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Pharmacogenetics of Drug Metabolizing Enzymes with Special Emphasis on Ethiopians

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ABSTRACT

Pharmacogenetics of drug metabolising enzymes has been well studied in Caucasians and Asians, but relatively little in Africans. The aim of this thesis was to investigate the molecular genetics of drug metabolising enzymes, in particular CYP2D6, CYP2C19, CYP2C9, CYP1B1, CYP1A2 and XO, with special emphasis on the Ethiopian population. Two different Ethiopian populations were investigated, healthy unrelated subjects living in Ethiopia (n=122) and Ethiopians living in Sweden (n=73). In the first group, it was found that only 1.8% were PMs for debrisoquine, whereas 29 % carried duplicated or multiduplicated *CYP2D6* genes, having higher enzyme activity. We identified new haplotypes carrying 4 or 5 *CYP2D6*2* gene copies on one allele. We found a significantly lower rate of CYP2D6 but not of CYP2C19-catalyzed reactions in Ethiopians living in Ethiopia as compared to Ethiopians living in Sweden of the same genotypes. We suggest that the dietary habits in Ethiopia have had an inhibitory effect on CYP2D6 and have contributed to dietary related selection for CYP2D6 duplication in Ethiopians.

We screened for new SNPs in the *CYP1B1* gene and detected 3 novel SNPs one of which (4360C>G in exon 3) was present at a frequency of 7 %, and causes an Ala443Gly (m5) amino acid substitution. In total, 5 missense SNPs were found yielding in total 32 possible *CYP1B1* haplotypes. However, we could only find 7 of them carrying 1-4 different missense SNPs. Functional characterization of these revealed that CYP1B1.6 and CYP1B1.7, having the Arg48Gly, Ala119Ser and Leu432Val mutations in common, had significantly decreased Vmax/Km for both the 2- and 4-hydroxylation of 17 β-estradiol whereas the other were similar to the CYP1B1.1 enzyme. It is emphasized that haplotypes rather than single SNPs are to be considered in e.g. molecular epidemiological studies.

Ethiopians living in Ethiopia (n=115) or in Sweden (n=73) were phenotyped with caffeine to evaluate any influence of environmental and gender related differences on XO and CYP1A2 activities. XO activity was significantly higher in Ethiopians living in Ethiopia compared with those living in Sweden. We suggest that the dietary habits in Ethiopia might have caused the elevated XO-expression.

Genomic DNA sequencing of *CYP1A2* gene from 12 subjects revealed the presence of a novel intron 1 SNP, -730C>T. Genotyping and molecular haplotyping methods for the intron 1 SNPs were developed and 4 different haplotypes were identified: *CYP1A2*1A* (wt for all SNPs); *CYP1A2*1F* (-164A); *CYP1A2*1J* (-740G and -164A) and *CYP1A2*1K* (-730T, -740G and -164A) with frequencies of 39.9, 49.6, 7.5 and 3.0 %, respectively. Subjects with *CYP1A2*1K* had significantly decreased CYP1A2 activity *in vivo* ($p<0.02$) and reporter constructs with this haplotype revealed significantly less inducibility with TCDD in human B16A2 hepatoma cells. EMSA analysis using nuclear extracts from B16A2 cells indicated that the -730 C>T mutation abolishes a binding site for a transcription factor of the Ets family. It is concluded that this polymorphism might be of great importance for individual sensitivity to carcinogen activation and rate of drug metabolism.

In conclusion unique novel alleles have been identified in the Ethiopian population and has given light to mechanisms behind their evolution and putative importance in carcinogen and drug metabolism.

Key words: drug metabolism, gene duplication, CYP2D6, CYP2C19, CYP2C9, CYP1B1, CYP1A2, genotyping, xanthine oxidase, ultrarapid metabolizers, polymorphism, caffeine, Ets, dietary selection.

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In memory of
Aklillu Gebreyes, my father
Fasika, Mesayet and Kidest, my sisters
I live missing you!

LIST OF PUBLICATIONS

I **Aklillu E**, Persson I, Bertilsson L, Johansson I, Rodrigues F, Ingelman-Sundberg M (1996) Frequent distribution of ultrarapid metabolizers of debrisoquine in an Ethiopian population carrying duplicated and multiduplicated functional *CYP2D6* alleles. *J Pharmacol Exp Ther* **278:** 441-446.

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III **Aklillu E**, Herrlin K, Gustafsson LL, Bertilsson L, Ingelman-Sundberg M (2002) Evidence for environmental influence on CYP2D6-catalysed debrisoquine hydroxylation as demonstrated by phenotyping and genotyping of Ethiopians living in Ethiopia or in Sweden. *Pharmacogenetics* **12:** 375-383.

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V Yasar U, **Aklillu E**, Canaparo R, Sandberg M, Sayi J, Roh H K, Wennerholm A (2002) Analysis of *CYP2C9*5* in Caucasian, Oriental and Black-African populations. Eur J Clin Pharmacol **58:** 555-558.

VI **Aklillu E**, Oscarson M, Hidestrand M, Leidvik B, Otter C, Ingelman-Sundberg M (2002) Functional analysis of six different polymorphic CYP1B1 enzyme variants found in an Ethiopian population. *Mol Pharmacol* **61:** 586-594.

VII **Aklillu E**, Carrillo JA, Makonnen E, Bertilsson L, Ingelman-Sundberg M (2003) Xanthine oxidase activity is influenced by environmental factors in Ethiopians. *Eur J Clin Pharmacol* (submitted).

VIII **Aklillu E**, Carrillo JA, Makonnen E, Bertilsson L, Ingelman-Sundberg M (2003) Genetic polymorphism of CYP1A2 in Ethiopians affecting induction and expression. Characterization of novel haplotypes with SNPs in intron 1. *Mol Pharmacol* (submitted)

CONTENTS

LIST OF ABBREVIATIONS

ADR	Adverse drug reaction
AhR	Aryl hydrocarbon receptor
Arnt	AhR nuclear translocator
Bp	Base pair
B[a]P	Benzo[a]pyrene
CYP	Cytochrome P450
DMBA	7,12-dimethylbenz(a)anthracene
DME	Drug metabolising enzyme
DNA	Deoxyribonucleic acid
EM	Extensive metabolizer
HPLC	High performance liquid chromatography
Kb	Kilo base
MR	metabolic ratio
nm	nanometer
PAH	Polycyclic aromatic hydrocarbon
PM	Poor metabolizer
PCR	Polymerase Chain Reaction
RNA	Ribonucleaic acid
RFLP	Restriction Fragment Length Polymorphism
SNP	Single nucleotide polymorphism
SSCP	Single Stranded Conformational Polymorphism
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
UM	ultrarapid metabolizer
Wt	wild-type
XO	Xanthine oxidase
XRE	Xenobiotic responsive element

GLOSSARY

Allele	any one of two or more alternative forms of a gene that occupy the same position (locus) on a chromosome.
Base pair	two nitrogenous bases (adenine and thymine or guanine and cytosine) held together by weak bonds.
Cancer	diseases that are characterised by uncontrolled, abnormal growth of cells.
Carcinogen	an agent capable of initiating development of malignant tumours. May be a chemical, a form of electromagnetic radiation or an inert solid body.
Carcinogenesis	the generation of cancer from normal cells, often used synonymously with transformation, tumourigenesis.
Chromosome	the self-replicating genetic structures of cells containing the cellular DNA that bears in its nucleotide sequence the linear array of genes.
Gene	the biologic unit of heredity, determined by a specific sequence of purine and pyrimidine bases in DNA, located at a definite position (locus) on a particular chromosome.
Genetic polymorphism	the occurrence in the same population of multiple discrete allelic states of which at least two have high frequency (conventionally of 1% or more).
Gene duplication	a class of DNA rearrangement that generates a supernumerary copy of a gene in the genome.
Genotype	the genetic constitution of an individual, either overall or at one or more specific loci, as distinct from its expressed features or phenotype.
Metabolism	conversion to another chemical species.
Mutation	a permanent transmissible change in the genetic material.
Pharmacogenetics	the convergence of pharmacology and genetics dealing with genetically determined responses to drugs.
Pharmacokinetics	the action of drugs in the body over a period of time, including the processes of absorption, distribution, localization in tissues, biotransformation and excretion.
Pharmacodynamics	the study of the biochemical and physiological effects of drugs and the mechanisms of their actions.
Phenotype	the total characteristics displayed by an organism under a particular set of environmental factors, regardless of the actual genotype of the organism.
Pseudogene	non-functional DNA sequences that is very similar to the sequences of known genes.
Oxidative Stress	oxygen or free radical mediated damage in living organisms.
Xenobiotics	chemical substances that are foreign to the biological system. They include naturally occurring compounds, drugs, environmental agents, carcinogens, insecticide.
XRE	a DNA motif responsible for the up regulation of gene induced by 3-methylcholanterine, dioxin, or ß-naphthoflavine.

1 GENERAL BACKGROUND

1.1 Introduction

All living organisms, including humans, are continuously exposed to chemicals present in food, drinks and the environment. Many naturally occurring compounds, such as mold aflatoxins, which are among the most potent carcinogens, are toxic (Ames et al., 1990). Exogenous substances, also called xenobiotics, include drugs, plant constituents, environmental pollutants, for instance halogenated hydrocarbons, arylamines, polycyclic aromatic hydrocarbons, combustion waste products, herbicides and pesticides etc. These compounds are hydrophobic and remain essentially water insoluble. After glomerular filtration in the kidney, they would be reabsorbed and remain in the body. Drug metabolizing enzymes are responsible for the biotransformation, detoxification and excretion of foreign chemicals such as drugs, from the body after their desired effect has been exerted in humans by converting them into more water-soluble products that could easily be excreted via urine or bile.

The biotransformation process occurs mainly in the liver in two steps (Nebert et al., 1999): In the first step (Phase I), chemical modification of the parent drug takes place, which usually involves cleavage of alkyl group or insertion of a polar group into the non polar parent compounds by oxidation, hydroxylation, reduction, hydrolysis etc. In the second step (Phase II), the polar groups of the intermediate metabolites arising from phase I are conjugated to highly water soluble groups such as glutathione, glucuronic acid, amino acids or acetylated rendering the whole molecule amenable for excretion. Phase I enzymes comprises the oxidases (i.e. cytochrome P450, flavin monoxygenases, xanthine oxidase), reductases, deaminases and hydrolases such as epoxide hydrolase. CYPs are the most important DME among Phase I enzymes. Phase II enzymes include glutathione-S-transferases (GSTs), sulfotranferases, N-acetyl transferases (NATs), UDP-glucuronosyl transferases (UGTs) etc. The metabolic products are often inactive or less active than the parent drug, but some of the metabolites generated contains reactive oxygen species, such as oxygen or hydroxy radicals and are more toxic than the parent compound to form DNA and protein adducts which initiate and promote cancer (Nebert, 1997). Thus, whether biotransformation results in detoxification or bioactivation of xenobiotics is, dependent up on the relative activity of the parent drug versus the metabolite.

1.2 Population genetics

Population genetics is the study of genetic influences on the components of cause and effect in the somatic characteristics of populations. During human migration and evolution, the global distribution of different polymorphic genes is explained by random genetic drift and by natural selection. Genetic drift is the random change of the occurrence of a particular gene in a population. It occurs rapidly in small populations, particularly in those that are also isolated, these groups quickly accumulate distinctive allele frequencies. These alleles are geographically restricted owing to genetic drift or region-specific selection. Natural selection is the genotype environment interactions occurring at the phenotypic level leading to differential reproductive success of individuals and hence to modification of the gene pool of a population. Through natural selection, a single mutant becomes the most common in

the population, if it is advantageous to the individual carrying it. Genetic distances is a way of measuring the amount of evolutionary divergence in two separated populations of a species by counting the number of allelic substitutions per locus that have cropped up in each population. Genetic distances are used to measure the global genetic differences between populations. Figure 1 shows the phylogenetic tree of human population based on archeological, genetic and linguistic data.

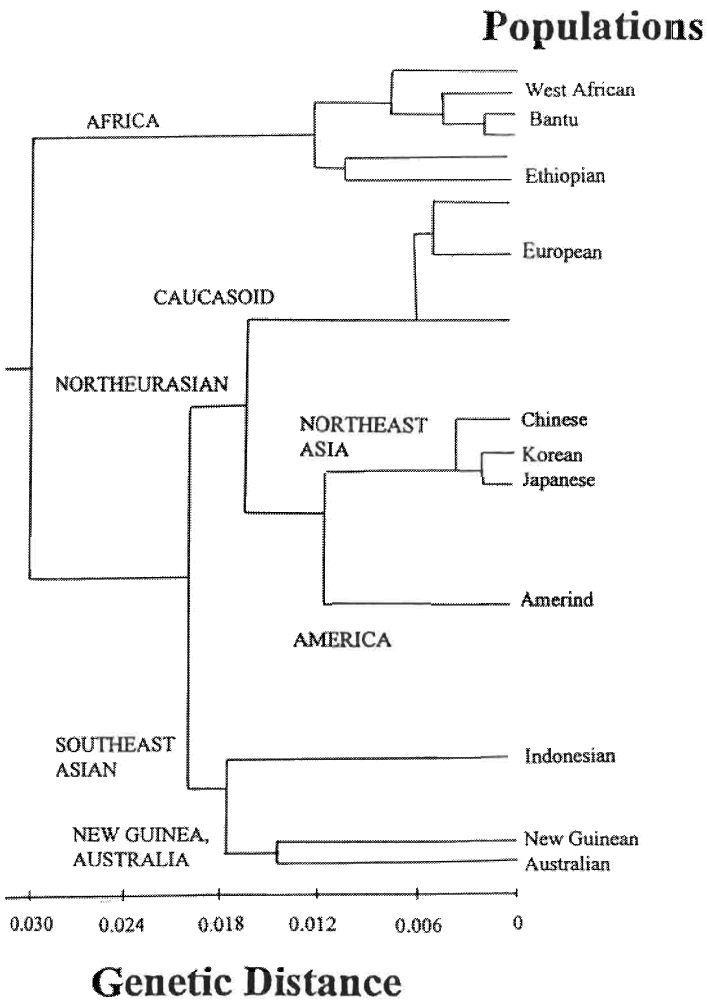

Figure 1. The phylogenetic tree of human population based on archeological, genetic and linguistic data from (Cavalli-Sforza et al., 1994).

1.2.1 African population

Africa is the second largest continent, after Asia, covering about one-fifth of the total land surface of the Earth. It is one of the most ethnically and genetically diverse regions of the world and is thought to be the ancestral homeland of all modern humans (Jorde et al., 1998; Tishkoff and Williams, 2002). Mitochondrial DNA markers indicate eastern Africa as the origin of both African and Eurasian expansion as well as a route for early exit of Homo sapiens from Africa (Jorde et al., 1998; Quintana-Murci et al., 1999; Tishkoff and Williams, 2002). East Africa has been the site of the most interesting recent discoveries of fossils in the human line and is considered the place for the most ancient living beings, the genus, Homo. East Africa attracted the attentions of anthropologists after the discovery of an ancient skeleton, "Lucy" that belongs to the *Australopithecus afarensis,* dated between 3 and 4 millions years ago, in the Afar regions of Ethiopia (Johanson and Taieb, 1976). *A. afarensis* are intermediate between those of modern humans and extant African apes. The Hadar site in Ethiopia is a prolific source of hominid fossils attributed to the species *A. afarensis*, which spans the period 3.4-3.0 million years. Also numerous fossils of *A. afarensis*, which is widely thought to be ancestral to Homo, have been recovered from both the Middle Awash and other sites in Ethiopia, Kenya and Tanzania (Asfaw et al., 1999; Brown et al., 2001; White et al., 1994; White et al., 1993).

Presently, Africa is inhabited by aboriginal groups; Caucasoids in the north down to the southern border of Sahara, and Negroids in sub-Saharan Africa. In the east, however, and especially in Ethiopia and Somalia, people have lighter skin and are considered as Negroids by some and Caucasoids by others (see Cavalli-Sforza et al. 1994, p 167). The population of sub-Saharan Africa has been greatly affected by the Bantu expansions that originated near the confluence of the Niger and Benue rivers. Figure 2 illustrates the different populations of Africa and the respective countries in which they predominantly reside. Both linguistic and genetic studies show that most sub-Saharan populations are closely related to each other where as Pygmy, Khoisan, and eastern African populations are the most differentiated (Cavalli-Sforza et al. 1994, p. 169-171). Pygmies appear to be the most divergent, although some Pygmy groups show a certain degree of admixture with neighboring populations (Chen et al., 1995) (Cavalli-Sforza 1986; Chen et al. 1995). Interestingly, Khoisan and Ethiopians display both great differences and important similarities with respect to each other. In contrast to the situation for Bantu speakers, it has been proposed that both Khoisan and Ethiopians share some Caucasoid features, which were acquired at different times. Genetic findings confirm the ancestral affinity between the Ethiopians and the Khoisan and these populations share an ancestral paternity (Scozzari et al., 1999; Semino et al., 2002). However, linguistic data indicate separate evolution of these two populations. Khoisans speak unique languages that make use of click sounds (Cavalli-Sforza et al. 1994, p. 164), whereas most Ethiopians speak Afro-Asiatic (Cushitic, Omotic, and Semitic) languages. Intermediary Bantu-speaking populations currently separate these two groups geographically and Khoisans are located in the South Africa.

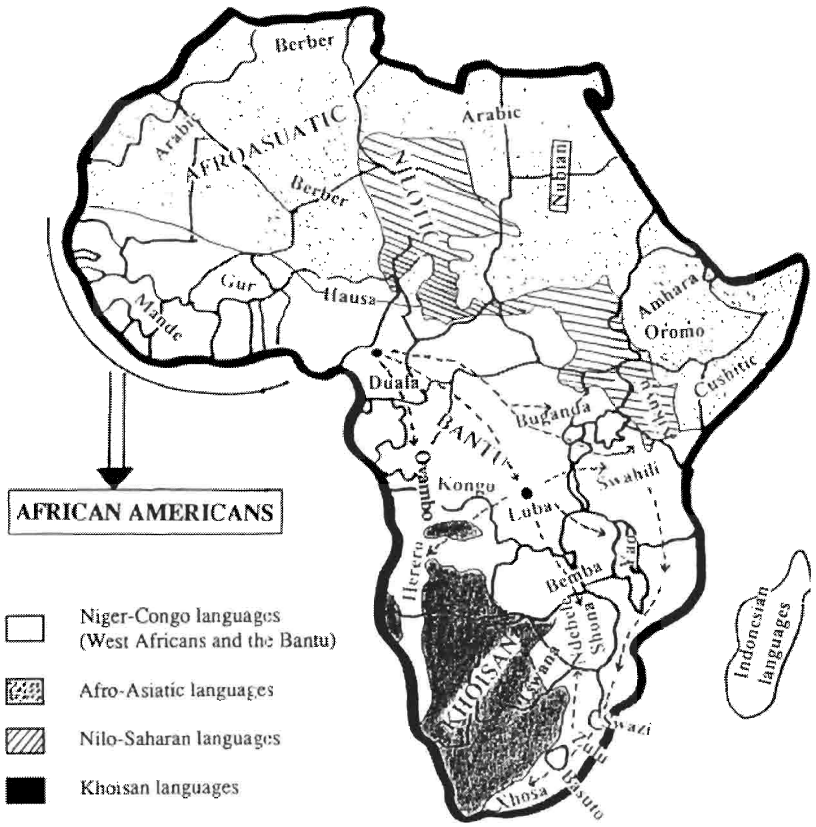

Figure 2. A map of Africa depicting the various populations groups and the respective countries in which they predominantly reside. The Bantu migration is indicated by (---) in the map. With permission from (Masimirembwa, 1995)

African populations show the largest amount of genetic diversity. In an attempt to study population history and natural selection in shaping genetic diversity in *CYP1A2*, Wooding *et al*. 2002, reported that the African population had the highest level of nucleotide diversity and the lowest level of linkage disequilibrium and the haplotypes found outside Africa were mostly a subset of those found within Africa (Wooding et al., 2002). The study of the levels and patterns of genetic diversity among the ethnically diverse African populations will help to understand human evolutionary history and the genetic basis of phenotypic variation. Complex disease genes that are mapped in African populations by association between SNPs and disease phenotype could have important implications for understanding the genetic risk factors for many ethnic groups (Tishkoff and Williams, 2002).

1.2.2 Ethiopians

Ethiopia, located in the East Africa, is one of the largest and most populace countries in Africa with about 65 million people. The first known inhabitants of Ethiopia were hunting people whose scattered descendants remained in southern Ethiopia. As early as the 8th millennium B.C., a Negroid element appeared, probably only in the southern part of the country, and mingled with people arriving later (Encyclopedia Britannica 1964, vol. 8, p. 782). By about 7000 B.C, linguistic evidences indicate that both Cushitic and Omotic speakers were present in Ethiopia. They probably derived from the Sahara or from Arabia (Encyclopedia Britannica 1964, vol. 8, p. 782), although a local Ethiopian origin of Afro-Asiatic languages has also been hypothesized.

On the basis of historical, linguistic, and genetic data, it has been suggested that the Ethiopian population has been strongly affected by Caucasoid migrations since Neolithic times. During the first millennium B.C. and possibly even earlier, various Semitic-speaking groups from Southwest Arabia began to cross the Red Sea and settle along the coast and in the nearby highlands of Ethiopia. One of the most important occurred in the second half of the 1st millennium B.C., when southern Arabians brought Sabean culture into Ethiopia and originated the protoGeeze-speaking group, from which Amhara, Tigre, and Gurage derived. A fusion of the newcomers with the indigenous inhabitants produced a culture known as pre-Aksumite. By the early fourth century, the Aksum Empire (a Kingdome from north Ethiopia) extended to parts of Yemen, Southwest Arabia and up to the southern coast of the Gulf of Aden. Aksum served as an important pathway for the ancient commercial trade routes between Africa, Arabia, and India. Evidence exists of a close cultural exchange between Aksum and the Arabian Peninsula (Encyclopedia Britannica, 2000).

The early Ethiopians underwent a strong diversification, and, by 2,000 B.C., constituted the Omotic speakers, the Cushites and the Semites. Further Semitic migrations took place: Jews (in the first centuries A.D.), Syrian Christians (4th-6th century A.D.), and Arabian Muslims (11th-12th centuries A.D.); these influenced the Ethiopian groups and cultures. The presence of Semitic languages in Ethiopia reflects the historical Arabian contacts. Oromo, Amahara, Tigre and Guarge are the major ethnic groups in Ethiopia consecutively and mtDNA and Y-chromosome polymorphisms analysis carried out on Cushitic speakers (Oromo) and Semitic speakers (Amhara, Tigres and Gurage) indicated that they are a rather homogeneous group (Passarino et al., 1998; Scozzari et al., 1999).

On the basis of autosomal polymorphic loci, it has been estimated that 60% of the Ethiopian gene pool has an African origin, whereas 40% is of Caucasoid derivation (Cavalli-Sforza et al. 1994, p. 174). Interestingly, Asian alleles, not found else where in Africa, are present in Ethiopians (Cavalli-Sforza et al. 1994, p. 193). An Asian specific mtDNA haplotype, which has never been observed in mtDNA molecules of the other African or Caucasoid lineages, was found in Ethiopians with a frequency of 20% (Chen et al., 1995; Passarino et al., 1996; Passarino et al., 1998). Similarly in this thesis, we found the presence of Asian specific P450 alleles, *CYP2D6*10* and *CYP2C19*3*, in Ethiopians.

1.3 Pharmacogenetics

Pharmacogenetics represents "the study of variability in drug response due to genetic variations or heredity". Pharmacogenetics utilizes genetic information of drug transporters, DMEs and drug receptors in order to optimize drug treatment and reduce ADRs (Ingelman-Sundberg, 2001). Several drug transporters and DMEs are genetically polymorphic. It is anticipated that pharmacogenetics will be used to identify individuals at a greater risk for specific drug dependencies, provide information that can lead to novel treatment and prevention approaches as well as provide guidance for individualization of treatment choice.

1.3.1 Factors affecting drug metabolism

Several factors influence concentrations and activity of drug metabolizing enzymes and hence metabolism of a drug. Pharmacokinetics and pharmacodynamics of drugs are affected by physiological, pathological, environmental and genetic factors thus cause variability in drug metabolism. Gender difference in the expression of DMEs is known. Women generally have a lower lean body mass, a reduced hepatic clearance and this may influence activity of DMEs. The expression of several P450s is influenced by endogenous factors such as hormones, and environmental factors such as nutritional state, exposure to toxicants, drugs and dietary constituents. Problems can arise when combinations of drugs are administered. Some drugs may act to increase or decrease the activity of one or more of the cytochrome P450s, which, in turn, may have an effect on how another drug is metabolized. In addition, it is known that certain individuals and populations in the world have different forms of these enzymes and hence react differently to the same drugs.

1.3.2 Genetic polymorphism of drug metabolizing enzymes

The term genetic polymorphism defines a monogenic trait resulting in at least two phenotypes, neither of which is rare, that exists in a normal population; the rarest phenotype still occurs at a frequency > 1% (Vogel and Motulsky, 1986). Strikingly exaggerated response or lack of effectiveness of drugs administered in the usual dosage has been observed as manifestation of inherited genetic polymorphism with respect to drug metabolism. Genetic polymorphism arises from variation in DNA sequence due to insertion/deletion or substitution of a single base (SNP) or starches of bases or repetitive tandem repeats, microsatellites or Alu segments. The interindividual variability in drug metabolism is caused mainly by interindividual variation in amount and/or activity of specific drug metabolizing enzymes. These variations are governed by genetic and environmental factors. Pharmacogenetic polymorphism is a monogenic trait that is caused by the presence in the same population of more than one allele at the same locus and more than one phenotype in regard to drug interaction with the organism and the frequency of the least common allele is >1% (Meyer, 1991).

Genetic polymorphism in drug metabolism gives rise to distinct populations differing in their ability to perform a certain drug biotransformation reaction, poor metabolizers (PM) and extensive metabolizers (EM) and ultrarapid metabolizers (UM). The PM phenotype is monogenically inherited as an autosomal recessive trait and is due to

complete absence or marked decrease in the amount and/or activity of the enzyme. The UM phenotype is due to the presence of duplicated genes resulting in expression of increased amount of enzyme (Bertilsson et al., 1993; Ingelman-Sundberg, 1999; Johansson et al., 1993). Most drug metabolizing P450 genes such as *CYP2D6, 2C19, 3A4, 2A6, 2C9, 1B1, 2E1* are polymorphic. Genetic polymorphism, which affects drug metabolism, in these enzymes is well identified and characterized in Caucasians and Asians but not so much is known about Africans. In the present thesis, genetic polymorphism of *CYP2D6*, *CYP2C19*, *CYP2C9*, *CYP1B1, CYP1A2* and XO was investigated with special emphasis on Ethiopians. Particularly, *CYP2C9*, *CYP1A2* and *CYP1B1* genetic polymorphism has never been studied before in Africans and for the first time genetic polymorphism of these enzymes in an African population was investigated in the present thesis.

1.3.3 Clinical consequences of genetic polymorphism

The clinical consequence of the UM phenotype is inability of reaching therapeutic plasma concentration of drugs administered at a normal dose due to fast elimination of the drug from the body, resulting in a therapeutic failure (Bertilsson et al., 1993). On the other side, a serious side effects and toxicity of drugs are manifested in PMs. Ultrarapid metabolizers of CYP2D6 carry more than two active gene copies and cannot achieve therapeutic plasma drug levels at ordinary dosage (Bertilsson et al., 1993; Dahl et al., 1995) resulting in loss of therapeutic efficacy. A pronounced relationship between number of *CYP2D6* gene copies and clearance of nortiptyline in gene dose dependent manner has been reported (Dalen et al., 1998). Genotyping of subjects with multiple copies of functional genes seems to be of great value for differentiating UM from patients who do not comply with the normal dose. For instance, lower therapeutic efficacy of haloperidol with increasing number of active *CYP2D6* genes and severe adverse effect in PMs is reported suggesting that treatment with haloperidol should be avoided in extremely slow and extremely rapid metabolizers of CYP2D6 substrates (Brockmoller et al., 2002).

Conversely, if the therapeutic effect is inherent in the metabolite, administration of a prodrug causes therapeutic failure in PMs and toxicity in UMs. For instance 10% of orally administered codeine is O-demethylated to morphine, which is the main active principle and thus codeine cannot be recommended as analgesic for PM patients, since sufficient morphine is not generated (Poulsen et al., 1996). On the other hand severe abdominal pain and side effect was observed in an UM patient after administration of a normal dose of codeine (Dalen et al., 1997). For those drugs that have a narrow therapeutic index, such as chemotherapeutic drugs, a slight change in plasma concentration can result in life-threatening toxicity. For instance, Sallee et al., (Sallee et al., 2000) reported a fluoxetine-related death of a 9 years old child with a mutated *CYP2D6* gene that results in poor metabolism of fluoxetine. Therefore it is apparent that analysis of genetic polymorphisms of drug metabolizing enzymes are of great importance in the therapeutic efficacy as well as in preventing toxicity of drugs and adverse drug reaction (Ingelman-Sundberg et al., 1999).

1.3.4 Adverse drug reaction

Serious ADRs occur amongst 6.7% of all hospitalized patients and that 0.32% of all hospitalized patients develop fatal adverse reactions, causing more than 100 000

deaths annually in the US, which is considered as between the fourth and sixth leading cause of death in USA and costs 100 billion USD (Lazarou et al., 1998). However, this figure is criticized for over estimation. As much as 12% of hospital admission to a clinic of internal medicine in Sweden take place due to ADR and preventable ADRs is estimated to reduce patient admission and costs for internal medicine by 4% (Mjorndal et al., 2002). Not all ADRs are preventable since multiple factors contribute to this phenomenon. Examples of non-preventable ADRs include severe allergic reactions to antibiotics in patients who have had no previously noted reactions and acute renal failure associated with drugs in seriously ill patients. Amongst the known risk factors for adverse reactions are higher age, polypharmacy, liver and renal disease as well as gender. Identifying those factors, which may predispose to ADRs is essential for risk management. If the genetic polymorphism is monogenic, predictive genotyping or phenotyping for known genetic polymorphisms is estimated to prevent the occurrence of 10-30 % of ADRs (Bush, 1998; Ingelman-Sundberg, 2001). Pharmacogenetics is the discipline, which takes the patient's genetic information of drug transporters, drug metabolizing enzymes and drug receptors into account to allow for an individualized efficient drug therapy leading to optimal choice and dose of the drugs as well as reducing cost of treatment.

1.3.5 Pharmacogenetic tools available for detection of variability in drug metabolism

Predictive genotype or phenotype should be valuable in improving therapeutic efficacy and reducing adverse effects of drugs. The following general methods are used to identify efficiency of drug metabolism in individuals and avoid drug related toxicity.

1.3.5.1 Phenotyping

DME metabolic status has traditionally been determined *in vivo* by administering a probe drug that is known to be a specific substrate. After a certain period of time urine, saliva or plasma samples are collected and the molar concentrations of the parent drug and its metabolites is determined by high-performance liquid chromatography or gas chromatography (Lennard et al., 1977; Sanz et al., 1989; Wedlund et al., 1984). There are various methods of phenotyping procedures for the different polymorphic CYPs using different probe drugs (Streetman et al., 2000a). The molar concentration ratio of the parent drug versus the metabolite is used as an index to assess the *in vivo* activity of that specific drug. Phenotyping of several CYPs using a cocktail of probe drugs is also developed (Frye et al., 1997; Streetman et al., 2000b).

1.3.5.2 Genotyping

Genotyping is defined as the genetic analysis of functionally important mutations or polymorphisms in the gene encoding a specific enzyme. Genotyping requires only a single sample, often a very small amount of material, such as blood from vein or a single finger prick or a buccal swab, for genetic analysis using for instance allele-specific PCR, PCR-RFLP, RFLP, SSCP etc. High throughput, automated methods such as DASH, Taqman etc are being developed that allow rapid screening for microsatellite alleles and SNPs. SNP or alleles may be distinguished by any one of several methods, including single nucleotide primer extension, allele-specific hybridization, allele-specific primer extension, oligonucleotide ligation assay, and

invasive signal amplification. Newer methods require less sample manipulation, have higher sensitivity, allow more flexibility, and a decreased reagent cost. Currently, genotyping chips are available for simultaneous genotyping of several P450 alleles but are relatively expensive.

1.3.5.3 Comparisons of genotype versus phenotype approach

Phenotyping reveals the activity of DME at a particular time point, indicates the actual metabolic capacity of the patient, and is more useful to predict optimal dosage adjustment based on the results. The disease condition of the patient, especially liver disease, and concomitant administration of drugs compromise enzyme activity. Studies on HIV+/AIDS patients indicated that advanced disease produces discordances between genotype and phenotype, indicating a reduction in the metabolic capabilities of individuals (Williams and Wainer, 2002). Although the phenotyping methodology is reliable, some problems are associated. Since drug metabolism is affected by pathophysiological and environmental conditions, the data may not be reproducible over time and hence significant intra-individual variations may arise. Reliable predictive genotyping approach using simple methods such as PCR, RFLP, and SSCP are increasingly substituting the traditional phenotyping approach to evaluate profile of genetic polymorphism. A major advantage of genotyping compared with phenotyping is that the former may be performed in blood samples collected at single time from patients irrespective of treatment, intraindividual variability is not an issue and results can be obtained more quickly. On the other hand, some problems provide a discrepancy between allelic heterogeneity and phenotypic different expression of the gene, such as mRNA level and enzyme activity. Sequentially, these genetic approaches are also hampered by multiple allelic heterogeneities in some cases, and then such complexities can render genotype interpretation difficult. In general, genotyping of individuals before undergoing therapy could in the future, lead to the optimization of therapeutic doses on individual basis.

1.4 The cytochrome P450 system.

Omura and Sato first characterized the cytochrome P450 system in 1964 from rat liver microsomes (Omura and Sato, 1964). A pigment (P) was named Cytochrome P450 due to its spectral properties when reduced and bound to carbon monoxide giving absorption maximum at a wavelength of 450 nm. Mammalian cytochrome P450s are membrane bound haem-thiolate proteins, which are localized on the cytosolic side of the smooth endoplasmic reticulum, although some P450s are also found in the mitochondrial inner membrane. They require molecular oxygen and coupling to a NADPH reductases for their catalytic activities. These enzymes are called mixed function oxidases or monooxygenases, because they incorporate one atom of molecular oxygen into the substrate and one atom into water.

The cytochrome P450s belongs to a superfamily, which metabolize foreign chemicals as well as a number of endogenous compounds. Mammalian cytochrome P450 can be divided into two major categories based up on their substrates: 1. P450s that are involved in the oxidative metabolism of endogenous substances such as fatty acids, prostaglandins, steroids etc. and 2: P450s that are responsible for the metabolism of exogenous substances such as drugs, carcinogens and environmental pollutants. The former class of P450s shows very high affinities for their substrates and very well conserved within species. In contrast, members of the latter category have much

wider substrate specificity and exhibit a relatively poor species structural conservation.

1.4.1 Cytochrome P450 nomenclature

Multiple isozymes of P450 have evolved from a common ancestral gene via divergent evolution through gene duplication, conversion, amplification and SNPs (Gonzalez and Nebert, 1990) as defense mechanisms to protect organisms from environmental toxicants. Based on amino acid sequence homology, the cytochrome P450 enzymes have been classified by family, subfamily, and isoform (Nebert and Nelson, 1991). P450 proteins that have 40% or less amino acid sequence similarities are considered to belong to different families. Each gene family is further subdivided into gene subfamilies. Members of each subfamily display more than 55% amino acid sequence similarity. Sequence similarity does not necessarily imply functional similarity. Allelic variants of a gene display more than 97 % amino acid sequence similarity. The root symbol "CYP" for human ("cyp" for mouse), denotes "cytochrome P450". The root symbol is followed by an Arabic numeral, designating the P450 family; a capital letter indicating the subfamily, and an Arabic numeral representing the individual gene within the subfamily. Numbers of the individual genes usually assigned in the order of their identification. Italicized names denote genes and cDNA and non-italicized names indicate the corresponding mRNA or protein. A web site, http://www.imm.ki.se/cypalleles/, is created and being used for listing of the different human CYP alleles involved in xenobiotic metabolism and naming of new CYP alleles or haplotypes according to recommendations described by Ingelman-Sundberg *et al*, (Ingelman-Sundberg et al., 2000; Ingelman-Sundberg et al., 2001).

The term "wild-type allele" has been used previously to designate a fully functional allele that exists at a very high frequency or a major allele in the population. However, now it is used to refer for the first allele sequenced (or sequences that are similar in sequence) and enzyme activity to that of a reference allele, to be designated as *1 (Ingelman-Sundberg et al., 2000). The consensus or reference sequence (*1 allele or wt) generally encodes an enzyme with efficient metabolic activity. The variant alleles encode enzymes with no activity or higher activity resulting in PM or UM phenotype respectively. A variant may also encodes an enzyme with altered or relatively the same activity as the wt variant allele. At present, the human CYP super family consists of 57 genes and 33 pseudogenes arranged into 18 families and 42 subfamilies (Nebert and Russell, 2002).

1.4.2 CYP gene regulation

CYP enzyme expression is regulated in various ways and at different cellular levels. Some P450s display tissue-specific expression, such as liver specific or extrahepatic P450s. They are regulated by endogenous mediators such as hormones and cytokines or are induced by foreign chemicals, for instance by their substrates to initiate gene transcription. Many of the P450s involved in drug metabolism, but not all, are transcriptionally activated by xenobiotics. The regulation of gene expression of the various P450s generally depends on the interaction of the xenobiotics with the receptors. The transcriptional activation of their promoters generally leads to the increased expressions of their mRNA. Induction is a protective mechanism whereby

the cell can process lipophilic compounds that might otherwise accumulate to harmful levels. However, since DME metabolizing P450s have broad substrate specificities, enzyme induction by one compound may lead to increased metabolism of a second, producing loss of drug effect leading to drug-drug interaction (Guengerich, 1997). In addition, cytochrome P450 induction can produce an imbalance between detoxification and activation, leading to accumulation of reactive metabolites beyond the capacity of cellular defenses, thereby producing toxicity or cancer (Conney, 1982). Smoking is most likely associated with several human cancers through the mechanistic induction of CYP1 family. Interestingly, induction exhibits genetic polymorphisms, and induction-defective mutants are available resulting in non-responder phenotypes to the stimuli.

The CYP1 family isoenzymes are inducible by polycyclic aromatic hydrocarbons (PAH) such as benzo[a]pyrene, which is a significant source from cigarette smoke, 3-methylcholanthrene and TCDD, industrial pollutants or food derived hetrocyclic and aromatic mutagens (Safe, 1995). The activation involves a specialized receptor called the Ah (aryl hydrocarbon) receptor, which acts as a nuclear ligand-induced transcription factor that interacts with xenobiotic or xenobiotic responsive elements located in 5'-flanking regions of responsive genes. PAHs induce their own metabolism and are ligand to the Ah receptor. The Ah receptor becomes activated by binding with an aromatic hydrocarbon ligand in the cytosol. Then it translocates from the cytosol to the nuclei, heterodimerizes with AhR nuclear translocator (Arnt), binds to the XRE sequence and subsequently transactivates gene expression. TCDD is a high-affinity agonist for AhR and is the most potent known inducer of CYP1A1, 1A2 and 1B1 transcription. These enzymes participate in the metabolic activation of PAHs to mutagenic and carcinogenic derivatives. Therefore, genetic polymorphisms in the regulatory regions might influence susceptibility to PAH-induced disease, such as smoking-induced cancer.

The steroid family of orphan receptors, the constitutive androstane receptor (CAR) and pregnane X receptors (PXR), heterodimerize with the retinoid X receptor (RXR), transcriptionally activate the promoters of CYP2B and CYP3A gene expression by xenobiotics such as phenobarbital-like compounds (CAR) and dexamethasone and rifampicin-type of agents (PXR). The human CYP3A family is transcriptionally induced by a variety of structurally unrelated xenobiotics, including the antibiotic rifampicin. Rifampicin also induces several CYP2C enzymes. This induction not only increases rifampicin metabolism but it also increases clearance of drugs that are substrates of CYP2C and CYP3A. Ligand-activated PXR and CAR regulate CYP3A4 expression by binding to hormone response elements (HREs) in the 5'-flanking region of the CYP3A4 gene as a heterodimer RXR(Goodwin et al., 2002; Goodwin et al., 1999). The hCAR-binding motifs in the proximal promoter and distal enhancer regions of CYP3A4 were mapped and shown to be identical to those described previously for hPXR (Goodwin et al., 2002). These elements are capable of binding both hCAR-hRXR and hPXR-hRXR heterodimers with high affinity. The convergence of hCAR- and hPXR-mediated signaling pathways at common response elements in the CYP3A4 gene clearly demonstrates that cross talk between these two nuclear receptors is probably an important factor in the regulation of this gene. Both CAR and PXR receptors are expressed in the liver and intestine where enhanced transcription of CYP3A occurs in response to xenobiotic exposure. A liver-specific orphan nuclear receptor hepatocyte nuclear factor-4alpha (HNF4 α) is involved in the PXR- and CAR-mediated transcriptional activation of CYP3A4 (Tirona et al., 2003). Binding of HNF4 α to a specific cis-acting element in the CYP3A4 gene enhancer

permits PXR- and CAR-mediated gene activation and mice with conditional deletion of HNF4 had reduced or absent expression of CYP3A (Tirona et al., 2003). The peroxisome proliferator activated receptor (PPAR) also dimerizes with RXR and it is activated by lipid lowering agent of fibrate-type of compounds leading to transcriptional activation of the promoters on the CYP4A genes.

Cytokines such as TNFα, interferon α and γ and various interleukins that become activated during infection and inflammation are known to be involved in down regulation of P450 expressions (Morgan, 1997). Suppression of xenobiotic inducible P450s occurs primarily at the level of gene transcription (Waxman, 1999) but the exact underlying mechanisms remain to be fully characterized.

1.4.3 Polymorphic drug metabolizing enzymes investigated in this thesis.

Human cytochrome P450s that metabolise exogenous substances are exclusively those, which belong to the CYP1, CYP2, and CYP3 and to a lesser extent CYP4. The human CYP1 family contains 3 subfamilies, 3 genes and 1 pseudogene; CYP2 family contains 13 subfamilies, 16 genes and 16 pseudogenes; CYP3 family contains 1 subfamily, 4 genes and 2 pseudogenes (http://drnelson.utmem.edu/P450lect.html). Six human cytochrome P450s, CYP3A4, CYP2E1, CYP1A2, CYP2C9, CYP2C19, CYP2D6 account for the metabolism of nearly all clinically useful medications. The relative importance theses enzymes with respect to drug metabolism are illustrated in Figure 3 In the present thesis genetic polymorphism of CYP2D6, CYP2C19, CYP2C9, CYP1B1 and CYP1A2 was investigated.

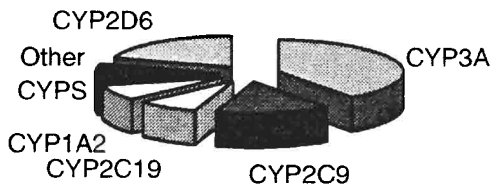

Figure 3. Relative proportions of drugs metabolized by human P450s (Rendic and Di Carlo, 1997)

1.4.3.1 CYP1 family

The human CYP1 gene family is one of the major cytochrome P450 families involved in the metabolism of endogenous and exogenous procarcinogens and endogenous substance such as estrogens. This family is comprised of three known isoenzymes, CYP1A1, CYP1A2 and CYP1B1. The two *CYP1A* subfamily members share 70% amino acid sequence identity and are about 40% identical with *CYP1B1*. *CYP1A1* and *CYP1A2* are located on the same chromosome in opposite direction and suggested to share the same 5′ regulatory sequences. CYP1A2 is expressed primarily only in liver, but CYP1A1 and 1B1 are both predominantly expressed in extrahepatic tissues. Both CYP1A1 and 1B1 are mainly involved in the metabolism of polycyclic aromatic

hydrocarbon but CYP1A2 preferentially metabolises arylamines and N-hetrocyclics as well as some drugs.

1.4.3.1.1 CYP1A2

Endogenous substrates of CYP1A2 include estradiol, uroporphyrinogen and melatonin. CYP1A2 hydroxylates estradiol to 2-hydroxy estradiol and oxidizes uroporphyrinogen to uroporphyrin in heme metabolism. It is the most conserved P450 among the P450s involved in drug metabolism. Exon 2-6 is highly conserved between human, mouse and rat CYP1A2. Conserved regions were also found in intron1 of *CYP1A2* in these species (Ikeya et al., 1989), suggesting a possible regulatory role of intron 1. However, CYP1A2 shows a 40-fold variation in expression among humans (Schweikl et al., 1993). Striking differences (greater than 15-fold) in levels of *CYP1A2* mRNA expression from human liver (Ikeya et al., 1989) and polymorphic metabolism of pro-carcinogens by human liver microsomes has been reported (Butler et al., 1989; Minchin et al., 1985). Several studies indicated the presence of a wide interindividual and racial/ethnic group differences in CYP1A2 activity using caffeine as a probe drug (Butler et al., 1992; Ilett et al., 1993; Nakajima et al., 1994). Unlike other drug metabolizing P450s such as CYP2D6 and CYP2C19, no nucleotide differences that could clearly explain the phenotypic variability in *CYP1A2* gene has been identified. Beside variation in constitutive expression and activity of CYP1A2, genetic polymorphism has also been identified in enzyme inducibility by smoking (Nakajima et al., 1999; Sachse et al., 1999; Schrenk et al., 1998). Two single nucleotide polymorphic sites (SNPs), -164 G>A and -740T>G, have previously been reported in the intron 1 the of human *CYP1A2* gene (see http://www.imm.ki.se/CYPalleles/cyp1a2.htm. A -164C>A SNP has been suggested to be associated with higher enzyme inducibility in Caucasian smokers (Sachse et al., 1999) and the -740T>G has not been functionally characterized.

1.4.3.1.2 CYP1B1

Human CYP1B1 differs from its two most closely related members of the CYP superfamily, CYP1A1 and CYP1A2, in the number of exons 3 versus 7 and chromosome location 2 versus 15. CYP1B1 is constitutively expressed mainly in extrahepatic tissues, including kidney, prostate, mammary gland, thymus, spleen, adrenal, colon, ovary, uterus, brain, heart, lung, intestine and testis.

Several different deleterious or truncating mutations in the human *CYP1B1* gene, identified in several populations, were shown to co-segregate with primary congenital glaucoma (PCG), an autosomal recessive disorder characterized by increased intraocular pressure resulting in enlargement of cornea and damage to the optic nerve that may lead to blindness (Bejjani et al., 2000; Kakiuchi-Matsumoto et al., 2001; Plasilova et al., 1999; Stoilov et al., 1998; Stoilov et al., 1997; Stoilov et al., 2002). Homozygosity for these deleterious mutations was shown to be associated with severe PCG. Several CYP1B1 mutations in various ethnic backgrounds have been implicated in the pathogenesis. Cyp1b1 is expressed in the developing and mature ciliary body of the mouse eye and it has been speculated that mutation in this gene may directly contribute to the abnormal elevation of the intraocular pressure in the PCG patients (Bejjani et al., 2002). However, Cyp1b1 knockout mice did not show a glaucoma phenotype (Buters et al., 1999), species difference in substrate specificity and tissue distribution between the human CYP1B1and mouse cyp1B1 may account

for this. The molecular mechanism leading to the development of PCG in human is yet unknown.

Seven common *CYP1B1* alleles have been identified so far, of which three alleles were identified in this thesis. Nine SNPs of which 5 causing amino acid substitution have been found in human and several epidemiological studies have been performed to investigate the association of individual SNP with incidence of cancer e.g., lung, breast, endometrial cancer. However, no study has been done before aimed to analyze in what combination theses SNPs do exist on the chromosome and the haplotypes distributed in the population. Interindividual differences in susceptibility for cancer might to a certain extent be inherited in different functional variants of this enzyme.

1.4.3.2 CYP2 family

The CYP2 gene family comprises of 13 subfamilies and 16 genes, that makes it the largest P450 family. CYP2 P450s are involved in the metabolism of drugs and other xenobiotics as well as arachidonic acids and some steroids. However the function of CYP2R1, 2S1, 2U1 and 2W1 are not yet identified (Nebert and Russell, 2002). The CYP2C subfamily constitutes approximately 20 % of the total human liver microsome P450 content and is the second most abundant P450 in human liver (Shimada et al., 1994). The human *CYP2C* subfamily appears to principally metabolize a number of clinically used drugs. Four members of this subfamily have been identified in humans: *CYP2C8, CYP2C9, CYP2C18,* and *CYP2C19*.

1.4.3.2.1 CYP2C9

CYP2C9 is the major CYP2C isoform found in the human liver. It is important in the metabolism of certain therapeutically used drugs including the anticoagulant drug warfarin and a number of non-steroidal anti-inflammatory drugs. Twelve variants of the *CYP2C9* allele have been found so far. However only **2*, **3* and **5* are functionally characterized. Several studies indicate that the allelic variants $Arg_{144}Cys$ (*CYP2C9*2*) and especially, $Ile_{358}Leu$ (*CYP2C9*3*) encode enzymes with reduced substrate turnover. *In vivo*, the *2/*3 and *3/*3 genotypes are associated with the tolbutamide PM phenotype (Sullivan-Klose et al., 1996) and the *3/*3 genotype is clearly linked to warfarin sensitivity (Steward et al., 1997) and impaired metabolism of losartan (Yasar et al., 2002). There is a strong association between *CYP2C9* variant alleles, especially *CYP2C9*3,* and low warfarin dose requirement and *CYP2C9* genotyping may identify a subgroup of patients who have difficulty at induction of warfarin therapy and are potentially at a higher risk of bleeding complications (Aithal et al., 1999; Steward et al., 1997)

*CYP2C9*2* is more frequent in Caucasians while it is absent in Asians and is very rare among African-Americans. Investigation of the *CYP2C9* genetic polymorphism has not been done in Africans before (Genetic status of an African population was studied for the first time in this thesis). Global distribution of *CYP2C9*2* and **3* is depicted in Figure 4. *CYP2C9*4* and **5* have recently been identified in Japanese and African Americans, respectively. The *CYP2C9*5* variant is specific for Black population and codes for an enzyme with a reduced activity (Dickmann et al., 2001).

Figure 4. Global distribution of the variant *CYP2C9*2* (A) and *CYP2C9*3* (B) allele frequencies in %. (Scordo et al., 2001)

1.4.3.2.2 CYP2C19

CYP2C19 is an important enzyme that metabolizes many clinically important drugs (Bertilsson, 1995). About 3-6 % of Europeans (Alvan et al., 1990) and Africans (Bathum et al., 1999; Herrlin et al., 1998; Masimirembwa et al., 1995) are poor metabolizers of S-mephenytoin. Significantly higher proportion (13-23 %) of Asians demonstrates complete deficiency of CYP2C19 activity compared to Africans and Caucasians (Bertilsson et al., 1992; de Morais et al., 1995).

Poor metabolizers are unable to 4'-hydroxylate the S- mephenytoin and several drugs metabolized by CYP2C19. To date, eleven variant alleles of *CYP2C19* has been identified so far of which *CYP2C19*2* and **3* are the most common defective alleles. *CYP2C19* genetic polymorphism indicates an impressive interracial variation. The PM frequency ranges 2-6 % in Caucasians and Black population, 13-23 % in Orientals and > 60 % in two malarious islands of Vanuatu in eastern Melanesia (Kaneko et al., 1999). The *CYP2C19*3* allele is very rare in Caucasians and is absent in South Africans and Zimbabweans (Dandara et al., 2001) and *CYP2C19*2* accounts for 78 % of the PMs in these populations, whereas both *2 and *3 together accounts for 100 % of PM phenotype in Orientals. The *CYP2C19* genetic polymorphism is increasingly emphasized due to its clinical relevance in a geographic area like Africa, where malaria is endemic since CYP2C19 is responsible for the metabolic activation of proguanil, an anti-malarial drug, into its active metabolite, cycloguanil. Apparently, reduced activity of CYP2C19 among Africans will limit the therapeutic efficacy of proguanil for malaria treatment in this population.

1.4.3.2.3 CYP2D6

CYP2D6 is one of the most studied cytochrome P450 with respect to its genetic polymorphism in a population and the second most important hepatic P450 in terms of drug metabolism. It metabolizes more than 75 clinically important drugs such as tricyclic antidepressants, neuroleptics, antiarrythimic drugs and ß-adreno-receptor blockers. It metabolizes about 20% of all commonly prescribed drugs. Clinical relevance of *CYP2D6* genetic polymorphism and its phenotyping/genotyping in patients receiving antipsychotics has been well reviewed (Bertilsson et al., 2002; Bertilsson et al., 1997; Dahl, 2002).

Over 70 CYP2D6 allelic variants are described so far (see http://www.imm.ki.se/CYPalleles/cyp2d6.htm) of which the *CYP2D6*3*, **4, *5, *10*, **17* and **41* are the major alleles that accounts for the observed phenotypes. These alleles are distributed in a population specific manner. *CYP2D6*3*, and *6, frame shift mutations, exist specifically in Caucasians. While *CYP2D6*4*, a splicing defect, is the most common mutant allele in Caucasians with an allele frequency of 21% and accounts for more than 75% of the mutant alleles in this population (Ingelman-Sundberg et al., 1999). *CYP2D6*10*, encoding an unstable protein with a decreased enzyme activity (Johansson et al., 1994) is present at a very high frequency (about 50%) in Chinese. The *CYP2D6*17* was first identified in Zimbabweans (Masimirembwa et al., 1996b) and later shown to cause a decreased enzyme activity (Oscarson et al., 1997). *CYP2D6*17* is specifically present in the Black populations. The *CYP2D6*5* allele has an even distribution (4-6%) among Caucasians, Orientals and Black populations (Bertilsson et al., 2002).

About 7–10 % of Caucasians lack functional CYP2D6 (Alvan et al., 1990; Bertilsson et al., 1992) whereas the incidence of CYP2D6 PM among Asians and Africans is <2% (Aklillu et al., 1996; Alvan et al., 1990; Bertilsson et al., 1992; Masimirembwa et al., 1996a; Wennerholm et al., 1999). Although, the incidence of CYP2D6 PM phenotype is very low in Oriental and Black populations, the majority of EMs show relatively lower enzyme activity as compared to Caucasian, which is clearly observed by the right shiftiness of debrisoquine MR histogram in these populations. The higher frequency of *CYP2D6*10* and *CYP2D6*17* is mainly responsible for the observed lower CYP2D6 activity among Asians and Zimbabweans respectively (Johansson et al., 1994; Masimirembwa et al., 1996b; Oscarson et al., 1997).

The previously termed *CYP2D6*2* allele had been associated with higher and intermediate phenotypes. The recently discovered -1584 C/G promoter polymorphism (Raimundo et al., 2000) on this allele identified as another major factor for expression and function with the -1584G (*CYP2D6*2*) promoter type being consistently associated with significantly higher expression than the -1584C (*CYP2D6*41*) (Zanger et al., 2001).

Examination of the global distribution of alleles with duplicated *CYP2D6* genes reveals that it is most frequent in Ethiopia and Saudi Arabia (Figure 5). About 30 % of Ethiopians (Aklillu et al., 1996), 20% of Saudi Arabians (McLellan et al., 1997) carry duplicated or multi duplicated *CYP2D6* gene. In Tanzania (7%) (Wennerholm et al., 1999) as well as in South and West Africa this frequency is < 2% (Dandara et al., 2001; Griese et al., 1999; Masimirembwa et al., 1996a; Panserat et al., 1999). In the Mediterranean area, about 10 % of the populations have duplicated *CYP2D6* genes (Agundez et al., 1995; Bernal et al., 1999). The corresponding prevalence in Northern Europe and Asians is generally less than 2% (Alvan et al., 1990; Dahl et al., 1995; Ingelman-Sundberg et al., 1999). There is a North to South gradient in *CYP2D6* duplicated gene frequency across Europe and Africa (Figure 5). Probably, as a consequence of the Muslim immigration to the Mediterranean area about 700 AD, these alleles have been transferred from East Africa or Meddle East to Europe and explain the relative high frequency of duplicated *CYP2D6* genes in this region (10 %) as compared to Northern Europe (1 %) (Ingelman-Sundberg, 1999).

Figure 5. Interethnic distribution of alleles containing duplicated active *CYP2D6* genes. Data are from Bernal et al. (Bernal et al., 1999), Nishida et al., Garcia-Barcelo et al., (Garcia-Barcelo et al., 2000) as well as from Ingelman-Sundberg et al., (Ingelman-Sundberg, 1999; Ingelman-Sundberg and Evans, 2001) and references therein; Global distribution of *CYP2D6* gene duplication.

1.4.3.3 Xanthine oxidase

XO is a cytosolic enzyme that mainly is responsible for the metabolism of endogenous purines and pyrimidines as well as several drugs such as thiopurines and methyl-xanthines with a wide range of substrates (Krenitsky et al., 1974). Major sites of expression in mammals are the liver and intestine, but relative to other species, humans express low levels of XO (Pritsos, 2000; Sarnesto et al., 1996). XO activates several clinically important anthracycline antibiotics including doxorubicin,

daunomycin and marcellomycine as well as a number of anticancer drugs into their reactive metabolites [Pan, 1980; Dodion, 1987; Pan, 1981]. Activation of an antiviral pro-drug, 6-deoxyacylovir to a therapeutically active from, acyclovir is carried out by XO (Krenitsky et al., 1984).

The highest XO activities are found in human liver and intestine compared to other tissue (Pritsos, 2000) and human liver XO has been purified and characterized (Krenitsky et al., 1986). High levels of XO have been associated with tissue injury in certain diseases and is considered to contribute to oxidative damage of cells through the generation of cytotoxic oxygen radicals, which are implicated as important pathologic mediators in many clinical disorders, including ischemia-reperfusion injury, myocardial infarction, hypertension, and atherosclerosis (Grace, 1994; Jeroudi et al., 1994; Nemeth et al., 2002). Myocardial XO level is increased in patients with chronic heart failure and XO inhibition by allopurinol lowered myocardial oxygen consumption and improved myocardial efficiency and patient condition (Cappola et al., 2001). Being implicated as an important generator of reactive oxygen radical (Hille and Nishino, 1995) and its inhibition showing protective effects towards both *in vitro* and *in vivo* models (Albuquerque et al., 2002), XO is considered as a potential target of drug therapy in various human diseases.

There is an interindividual variation in XO activity from human liver and about 20 % displayed relatively lower enzyme activity (Guerciolini et al., 1991). Population phenotyping studies using caffeine indicated marked interindividual and interethnic differences in XO activity (Carrillo and Benitez, 1994; Saruwatari et al., 2002). XO polymorphism has been well characterized among Caucasians and Asians but not in Africans.

1.4.4 CYPs and pharmaceutical industry

Cytochrome P450s are the most prominent group of drug-metabolizing enzymes in humans, and consequently are of great importance to the pharmaceutical industry. Since cytochrome P450s were first linked to drug metabolism in the 1970s, many drugs have had to be withdrawn from the market or stopped in clinical trials due to adverse side effects from their interactions with these enzymes.

The pharmaceutical industry is very interested in P450 crystal structures with drugs bound for improvement of drug design. Very recently the 3-dimensional crystal structure of human CYP3A4 and CYP2C9 has been discovered, a breakthrough and important discovery which will enable drugs to be rationally designed to reduce metabolic and toxicity problems caused by their interactions with CYP3A4 and 2C9. Since these enzymes are involved in the metabolism of several drugs, finding of their crystal structure is a grate advantage for the pharmaceutical companies. The structure-based drug discovery using high throughput X-ray crystallography will facilitate the rapid identification of novel drug candidates. With regard to the process of drug development, it would be useful to know as early in the development process as possible which CYPs are likely to metabolise the candidate drug, the likely interindividual variation in the metabolism, which CYP activities are likely to be altered and to what magnitude by the drug. This information will be useful in predicting drug interactions between the candidate drug and currently available drugs.

1.4.5 CYPs and cancer

Environmental and dietary carcinogens initiate a significant proportion of human cancers. Although P450s play an important role in detoxification of many drugs and chemical carcinogens, they are also responsible for catalyzing metabolic activations of some procarcinogens into highly reactive intermediates that attack cellular molecules such as DNA or proteins to initiate carcinogenic events and cytotoxicity. Numerous procarcinogens are known to require P450 dependent metabolic activation before they become carcinogenic, highly toxic intermediate metabolite that damage and kill the cells and this property is being exploited for cancer treatment.

Preclinical studies have shown that P450 gene transfer, which confers the capability to activate the prodrug directly within the target tissue, can dramatically increase the chemosensitivity of tumors to these prodrugs. CYP based cancer gene therapy is based on combination of P450 gene with a cancer chemotherapeutic prodrug that is activated by the P450 gene (Waxman et al., 1999) which greatly enhances the therapeutic effect of P450-activated anti-cancer prodrugs without increasing host toxicity associated with systemic distribution of active drug metabolites formed by the liver. Tumor-selective gene delivery include the use of tumor targeted cellular vectors and tumor-selective oncolytic viruses containing a P450 gene is injected into tumor cells, thereby increasing the amount of P450 expressed locally. Then, a non-toxic pro-drug, which is activated by the P450 into a toxic compound, is administered with the intention to selectively increase tumor cell exposure to cytotoxic drug metabolites that kills the cells. Oncolytic herpes simplex virus type 1 (HSV-1) vectors expressing "suicide" genes, rat CYP2B1 transgene, responsible for the bioactivation of the prodrugs, cyclophosphamide and ifosfamiderat, have been constructed to maximize tumor destruction through multimodal therapeutic mechanisms (Chase et al., 1998; Varghese and Rabkin, 2002). Anticancer drugs that are metabolized to therapeutically active drug and cytotoxic towards tumor cells include cyclophosphamide, ifosfamide, procabazine and dacarbazine (Waxman et al., 1999). Retroviral and adenoviral vector-mediated gene transfer of cytochrome CYP2B1 and co administration of cyclophosphamide, has improved effective transduction of the P4502B1 gene and increasing the concentration of cyclophosphamide delivered to the tumors in brain tumor gene therapy *in vitro* and *in vivo* (Manome et al., 1996; Wei et al., 1994). Transfer of a liver cytochrome P450 gene, CYP2B1, into human breast MCF-7 cancer cells and a panel of human tumor cell lines such as glioma and prostate cancer cells, rendered each of the cell lines highly sensitive to cyclophosphamide and its isomer ifosfamide (Chen et al., 1996). The CYP1 family of P450s is of special interest with respect to carcinogenesis since they can activate compounds to carcinogens. Certain P450s such as CYP1B1 are overexpressed in tumor tissues and this could be used to design anticancer drugs.

Although polymorphisms in xenobiotic enzymes would be considered cancer modifiers and would be associated only with low relative risks, their higher prevalence in the population makes them of great importance to public health since they would be expected to contribute to a large number of cancers. This is in contrast to cancer genes such as BRCA1 and APC that are high risk but of low prevalence. Studying genetic polymorphism and role of CYP1 in cancer susceptibility and the relationships between polymorphisms in these enzymes and environmental/dietary exposures to chemicals might perhaps be used to identify cancer susceptible

individuals to limit carcinogen exposure. Such information would be used to advise a cancer susceptible individual to limit a dietary, industrial, or environmental exposure.

1.4.5.1 CYP1A2 and its role in chemical carcinogenesis

CYP1A2 is known to metabolizes various chemical procarcinogens, such as food derived hetrocyclic and aromatic mutagens, N-hetrocyclics found in tobacco smoke as well as difuranocoumarins to reactive carcinogens (Landi et al., 1999; Zhao et al., 1994). The carcinogenesis of aromatic amines has been well established in both humans and animal models. CYP1A2 is also known to be involved in the activation of arylamines and hetrocyclic amines implicated in the genesis of colon and bladder cancer (McManus et al., 1990). Besides its significant role in chemical carcinogenesis (Eaton et al., 1995), CYP1A2 is induced by its substrates and shows interindividual variation in its capacity to activate procarcinogens (Butler et al., 1989; Minchin et al., 1985). CYP1A2 is inducible by cigarette smoking (Schrenk et al., 1998). High levels of CYP1A2 due to cigarette smoking have been linked to an increased risk of colorectal cancer (Le Marchand et al., 2002) and hepatocarcinogenesis (Nishikawa et al., 2002). Hetrocyclic aromatic amines (HAAs) are produced during cooking of meat and fish and their amount increases with the time of cooking and they are inducers of CYP1A2. In humans, several HAAs are activated *in vivo* by CYP1A2 to mutagens or carcinogens (Lynch et al., 1995; Raza et al., 1996). Nicotine derived nitrosoamine, 4-methyl-nitrosamino-1-(3-pyridyl)-1-butanone (NNK) is the most potent carcinogen and has therefore been implicated as a significant cause of tobacco associated cancers (Hecht and Hoffmann, 1988). NNK is metabolized to a potentially carcinogenic metabolite by CYP1A2 (Crespi et al., 1991).

Hepatocellular carcinoma (HCC) is a common neoplasm, especially in Africa and East Asia. The disease is multifactorial in etiology, the possible etiological agents including hepatitis B virus and a number of chemical carcinogens, among which the most important appear to be the aflatoxins and N-nitroso compounds. The high incidence of HCC in Africa is caused by the intake of aflatoxin (Uwaifo and Bababunmi, 1984) and in Asia by hepatitis virus B infection (Saito et al., 1990). The aflatoxins constitute a group of closely related difurnocoumarine mycotoxins, which are important public health problem in regions where high heat and humidity favor the mold growth.

Aflatoxin B (AFB), which is among the most potent hepatocarcinogene ever identified (Busby and Wogan, 1984), is metabolized to a reactive metabolite mainly by CYP3A4 and 1A2 (Gallagher et al., 1994). The affinity of these enzymes towards AFB varies with substrate concentration. CYP1A2 is reported to have a more important role than CYP3A4 in the bioactivation at low aflatoxin concentrations, which is reflective of dietary exposures in human liver microsomes (Gallagher et al., 1996) and in human lung cells expressing CYP1A2 (Van Vleet et al., 2002). Carcinogenesis is due to the formation of aflatoxin B1-*exo*-8, 9-epoxide. At the concentrations of AFB that could result in human liver following ingestion of even highly AFB- contaminated diets, CYP1A2 is likely to be a single most important CYP enzyme in human liver for the activation of AFB to a carcinogenic metabolite (Eaton and Gallagher, 1994). An anti parasitic drug oltipraz, which is currently being investigated as a chemopreventive agent especially with respect to aflatoxin associated hepatocarcinogenesis has been shown to be a potent inhibitor of CYP1A2 (Sofowora et al., 2001). As CYP1A2 appears to mediate most of the activation of

AFB to exo-AFBO in human liver at low dietary concentrations of AFB encountered in the human diet, much of the putative protective effects of oltipraz could be mediated via inhibition of CYP1A2 rather than induction of GSTs.

Interindividual differences in response to chemoprotection against aflatoxin-induced hepatocarcinogenesis are suggested to be due to differences in biotransformation (Eaton et al., 2001). It has been suggested that greater CYP1A2 activity, due to induction either by smoking or food-derived heterocyclic amines, produces higher levels of DNA adducts thereby increasing the likelihood of cancer initiation. High CYP1A2 activity in conjunction with high N-acetyltransferase activity has been associated with elevated risk of colon cancer, especially in those subjects that have a diet rich in meats that are cooked well done (Lang et al., 1994). Lower levels of DNA adduct formation in the Cyp1a2 knockout mice as compared to the wt mice was observed after treating the mice with heterocyclic amines found in cooked meat (Snyderwine et al., 2002). Since several procarcinogens are metabolized by CYP1A2 and it is induced by some of its substrate, inter individual differences in its activity may influence individual susceptibility to cancer risk and therapeutic efficacy of some drugs. Thus, genetic difference in constitutive and/or inducible CYP1A2 gene expression may play an important role in individual risk of environmental toxicity or cancer.

1.4.5.2 CYP1B1 and its role in chemical carcinogenesis

CYP1B1 is a tumor-related form of cytochrome P450, which is over expressed in a wide range of human cancers including cancers of the breast, colon, lung esophagus, skin, lymphnode, brain and testes (Murray et al., 1997) of different histological type. The presence of CYP1B1 may be of importance in the modulation of these tumors to anti-cancer drugs. CYP1B1-mediated metabolism usually results in reduced activity or inactivation of the anticancer drugs, but in some cause bioactivation to a more cytotoxic metabolite occurs. Several anticancer agents inhibit CYP1B1 activity and probably are substrates of CYP1B1 and their metabolism by CYP1B1 may results in reduced activity or inactivation of the anticancer drugs; thus drug inactivation by CYP1B1 may represent a novel mechanism of resistance, influencing the clinical outcome of chemotherapy (McFadyen et al., 2001b; Rochat et al., 2001).

CYP1B1 is the major form of cytochrome P450 expressed in breast cancer (McKay et al., 1995) and ovarian cancer (McFadyen et al., 2001a), which is usually an estrogen-dependent tumor. Human CYP1B1 expressed in yeast shows high specific activity towards the 4-hydroxylation of 17ß-estradiol (Hayes et al., 1996) whereas CYP1A1 and CYP1A2 oxidize preferentially at the 2- position (Yamazaki et al., 1998). Indication of considerably higher 4-hydroxylation of 17β-estradiol in breast cancer microsomes as compared to with a very low level of 4-hydroaxylation in normal breast tissue (Liehr and Ricci, 1996) has received particular attention due to the fact that 4-hydroxyestradiol but not 2-hydroxy estradiol is carcinogenic in human myometrium (Liehr et al., 1995) and breast (Liehr and Ricci, 1996). Elevated rates of 4-hydroxylation of estradiol and expression of CYP1B1 may be markers of malignant phenotype. The mechanism behind overexpression of CYP1B1 in cancer tissues as compared to the normal tissues remains to be elucidated. Reservitol, a cancer preventive agent found in red wine, is converted to the anticancer agent, piceatannol

by CYP1B1 (Potter et al., 2002). Overexpression of CYP1B1 in tumor cells can be utilized for cancer treatment by introducing a protoxic drug whose metabolism is performed by 1B1 to a toxic metabolite that can kill tumor cells but not the normal cells there by limiting the chemotherapy to cancer tissues.

The importance of CYP1B1 in chemical carcinogens has been illustrated using *Cyp1b1* null mice. Embryonic fibroblast cells derived from *Cyp1b1* null mice were found to be resistant to DMBA mediated toxicity in contrast to cells from the wild type animals (Buters et al., 1999). The *Cyp1b1* null mice were protected from DMBA-induced malignant lymphomas (Buters et al., 1999) as well as bone marrow cytotoxicity and preleukemia (Heidel et al., 2000), in comparison to their wild-type counterparts. These data suggest that CYP1B1 is an essential enzyme for metabolic activation and thus the carcinogenic potential of DMBA. Upon treatment of mice with dibenzo[a,l]pyrene, the wt mice were diagnosed with both benign and malignant tumors of the ovaries, lymphoid tissues, as well as with skin and endometrial hyperplasias, whereas CYP1B1-null mice developed only lung adenomas and endometrial hyperplasias (Buters et al., 2002). DNA binding studies using embryonic fibroblasts isolated from these animals indicated that formation of DNA adducts was the critical step in dibenzo[a,l]pyrene-mediated carcinogenesis in mice, and probably also in man.

Several epidemiological studies have been conducted to investigate the relationship between SNPs present in the human *CYP1B1* gene and incidence of various cancer forms in different populations (Bailey et al., 1998; Tanaka et al., 2002; Tang et al., 2000; Watanabe et al., 2000; Zheng et al., 2000). Some studies have revealed a significant relationship between the presence of the Ala119Ser variant and the incidence of breast cancer as well as squamous cell carcinoma in the lung (Watanabe et al., 2000) and prostate cancer (Tanaka et al., 2002). An increased risk for prostate cancer was also reported in subjects homozygous for the Leu432Val form (Tang et al., 2000). However, these studies focused on trying to establish a relationship with a single SNP and no thorough investigations of the haplotypes versus incidence of cancer has been done. It is anticipated that these polymorphisms would cause an altered function of the enzyme in order to provide a role for the CYP1B1 polymorphism in determining interindividual differences in susceptibility for carcinogenesis. Since estradiol is activated by CYP1B1 to a carcinogenic metabolite and it is overexpressed in mammary tumors, subjects with higher CYP1B1 activity and exposed to exogenous estradiol therapy might be at a higher risk in developing mammary carcinoma. Individual differences in CYP1B1 activity may thus influence individual susceptibility to cancer risk.

1.4.5.3 P450 knockout mice as a model to study chemical carcinogenesis

The biological role of DME P450s in acute and chronic toxicities, chemical carcinogenesis, cancer susceptibility and toxicity upon exposure to procarcinogens has been actively investigated using gene knockout mice to determine whether polymorphisms in these genes are involved in cancer susceptibility. The use of null-mice allows determination of the roles of individual enzymes in drug metabolism and elimination, toxicities and cancer in an intact animal model. Transgenic mice lacking functional Cyp1a1, Cyp1b1, Cyp1a2, and Cyp2e1 has been produced by targeted gene disruption and used to determine the roles of individual P450s in intact animals and their contributions to the mechanisms of toxicity and carcinogenesis (Gonzalez and Kimura, 2003). No deleterious phenotypic difference and abnormality was

observed when the null mice were born indicating that theses P450s are not crucial for the mammalian development and physiological homeostasis (Gonzalez, 2002). However, the null mice lacking the expression of theses P450s showed altered responses to the toxic and carcinogenic effects of chemicals as compared with wild-type mice. Several studies reported that the wt- mice were sensitive for development of various types of malignancy and tissue cancer where as the knockout mice were resistant after treating the mice with various PAHs (Buters et al., 2002; Buters et al., 1999; Heidel et al., 2000). Furthermore, the level of DNA adduct formation by embryonic cells derived from the wt-mice was reported to be significantly higher as compared to cells from the Cyp1b1 or Cyp1a2 knockout mouse (Buters et al., 2002; Buters et al., 1999; Heidel et al., 2000; Snyderwine et al., 2002).

2 THE PRESENT STUDY

2.1 Aims of the study

The genetic polymorphism of drug metabolizing enzymes and clinical consequence of their defective allelic variants has been well studied in Caucasians and Orientals, whereas not much is known about the distribution of their variants and clinical effects among Africans. The aim of this thesis was

1. To analyze the pharmacogenetics of CYP2D6, CYP2C19, CYP2C9, CYP1B1 CYP1A2 and xanthine oxidase with special emphasis on Ethiopians and compare the result with other ethnic groups studied like Caucasians, Orientals, African Americans and other African populations with highlighting on optimization of drug therapy. The genetic status of *CYP1B1*, *CYP2C9* and *CYP1A2* among African black population is studied for the first time by this project.

2. To investigate *CYP1B1* and *CYP1A2* genes for the presence of known as well as novel SNPs in the coding and regulatory regions and determine the haplotypes distributed in the population with the emphasis on developing molecular methods to identify susceptibility factors on the genetic levels and their biological functions as well as disease risk e.g. cancer.

3. To study clinical consequence of novel SNPs and haplotypes *in vivo* by examining the correlation with the phenotype analysis where it is applicable.

4. Functional characterizations of novel SNPs and haplotypes *in vitro* and study their effect on the activity of the corresponding enzyme.

5. To evaluate influence of environmental factors such as differences in dietary habits on the activity of CYP2D6, CYP2C19, CYP1A2 and XO.

6. To study effects of gender and smoking habit related differences on the activity of CYP1A2 and XO.

2.2 Materials and methods

2.2.1 Study populations and ethics approval

Healthy unrelated Ethiopians living in Ethiopia or Sweden participated in the study. In addition to Ethiopians, Italians in paper IV; Swedes in paper III and V; Tanzanians and Koreans in paper V participated. The studies were approved by the Ethics Committees at Karolinska Institute, Main campus, at Huddinge University Hospital, Karolinska Institutet, Stockholm, Sweden and by the National Ethics committee of The Ethiopian Science and Technology commission, Addis Ababa, Ethiopia. Ethical approval for the other populations participated in the study was obtained from their respective ethics committees.

2.2.2 Genotyping (*paper I-VI, VIII*)

A 10-ml venous blood sample was taken from each subject into an EDTA-containing Vacutainer tube and DNA was isolated from peripheral leukocytes using a guanidinium-isothiocynate method for genotyping and genomic DNA sequencing. Several molecular biology techniques such as allele-specific PCR, long PCR, SSCP, RFLP, and Southern blot were used for genotyping. Previously established genotyping methods as well as newly developed genotyping methods were used to analyze the frequency of different variant alleles in the populations. We developed molecular haplotyping methods using SNP-specific long PCR- RFLP, to map the different SNPs on the chromosome and determine the CYP1B1 and CYP1A2 haplotypes.

2.2.3 Phenotyping (*paper I- III, VII, VIII*)

Debrisoquine, S-mephenytoin, and caffeine were used as probe drugs to determine the *in vivo* activities of CYP2D6, CYP2C19 and CYP1A2 and XO respectively. Subjects were allowed to take the probe drugs just before bedtime after voiding their bladder and subsequent 0-8 hr urine was collected. Twenty ml aliquots of urine were taken and kept at -20 ºC until the time of phenotype analysis. The urinary molar concentrations of the parent drugs and their respective metabolites were analyzed using HPLC or gas chromatography. Parent to metabolite concentrations were used to calculate the respective metabolic ratio as an index to estimate *in vivo* activities of the respective enzyme.

2.2.4 Genomic DNA sequencing of *CYP1B1* and *CYP1A2* (*paper VI and VIII*)

The entire coding region of CYP1B1 was amplified by long PCR from 5 individuals and was used as a template to sequence each exon and exon intron boundaries to screen for new polymorphic sites in the gene. The *CYP1A2* gene was PCR amplified into two different fragments from 12 different subjects previously phenotyped with caffeine. The PCR products from each DNA samples to be sequenced were pooled and purified using the Wizard® PCR Preps DNA Purification kit. The entire coding regions as well as intron exon junctions were sequenced in both directions using the PCR products as a template with forward and reveres sequence primers. DNA sequencing was performed using the ABI PRISM™ Big Dye terminator cycle sequencing ready reaction kit (Applied Bio systems) and analyzed on an ABI Prism 377 DNA sequencer.

2.2.5 Site directed mutagensis and cloning (*paper VI, VIII*).

The *CYP1B1* wt cDNA, cloned into the pYeDP60 expression plasmid, was used as a template to introduce the different SNPs and generate the different *CYP1B1* variants by site-directed mutagenesis using QuikChange™ Site-Directed Mutagenesis Kits according to the manufacturer's instructions. The *CYP1A2*1A* promoter region containing the intron 1, exon 1 and part of the 5′flanking region was amplified by PCR and cloned into the pGL3 expression vector. This plasmid was used as a template to introduce the different SNPs to generate *CYP1A2*1F, *1J* and **1K* by site-directed mutagenesis. All plasmids were sequenced using the ABI Prism Big Dye terminator cycle sequencing kit and analyzed with ABI Prism 377 DNA sequencer to ensure the correct constructs and to exclude any potential PCR artifacts.

2.2.6 Expression in yeast and kinetic analysis (*paper VI*)

The Saccaromyces cerevisiae yeast strain INVSc1-HR *MAT α his3Δ1 leu2 trp1-289 ura3-52* (*pFL-35 human reductase*) expressing human cytochrome P450 reductase was used to express the different CYP1B1 enzyme variants. Yeast cells were transfected with the pYeDP60-CYP1B1 expression plasmids and were subsequently inoculated into selective medium and grown and microsomes were prepared using differential centrifugation. Rates of CYP1B1-dependent metabolism of estradiol in yeast microsomes were analyzed by incubating microsomes with different concentrations of estradiol. The molar concentrations of 2-and 4-hydroxy estradiol were measured by HPLC using radioactive detector. Km and Vmax values were calculated for each variant enzyme and compared.

2.2.7 Electron mobility shift assay (*paper VIII*)

Functional significance of the different CYP1A2 haplotypes and intron 1 SNP was anysed *in vitro* by EMSA assay using nuclear extracts from a B16A2 hepatoma cell line. Oligonucleotide probes spanning the polymorphic sites, both the wt and mutant variants, were prepared and used in EMSA. DNA-protein complex formation was analysed by incubating the radio labelled wt probe with the nuclear protein extracted from a hepatoma cell line, B16-A2. The effect of the intron 1 SNPs on DNA-protein binding was analysed by using unlabeled mutant oligonucleotides as competitors in 100-fold molar excess. A supershift or interference assay was done using polyclonal or monoclonal antibodies specific for Ets 1, Ets1/Ets 2, PU.1, PEA3, Elk-1 and GABP-a. Complexes were resolved by electrophoreses in 4% polyacrylamide, 0.5 X TBE gel.

2.2.8 Cell transfection and reporter gene assay (*paper VIII*)

Cell transfection studies were performed to determine the effect of intron 1 SNPs and haplotypes on the transcriptional regulation, induction and expression of *CYP1A2* using reporter gene assays. All DNA plasmids used in transient transfection studies were purified using QIAGEN plasmid Maxi kits. Reporter gene assays were performed after cloning of the 5′flanking regions of *CYP1A2* in pGL3 vector, which contains a firefly luciferase gene as a reporter. A human hepatoma cell line, B16-A2, were transfected with the various *CYP1A2* haplotype constructs. Transfection assay was done in quadruplicate and each transfection was performed using 5 μg of the different the plasmid constructs, co-transfected with 0.1 μg of pRL-TK plasmid

containing the *Renilla* luciferase reporter gene, to provide as an internal control of the transfection efficiency and DMRIE-C as a transfection reagent. Effects of intron 1 haplotypes on enzyme inducibility were performed by treating cells with TCDD. The firefly and *Renilla* luciferase activities were measured using the Dual Luciferase reporter assay system (Promega) and firefly/*Renilla* ratio was determined.

2.3 Results

2.3.1 Genetic polymorphisms of *CYP2D6* and *CYP2C19* in Ethiopians (*paper I and II*)

One hundred and twenty two unrelated healthy Ethiopians were genotyped for *CYP2D6*3, *4, *5, *10* and **17* of which 115 of them were phenotyped with debrisoquine. Two individuals (1.8%) were classified as PM but none of them was homozygous for the defective alleles. Seven % of the population had MR values < 0.2 and the median MR in the general population being 0.8.

The presence of *CYP2D6* duplication and number of gene copies were determined using *Xba*I and *EcoR*I RFLP. The *Xba*I RFLP indicates variation in *CYP2D6* gene fragment length. Twenty-nine % of the subjects carried duplicated or multi-duplicated *CYP2D6* genes. Three subjects were homozygous for *CYP2D6* duplication (*Xba*I 42/42 kb). Three subjects with *Xba*I 54 kb, two subjects with *Xba*I 66 kb and one individual with *Xba*I 78 kb haplotypes were found indicating the presence of three, four and five *CYP2D6* gene copies on the same allele respectively. We further verified the number of extra *CYP2D6* gene copies with *Eco*R I RFLP where 12.1 kb band intensity corresponds with the number of extra *CYP2D6* genes. The southern blot films from *Eco*RI RFLP were scanned using densitometer. The numbers of extra *CYP2D6* genes were quantified by comparisons of band intensities of the 12.1 kb band with that of 9.4 kb (containing one *CYP2D6* gene). The highest *Eco*RI band intensity was from a subject with *Xba*I 78/42 kb haplotype having five CYP2D6 genes in one allele and two *CYP2D6* genes on the other one, i.e. in total seven *CYP2D6* genes. Subjects with 4 and 5 copies of *CYP2D6* on the same allele were found for the first time in this study. All individuals with *CYP2D6* duplication had the highest rate of debrisoquine metabolism indicating gene dose dependent response. Interestingly, a relatively higher *CYP2D6*10* and lower *CYP2D6*17* (allele frequency of 8.6 and 9.0 % respectively) was observed in Ethiopians as compared to other African populations.

Genetic polymorphism of *CYP2C19* and the polymorphic metabolism of S-mephenytoin were analyzed among 114 Ethiopians. Six subjects (5.2%) were PMs of which 3 of them were *CYP2C19*2/*2* and the remaining 3 were *CYP2C19*2/*3* thus *2 and *3 accounts for all the defective allele. The allele frequency of *2 and *3 were 14 % and 2 % respectively.

2.3.2 Influence of environmental differences on CYP2D6 and CYP2C19 activity (*paper III*)

In paper I and paper II we found out that Ethiopians showed lower metabolic activity for debrisoquine and S-mephenytoin as compared to Caucasians having the same *CYP2D6* and *CYP2C19* genotypes respectively. Thus, we investigated if environmental influences contributed for the observed differences in the metabolic activity. For this purpose, phenotyping with debrisoquine and S-mephenytoin was repeated among Ethiopians living in Sweden (n=70) and compared the results with those living in Ethiopia and Swedish Caucasians (n=134). Genotyping for *CYP2D6* *2, **3, *4, *5, *6, *10, *17*and **41* was performed. We also analyzed the DNA

samples from Swedes for the -1584C>G polymorphism to differentiate between the *CYP2D6*2* and *CYP2D6*41* alleles.

There was no significant difference in *CYP2C19* genotype or phenotype as assessed by the mephenytoin S/R ratio between Ethiopians living in Sweden or in Ethiopia. However, Swedes were significantly more rapid for CYP2C19 activity than both Ethiopian groups. However, a comparison of the debrisoquine MR among individuals of the same *CYP2D6* genotype revealed a significantly higher activity among Ethiopians living in Sweden ($p<0.02$, Univariate test, ANOVA) as compared to Ethiopians living in Ethiopia.The allele frequencies of *CYP2D6*2, *4, *5, *10* and **17* among the Ethiopians living in Sweden were 13.6, 5.9, 3.1, 8.0 and 14.9 %, respectively. *CYP2D6*3* and **6* are absent in Ethiopians. The frequency of **41* among Ethiopians in Sweden, Ethiopians in Ethiopia and Swedes were 21.6, 25.3 and 10 % respectively. The major variant allele among Ethiopians with duplicated *CYP2D6* gene was **41* where as in Swedes it was the **2* allele. Our results indicate that the CYP2D6-dependent metabolism but not CYP2C19-dependent oxidation of S-mephenytoin was higher among Ethiopians in Sweden as compared to Ethiopians in Ethiopia of the same genotype.

2.3.3 Genetic polymorphism of *CYP2C9* in different populations *(paper IV and V)*

We examined the frequencies of *CYP2C9*1*, *CYP2C9*2* and *CYP2C9*3* alleles in 157 Italians and 150 Ethiopians. The frequencies of *CYP2C9*1, *2* and **3* in Italians were 80, 11 and 9 %. However in the Ethiopian population *CYP2C9*1*, *CYP2C9*2* and *CYP2C9*3* were present at 94, 4 and 2 % frequency, respectively. There was a significant difference in the *CYP2C9* allele frequencies between Ethiopians and Italians. The 95 % confidence intervals in *CYP2C9*1*, *CYP2C9*2* and *CYP2C9*3* between Italians and Ethiopians were 0.098, 0.176; 0.040, 0.098 and 0.040, 0.098 respectively. Among the Italian volunteers, 9 individuals (5.7%) were found to carry two mutated alleles, four subjects were homozygous for *CYP2C9*2*, two homozygous for *CYP2C9*3*, and three subjects were *CYP2C9*2/*3*. In contrast, no individual was found to be homozygous for the defective alleles among Ethiopians.

We developed a genotyping method for the recently described *CYP2C9*5* allele and compared the allele frequency in 200 Swedes, 150 Koreans, 150 Ethiopian and 183 Tanzanians. The *CYP2C9*5* allele was found in three Tanzanians (allele frequency, 0.0082) but not in Ethiopians, Swedes or Koreans.

2.3.4 Functional characterizations of novel *CYP1B1* haplotypes found in Ethiopians *(paper VI)*

We sequenced the complete open reading frame of the *CYP1B1* genes from 5 different subjects and identified 3 novel SNPs, one of which (4360C>G in exon 3) is present at a frequency of 7 %, and causes an Ala443Gly (m5) amino acid substitution. The allele frequencies of the 4 previously described polymorphisms, Arg48Gly, Ala119Ser, Leu432Val, Asn453Ser and the novel *CYP1B1* SNP, Ala443Gly (m5) in 150 Ethiopians were of 51, 50, 53, 2 and 7 % respectively, yielding in total 32 possible *CYP1B1* haplotypes. We developed molecular haplotyping methods and identified seven *CYP1B1* haplotypes, namely *CYP1B1*1, *2, *3, *4, *5, *6* and **7* with allele frequencies of 8, 37, 39, 2, 0.7, 6 and 7 %, respectively. The last three

haplotypes were newly identified in this study. In total, we detected 18 different combinations of haplotypes in the Ethiopian population.

We performed functional characterization the different CYP1B1 haplotypes (except CYP1B1.5) as well as of the Leu432Val + Asn453Ser and Leu432Val +Ala443Gly variants after cloning and expressing them in yeast, *Saccaromyces cerevisiae*. Kinetic analyses were done using 17β-estradiol as a substrate. Two of the haplotypes, CYP1B1.6 and CYP1B1.7 exhibited altered kinetics with significantly increased apparent Km and lowered Vmax for both the 2- and 4-hydroxylation of estradiol, whereas the other constructs were comparable to the CYP1B1.1 in their activity.

2.3.5 Influence of environmental factors on XO and CYP1A2 activity *(paper VII, VIII)*

The aim of this study was to assess effect of gender, smoking habit and environmental factors in CYP1A2 and xanthine oxidase (XO) activity among Ethiopians living in Sweden or in Ethiopia using caffeine as a probe. The activity of CYP1A2 was not affected by gender or environmental difference, but increased by smoking. No significant differences in activity were observed in XO MR between men and women or between smokers and non-smokers ($p>0.05$). However, the XO activity was significantly higher in Ethiopians living in Ethiopia ($p<0.004$, Kruskal-Wallis ANOVA, median test) compared to those living in Sweden. The 95% CI for the difference between the two means was (0.012; 0.044).

2.3.6 Characterizations of new *CYP1A2* intron 1 haplotypes *(Paper VIII)*

We sequenced *CYP1A2* gene using genomic DNA from 12 subjects and revealed a novel intron 1 SNP, -730C>T. No SNP that causes amino acid substitution in the coding regions were found. With the three SNPs in intron 1 of the *CYP1A2* gene theoretically $2^3 = 8$ possible different haplotypes could exist. We developed SNP-specific PCR-RFLP genotyping and molecular haplotyping methods for the intron 1 SNPs and four different haplotypes were identified: *CYP1A2*1A* (wt for all SNPs); *CYP1A2*1F* (-164A); *CYP1A2*1J* (-740G and -164A) and *CYP1A2*1K* (-730T and -740G and -164A) having frequencies of 39.9, 49.6, 7.5 and 3.0 % respectively. The last two haplotypes were novel, identified by this study. No individual was homozygous for the *CYP1A2*1K*.

Subjects with *CYP1A2*1K* had significantly reduced CYP1A2 activity as compared to those carrying *CYP1A2*1A, CYP1A2*1F* or *CYP1A2*1J* as revealed by lower caffeine MR ($p<0.02$, two way Kruskal-Wallis ANOVA). Of the 3 SNPs linked in *CYP1A2*1K*, we identified which one of them could be responsible for the altered enzyme activity *in vivo*. The functional significance of the different intron 1 haplotypes was analyzed using EMSA and reporter gene assays. In EMSA analysis, the presence of the -730C>T completely abolished a binding site for a nuclear protein extracted from B16-A2 cells. Competition was also obtained using Ets consensus probes, indicating that possibly a binding site for a member of Ets transcription factor family was abolished by the -730C>T. However, the other intron 1 SNPs, -740T>G or -164C>A, did not affect DNA-protein complex formation. Reporter gene assays indicated that B16-A2 cells transfected with the *CYP1A2*1K* plasmids showed significantly less inducibility with TCDD where as the other three haplotypes, *CYP1A2*1A, *1F* or **1J* did not affect inducibility.

2.4 General Discussion

***CYP2D6* and *CYP2C19* genetic polymorphism in Ethiopians**

The percentage of CYP2D6 PMs and frequency of *CYP2D6* defective alleles were low in Ethiopians, which is similar to findings in other Africans. However, the absence of *CYP2D6*3*, the Caucasians specific defective allele, and lower % of *CYP2D*4* indicates a difference between Ethiopians and Caucasians. The most interesting finding of paper I was that about 30 % of Ethiopians carry duplicated or multiduplicated *CYP2D6* genes, which makes Ethiopians unique with respect to *CYP2D6* locus. Not only the frequency of *CYP2D6* gene duplication was higher, but also the number of *CYP2D6* gene copies duplicated on the same locus varies. The presence of 4 and 5 copies of the *CYP2D6* gene on one allele have so far only been described in Ethiopians. Twelve extra copies of a functional *CYP2D6* gene were identified in one Swedish family (Johansson et al., 1993). The clinical out come of carrying *CYP2D6* gene duplication is that the subjects become UM and may require higher doses than usually recommended of drugs that are CYP2D6 substrates in order to achieve therapeutic plasma concentrations of the drug.

The molecular mechanism for the generation of *CYP2D6* gene deletion/duplication is suggested to be an unequal cross over event between two *CYP2D6*2* genes during meiosis; the result being deletion of the whole gene (*Xba*I 11.5 kb haplotype) on one allele and duplication of *CYP2D6* gene on the other allele (XbaI 42 kb haplotype) (Ingelman-Sundberg, 1999; Lundqvist et al., 1999). Chromosome 22, where *CYP2D6* gene is located, appears to be non-conserved since there are varied number of genes and pseudogenes residing on the chromosome. The presence of *CYP2D* pseudogenes, which have a high sequence similarity with the *2D6* gene, might have served as a hot-site region for the unequal cross over event. Unequal cross over mechanisms have been proposed for the generation of variant alleles and for the occurrence of duplication/deletion in *CYP2A* locus (Oscarson et al., 2002) and Glutathione S-transferase M1 (Ingelman-Sundberg, 1999).

The finding of 1, 2, 3, 4 and 5 copies of *CYP2D6* gene in Ethiopians and their frequencies, indicate that *CYP2D6* gene duplication has occurred in this population many years ago through evolution. Possibly, genetic selection due to a dietary pressure has been of importance for the occurrence of high frequency of *CYP2D6* gene duplications in Ethiopia and, at the same time, very few inactive *CYP2D6* gene variants. Gene duplications are not stable if it is not beneficial for the organism (Clark, 1994). The higher frequency of *CYP2D6* duplication in Ethiopians most likely is in response to dietary components that have resulted in a selection of alleles with multiple genes thereby increasing the amount of enzyme for efficient role of detoxification of dietary products and evolving plant metabolites. As observed in paper III, Ethiopians living in Ethiopia had lower CYP2D6 activity compared to those living in Sweden having the same *CYP2D6* genotype, indicating the influence of environmental factors on CYP2D6 activity. There are numerous plants and plant products containing alkaloids, for instance khat (*Cata edulis*), which are consumed for dietary and non-dietary purpose, specifically in Ethiopia and the surrounding region. CYP2D6 is known to metabolise several plant alkaloids. Cathinone, an alkaloid and active principle of khat, was found to be a "natural amphetamine" and substrates of CYP2D6 (Bach et al., 1999). Thus suppression of enzyme activity by inhibitors present in the diet might have imposed a selective pressure for preservation

of alleles with duplicate *CYP2D6* gene. It might be suggested that these specific food constituents have an inhibitory action on the CYP2D6 activity seen among Ethiopians in Ethiopia and might possibly have contributed in the past for selection of subjects with multiple *CYP2D6* gene copies. Carriers of multiple *CYP2D6* gene copies would be able to detoxify more alkaloids and therefore be able to digest more sorts of plants than other individuals.

Examination of the global distribution of alleles with duplicated *CYP2D6* genes (Figure 5) reveals that these are most frequent in Ethiopia and Saudi Arabia (Ingelman-Sundberg, 1999). In Tanzania as well as in South and West Africa this frequency is much lower. Probably, duplication of *CYP2D6* originated around the Red Sea neighbouring countries and as a consequence of the Muslim migration to the Mediterranean area about 700 AD, these alleles have been transferred and explain the relative high frequency of duplicated *CYP2D6* genes in this region (10 %) as compared to Northern Europe (1 %) (Ingelman-Sundberg, 1999). An alternative hypothesis would be a gene flow to Arabs and the surrounding area due to an earlier Ethiopians migration to the Arabian Peninsula.

We found a lower percentage of S-mephenytoin PMs among Ethiopians. The frequency of S-mephenytoin PM among Ethiopians is similar to Caucasians, Black Americans and Zimbabweans but much lower than in Asians. The frequency of *CYP2C19*2* in Ethiopians is similar to what is found among Zimbabweans. The presence of *CYP2C19*3* was identified for the first time in a Black population, and it accounts for 25 % of the defective alleles in Ethiopian PMs. The *CYP2C19*3* allele is very rare in Caucasians and is absent in South Africans and Zimbabweans (Dandara et al., 2001). *CYP2C19*2* accounts for 78 % of the PMs in these populations where as both *2 and *3 accounts 100 % in Orientals.

The presence of the Asian specific alleles, *CYP2C19*3* and *CYP2D6*10* in Ethiopians but not in other African population is difficult to explain in terms of population interaction, since there is no specific interaction of Ethiopians with far Eastern Oriental people which lead to genetic mixture in the literature. Similarly, mtDNA and Y-chromosome polymorphism analysis indicated the presence of Asian specific mitochondrial DNA haplotype, which does not exist in other black population or Caucasians, in Ethiopians (Chen et al., 1995; Passarino et al., 1996; Passarino et al., 1998). This haplotype reaches a frequency of 20% in Ethiopia and has never been observed in mtDNA molecules of the other African or Caucasoid lineages.

Influence of environmental differences on CYP2D6, 2C19, 1A2 and XO activities in Ethiopians.

Ethiopians with active *CYP2D6* and *CYP2C19* genes metabolize drugs at a relatively lower rate compared to Caucasians of the same genotype. Even the activity of the enzyme with extra copy of *CYP2D6* gene as well as the wild type variant seems to be lower as compared to the corresponding genotype among Swedes. Several studies have shown lower rates of CYP2D6 and CYP2C19 dependent drug metabolism in Black Africans as compared to Caucasian populations of the same apparent genotype, as indicated by the right shift of debrisoquine and mephenytoin MR histogram in these populations compared to Caucasians (Bathum et al., 1999; Herrlin et al., 1998; Herrlin et al., 2000; Masimirembwa et al., 1995; Masimirembwa et al., 1996a; Wennerholm et al., 1999). Despite a lower prevalence of CYP2D6 PMs and

frequency of non-functional alleles, a reduced metabolic clearance for CYP2D6 substrates was observed in Ghanaians (Griese et al., 1999), Zimbabweans (Masimirembwa et al., 1996a) and Gabonese populations (Panserat et al., 1999) and Tanzanians (Wennerholm et al., 1999). The frequent distribution of the *CYP2D6*17* allele explained the lower CYP2D6 activity in Zimbabweans but not in Tanzanians and Ghanaians. Similarly the frequency of *CYP2D*17* in Ethiopians is relatively low and cannot explain for the lower CYP2D6 activity of this population.

Genetic factors resulting in expression of enzymes with lower amount/activity or environmental factors such as dietary habits, which may induce enzyme expression or inhibit enzyme activity, might be responsible for the observed differences in enzyme activity. In order to observe the influence of environmental factors, we compared the phenotype data obtained from Ethiopians living in Ethiopia or Sweden. The profound effect of differences in environment such as, dietary habits on DMEs has not been investigated before. The result in paper III indicates the presence of a significant influence of environmental factors as an explanation for the difference in capacity for CYP2D6 but not for CYP2C19 metabolism between Caucasians and Ethiopians. We assume that the dietary habits in Ethiopia have an inhibitory effect on CYP2D6 and might have triggered for duplication of these gene in the past, as discussed above. Other genetic factors, for instance yet unidentified mutation in the *CYP2C19* gene or *5´* regulatory regions, which cause lower amount of enzyme to be expressed or expression of enzyme with lower activity could be responsible for the observed variation in CYP2C19 enzyme activity between Caucasians and Ethiopians.

In the contrary, in paper VII, Ethiopians living in Ethiopia showed higher XO activity compared to those living in Sweden. Induction of XO by endogenous and exogenous substances has been reported before. Increased tissue-specific expression of XO has been reported by several cytokines and dexamethasone (Dupont et al., 1992; Falciani et al., 1992; Pfeffer et al., 1994). Induction of hepatic XO expression by treatment of rats with xenobiotic agents such as carbon tetracholrides, ethacrynic acid and butionine sulphoximine was reported (Cassidy et al., 1997). Thus since XO is inducible, we suggest that perhaps the dietary habits in Ethiopia might induce XO expression resulting in higher enzyme activity. Neither gender nor smoking habit related differences influenced XO activity. However, the CYP1A2 activity like CYP2C19 was not different between the two Ethiopian groups indicating that the interindividual variation in CYP2C19 and CYP1A2 activities are mainly controlled by genetic factors. As shown before, smokers had higher CYP1A2 activity.

***CYP2C9* genetic polymorphism in different populations**

Interethnic differences in *CYP2C9* allele distribution have been described between Caucasians and Orientals. However, no data were available from black African populations (Sullivan-Klose et al., 1996; Wang et al., 1995). The allele frequency of **2* and **3* in Italians were comparable to that described for other Caucasians but significantly higher than Ethiopians. However, the *CYP2C9*2* allele frequency found among Ethiopians (0.043), was lower than Caucasians (0.112), but higher than African-Americans (0.01). By contrast, the *CYP2C9*2* allele doesn't exist in Asian populations and very low in African-Americans. These findings suggest that the *CYP2C9*2* allele occurred quite recently, after the separation of the Caucasian and Asian racial groups. Similarly the allele frequency of *CYP2C9*3* among Ethiopians we found (0.023) was comparable to that found in Japanese (0.021) (Nasu et al.,

1997) and Chinese (0.026)(Wang et al., 1995) populations but much higher than in African Americans (0.005) (Sullivan-Klose et al., 1996).

A new *CYP2C9* variant allele *CYP2C9*5* and *6** were identified in African Americans (Dickmann et al., 2001; Kidd et al., 2001). We analysed the frequency distribution of *CYP2C9*5* in 3 different populations, Caucasian, Asian and Black African Populations. *CYP2C9*5* was found so far only in Tanzanians apart from African Americans and does not exist in Caucasians or Asians. Although the allele frequency of **2* and **3* found in Ethiopians were higher than in African Americans, the *CYP2C9*5,* which is considered as specific for African Americans and hence, Black population, was absent in Ethiopians. The finding of *CYP2C9*5* in Tanzanians but not in Ethiopians, indicates the heterogeneous nature of African populations with respect to *CYP2C9* genetic polymorphism. Our results indicate a unique relative distribution of the *CYP2C9* alleles in the Ethiopian population, which is not similar to any other ethnic group described so far. The characterization of *CYP2C9* genetic polymorphism among different races mi ght contribute to the optimization of therapy with a range of clinically important drugs.

Functional characterization of CYP1B1 haplotypes found in Ethiopians.

We analyzed the functional consequences of the different CYP1B1 haplotypes by expressing the corresponding cDNAs in yeast and found out that two of the haplotypes, CYP1B1.6 and CYP1B1.7 having 3 amino acid substitutions in common showed very low activity towards estradiol metabolism. These results indicate that the true haplotype is important in determining the overall function of the specific form of CYP1B1, indicating that combinations of SNPs would influence the structure of the active site after protein folding. Similarly, the CYP2D6.17 variant having three amino acid substitutions Thr107Ile, Arg296Cys and Ser486Thr, showed altered enzyme kinetics compared to the wt enzyme. Incorporation of these polymorphisms separately into wt *CYP2D6* cDNA did not influence the catalytic function. However, constructs having both the Thr107Ile and Arg296Cys substitutions, displayed a reduced affinity for the CYP2D6 substrates bufuralol and codeine as compared to the wt enzyme (Oscarson et al., 1997). These findings are in agreement with phenotype analysis *in vivo*, where the activity of CYP2D6.17 has been found to be severely impaired when debrisoquine was given as a probe drug (Masimirembwa et al., 1996b). Because of lack of established CYP1B1 probe drug, we could not investigate the *in vivo* effects of these haplotypes.

Variations in the pattern of CYP1B1 disease causing mutations and benign variants was reported to exist in Primary congenital glaucoma (PCG) patients from different ethnic backgrounds (Sitorus et al., 2003). In this study, in contrast to European patients who showed truncating mutations, Indonesian patients exhibited the missense mutation as the most common mutation. Furthermore interethnic variations in the pattern of SNP distributions among PCG patients was indicated suggesting that determination *CYP1B1* haplotypes might contribute for the identification of subjects with high PCG susceptibility.

Several studies have been devoted to the investigation of a potential relationship between SNPs present in the human *CYP1B1* gene and incidence of various cancer forms (Bailey et al., 1998; Tanaka et al., 2002; Tang et al., 2000; Watanabe et al., 2000; Zheng et al., 2000) and some of them have reported a positive association between the presence a single SNP and higher incidence of the cancer type

investigated for. However these studies tried to look for associations of individual SNP with the cancer incidence. We have shown the existence of linkage disequilibrium between *CYP1B1* SNPs resulting in an altered enzyme activity of the resulting haplotypes. Since CYP1B1 is involved in the metabolism of several procarcinogens, the *CYP1B1* genetic polymorphism may contribute to interindividual and interethnic variation in susceptibility towards the development of cancer upon exposure of procarcinogens or estrogen over a long period of time. However, an accurate investigation regarding the genetic influence of the polymorphic CYP1B1 on the susceptibility of different forms of cancer requires determination of the *CYP1B1* haplotypes.

Characterization of a novel *CYP1A2* intron 1 SNP and haplotypes affecting expression and induction.

Unlike other drug metabolizing enzymes such as CYP2D6, 2C9 or 2C19, CYP1A2 is relatively conserved and no deleterious mutations in the coding regions have been found in Ethiopians or other populations so far. This indicates the occurrence of selective pressure in the *CYP1A2* gene to maintain active CYP1A2 enzyme perhaps due to its importance in the metabolism of endogenous substances. Absence of difference in CYP1A2 activity between Ethiopians living in Ethiopia or Sweden might perhaps indicate that variations in CYP1A2 activity are mainly due genetic factors, most likely by polymorphism in the regulatory regions affecting enzyme expression or inducibility. Accordingly, a recent study on twins indicated that the CYP1A2 activity is mainly governed by genetic factors (Rasmussen et al., 2002).

Several studies were conducted to investigate the importance of the -164 C>A polymorphism and enzyme inducibility by smoking or its association with a severity of tardive dyskinesia in schizophrenic smoking patients from different populations. In contrast to Sachse, *et.al*, (Sachse et al., 1999), we did not observe significant difference in enzyme activity between *1A/*1A and *1F/*1F in neither smokers nor non-smokers. In accordance to our results, lack of impact of the -164 SNP genotypes on the plasma concentration of haloperidol in smoking male Japanese with schizophrenia (Shimoda et al., 2002) and in smoking Swedish pregnant women (Nordmark et al., 2002) have recently been reported. A contradictory result were reported in studies attempted to investigate the association of the -164 C>A SNP and severity of tardive dyskinesia in smoking schizophrenic patients in US (Basile et al., 2000) and German population (Schulze et al., 2001). Most of these studies had anysed the individual SNPs independently. Thus, a possible explanation for the discrepant findings could be incomplete determination of the different haplotypes existing in the population. Our haplotype analysis indicates that -164 SNP can be in linkage disequilibrium with other intron 1 SNPs resulting in at least 4 different haplotypes having different functional impact and it is thus important to take the complete haplotypes into consideration when investigating associations of phenotype rather than focusing on single SNPs. This is in accordance with a very recent report, where the common *CYP1A2* polymorphisms were found to be in strong linkage disequilibrium and the extent of linkage observed in their study suggests a change in the current nomenclature of CYP1A2 alleles might be appropriate (Sachse et al., 2003). Some of the SNPs defining alleles in the current nomenclature of CYP1A2 seem not to occur on their own, but together with other SNPs. For instance, the -740 T>G termed *CYP1A2*1E* in the present nomenclature, is always in combination with the -164 C>A and the novel SNP -730C>T is always in linkage disequilibrium with

the -164 C>A and -740 T>G, at least in Ethiopians. However, some linkage disequilibrium observed in Ethiopians may not appear in the same way in another ethnic group.

Among the four CYP1A2 intron 1 haplotypes exist in Ethiopians, only *CYP1A2*1K*, is associated with lower enzyme activity and inducibility. The only additional SNP in *CYP1A2*1K*, as compared to the others, is -730 C>T and thus the presence of this SNP is apparently critical for a decreased CYP1A2 activity *in vivo*. We analyzed *CYP1A2*1K,* to identify which of the three linked SNPs that would be responsible for the observed lower CYP1A2 activity *in vivo*. EMSA assays indicated that the novel intron 1 SNP, -730C>T abolishes a binding site for a nuclear protein in B16A2 cells, a member of an Ets transcription factor family. The Ets family members are important transcription factors involved in development and are implicated in several types of cancer and other human diseases (Dittmer and Nordheim, 1998). However, the presence of the other intron 1 SNPs, -740 T>G or -164 C>A, did not affect DNA-protein binding complex formation. Cell transfection assay indicated that the -730C>T causes a significantly reduced induction with TCDD. Induction of cells transfected with the other haplotypes were comparable with the wt. Unfortunately, there was only one individual who was heterozygous for *CYP1A2*1K* among smokers and he had the lowest log caffeine MR in this group. Therefore, further investigations need to be done to determine the influence of *CYP1A2*1K* on enzyme inducibility *in vivo*. Both smoking and administration of omeprazole, a gastric proton-pump inhibitor, induce selectively human liver CYP1A2 *in vivo* (Rost et al., 1994). Our future work aims to elucidate the influence of *CYP1A2* haplotypes on enzyme inducibility *in vivo*. This can be done either by including large number of smokers or alternatively, by administration of omeprazole to non-smoker subjects with different *CYP1A2* genotypes and determine their caffeine MR. However, since omeprazole is metabolized by CYP2C19, genotyping for *CYP2C19* variant alleles is essential to eliminate variations in the metabolism of omeprazole by CYP2C19, which otherwise affects CYP1A2 inducibility. Recently, the influence of *in vivo* CYP1A2 induction by omeprazole in subject with *CYP1A2*1C/*1F* and *CYP1A2*1F/*1F* genotypes and who were *CYP2C19*1/*1* has been reported (Han et al., 2002).

In summary, the -730C>T present in *CYP1A2*1K* but not -740T>G or -164C>A is responsible for the altered activity *in vivo* and affects CYP1A2 expression and induction. We found a novel intron 1 that is involved in *CYP1A2* gene regulation and affects the level of expression and enzyme induction by abolishing a binding site for a member of Ets nuclear transcription factor family. The results indicate that polymorphism in intron 1 of the *CYP1A2* gene might be critical for CYP1A2 inducibility and that transcriptional factors of the Ets family are of importance in this respect. Furthermore, the altered activity by the presence of combinations of SNPs in CYP1B1 and CYP1A2 indicates the importance of a complete haplotype analysis rather than individual SNP investigation in epidemiological studies trying to investigate association genotypes with a disease phenotype, for instance, incidence of cancer.

2.5 Conclusions

- Lower incidence of defective *CYP2D6* alleles in Ethiopians, compared to Caucasians, accounts for the decreased percentage CYP2D6 PM phenotypes. About a third of Ethiopians carry the duplicated and multiduplicated functional *CYP2D6* gene, which is the highest percentage compare to any other ethnic group registered so far.

- The incidence of CYP2C19 PMs and defective alleles found in Ethiopians resembles that of Caucasians and Africans, but lower than Asians. The Asian specific mutation, *CYP2C19*3* is present in Ethiopians.

- Activity of CYP2D6 but not CYP2C19 is influenced by environmental factors. We assume that the dietary habits in Ethiopia have an inhibitory effect on CYP2D6 and might have triggered for duplication of this gene in the past. Additional genetic and perhaps environmental factors have to be identified to fully explain the differences in the rate of CYP2C19 mediated drug metabolism between Black Africans and Caucasians of the same apparent genotype.

- The frequencies of *CYP2C9*1, *2* and **3* found in the Italian population were similar to other Caucasian groups. However, in the Ethiopian population incidence of the *CYP2C9* defective alleles are significantly lower. Accordingly, the *CYP2C9*5* allele is absent in Ethiopians, Caucasians or Orientals but present in Tanzanians.

- CYP1B1 is polymorphic in Ethiopians with five different SNPs in the gene resulting in seven different haplotypes and CYP1B1 enzyme variants, of which CYP1B1.6 and CYP1B1.7 exhibit altered kinetic properties for estradiol hydroxylation. Consequently, the *CYP1B1* genetic polymorphism may contribute to interindividual and interethnic variation in susceptibility towards the development of cancer upon exposure of procarcinogens or estrogen over a long period of time.

- Activity of XO is affected by environmental factors in Ethiopians perhaps the dietary habit in Ethiopia might induce higher expression of this enzyme and thus might explain the higher XO activity in Ethiopian living in Ethiopia.

- A novel *CYP1A2* intron 1 SNP, -730 C>T was identified and is likely to be involved in gene regulation and responsible for lower enzyme inducibility and expression of CYP1A2, possibly by abolishing a biding site for a nuclear protein factor.

- Ethiopians, in comparison to Caucasians, Oriental and other African populations somehow are different with respect to the genetic polymorphisms of *CYP2D6*, *2C19* and *2C9*, *CYP1B1* locus. African populations are heterogeneous in this respect and unlike Asians and Caucasians, generalization about the drug metabolic capacity cannot be made in Africans.

- The results emphasize the necessity of a complete haplotype analysis of enzyme variants rather than individual SNPs for analyses of genetic polymorphisms and evaluation of functional consequences *in vivo* in

epidemiological studies to associate a disease phenotype or to identify cancer risk.

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