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IMPORTANCE OF PHARMACOGENETIC AND ENVIRONMENTAL FACTORS FOR VARIATION IN CAFFEINE DISPOSITION: WITH SPECIAL EMPHASIS ON CYP1A2, CYP2A6, NAT2 AND XO

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Stockholm 2012

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ISBN 978-91-7457-681-8

ABSTRACT

Inter-individual variation in response to drugs conveys considerable risk of either therapy failure or drug toxicity. Caffeine, as one of the most frequently used psychoactive substances in the world, is not an exception, and both lack of effects and adverse reactions have been observed after usual doses. In addition to inter-individual differences at the drug-receptor level and drug-drug interactions, variability in caffeine metabolism has been proposed as a possible explanation. The objective of this study was to investigate the influence of pharmacogenetic and environmental factors, which are known to be of importance for inter-individual variation in drug disposition, on the activity of enzymes involved in caffeine metabolism, namely cytochrome P450 1A2 (CYP1A2) and 2A6 (CYP2A6), *N*-acetyltransferase-2 (NAT2) and xanthine oxidase (XO).

The study involved unrelated healthy volunteers from three ethnically distinct populations: Serbian (n=140), Swedish (n=190) and Korean (n=150). Phenotyping of CYP1A2, CYP2A6, NAT2 and XO was performed using caffeine as a probe drug and measuring concentration of parent compound and its metabolites in plasma or urine samples. CYP1A2 activity was estimated by 17X/137X plasma ratio, and CYP2A6, NAT2 and XO activities were determined by 17U/17X, AFMU/(AFMU+1X+1U) and 1U/(1U+1X) urinary ratios, respectively. In addition, subjects were genotyped for the most important *CYP1A2*, *CYP2A6* and *NAT2* polymorphisms.

Daily consumption of at least three cups of coffee in non-OC (oral contraceptive) users significantly increased CYP1A2 enzyme activity in both Swedes ($P<0.0001$) and Serbs ($P=0.0002$). When additionally controlling for smoking, the observed difference remained significant in both populations ($P\leq0.02$). Significant association of heavy coffee consumption with high CYP1A2 enzyme activity was observed only in carriers of −163 A/A, and increasing effect of −163C>A on CYP1A2 inducibility was found in both Serbian ($P=0.022$) and Swedish ($P=0.016$) nonsmoking heavy coffee consumers. There was no significant difference in CYP1A2 enzyme activity among genotypes in non–heavy coffee consumers. Controlling for the effect of smoking, heavy coffee consumption habit and OC use, significantly lower 17X/137X ratio was observed in Serbs than in Swedes ($P=0.0003$). Comparison between Swedes and Koreans revealed that functional *CYP2A6* alleles were more frequent in former, whereas the defective were more frequent in latter ($P\leq0.002$). *CYP2A6* genotype significantly affected enzyme activity in both populations ($P=0.004$), while no effect of sex, age, cigarette smoking or OC use was observed. CYP2A6 activity was higher in Swedes compared to Koreans, with 3.16% of Swedes and 18.75% of Koreans being slow metabolizers ($P=0.0001$). The observed differences between the two populations remained significant when controlling for the genotype effect, i.e. within rapid ($P=0.0007$) and intermediate ($P=0.04$) genotype groups. Rapid acetylator was the predominant NAT2 phenotype in Serbs, present in 55% of the population. Significant NAT2 genotype-phenotype association was detected in Serbs ($P<0.0001$), Swedes ($P=0.03$) and Koreans ($P=0.008$). Partial NAT2 genotype-phenotype discordance in Serbian rapid acetylators could not be explained by *NAT1* gene polymorphism. Koreans displayed significantly

higher NAT2 activity compared to Swedes, and the difference remained significant when controlling for the influence of cigarette smoking, sex, or OC use ($P < 0.0001$), as well as *NAT2* genotype ($P = 0.016$). Swedes and Koreans significantly differed in terms of *NAT2* genotype groups distribution ($P < 0.0001$). Stratified by genotype, among carriers of at least one wild type *NAT2*4* allele Koreans displayed higher enzyme activity compared to Swedes ($P = 0.004$). No significant influence of smoking or sex on NAT2 activity was observed in Serbs, Swedes or Koreans. OC use significantly increased NAT2 activity in Swedish women ($P = 0.007$). In terms of XO activity, no significant difference between Swedes and Koreans was detected. In Swedes, higher XO activity was observed in women compared to men ($P = 0.003$), and the effect remained significant after controlling for OC use ($P = 0.01$). OC use and cigarette smoking did not affect XO activity in either Swedes or Koreans.

In conclusion, habitual heavy coffee consumption induces CYP1A2 enzyme activity in carriers of *CYP1A2* −163 A/A genotype. *CYP2A6* genotype, but not sex, age, cigarette smoking and OC use, significantly affect CYP2A6 enzyme activity. *NAT2* genotype, but not sex and cigarette smoking, significantly affect NAT2 enzyme activity. Cigarette smoking and OC use do not affect XO enzyme activity. In Swedes, female sex and oral contraceptive use are associated with higher XO and NAT2 enzyme activities, respectively. Swedes display significantly higher CYP1A2 and CYP2A6 activities compared to Serbs and Koreans, respectively. Koreans display significantly higher NAT2 enzyme activity compared to Swedes. Serbs differ from other Caucasians in terms of *N*-acetylation capacity, due to unprecedented high prevalence of rapid acetylator phenotype. There is no inter-ethnic difference between Swedes and Koreans in terms of XO enzyme activity.

LIST OF PUBLICATIONS

I. **Djordjevic N**, Ghotbi R, Jankovic S, Bertilsson L, Aklillu E. Induction of CYP1A2 by heavy coffee consumption in Serbs and Swedes. Eur J Clin Pharmacol 2008; 64(4): 381-5.

II. **Djordjevic N**, Ghotbi R, Jankovic S, Aklillu E. Induction of CYP1A2 by heavy coffee consumption is associated with the CYP1A2 −163C>A polymorphism. Eur J Clin Pharmacol 2010; 66(7):697-703.

III. **Djordjevic N**, Carrillo JA, Ueda N, Gervasini G, Fukasawa T, Suda A, Jankovic S, Aklillu E. N-acetyltransferase-2 (NAT2) gene polymorphisms and enzyme activity in Serbs: unprecedented high prevalence of rapid acetylators in a white population. J Clin Pharmacol 2011; 51: 7: 994-1003.

IV. **Djordjevic N**, Carrillo JA, Roh HK, Karlsson S, Ueda N, Bertilsson L, Aklillu E. Comparison of N-acetyltransferase-2 enzyme genotype-phenotype and xanthine oxidase enzyme activity between Swedes and Koreans. J Clin Pharmacol 2011; DOI: 10.1177/0091270011420261

V. **Djordjevic N**, van den Broek MPJ, Carrillo JA, Roh HK, Bertilsson L, Aklillu E. Comparisons of CYP2A6 genotype and enzyme activity between Swedes and Koreans. In manuscript.

CONTENTS

LIST OF ABBREVIATIONS

CYP1A2	Cytochrome P450 1A2
CYP2A6	Cytochrome P450 2A6
NAT2	N-acetyltransferase-2
XO	Xanthine oxidase
137X	Caffeine (1,3,7-trimethylxanthine)
17X	Paraxanthine (1,7-dimethylxanthine)
17U	1,7-dimethyluric acid
AFMU	5-acetylamino-6-formylamino-3-methyluracil
1X	1-methylxanthine
1U	1-methyluric acid
OC	Oral contraceptive
PCR	Polymerase chain reaction
RFLP	Restriction fragment length polymorphism
AS	Allele specific
SNP	Single nucleotide polymorphism
HPLC	High performance liquid chromatography
bp	Base pair
AHR	Aryl hydrocarbon receptor
ARNT	Aryl hydrocarbon receptor nuclear translocator
PAHs	Polycyclic aromatic hydrocarbons
XRE	Xenobiotic response element

1 INTRODUCTION

1.1 VARIATION IN DRUG DISPOSITION

Inter-individual variability in response to drugs represents an important problem in clinical practice, as it conveys considerable risk of either lack of efficacy or development of adverse effects and toxicity in patients on generally accepted and recommended therapy regimen (1-3). This could be due to the differences in drug disposition, i.e. pharmacokinetics (absorption, distribution, metabolism and elimination), in drug pharmacodynamics, which is related to drug target, or both (4, 5). Although all drugs exhibit certain variability, the highest potential for clinically significant event is observed in those with narrow therapeutic range and extensive metabolism (4-6).

To ensure maximal efficacy and safety of drug therapy, physicians need to choose and prescribe an appropriate medication at the right dose for each patient (5, 7). Due to inter-individual variability in drug disposition and response, numerous factors, classified as either intrinsic or extrinsic, have to be taken into account. Typical intrinsic factors are related to the individual, and include body weight, age, gender, concomitant diseases, and genetics. On the other hand, typical extrinsic factors are related to the environment that the individual is exposed to, including concomitant drug use, dietary intake, as well as exposure to certain substances such as tobacco and alcohol (3, 4, 8-10).

Although recognized as building bricks of inter-individual differences, certain intrinsic and extrinsic factors could be shared among individuals, assembling them into populations that differ among each other in terms of drug disposition and response (11, 12). Populations that share “common traits and customs”, such as genetic heritage, social background and environment, are regarded as ethnic populations (13, 14). Therefore, ethnicity has been accepted as a multidimensional determinant of described inter-individual variability (3, 9, 14-16).

> Swedes belong to Scandinavian i.e. northern European population and live on the eastern side of the Scandinavian Peninsula, located at the far north of the Europe (17, 18). Serbs belong to the South Slavic Europeans that primarily inhabit central and western part of the Balkan Peninsula, known as the gate between the Europe and the Near East (19-21). Both Swedish and Serbian populations belong to Caucasians (20, 22). On the other hand, Korean population belongs to Asians and populates Korean Peninsula, which extends toward the south from the east part of the Asian continent (22, 23).

1.1.1 Pharmacogenetics

Genetic polymorphisms are naturally existing variants of the gene that are present in at least 1% of the population (24). Three major types of genetic polymorphisms are single nucleotide polymorphisms (SNPs), insertions/deletions (indels), and copy number variations (CNVs). Their functional effects depend on their localization within the gene or its flanking regions, and range from complete lack of protein production to induction of gene expression (5, 25). It has been estimated that up to 95% of inter-individual variability in drug disposition and response could be explained by genetic

polymorphisms alone, or by its interaction with other intrinsic and extrinsic factors (24).

Genetics can affect efficacy and safety of any drug whose pharmacokinetics or pharmacodynamics are regulated by proteins encoded by polymorphically expressed genes (6, 8, 24). Nevertheless, probably the most important source of phenotypically evident genetic variability lies within drug metabolizing enzymes that control phase I or phase II reactions (5-8, 25). Depending on the existing variants of the metabolizing enzyme coding gene, resulting phenotype can range from poor metabolizer, which can cause drug accumulation and toxicity, to ultrarapid metabolizer, leading to fast drug elimination and likely failure of the therapy (6, 10). The science that deals with the role of genetics in drug response is called pharmacogenetics (6).

1.1.2 Environmental factors

To date, numerous environmental factors influencing drug disposition and response have been identified, including nutrition components, cigarette smoking, alcohol, drugs and herbal products, as well as different xenobiotics present in food, water, soil or air, such as polycyclic aromatic hydrocarbons, dioxins, organic solvents, and organophosphate insecticides (26). Environmental factors may interact with drugs at different levels, increasing or decreasing their activity or toxicity. Yet, the most common interactions involve drug metabolizing enzymes and result in metabolism induction or inhibition (5).

The mechanism behind enzyme induction involves enhanced synthesis by activation of ligand-regulated transcription factors (e.g. induction of CYP1A2 by cigarette smoking) (27), or enzyme stabilization following transcription (e.g. induction of CYP2E1 by alcohol intake) (28). On the other hand, enzyme inhibition refers to competition for the enzyme's active site (e.g. inhibition of CYP3A4 by indinavir) (29), or binding to a separate site on the enzyme or to enzyme-substrate complex, making the enzyme nonfunctional (e.g. inhibition of CYP3A4 by erythromycin) (26). The clinical consequences of metabolism induction or inhibition by environmental factors range from therapeutic failure to adverse drug effects and drug toxicity (30).

1.2 CAFFEINE

Caffeine (1,3,7-trimethylxanthine) is an alkaloid naturally present in seeds, leaves or fruit of plants such as coffee (*Coffea sp.*), tea (*Camellia sinensis*), cocoa (*Theobroma cacao*), kola (*Cola acuminate*), yerba mate (*Ilex paraguariensis*), and guarana (*Paullinia cupana*) (31). History of caffeine use dates back thousands of years: first tea consumption has been attributed to Chinese emperor Shen Nung and his accidental discovery of the drink in 2737 B.C, while indulging in coffee originates from Ethiopia and is credited to goat herder Khaldi, who lived around 850 A.D (32-34). Not until 16th and 17th century caffeine consumption became widespread, yet today it represents one of the most frequently used psychoactive substances in the world (35). Variety of common beverages, products containing cocoa or chocolate, as well as certain medications, contains caffeine (31, 35, 36). Nevertheless, its primary source is coffee, delivering approximately 70% of all consumed caffeine to about one-third of the world population on a daily basis (34, 37).

Caffeine acts through multiple mechanisms, including inhibition of phosphodiesterase and blockade of γ-aminobutyric acid (GABA) receptors, but its competitive antagonism of adenosine effects is the most important at typical plasma concentrations (32, 36, 38, 39). Adenosine is an endogenous purine nucleoside that at physiological levels preferentially activates its A_1 and A_{2A} receptors, primarily localized in the brain (32, 40-42). Blockade of both A_1 and A_{2A} receptors prevents adenosine-induced GABA inhibition of dopaminergic system and increases the release of various neurotransmitters, and it is responsible for stimulating effects of caffeine (35, 40, 42).

In moderate doses, caffeine increases vigilance, endurance and neuromuscular coordination, improves mood and cognitive performance and decreases anxiety and fatigue (32, 35, 38, 39, 41, 43). In addition, it elevates circulating catecholamine levels and basal metabolism, promote lipolysis and help in weight loss, while in sensitive individuals may even raise systolic blood pressure (32, 35, 42). In clinical practice, caffeine is used as an analgesic in combination with aspirin, acetaminophen, codeine or ergotamine, but also as a short-term treatment of neonatal apnea of prematurity (44). With chronic caffeine use, the tolerance to its effects can develop within hours or days, and the reason is considered to be upregulation of A_1 receptors (32, 39). When the regular consumption of coffee is discontinued, the same mechanism causes the caffeine withdrawal syndrome, characterized by headache and fatigue as the most prominent symptoms, followed by irritability, nausea and vomiting. Withdrawal symptoms typically begin at 12-24 hours and peak at 20-48 hours after cessation of caffeine consumption, disappear within the three days after the last caffeine dose, but may persist for a week (32, 35, 40, 41, 43).

Very high doses of caffeine could lead to tachycardia, nausea, anxiety, restlessness and tremors (42), while the acute toxic dose of caffeine in adults has been estimated to approximately 10 grams per day (32, 42). On the other hand, moderate daily consumption of up to 400 mg of caffeine, except in childhood and pregnancy, should pose no risks to human health. Furthermore, beneficial effects of regular caffeine intake, including prevention of Parkinson's disease, diabetes mellitus or skin cancer, have been discovered (39, 42). However, not everyone respond to caffeine as described, so both lack of effects and adverse reactions have been observed after usual doses. In addition to inter-individual differences at the drug-receptor level and drug-drug interactions, variability in caffeine pharmacokinetics has been proposed as a possible explanation (1, 35).

Pharmacokinetics of caffeine has been widely investigated, and its basic principles are already well known. After ingestion, caffeine undergoes rapid and complete absorption from the gastrointestinal tract, reaching 100% bioavailability and the peak plasma level between 30 and 75min (31, 36). Due to its lipophilic properties and low plasma protein binding, caffeine easily penetrates all biologic membranes, including placental and blood-brain barrier (32, 38). It has an average half-life of about 4 to 6 hours and a total clearance of approximately 2ml/min/kg (36, 38). Only 2-3% of ingested dose is excreted unchanged in urine (36, 45). Metabolism of caffeine exhibits linear pharmacokinetics, which becomes saturated at doses higher than 250mg. It is mainly restricted to liver, and involves several drug-metabolizing enzymes, including

cytochrome P450 1A2 and 2A6, *N*-acetyltransferase-2 and xanthine oxidase (45-49) (Figure 1). Since it is safe and inexpensive, with rapid and complete absorption and metabolism short half-life, caffeine is considered an ideal probe drug for estimation of *in vivo* activity of these enzymes (47).

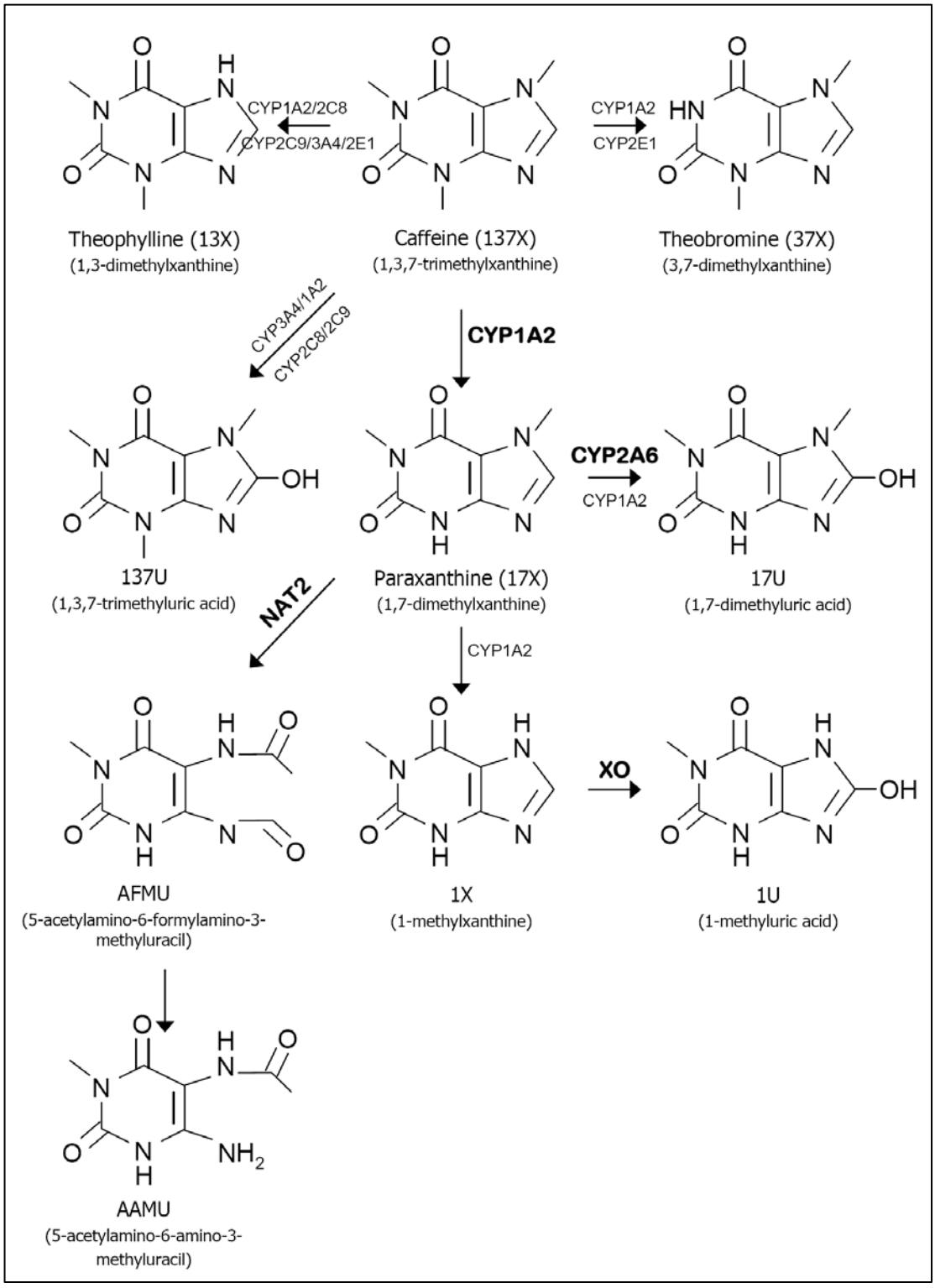

Figure 1. Caffeine metabolism in humans.

1.2.1 CYP1A2

Cytochrome P450 1A2 (CYP1A2) is an important phase I hepatic enzyme that metabolizes various endogenous substrates, such as steroid hormones, arachidonic acid and melatonin, and activates several procarcinogens, e.g. arylamines and aflatoxin B_1. Moreover, it is involved in biotransformation of more than 100 clinically used drugs, including antipsychotics clozapine, olanzapine and haloperidol, antidepressants amitriptyline and imipramine, sedative diazepam, analgesic acetaminophen, cardiovascular drugs propranolol, verapamil, anticoagulant warfarin, as well as theophylline and caffeine (48, 50-52).

CYP1A2 gene is located on chromosome 15 (15q22 – q24), spans about 7.8 kb and comprises 7 exons (50, 53). To date, numerous variations of *CYP1A2* gene have been reported (http://www.cypalleles.ki.se/cyp1a2.htm). However, only few of them have been associated with alteration in enzyme activity: substitution C>A at position −163 (rs762551), which tags *CYP1A2*1F* allele, leads to increased enzyme inducibility (54), while carriers of *CYP1A2*1C* (−3860G>A, rs2069514) display decreased CYP1A2 activity (55). Single nucleotide polymorphism (SNP) -163C>A represents common *CYP1A2* polymorphism, observed at 60-70% frequency in many Caucasian, Asian and African populations (27, 56, 57). On the other hand, −3860G>A was found to be mainly present in Asia (the frequency of about 25%), while it is extremely rare in other populations (27, 56, 57).

CYP1A2 activity has been reported to vary significantly among individuals, resulting in up to 40-fold difference in steady-state plasma concentration of substrate (52, 58). This wide variation has been attributed to either genetic or environmental factors (58). It has been shown that 42, 38, and 33% of the catalytic activity, protein expression, and mRNA levels, respectively, seem to be due to genetic variation of CYP1A2 (52). Environmental factors of influence include cigarette smoking (27), charcoal-grilled meat (59), omeprazole (56) and carbamazepine (60) as inducers, and oral contraceptives (27), fluvoxamine (61) and fluoroquinolone antibiotics (52) as inhibitors. Investigations of gender difference in CYP1A2 activity (27, 62, 63) or possible influence of caffeine consumption (63, 64) gave inconclusive results. Inter-ethnic differences in CYP1A2 activity have been reported as lower enzyme activity in black compared to white populations (62, 65).

As the metabolism of caffeine is more than 90% dependent on CYP1A2 (transformation of caffeine into its main metabolite 17X), its clearance is considered the standard probe for assessment of CYP1A2 activity (48, 52, 58). Many urine caffeine metabolic ratios, such as (17X+17U)/137X (66) and (AFMU+1X+1U)/17U (64), have been used, but plasma or saliva 17X/137X ratio has been suggested as the most appropriate for estimation of *in vivo* CYP1A2 activity (46, 67, 68).

1.2.2 CYP2A6

Cytochrome P450 2A6 (CYP2A6) is another phase I metabolizing enzyme, expressed mainly in the liver, which is important for metabolism of steroids and fatty acids and activation of procarcinogenes (e.g. nitrosamines and aflatoxin B1). In addition, it plays

an essential role in biotransfomation of certain drugs, including antiepileptic valproic acid, anestetics halothane and methoxyflurane, aldehyde dehydrogenase inhibitor disulfiram, chemotherapeutic drugs cyclophosphamide, iphosphamide and tegafur, well as nicotine and caffeine (50, 69-72).

CYP2A6 gene has been mapped to chromosome 19 (19q12 – q13.2), spans a region of about 6 kb and comprises 9 exons (69), and it is highly polymorphic (http://www.cypalleles.ki.se/cyp2a6.htm). Many of the described variants are nonsynonimous, e.g. *CYP2A6*4* (gene deletion) and *CYP2A6*5* (1436G>T, rs5031017) completely lack enzyme activity, *CYP2A6*7* (1412T>C, rs5031016), *CYP2A6*8* (1454G>T, rs28399468) and *CYP2A6*9* (-48T>G, rs28399433) decrease CYP2A6, while *CYP2A6* duplication corresponds to the presence of three copies of the gene and thus provides about 1.4-fold higher enzyme activity (73-77).

Wide inter-individual variations in CYP2A6 activity (more than 100-fold) that have been observed seem to be affected by a variety of factors (69, 72). It has been shown that certain drugs, including rifampicin, dexamethasone, phenobarbital and oral contraceptives (50), act as inducers, whereas liver diseases, such as viral hepatitis A or alcohol-induced cirrhosis (69), as well as consumption of grapefruit juice and intake of ketokonazole (50, 78, 79), decrease CYP2A6 activity. Several previous studies described inhibitory effect of cigarette smoking on CYP2A6 enzyme activity (80, 81), while some researchers (82, 83), but not all (84, 85), reported sex as a factor of influence. Nevertheless, genetic polymorphisms of the *CYP2A6* gene are considered the major cause for the observed inter-individual variability of CYP2A6 enzyme activity (69, 75, 86). Pronounced inter-ethnic variations in CYP2A6 activity and expression have been observed as well, usually due to the well-known difference in the frequency of *CYP2A6* alleles among populations (69).

In the metabolism of caffeine, CYP2A6 participates in the hydroxylation of the caffeine metabolite 17X to yield 17U (48). Due to its favorable pharmacokinetics properties and proven safety, caffeine test is considered valid and useful method for estimation of *in vivo* CYP2A6 activity (72, 84). To assess enzymatic activity of CYP2A6, urinary metabolic ratios 17U/(17U+17X/137X+1U+1X+AFMU) (85) or 17U/17X (87) have been used successfully.

1.2.3 NAT2

Arylamine *N*-acetyltransferase 2 (NAT2) is a phase II enzyme with predominant localization in the liver, where it participates in metabolism of many xenobiotics (88). Substrates for NAT2 include commonly used drugs, such as antibacterials isoniazid, sulfamethoxazole and dapsone, chemotherapeutic drug aminoglutethimide, antiarrhythmic procainamide, anti-inflammatory drug 5-aminosalicylic acid, antihypertensives hydralazine and acebutolol, MAO inhibitor phenelzine, as well as caffeine (89-93). In addition, it metabolizes several environmental toxicants, including potentially carcinogenic heterocyclic amines present in well-cooked red meat and cigarette smoke (92).

NAT2 gene is located on chromosome 8 (8p21.3-23.1) and consists of one intronless open reading frame of 870 bp (92-94). More than 60 variant alleles of NAT2 gene have been identified so far (http://louisville.edu/medschool/pharmacology/consensus-human-arylamine-n-acetyltransferase-genenomenclature/nat_pdf_files/Human_NAT2_alleles.pdf/view). The presence of at least one wild-type *NAT2*4* or variant **11* (481C>T, rs1799929), **12* (803A>G, rs1208), **13* (282C>T, rs1041983) and **18* (845A>C, rs56054745) allele provides normal enzyme activity (92, 95). The rest of the *NAT2* allelic variants usually share one or more common missense single nucleotide polymorphisms, resulting in decreased expression, low activity, or enzyme instability (89, 92, 95).

Wide range of *N*-acetylation activity has been observed, classifying individuals as slow, intermediate, or rapid acetylators (92, 93). NAT2 is not inducible to a significant extent *in vivo* (79, 89). Instead, *NAT2* genetic polymorphism is considered the most important underlying cause of the described variability (89, 92, 95, 96). It has been shown that frequencies of *NAT2* SNPs differ among ethnic populations, leading to substantial inter-ethnic variability in *N*-acetylation capacity (79, 88, 90, 92, 93, 97).

NAT2 participates in metabolism of caffeine by converting 17X into AFMU (48, 49). Since it is much safer than other NAT2 probe drugs, e.g. sulfamethazine or isoniazid, caffeine test is considered the most appropriate for the assessment of *in vivo N*-acetylation status (92). Both AFMU/1X and AFMU/(AFMU+1X+1U) urinary ratios are proved to be reliable indicators of NAT2 enzyme activity (62, 84, 92, 98).

1.2.4 XO

Xanthine oxidase (XO) is an important phase I enzyme, in humans predominantly expressed in the liver and intestine (99-101). It catalyzes essential constitutive reactions involving endogenous purines, pyrimidines and aldehydes, with the conversion of hypoxanthine to xanthine and further to uric acid as one of its primary roles (100, 102-107). In addition, XO is responsible for biotransformation of several xenobiotics, including clinically important drugs such as allopurinol, acyclovir, mercaptopurine, azathioprine, pyrazinamide, methotrexate, fluorouracil, doxorubicin, daunomycin, and mitomycin C, as well as caffeine (99-101, 103, 106-108).

The human *XO* gene is localized on chromosome 2 (2p22), it consists of 36 small exons spanning a region of over 60 kbp (100, 104). Variations of the gene have been described, but they rarely proved to be functional (91, 101).

There are marked inter-individual and inter-ethnic differences in XO activity, with approximately 20% of Caucasians, 11% of Japanese and 4% of Ethiopians displaying lower enzyme activity (62, 84, 101, 109, 110). In addition, XO can be up- or down-regulated under a variety of non-genetic factors (104). It has been observed that its level and activity increases during certain diseases, such as arthritis, asthma, viral hepatitis, and hypoxia-reperfusion injury (104-108, 111, 112), and the inducing effect has been attributed to cytokines, including tumor necrosis factor-α, interleukin-1β, and interferon-γ (104, 105). XO activity can be induced by xenobiotics too, both drugs (dexamethasone, ethacrynic acid and iron) and hepatotoxic agents, such as halothane

alcohol, carbon tetrachloride and 2,3,7,8-tetrachlorodibenzo-p-dioxin (91, 101, 106, 107, 113). On the other hand, XO can be inhibited by allopurinol and its metabolite oxypurinol, and this effect serves as a foundation in the treatment of hyperuricemia and its complications (91, 106, 107). It is generally accepted that cigarette smoking does not affect XO activity (84, 108, 110), but numerous investigations of possible sex differences yielded contradictory results (62, 84, 99, 102, 108, 110).

XO participates in the metabolism of caffeine by converting 1X to 1U (47). Thus, caffeine as a probe and urinary (1U)/(1X+1U) ratio has been accepted as appropriate for estimation of *in vivo* XO enzyme activity (47, 91, 99, 101, 103).

2 OBJECTIVES

The general objective of the study was to investigate the influence of pharmacogenetic and environmental factors on the activity of drug metabolizing enzymes involved in caffeine disposition, namely CYP1A2, CYP2A6, NAT2 and XO, in three ethnically distinct populations: Serbs, Swedes and Koreans.

Specific objectives of the study were as follows:

1. To investigate the effect of regular heavy coffee consumption, *CYP1A2* genotype and ethnicity on CYP1A2 enzyme activity **(papers I, II)**.
2. To investigate the effect of sex, age, cigarette smoking, OC use, *CYP2A6* genotype and ethnicity on CYP2A6 enzyme activity **(paper V)**.
3. To investigate the effect of sex, cigarette smoking, OC use, *NAT2* genotype and ethnicity on NAT2 enzyme activity **(papers III, IV)**.
4. To investigate the effect of sex, cigarette smoking, OC use and ethnicity on XO enzyme activity **(paper IV)**.

3 MATERIALS AND METHODS

3.1 SUBJECTS

The study involved unrelated healthy volunteers of three distinct ethnic origins: Serbian, Swedish and Korean. Serbs (n=140) were 18 to 46 years old (median age: 27 years), and the group included 72 men, 38 smokers (smoking on average two or more cigarettes per day), 47 heavy coffee consumers (regularly drinking three or more cups of coffee per day), and no oral contraceptive (OC) users. Swedes (n=190) were 18 to 60 years old (median age: 29 years), and in the group there were 79 men, 39 smokers, 63 heavy coffee consumers and 44 OC users. Koreans (n=150) were 20 to 46 years old (median age: 25 years) and included 74 men, 28 smokers and only one OC user, while the data on regular coffee consumption were not available. None of the study subjects used any medications, were pregnant or breast-feeding.

As some of the data, including genotyping and phenotyping results, were missing at random (could not be obtained for some of the subjects), subjects with missing data were excluded from certain analyses, i.e. complete case analysis approach was introduced. In addition, some of the analyses required specific exclusion criteria to be met, e.g. in paper II the effect of heavy coffee consumption could not be tested in smokers. Therefore, the sample size differed: paper I included 100 Serbs and 149 Swedes, paper II were based on the data obtained from 126 Serbs and 114 Swedes, paper III involved 140 Serbs, 113 Swedes and 150 Koreans participated in paper IV, while 190 Swedes and 144 Koreans took part in paper V.

3.2 STUDY DESIGN

All study subjects were advised to abstain from any form of caffeine intake, including coffee, tea, chocolate and other caffeine-containing food or beverage consumption, for at least (114) 24 h prior to and throughout the study. After completing the wash-out period, subjects received single oral dose of 100 mg caffeine (Koffein Recip; Recip AB, Årsta, Sweden) and answered a detailed questionnaire about their smoking habit, regular coffee consumption and OC use (115).

Four hours after caffeine intake (68), a 20-ml venous blood sample was drawn into EDTA-containing vacutainer tubes (Sarstedt, Nümbrecht, Germany): 10ml was kept as a whole blood sample, and 10ml was centrifuged and plasma separated. In addition, 8-hours urine was collected, pH adjusted to 3.5 with 0.1 M HCl to ensure the stability of AFMU (85) and 20-ml aliquots were stored.

All samples were frozen at −80°C. Blood and plasma samples collected outside Stockholm (in Serbia and Korea) were packed on dry ice and sent to Karolinska University Hospital, Huddinge, Sweden, where genotyping and CYP1A2 phenotyping analyses were performed. Urine samples were sent to Extremadura University in Badajoz, Spain, for CYP2A6, NAT2 and XO phenotype analyses.

3.3 LABORATORY ANALYSES

3.3.1 Genotyping

Genomic DNA was extracted from the whole blood samples using QIAamp DNA Mini Kit (QIAGEN GmbH, Hilden, Germany). DNA concentration and purity were measured using DU®530 Life Science UV/Visible Spectrophotometer (Beckman Coulter, Fullerton, CA).

Genotyping for ***CYP1A2* polymorphisms (paper II)** -3860G>A (rs2069514), -739T>G (rs2069526), -729C>T (rs12720461), -163C>A (rs762551), 2159G>A (rs2472304), 4795G>A (rs12904742) was carried out using the tag-array minisequencing method according to Lindros et al.(116). In brief, DNA fragments spanning *CYP1A2* polymorphic sites of interest were amplified by multiplex PCR reaction, using six PCR primer pairs on GeneAmp® PCR System 2700 (Applied Biosystems, Foster City, CA). After removing excess of PCR primers and dNTPs, products of multiplex PCR reaction were subjected to minisequencing reaction on PTC-225 Peltier Thermal Cycler (Bio-Rad, Hercules, CA, USA), with the extension mix that contained fluorophore-labelled ddNTPs and tagged minisequencing primers. Multiplex PCR and minisequencing primers were designed by OligoPerfect Designer (www.invitrogen.com) and Auto-Primer software (http://www.autoprimer.com/), respectively, and purchased from Integrated DNA Technologies (IDT, Coralville, IA, USA). Products of minisequencing reaction were then pipetted onto the microarray, spotted with oligonucleotides complementary to the "tag" sequence of the minisequencing primers. After completed hybridization, fluorescence signals from microarray were measured using ScanArray Express HT® microarray scanner (Perkin Elmer Life and Analytical Sciences, Inc., Waltham, MA) and QuantArray® 2001 software, version 3.0.0.0 (Packard BioScience Company, Meriden, CT). The QuantArray file was exported and analysed using the SNPSnapper analysis software, version 4.0 beta, (http://www.bioinfo.helsinki.fi/SNPSnapper). SNPs were detected based on the type of the fluorescence signal (Texas-Red–ddATP, Tamra–ddCTP, R110–ddGTP, Cy5–ddUTP) received from the specific position on the microarray.

In addition, *CYP1A2* polymorphism −2467delT (rs35694136) was determined by PCR-RFLP method described by Chida et al. (117). In short, the fragment of *CYP1A2* spanning the SNP was amplified in the PCR reaction on GeneAmp® PCR System 9700 (Perkin-Elmer Applied Biosystems, Foster City, CA) and then subjected to the restriction enzyme NdeI (New England Biolabs, Ipswich, MA). As enzyme cuts only wild type allele to fragments of 149 bp and 18 bp, SNP detection was conducted using electrophoresis on 2% agarose gel.

Genotyping for ***CYP2A6* polymorphisms (paper V)** gene deletion, gene conversion in the 3′ region, 1436G>T (rs5031017), 1412T>C (rs5031016), 1454G>T (rs28399468) and gene duplication was carried out using the AS-PCR methods, as previously described in the literature. In brief, gene deletion was detected using nested AS-PCR reaction (118), with the first PCR reaction used to amplify the region common for both *CYP2A6* and *CYP2A7* genes, followed by a second amplification with primers specific for either wild type or *CYP2A6del* allele. Gene conversion in the 3′ region and

1436G>T were also detected by nested AS-PCR reaction (119), where the first PCR reaction was common for both SNPs, and the allelic discrimination was performed separately with different primer pairs. Similar nested AS-PCR methods was used for separate genotyping of 1412T>C (rs5031016) and 1454G>T (rs28399468) (120). Genotyping for gene duplication was performed by one-step AS-PCR method, with either *CYP2A7*-specific or CYP2A6-specific forward primer in addition to *CYP2A7* reverse to distinguish between the wild-type *CYP2A7* and the duplicated *CYP2A6*, respectively (76). All PCR reactions were performed on GeneAmp PCR System 2700 (Applied Biosystems, Foster City, CA), and the products were detected by electrophoresis on 1.2% agarose gel.

CYP2A6 polymorphism −48T>G (rs28399433) was determined using newly designed nested AS-PCR method. First PCR reaction was performed with 5'-TGGCTGTGTCCCAAGCTAGGCA-3' as forward and 5'-CTTCATGAGGGAGTT GTACATC-3' as reversed primer, in the following conditions: initial denaturation at 95°C for 1 min; 35 cycles of denaturation at 95°C for 15 s, annealing at 56°C for 20 s, extension at 72°C for 90 s; final extension at 72°C for 7 min. In AS-PCR reaction, allele-specific forward primer 5'-CTTTTTCAGGCAGTAT-3' or 5'-CTTTTTCAG GCAGTAG-3' was used with the common 5'- CTTCATGAGGGAGTTGTACATC-3' reversed primer, and the reaction conditions were as follows: initial denaturation at 95°C for 1 min; 15 cycles of denaturation at 95°C for 15 s, annealing at 54°C for 20 s, and extension at 72°C for 45 s; and final extension at 72°C for 7 min. PCR reactions were performed on GeneAmp PCR System 2700 (Applied Biosystems, Foster City, CA). PCR products were detected by electrophoresis on 1.2% agarose gel.

Genotyping for ***NAT2* polymorphisms (papers III, IV)** 190C>T (rs1805158), 403C>G (rs12720065), 411T>A (rs4986997), 590G>A (rs1799930), 803A>G (rs1208), and 857G>A (rs1799931) were carried out using the tag-array minisequencing method according to according to Lindros et al.(116), as described above. *NAT2* polymorphisms 191G>A (rs1801279), 282C>T (rs1041983), 341T>C (rs1801280), and 481C>T (rs1799929) were detected using restriction fragment length polymorphism (RFLP) or allele-specific (AS) PCR reaction, as reported by Anitha and Banerjee (121). Briefly, after amplifying approximately 1.2 kb of *NAT2* gene region of interest, PCR products were separately digested by the restriction enzymes MspI, FokI and KpnI (New England Biolabs, Ipswich, MA) and the pattern of restrictions were examined to detect 191G>A, 282C>T and 481C>T, respectively. On the other hand, to distinguish between T and C at the position 341, two separate PCR reactions with either wild- or variant-specific forward primer and the common reverse primer were performed on GeneAmp PCR System 2700 (Applied Biosystems, Foster City, California). Both RFLP- and AS-PCR products were analyzed by gel electrophoresis on 1.2% agarose gel.

Furthermore, coding regions of the *NAT2* gene were amplified, purified and sequenced in both forward and reverse directions, using the ABI PRISM™ BigDye terminator cycle sequencing ready reaction kit version 3.1 (Applied Biosystems, Foster City, California) and ABI Prism 377 DNA sequencer. *NAT2* sequence chromatograms were analyzed using software FinchTV version.1.4.0 (http://www.geospiza.com) and aligned

with the NAT2 reference sequence (http://www.ncbi.nlm.nih.gov; GenBank reference: NM 000015.2) for SNPs identification.

In addition, Serbian subjects (paper III) were genotyped for three common *NAT1* polymorphisms coding for the NAT1 fast acetylators phenotype, namely 445G>A, 640T>A and 1088T>A. Genotyping for 445G>A and 640T>A was performed by real-time PCR and the Taqman genotyping assays using the ABI 7500 FAST (Applied Biosystems, Foster City, California) (122). Genotyping for 1088T>A was performed as described by Henning et al. (123), using two-step (nested) AS-PCR reaction: first amplification that used one pair of primers was followed by AS-PCR for allelic discrimination, i.e. two separate PCR reactions with different forward primers and the common reverse primer. AS-PCR products were analyzed by gel electrophoresis on 1.2% agarose gel.

Genotyping of ***XO*** was not performed, since this gene is not considered significantly polymorphic (91, 101).

3.3.2 Phenotyping

Phenotyping of CYP1A2, CYP2A6, NAT2 and XO was performed using caffeine (Koffein; ACO AB, Helsingborg, Sweden) as a probe drug and measuring concentration of parent compound and its metabolites in plasma or urine samples.

To estimate *in vivo* **CYP1A2 enzyme activity (papers I, II)**, molar concentrations of caffeine (1,3,7-trimethylxanthine or 137X) and its metabolites paraxanthine (1,7-dimethylxanthine or 17X) were determined in plasma samples according to Ghotbi at al. (27), using reversed-phase HPLC with UV detection. In short, caffeine and paraxanthine were extracted from 100μ plasma samples with 300 ml acetonitrile, after addition of 25 μl of 50 μM internal standard (β-hydroxyethyl-theophiline). The organic phase was evaporated to dryness at the room temperature under nitrogen. After resolving the residue in 50 μl mobile phase, 15 μl was injected into the column. All samples, standards and quality controls were measured in one run and in duplicate. The flow rate was 1.0 ml/min and the compounds were monitored at 273.0 nm. The retention time was 5.5 - 7.6 min and 11.5 – 16.7 min for paraxanthine and caffeine, respectively. Calibration curves were linear, with the coefficients of correlation higher than 99%. At 16.3 μM for caffeine and 10.3 μM for paraxanthine, withinday and between-day coefficients of variations were less than 10 and 5% respectively. The HPLC equipment for the caffeine assay included 402 Dilutor-Dispenser (Gilson Inc., Middleton, WI), 231 XL Sample Injector (Gilson Inc., Middleton, WI), Dionex P580 Pump P480 (Dionex, Sunnyvale, CA) and 1010 Model Lambda - UV/VIS-Detektor (Bischoff, Leonberg, Germany). For the separation of molecules we used C18 Luna column (100 × 4.6 mm ID, 3 μm), and the precolumn Security Guard Cartridges C18, 4 x 3 mm, both purchased from Phenomenex Inc., Torrance, CA. Chromatographic data were registered and processed by ELDS Win Pro v 1.1 software (Chromatography Data System, Svartsjö, Sweden). *In vivo* CYP1A2 enzyme activity was estimated by the 17X/137X ratio (124).

To estimate *in vivo* **CYP2A6 enzyme activity (paper V),** high-performance liquid chromatography (HPLC) was used to determine the molar concentrations of caffeine metabolites 17X (1,7-dimethylxanthine or paraxanthine) and 17U (1,7-dimethyluric acid) in collected urine samples, according to Carillo et al. (125). In brief, 17X and 17U were extracted simultaneously with 4 ml chloroform/isopropanol, after the addition of 6 μg/50 μl internal standard (N-acetyl-p-aminophenol) and 120 mg ammonium sulfate. The organic phase was evaporated to dryness at 45°C under nitrogen, the residue reconstituted in 200 μl mobile phase, and 50 μl injected onto a Ultrasphere-IP (5 μm particle size, 250 × 4.6 mm ID) reversed-phase column (Beckman Instruments, Madrid, Spain), eluted isocratically with acetic acid/acetonitrile/tetrahydrofuran/water (1:40:2:957 v/v). The flow rate was 1.0 ml/minute and the detection wavelength was 280 nm. Standard curves were with the coefficients of correlation higher than 99%. The limits of detection for the metabolites were within the range of 0.1 to 0.3 μmol. Withinday and intra-assay coefficient of variation (CV) was less than 10% at all concentration levels and for all metabolites, and the accuracy was between 96% and 105%. System Gold Microbore HPLC with 32 Karat Software (Beckman Instruments, Madrid, Spain) was used to process the HPLC data. *In vivo* CYP2A6 enzyme activity was estimated by the 17U/17X ratio (81, 87).

In vivo **NAT2 and XO enzyme activities (papers III, IV)** were assessed by the caffeine urinary test, according to Carrillo et al. (125). Molar concentrations of caffeine metabolites AFMU (5-acetylamino-6-formylamino-3-methyluracil), 1X (1-methyl xanthine) and 1U (1-methyluric acid) were determined by HPLC method, as described above. *In vivo* NAT2 and XO enzyme activities were estimated by the AFMU/(AFMU +1X+1U) and 1U/(1U+1X) ratios, respectively (84, 110, 126).

3.4 STATISTICAL ANALYSES

All statistical analyses were performed with Statistica, version 7.1 (StatSoft Inc, Tulsa, OK, USA). For all statistical procedures, $P<0.05$ was considered as significant.

The 17X/137X, 1U/(1U+1X), AFMU/(AFMU+1X+1U) and 17U/17X ratios were log-transformed before statistical analyses, and their normality of distribution was assessed by Kolmogorov-Smirnov or Shapiro-Wilk test **(papers I-V)**. Student's t-test, Mann-Whitney test or one-way analysis of variance (ANOVA) followed by the post-hoc analyses were used to determine the influence of different factors on CYP1A2 enzyme activity **(papers I, II)**. Haplotype analysis was carried out using the population genetic software program Arlequin, version 3.11 (http://cmpg.unibe.ch/software /arlequin3), and chi-square test was used to compare obtained with expected allele frequencies, i.e. to test the consistency with the Hardy-Weinberg equilibrium **(papers II-V)**. Kruskall-Wallis analysis, ANOVA followed by the post-hoc analyses or multiple linear regression were used to assess the effect of possible influencing factors on NAT2 and XO activity **(papers III, IV)**. Effect on CYP2A6 enzyme activity was tested by multiple regression analysis **(paper V)**.

3.5 ETHICAL CONSIDERATIONS

All subjects participated voluntarily in the studies after signing written informed consent. The study was conducted in accordance with the Declaration of Helsinki and its subsequent revisions, and approved by the ethics committees at the Karolinska Institutet, Stockholm, Sweden, No.2009/219-32 **(papers I-V)**, the Medical Faculty, University of Kragujevac, Serbia, No.01-1995, No.01-4502, No.01-6427/6 **(papers I-III)**, and at the Inha University Hospital, Korea **(papers IV, V)**.

4 RESULTS

4.1 CYP1A2

Having analyzed only non-OC users, significantly higher CYP1A2 enzyme activity in heavy coffee consumers compared to non-heavy coffee consumers was observed in Swedish (n=149, P<0.0001; 95% CI of the mean difference 0.06, 0.16) and Serbian (n=100, P=0.0002; 95% CI of the mean difference 0.08, 0.21) subjects. In both populations, the observed difference remained significant when additionally controlling for smoking, i.e. when analysis was performed among Swedish (n=114, P=0.02; 95% CI of the mean difference 0.01, 0.12) and Serbian (n=72, P=0.003; 95% CI of the mean difference 0.04, 0.18) non-smokers. In smokers, however, only Swedes displayed higher CYP1A2 activity if heavy coffee consumers (n=35, P<0.0001; 95% CI of the mean difference 0.13, 0.33), while no significant difference was observed among Serbian smokers (n=28, P=0.77) **(paper I)**.

Effect of *CYP1A2* genotype on enzyme activity was analyzed among non-OC users and non-smokers, and −163C>A polymorphism was detected as the only predictor. Significantly higher CYP1A2 activity was observed in carriers compared to non-carriers of −163C>A polymorphism in both Swedish (n=42, P=0.016) and Serbian (n=17, P=0.022) heavy coffee consumers. The highest enzyme activity among heavy coffee consumers was detected in the −163A/A genotype group in both populations, observed as significantly higher compared to homozygous carriers of variant type allele among Swedes (P=0.036; 95% CI of the mean difference 0.01, 0.23) and Serbs (P=0.001; 95% CI of the mean difference 0.09, 0.36). However, among non-heavy coffee consumers no significant effect of −163C>A polymorphism on CYP1A2 enzyme activity was observed in either Swedes (n=72, P≥0.31) or Serbs (n=47, P≥0.15). In addition, the inducing effect of heavy coffee consumption on CYP1A2 activity was observed only in carriers of −163A/A genotype in both Swedes (n=46, P<0.0001; 95% CI of the mean difference 0.08, 0.22) and Serbs (n=22, P=0.005; 95% CI of the mean difference 0.06, 0.28) **(paper II)**.

When controlling for the effect of cigarette smoking, heavy coffee consumption and OC use, significantly higher CYP1A2 enzyme activity (P=0.0003; 95% CI of the mean difference -0.14, -0.04) was observed in Swedes (n=72) compared to Serbs (n=54) **(paper I)**.

4.2 CYP2A6

Except for the 1436G>T polymorphism that was absent in both populations, the distribution of *CYP2A6* variant alleles and genotypes was significantly different between Swedes and Koreans (P≤0.002). The distribution of genotype groups, appointed according to the presence of functional (**1A*, **1x2*, or **1B1*) or less functional/non-functional *CYP2A6* alleles (**4*, **7*, **8*, **9* and **10*), also significantly differed between the populations (P<0.0001), revealing rapid (95% CI of the difference in proportions 0.44, 0.62) and slow (95% CI of the difference in proportions -0.32, -0.18) genotype groups as the most prevalent among Swedes and Koreans, respectively.

CYP2A6 genotype significantly affected enzyme activity in both populations (P=0.004), while no effect of sex (P=0.14), age (P=0.32), cigarette smoking (P=0.39) or OC use (P=0.59) was observed **(paper V)**.

The 17U/17X ratios in Swedes (n=190) ranged from 0.00 to 2.81 (median: 0.50), while in Koreans (n=144) were between 0.00 and 1.96, with the median value of 0.22. The probit transformation indicated common antimode at 0.01, with 3.16% of Swedes and 18.75% of Koreans having phenotype of slow metabolizer (P<0.0001; 95% CI of the difference in proportions -0.22, -0.09). CYP2A6 enzyme activity was significantly higher in Swedes compared to Koreans (P=0.0001; 95% CI of the median difference 0.23, 0.46). The observed differences between the two populations remained significant when controlling for the genotype effect, i.e. within rapid (P=0.0007; 95% CI of the median difference 0.08, 0.40) and intermediate (P=0.04) genotype groups **(paper V)**.

4.3 NAT2

The AFMU/(AFMU+1X+1U) ratios in Serbs ranged from 0.05 to 0.64 (median: 0.33), with clear antimodes at 0.09 and 0.25 that appointed 9% (n=9), 36% (n=36) and 55% (n=55) of Serbian subjects for slow, intermediate and rapid acetylators, respectively **(paper III)**. The AFMU/(AFMU+1X+1U) ratios in Swedes and in Koreans were within the range from 0.00 to 0.53 (median: 0.02) and from 0.00 to 0.44 (median: 0.06), respectively. The antimode at 0.16 observed in Koreans denoted 25.9% of Korean subjects as rapid metabolizers, while in only 6.2% of Swedes the AFMU/(AFMU+1X+1U) ratios were above the same value (P<0.0001; 95% CI of the difference in proportions -0.28, -0.11) **(paper IV)**.

Comparison between Swedes and Koreans revealed latter to have significantly higher NAT2 activity, and the difference remained significant when controlling for the influence of cigarette smoking, sex, or OC use (P<0.0001), as well as *NAT2* genotype (P=0.016). Stratified by genotype, Koreans display higher enzyme activity compared to Swedes among carriers of at least one wild type *NAT2*4* allele (P=0.004), while similar tendency of higher enzyme activity in Koreans than in Swedes was observed among slow acetylators (P=0.31) **(paper IV)**.

With the frequency of 43%, the most common NAT2 haplotype in Serbs was *NAT2*5*, followed by *NAT2*6* that was present in 28% of Serbian subjects **(paper III)**. Similar distribution was observed in Swedes, with 47% and 28% of subjects having *NAT2*5* and *NAT2*6* haplotype, respectively **(paper IV)**. On the other hand, 65% of Koreans were carriers of wild type *NAT2*4*, *NAT2*6* had a frequency of 23%, while *NAT2*5* was extremely rare (0.2%) **(paper IV)**.

When stratified based on the presence or absence of functional *NAT2*4*, *NAT2*12* or *NAT2*13* allele, Swedes and Koreans significantly differed in terms of distribution of genotype groups (P<0.0001). In Swedes, carriers of two, one and no functional alleles occurred with the frequencies of 8.85%, 29.20%, and 61.95%, respectively, whereas in Koreans the respective percentages were 40.67%, 48.67%, and 10.67% **(paper IV)**. In Serbs, however, both alleles were functional in 4.9% of subjects, genotypes containing

only one functional allele had a frequency of 45.5%, while no functional alleles had 49.6% of the population **(paper III)**.

Significant NAT2 genotype-phenotype association was detected in all three populations: in Serbs (P<0.0001) **(paper III)**, Swedes (P=0.03) and Koreans (P=0.008) **(paper IV)**. Due to unexpectedly high frequency of rapid acetylators observed in Serbs, genotype-phenotype correlation was investigated in each of the Serbian subjects separately. It was observed that, of all slow/intermediate acetylators (AFMU/(AFMU+ 1X+1U) ratio bellow 0.25) who had their genotype determined (n=44), in 93.2% observed phenotype was in agreement with the genotype, i.e. subjects were homozygous for the defective variant alleles. However, only 84.9% concordance with the genotype was observed within the rapid acetylator group (AFMU/(AFMU+1X+1U) ratio above 0.25, n=53), that is, there were 8 subjects carrying both defective alleles, yet displaying high enzyme activity. Described NAT2 genotype-phenotype discordance could not be explained by *NAT1* gene polymorphism, as there was no significant differences in *NAT1* alleles and genotypes frequency distribution between NAT2 phenotype (P=0.36) or genotype groups (P=0.07) **(paper III)**.

No significant influence of smoking on NAT2 activity was observed in either Serbs (P=0.57) **(paper III)**, Swedes (P=0.14) or Koreans (P=0.05) **(paper IV)**. Similarly, there was no significant difference between men and women within Serbian (P=0.94) **(paper III)**, Swedish (P=0.74) and Korean (P=0.39) population **(paper IV)**. On the other hand, significant correlation of OC use and NAT2 activity was observed in Swedish women (P=0.007; 95% CI of the median difference -0.53, -0.21), users (n=33, median: 0.04) having significantly higher enzyme activity compared to non-users (n=38, median: 0.02) **(paper IV)**. This effect could not be evaluated in other two populations, as none and only one woman used oral contraceptives in Serbs and Koreans, respectively **(paper III, IV)**.

4.4 XO

The 1U/(1U+1X) ratios in Swedes and Koreans ranged from 0.01 to 0.98 in former and from 0.00 to 0.98 in latter, with the median values of 0.46 and 0.48, respectively. The common antimode at 0.08 identified 6.4% of Swedes and 9.5% of Koreans as slow metabolizers. In terms of XO activity, no significant difference between the two populations was detected (P=0.40) **(paper IV)**.

XO activity was significantly influenced by sex in Swedes (P=0.003; 95% CI of the median difference 0.06, 0.32), but not in Koreans (P=0.43). In Swedes, higher 1U/(1U+1X) ratio was observed in women (n=70, median: 0.53) compared to men (n=39, median: 0.34), and the effect remained significant after the OC users were excluded from the comparison (P=0.01). OC use (P=0.09) and cigarette smoking (P=0.15) did not affect XO activity in either of the populations **(paper IV)**.

5 DISCUSSION

5.1 CYP1A2

In the present study, the main findings related to CYP1A2 activity are enzyme induction by habitual heavy coffee consumption in carriers of −163C>A *CYP1A2* polymorphism, and higher CYP1A2 enzyme activity in Swedish compared to Serbian population **(paper I, II)**.

Effect of heavy coffee consumption has been investigated in Serbian and Swedish healthy volunteers, none of which was using any medications, including oral contraceptives. Possible competitive inhibition was avoided by abstaining from intake of any caffeine-containing food, beverage and medication for at least 24 h before and during the study (114, 124). The results demonstrated that daily consumption of at least three cups of coffee increased CYP1A2 enzyme activity in all Swedes and Serbs. Since it is known that cigarette smoking induces CYP1A2 enzyme activity (52), effect of heavy coffee consumption among Swedes and Serbs was additionally analyzed when controlling for this confounding factor. Among non-smokers in both populations, heavy coffee consumers displayed higher enzyme activity compared to non-heavy coffee consumers. Similar results were obtained among Swedish smokers, but we were not able to show the same effect in Serbian smokers due to the low number of subjects in this group **(paper I)**.

The general mechanism of CYP1A2 induction is well known and includes ligand-dependent activation of aryl hydrocarbon receptor (AHR) - aryl hydrocarbon receptor nuclear translocator (ARNT) pathway (48, 127). Upon ligand binding, AHR translocates from cytoplasm into the nucleus, and with ARNT protein, already present in the nucleus, forms a heterodimer. This complex then recognize and binds to one of the several xenobiotic response elements (XRE), localized within the promoter region of *CYP1A2* gene, and in turn induce transcription activation (52, 128, 129). The ligands that activate AHR could be certain environmental chemicals, of which the most common are polycyclic aromatic hydrocarbons (PAHs) (127). PAHs are produced by incomplete combustion processes of organic material at high temperatures (130, 131), and could be found in cigarette smoke and grilled meat – both known inducers of CYP1A2 activity (48, 52, 132).

Coffee is a complex chemical mixture of over 1000 different chemicals, with caffeine, its best-known component, participating with only 1 % (34). According to previous investigations, caffeine has a very low affinity for the AHR (133). Furthermore, CYP1A2 induction was observed to be more dependent on a regular coffee consumption than on an overall caffeine intake (63), suggesting that effect of some other coffee constituent might be more important than the effect of caffeine itself. In addition, in vitro studies reported that coffee consumption up-regulate glucuronidation by AHR signaling, but that this process takes place independently of caffeine (134). At the same time, all types of coffee beans have to be roasted before preparation and the temperature during roasting reaches up to 260°C (34, 135). Thus, the concentration of PAHs, which are already present in green coffee beans, increases during the roasting

process (135), and reach up to 1.8 μg of PAHs per liter of coffee brew samples (136). According to our results, regular heavy coffee consumption induces CYP1A2 activity. Based on the data from the literature, we presume polycyclic aromatic hydrocarbons to be at least partly responsible for this effect.

CYP1A2 is known to be polymorphic, with several polymorphisms having an impact on drug metabolism (48, 52). Of those, substitution C>A at position −163 is one of the most common in Caucasians, with frequencies that range from 33.8% in British (137) to 71.4% in Swedish population (27). Its importance for CYP1A2 enzyme activity was first observed by Sachse et al. (54) as higher enzyme inducibility by cigarette smoking in the presence of −163A/A genotype. Several subsequent studies (27, 56, 138, 139), but not all (57, 140, 141), confirmed this observation by reporting higher CYP1A2 enzyme activity in the presence of CYP1A2 inducers, such as cigarette smoking or omeprazol.

To investigate the possible effect of *CYP1A2* genetic polymorphism on enzyme induction by heavy coffee consumption, we compared enzyme activity among Serbian and Swedish heavy and non-heavy coffee consumers, controlling for the influence of cigarette smoking and OC use. In heavy coffee consumers, the highest CYP1A2 enzyme activity was detected in carriers of -163A/A genotype. The inducing effect of heavy coffee consumption was observed only in subjects homozygous for the variant -163A allele. In non-heavy coffee consumers, influence of -163C>A was not observed **(paper II)**.

To the best of our knowledge, this is the first study to report increased CYP1A2 inducibility by heavy coffee consumption in carriers of −163A/A genotype. Significant genotype-phenotype association was analyzed and confirmed independently in two different populations, regardless of possible linkage disequilibrium with other *CYP1A2* polymorphisms. It is interesting to note that among all heavy coffee consumers inducing effect was observed only in carriers of −163A/A genotype, while among all carriers of −163A/A genotype higher enzyme activity was observed only in heavy coffee consumers. Based on these observations, both −163A/A genotype and an inducer, such as heavy coffee consumption, seem to be necessary for the CYP1A2 induction. Our results suggest that regular heavy coffee consumption might be a possible confounding factor for the discrepant findings in studies investigating the association of −163C>A *CYP1A2* polymorphism with CYP1A2 enzyme induction.

Previous studies, comparing black with white populations (62, 65), or Koreans with Swedes (27), reported the influence of ethnicity on CYP1A2 activity. Within Caucasian population, significantly higher CYP1A2 activity has been observed among residents of Germany compared to subjects living in Bulgaria and Slovakia (63). In the present study, two populations, both of Caucasian origin but representing different ethnic groups, were compared in terms of CYP1A2 enzyme activity. The comparison revealed significantly higher enzyme activity in Swedes compared to Serbs **(paper I)**.

The effects of ethnicity on drug metabolism can be determined by both genetic and environmental factors, including diet, lifestyle and concomitant use of medications (9). In the present study, inter-ethnic variation in CYP1A2 enzyme activity was investigated

by comparing healthy unrelated Swedish and Serbian volunteers, who were not using any medications, including OC. The frequency of −163C>A CYP1A2 polymorphism was similar between the population, and the influence of cigarette smoking and heavy coffee consumption was ruled out by excluding smokers and heavy coffee consumers from the comparison. The reason for the observed difference remains to be additionally investigated, with other *CYP1A2* genetic variants, epigenetic regulation or unreported intake of herbal preparations as possible explanations (25, 52, 127, 142, 143).

5.2 CYP2A6

Regarding CYP2A6, the most important observation of this study is the difference between Swedish and Korean population in terms of both *CYP2A6* genotype and enzyme activity. Unlike sex, age, cigarette smoking and OC use, *CYP2A6* genotype significantly affected CYP2A6 enzyme activity **(paper V)**.

Based on the earlier investigations, *CYP2A6* genetic variation has been suggested to be the leading cause for the variability in enzyme activity (69, 74, 75, 86). In the present study, Swedish and Korean subjects were genotyped for functional variant alleles previously found in Caucasians and/or Asians, and the results corresponded well with the earlier reports (75-77, 144-149). In Swedes, the most frequently found were rapid genotypes, consisting of both functional alleles, whereas in Koreans the most numerous were the carriers of slow genotype with both alleles nonfunctional. As expected (73, 150), the effect of genotype on CYP2A6 activity was confirmed in both populations **(paper V)**.

Inter-ethnic differences in CYP2A6 activity have already been studied, and significantly lower enzyme activity was observed in African Americans and Asians compared to Caucasians in general (151, 152). Additionally, when comparing with black and white populations and between each other, Japanese displayed the lowest and Koreans the highest CYP2A6 activity (146, 148). In the present study we detected higher CYP2A6 activity in Swedes compared to Koreans **(paper V)**. Since rapid *CYP2A6* genotypes were more frequent in Swedes and slow in Koreans, the observed difference in enzyme activity between the populations could be explained by genotype effect. Nevertheless, after stratification according to the genotype Swedes still displayed higher enzyme activity, indicating that, although it contributes to, the investigated *CYP2A6* genetic polymorphisms cannot fully explain inter-ethnic variability in CYP2A6 enzyme activity. Possible reasons for the observed difference could include other *CYP2A6* genetic variants, epigenetics or diet (146, 148, 153).

Considerable controversy about possible effect of sex on CYP2A6 activity dwells in the available literature. Some of the previous studies, investigating Caucasians (75, 82, 152, 154), Africans (83, 148), and Asians (155), reported higher enzyme activity in women compare to men. Since increased enzyme activity have been observed in OC users too (72, 82), female sex hormones were suggested as responsible for the inducing effect of on CYP2A6 activity (82). However, other studies failed to find any significant sex differences in enzyme activity in either Caucasians (81, 84, 148, 156) or Asians (157). Similarly, in the present study we did not observe any effect of either sex or OC use on CYP2A6 activity in Swedes or in Koreans **(paper V)**. It is known that sex-

related differences in pharmacokinetics could be important determinants of inter-individual variation in drug response (3, 4, 8-10), with body mass index and hormonal status as the most likely underlying cause (158). Yet, many of the studies on sex-based inter-individual differences in drug metabolizing enzyme activity have been performed in small number of subjects, thus producing inconclusive results (158). Regarding CYP2A6, the influence of sex and OC use on enzyme activity remains controversial, thus future studies, taking account both sample size and possible confounders, such as body weight or hormonal replacement therapy, will be needed to resolve this issue.

Although the hypothesis that activity of CYP2A6 activity could change with age was not widely investigated, some of the studies did describe significantly higher enzyme activity in older individuals (72, 154, 156). Consistently with the opposite reports, in the present study, involving subjects from 18 to 60 years old, no association of CYP2A6 activity with age neither in Swedes nor in Koreans was observed **(paper V)**. Our finding is in accordance with the experimental data demonstrating that the content and activities of various CYP450 enzymes practically do not decline with age in healthy subjects (159-162). Most probably, seeming age effect on CYP2A6 activity observed in several previous studies might be the due to unrecognized chronic liver disease or unreported intake of CYP2A6 inducers or inhibitors (159-161).

The effect of cigarette smoking on CYP2A6 activity, assuming competitive enzyme inhibition by nicotine, was much more intensively studied (70, 81). Some of the studies identified expected decrease in enzyme activity in smokers (75, 81, 83, 145), while others did not observe any influence of cigarette smoking habit on CYP2A6 (72, 84, 156). The results of the present study are consistent with the latter reports, as sought effect of smoking was not detected in either Swedes or Koreans **(paper V)**. It is well known that nicotine is almost exclusively metabolized by the CYP2A6 enzyme (86), thus the competitive inhibition of caffeine metabolism by cigarette smoking could be expected. Yet, since the conversion of 17X to 17U, which depends on CYP2A6, is not saturable in physiological conditions (163, 164), the nicotine effect, if exists, might not be very significant, necessitating larger sample size for detection. In addition, unreported intake of herbal preparations or nicotine replacement therapy should be considered as confounders.

5.3 NAT2

The most important findings of this study related to NAT2 include higher enzyme activity in Koreans compared to Swedes **(paper IV)**, and unprecedentedly high prevalence of rapid acetylators in Serbian population **(paper III)**. All three populations differed in terms of *NAT2* genotype distribution, but in all significant genotype-phenotype association was detected **(paper III, IV)**. Yet, in Serbs partial discordance between rapid acetylator phenotype and *NAT2* genetic background was observed, which could not be explained by *NAT1* gene polymorphism either **(paper III)**. Sex and cigarette smoking did not affect NAT2 activity in either of the populations **(paper III, IV)**, while in Swedish women OC users had higher enzyme activity **(paper IV)**.

NAT2 gene is known to be highly polymorphic (92, 95). In the present study, the most important nonsynonimous gene polymorphisms have been determined, and subjects

were stratified according to the genotype to either fast (carriers of at least one *NAT2* functional allele) or slow genotype group (165, 166). Additionally, fast genotype group was further divided into intermediate and rapid group, consisting of carriers of one and two functional alleles, respectively. The allele frequencies observed in Serbs, Swedes and Koreans were as expected for populations belonging to Caucasians and Asians, respectively. (166-170). Comparison among the populations revealed significant difference in the distribution of the *NAT2* genotypes. Namely, slow genotype was the most prevalent in Swedes and fast genotype in Koreans, while in Serbs both slow and fast genotypes were equally distributed **(paper III, IV)**.

Significant correlation of *NAT2* genotype with *N*-acetylation capacity has already been reported by a number of studies, designating genetics as the most important determinant of inter-individual variation in NAT2 activity (89, 95, 98, 167). As expected, our study confirmed significantly high NAT2 genotype-phenotype correlation in all three investigated populations **(paper III, IV)**. Nevertheless, certain discordance was observed in Serbian population, as the genotype did not correspond to observed acetylation status in all subjects. Namely, there were subjects having both functional alleles and displaying intermediate phenotype, as well as subjects carrying both defective alleles, yet displaying rapid phenotype **(paper III)**. Although unusual, similar NAT2 genotype-phenotype discordance has already been reported in up to 10% of the population (89, 95, 96, 167). Other variations of *NAT2* gene were suggested as a plausible explanation for intermediate acetylators with rapid genotype finding (167), but sequencing of the *NAT2* coding region did not reveal any additional nonsynonymous polymorphism. Thus, either unknown variation within promoter region (97) or factors other than genetic variations, such as epigenetic influences (98) diet (79), or unreported traditional medications (171), might be responsible for the observed deviation within intermediate acetylator group. On the other hand, the presence of less functional allele renders NAT2 genotype slow regardless of other polymorphisms, thus additional *NAT2* genotyping could not explain discordance observed in rapid acetylator group. However, as NAT1 participates in caffeine metabolism by contributing to AFMU formation (166), *NAT1* genotyping was considered to be of importance: over-representation of *NAT1* fast alleles might have increased formation of AFMU and consequently AFMU/(AFMU+1X+1U) ratio, thus causing misclassification of NAT2 acetylators phenotype as rapid. To test the hypothesis, subjects were additionally genotyped for SNPs in the *NAT1* gene responsible for the fast NAT1 acetylation activity (95, 172-174). Nevertheless, fast *NAT1* alleles were similarly distributed among both NAT2 genotype-phenotype concordant and discordant subjects, and did not affect AFMU/(AFMU+1X+1U) ratio. Therefore, *NAT1* genetic polymorphism was excluded as an underlying cause for the observed NAT2 genotype-phenotype discordance within rapid acetylator group, and unreported environmental factors were suggested instead.

N-acetylation activity within populations usually follows bimodal or trimodal pattern, discriminating slow and intermediate from the rapid acetylator phenotype (62, 84, 166, 167, 175). In our study, Swedes displayed unimodal and Koreans bimodal NAT2 activity distribution, with lower *N*-acetylation capacity in Swedes compared with Koreans **(paper IV)**. The observed difference was in accordance with the population-specific *NAT2* genotype distribution patterns, as the frequency of defective variant

alleles coding for the slow acetylator phenotype was higher in Swedes compared with Koreans. Inter-ethnic variations in NAT2 enzyme activity have already been observed (62, 79, 89, 90), including report of higher *N*-acetylation capacity in Caucasians compared to Asians (176), but the genotype effect was rarely considered. When we compared NAT2 activity between Swedes and Koreans while controlling for the *NAT2* polymorphism, the difference remained significant among carriers of fast genotype, while a similar tendency was observed between slow genotype groups **(paper IV)**.

On the other hand, Serbs display trimodal distribution of NAT2 enzyme activity, with lower and upper antimode values at 0.09 and 0.25, respectively **(paper III)**. Previous studies, using the same metabolic ratio as an index of NAT2 activity, set the lower limit for the rapid acetylators' phenotype between 0.2 and 0.3, regardless of the distribution pattern (62, 175, 177). This common cut-off point allowed us to compare our results with the previous reports for Caucasians, where the frequency of rapid acetylators, exceeding the similar cut-off point, was 44.9% or less (62, 84, 166), and slow acetylators phenotype was predominant. In contrast, our study revealed slow acetylator as the least common phenotype in Serbs, while 55% of subjects were designated as rapid acetylators, having AFMU/(AFMU+1X+1U) urinary ratio above upper antimode value of 0.25 **(paper III)**. This unprecedented high prevalence of rapid acetylators in a white population could not be explained by either *NAT2* or *NAT1* genotypes, as both were found at frequencies consistent with the previous reports (122, 166-168, 178, 179). Our results of both comparison between Swedes and Koreans and phenotyping of Serbian population indicate that, in addition to *NAT2* genotype, ethnicity should be considered an important factor in treating patients with NAT2 metabolized drugs (169, 180). Inter-ethnic difference in *N*-acetylation capacity might be due to epigenetics, diet, or traditional medications (79, 98, 169, 175).

In agreement with the earlier reports (62, 79, 98, 181), sex or cigarette smoking did not affect NAT2 acetylation capacity in Serbs, Swedes or Koreans **(paper III, IV)**. On the other hand, association between OC use and higher NAT2 activity was observed in Swedish women. Since only one Korean and none of the Serbian women used oral contraceptives, the finding could not be confirmed. It is known from previous animal studies that certain hormones can induce NAT2 via hormone-responsive elements (89). Yet, previous human study on OC use and NAT2 activity failed to find any significant association (181). Thus, to investigate the effect of steroid hormones on acetylation capacity in humans, as well as to explain the underlying mechanism of induction, additional studies will be necessary.

5.4 XO

The main findings of the present study related to XO comprise the lack of inter-ethnic difference between Swedes and Koreans and higher enzyme activity in Swedish women compared to men. Cigarette smoking and OC use did not affect XO activity in either of the populations **(paper IV)**.

As a multifunctional enzyme involved in metabolism of both endogenous and exogenous substrates (104), XO has been widely investigated (62, 84, 108-110, 175, 181). Yet, although lower XO activity has been observed in black compared to

Caucasians (62), inter-ethnic differences were mainly reflected in the enzyme activity distribution pattern. Namely, in Caucasian populations XO activity were usually unimodally distributed (81, 84, 181), while others displayed bimodal distribution, with poor metabolizers ranging from 4% in Spaniards and Ethiopians (109, 110) up to 11% in Japanese (175). In the present study, in accordance with earlier studies comparing Caucasians and Chinese (64), comparison between Swedes and Koreans revealed the lack of interethnic variability in XO activity. Nevertheless, both Swedes and Koreans displayed bimodal distribution of XO activity **(paper IV)**. In Koreans as an Asian population poor metabolizer phenotype was expected to be present (175), and its frequency corresponded well with the previously reported data. On the other hand, additional investigations, taking into account both XO genetic polymorphism (101) and environmental factors, will be needed to explain the slow metabolizer phenotype in the Swedish population.

Most of the previous studies did not observe any correlation between sex and XO enzyme activity (64, 108, 110, 175, 181). Nevertheless, Relling et al. (62) and Guerciolini et al. (102) reported higher and lower enzyme activity in women, respectively. In addition, Brown et al (182) described a positive association between female hormone levels and OC use with XO activity in human breast milk. In the present study, women displayed higher XO activity in Swedes but not in Koreans, while the influence of OC use was not observed **(paper IV)**. Our results suggest sex as a factor to be considered for dose adjustments in Swedish patients treated with drugs metabolized by XO. On the other hand, it is generally accepted that cigarette smoking does not affect XO activity (108, 110, 175, 181). As expected, cigarette smoking did not affect XO activity in either Swedes or Koreans **(paper IV)**.

6 CONCLUSIONS

- Habitual heavy coffee consumption induces CYP1A2 enzyme activity in carriers of *CYP1A2* −163A/A genotype.
- Swedes display significantly higher CYP1A2 activity compared to Serbs, and the observed difference is not due to *CYP1A2* genetic polymorphism, cigarette smoking, oral contraceptive use or heavy coffee consumption.

- *CYP2A6* genotype, but not sex, age, cigarette smoking and OC use, significantly affect CYP2A6 enzyme activity.
- Swedes display significantly higher CYP2A6 activity compared to Koreans, and the observed difference is mainly, but not entirely due to lower frequency of defective variant alleles in former compared to latter.

- *NAT2* genotype, but not sex and cigarette smoking, significantly affect NAT2 enzyme activity. In Swedish women, oral contraceptive use is associated with higher NAT2 enzyme activity.
- Koreans display significantly higher NAT2 enzyme activity compared to Swedes and the observed difference is mainly, but not entirely due to lower frequency of defective variants alleles in former compared to latter.
- Serbs differ from other Caucasians in terms of *N*-acetylation capacity, due to unprecedented high prevalence of rapid acetylator phenotype in the population. Partial NAT2 genotype-phenotype discordance observed in Serbian rapid acetylators could not be explained by *NAT1* gene polymorphism.

- Cigarette smoking and OC use do not affect XO enzyme activity. In Swedes, female sex is associated with higher XO enzyme activity.
- There is no inter-ethnic difference between Swedes and Koreans in terms of XO enzyme activity.

7 ACKNOWLEDGEMENTS

Many people in different ways contributed to this thesis, and I am truly grateful to all of them. Yet, my deepest appreciation undoubtedly belongs to my main supervisor, *Eleni Aklillu*, for accepting me as her student, always believing in me, encouraging me, supporting me and guiding me on my journey through pharmacogenetics. I am also most grateful to both of my co-supervisors: *Leif Bertilsson*, for generously sharing his great knowledge, experience and enthusiasm with me, and *Juan Antonio Carrillo*, for exciting and fruitful collaborative work.

I would also like to express my sincere gratitude to: *Stefan Lundgren*, for introducing me to the world of pharmacogenetics; *Anders Rane*, *Folke Sjökvist* and *Georgios Panagiotidis*, for giving me the opportunity to join KI; *Lilleba Bohman*, *Jolanta Widen*, *Birgitta Ask*, *Lena Ekström*, *Margit Ekström*, *Yvonne Sjölind* and *Margarita Mahindi*, for their kindness and invaluable help in the lab and with the administration; *Takashi Fukasawa*, *Ksenia Goryachkina*, *Mandana Shirazi*, *Parvaneh Afsharian* and *Aleksandra Trifunovic*, for their friendship and support, *Sarah Nanzigu*, *Roza Ghotbi*, *Lili Milani*, *Guillermo Gervasini*, *Hyung-Keun Roh*, *Sara Karlsson*, *Nobuhisa Ueda*, *Akira Suda*, *Slobodan Jankovic* and *Marieke PJ van den Broek*, for successful collaboration; *Rudolf Poredos*, for unceasing inspiration and support; as well as all the Serbian, Swedish and Korean volunteers, for their participation in the study.

Last, but not least: I would like to thank *my Dejan* and *my parents*, for loving me for who I am, always being by my side and supporting me selflessly.

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