 3 hours at 37°C. An uncut control containing 1X NEBuffer 4, but no enzyme, was also incubated for the same amount of time.

Following digestion, 20 μl of cut and 20 μl of uncut PCR product were analysed on a 1.2% agarose gel. Digestion of samples which did not carry the *CYP2D6* $C^{188}T$ mutation produced fragments of 477 and 40 bp. Samples homozygous for the $C^{188}T$ mutation yielded fragments of 377, 100 and 40 base pairs. Finally, samples which were heterozygous, having both the C^{188} and T^{188}, resulted in fragments of 477, 377, 100 and 40 base pairs.

CYP2C19 Genotype

Genomic DNA was analysed for the *CYP2C19*2* and *CYP2C19*3* null mutant alleles which have previously been described (deMorais *et al.*, 1994a & 1994b).

I. *CYP2C19*2* Null Mutant Allele

The *CYP2C19*2* variant is characterized by a single base (G to A) substitution in exon 5 of the CYP2C19 gene. The detection of the *CYP2C19*2* allele involved the PCR/RFLP method described by de Morais *et al.* (1994a) (Figure 3.5).

PCR was carried out in a 60 μl reaction mixture containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 0.2 mM of each dNTP, 4.8% DMSO, 0.54 μM of each primer, and 2 U of Taq polymerase (Gibco BRL). An initial denaturation at 94 °C for 90s was followed by 35 cycles of 94 °C for 60s,

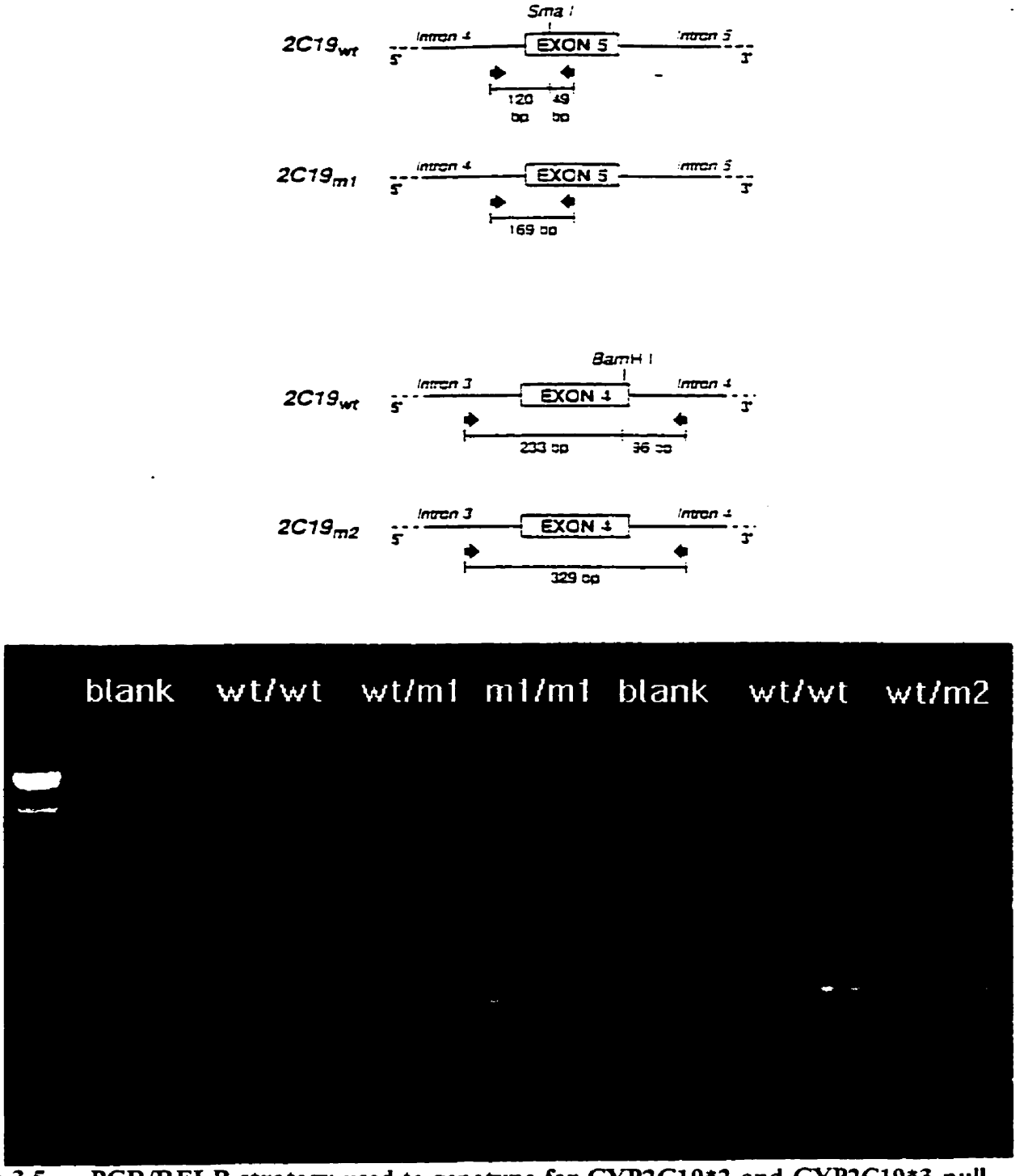

Figure 3.5 **PCR/RFLP strategy used to genotype for CYP2C19*2 and CYP2C19*3 null mutant alleles**. CYP2C19*2 is detected using a PCR amplification of exon 5 followed by digestion with SmaI. Since the CYP2C19*2 allele removes the SmaI cutting site, CYP2C19*2 homozygotes are not digested by SmaI. CYP2C19*3 is detected using a PCR amplification of exon 4 followed by digestion with BamHI. CYP2C19*3 homozygotes also result in uncut PCR product (de Morais *et al.*, 1994b). Digestion products for five genotype controls are shown.

52 °C for 90s, 72 °C for 90s and a final denaturation step for 7 minutes at 72 °C (Chang *et al.*, personal communication). Following amplification, PCR product was aliquoted into three tubes, each containing 20 μl of PCR product. Two tubes were used for digestion reactions with Sma I (Pharmacia Biotech) and BstN I (New England Biolabs), while the third was used as an uncut control (Table 3.2).

Sma I digestion was performed in a reaction mixture of 25 μl containing One-Phor All buffer (100 mM Tris-HCl (pH 7.5), 100 mM magnesium acetate, 500 mM potassium acetate), 5 U of Sma I, and 20 μl of PCR product. Reaction mixtures were incubated overnight at 30°C using a water bath or the MJ DNA Engine.

Table 3.2 Digestion conditions for CYP2C19 genotyping.

Mutant	Enzyme (Units)	Digestion Volume	Buffer	Temperature/ Incubation Time	Digestion Specificity
*CYP2C19*2*	Sma I (5 U)	25 μl	One-Phor-All	30°C/overnight	exon 5 wt
	BstN I (3U)	25 μl	NEBuffer #2	60°C/5 hrs.	exon 5 *2
	none	25 μl	One-Phor-All	30°C/overnight	
*CYP2C19*3*	BamHI (12U)	25 μl	One-Phor-All	37°C/3 hrs.	exon 4 wt
	None	25 μl	One-Phor-All	37°C/3 hrs.	

The causative mutation of the *CYP2C19*2* allele, a G to A substitution in exon 5, results in the deletion of the Sma I digestion site (de Morais *et al.*, 1994a). Consequently, homozygous digestion of the PCR product by Sma I is indicative a wild-type sequence at that locus. The banding patterns of control samples is illustrated in Figure 3.5. Following digestion with Sma I, the 169 bp PCR product yields fragments of 120 and 49 bp (de Morais, 1994a).

Upon analysis of the *CYP2C19*2* DNA sequence in exon 5, we found that the G to A mutation in exon caused the formation of a BstN I digestion site which is not found in the wild-type

CYP2C19 sequence. As a result, digestion with BstNI was added to the genotyping protocol, thereby providing a more accurate RFLP assay which consisted of both a wildtype-positive (at this locus) and a CYP2C19*2-positive digestion in exon 5.

Samples were digested with BstNI in a 25 μl mixture containing NEBuffer 2 (50 mM NaCl, 10 mM Tris-HCL, 10 mM $MgCl_2$, 1 mM DTT, pH 7.9 @ 25°C) 3 U of BstNI and 20 μl of PCR product at 60 °C for 5 hours (Table 3.4).

II. *CYP2C19*3* Null Mutant Allele

*CYP2C19*3* is characterized by a G636A substitution in exon 4 of the CYP2C19 gene (de Morais *et al.*, 1994b). Once again, a PCR/RFLP approach described by de Morais *et al.*, (1994b) was used to detect this mutation in the Canadian Native Indian population.

PCR reactions were carried out in a 60 μl mixture containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 0.08 of each dNTP, 3.75 $MgCl_2$, 4% DMSO, 0.42 μM of each primer and 2 U of Taq polymerase (Gibco BRL). The resulting PCR product (329 bp) was digested with 12 U of BamHI (Pharmacia Biotech) which results in a wildtype-positive cut in exon 4. The digestion mixture was incubated for 3 hours at 37°C. Digested products were analysed on ethidium bromide-stained 3% agarose gels. Wildtype homozygotes, wt/CYP2C19*3 heterozygotes, and *CYP2C19*3* homozygotes yielded banding patterns of 233/96 bp, 329/233/96 bp and 329 bp, respectively (Figure 3.5).

CYP2A6 Genotype

CYP2A6 genotype was determined using nested PCR and RFLP as described by Fernandez-Salguero *et al* (1995). The first amplification, which is CYP2A6 gene-specific, was used to increase the specificity of the second amplification. Exon 3 was utilized in the second amplification because

both the *CYP2A6*2* and *CYP2A6*3* mutant alleles contain nucleotide changes leading to amino acid changes in this region of the CYP2A6 gene (Figure 3.6).

The first amplification was performed using the XL-PCR kit (Perkin-Elmer Co., Norwalk, Connecticut). A 100 μl reaction mixture of 0.2 μM of primer F4 and R4 (Table 3.1), 200 μM dNTPs, 0.8 mM magnesium acetate, and 2 U of rTth1 DNA polymerase and 400 to 600 ng of genomic DNA used. The amplification was performed in a MJ DNA Engine (MJ Research, Inc., Watertown, Massachusetts) at 93°C for 1 minute, 66°C for 6 minutes and 30 seconds for 31 cycles.

At the time that the assay was being set up in our laboratory, 0.7 % ethidium-stained agarose (Gibco BRL) gels were used to detect first amplification PCR products. However, it was soon realized that the strength and/or presence of the 7.8 Kb band on the agarose gel was not predictive of second amplification efficacy. As a result, this step was abolished for genotyping of sample DNAs.

The second amplification was performed in a reaction mixture containing 0.5 μM of primers E3F and E3R (Table 3.1), 200 μM dNTPs, 1.5 mM $MgCl_2$, 2.5 U of Taq DNA polymerase (Gibco BRL, Life Technologies, Burlington, Ontario), and 2.5 μl of first amplification product, which was the template for the reaction. The reaction conditions were as follows: 94°C for 3 minutes, followed by 31 cycles of 94°C for 1 minute, 60°C for 1 minute and 72°C for 1 minute.

The second amplification yielded a PCR product 201 bp in length which was digested with Xcm I (New England Biolabs) and Dde I (New England Biolabs and Pharmacia Biotech) to detect the *CYP2A6*2* and *CYP2A6*3* mutations, respectively. Concentrations of enzymes and PCR product, total volume and digestion time were determined empirically to optimize cutting efficiency with a minimal amount of time and enzyme. Xcm I digestion reactions were carried out at 37°C for 2 hours in a 30 μl reaction mixture containing 1X NEBuffer 3 (100 mM NaCl, 50 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM DTT pH 7.9 @ 25°C), dH_20, and 2 U of Xcm I. Dde I digestions were carried

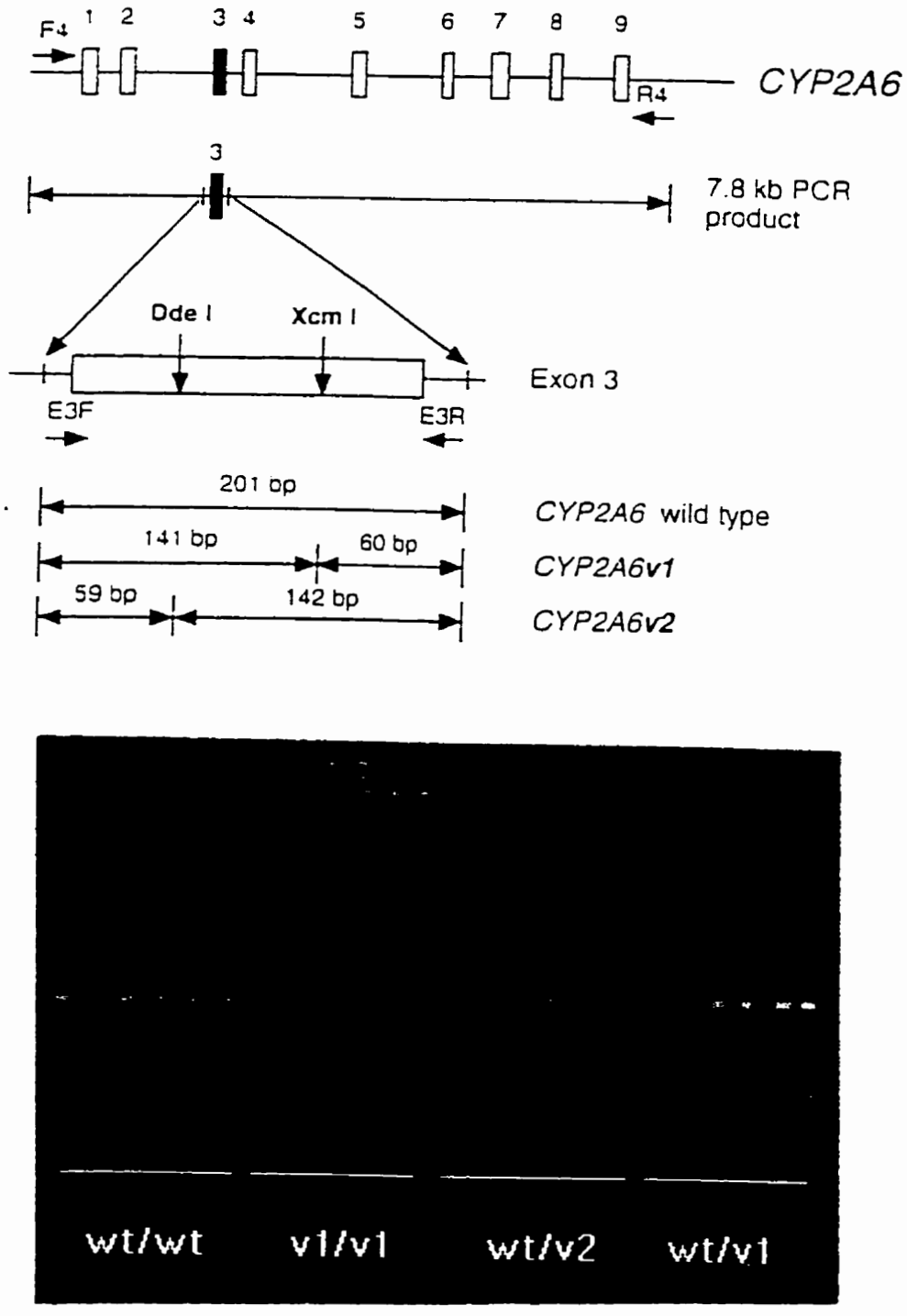

Figure 3.6 **Strategy used to genotype for the CYP2A6*2 (v1) and CYP2A6*3 (v2) mutant allele.** A CYP2A6-specific PCR amplification is followed by an amplification of exon 3 and subsequent digestions with XcmI and DdeI to detect the CYP2A6*2(v1) and CYP2A6*3 (v2) mutant alleles, respectively. Banding patterns for four control samples are shown. XcmI digestion results in bands 60 bp and 141 bp in length. DdeI digestion results in fragments of 59 bp and 142 bp respectively (Fernandez-Salguero *et al.*, 1995).

out at 37°C for 2 hours in a 30 μl reaction mixture containing One-Phor-All (OPA) buffer (Pharmacia Biotech) and 2 U of Dde I. All digestions were performed with uncut controls and sequenced controls obtained from F.J. Gonzalez (National Institute of Health, Bethesda, MD, USA) and H. Raunio (University of Oulu, Finland). Digestion products were analysed on ethidium-stained 3% agarose gels. Banding patterns for the CYP2A6 RFLP are illustrated in figure 3.6 (Fernandez-Salguero *et al.*, 1995).

Statistical Analysis

Significance of differences in dextromethorphan log ODMRs was calculated using the two sample t-test (Microsoft Excel v5.0). Allele frequencies, phenotype frequencies and associations of genotype with drug dependence were statistically analysed and compared using the Chi-square test (Easystat). In some cases, the Chi-square test could not be used due to a low number of observations in any given cell. In such cases, the Fisher's exact test (Easystat)was used. The Fisher's exact test provides a correction factor for cases where the number of observations are low.

Results

One hundred and fifty-nine unrelated Canadian Native Indian volunteers were recruited in the Metropolitan Toronto area. Based on the family histories provided, blood quantum factors were calculated and 115 volunteers were determined to have blood quantum factors of 100 indicating that they were descendants of four Canadian Native Indian grandparents. The remaining 44 volunteers had blood quantum factors ranging from 25 to 87.5 indicating that at least one parent or grandparent was not of Canadian Native Indian ancestry (Table 4.1). Thirty-eight of the mixed-ancestry subjects reported partial Caucasian ancestry, four did not know the ethnic background of their non-Native ancestors and two reported mixed Canadian Native Indian, Caucasian and unknown ancestry.

Table 4.1 Ethnic Backgrounds of Study Volunteers.

Number of Canadian Native Indian Grandparents	Blood Quantum Factor	Code	Males/Females	Number of Subjects
4	100	4N	85/30	115
3	75-87.5	3N1W	10/8	18
2	50	2N2W	12/12	24
1	25-37.5	1N3W	2/0	2

The study volunteers consisted of 109 males and 50 females ranging from 16 to 62 years of age with a mean age of 33.8 $\pm$ 9.9 years (mean $\pm$ S.D).

Drug Dependence

The prevalence of drug dependence was determined using DSM-IV criteria. Ninety-nine (62.3%) and 109 (68.6%) subjects were dependent on alcohol and nicotine, respectively. Seventy-nine subjects (49.7%) were dependent on both alcohol and nicotine. Thirty-four (21.4%) subjects

were dependent on cocaine at some point in their life. Eight (5.0%) subjects were dependent on codeine and four (2.5%) were dependent on amphetamines. Twenty-six (16.4%) subjects were dependent on three or more of the compounds mentioned above. Nineteen (11.9%) individuals were never dependent on alcohol, nicotine, cocaine, opiates, or amphetamines.

Medication Use History

All subjects were asked to provide a detailed drug use history. Any subjects taking medications which might interfere with the CYP2D6 phenotyping (eg. fluoxetine, codeine, etc.) were excluded from the study. As a result, none of the 159 Canadian Native Indian volunteers involved in this study reported taking any known CYP2D6 substrates or inhibitors at the time of the phenotyping test.

CYP2D6 Phenotype

All Canadian Native Indian volunteers were phenotyped for CYP2D6 activity using a 30 mg dextromethorphan capsule. Phenotyping compliance among the full Canadian Native Indian volunteers was 83% (95/115). HPLC analysis revealed that 20 urine samples did not contain dextromethorphan (DEX) or any of its three metabolites (3MM, 3HM, DOR) suggesting that 20 subjects did not take the dextromethorphan capsule provided.

Only 1 of the 95 full Canadian Native Indian subjects was determined to be a poor metabolizer of dextromethorphan. This translates to a CYP2D6 PM frequency of 1.1% which was significantly lower ($p=0.03$) than the PM frequency observed in Caucasians (6.2%) and was not significantly different ($p=0.7$) from the PM frequency observed in the Chinese population (1.2%) (Figure 4.1; Figure 4.2; Table 4.2).

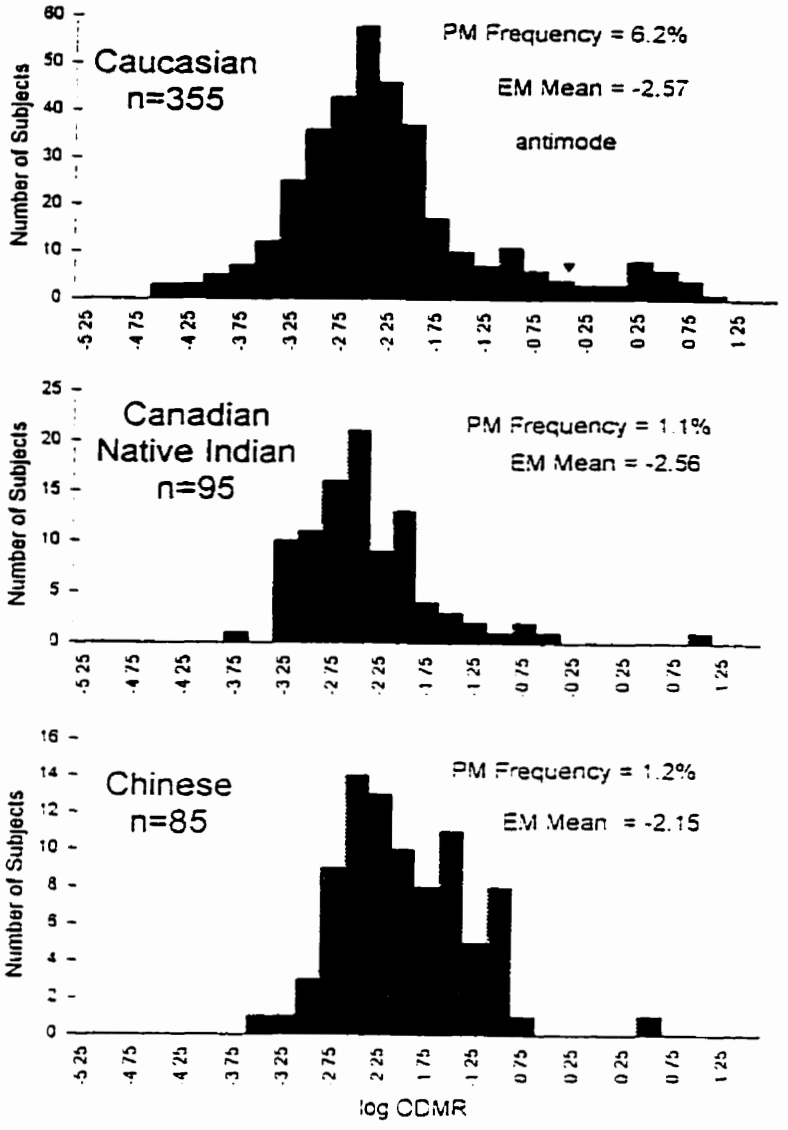

Figure 4.1 **Frequency distributions of ODMR values for Caucasian, Canadian Native Indian and Chinese populations**. The Canadian Native Indians resemble Caucasians with respect to mean CYP2D6 metabolic activity but differ with respect to CYP2D6 PM frequency. In comparison to the Chinese, the Canadian Native Indians have a similar PM frequency, but have a higher mean metabolic activity

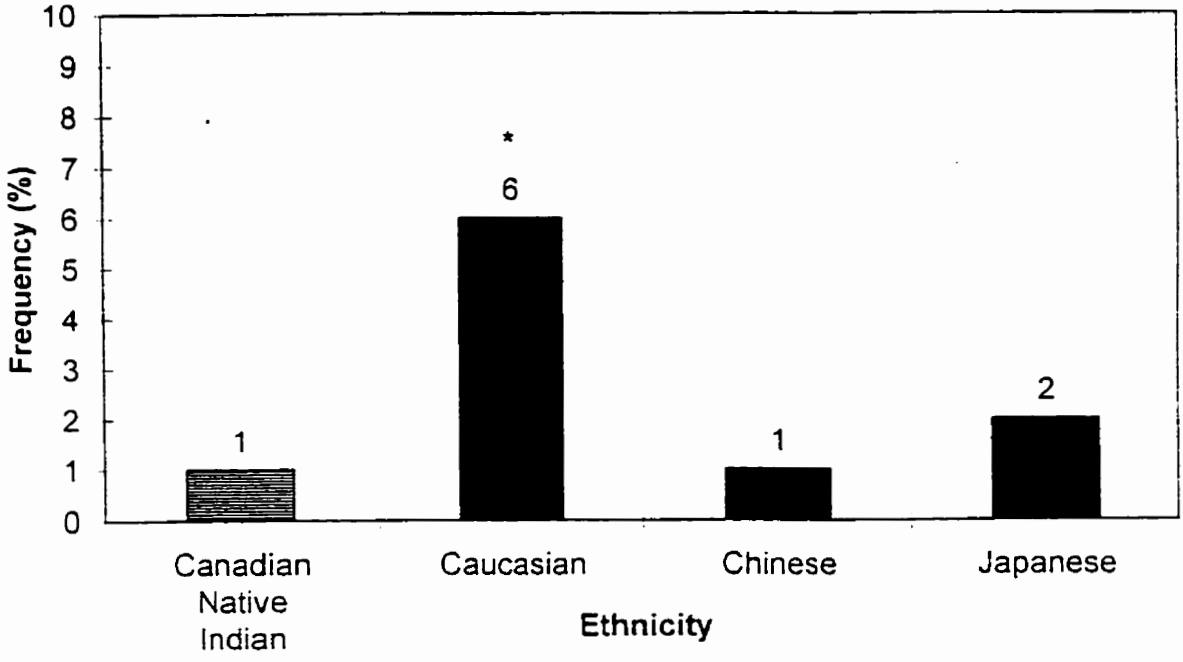

Figure 4.2 **Comparison of CYP2D6 PM frequencies in four ethnic groups**. The CYP2D6 PM frequency in Canadian Native Indians is significantly lower than in Caucasians but is not significantly different from Chinese or Japanese (Ishizaki et al., 1987) populations (*$p<0.05$).

The dextromethorphan log ODMR means of Canadian Native Indian, Caucasian and Chinese CYP2D6 EM subjects were compared to determine whether there were any inter-ethnic differences in the mean metabolic activity of CYP2D6. The mean log ODMR of Canadian Native Indian EM subjects was -2.56 $\pm$ 0.60. This value was not significantly ($p=0.4$) different from the mean log ODMR of -2.57 $\pm$ 0.77 observed in Caucasian EM (n=333) subjects, but was significantly lower ($p<0.001$) than the mean log ODMR of -2.15 $\pm$ 0.67 in Chinese (n=84) EM subjects (Figure 4.1).

CYP2D6 Genotype

*CYP2D6*3* and *CYP2D6*4* Null Mutant Alleles

The *CYP2D6*3* null mutant allele was not detected in Canadian Native Indian subjects (n=115). This translated to a *CYP2D6*3* null mutant allele frequency of 0% which was significantly lower than the 2% reported in Caucasian subjects by Sachse *et al* ($p<0.02$; n=589; 1997) and was also significantly lower than the 1.8% frequency observed in Caucasians genotyped in our laboratory ($p=0.03.4$; n=276; Tyndale *et al.*, 1997 in press). The Canadian Native Indian *CYP2D6*3* null mutant allele frequency was not significantly different ($p=0.99$) from Chinese subjects (0%) studied by Lee and Jeyaseelan (n=93; 1994) or Chinese (0%) subjects genotyped in our laboratory (n=54) (Figure 4.3).

The *CYP2D6*4* null mutant allele was present in Canadian Native Indians (n=115) at a frequency of 3% which was significantly lower than the frequency observed in Caucasians studied by Sachse *et al* (20.7%, n=589, $p<0.001$; 1997) and Tyndale *et al* (16.8%, n=276, $p<0.001$, 1997, in press), but was not significantly different from Chinese populations (0%, n=93, $p=0.2$) (Lee and Jeyaseelan, 1994) and (0%, n=54, $p=0.2$) (Figure 4.3). Neither the *CYP2D6*3* nor the *CYP2D6*4* alleles were detected in full Canadian Native Indian CYP2D6 PMs (log ODMR=0.77; figure 4.4).

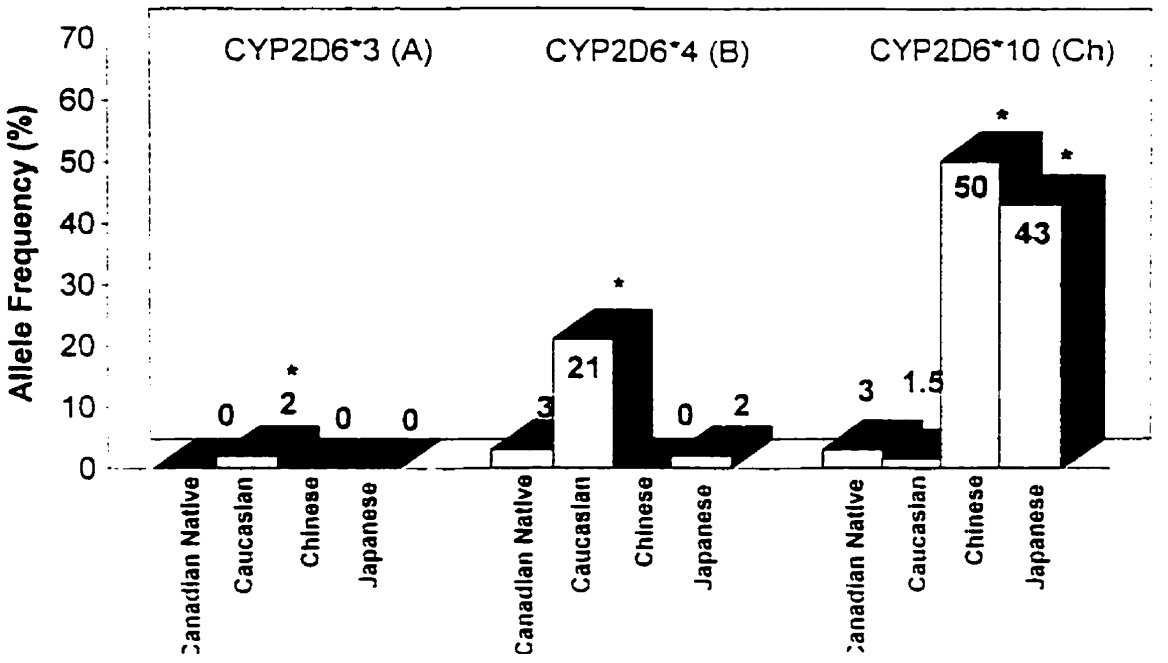

Figure 4.3 **Comparison of CYP2D6 mutant allele frequencies in four ethnic groups**. The Canadian Native Indian population resembles Asians (p=0.99) and differs from Caucasians (p<0.02) with respect to *CYP2D6*3*. The *CYP2D6*4* mutant allele frequency among Canadian Native Indians is significantly lower (p<0.001) than in previously studied Caucasian populations but is not significantly different (p=0.2) from previously studied Chinese and Japanese populations. Finally, the *CYP2D6*10* mutant allele was significantly lower (p<0.001) in Canadian Native Indians than in Asian populations, but was not significantly different (p=0.54) from Caucasians (*p<0.05).

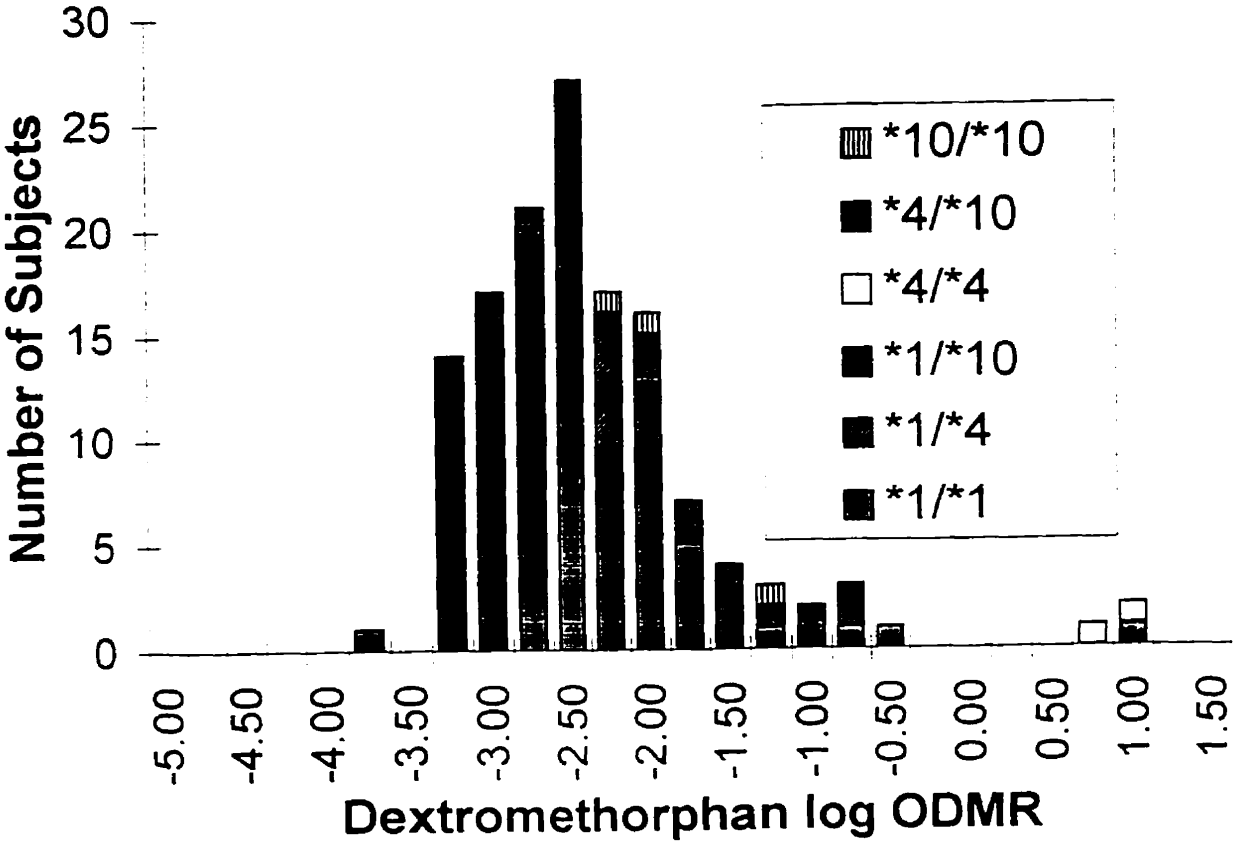

Figure 4.4 **Dextromethorphan log ODMR frequency distribution of 136 Canadian Native Indians in relation to their CYP2D6 genotype**. The figure illustrates the effect of the *CYP2D6*4* and *CYP2D6*10* mutant alleles on CYP2D6 phenotype. Subjects who carry these alleles exhibit significantly higher log ODMR values than subjects who are homozygous for the CYP2D6 wild-type allele.

*CYP2D6*10* Mutant Allele

The *CYP2D6*10* mutant allele frequency in full Canadian Native Indians (n=115) was 3%, which was not significantly different (p=0.5) from previously studied Caucasians (1.5%, n=589; Sachse *et al.*, 1997), but was significantly lower from Chinese subjects (50.7%, n=113, p<0.001) studied by Johansson *et al* (1994) and Chinese subjects (73%, n=54, p<0.001) genotyped in our laboratory. The frequencies of *CYP2D6* mutant alleles in different ethnic populations are illustrated in Figure 4.3.

Table 4.2 **CYP2D6 phenotype and allele frequencies in 159 Canadian Native Indian subjects**. Asterisks (*) indicate significant difference from full Canadian Native Indian subjects.

BQF	n=	*CYP2D6*3* Frequency (%)	*CYP2D6*4* Frequency (%)	*CYP2D6*10* Frequency (%)	Number of PMs (%)†	mean log ODMR
100	115	0	2.6	2.6	1 (1.1)	-2.56
75-87.5	18	0	22.2***	0	1 (6)	-2.47
50	24	0	25***	12.5*	1 (4)	-2.28
25	2	0	0	0	0	-2.15
Total Mixed Ancestry	44	0	22.7***	6.8**	4.9	-2.35
Total	**159**	**0**	**8.2**	**3.8**	**2.5**	**-2.47**
Caucasians ††	569	20.7	2	1.5	7.2	NA

† CYP2D6 PM frequencies were calculated from the number of subjects phenotyped (ie. 95 4N and 41 mixed-ancestry subjects)

†† Caucasian data taken from Sachse et al., 1997
(*p<0.05; **p<0.01, ***p<0.001)

Table 4.3 CYP2D6 Genotypes in 159 Canadian Native Indian Subjects.

Blood Quantum Factor	2D6*1/ 2D6*1	2D6*1/ 2D6*4	2D6*1/ 2D6*10	2D6*10/ 2D6*10	2D6*4/ 2D6*10	2D6*4/ 2D6*4
100%	107 (67.3%)	3 (1.9%)	1 (0.6%)	2 (1.2%)	1 (0.6%)	1 (0.6%)
75-87.5%	11 (6.9%)	6 (3.8)	0	0	0	1 (0.6%)
50%	11 (6.9%)	7 (4.4%)	1 (0.6%)	1 (0.6%)	3 (1.9%)	1 (0.6%)
25%	2 (1.2%)	0	0	0	0	0
Total	**131 (82.3%)**	**16 (10.0%)**	**2 (1.2%)**	**3 (1.9%)**	**4 (2.5%)**	**3 (1.9%)**

Effect of Caucasian Admixture on CYP2D6 Phenotype and Genotype

Forty-one mixed-ancestry Canadian Native Indian subjects were phenotyped and all 44 subjects were genotyped. One hundred and fifteen full Canadian Native Indians were compared to the 44 mixed-ancestry Canadian Native Indians with respect to PM frequency, mean CYP2D6 metabolic activity and *CYP2D6* mutant allele frequencies. No significant differences were observed between the two groups with respect to mean log ODMR ($p=0.2$), CYP2D6 PM frequency ($p=0.2$), or *CYP2D6*3* null mutant allele frequency ($p=0.99$) (Figure 4.5 and Table 4.2). However, subjects with mixed Caucasian and Canadian Native Indian ancestry displayed significantly higher allele frequencies of *CYP2D6*4* ($p<0.001$) and *CYP2D6*10* ($p<0.01$) mutant alleles.

CYP2D6 Genotype and Drug Dependence

CYP2D6 genotype data were analyzed to determine whether there was an association between drug dependence and CYP2D6 genotype. There were no significant differences in proportion of CYP2D6 EMs (*1/*1), IMs (intermediate metabolizers: *1/*4, *1/*10, *10/*10 and *4/*10) and PMs (*4/*4) between subjects who were dependant and non-dependant on alcohol ($p=0.3$), nicotine

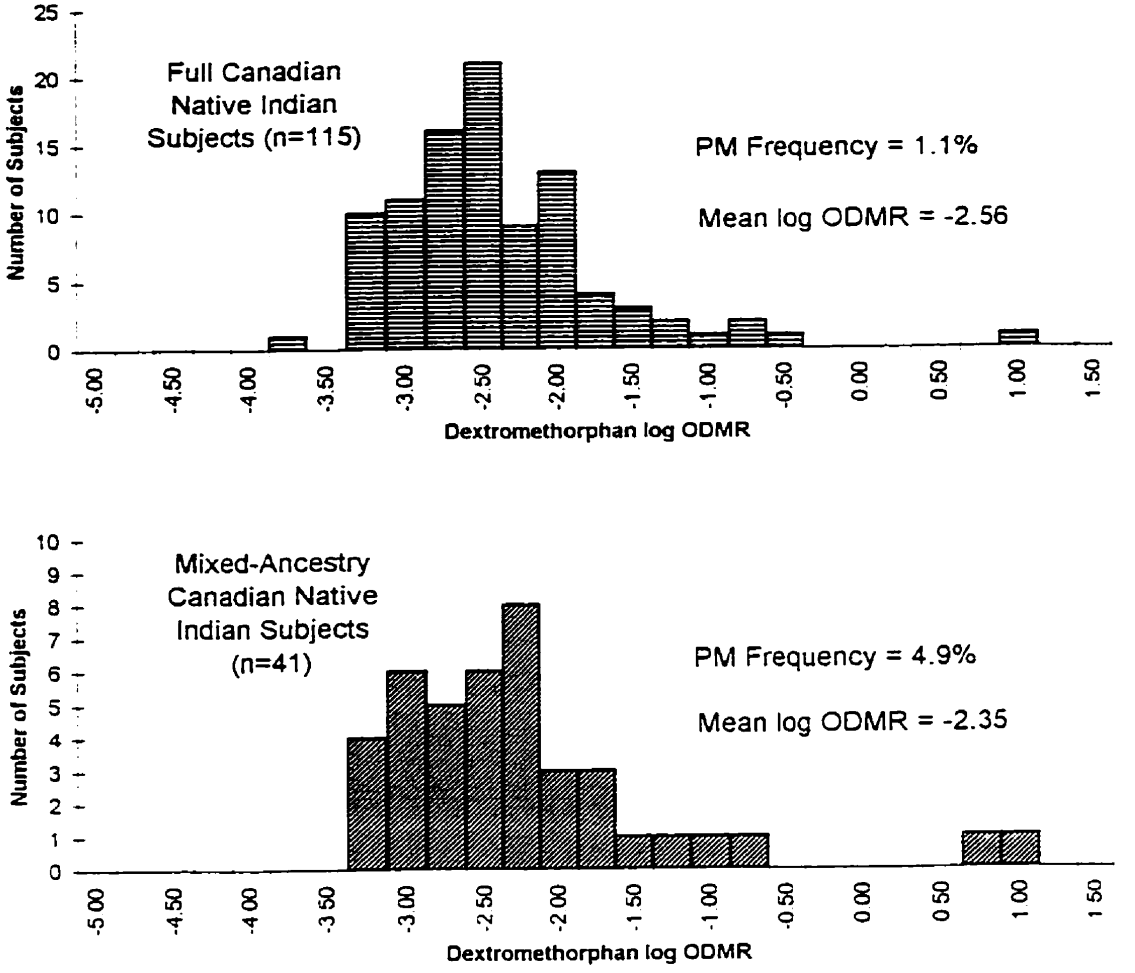

Figure 4.5 **Comparison of CYP2D6 phenotype results in full and mixed-ancestry Canadian Native Indian subjects**. There are no significant differences in either the PM frequency ($p=0.2$) or the mean log ODMR ($p=0.2$) between full and mixed-ancestry Canadian Native Indians.

(p=0.6), codeine (p=0.2), cocaine (p=0.6) and amphetamine (p=0.8).

CYP2D6 Genotype and Gender

There was no association between gender and CYP2D6 genotype (p=0.6).

CYP2C19 Genotype

The *CYP2C19*2* null mutant allele frequency in full Canadian Native Indians (n=115) was 19.1%. This was significantly higher than the frequency reported by Ferguson in Caucasians (13%, n=173, p=0.03), but was significantly lower than the CYP2C19*2 frequency observed in Chinese described by Xiao *et al* (25.7%, n=202, p=0.04), Japanese described by Takakubo *et al.* (27%, n=217, p<0.02) and Chinese genotyped in our laboratory (29%, n=69, p<0.01) (Figure 4.6).

The *CYP2C19*3* null mutant allele was not found in the full Canadian Native Indian subjects (n=115). This was not significantly different from the frequency observed in Caucasians (0.3%, n=173, p=0.9) by Ferguson *et al,* but was significantly lower than the frequencies observed in previously studied Chinese (5.5%, n=202, p<0.001) and Japanese (10%, n=217, p<0.001) populations (Xiao *et al.*, 1997; Takakubo *et al.*, 1996). It was also significantly lower (p<0.001) than the *CYP2C19*3* frequency observed in Chinese (7%, n=70, p<0.001) in our laboratory (Figure 4.6).

Eight Canadian Native Indians (n=115) were CYP2C19 PMs. The resulting CYP2C19 PM genotype frequency of 7% is significantly higher than in Caucasians (3.3%, n=488, p<0.02) but is significantly lower than in Chinese (13.4%, n=202, p<0.02) and Japanese (17.3%, n=217, p<0.02) populations (Bertilsson *et al.*, 1992; Xiao *et al.*, 1997; Takakubo *et al.*, 1996) (Table 4.4). It is also significantly lower (p<0.02) than the CYP2C19 PM genotype frequency of 17.8% observed in Chinese subjects in our laboratory.

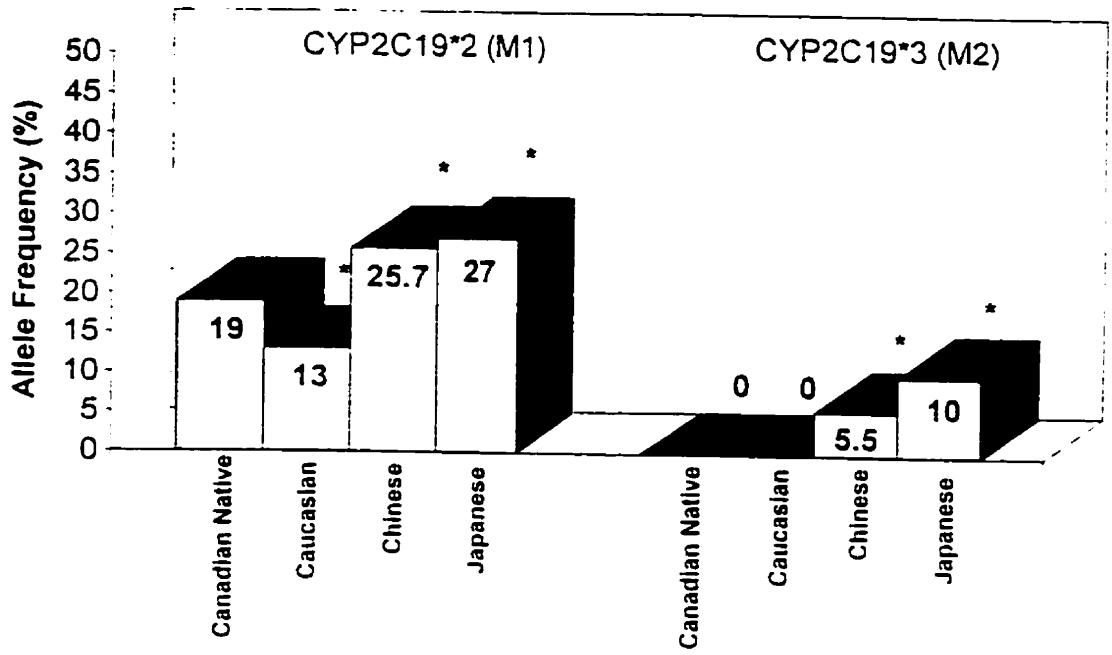

Figure 4.6 **Comparison of CYP2C19 mutant allele frequencies in four ethnic groups.** The frequency of the *CYP2C19*2* mutant allele in Canadian Native Indians is intermediate between the frequencies observed in Caucasian and Asian populations. The frequency of the *CYP2C19*3* mutant allele among Canadian Native Indians resembles the frequencies in previously observed Caucasian populations but is significantly lower the frequencies observed in previously studied Asian populations (*$p<0.05$).

Effect of Caucasian Admixture on CYP2C19 Mutant Allele Frequencies

Unlike CYP2D6, CYP2C19 mutant allele and PM frequencies did not exhibit a gene dose effect. There were no significant differences in the *CYP2C19*2* mutant allele frequency between the full Canadian Native Indians and the subjects with blood quantum factors of 75% (p=0.08), 50% (p=0.6), 25% (no stats, n=1) or total (75% + 50% + 25%) mixed-ancestry (p=0.2) Canadian Native Indian subjects combined. There was also no significant difference in the CYP2C19 PM (p=0.3) and CYP2C19*3 (p=0.99) frequencies between full and mixed-ancestry Canadian Native Indian subjects (Figure 4.4).

CYP2C19 and Drug Dependence

The CYP2C19 genotype data were analyzed to determine whether the CYP2C19*2 mutant allele or genotype was associated with an increased risk of drug dependence. No association was found between *CYP2C19*2* mutant allele frequency and dependence on alcohol (p=0.5), nicotine (p=0.06), cocaine (p=0.5) or codeine (p=0.13). In addition, no associations were found between CYP2C19 genotype (*1/*1 vs *1/*2 vs *2/*2) and dependence on alcohol (p=0.96), nicotine (p=0.1), cocaine (p=0.99) and codeine (p=0.05).

CYP2C19 and Gender

No significant differences were observed between males and females with respect to CYP2C19*2 mutant allele frequency (p=0.3) and CYP2C19 PM genotype frequency (p=0.6).

Table 4.4 CYP2C19 Mutant Allele Frequencies in Canadian Native Indians.

Blood Quantum Factor	Subjects Analyzed	*CYP2C19*2* Frequency (%)	*CYP2C19*3* Frequency (%)	Number of Homozygous Mutants	CYP2C19 PM Genotype Frequency (%)
100	115	19.1	0	8	7
75	18	8.3	0	0	0
50	24	18.8	0	1	4
25	2	0	0	0	0
Total	**159**	**17.6**	**0**	**9**	**5.7**

CYP2A6 Genotype

One hundred and fifty Canadian Native Indians were genotyped for the *CYP2A6*2* and *CYP2A6*3* mutant alleles. The *CYP2A6*2* and *CYP2A6*3* mutant allele frequencies in full Canadian Native Indians were 0.9% and 13.9%, respectively. The frequency of the *CYP2A6*2* mutant allele was not significantly different from the frequency observed in Caucasian (2.2%, n=270, p=0.2) and Chinese (0%, n=67, p=0.4) subjects genotyped in our laboratory and the Japanese (0%, n=20, p=0.7) subjects studied by Fernandez-Salguero *et al.* (1995). However, it was significantly lower than the CYP2A6*2 frequencies observed in Caucasian (16.7%, n=42, p<0.001), Taiwanese (11%, n=79, p<0.001) subjects reported by Fernandez-Salguero *et al.*, 1995) (Figure 4.7).

The frequency of the *CYP2A6*3* mutant allele in the Canadian Native Indian subjects (13.9%) was significantly higher than in Caucasians studied by Fernandez-Salguero (4.7%, n=42, p<0.02) and Caucasians genotyped in our laboratory (6.5%, n=270, p<0.001), but was not significantly different (12%, n=67, p=0.4) from the frequency observed in Chinese subjects (Figure 4.7)

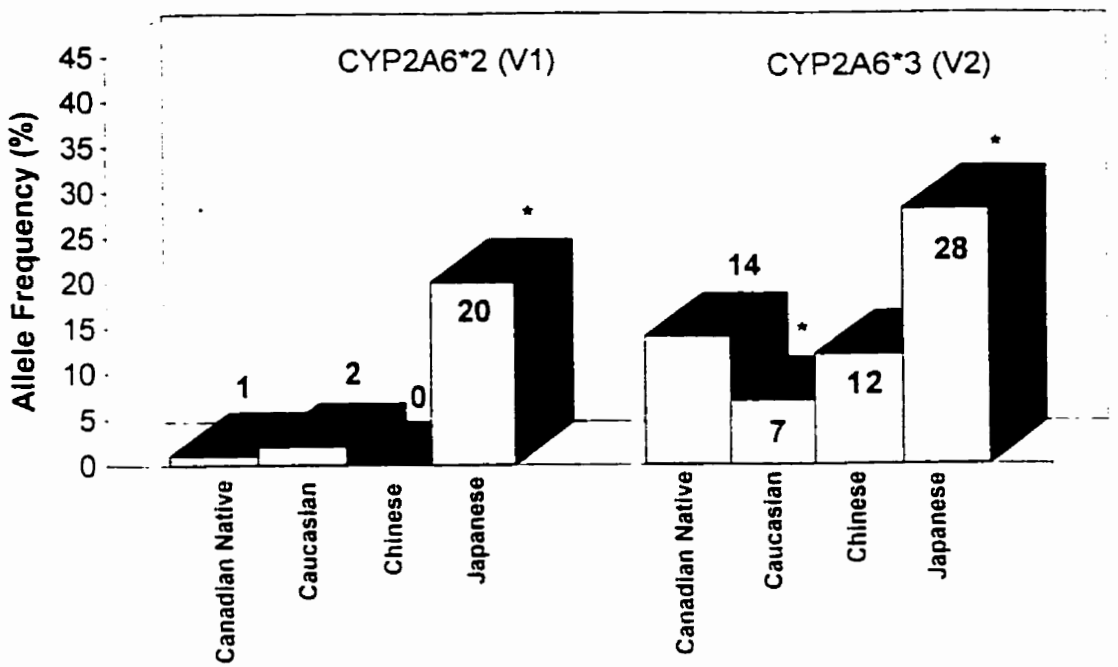

Figure 4.7 **Comparison of CYP2A6 mutant allele frequencies in four ethnic groups**. The *CYP2A6*2* mutant allele frequency in Canadian Native Indian subjects resembles the frequencies observed in our Caucasian and Chinese populations, but is significantly lower than the frequency observed in Japanese subjects studied by Fernandez-Salguero (1995). The frequency of the *CYP2A6*3* mutant allele in Canadian Native Indians resembles the frequency observed in Chinese subjects, but is significantly higher than the frequencies observed in both Caucasian and Japanese (Fernandez-Salguero, 1995) subjects (*$p<0.05$).

Effects of Caucasian Admixture on CYP2A6 Allele Frequencies

There were no significant differences in *CYP2A6*2* (p=0.3) and *CYP2A6*3* (p=0.5) mutant allele frequencies between the full and mixed-ancestry Canadian Native Indians. More in-depth analysis revealed no significant differences in the frequencies of these two alleles between full Canadian Native Indians and 75% (p=0.6 for *2, p=0.4 for *3), 50% (p=0.09 for *2, p=0.4 for *3) and 25% (p=0.9 for *2, p=0.7 for *3).

Table 4.5 CYP2A6 Mutant Allele Frequencies in Canadian Native Indians.

Blood Quantum Factor	Samples Analyzed	*CYP2A6*2* Frequency (%)	*CYP2A6*3* Frequency (%)	Number of Homozygous Mutants	Genotype-predicted PM Frequency (%)
100	108	0.9	13.9	0	0
75	24	0	10.4	0	0
50	17	6	17.6	1	6
25	1	0	0	0	0
Total	**150**	**1.3**	**13.6**	**1**	**0.7**

Tobacco Use

Since CYP2A6 is involved in nicotine metabolism, genotype and tobacco-use data were analyzed to determine whether individuals carrying CYP2A6 mutant alleles differed from CYP2A6 wild-type individuals with respect to tobacco-smoking behavior. *CYP2A6*1* homozygous subjects were compared to individuals carrying CYP2A6 mutant alleles (including one homozygous CYP2A6*2/*2) with respect to a number of tobacco variables obtained from the GAQ questionnaire.

There was no association between CYP2A6*2 frequency and dependence on tobacco (p=0.4), alcohol (p=0.15), cocaine (p=0.06), codeine (p=0.6) and amphetamine (p=0.7). No significant

differences (p=0.7) were observed in the ages at which subjects first smoked a cigarette. The means (mean ± S.D.) for *CYP2A6*1* homozygous subjects and heterozygotes were 14.3 ± 3.8 and 14.1 ± 4.1, respectively. There were also no significant differences between CYP2A6 wild-type homozygous subjects and mutant heterozygotes in the number of cigarettes smoked per day in the past 90 days (13.9 vs. 13.2; p=0.83), proportion of individuals who have tried smoking and gone on to smoke regularly (85.2% vs 72.8%; p=0.16), proportion of individuals who tried smoking but did not experience desirable effects (44.4% vs 50.0%; p=0.62), proportion of individuals who tried smoking but did not continue due to undesirable effects (44.4% vs 0%; p=0.09), number of cigarettes smoked per day among regular smokers (24.1 ± 14.0 vs. 20.9 ± 16.3; p=0.43), maximum number of cigarettes smoked in any 24 hour period among smokers (34.4 ± 16.8 vs 42.4 ± 29.1; p=0.3), how soon after waking up did the individual smoke the first cigarette (p=0.53) and in the proportion of smokers who have difficulty in refraining from smoking in places where it is forbidden (51.9% vs 37.5%; p=0.24).

The only significant differences between CYP2A6 wild-type homozygous subjects and mutant heterozygotes in the parameters studied was the proportion of individuals who smoked even when they were ill. A significantly higher (p=0.04) proportion of heterozygotes (53.8%) smoked when they were ill compared to CYP2A6 homozygous subjects (25.0%).

Association between CYP2D6, CYP2C19 and CYP2A6 Genotype Results

The genotype results for the three genes were analyzed in 108 Canadian Native Indian subjects. Thirteen subjects carried mutant alleles for more than one of the enzymes. Nine subjects were genotyped as CYP2C19*1/CYP2C19*2 and CYP2A6*1/CYP2A6*3. Two subjects were genotyped as CYP2C19*1/CYP2C19*2 and CYP2A6*1/CYP2A6*2 and two were genotyped as CYP2C19*2/CYP2C19*2 and CYP2A6*1/CYP2A6*3 (Table 4.6).

Table 4.6 Genotype combinations for the CYP2D6, CYP2C19 and CYP2A6 for 108 full Canadian Native Indians.

CYP2D6 Genotype				CYP2C19 Genotype			CYP2A6 Genotype			
1/1	1/4	1/10	4/4	1/1	1/2	2/2	1/1	1/2	1/3	n=
x				x			x			48
x				x				x		2
x				x					x	19
x					x		x			16
x					x				x	9
x					x			x		2
x						x	x			5
x						x			x	2
		x		x			x			1
	x			x			x			3
			x	x			x			1
										108

A similar analysis was performed on 43 mixed ancestry subjects. One mixed ancestry subject tested positive for mutant alleles of all three genes. The CYP2D6, CYP2C19 and CYP2A6 genotypes for this subject were *CYP2D6*1/CYP2D6*4*, *CYP2C19*2/CYP2C19*2* and *CYP2A6*1/CYP2A6*3*., respectively (Table 4.7).

Table 4.7 **Genotype combinations for the CYP2D6, CYP2C19 and CYP2A6 for 43 mixed-ancestry Canadian Native Indians.**

CYP2D6 Genotype						CYP2C19 Genotype			CYP2A6 Genotype			
1/1	1/4	1/10	10/10	4/4	4/10	1/1	1/2	2/2	1/1	2/2	1/3	n=
x						x			x			16
x						x					x	3
x							x		x			2
x							x				x	2
		x					x				x	1
	x					x			x			7
	x					x					x	1
	x					x				x		1
	x						x		x			3
	x							x			x	1
			x				x		x			1
					x	x					x	2
					x		x		x			1
				x		x			x			1
				x		x					x	1
												43

Discussion

Canadian Native Indians are descendants of Asian populations which migrated to North America between 13,000 and 30,000 years ago. They are a unique population which was genetically isolated in the North American environment for thousands of years; an environment which differs from that of Asia with respect to climate, flora and fauna. Since cytochrome P450 enzymes are involved in "plant-animal warfare", these environmental differences, including diet, may have resulted in unique selective pressures which, ultimately, may have had an impact on the genotype patterns of cytochrome P450 enzymes in Native North American populations. Since these enzymes are involved in the metabolism of many commonly used drugs, understanding their genotype in these populations may have important clinical implications. Moreover, understanding the genotype of these enzymes in Canadian Native Indians may also provide insight into the evolutionary histories of these populations.

Subject Characteristics

The study population consisted on 109 male and 50 female Canadian Native Indian volunteers ranging from 16 to 62 years of age with a mean age of 33.8 $\pm$ 9.9 years (mean $\pm$ S.D.). One hundred and fifteen subjects reported full Canadian Native Indian ancestry and 44 subjects reported mixed Canadian Native Indian ancestry.

CYP2D6 Phenotype

The log ODMR frequency distribution of full Canadian Native Indians revealed a CYP2D6 PM frequency of 1.1%. This resembled the CYP2D6 PM frequency in the Chinese population, but was significantly lower than the CYP2D6 PM frequency observed in the Caucasian population. The

CYP2D6 PM frequency observed in Canadian Native Indian subjects agreed with our original hypothesis which stated that due to their Asian origins, Canadian Native Indians would resemble the Chinese with respect to the low prevalence of the CYP2D6 PM phenotype.

In contrast, the mean log ODMR of -2.56 in the Canadian Native Indian population did not resemble the mean of -2.15 observed in Chinese subjects. It did, however, resemble the Caucasian population which exhibited a mean log ODMR of-2.57. Unlike the Chinese, the full Canadian Native Indian subjects did not exhibit a right-ward shift in the log ODMR frequency distribution relative to Caucasian populations, which would be indicative of decreased CYP2D6 metabolic activity among EM subjects. As a result, Canadian Native Indian EM subjects appear to have a 'normal' CYP2D6 metabolic activity relative to Caucasian populations.

Possible Genetic Basis for Phenotype Results

In interpreting these phenotype data, we have proposed a genetic basis for low CYP2D6 PM frequency and the absence of a right-ward shift among Canadian Native Indian subjects.The low prevalence of the CYP2D6 PM phenotype suggests that Canadian Native Indians may exhibit low frequencies, or altogether lack, one or both of the *CYP2D6*3* and *CYP2D6*4* null mutant alleles. These two mutations account for approximately 90% of Caucasian CYP2D6 PM subjects. In addition, the absence of a right-ward shift in the log ODMR frequency distribution of Canadian Native Indians may indicate that the *CYP2D6*10* mutant allele is present at low frequencies, or is altogether absent, in this population. *CYP2D6*10* is present in Asian populations at frequencies of up to 70%. It is responsible for decreased mean metabolic activity of the CYP2D6 enzyme in these populations. However, since the Canadian Native Indian population does not appear to exhibit decreased CYP2D6 activity among EM subjects, these data suggest that the *CYP2D6*10* mutant

allele is not prevalent in this population.

CYP2D6 Genotype

To investigate these possibilities, Canadian Native Indian subjects were genotyped for the *CYP2D6*3*, *CYP2D6*4* and *CYP2D6*10* mutant alleles using a combination of PCR and RFLP. Genotyping revealed that the *CYP2D6*3* null mutant allele was absent in the Canadian Native Indian population while *CYP2D6*4* was detected at a frequency of 3%. As hypothesized, both the *CYP2D6*3* and *CYP2D6*4* null mutant alleles were present at frequencies which were similar to those observed in Asians, but lower than those observed in Caucasians.

With a CYP2D6*10 mutant allele frequency of 3%, Canadian Native Indians resembled Caucasians with respect to CYP2D6*10. However, this frequency was significantly lower than the 47-70% observed in previously studied Asian populations. Although this result did not agree with our initial hypothesis regarding CYP2D6*10, it was supported by the phenotype results which did not show a right-ward shift of log ODMR values in Canadian Native Indian subjects.

Concordance Between CYP2D6 Phenotype and Genotype

One hundred and thirty-five of the 136 subjects who were phenotyped displayed full concordance between *CYP2D6* phenotype and genotype. The phenotypes and genotypes of 136 subjects are illustrated in figure 4.4. This figure demonstrates the effects of the *CYP2D6*4* and *CYP2D6*10* mutant alleles on *CYP2D6* phenotype. The subjects who were positive for the CYP2D6*4 and/or CYP2D6*10 mutant alleles exhibited mean log ODMR values which were above the mean of -2.56, indicating decreased CYP2D6 metabolic activity. This is in agreement with previous studies which have shown that CYP2D6*10 homozygotes and heterozygotes, as well as

CYP2D6*4 heterozygotes, tend to exhibit a lower CYP2D6 metabolic activity than individuals who are CYP2D6*1 (wild-type) homozygous.

The lone full Canadian Native Indian PM subject, who had a log ODMR of 0.77, could not be accounted for by either the *CYP2D6*3* or the *CYP2D6*4* mutant alleles. Consequently, there was a lack of concordance between phenotype and genotype with respect to this individual as the EM genotype result does not agree with the PM log ODMR of 0.77. We suspect that this individual may have been classified as a *CYP2D6* PM as a result of one of the following: 1) enzyme inhibition due to concomitant use of medication which may be a *CYP2D6* inhibitor and was not reported during the medical GAQ interview, 2) concomitant use of an as yet unidentified CYP2D6 inhibitor, 3) presence of rare mutant alleles such as *CYP2D6*6, *7, *8, *11, *12, *13, *14, *15, *16 or others which were not tested for in this study, or 4) presence of novel mutant allele(s) which result in a PM phenotype. We have attempted to contact this individual in order to phenotype her again, however she could not be reached.

In addition to the full Canadian Native Indian PM, phenotyping also revealed two mixed-ancestry subjects who were poor metabolizers of dextromethorphan. Both of these subjects were genotyped as *CYP2D6*4/CYP2D6*4* homozygotes.

Effects of Caucasian Admixture on CYP2D6 Expression in Mixed-Ancestry Subjects

Comparisons of the *CYP2D6* PM frequency and the mean log ODMR between full and mixed-ancestry Canadian Native Indians did not reveal any statistically significant differences between the two groups. However, significant differences in *CYP2D6*4* and *CYP2D6*10* mutant allele frequencies were observed between full and mixed-ancestry Canadian Natives. The *CYP2D6*4* allele was present at a frequency of 3% in full ancestry Canadian Native Indians and 22.7% in mixed-

ancestry subjects. Statistical analysis showed this difference to be significant at the 0.001 level.

In fact, the observed increase in *CYP2D6*4* allele frequency in mixed-ancestry subjects was greater than would be expected based on the *CYP2D6*4* allele frequencies reported in previously studied Caucasian populations (20-23%). Given that just over 40% (36/88) of the gene pool among the mixed-ancestry subjects was Caucasian, we would expect the mixed-ancestry subjects to exhibit a *CYP2D6*4* allele frequency of approximately 9.7% [n=36(0.20) + n=52 (0.026) = 8.55; 8.55/88 alleles=9.7%). It is unclear why the mixed ancestry subjects exhibited such a high *CYP2D6*4* null mutant allele frequency. This may be due to a small number of Caucasian alleles in the mixed-ancestry group (n=36) or due to Caucasian admixture many generations ago which would not have been reported by study subjects.

The *CYP2D6*10* mutant allele was detected in mixed-ancestry subjects at a frequency of 6.8% which was higher than the 2.6% observed in their full Canadian Native Indian counterparts. This difference was statistically significant at the 0.01 level indicating that Caucasian admixture was also associated with the increased *CYP2D6*10* mutant allele frequency. Once again this frequency is higher than what we would expect based on previously reported frequencies of *CYP2D*10* in Caucasian populations (n=36 [0.015] + n=52[0.03] = 2.1; 2.1/88=2.4%).

The *CYP2D6*3* mutant allele was not present in the mixed-ancestry subjects. This result was expected given that this allele accounts for only 2% of Caucasian *CYP2D6* alleles.

CYP2D6 Summary

The phenotype results have shown that Canadian Native Indians resemble Asians with respect to CYP2D6 PM frequency, which is consistent with the low frequencies of the *CYP2D6*3* and *CYP2D6*4* null mutant alleles, but differ from Caucasians with respect to these parameters. In

contrast, Canadian Native Indian subjects resemble Caucasians with respect to mean CYP2D6 metabolic activity as determined by dextromethorphan O-demethylation and low *CYP2D6*10* mutant allele frequency. These results are interesting given that previous studies have shown that, when compared to Caucasians, populations of Asian origin exhibit both a decreased *CYP2D6* PM frequency and a decreased CYD2D6 metabolic activity among Asian EM subjects.

Table 5.1 **Summary of *CYP2D6* Results**. Comparison of Canadian Native Indian results to Caucasian and Asian data. Characteristics which resemble those of Canadian Native Indians are presented in bold.

	Ethnic Group		
***CYP2D6* Characteristic**	**Canadian Native Indian**	**Chinese**	**Caucasian**
PM Frequency	**1.1%**	**1.2%**	6.2%
Mean log ODMR	**-2.56**	-2.15	**-2.57**
*CYP2D6*3* Frequency	**0%**	**0%**	2%
*CYP2D6*4* Frequency	**3%**	**0%**	20-23%
*CYP2D6*10* Frequency	**3%**	50%	**1.5-5%**

Possible Explanations for Low *CYP2D6*10* Frequency in Canadian Native Indians

It is unclear why Canadian Native Indians resemble Asian populations with respect to *CYP2D6* PM frequency, *CYP2D6*3* and *CYP2D6*4* allele frequencies, but not with respect to *CYP2D6* mean metabolic activity and *CYP2D6*10* frequency. Consistent with our hypothesis, Canadian Native Indians resemble Asians with respect to: 1) the frequency of the CYP2D6 PM phenotype, 2) the allele frequency of *CYP2D6*3* and 3) the allele frequency of *CYP2D6*4*. These results were expected as both Asians and Canadian Native Indians share common genetic ancestry.

On the other hand, the fact that Canadian Native Indians resemble Caucasians, and not Asians, with respect to CYP2D6 mean metabolic activity and *CYP2D6*10* allele frequency was not predicted

by our hypotheses. This discrepancy may be explained by the separate and distinct evolutionary histories of Canadian Native Indian and Asian populations over tens of thousands of years. According to Hardy-Weinberg principle, "both the allele and genotype frequencies in a large, random-mating population will remain constant from generation if there is no mutation, no migration, and no selection" (Raven & Johnson, 1989). The Hardy-Weinberg principle states that a number of factors can alter allele frequencies. The factors which might best explain our observations include mutation, genetic drift, and selection.

Mutation is unlikely to play an important role in this discussion as mutation rates occur at such slow rates that they have little impact on the frequencies of common alleles (e.g. *CYP2D6*10)* (Raven & Johnson, 1989). For example, the mutation rate of mammalian P450 genes has been estimated at 1% in amino acid sequence every 4 million years (Nelson and Strobel, 1987). Given that Canadian Native Indians and Asians have been separated for no more than 30,000 years, mutation probably had minimal influence on the differences in CYP2D6*10 frequencies between the two groups.

The large difference in *CYP2D6*10* allele frequencies between modern Asian and Canadian Native Indian populations may, however, be explained by two forms of genetic drift, the founder principle and the bottleneck effect. Genetic drift is defined as "a random change in the frequency of alleles at a locus." (Raven & Johnson, 1989). Although genetic drift has negligible impact on large populations, in small populations it may lead to the increase or decrease of certain alleles. One type of genetic drift is known as the founder principle; a process whereby the frequencies of certain alleles become enhanced or decreased when small populations migrate and become founders of a new population. In such cases, the alleles which the new founders carry are of special significance given that the newly arising populations are often dominated by alleles which were present in the original population.

The founder effect may be applicable to modern Canadian Native Indian populations which were founded by migrating Asian populations tens of thousands of years ago. If the founding Asian populations were small and carried low frequencies of the *CYP2D6*10* mutant allele, it is quite possible that the differences in *CYP2D6*10* frequencies between modern Canadian Native Indian and Asian populations may be attributed to the founder principle.

Another form of genetic drift is known as the bottleneck effect. Unlike the founder effect, which involves the migration of small founding populations to new locations, the bottleneck effect is used to describe populations which have experienced drastic reductions in size due to events such as floods, fires, earthquakes, disease or other more progressive changes in environment. Any one of these events may result in a small surviving population which is a random sample of the original population (Raven & Johnson, 1989). As a result, the alleles present in the surviving population may be over-propagated relative to the original population.

The bottleneck effect may also be applicable to modern Canadian Native Indian populations. If we were to accept the bottleneck effect as an explanation for allele frequency differences between modern Asian and Canadian Native Indian populations, we would hypothesize that the Asian population which migrated across the Bering Strait was genetically similar to other Asian populations. Following their arrival in North America, the founding population experienced conditions which resulted in a drastic reduction in its numbers. This may have been a result of extreme cold, drought, inadequate diet, or other factors such as small pox, which affected many North American Indians. By chance, the surviving population carried a low frequency of the *CYP2D6*10* allele which, in turn, resulted in a low *CYP2D6*10* mutant allele frequency in modern Canadian Native Indians.

The final explanation for the differences in *CYP2D6*10* frequencies observed between Canadian Native Indian and Asian populations involves natural selection. Since selection acts on

phenotype, we would hypothesize that some environmental factor(s) in the North American environment, which was/were absent in Asia, conferred a genetic advantage to individuals with high *CYP2D6* metabolic activity. For example, if one accepts the "plant-animal warfare" concept proposed by Gonzalez and Nebert (1990), one can imagine that some North American plants, which were not present in Asia, may have produced xenobiotic compounds (phytoalexin) which were harmful to individuals with decreased *CYP2D6* activity. As a result, the *CYP2D6*10* allele frequency was drastically decreased in these populations over tens of thousands of years. Such a phenomenon is often termed stabilizing or directional selection. It acts to eliminate phenotypes which deviate from the norm thereby resulting in a phenotypically homogenous population with respect to the trait being selected for.

The difference between the proposed mechanisms by which the frequency of the *CYP2D6*10* allele was decreased in the Canadian Native Indians is that mutation, the founder effect and the bottleneck effect are neutral from an evolutionary perspective in that they occur in a random fashion and therefore do not offer any genetic advantage to the population. In contrast, natural selection acts to increase fitness and survival potential of the affected populations. Therefore, if variations in cytochrome P450 enzymes, and more specifically *CYP2D6*, increase survival potential then the latter explanation is the most likely.

CYP2D6 Polymorphism in a Canadian Inuit Population

A recent study examining CYP2D6 in Canadian Inuit subjects has shown that this population resembles Canadian Native Indians with respect to the CYP2D6 (Jurima-Romet *et al.*, 1997).The *CYP2D6*3* null mutant allele was absent in Canadian Inuits. This was not significantly different ($p=0.99$) from the Canadian Native Indian results. The *CYP2D6*4* null mutant allele was present at

a frequency of 6.7-8.3%. The difference in CYP2D6*4 mutant allele frequencies between the two groups barely reached significance (p=0.0489) (Table 5.2). *CYP2D6*10* was found in the Canadian Inuit population at a frequency of 2.2%. This was not significantly different (p=0.4) from the 3% observed among Canadian Native Indian subjects.

The mean log ODMR of the Inuit subjects was not compared to Asian and Caucasian populations, however, the low *CYP2D6*10* mutant allele frequency in this population would suggest that Canadian Inuit EM subjects probably resemble Caucasian EMs with respect to mean *CYP2D6* activity. The PM frequency in the Inuit population was determined to be 3.3% which is also not significantly different (p=0.3) from the 1.1% observed among Canadian Native Indians.

Table 5.2 Comparison of Canadian Native Indian and Canadian Inuit populations with respect to the CYP2D6 polymorphism.

Ethnic Group	Number of Subjects	CYP2D6 PM Frequency	*CYP2D6*3* Frequency	*CYP2D6*4* Frequency	*CYP2D6*10* Frequency
Native Indian	115	1.1%	0%	3%	3%
Inuit	90	3.3%	0%	7-8%	2%
Caucasian	569	5-10%	2%	21%	1.5%
Chinese	93 & 113	1%	0%	0%	51%

In summary Canadian Native Indians resemble Canadian Inuits with respect to the CYP2D6 polymorphism. The similarities in the frequency of the *CYP2D6*10* mutant allele is especially interesting as it lends support to the role of natural selection in decreasing *CYP2D6*10* mutant allele frequencies among Native North American populations.

According to Greenberg's hypothesis, modern Native North American populations, namely

the Na Dene, the Eskimo-Aleut (eg. Inuit) and Indians (eg. Canadian Native Indians), arrived in North America as three distinct groups in three separate migrations across the Bering Strait. Since these populations originated from Asia, it is probable that they carried relatively high frequencies of the *CYP2D6*10* mutant allele. It is unlikely that the *CYP2D6*10* mutant allele frequency was decreased in both the Canadian Native Indian and Inuit populations though random events such as a founder effect or a bottleneck effect. The fact that both of these North American populations exhibit low frequencies of *CYP2D6*10* suggests that natural selection may have been involved.

Drug Metabolism

From the view of clinical pharmacology, Canadian Native Indians may represent a population which is less likely to experience concentration-related toxicity reactions to drugs which are extensively deactivated to an inactive metabolite by CYP2D6. Since CYP2D6 PM subjects are less common than CYP2D6 EM subjects, they are more susceptible to such side-effects due to the fact that, through chance alone, new drugs are tested on populations which are composed primarily of CYP2D6 EMs.

Theoretically, approximately 5-10% of Caucasian and 1% of Asian subjects are at an increased risk of experiencing concentration-dependent side effects to drugs which are metabolized by CYP2D6. This is due to a deficient CYP2D6-mediated metabolism. In addition, a large proportion of Asian patients are also at an increased risk of developing similar complications due to their decreased mean CYP2D6 activity compared to Caucasians. Consequently, Asian patients may experience a higher incidence of adverse reactions to CYP2D6-substrate drugs in comparison to Caucasian EM subjects as a result of decreased CYP2D6-mediated metabolism of these drugs.

Our results suggest that Canadian Native Indian patients may have a lower genetic

susceptibility to concentration-related toxicity effects of CYP2D6-metabolized drugs in comparison to Caucasian and Asian populations. This implies that low prevalence of the CYP2D6 PM phenotype and absence of a right-ward shift in CYP2D6 mean metabolic activity observed in Canadian Native Indians may prove to be advantageous in clinical situations where CYP2D6 substrate drugs are prescribed.

Implications in Disease Susceptibility

Our phenotype and genotype results suggest that Canadian Native Indians might be genetically less susceptible than Caucasians to diseases associated with deficient *CYP2D6* activity and more susceptible to diseases in which *CYP2D6* is involved in the bioactivation of precursors into active toxins. For example, since individuals with decreased/deficient *CYP2D6* activity are relatively rare in Canadian Native Indians, Parkinson's disease (PD) and Alzheimer's disease (AD) may be less prevalent among Canadian Native Indian populations than among Caucasian populations. This is assuming that environmental conditions and diet are similar among the two populations. Although we have not been able to find epidemiological data on PD in North American Indian populations, several studies have suggested North American Indians may have a decreased risk of developing AD.

A study involving Cherokee Indians in northeastern Oklahoma has shown an inverse relationship between the genetic degree of Cherokee ancestry and the prevalence of AD, such that as the blood quantum of Cherokee ancestry decreases, the risk of developing AD increases. The study also found that, at age 65, a decrease of 10% in Cherokee ancestry resulted in a nine-fold higher risk of developing AD (Rosenberg *et al.*, 1996). Another study involving Cree Indians in northern Manitoba found the prevalence of AD among Cree Indians to be 0.5%, which was significantly lower ($p<0.001$) than the prevalence of 3.5% observed among Caucasian subjects in Winnipeg, Manitoba

(Hendrie *et al.*, 1993). Although both of these studies have shown that North American Indians may be less susceptible to AD than Caucasians, the results should be interpreted with caution as many North American Indians reside in different physical environments and consume traditional foods, which are different from those of other North Americans. These differences may partially account for the differences in AD susceptibility.

Since 99% of Canadian Native Indian subjects are CYP2D6 extensive metabolizers who do not exhibit a decreased CYP2D6 activity, it could be hypothesized that Canadian Native Indians are genetically more susceptible than Caucasians to cancers which have been associated with the CYP2D6 EM phenotype. These include cancers of the lung, bladder, liver and colon.

At the turn of the century, cancer was rare among Native North American populations (Hrdlicka, 1908). Today, cancer rates among most North American Indian populations are significantly lower than in the general populations of Canada and the United States (Sievers and Fisher, 1983; Mahoney and Michalek, 1991; Cobb, 1996; Baquet, 1996; Byers, 1996; Gaudette *et al.*, 1991; Young and Choi, 1985; Young and Frank, 1989). However, rates of some cancers are rising steadily among North American Natives, thereby approaching the rates observed in the non-Native population. In fact, Alaska Natives exhibit cancer rates which exceed those observed in non-Native American and Canadian populations (Mahoney and Michalek, 1995).

There is growing concern that these increases in cancer rates among Native communities are due to changes in diet and environment which include: increased tobacco use, decreased physical activity and a transition from a traditional diet rich in fruits and vegetables to a Western diet poor in fruits and vegetables and rich in fat (Cobb, 1996; Byers, 1996). For example, lung cancer rates are increasing dramatically as a large number of Native youth take up smoking. Southwest tribes, where smoking rates have been very low (~20%), exhibit lung cancer death rates of 7.6 per 100,000 whereas

tribes in the Northern Plains and Alaska, which exhibit smoking rates of approximately 70% exhibit lung cancer death rates of 74 deaths per 100,000 each year (Cobb, 1996). Since 87% of all lung cancers are caused by cigarette smoking, the easiest way of decreasing lung cancer death rates is to decrease tobacco use among Native youth (US Department of Health and Human Services, 1989).

The rate of regular tobacco use among the Canadian Native Indian subjects in this study was 68.6%. This is more than double the 33% reported for the overall Canadian population (Gaudette *et al.*, 1991). Large differences in lifestyle exposures between these populations make it extremely difficult to discern between the environmental and genetic factors which affect cancer rates. This is especially true in light of the fact that 70-80% of cancers in the United States are believed to be attributed to environmental exposure (Doll and Peto, 1981).

In summary, although we have hypothesized that Canadian Native Indians may be genetically more susceptible to certain types of cancers than Caucasians, this is difficult to support with epidemiological data due to the vast differences in environmental and lifestyle exposures between the two groups. These include differences in tobacco-use, drug use, diet and physical environment.

CYP2C19 Polymorphism

The CYP2C19 polymorphism was investigated in all 159 Canadian Native Indian subjects. CYP2C19 phenotype results were not available for these subjects. All Canadian Native Indian subjects were genotyped for the two CYP2C19 mutant alleles described by de Morais *et al*, *CYP2C19*2* and *CYP2C19*3* (de Morais *et al.*, 1994a & 1994b). The CYP2C19 PM genotype frequencies were determined by calculating the proportion of subjects who were homozygous for CYP2C19 mutant alleles.

CYP2C19 Genotype

The *CYP2C19*2* mutant allele was present at a frequency of 19.1% , while the *CYP2C19*3* mutant allele was not detected among the full Canadian Native Indians. Therefore, it appears that Canadian Native Indians do not resemble Asian populations with respect to either *CYP2C19*2* or *CYP2C19*3*. Both of these alleles are present at significantly higher frequencies in Asian populations. Canadian Native Indians exhibit a *CYP2C19*3* null mutant allele frequency of 0% which resembles the frequency observed in previously studied Caucasians (Ferguson et al., 1997)

The frequency of the *CYP2C19*2* mutant allele in Canadian Native Indians was significantly higher than in Caucasians but significantly lower than in Asians. As a result, it appears that Canadian Native Indians exhibit a *CYP2C19*2* allele frequency which is intermediate between the *CYP2C19*2* null mutant allele frequencies observed in Asian and Caucasian populations.

The frequency of the CYP2C19 PM genotype in the Canadian Native Indian population was 7% (8/115). Once again this value is intermediate between the 2% PM frequency observed in Caucasian populations and the 14% observed in Chinese populations.

In summary, these data suggest that Canadian Native Indians resemble Caucasians with respect to *CYP2C19*3* mutant allele frequency and are intermediate between Caucasian and Asian populations with respect to *CYP2C19*2* and CYP2C19 PM frequencies.

Effect of Caucasian Admixture on CYP2C19 Mutant Allele Frequencies

The mixed-ancestry subjects exhibited a *CYP2C19*2* mutant allele frequency of 13.6% compared to 19.1% observed in full Canadian Native Indian subjects. This difference was not significant at the 0.05 level. Since the *CYP2C19*3* allele was absent in both Caucasian and Canadian

Native Indian populations, no gene dose effect was observed in the frequency of this allele between full and mixed-ancestry Canadian Native Indians. Finally, there were no significant differences in the CYP2C19 PM genotype frequencies between full and mixed-ancestry Canadian Native Indians.

In summary, a gene dose effect in CYP2C19 mutant allele and CYP2C19 PM frequencies was not observed in the mixed ancestry subjects.

CYP2C19 in Canadian Inuit population

A recent study of the CYP2C19 polymorphism in 90 Canadian Inuit subjects revealed *CYP2C19*2* and *CYP2C19*3* mutant allele frequencies of 12% and 0%, respectively. The PM frequency in this population was 3.3% (Jurima-Romet, 1996). As was the case with *CYP2D6*, the CYP2C19 results in Inuits resemble our observations in Canadian Native Indians.

First, both populations lack the *CYP2C19*3* mutant allele. Second, the PM frequencies are significantly lower in these populations when compared to other populations of Asian origin. Finally, both populations exhibit *CYP2C19*2* mutant allele frequencies which are closer to the frequencies observed in Caucasians than Asians.

The fact that these two populations: 1) are both descendants of North Asian populations, 2) arrived in North America independently during two separate migratory waves and 3) both exhibit CYP2C19 mutant allele frequencies which are significantly different from those observed in other Asian populations, all suggest that the differences in CYP2C19 gene frequencies between Asians and Native North Americans may also be attributed to natural selection. As with CYP2D6, it appears that higher CYP2C19 activity provides an evolutionary advantage compared to decreased/deficient CYP2C19 activity.

Clinical Implications

Although CYP2C19 has not yet been implicated in disease susceptibility, it has been shown to have significant consequences with respect to drug side effects. For example, while the frequency of reported side effects to mephobarbital, a CYP2C19 substrate, is approximately 20% in Japanese populations, it is only 3.5% in Caucasian populations (Nakamura *et al.*, 1985). It is believed that this six-fold difference in the incidence of mephobarbital side effects is due to a roughly six-fold difference in CYP2C19 PM phenotype frequencies between the two populations.

Since Canadian Native Indians more closely resemble Caucasians than Asians with respect to the CYP2C19 polymorphism, it is reasonable to assume that this population is not at an increased risk of developing toxicity-related side effects to drugs which are metabolized primarily by CYP2C19. Unlike Asians subjects, which require lower doses of these drugs, Canadian Native Indians can be given doses of CYP2C19-substrate drugs which are comparable to those administered to Caucasian patients.

CYP2A6 Polymorphism

One hundred and fifty Canadian Native Indian subjects were genotyped for the *CYP2A6*2* and *CYP2A6*3* mutant alleles. The frequency of the *CYP2A6*2* mutant allele in Canadian Native Indian subjects (n=108) was 0.9%. This frequency was significantly lower than the frequencies observed in Caucasian, Taiwanese and Japanese populations by Fernandez-Salguero (1995). The frequency of the *CYP2A6*3* mutant allele in Canadian Native Indians was 13.9%. This was significantly higher than the frequencies reported in Caucasian and Taiwanese populations, but was significantly lower than reported in a Japanese population studied by Fernandez-Salguero (1995). These results suggest that Canadian Native Indians do not resemble any of the previously studied

populations with respect to CYP2A6 mutant allele frequencies.

However, Caucasians previously genotyped by Cholerton and Idle revealed a *CYP2A6*2* mutant allele frequency of 1% (unpublished data, referred to in Fernandez-Salguero and Gonzalez, 1995). We have genotyped 270 Caucasian and 67 Chinese subjects and found *CYP2A6*2* frequencies of 2.2% and 0%, respectively. Both of these frequencies resemble the frequency observed by Cholerton and Idle. Taken together, these data suggest that the allele frequencies published by Fernandez-Salguero may be over-estimated. This may be due to the small sample size examined (e.g. $n_{Finnish}=13$, $n_{English}=29$).

Statistical comparisons of Canadian Native Indians and our Caucasian and Asian control populations revealed that Canadian Native Indians (0.9%) resembled both Asian (0%) and Caucasian (2.2%) subjects with respect to *CYP2A6*2*. The frequency of the *CYP2A6*3* mutant allele among Canadian Native Indian (13.9%) subjects was not significantly different from Asians (12.0%) and was significantly higher than the frequency observed among Caucasians (6.5%).

Effects of Caucasian Admixture on CYP2A6 Mutant Allele Frequencies

No significant differences were observed in *CYP2A6*2* mutant allele frequencies between full and mixed-ancestry Canadian Native Indian subjects, suggesting that Caucasian admixture had no effect on allele frequencies. This result was expected for the *CYP2A6*2* mutant allele since Caucasians and Canadian Native Indians genotyped in our laboratory did not differ significantly in the allele frequency of *CYP2A6*2*. There were also no significant differences in *CYP2A6*3* mutant allele frequencies between the full and mixed-ancestry Canadian Native Indian subjects.

One mixed-ancestry subject was genotyped as a possible CYP2A6 deficient individual. This individual's genotype was CYP2A6*2/*2 which is indicative of a CYP2A6 PM phenotype.

Possible Clinical Implications

In vivo studies have shown that CYP2A6 plays an important role in nicotine metabolism in humans. Approximately 70-80% of nicotine is metabolized to the inactive metabolite cotinine via CYP2A6 (Nakajima *et al.*, 1996; Messina *et al.*, in press). Therefore, we hypothesize that CYP2A6 activity may be an important risk factor in tobacco dependence. Individuals who exhibit deficient CYP2A6 metabolism may be at a lower risk of becoming dependant on tobacco. Upon trying smoking for the first time, CYP2A6 PM subjects may obtain higher plasma levels of nicotine compared to CYP2A6 EM subjects. As a result, CYP2A6 PMs may experience greater aversive effects to nicotine which may make them less likely to continue smoking. In addition, CYP2A6 PM smokers may smoke less than CYP2A6 EM smokers due to decreased elimination of nicotine via the CYP2A6 pathway.

Our data indicate that there were no significant differences in CYP2A6 mutant allele frequencies between smokers and nonsmokers. Since our study population contained only one CYP2A6 PM individual, we have compared the smoking histories between CYP2A6 wild-type homozygous and heterozygous subjects to determine whether CYP2A6 heterozygous subjects exhibited different smoking patterns compared to CYP2A6 wild-type homozygous individuals. We found no significant differences between the two groups with respect to: age first tried, average number of cigarettes smoked per day during last 90 days, proportion of individuals who have tried smoking and gone on to smoke regularly, proportion of individuals who tried smoking but did not experience desirable effects, proportion of individuals who tried smoking but did not continue due to undesirable effects, number of cigarettes smoked per day among regular smokers, maximum number of cigarettes smoked in any 24 hour period among regular smokers, how soon after waking up the individual smoked the first cigarette and finally, the proportion of smokers who had difficulty

refraining from smoking in places where it was forbidden. We did, however, find a significant difference (p=0.04) in the proportion of individuals who smoked even when they were ill. In summary, there does not appear to be a difference in the smoking behaviours of CYP2A6 wild-type homozygous and mutant heterozygous subjects.

Association Between CYP2D6, CYP2C19 and CYP2A6 Genotypes

Genetic analysis of 108 Canadian Native Indian subjects revealed that 13 individuals were positive for both the *CYP2C19* and *CYP2A6* mutant alleles (Figure 4.6). Since the frequencies of CYP2C19 and CYP2A6 mutant alleles in full Canadian Native subjects were 19.1% (19.1 + 0) and 14.8% (13.9 + 0.9) one would expect that 3/108 (2.8%) of subjects would test positive for both alleles. Our results show that 13/108 (12.0%) full Canadian Native Indian subjects exhibit this combination of genotypes. This difference is significant at the 0.01 level. This suggests that there is an association between the presence of CYP2A6 and CYP2C19 mutant alleles. This may be explained by the presence of selective pressures which favour the presence of these alleles. For instance,

A similar analysis was performed on 43 mixed ancestry subjects. The predicted rate of finding an individual with both *CYP2C19* and *CYP2A6* mutant alleles in these subjects was 1.78% (1/43) subjects. Our results show that four subjects were positive for both *CYP2C19* and *CYP2A6* mutant alleles. This is not significantly different (p=0.2) from the predicted rate.

Neither the full or mixed-ancestry subjects exhibited any association between CYP2D6 and CYP2C19 or CYP2A6 mutant alleles.

Discussion Summary

Canadian Native Indians do not completely resemble Caucasians or Asians with respect to P450 polymorphisms. They exhibit patterns of expression of CYP2D6, CYP2C19 and CYP2A6 which are unlike those of previously studied populations, with the exception of Canadian Inuits. These differences have probably arisen due to the selective pressures which were absent in the Asian environment.

We have shown that although Canadian Native Indians resemble Asians with respect to the frequencies of the *CYP2D6*3* and *CYP2D6*4* null mutant alleles, they do not resemble Asians with respect to the allele frequencies of *CYP2D6*10*, *CYP2C19*2*, *CYP2C19*3*, and *CYP2A6*3*. While some of these allele frequencies resemble those of Caucasian populations, others seem to be intermediate between Caucasian and Asian populations. These differences in P450 expression may have important implications with respect to disease susceptibility, drug dosing and drug-taking behaviour.

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Appendix I

VOLUNTEERS NEEDED FOR RESEARCH ON ENZYMES

COMPENSATION $30.00

PHASE II - Canadian Native People Only

Participation involves - brief questionnaire
- urine sample
- 12ml blood sample taken by experienced nurse

Call now.
LINDA
595-6815

ARF Addiction Research Foundation / Fondation de la recherche sur la toxicomanie

Appendix II

INSTRUCTIONS

1. Complete the consent form, general questionnaire and Use of Psychoactive Substances Questionnaire.

2. At bedtime, empty your bladder and swallow the dextromethorphan capsule with water.

3. When you first get up the next morning (about eight hours after you swallowed the dextromethorphan), collect ALL of your overnight urine in the bottle provided.

4. Print your name on the bottle.

5. Return urine, consent form and questionnaires that same morning as instructed.

PRODUCT MONOGRAPH FROM CPS:

ORNEX•DM© *SK&F*

Dextromethorphan ***Antitussive***

Indications: Symptomatic relief of dry, hacking (nonproductive) cough.

Contraindications: Dextromethorphan sensitivity, concurrent MAO inhibitor therapy.

Precautions: Use with caution in patients with liver disease, asthma or depression.

Children: Ornex•DM 15 is not recommended for children under 1 year of age. Ornex•DM 30 is not recommended for children 12 years and under, except on the advice of a physician.

Pregnancy and Lactation. As with any drug, the use of this product is not recommended during pregnancy, or when nursing, unless the physician is convinced that the potential benefits outweigh the possible risk to mother and child.

Do not exceed recommended dosage, and do not continue medication beyond 7 days, except on the advice of a physician.

If cough persists beyond 7 days, or is accompanied by high fever, see a physician.

Adverse Effects: Rarely, headache, nausea, vomiting, slight drowsiness or dizziness may occur.

Overdose: Symptoms: Drowsiness or dizziness; nausea and/or vomiting; headache; excitation (especially in children) or mental confusion. Very high doses may produce respiratory depression.

Treatment: Gastric lavage, repeated several times.

Respiratory depression should be treated promptly by the use of oxygen and stimulants.

If marked excitement is present, a short-acting barbiturate may be used. Otherwise, do not administer sedation.

Force fluids by mouth or, if necessary, by i.v. administration.

Dosage: Ornex•DM 15 Liquid. Adults: 5 to 10 mL 3 or 4 times a day. Children: 6 to 12 years, 2.5 to 5 mL 3 or 4 times a day; 1 to 5 years, 1.25 to 2.5 mL 3 or 4 times a day.

Ornex•DM 30 Capsules: Adults and children over 12 years: 1 capsule 3 or 4 times a day. Not recommended for children 12 years or under.

Supplied: Ornex•DM 15 Liquid: Each 5 mL of clear, purple, grape-flavored liquid contains: dextromethorphan HBr 15 mg. Sodium: <1 mmol (14.425 mg)/5 mL. Energy: 40.66 kJ (9.68 kcal)/5 mL. Bottles of 100 mL.

Ornex•DM 30 Capsules: Each hard gelatin capsule with a blue cap and white opaque body, sealed with a yellow gelatin band, monogrammed ORNEX•DM SKF on both cap and body, contains: dextromethorphan HBr 30 mg. Sodium: <1 mmol (0.032 mg). Lactose: 50 mg. Energy: 2.41 kJ (0.5748 kcal). Cartons of 12 and 24.

(Shown in Product Recognition Section)

Reviewed 1988

CONSENT FORM

I _____. hereby consent to participate in the research project entitled "Dextromethorphan Oxidation Phenotype Distribution" being conducted at the Clinical Research and Treatment Institute of the Addiction Research Foundation and University of Toronto under the direction of E.M. Sellers, M.D., Ph.D., R.F. Tyndale, Ph.D. and M. Nowak. The purpose of this research, the procedures to be followed, and possible risks of this research have been explained to my by _____. In consenting to participate, I understand that:

1. The purpose of this study is to determine my pattern of metabolizing a substance called dextromethorphan.

2. Dextromethorphan is a widely used non-prescription anti-cough medication. Dextromethorphan has been given safely in the dose to be used in this study to millions of individuals without apparent risk or hazard. At the dose I will receive, the side effect that might occur is a slight degree of drowsiness.

3. As part of this study, I will provide information about myself including medical history and current medication use (including non-prescription drug use).

4. As part of this study, I will take dextromethorphan 30 mg in a capsule. Before doing this I will empty my bladder. After taking the drug I will collect all my urine for the next 4 to 8 hours in the container provided. This procedure may be repeated on other occasions up to a maximum of 4 additional times.

5. I may decline to answer any particular questions asked of me. If this refusal makes my participation in this study of no scientific value, my participation can be terminated.

6. I can expect no therapeutic benefit from participation in this study and may freely choose to not participate in any repeat testing of this procedure.

7. The data I provide will be kept strictly confidential and secure, available only to the researchers involved in this study and for the purposes set out in paragraph #1 above. Neither my name nor any pieces of identifying information will be kept together with the other data that I may provide. My records will be treated with the same confidentiality afforded medical records.

8. The results of this study may be published and if so will be published in such a manner that I will not be identifiable. Published reports will refer to group data and not to a particular individual.

9. I have had an opportunity to ask questions, and my questions have been satisfactorily answered.

10. I will be given a copy of the consent form at the time I sign it.

Dated at Toronto, this _____ day of _____, 19 _____.

_____ _____

Signature _____

_____

_____

_____ _____

Print Name Address

Date of Birth: _____

day / month / year

_____ _____

Witness Signature Date

This consent form was read in my presence by _____ who has informed me that he/she carefully considered and understood each point above. I hereby confirm that the study will be conducted in accordance with the conditions and procedures set out above.

_____ _____

Print Name Classification

_____

Signature

Appendix III

CYP2D6 POPULATION STUDY

Genetics of Addiction Questionnaire

0 9 SUBJECT
G E N 1 0 0 1 0 0
SYSTEM FORM PER
DATE: YR. MO. DA.

1. Year of Birth: ____ Interviewers Initials ____

2. Sex: 1. ___ Female 2. ___ Male

3. Country of birth: _____ (specify) ____

Briefly describe your family's ethnic origins as far back as you are aware: _____

_____

_____

Coding:

Parents			Grandparents
maternal	____	_____	maternal ____
			paternal ____
paternal	____	_____	maternal ____
			paternal ____

(01)	African	(13)	English	(25)	Metis
(02)	American (U.S.A.)	(14)	French Canadian	(26)	Native Indian
(03)	Arab	(15)	French	(27)	Other Native American
(04)	Black-African	(16)	German	(28)	Other South East Asian
(05)	Black-Caribbean	(17)	Greek	(29)	Polish
(06)	Black-North American	(18)	Indo-Pakistani	(30)	Portuguese
(07)	Canadian	(19)	Inuit	(31)	Scottish
(08)	Caribbean	(20)	Irish	(32)	Spanish
(09)	Latin American	(21)	Italian	(33)	Ukrainian
(10)	Chinese	(22)	Japanese	(34)	Vietnamese
(11)	Dutch	(23)	Jewish	(35)	Other-specify: _____
(12)	East European	(24)	Korean	(55)	Unknown

4. Are you currently taking any medications? 1. ___ Yes 2. ___ No

If yes, complete the following:

Reason			Drug Name
Heart	1. ___ Yes	2. ___ No	_____
Blood Pressure	1. ___ Yes	2. ___ No	_____
Diabetes	1. ___ Yes	2. ___ No	_____
Thyroid	1. ___ Yes	2. ___ No	_____
Seizure Disorder	1. ___ Yes	2. ___ No	_____
Asthma	1. ___ Yes	2. ___ No	_____
Cough/Cold/Allergy	1. ___ Yes	2. ___ No	_____
Stomach	1. ___ Yes	2. ___ No	_____
Constipation	1. ___ Yes	2. ___ No	_____
Pain	1. ___ Yes	2. ___ No	_____
Infection	1. ___ Yes	2. ___ No	_____
Birth Control	1. ___ Yes	2. ___ No	_____
Depression	1. ___ Yes	2. ___ No	_____
Anxiety	1. ___ Yes	2. ___ No	_____
Withdrawal	1. ___ Yes	2. ___ No	_____
Cholesterol Control	1. ___ Yes	2. ___ No	_____
Other _____	1. ___ Yes	2. ___ No	_____

CYP2D6 POPULATION STUDY
Genetics of Addiction Questionnaire

0 9
SUBJECT

5.

DRUG TYPE	Ever Used 1 = no 2 = yes	Age First Used	Total Years Used 85 = 1-3 mon 86 = 4-7 mon 87 = 8-11 mon 00 = infrequent	Most Typical Route of Administration 1 = oral 2 = sniffed 3 = injected 4 = smoked 5 = inhaled 6 = other	Year Last Used	Typical * Frequency of Use in Past 90 Days	Typical Amount Used Per Day (on days used) in Past 90 Days
ALCOHOL					19		
CANNABIS: (e.g. Marijuana, hashish hash oil)					19		
STIMULANTS: Cocaine/Crack					19		
STIMULANTS: Methamphetamine, amphetamines (e.g. speed, ice)					19		
STIMULANTS: Others (e.g. ritalin, diet pills)					19		
DEPRESSANTS: Anti-anxiety drugs/ Sedative-Hypnotics (e.g. Valium, Librium, Seconal, Amytal)					19		
NARCOTICS: Heroin					19		
NARCOTICS: Methadone					19		
NARCOTICS: Pentazocine (Talwin, T's)					19		
NARCOTICS: Propoxyphene (Darvon)					19		
NARCOTICS: Meperidine (Demerol)					19		
NARCOTICS: Codeine (Tylenol 1, 2, 3, 4, 222's, AC & C, 282's, 292's					19		
NARCOTICS: Hydrocodone (Hycodan, Novahistex DH, Tussionex)					19		
NARCOTICS: Hydromorphone (Dilaudid)					19		
NARCOTICS: Morphine					19		
NARCOTICS: Oxycodone (Percodan, Percocet)					19		
INHALANTS: Glue, solvents, aerosols, liquid paper, volatile nitrates					19		
HALLUCINOGENS: LSD, PCP, STP, MDA, angel dust, mescaline, psilocybin, peyote, mushrooms.					19		
TOBACCO					19		
OTHER (Specify) _____					19		

NOTE: If "EVER USED" is **NO** (1) for any given line, the remainder of the line should be left blank.

*** Frequency Code**

0 = no use
1 = <1/month
2 = 1 x /month
3 = 2 to 3 x/month
4 = 1 to 2 x /week
5 = 3 to 6 x /week
6 = daily

If shaded boxes are coded "2", the corresponding module must be completed.

CYP2D6 POPULATION STUDY

Genetics of Addiction Questionnaire

SUBJECT 0 9

SYSTEM G E N | FORM 0 0 | PER 2 0 0

DATE: YR. MO. DA.

OPIATE MODULE

1. How much have you used each of these opiates?

A. codeine	1. ___ used regularly	2. ___ tried, never used regularly	3. ___ never tried
B. hydrocodone	1. ___ used regularly	2. ___ tried, never used regularly	3. ___ never tried
C. hydromorphone	1. ___ used regularly	2. ___ tried, never used regularly	3. ___ never tried
D. morphine	1. ___ used regularly	2. ___ tried, never used regularly	3. ___ never tried
E. oxycodone	1. ___ used regularly	2. ___ tried, never used regularly	3. ___ never tried

If answered "used regularly" to any of these opiates, proceed to Question 3.

2. If you have not used any of these opiates regularly, which of the following statements applies to you?

A. ___ no reason to use them
B. ___ did not experience desirable effects
C. ___ experienced undesirable effects
D. ___ concerned about potential drug effects on health/behaviour
E. ___ significant others (family/friends/employer) would disapprove
F. ___ too expensive
G. ___ not available
H. ___ other _____

STOP!

This is the end of the opiate module for those who have never used opiates regularly.

PLEASE GO TO PAGE 7

3. For those you have used regularly, during what time period were you using the most? (code 00 for the month, if not known)

	From (M Y)	To (M Y)
A. codeine		
B. hydrocodone		
C. hydromorphone		
D. morphine		
E. oxycodone		

OPIATE MODULE cont'd.

4. What was your typical frequency of use during this period?

A. codeine	⎵	
B. hydrocodone	⎵	
C. hydromorphone	⎵	
D. morphine	⎵	
E. oxycodone	⎵	

<u>Frequency codes</u>:
0 = never
1 = < 1 x/mo
2 = 1 x /mo
3 = 2-3 x/mo
4 = 1-2 x/wk
5 = 3-6 x/wk
6 = daily

5. Approximately, how many tablets (teaspoonsful) of these opiates would you take on the days you used them during this period? Indicate strength for product used and total mg/day.
(i.e. ⎵⎵⎵ # tabs x ⎵⎵ strength/tab = ⎵⎵⎵ mg total/day)

	tabs		strength		mg total/day	Specify Product	
A. codeine	⎵⎵⎵	x	⎵⎵	=	⎵⎵⎵⎵	____________	⎵⎵
	⎵⎵⎵	x	⎵⎵	=	⎵⎵⎵⎵	____________	⎵⎵
	⎵⎵⎵	x	⎵⎵	=	⎵⎵⎵⎵	____________	⎵⎵
	⎵⎵⎵	x	⎵⎵	=	⎵⎵⎵⎵	____________	⎵⎵
	⎵⎵⎵	x	⎵⎵	=	⎵⎵⎵⎵	____________	⎵⎵
B. hydrocodone	⎵⎵⎵	x	⎵⎵	=	⎵⎵⎵⎵		
C. hydromorphone	⎵⎵⎵	x	⎵⎵	=	⎵⎵⎵⎵		
D. morphine	⎵⎵⎵	x	⎵⎵⎵	=	⎵⎵⎵⎵		
E. oxycodone	⎵⎵⎵	x	⎵⎵	=	⎵⎵⎵⎵		

6. What is the maximum number of tablets (teaspoonful) you have ever used in one 24 hour period? Indicate strength for product used and total mg/day.
(i.e. ⎵⎵⎵ # tabs x ⎵⎵ strength/tab = ⎵⎵⎵ mg total/day)

	tabs		strength		mg total/day	Specify Product	
A. codeine	⎵⎵⎵	x	⎵⎵	=	⎵⎵⎵⎵	____________	⎵⎵
	⎵⎵⎵	x	⎵⎵	=	⎵⎵⎵⎵	____________	⎵⎵
	⎵⎵⎵	x	⎵⎵	=	⎵⎵⎵⎵	____________	⎵⎵
	⎵⎵⎵	x	⎵⎵	=	⎵⎵⎵⎵	____________	⎵⎵
	⎵⎵⎵	x	⎵⎵	=	⎵⎵⎵⎵	____________	⎵⎵
B. hydrocodone	⎵⎵⎵	x	⎵⎵	=	⎵⎵⎵⎵		
C. hydromorphone	⎵⎵⎵	x	⎵⎵	=	⎵⎵⎵⎵		
D. morphine	⎵⎵⎵	x	⎵⎵⎵	=	⎵⎵⎵⎵		
E. oxycodone	⎵⎵⎵	x	⎵⎵	=	⎵⎵⎵⎵		

OPIATE MODULE cont'd.

7. What was your primary reason for first using these opiate products?

1. ___ to treat headaches
2. ___ to treat back pain
3. ___ to treat other pain _____
4. ___ for the pleasurable effects
5. ___ other _____

8. What was your primary reason for using these opiates during your period of heaviest use?

1. ___ to treat headaches
2. ___ to treat back pain
3. ___ to treat other pain _____
4. ___ for the pleasurable effects
5. ___ other _____

9. If you have used these opiates primarily for pain, was there ever a time when you have used them when you did not have pain, that is, to get other effects?

1. ___ Yes Specify _____
2. ___ No
3. ___ Not applicable

10. If you have used codeine regularly, which type of products did you use?

1. ___ always OTC
2. ___ usually OTC, sometimes prescription
3. ___ equal frequency OTC and prescription
4. ___ usually prescription, sometimes OTC
5. ___ always prescription
6. ___ not applicable

11. If you have used a prescription drug regularly, how did you generally obtain the drug? (Check all that apply)

A. ___ prescription from physician
B. ___ family
C. ___ friends
D. ___ purchased off the street
E. ___ forged prescriptions
F. ___ other, specify _____
G. ___ not applicable

CYP2D6 POPULATION STUDY
Genetics of Addiction Questionnaire

0 9 SUBJECT

OPIATE MODULE cont'd.

12.

	DSM CRITERIA	During Period of Heaviest Use	Currently (past month)
1.	Did you often find that when you started using opiates you ended up taking much more of them than you were planning to or over a longer period of time?	1 2 3	1 2 3
2.	Did you try to cut down or stop using opiates? Were you successful? Did you want to stop or cut down (did you worry about it?)	1 2 3	1 2 3
3.	Did you spend a lot of time taking opiates or doing whatever you had to do to get them? Did it take you a long time to get back to normal?	1 2 3	1 2 3
4. a.	Did you ever use opiates while doing something where it might have been dangerous? (Did you ever drive while you were really too stoned or high to be driving?)	1 2 3	1 2 3
4. b.	Was there ever a time when you were often using opiates or hung over from opiates when you were doing something important, like being at school or work, or taking care of children? (What about missing something important?)	1 2 3	1 2 3
5.	Did you use opiates so often that you started to use opiates instead of working or spending time on hobbies or with your family or friends?	1 2 3	1 2 3
6. a.	Did your use of opiates cause problems with other people, such as with family members or people at work?	1 2 3	1 2 3
6. b.	Did your use of opiates cause psychological or physical problems or make them worse?	1 2 3	1 2 3
7.	Did you find that you needed to use a lot more opiates in order to get high than you did when you first started using it, or when you used the same amount, it had much less effect than before?	1 2 3	1 2 3
8.	Have you ever had withdrawal symptoms, that is, felt sick when you cut down or stopped using opiates? (refer to list of symptoms at end of questionnaire)	1 2 3	1 2 3
9.	IF YOU HAD WITHDRAWAL SYMPTOMS: After not using opiates for a few hours or more, did you often use it to keep yourself from getting sick with withdrawal symptoms?	1 2 3	1 2 3
10.	Have you had any legal problems related to your use of opiates?	1 2 3	1 2 3
	1 = absent or false 2 = subthreshold 3 = threshold or true		

13. For how long a time were you having some symptoms of drug dependence or abuse?

1. ___ 0 - 1 month
2. ___ ≥ 1 month

14. Please indicate how long it has been since the individual met any (even one) of these criteria?

1. ___ 0 - 1 month (meets at least one criteria currently)
2. ___ > 1 - < 6 months
3. ___ ≥ 6 months - < 12 months
4. ___ ≥ 12 months
5. ___ N/A (i.e they have never met any criteria)

Note: For your reference and clarification, the DSM-IV criteria for psychoactive substance abuse and dependence are listed on the last page of this questionnaire.

CYP2D6 POPULATION STUDY

Genetics of Addiction Questionnaire

0 9 SUBJECT
G E N 1 0 0 3 0 0
SYSTEM FORM PER
DATE: YR. MO. DA.

ALCOHOL MODULE

What are your drinking habits like? (How much do you drink?)

Was there ever a period in your life when you drank too much?

Has alcohol ever caused problems for you? ___ What? _____

_____

Has anyone ever objected to your drinking? ___ Why? _____

_____

1. Is there any suggestion that the person drinks or drank excessively or had problems with alcohol as determined from above screening items?

   1. ___ Yes If yes, then proceed to question 3.
   2. ___ No If no, then proceed to question 2.

2. If you have never experienced problems with drinking alcohol, which of the following reason(s) apply to you? (Check all that apply).

   A. ___ no reason to drink more
   B. ___ did not experience desirable effects
   C. ___ experienced undesirable effects
   D. ___ concerned about potential effects on health and behaviour
   E. ___ significant others (family/friends/employer) would disapprove
   F. ___ too expensive
   G. ___ other _____

STOP!

This is the end of the alcohol module if the screening items produced a no response.

PLEASE GO TO PAGE 10

ALCOHOL MODULE cont'd.

3. During what period in your life were you drinking the most? (code 00 for the month if not known)

From |_|_|_|_| To |_|_|_|_|
M Y M Y

4. During this period, approximately how frequently would you drink?

|_| Frequency codes :
0 = never
1 = < 1 x/mo
2 = 1 x /mo
3 = 2-3 x/mo
4 = 1-2 x/wk
5 = 3-6 x/wk
6 = daily

5. a. On the days that you would drink during this period, approximately how many drinks would you have? (Please refer below for Standard Drink Conversions).

|_|_|_| # of drinks/day

b. During this period, approximately how many drinks would you have in any one week? (Please refer below for Standard Drink Conversions).

|_|_|_|_| # of drinks/week

Standard Drink Conversion :
12 ounces of beer (5%)
5 ounces of wine (12-17%)
1 ½ ounces of hard liquor (80% proof)

6. What is the maximum number of drinks you have ever had in one 24 hour period?

|_|_|_| # drinks

ALCOHOL MODULE cont'd.

7.

	DSM CRITERIA	During Period of Heaviest Use	Currently (past month)
1.	Did you often find that when you started drinking you ended up drinking much more than you were planning to or over a longer period of time?	1 2 3	1 2 3
2.	Did you try to cut down or stop drinking? Were you successful? Did you want to stop or cut down (did you worry about it?)	1 2 3	1 2 3
3.	Did you spend a lot of time drinking alcohol or doing whatever you had to do to get it? Did it take you a long time to get back to normal?	1 2 3	1 2 3
4. a.	Did you ever drink while doing something where it might have been dangerous? (Did you ever drive while you were really too intoxicated to be driving?)	1 2 3	1 2 3
4. b.	Was there ever a time when you were often drinking or hung over from alcohol when you were doing something important, like being at school or work, or taking care of children? (What about missing something important?)	1 2 3	1 2 3
5.	Did you drink so often that you started to drink instead of working or spending time on hobbies or with your family or friends?	1 2 3	1 2 3
6. a.	Did your use of alcohol cause problems with other people, such as with family members or people at work?	1 2 3	1 2 3
6. b.	Did your use of alcohol cause psychological or physical problems or make them worse?	1 2 3	1 2 3
7.	Did you find that you needed to drink a lot more in order to get intoxicated than you did when you first started drinking, or when you drank the same amount, it had much less effect than before?	1 2 3	1 2 3
8.	Have you ever had withdrawal symptoms, that is, felt sick when you cut down or stopped drinking? (refer to list of symptoms at end of questionnaire)	1 2 3	1 2 3
9.	IF YOU HAD WITHDRAWALSYMPTOMS: After not drinking for a few hours or more, did you often use it to keep yourself from getting sick with withdrawal symptoms?	1 2 3	1 2 3
10.	Have you had any legal problems related to your drinking?	1 2 3	1 2 3
	1 = absent or false 2 = subthreshold 3 = threshold or true		

8. For how long a time were you having some symptoms of drug dependence or abuse?

1. ___ 0 - 1 month
2. ___ ≥ 1 month

9. Please indicate how long it has been since the individual met any (even one) of these criteria?

1. ___ 0 - 1 month (meets at least one criteria currently)
2. ___ > 1 - < 6 months
3. ___ ≥ 6 months - < 12 months
4. ___ ≥ 12 months
5. ___ N/A (i.e they have never met any criteria)

Note: For your reference and clarification, the DSM-IV criteria for psychoactive substance abuse and dependence are listed on the last page of this questionnaire.

CYP2D6 POPULATION STUDY

Genetics of Addiction Questionnaire

SUBJECT 0 9
SYSTEM G E N 1 FORM 0 0 4 PER 0 0
DATE: YR. MO. DA.

TOBACCO MODULE

1. Have you ever smoked regularly?

1. ___ Yes — If your answer is yes, then proceed to question 3.
2. ___ No — If your answer is no, then proceed to question 2.

2. If you have never smoked regularly, which of the following reason(s) apply to you? (Check all that apply)

A. ___ no reason to smoke more
B. ___ did not experience desirable effects
C. ___ experienced undesirable effects
D. ___ concerned about potential effects on health
E. ___ significant others (family/friends/employers) would disapprove
F. ___ too expensive

STOP!

This is the end of the tobacco module for those who have never used tobacco regularly

PLEASE GO TO PAGE 13

3. During what period in your life were you smoking the most cigarettes? (code 00 for the month, if not known)

From |_|_|_|_| (M Y) To |_|_|_|_| (M Y)

4. During this period, typically how frequently would you smoke?

|_| Frequency codes:

0 = never
1 = < 1 x/mo
2 = 1 x /mo
3 = 2-3 x/mo
4 = 1-2 x/wk
5 = 3-6 x/wk
6 = daily

TOBACCO MODULE cont'd.

5. a. During the above period of time, approximately how many cigarettes would you smoke in a day ?

|_|_|_| # of cigarettes

b. How many in any one week period?

|_|_|_|_| # of cigarettes

6. What is the maximum number of cigarettes you have ever smoked in one 24 hour period?

|_|_|_| # of cigarettes

7. How soon after you wake up would you smoke your first cigarette?

1. ___ within 30 minutes
2. ___ more than 30 minutes, less than 2 hours
3. ___ greater than 2 hours

8. Would you find it difficult to refrain from smoking in places where it is forbidden?

1. ___ Yes
2. ___ No

9. Would you smoke even if you are very ill?

1. ___ Yes
2. ___ No

10. Would you inhale when you smoke?

0. ___ never
1. ___ sometimes
2. ___ always

11. Would you avoid socializing with non-smokers if you couldn't smoke in their company?

1. ___ Yes
2. ___ No

TOBACCO MODULE cont'd.

12.

	DSM CRITERIA	During Period of Heaviest Use	Currently (past month)
1.	Did you often find that when you started smoking you ended up smoking more than you were planning to or over a longer period of time?	1 2 3	1 2 3
2.	Did you try to cut down or stop smoking? Were you successful? Did you want to stop or cut down (did you worry about it?)	1 2 3	1 2 3
3.	Did you spend a lot of time smoking tobacco? (Or doing whatever you had to do to get it? Did it take you a long time to get back to normal?	1 2 3	1 2 3
4. a.	Did you ever smoke while doing something where it might have been dangerous? (i.e. falling asleep with a lit cigarette in bed)	1 2 3	1 2 3
4. b.	Was there ever a time when your smoking interferred with doing something important, like being at school or work, or taking care of children? (What about missing something important?)	1 2 3	1 2 3
5.	Did you smoke so often that you would smoke instead of working or spending time on hobbies or with your family or friends?	1 2 3	1 2 3
6. a.	Did your smoking cause problems with other people, such as with family members or people at work?	1 2 3	1 2 3
6. b.	Did your smoking cause psychological or physical problems or make them worse?	1 2 3	1 2 3
7.	Did you find that you needed to smoke a lot more than when you first started smoking? (Or when you used the same amount, it had much less effect than before?)	1 2 3	1 2 3
8.	Have you ever had withdrawal symptoms, that is, felt sick when you cut down or stopped smoking? (refer to list of symptoms at end of questionnaire)	1 2 3	1 2 3
9.	IF YOU HAD WITHDRAWALSYMPTOMS: After not smoking for a few hours or more, did you often smoke to keep yourself from getting sick with withdrawal symptoms?	1 2 3	1 2 3
10.	Have you had any legal problems related to your smoking?	1 2 3	1 2 3
	1 = absent or false 2 = subthreshold 3 = threshold or true		

13. For how long a time were you having some symptoms of drug dependence or abuse?

1. ___ 0 - 1 month
2. ___ ≥ 1 month

14. Please indicate how long it has been since the individual met any (even one) of these criteria?

1. ___ 0 - 1 month (meets at least one criteria currently)
2. ___ > 1 - < 6 months
3. ___ ≥ 6 months - < 12 months
4. ___ ≥ 12 months
5. ___ N/A (i.e they have never met any criteria)

Note: **For your reference and clarification, the DSM-IV criteria for psychoactive substance abuse and dependence are listed on the last page of this questionnaire.**

CYP2D6 POPULATION STUDY

Genetics of Addiction Questionnaire

0 9 SUBJECT
G E N 1 0 0 5 0 0
SYSTEM FORM PER
DATE: YR. MO. DA.

COCAINE MODULE

1. Have you ever used cocaine regularly?

1. ___ Yes Proceed to question 3
2. ___ No Proceed to question 2

2. As someone who had never used cocaine regularly, which of the following reasons apply?

A. ___ no reason to use it
B. ___ did not experience desirable effects
C. ___ experienced undesirable effects
D. ___ concerned about potential effects on health/behaviour
E. ___ significant others (family/friends/employers) would disapprove
F. ___ too expensive
G. ___ not available
H. ___ illegal
I. ___ other _____

STOP!

This is the end of the cocaine module for those who have never used cocaine regularly.

PLEASE GO TO PAGE 15

3. During what period in your life were you using cocaine the most?
(code 00 for the month, if not known)

From |_|_|_|_| To |_|_|_|_|
M Y M Y

4. During this period what was your typical frequency of use?

|_| Frequency codes :
0 = never
1 = < 1 x/mo
2 = 1 x /mo
3 = 2-3 x/mo
4 = 1-2 x/wk
5 = 3-6 x/wk
6 = daily

5. What was your most typical route of administration during this period?

|_|
1. sniffed
2. injected
3. smoked

6. Approximately how many times would you use cocaine on the days you would use?
(code 88 if indicate "continuously")

|_|_|_| times

7. What was the most number of times you have ever used cocaine in a single day?
(code 88 if indicate "continuously")

|_|_|_| times

COCAINE MODULE cont'd.

8.

	DSM CRITERIA	During Period of Heaviest Use	Currently (past month)
1.	Did you often find that when you started using cocaine you ended up smoking much more of them than you were planning to or over a longer period of time?	1 2 3	1 2 3
2.	Did you try to cut down or stop using cocaine? Were you successful? Did you want to stop or cut down (did you worry about it?)	1 2 3	1 2 3
3.	Did you spend a lot of time using cocaine or doing whatever you had to do to get it? Did it take you a long time to get back to normal?	1 2 3	1 2 3
4. a.	Did you ever use cocaine while doing something where it might have been dangerous? (Did you ever drive while you were really too stoned or high to be driving?)	1 2 3	1 2 3
4. b.	Was there ever a time when you were often using cocaine or hung over from cocaine when you were doing something important, like being at school or work, or taking care of children? (What about missing something important?)	1 2 3	1 2 3
5.	Did you use cocaine so often that you started to use cocaine instead of working or spending time on hobbies or with your family or friends?	1 2 3	1 2 3
6. a.	Did your use of cocaine cause problems with other people, such as with family members or people at work?	1 2 3	1 2 3
6. b.	Did your use of cocaine cause psychological or physical problems or make them worse?	1 2 3	1 2 3
7.	Did you find that you needed to use a lot more cocaine in order to get high than you did when you first started using it, or when you used the same amount, it had much less effect than before?	1 2 3	1 2 3
8.	Have you ever had withdrawal symptoms, that is, felt sick when you cut down or stopped using cocaine? (refer to list of symptoms at end of questionnaire)	1 2 3	1 2 3
9.	IF YOU HAD WITHDRAWAL SYMPTOMS: After not using cocaine for a few hours or more, did you often use cocaine to keep yourself from getting sick with withdrawal symptoms?	1 2 3	1 2 3
10.	Have you had any legal problems related to your cocaine use?	1 2 3	1 2 3
	1 = absent or false 2 = subthreshold 3 = threshold or true		

9. For how long a time were you having some symptoms of drug dependence or abuse?

1. ___ 0 - 1 month
2. ___ ≥ 1 month

10. Please indicate how long it has been since the individual met any (even one) of these criteria?

1. ___ 0 - 1 month (meets at least one criteria currently)
2. ___ > 1 - < 6 months
3. ___ ≥ 6 months - < 12 months
4. ___ ≥ 12 months
5. ___ N/A (i.e they have never met any criteria)

Note: For your reference and clarification, the DSM-IV criteria for psychoactive substance abuse and dependence are listed on the last page of this questionnaire.

CYP2D6 POPULATION STUDY

Genetics of Addiction Questionnaire

SUBJECT 0 9
SYSTEM G E N FORM 1 0 0 PER 6 0 0
DATE: YR. MO. DA.

AMPHETAMINE MODULE

1. Have you ever used amphetamines regularly?

   1. ___ Yes Proceed to question 3
   2. ___ No Proceed to question 2

2. As someone who had never used amphetamines regularly, which of the following reasons apply?

   A. ___ no reason to use it
   B. ___ did not experience desirable effects
   C. ___ experienced undesirable effects
   D. ___ concerned about potential effects on health/behaviour
   E. ___ significant others (family/friends/employers) would disapprove
   F. ___ too expensive
   G. ___ not available
   H. ___ illegal
   I. ___ other _____

STOP!
This is the end of the amphetamine module
for those who have never used amphetamines regularly.
THIS IS THE END OF THE QUESTIONNAIRE

3. During what period in your life were you using amphetamines the most?
   (code 00 for the month, if not known)

   From |_|_|_|_| (M Y) To |_|_|_|_| (M Y)

4. Which amphetamine were you using the most? _____ |_|_|
   _____ |_|_|
   _____ |_|_|

5. During this period what was your typical frequency of use?

   |_| Frequency codes : 0 = never
   1 = < 1 x/mo
   2 = 1 x /mo
   3 = 2-3 x/mo
   4 = 1-2 x/wk
   5 = 3-6 x/wk
   6 = daily

6. What was your most typical route of administration during this period?

   |_| 1. oral
   2. injected
   3. smoked

7. Approximately how many times would you use amphetamines on the days you would use?

   |_|_|_| times

8. What was the most number of times you have ever used amphetamines in a single day?

   |_|_|_| times

AMPHETAMINE MODULE cont'd.

9.

	DSM CRITERIA	During Period of Heaviest Use	Currently (past month)
1.	Did you often find that when you started using amphetamines you ended up taking much more of them than you were planning to or over a longer period of time?	1 2 3	1 2 3
2.	Did you try to cut down or stop using amphetamines? Were you successful? Did you want to stop or cut down (did you worry about it?)	1 2 3	1 2 3
3.	Did you spend a lot of time taking amphetamines or doing whatever you had to do to get them? Did it take you a long time to get back to normal?	1 2 3	1 2 3
4. a.	Did you ever use amphetamines while doing something where it might have been dangerous? (Did you ever drive while you were really too stoned or high to be driving?)	1 2 3	1 2 3
4. b.	Was there ever a time when you were often using amphetamines or hung over from amphetamines when you were doing something important, like being at school or work, or taking care of children? (What about missing something important?)	1 2 3	1 2 3
5.	Did you use amphetamines so often that you started to use amphetamines instead of working or spending time on hobbies or with your family or friends?	1 2 3	1 2 3
6. a.	Did your use of amphetamines cause problems with other people, such as with family members or people at work?	1 2 3	1 2 3
6. b.	Did your use of amphetamines cause psychological or physical problems or make them worse?	1 2 3	1 2 3
7.	Did you find that you needed to use a lot more amphetamines in order to get high than you did when you first started using them, or when you used the same amount, it had much less effect than before?	1 2 3	1 2 3
8.	Have you ever had withdrawal symptoms, that is, felt sick when you cut down or stopped using amphetamines? (refer to list of symptoms at end of questionnaire)	1 2 3	1 2 3
9.	IF YOU HAD WITHDRAWALSYMPTOMS: After not using amphetamines for a few hours or more, did you often use them to keep yourself from getting sick with withdrawal symptoms?	1 2 3	1 2 3
10.	Have you had any legal problems related to your amphetamine use?	1 2 3	1 2 3
	1 = absent or false 2 = subthreshold 3 = threshold or true		

10. For how long a time were you having some symptoms of drug dependence or abuse?

1. ___ 0 - 1 month
2. ___ ≥ 1 month

11. Please indicate how long it has been since the individual met any (even one) of these criteria?

1. ___ 0 - 1 month (meets at least one criteria currently)
2. ___ > 1 - < 6 months
3. ___ ≥ 6 months - < 12 months
4. ___ ≥ 12 months
5. ___ N/A (i.e they have never met any criteria)

Note: For your reference and clarification, the DSM-IV criteria for psychoactive substance abuse and dependence are listed on the last page of this questionnaire.

THIS IS THE END OF THE QUESTIONNAIRE

- LIST OF WITHDRAWAL SYMPTOMS (FROM DSM-IV CRITERIA) -

Alcohol Withdrawal Symptoms

Two (or more) of the following, developing within several hours to a few days after Criterion A:

1. autonomic hyperactivity (e.g. sweating or pulse rate greater than 100)
2. increased hand tremor
3. insomnia
4. nausea or vomiting
5. transient visual, tactile, or auditory hallucinations or illusions
6. psychomotor agitation
7. anxiety
8. grand mal seizures

Opioids Withdrawal Symptoms

Three (or more) of the following, developing within minutes to several days after Criterion A:

1. dysphoric mood
2. nausea or vomiting
3. muscle aches
4. lacrimation or rhinorrhea
5. pupillary dilation, piloerection, or sweating
6. diarrhea
7. yawning
8. fever
9. insomnia

Nicotine Withdrawal Symptoms

Abrupt cessation of nicotine use, or reduction in the amount of nicotine used, followed within 24 hours by four (or more) of the following signs:

1. dysphoric or depressed mood
2. insomnia
3. irritability, frustration or anger
4. anxiety
5. difficulty concentrating
6. restlessness
7. decreased heart rate
8. increased appetite or weight gain

Cocaine or Amphetamine Withdrawal Symptoms

Dysphoric mood and two (or more) of the following physiological changes, developing within a few hours to several days.

1. fatigue
2. vivid, unpleasant dreams
3. insomnia or hypersomnia
4. increased appetite
5. psychomotor retardation or agitation

Psychoactive Substance Dependence

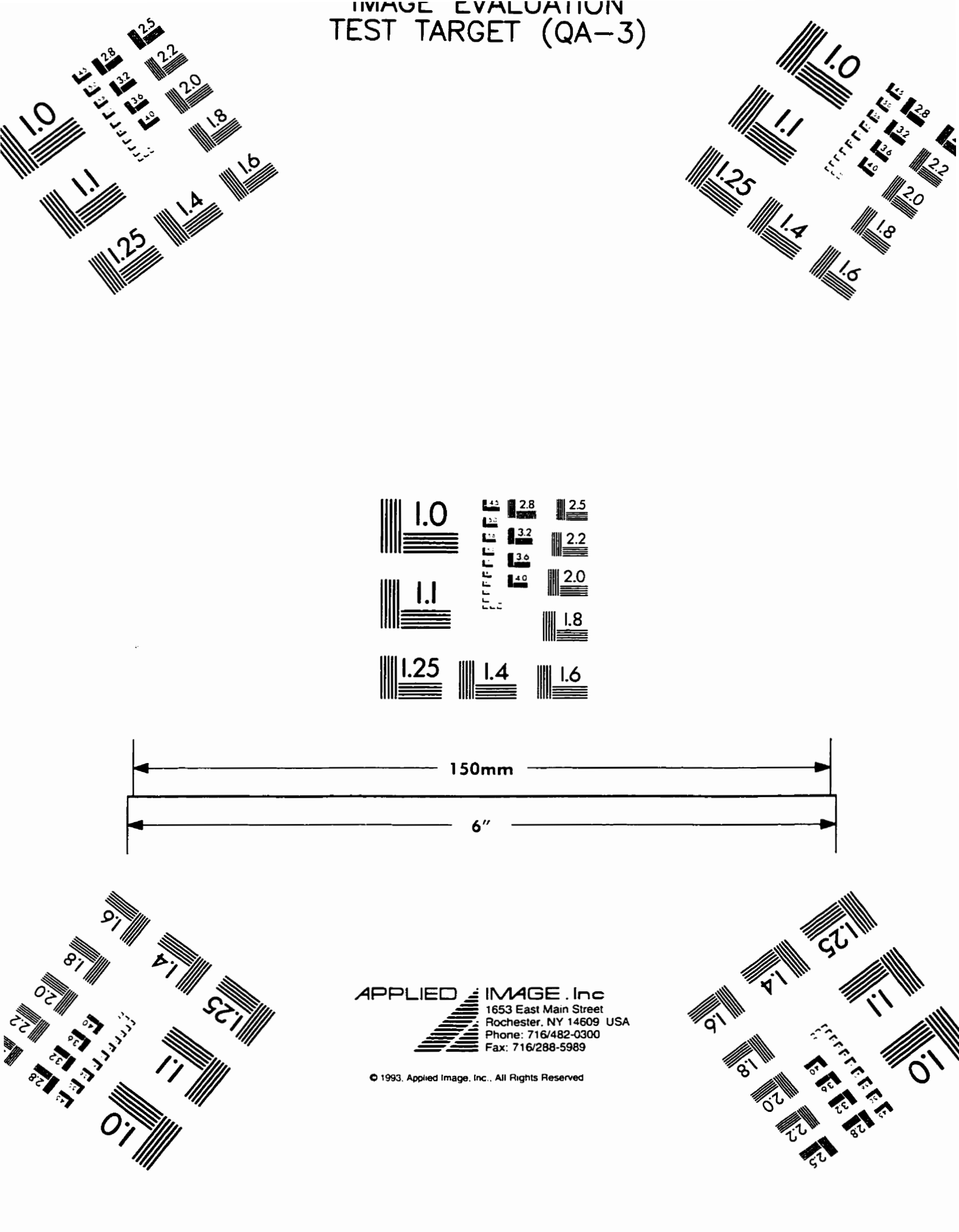
A maladaptive pattern of substance use, leading to clinically significant impairment or distress, as manifested by three (or more) of the following occuring at any time in the same 12-month period:

1. tolerance, as defined by either of the following:
   a) a need for markedly increased amounts of the substance to achieve intoxication or desired effect
   b) markedly diminished effect with continued use of the same amount of the substance
2. withdrawal, as manifested by either of the following:
   a) the characteristic withdrawal syndrome for the substance (refer to Criteria A and B of the criteria sets for Withdrawal from the specific substances)
   b) the same (or a closely related) substance is taken to relieve or avoid withdrawal symptoms
3. the substance is often taken in larger amounts or over a longer period than was intended
4. there is a persistent desire or unsuccessful efforts to cut down or control substance use
5. a great deal of time is spent in activities necessary to obtain the substance (e.g. visiting multiple doctors or driving long distances), use the substance (e.g. chain-smoking), or recover from its effects
6. important social, occupational, or recreational activities are given up or reduced because of substance use
7. the substance use is continued despite knowledge of having a persistent or recurrent physicial or psychological problem that is likely to have been caused or exacerbated by the substance (e.g. current cocaine use despite recognition of cocaine-induced depression, or continued drinking despite recognition that an ulcer was made worse by alcohol consumption)

Psychoactive Substance Abuse

A. A maladaptive pattern of substance use leading to clinically significant impairment or distress, as a manifested by one (or more) of the following, occurring within a 12-month period:
   1. recurrent substance use resulting in a failure to fulfill major role obligations at work school, or home (e.g., repeated absences or poor work performance related to substance use; substance-related absences, suspensions, or expulsions from school; neglect of children or household)
   2. recurrent substance use in situations in which it is physically hazardous (e.g. driving an automobile or operating a machine when impaired by substance use)
   3. recurrent substance-related legal problems (e.g. arrests for substance-related disorderly conduct)
   4. continued substance use despite having persistent or recurrent social or interpersonal problems caused or exacerbated by the effects of the substance (e.g. arguments with spouse about consequences of intoxication, physical fights)

B. The symptoms have never met the criteria for Substance Dependence for this class of substance.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