APPENDIX A6.1.3.18 CYP2A6 – FACTSHEET

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Cytochrome P450

Cytochrome P450 (CYP) enzymes are a superfamily of heme-containing mono-oxygenases that are found in all kingdoms of life, and which show extraordinary diversity in their reaction chemistry. In mammals, these proteins are found primarily in endoplasmic reticulum of hepatocytes and other cell types, where they oxidize steroids, fatty acids and xenobiotics, and are important for the detoxification and clearance of various compounds, as well as for hormone synthesis and breakdown, cholesterol synthesis and vitamin D metabolism [1]. In plants, these proteins are important for the biosynthesis of several compounds such as hormones, defensive compounds and fatty acids. In bacteria, they are important for several metabolic processes, such as the biosynthesis of antibiotic erythromycin in *Saccharopolyspora erythraea (Streptomyces erythraeus)* [2]. The knowledge of the human P450s enzymes has gained a lot of importance in the context of drug development, as the fate of candidate drugs are largely influenced by the CYP metabolism [3]. CYP-mediated drug metabolism can thus lead to altered drug efficacies, through inactivation of an active drug or, on the opposite, activation of a prodrug [4]. Similarly, toxic compounds may be detoxified through CYP-catalyzed biotransformation, and inert xenobiotics can be activated to become toxicants.

Organisms produce many different cytochrome P450 enzymes (at least 58 in humans), which together with alternative splicing can provide a wide array of enzymes with different substrate and tissue specificities. Individual cytochrome P450 proteins follow the nomenclature: CYP (superfamiliy), followed by a number (family), then a letter (subfamily), and another number (protein); e.g., CYP2A6 is the sixth protein in family 2, subfamily A. In general, family members should share >40% identity, while subfamily members should share >55% identity [2].

Cytochrome P450 enzymes use iron containing heme to oxidize their substrates, using protons derived from NADH (nicotinamide adenine dinucleotide) or NADPH (nicotinamide adenine dinucleotide phosphate) to split the oxygen so a single atom can be added to a substrate. They also require electrons, which they receive from a variety of redox partners. The most common reaction catalyzed by cytochromes P450 is a monooxygenase reaction, e.g., insertion of one atom of oxygen into the aliphatic position of an organic substrate (RH) while the other oxygen atom is reduced to water [1]:

$$RH + O_2 + NADPH + H^+ \rightarrow ROH + H_2O + NADP^+$$

In humans, only about a dozen enzymes belonging to the 1, 2, and 3 CYP-families are responsible for the metabolism of the majority of drugs and other xenobiotics [5]. These CYP enzymes are controlled by a number of different factors, including sex, age, ethnicity, disease, and diurnal influences [5].

CYP2A6 - Expression

CYP2A6 protein is mainly expressed in the liver, where it represents approximately 10% of the total liver CYP content, although with marked inter-individual variability [6]. Sensitive detection methods such as reverse transcriptase polymerase chain reaction (RT-PCR) have detected CYP2A6 mRNA in extrahepatic tissues, such as nasal mucosa and

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other areas of the respiratory tract [6, 7]. In the nasal mucosa and other respiratory tissues, considered as xenobiotics portal-of-entry organs, CYP2A6 may contribute to short- and long-term toxicity by compounds that are metabolized by these enzymes [4, 8]. Protein expression has also been detected in the stomach [9]. Very little is known about the ontogeny of CYP2A proteins in humans, but it appears that only a limited degree of CYP2A6 expression occurs in liver before birth, after which CYP2A6 expression gradually increases. The level of CYP2A6 activity, as measured by coumarin 7-hydroxylation capacity, reaches adult levels between 6 and 13 years of age [6].

CYP2A6 – Activity

CYP2A6 is a highly polymorphic gene. There are 45 variants of the CYP2A6 protein (status February 2014) that have been described and many additional single nucleotide polymorphisms (SNPs) in the Home Page of the Human Cytochrome P450 Allele Nomenclature Committee (www.cypalleles.ki.se). Many of the genetic variants have been shown to alter the expression, stability, and function of the CYP2A6 enzyme.
Individuals with different CYP2A6 variants can be grouped according to CYP2A6 enzyme activity as normal, intermediate (approx. 75% of normal), or slow (< 50% of normal) metabolizers. Substantial ethnic variations exist in the population frequencies of CYP2A6 alleles. CYP2A6*4, CYP2A6*7, CYP2A6*9, and CYP2A6*10 alleles are more prevalent in the Japanese population than Caucasians. The CYP2A6*4 allele is present in approximately 20% of Japanese. Some rare alleles, such as CYP2A6*18B in Caucasians, are found only in specific populations [6].

The same polymorphism can have a different effect on the metabolism of different substrates. For example, CYP2A6*18 activity against nicotine is similar to wildtype enzyme, but is reduced for coumarin and tegafur (or SM-12502, a platelet-activating factor receptor antagonist used as a chemotherapeutic fluorouracil prodrug) metabolism, in vitro, and conversely *17 activity against coumarin is similar to wildtype but significantly reduced for nicotine metabolism [10]. This suggests that the effect of CYP2A6 polymorphisms on metabolism should be investigated and interpreted in the context of individual substrates [11, 12].

CYP2A6 – Substrates, inhibitors and inducers

About 40% of human drug metabolism is carried out by the CYP polymorphic enzymes, which can result in altered drug pharmacokinetics [13]. Subjects carrying certain variant alleles may thus suffer from adverse effects of certain drugs because of the presence of defective alleles [13]. CYP2A6 being a highly polymorphic gene, the different drugs that act either as CYP2A6 substrates, inducers or inhibitors (Table 1) need to be dosed with care (e.g., tegafur) [13-15]. Similarly, the metabolism of nicotine can also be affected by the variant of CYP2A6 expressed by individual smokers [16-24]. CYP2A6 is also involved in the metabolism of several endogenous compounds, such as retinoic acid, testosterone, estradiol, and progesterone, but its contribution is minor in their overall metabolic pathways [6]. Nothing is, however, known about possible consequences of the altered metabolism of endogenous compounds due to CYP2A6 genetic polymorphism.

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The substrate structural characteristics of CYP2A6 are usually nonplanar low molecular weight molecules with two hydrogen bond acceptors, and include ketones and nitrosamines [5].

Table 1 Non-exhaustive list of compounds interacting with CYP2A6, either as substrates, inhibitors or inducers [5, 6, 25, 26]

CYP2A6 substrates	CYP2A6 inhibitors	CYP2A6 inducers
1,3-Butadiene	2-Bromonaphthalene	Amobarbital
1,7-dimethylaxanthine	(R)-(+) menthofuran	Artemisinin
2,6-dichlorobenzonitrile (DCBN)	1-Benzothiophene-3-carbaldehyde	Carbamazepine
4-methylnitrosoamino-1-(3-pyridyl)-1-butanone (NNK)	^{1}H-substituted imidazoles	Dexamethasone
Aflatoxin B_1	3-(2-methyl-^{1}H-imidazole-1-yl)pyridine	Estrogens
Bilirubin	3-(pyridine-3-yl)-^{1}H-(pyrazol-5-yl)methenamine (PPM)	PENTobarbital
Camphor	4-Methoxybenzaldehyde	PHENobarbital
Chloroform	8-methoxypsoralen	Rifampicin
Cotinine	Amiodarone	Rifampin
Coumarin	Chalepensin	Secobarbital
Dexmedetomidine	Decursinol angelate	
Efavirenz	Desipramine	
Fenchol	Genistein	
Halothane	Grapefruit juice	
Ifosfamide	Isoniazid	
Indole	Ketoconazole	
Letrozole	Letrozole	
Losigamone	Menthol	
Menthol	Methoxsalen	
Methoxyflurane	Miconazole	
Methyl tert-butyl ether (MTBE)	N1-(4-fluorophenyl) cyclopropane-1-carboxamide	
Nicotine	Organosulfur compounds	
N–nitrosodiethylamine (NDEA)	Pilocarpine	
N-nitrosodimethylamine (NMDA)	Selegiline	
Pilocarpine	Selegiline	
Quinoline	Tranylcypromine	
S-1 (tegafur, 5-chloro-2,4-dihydroxypyridine, potassium	Tryptamine	

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CYP2A6 substrates	CYP2A6 inhibitors	CYP2A6 inducers
oxonate)		
Tegafur		
Valproic acid		
SM-12502		

CYP2A6 in smokers - Nicotine metabolic ratio (NMR)

In humans, about 70–80% of nicotine is metabolized to cotinine. This transformation involves two steps [6, 16, 23]. The first is mediated primarily by CYP2A6 to produce nicotine-Δ1′ (5′)-iminium ion, which is in equilibrium with 5′-hydroxynicotine. The second step is catalyzed by a cytoplasmic aldehyde oxidase. CYP2A6 is the sole mediator of the conversion of cotinine to the metabolite trans-3'-hydroxycotinine (3HC) [27, 28]. Of the abbreviated list of harmful and potentially harmful constituents [29], besides nicotine, 1,3-butadiene, NNK (i.e. nicotine-derived nitrosamine ketone (NNK), also known as 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone) and NNN (i.e N-nitrosonornicotine) are also metabolized by CYP2A6, amongst other enzymes [30-32].

The availability of a phenotypic marker of CYP2A6 activity is important because of the wide variability in nicotine clearance among people with wild-type CYP2A6 genes [16], and only a small proportion of the genetic variability in nicotine clearance can be explained by known CYP2A6 gene variants, at least in Caucasians [22]. The trans-3′-hydroxycotinine/cotinine (3HC/COT) ratio can be used to phenotype nicotine metabolism and CYP2A6 enzyme in smokers while smoking their usual cigarettes, and has even been studied as predictor of response to non-nicotine cessation pharmacotherapy, such as bupropion [16, 19]. This 3HC/COT metabolite ratio (also known as the nicotine metabolic ratio, NMR) measured in human plasma is thus a reliable measure of in vivo CYP2A6 activity among regular smokers [33]. This ratio can be expressed in decimal or percentage. Because of the high genetic polymorphism of CYP2A6, NMR can serve as a genetically informed biomarker of CYP2A6 activity, incorporating environment influences on nicotine clearance [34]. NMR has recently been used to characterize novel CYP2A6 alleles [35]. This ratio can also be evaluated from saliva [27], as a non-invasive method, even though it has a better correlation with oral nicotine and cotinine clearance when using plasma. The saliva NMR does not vary with time of day [36], which could make it an ideal non-invasive matrix to assess NMR as a diagnostic biomarker to differentiate less dependent smokers from more dependent smokers and personalize their smoking cessation treatment accordingly, as different CYP2A6 activities have been shown to be able to distinguish different types of smoking behaviors [34]. Assessment of NMR from urine sample is also possible, however, a recent cross-laboratory and method comparison has shown that it is not as reliable as plasma measurement [33].

In PMI's studies, cytochrome P450 2A6 activity was calculated in plasma as the metabolic ratio of *trans*-3'-hydroxycotinine and cotinine, both expressed in molar equivalent (nmol/L).

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A conversion factor was applied as follows:

Cotinine: the molecular weight is 176.2178 g/mol [37]. For the conversion of cotinine from ng/mL to nmol/L, the result in ng/mL was multiplied by 5.675.

Trans-3'-hydroxycotinine: the molecular weight is 192.217 g/mol [38]. For the conversion of *trans*-3'- hydroxycotinine from ng/mL to nmol/L, the result in ng/mL was multiplied by 5.202.

The converted results were reported to 3 decimal places and the ratio (CYP2A6 or NMR) was reported to 2 decimal places.

CYP2A6 polymorphism and its influence on the NMR

NMR measurements have shown that some smokers have slower nicotine metabolism (NMR < 0.31) than others (NMR ≥ 0.31) [39]. Genetically slow nicotine metabolizers are less likely to be smokers, and as smokers, they are less dependent, and smoke fewer cigarettes per day [40, 41]. NMR is associated with a variety of smoking behaviors, including cessation [42-44] and early symptoms of withdrawal and craving [44]. Lower NMR is associated with lower cigarette consumption [11, 45, 46], dependence scores [47], nicotine-mediated reward [48], as well higher quit rates on placebo and nicotine patch [43, 44]. This observation has led to the search for selective CYP2A6 inhibitors, to be used as drugs to help smoking cessation [49]. Not all cessation therapies however target CYP2A6, as, for example, the effect of bupropion on relapse likelihood is largely unrelated to CYP2A6 activity [50]. On the other hand, Chen et al. have shown that NRT is more effective in individuals with fast, but not slow, CYP2A6-defined nicotine metabolism [50]. This finding thus seems to suggest that NMR does not necessarily predict cessation outcome amongst smokers given NRT, which appears to neutralize the relapse risk associated with faster nicotine metabolism, however, the differences with other studies are not really resolved, with the only explanation that study designs and experimental conditions differed (e.g. inclusion of a placebo group, type of behavioral counseling, inclusion of a treatment group other than nicotine, such as bupropion).

CYP2A6 polymorphism in different ethnicities and influence on smoking behavior

Smoking prevalence, cigarette consumption among smokers and the risk of developing smoking-related disease differ among racial groups [51]. It has been shown, for example, that Asian-Americans are less likely to become smokers, smoke fewer cigarettes per day and are less likely to develop lung cancer compared to Caucasians [52, 53]. Black Americans have a similar prevalence of smoking, smoke fewer cigarettes per day but are more likely to develop lung cancer compared to Caucasians [53, 54]. One factor influencing individual differences in smoking behavior is likely to be the rate of metabolism of nicotine. Compared to Caucasians, Asians and Black-Americans have a higher prevalence of *CYP2A6* gene alleles that are associated with slower metabolism of nicotine [12]. Slower metabolism of nicotine is expected to result in the need to smoke fewer cigarettes per day to achieve a desired level of nicotine in the body, and has been associated with greater rates of quitting compared to faster metabolizers. In the Japanese population, the allele frequencies of CYP2A6*4, CYP2A6*7, and CYP2A6*9 is

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relatively high. The CYP2A6*5, CYP2A6*6, CYP2A6*10, and CYP2A6*11 alleles are rare, and the CYP2A6*2 allele has not been not found [55]. Cotinine formation is impaired in the homozygotes of either CYP2A6*4, CYP2A6*7 and CYP2A6*10 (i.e., "slow" metabolizers). The CYP2A6*9 allele causes decreased expression levels of CYP2A6 mRNA and protein in vitro, and decreases the nicotine metabolism in vivo [55]. The high prevalence of slow metabolizers in the Japanese population could partially explain the longer than expected half-life of nicotine in plasma, as previously observed in smokers with an average CYP2A6 activity amounting to 50% of 'normal' activity [56].

In contrast to Asian populations, the CYP2A6*2 allele, which lacks the enzymatic activity, has been found in Europeans, European-Americans, and African-Americans, although the allele frequencies are not so high. CYP2A6*9 has also been found in these populations with moderate frequencies. Recently, novel alleles, CYP2A6*17 and CYP2A6*20, that are specific for the African-American population, have been found [55]. Since the CYP2A6*17 allele has a decreased enzymatic activity and the CYP2A6*20 allele (frameshift) lacks the activity, African-American subjects with these alleles showed low cotinine formation in vivo [55].

NMR stability

Because the elimination rate of 3-HC is limited by its formation, and given that cotinine has a relatively long half-life (averaging 16 h), the NMR is expected to be stable over time among smokers who maintain constant smoking consumption [57]. The NMR measured in various biological media is stable in ad libitum smokers over study periods ranging from a few days to 44 weeks [57]. Nonetheless, it is possible that the NMR may not reliably reflect the rate of nicotine metabolism in smokers engaged in smoking reduction. Previous studies have indeed shown that tobacco smoke inhibits nicotine metabolism and this inhibition could be mediated by the nicotine in smoke [58]. Therefore, NMR could be changing as smoke exposure is reduced. One study, however, reported that the urine NMR was relatively stable in smokers during a 12-week smoking reduction period, during which the smokers were allowed to use nicotine replacement therapy (NRT) ad libitum [59]. Similarly, the NMR in smokers with reduced nicotine intake over 24 weeks by smoking reduced nicotine content cigarettes (from 12 mg to 1 mg per cigarette in the tobacco blend) was also shown to be relatively stable over time, while nicotine levels declined in smokers [57].

No study assessing NMR during smoking cessation was found, however, because cotinine has a longer half-life than trans-3′-hydroxycotinine, the NMR in quitters may actually transiently increase, as compared to smokers who keep using products with similar amounts of nicotine.

NMR and other influencing factors of the NMR

Menthol is widely used as a flavorant in cigarettes. A moderate inhibition of CYP2A6-mediated nicotine metabolism in human liver microsomes by menthol and various related compounds has been reported [60]. This, however, remains somewhat controversial, as another group reported no *in vitro* evidence of menthol-mediated inhibition of CYP2A6 [61]. Menthol-mediated inhibition has been further supported by a crossover study in

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smokers, showing that mentholated cigarettes significantly inhibit metabolism of nicotine to cotinine and nicotine glucuronidation when compared to smoking non-mentholated cigarettes [62]. A recent study has further shown that daily menthol smokers have slower rates of nicotine metabolism than non-menthol smokers, as shown by a significantly lower NMR in menthol smokers, after adjusting for race/ethnicity, gender, body mass index and cigarette smoked per day [63].

Grapefruit juice inhibits CYP2A6, as evidenced by inhibition of coumarin metabolism [64]. The metabolism of nicotine to cotinine in nonsmokers who were given nicotine orally has been shown to be inhibited in a dose dependent manner, with evidence of a greater effect with larger doses of grapefruit juice [65]. Grapefruit juice also increased renal clearance of nicotine and cotinine by an unknown mechanism. It had, however, no significant effect on overall exposure to nicotine (area under the plasma concentration–time curve), probably because the effects of slowed metabolism were compensated by the effects on increased renal clearance.

Higher metabolism of nicotine has been seen in females, as compared to males, which could be linked to CYP2A6 activity, as it is induced by estrogen via the estrogen receptor [66]. CYP2A6 activity is, however, apparently not influenced by the menstrual cycle (follicular phase vs. luteal phase) [67].

CYP2A6 protein levels in elderly or nicotine metabolism in liver microsomes seem to remain stable with ageing, hence the slower metabolism of nicotine in elderly people is apparently not linked to CYP2A6 activity [68]. Among adolescent smokers, racial variations in rates of nicotine metabolism were similar to those that have been reported in adult smokers. In contrast to findings in adult smokers, the NMR did not vary significantly by gender or self-reported hormone use.

In a US study examining the NMR and multiple measures of smoking behavior and nicotine dependence in a large, national representative sample of young adults, it was found that females had higher NMRs than males, and that whites and Hispanics had higher NMRs than African Americans or Asians [69]. In another study, mean NMR was also found to be higher in Caucasians versus African Americans, and in females versus males [45]. Among females, birth control pill use and hormone replacement therapy were associated with higher mean NMR, respectively, albeit non-significantly [45]. As discussed previously, CYP2A6 polymorphism in different ethnicities largely influences the nicotine metabolism and explains these differences in NMR.

CYP2A6 polymorphism and link to tobacco-induced diseases

Not only have CYP2A6 polymorphisms been associated with extent of nicotine metabolism and effect on smoking behaviors (as discussed previously), but also with activation of carcinogens from tobacco and with xenobiotics [12], such as NNK; NNN and 1,3-butadiene. Activation of NNK and NNN by CYP2A6 can induce reactive metabolites that can bind to DNA to form adducts. CYP2A6 polymorphisms conferring lower enzyme activity are associated with reduced risk of lung, oral, head and neck, and upper aero-digestive cancers, particularly in tobacco users [12]. The association between CYP2A6 and lung cancer is especially prominent among those who smoke 20 or fewer

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cigarettes per day [41]. Higher CYP2A6 enzyme activity is associated with increased risk of pancreatic cancer (adjusted for smoking status) [70]. The allele frequency of CYP2A6*4 differed significantly between Asian and non-Asian populations, with CYP2A6*4 being more prevalent among Japanese individuals [71]. It is also relatively high among Koreans and Thais, whereas it is lower among Brazilians, French individuals and Canadians [12]. The lung cancer risk estimates in studies with European and African American populations are comparable [41]. Data suggest that the presence of the CYP2A6*4 allele might be associated with an increased lung cancer risk, especially for Asians [71]. African Americans possess different gene variants and more low frequency variants, with reduced / null activity which is associated with a reduction in lung cancer [72].

List of abbreviations

FDA	Food and Drug Administration
HC	Health Canada
IARC	International Agency for Research on Cancer
IUPAC	International Union of Pure and Applied Chemistry
MW	Molecular weight
NA	Not applicable
NMR	Nicotine metabolic ratio
NRT	Nicotine replacement therapy
PMI	Philip Morris International
RDT	Reproductive or developmental toxicant
RT	Respiratory toxicant
WHO	World Health Organization

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