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Genetically Determined Interindividual Variation in Cytochrome P450 Dependent Drug Metabolism; Molecular Basis and Clinical Implications

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To my husband
And to my son

ABSTRACT

Pharmacogenetics is the study of genetic variation that causes variation in drug levels (pharmacokinetics), drug response (pharmacodynamics) and adverse drug reactions. Pharmacogenetics aims at identifying biomarkers that allows a personalized drug treatment and thereby increased drug efficacy and reduced occurrence of adverse drug reactions. The highest genetic influence on interindividual variability in drug bioavailability is exerted by polymorphism in cytochrome P450 (*CYP*) genes encoding enzymes of critical importance for drug metabolism. In the current thesis, genetic factors behind the interindividual variation in CYP2C19 and CYP3A expression and activity have been investigated.

A yet unidentified member of the CYP3A subfamily, CYP3A43, was cloned and characterized. It was found that CYP3A43 expression was insignificant in human tissues and that the cDNA-expressed protein did not translate into detectable protein or catalytically active enzyme in a variety of heterologous expression systems. Thus, we concluded that CYP3A43 does not contribute to interindividual variation in CYP3A activity.

CYP3A5 has been claimed to be polymorphically expressed at very high levels in Caucasians and CYP3A5 expression has been suggested to account for a large part of the interindividual differences in CYP3A activity. However, using antibodies raised against the C-terminal peptide of CYP3A5 and a peptide-conjugate as standard, we could show that the average expression in Caucasian liver is only 2 % of total CYP3A, although some livers with higher expression were identified. By contrast, we found that the fetal form of CYP3A, i.e. CYP3A7, unexpectedly accounted for a similar amount of CYP3A protein as that of CYP3A5 in adult human livers. The expression was to some extent associated to the presence of the previously described *CYP3A7*1C* allele. It is concluded that additional genetic factors remain to be identified in order to explain the interindividual differences in CYP3A activity.

CYP2C19 catalyzes the metabolism of e.g. proton pump inhibitors and antidepressants and its genetic polymorphism significantly affects drug metabolism and pharmacokinetics. There is a high interindividual variability in homozygote carriers of wildtype alleles, the basis of which has not been known. We were able to identify a new common allele (*CYP2C19*17*) containing a -806C>T mutation that introduces a functional transcription factor element that results in binding of nuclear factors and increased transcriptional activity. Analyses in Ethiopians and Swedes revealed a significant influence of *CYP2C19*17* on drug pharmacokinetics and it was calculated that homozygous carriers will have about 40% lower omeprazole area under the plasma concentration-time curve after a single dose as compared to homozygotes for the wildtype allele. Thus, this is the first study to provide with a basis for the variability in CYP2C19 activity among subjects previously defined as homozygous for the wildtype allele. It is suggested that *CYP2C19*17* may contribute to a higher frequency of non-responders during antiulcer and possibly also antidepressant therapy.

PUBLICATIONS

I. Westlind A, **Malmebo S**, Johansson I, Otter C, Andersson TB, Ingelman-Sundberg M, Oscarson M. Cloning and tissue distribution of a novel human cytochrome P450 of the CYP3A subfamily, CYP3A43. *Biochem Biophys Res Commun*. 2001; **281**:1349-55.

II. Westlind-Johnsson A*, **Malmebo S***, Johansson A, Otter C, Andersson TB, Johansson I, Edwards RJ, Boobis AR, Ingelman-Sundberg M. Comparative analysis of CYP3A expression in human liver suggests only a minor role for CYP3A5 in drug metabolism. *Drug Metab Dispos.* 2003; **31**:755-61.
**These authors contributed equally to this work.*

III. **Sim SC**, Edwards RJ, Boobis AR, Ingelman-Sundberg M. CYP3A7 protein expression is high in a fraction of adult human livers and partially associated with the *CYP3A7*1C* allele. *Pharmacogenet Genomics*. 2005; **15**:625-31.

IV. **Sim SC**, Risinger C, Dahl ML, Aklillu E, Christensen M, Bertilsson L, Ingelman-Sundberg M. A common novel *CYP2C19* gene variant causes ultrarapid drug metabolism relevant for the drug response to proton pump inhibitors and antidepressants. *Clin Pharmacol Ther*. 2006; **79**:103-13.

APPENDIX

V. **Sim SC**, Ingelman-Sundberg M. The human cytochrome P450 allele nomenclature committee web site: submission criteria, procedures, and objectives. *Methods Mol Biol.* 2006; **320**:183-91.

ABBREVIATIONS

ADR	Adverse drug reaction
APL	Acute promyelocytic leukemia
atRA	All-*trans* retinoic acid
AUC	Area under the plasma concentration-time curve
CAR	Constitutive androstane receptor
CLA	Clarithromycin
CYP	Cytochrome P450
DHEA	Dehydroepiandrosterone
DPL	Drug package label
EM	Extensive metabolizer
EMSA	Electrophoretic mobility shift assay
EST	Expressed sequence tag
FDA	Food and drug administration
IM	Intermediary metabolizer
MDR1	Multidrug resistance 1
MDZ	Midazolam
MR	Metabolic ratio
MUT	Mutant
P450	Cytochrome P450
PCR	Polymerase chain reaction
P-gp	P-glycoprotein
PM	Poor metabolizer
PPI	Proton pump inhibitor
PXR	Pregnane X receptor
RA	Retinoic acid
RACE	Rapid amplification of cDNA ends
RFLP	Restriction fragment length polymorphism
SNP	Single nucleotide polymorphism
SSRI	Selective serotonin re-uptake inhibitor
TCA	Tricyclic antidepressant
TPMT	Thiopurine methyltransferase
UGT	UDP-glucuronosyltransferase
UM	Ultrarapid metabolizer
VKOR	Vitamin K epoxide reductase complex
WT	Wildtype

CONTENTS

INTRODUCTION

GENERAL INTRODUCTION

There is a large interindividual variability in drug levels, drug response and adverse drug reactions (ADRs) between individuals. Drug levels can vary substantially between different subjects with the same body weight and receiving the same drug dose, and this variation can be due to for example environmental or genetic factors. The variation in drug levels can in turn affect the frequency and magnitude of drug response and side effects. One class of drugs, the antidepressants, has a particularly high rate of non-responders that can vary between 30% to 70% depending on the study (1). In the US, the amount of people that will experience depression during their lifetime is 16% (2). Thus, the high rate of non-response to antidepressant treatment is a considerable problem both from an economical and an individual point of view. The occurrence of ADRs in drug treatment is a large problem that can lead to severe injury or secondary disease, such as hepatotoxicity, drug-induced hepatitis or even death. Not only can ADRs be a personal catastrophe for the patient, it also costs the society substantial amounts of money. About 5% of all hospital admissions in the US and up to 7% in Sweden and the UK are predicted to be caused by ADRs (3, 4).

Of all genetic factors that affect drug treatment, those affecting the activity of the drug-metabolizing enzymes cytochrome P450 (P450) are currently considered the quantitatively most important. This thesis aims at investigating genetic variation in *P450* genes of importance for drug treatment.

THE CYTOCHROME P450 SUPERFAMILY OF ENZYMES

The P450 superfamily of heme-containing enzymes is involved in the conversion of a wide variety of endogenous and exogenous compounds to more hydrophilic derivatives that are more easily excreted from the body. The name P450 refers to the "pigment at 450 nm" and is based on the absorbance at 450 nm after reduction of the heme iron and complexing with carbon monoxide. It is generally accepted that the human genome contains 57 functional *P450* genes (5).

The P450s are mainly expressed in the liver and intestine, but are also present at lower levels in other tissues. P450s are localized to the endoplasmic reticulum or the mitochondrial outer or inner membrane where they are membrane-anchored through their N-termini.

The families CYP1-4 (see below for definition) are involved in the metabolism of drugs and xenobiotics and many of them are inducible. For drug metabolism, CYP3A4, CYP2C9, CYP2C19 and CYP2D6 are considered to be the most relevant (6).

CYP NOMENCLATURE AND DEFINITIONS

The enzymes of the cytochrome P450 superfamily are classified according to amino acid sequence homology. The P450 nomenclature follows that of dividing isoenzymes into families (e.g. CYP3), subfamilies (e.g. CYP3A) and individual enzymes (e.g. CYP3A4). Separate alleles of the same gene are designated by an asterisk and a number (e.g. *CYP3A4*15*). To be designated a unique allelic number; the nucleotide variations should affect transcription, splicing, translation, posttranscriptional or posttranslational modifications, or result in at least one amino acid change (see www.cypalleles.ki.se/criteria). If additional variations are identified in an allele already given a specific number and they have a less dominant effect on function than the one already present, additional alleles are defined by additional letters (e.g., *CYP3A4*15B*), whereas the primary allele is designated by *A* (e.g., *CYP3A4*15A*). If the effects of additional variations are expected to be functionally similar, e.g. two variations giving rise to different amino acid substitutions, the combination can be designated by a new allelic number.

PHARMACOGENETICS

Pharmacogenetics is the study of genetic variation that causes variation in drug levels (pharmacokinetics), drug response (pharmacodynamics) and side effects. Genetic variation affecting drug concentration and response can be exerted at the level of for example drug-receptor binding (target), absorption rate (transport) or drug elimination (e.g. metabolism). Relevant genes to study in these respects are those encoding drug-metabolizing enzymes, as well as drug transporters and drug receptors. However, with a few exceptions, the genetic polymorphism of transporters and receptors has hitherto not been shown to exert important effects on drug levels and drug response (4). By contrast, polymorphic enzymes catalyzing drug metabolism have often been shown to affect drug bioavailability. Drug metabolism is mainly catalyzed by P450-mediated reactions and conjugations (4). About 15-30% of the variability in drug metabolism can be explained by genetic factors (4). Drug levels can relatively easily be monitored in plasma or serum and genetic association studies be carried out, but there is not always a clear drug concentration-effect relationship and investigations of the impact of gene variants on treatment outcome are thus more difficult.

Phenotype categories

Dividing individuals into groups based on the activity (phenotype) of P450 metabolism can be done by the use of P450 isoform-specific probe drugs. Consequently, individuals can be classified as ultrarapid metabolizers (UM), extensive metabolizers (EM), intermediate metabolizers (IM) and poor metabolizers (PM). Ultrarapid metabolism is usually caused by gene duplications such that more than one gene copy occur on the same chromosome. Intermediate metabolizers normally carry one defective allele, whereas poor metabolizers carry two.

Cytochrome P450 enzymes of particular importance for drug metabolism

CYP2D6 is one of the most studied P450s in the literature, due to its polymorphic expression and its importance in drug metabolism that equals to 20-25% of all drugs in clinical use (7). Examples of classes of CYP2D6 substrates are antidepressants, antipsychotics and neuroleptics. The CYP2D6 phenotype can be divided into four clear categories that are caused by polymorphism of the *CYP2D6* gene. Defective alleles such as *CYP2D6*4* give rise to the IM and PM phenotypes. Alleles produced by duplication of the *CYP2D6* gene (e.g. *CYP2D6*1XN* where N indicates two or more gene copies) give rise to the UM phenotype (see www.cypalleles.ki.se). Individuals with the UM phenotype are shown to suffer from a reduced response to drug treatments such as those with certain antidepressants (8, 9). As a consequence, much higher than the standard antidepressant dose can be recommended for UM phenotypes, who correspond to approximately 5% of the European population (3, 10). In contrast, PM individuals would require down to 30% of the standard dose of antidepressants in order to achieve the same plasma concentrations (10), and this would be relevant for about 7% of the European population (3). CYP2D6 UMs are at increased risk of experiencing morphine intoxication due to the high rate of bioactivation of codeine to morphine. In fact, infants being breast fed by mothers with a CYP2D6 UM phenotype that are taking codeine are at the risk of fatal intoxication due to the high amount of morphine being transferred to the infant via breast milk (11). CYP2D6 PMs are on the other hand at an increased risk of experiencing ADRs due to increased plasma concentration at standard drug dosage due to inefficient metabolism in these individuals (3).

The **CYP3A** subfamily metabolizes more than 40% of clinically used drugs (3, 12) and is thus quantitatively the most important enzyme route for drug metabolism. The variation in CYP3A metabolism between individuals is large, thus potentially affecting drug levels for a substantial number of drugs. CYP3A activity can be affected by induction and inhibition events, thereby increasing and decreasing CYP3A drug metabolism, respectively. For example, the antibiotic rifampicin is a potent inducer, whereas inhibition can be caused by antiretroviral drugs used in

the treatment of HIV infection. Examples of substrates are antihypertensive medications and antimalaria drugs.

CYP2C9 metabolizes about 10% of clinically used drugs (3) and is an important enzyme due to the metabolism of for example warfarin that is used in the treatment of thrombosis. CYP2C9 is also involved in the metabolism of antidiabetic drugs and non-steroidal anti-inflammatory drugs (NSAID), which are common in the treatment of pain, fever and inflammation. Defective *CYP2C9* alleles (see www.cypalleles.ki.se) cause to increased drug levels of CYP2C9 substrate drugs and an increased risk of ADRs.

CYP2C19 is involved in the metabolism of about 5% of drugs (3). CYP2C19 plays an important role in the metabolism of antiulcer drugs and antidepressants. The CYP2C19 PM phenotype occurs with a frequency of about 5% in Caucasian populations and it is well supported that patients with the PM phenotype benefits from antiulcer treatment using standard doses due to increased drug levels and thus improved healing of gastric ulcers (13).

CYP1A2 metabolizes about 5% of all drugs (3), but is also involved in the activation of procarcinogens (14). CYP1A2 is induced by smoking and several dietary components, and is involved in the metabolism of caffeine (14). Induction and inhibition of the CYP1A2 enzyme are the main mechanisms behind variation in activity, since genetic polymorphism giving rise to interindividual differences in the activity level are rare (see www.cypalleles.ki.se).

CYP2A6 is quantitatively the least important P450 of those mentioned in this section, since it accounts for the metabolism of only 2% of all drugs (3). However, CYP2A6 metabolizes some important drugs such as the antithrombotic medication coumarin, and is also involved in the metabolism of nicotine (15).

Antidepressants as an example of genetic polymorphism affecting target, transport and metabolism of drugs

As mentioned, drug levels and response can be affected by genetic variation in drug target, drug transport and drug metabolism. The antidepressant class of drugs, which encompasses tricyclic antidepressants (TCA) and serotonin re-uptake inhibitors (SSRI), is a good example for which genetic variation at all three levels appears to have an impact. Antidepressants are partially or selectively blocking the uptake of serotonin from the synapse, and polymorphism in a gene encoding the **target**, i.e. the serotonin transporter, has been shown to affect treatment response due to differences in transcription level of the target transporter (16). Consequently, alleles giving rise to higher levels of the serotonin transporter could lead to higher response rates in

antidepressant treatment. In addition, polymorphism in the gene multidrug resistance 1 (*MDR-1*) that encodes the **drug transporter** P-glycoprotein (P-gp, ABCB1) appears to affect the amount of antidepressant reaching the brain by controlling the transport over the blood-brain barrier (16). Thus, low-activity *MDR-1* alleles could infer a higher drug response due to increased drug concentration in the brain. Finally, the **drug-metabolizing** enzyme CYP2D6 has as mentioned been implicated in the response rate and the occurrence of side effects during antidepressant treatment, whereby UM phenotypes experience a lower frequency of response due to lowered antidepressant levels (8, 9), and PM phenotypes are predisposed for the occurrence of side effects (17, 18).

Examples of aspects for evaluating pharmacogenetic importance

The main goal of pharmacogenetic studies is to improve drug treatment. Concerning drug-metabolizing enzymes, Gardiner and Begg (6) have listed the following general aspects for addressing the pharmacogenetic importance of genetic variations:

a) does the polymorphism significantly affect total concentration of active moieties (parent drug and metabolite)?
b) is there a clear concentration-response relationship?
c) is the therapeutic index low?
d) are the concentration-related side effects significant?

If a particular enzyme metabolism leads to an active drug metabolite, polymorphism in the gene encoding the enzyme would not be expected to affect the outcome of drug treatment. In addition, if several enzymes are involved in the metabolism of a drug, genetic polymorphism in one enzyme pathway would not significantly alter drug levels and drug response. By using an algorithm for the pharmacogenetic effect on drugs, and based on the above aspects and data supportive of pharmacoeconomic benefits, Gardiner and Begg (6) identified only two enzymes, thiopurine methyltransferase (**TPMT**) and butyrylcholinesterase, that could be rated as being of "definite importance" for genotype-adjusted drug dosing in clinical practice using immunosuppressants and muscle relaxants, respectively. The clinical value of genotyping the *TMPT* gene is due to the strong association of low activity alleles with severe myelosuppression during treatment with particular immunosuppressant drugs (6-mercaptopurine, azathioprine) (4). However, since many cases of myelosuppression occur also in patients with high activity alleles, genotyping is not expected to replace blood monitoring (6). In addition, phenotyping red blood cells for the capacity to produce the bioactivated azathioprine appears to efficiently and reliably predict clinical outcome in Crohn's disease (19), whereby genotyping is of minor importance.

Based on the algorithm, it was also found that warfarin and *CYP2C9* genotype, and omeprazole and *CYP2C19* genotype, belong to the category of "possible importance" in prospective genotyping (6) (see further discussions in sections below).

Despite the relevance of every single aspect mentioned above, an overall rating based on the sum of scores is not applicable. Specifically, a high total rating based on high scores of most aspects but a low score in one aspect is not necessarily practically relevant. For example, if there is a lack of drug concentration-response relationship, fulfilling the other aspects is not sufficient to support genotype-adjusted drug dosing, despite potentially high total scores. In addition, a relatively low total rating due to a wide therapeutic window does not necessarily mean that genotype-based drug dosing is irrelevant, as will be further discussed below. Thus, separate aspects are indeed important as such, but the weighted rating based on all aspects is not an easy task. In addition, the occurrence of ADRs is a very important aspect to consider when evaluating the significance of pharmacogenetic testing. This has not been listed as one major aspect by Gardiner and Begg (6), but appears still to be considered in their algorithm (see TPMT above). It needs however to be emphasized that avoidance of ADRs due to genotyping analyses is a very important aspect in pharmacogenetics.

A cost-benefit evaluation of any genotype-adjusted drug dosage must be taking several sources of expenses and advantages into consideration. The cost of genotyping and the cost of implementing a more complicated dosing schedule must be weighted against a lower incidence of ADRs and thus savings in the treatment of those, and an increased treatment efficacy, which thus reduces the cost due to a reduced necessity of second-line treatments. The frequency and severity of ADRs is of course variable depending on the drug, as is also the possibility to affect treatment efficacy. Thus, the cost-benefit relationship needs to be specifically investigated for every drug.

Non-genetic sources of variation

Genetic variation must be impacting more on the drug level and response than other sources of variation in order to be clinically important. Other sources of variation are for example age, sex, height, weight, poor compliance and disease states. Drug-drug interactions are also important sources of variation, giving rise to enzyme induction and/or inhibition events. Thus, despite a good association of genotype with drug levels, variability exerted at other non-genetic levels may be large, thus making drug dosing based on only genotyping difficult.

Impact of therapeutic index

The therapeutic index is largely but not determinately affecting the relevance of genotyping tests. A narrow therapeutic index leading to severe side effects or lack of response of a severe condition if the drug concentration is slightly increased or decreased, respectively, justifies genetic testing in order to avoid unwanted effects. However, a wide therapeutic index, where a variation in drug concentration does not significantly affect side effects or drug response, does in general not support genetic testing. Despite the difference in therapeutic index, the following drugs and P450 enzymes have relatively strong implications for future genotype-based drug dosing:

- Proton pump inhibitors (PPIs) such as **omeprazole** are used in combination with antibacterial agents to treat *H. pylori*-positive gastric and duodenal ulcers and they have a wide therapeutic index. The several fold higher omeprazole area under the plasma concentration-time curve (AUC) observed in **CYP2C19** PMs compared to EMs does not result in any significant side effects (20). The PPI drug level and effect (acid inhibition and cure rate of *H. pylori* infection) is however dependent on *CYP2C19* genotype, and PMs have a higher cure rate of acid-related ulcers than EMs at standard doses (21).

- **Warfarin** is an antithrombotic drug with a narrow therapeutic index and the variation in dose required for achieving desired therapeutic effect is large, whereby monitoring of the anticoagulative effect is necessary. **CYP2C9** is the main enzyme involved in warfarin metabolism and defective *CYP2C9* alleles are associated with lower dose requirement and increased risk of bleeding (22). Warfarin reduces coagulation by inhibiting the vitamin K epoxide reductase complex (**VKOR**) that is necessary for the Vitamin-K dependent clotting cascade, and defective *VKORC1* gene variants affect warfarin response. Polymorphisms in the *CYP2C9* gene (metabolism) as well as the *VKORC1* gene (target) are relatively common and it appears that genotyping of both together with age and height parameters can predict 55% of the factual doses (23).

It is interesting that both of the above examples are likely to benefit from a *P450* genotype-based drug dose adjustment, despite their difference in the size of the respective therapeutic windows. Genotyping for CYP2C19 in omeprazole treatment would not be based on any risk of side effects, but rather aims at increasing drug response to minimize treatment costs. Indeed, it seems clear that the cost of antiulcer treatment would be reduced by individualizing drug dose based on *CYP2C19* genotype, at least for the treatment of duodenal ulcer (24). In addition to individualizing drug dose, choice of therapy could also be improved since a dual therapy of a PPI and an antibacterial agent would be sufficient for PM individuals, and triple therapy using a PPI and two antibacterial agents would be reserved for EM genotypes, thereby minimizing the

risk of bacterial resistance (20). For CYP2C9 and warfarin treatment, the risk of severe reactions due to side effects or too low therapeutic effect is substantial. Genotyping-based therapy would not only have the potential to reduce treatment cost and to facilitate faster adjustement of warfarin dosing, but could also be very important to improve quality of life and reduce the risk of long-term or infinite disability for the patients.

Commercial P450 genotyping and clinical implications

In 2004, the first pharmacogenetic DNA microarray assay, which determines several *CYP2D6* and *CYP2C19* alleles in order to predict phenotype of drug metabolism, was approved by the US Food and Drug Administration (FDA) (25). Together, CYP2D6 and CYP2C19 metabolize approximately 25-30% of all clinically prescribed drugs. Among them are several clinically important and commonly used drugs such as antidepressants and antipsychotics (25). The high frequency of *CYP2D6* and *CYP2C19* genetic variants (26, 27) together with the importance of the corresponding enzymes in the metabolism of drugs make them good target genes in pharmacogenetic testing for the aim of individualizing drug dosage:

- **CYP2D6** is non-inducible (27), whereby potential environmental factors modulating enzyme activity is diminished. CYP2D6 is well characterized regarding genetic variation. The level of CYP2D6 activity can as mentioned be resolved into 4 phenotype groups; UM, EM, IM and PM, and these phenotype groups can be predicted rather accurately by genotyping analyses.

- **CYP2C19** is genetically well characterized, but is inducible by for example rifampicin and phenobarbital that thus can account for a non-genetic basis of variation (28). The phenotype categories of EM, IM and PM can be relatively well separated. In addition, two defective alleles explain the majority of phenotype groups.

When aiming at determining genotype to predict phenotype, one need to keep in mind that genotype-phenotype predictions are probe-specific, i.e. they are based on a particular P450 substrate to monitor the enzyme activity. Since the specific enzyme activity can vary with the probe drug of choice, the outcome of a genotype-phenotype prediction is dependent on the probe drug used for the analyses. Thus, the outcome of the drug metabolism phenotype prediction may not apply for all drugs metabolized by the enzyme, and thus needs to be addressed for particular drugs. Also, known or unknown rare allelic variants are not/can not be genotyped, whereby a 100% accuracy in the prediction never can be achieved.

When addressing the relationship between drug package label (DPL) and pharmacogenetic data in the literature, Zineh *et al.* (29) found that among the 174 most used drugs only 22 (13%) had pharmacogenetic information in their DPLs and that this information was not related to the relevance of the pharmacogenetic data in the literature. Thus, there appears to be a gap between knowledge and clinical application. The US FDA (www.fda.gov/cder/genomics) states that pharmacogenetic information (genetic biomarkers) is present in about 10% of DPLs for drugs approved by the FDA. Only a few of these biomarkers have been ranked as "genetic test required" or "genetic test recommended", most of which are drug targets:

Required

- **Epidermal growth factor receptor** (EGFR) - high or low expression (genotype-based) is immunohistochemically determined for the treatment of colorectal cancer by the monoclonal antibody **cetuximab**, and based on the necessity of overexpressing the receptor to benefit from treatment (30).

- **HER2/neu receptor** – gene duplication is determined by genetic test for the treatment of breast cancer using the monoclonal antibody **trastuzumab/herceptin**, due to the necessity of overexpressing the receptor to benefit from treatment (31).

Recommended

- **Activated protein C deficiency** – the disorder is detected by genetic test for the treatment of thrombosis by **warfarin**, due to the increased risk of skin necrosis during treatment (32).

- **TPMT** – defective variant alleles are detected by genetic test for the treatment of for example organ transplantation and autoimmune diseases by **azathioprine**, due to the increased risk of myelosuppression during treatment (see also above) (4).

- **UDP-glucuronosyltransferase 1A1** (UGT1A1) – low-activity alleles are detected by genetic test for colon cancer treatment by **irinotecan**, due to the increased risk of neutropenia during treatment (33).

Current status of clinical genotyping tests in Sweden

In my work, I have received several enquires to genotype patients classified as non-responders in drug treatment. In addition, referral forms used in the Swedish clinics include preprinted options for genotyping the drug metabolizing enzymes CYP2C9, CYP2C19, CYP2D6, *N*-acetyltransferase-2 (NAT-2) and UGT1A1. These genotyping tests are generally used to explain side effects or lack of response, rather than prospective drug dosing. Methylenetetrahydrofolate reductase (MTHFR) is also included in the referral form, due to the function of this enzyme in the folate homeostasis and the association of low-activity alleles with a potentially increased risk of thrombosis, atherosclerosis, miscarriage and fetal defects (34), whereby increased intake of folate can be recommended to carriers of defective alleles.

REGULATION OF CYTOCHROME P450

There seems to be a complex network of receptors protecting the organism from toxic levels of potentially harmful compounds by inducing enzyme levels and thereby increasing the enzymatic metabolism. Receptors regulating *CYP2B*, *CYP2C* and *CYP3A* genes have been shown to share dimerization partner, ligands, DNA response elements and response genes in an overlapping manner (35), and the receptors involved are mainly the pregnane X receptor (PXR) and the constitutive androstane receptor (CAR), but also the vitamin D3 receptor and the glucocorticoid receptor have been implicated (36-41). Additional receptors involved in the regulation of drug-metabolizing P450s are the aryl hydrocarbon receptor (AhR) and the peroxisome proliferator activated receptors (PPARs), which are regulating CYP1 and CYP4 expression, respectively (42).

THE CYP3A SUBFAMILY

The CYP3A subfamily is composed of four members; CYP3A4, CYP3A5, CYP3A7 and the recently discovered CYP3A43 that was identified and characterized in the current study (paper I). The *CYP3A* gene cluster is located on chromosome 7 (Fig. 1). CYP3A is the most important subfamily in drug metabolism due to the wide range of different substrates and the high expression of CYP3A in the liver and intestine. This is manifested by the involvement of CYP3A in the metabolism of 40-60% of drugs (3, 12), and a protein expression level that accounts for 30% and 40% of total P450 in human liver and intestine, respectively (43, 44). There is a substantial interindividual variation in CYP3A activity both *in vivo* and *in vitro* (45, 46), and by measuring the variability in CYP3A metabolism between and within individuals it has been estimated that up to 90% of the variation could be explained by genetic factors (47). Hitherto,

however, any major genetic factors affecting CYP3A variability has not been identified. Thus, the first aim of this study is to investigate and evaluate genetic factors of potential importance for interindividual variation in CYP3A expression and activity.

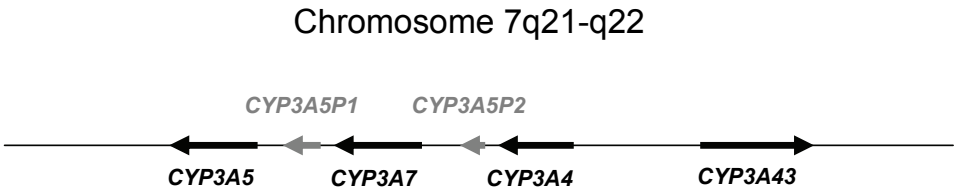

Figure 1. The *CYP3A* gene cluster spans approximately 220 kb and is located on chromosome 7q21-q22. The cluster encompasses *CYP3A5*, *CYP3A7* and *CYP3A4* that are located in tandem, and *CYP3A43* that lies in reverse order compared to the other *CYP3A* genes. Two pseudogenes are present within the cluster (indicated in grey). Reproduced from NCBI Entrez Gene (www.ncbi.nlm.nih.gov/entrez).

Several studies have tried to clarify the difference in substrate specificity, regioselectivity and activity between the different CYP3A isoenzymes. For example, CYP3A4 and CYP3A5 hydroxylate midazolam (MDZ) at the 4-position at a similar rate, whereas the 1-hydroxylation occurs at different rates (48). Furthermore, CYP3A7 seems to lack catalytic activity for the 1-hydrozylation of MDZ (49). In a comparative study of the catalytic activities of CYP3A4, CYP3A5 and CYP3A7 for ten different endogenous and exogenous substrates, CYP3A4 possessed on average a 3 to 4-fold lower K_m than CYP3A5 and CYP3A7 (50). Furthermore, there was an apparent difference in regioselectivity for the CYP3As for some of the substrates and a general rank order in clearance of substrates in the order of CYP3A4>CYP3A5>CYP3A7 (50). CYP3A7 is however considerably more effective than CYP3A4 in the 16α-hydroxylation of dehydroepiandrosterone (DHEA) (49, 51). On the contrary, CYP3A4 catalyses the conversion of testosterone to its 6β-hydroxylated derivative at a rate significantly higher than that of CYP3A7 (49, 51). The CYP3A isoforms are also differently susceptible to inhibition by drugs. For example, ketoconazole and fluconazole inhibits CYP3A4 more efficiently than CYP3A5 (52).

Cytochrome P450 3A4

CYP3A4 is considered to be the most important enzyme involved in the metabolism of drugs due to the involvement of CYP3A4 in the oxidation of a large range of substrates and due to the high amounts of CYP3A4 present in the liver (53). It has been shown that CYP3A4 mRNA accounts on average for 95% of all CYP3A transcripts in liver (54).

Currently, there are 19 different *CYP3A4* alleles leading to amino acid substitutions or frameshifts (www.cypalleles.ki.se). However, several of these alleles are rare and several of them have not been demonstrated to affect enzyme activity (55).

CYP3A4 is partially co-regulated with P-gp (56), which serves as an efflux transporter of drugs in the liver and in the intestine, thereby affecting drug bioavailability. The effect of P-gp on bioavailability is however more relevant at low drug concentrations due to saturation of P-gp at high concentrations (57, 58). There is an overlapping substrate specificity between CYP3A4 and P-gp, and together with similarities in P-gp and CYP3A4 inhibitors and inducers it may form a basis for drug interactions (59). It seems that the concerted action of inducers to upregulate both CYP3A4 and P-gp is a mechanism by which the body reduces the amount of high or even toxic levels of endogenous and exogenous substances by increased metabolism and by increased efflux, respectively.

Cytochrome P450 3A5

It has long been recognized that CYP3A5 protein is expressed at significant amounts in 10-30% of adult and fetal human livers (60-64). It was reported that this polymorphic CYP3A5 expression was associated with the occurrence of the *CYP3A5*1* allele and that this allele could lead to an increased total level of hepatic CYP3A protein (65). The *CYP3A5*3* allele, which genetically explains the majority of livers lacking CYP3A5 expression (65), carries an intronic single nucleotide polymorphism (SNP) that gives rise to a cryptic splice site and a mRNA with a premature stop codon.

Cytochrome P450 3A7

CYP3A7 has been considered a fetus-specific isoform that accounts for up to 50% of total P450 in fetal human livers (66). In adult livers, the *CYP3A7* mRNA level is on average only 2% of that of fetal livers (67). There is a shift in hepatic CYP3A7 expression to CYP3A4 expression after birth, as evident by a decrease in CYP3A7 activity (16α-hydroxylation of DHEA), an increase in

CYP3A4 activity (6β-hydroxylation of testosterone), and an increase in *CYP3A4* mRNA (49). However, a relatively common phenotype of increased *CYP3A7* mRNA levels in 11% of adult livers indicates the presence of significant CYP3A7 mRNA expression also in adult life (40). Two alleles with polymorphisms in the 5'-flanking region of the *CYP3A7* gene, *CYP3A7*1C* and *CYP3A7*1B*, were shown to explain 50 and 15% of livers with increased *CYP3A7* mRNA levels, respectively (40). The *CYP3A7*1C* allele is a product of a gene conversion event between the *CYP3A4* and *CYP3A7* genes that lead to the insertion of the proximal PXR/CAR response element of *CYP3A4* into the proximal promoter of *CYP3A7* (65).

CYTOCHROME P450 2C19

CYP2C19 is a member of the CYP2C subfamily of P450 enzymes that also includes CYP2C8, CYP2C9, and CYP2C18 (Fig. 2).

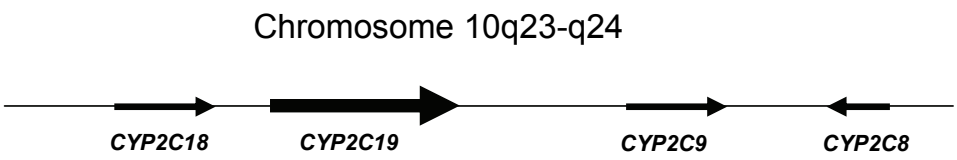

Figure 2. The *CYP2C* gene cluster spans approximately 500 kb and is located on chromosome 10q23-q24. *CYP2C19* is indicated by the thicker arrow. *CYP2C18*, *CYP2C19* and *CYP2C9* are located in tandem, with the *CYP2C8* gene in reverse order compared to the other *CYP2C* genes.
Reproduced from NCBI Entrez Gene (www.ncbi.nlm.nih.gov/entrez).

Classical CYP2C19 substrates and commonly used probe drugs are omeprazole and mephenytoin (68). The PM phenotype of CYP2C19 activity occurs at a frequency of 3-5% in Caucasians and Africans, and 13-23% in Asians, and is the result of two defective *CYP2C19* gene variants (69-72). The defective *CYP2C19*2* and *CYP2C19*3* alleles account for most of the PM phenotypes (73, 74). Consequently, in Japanese and Ethiopian individuals, the *CYP2C19*2* allele together with the less frequent *CYP2C19*3* allele explains 100% of PMs (72, 74). In Caucasians however, the *CYP2C19*2* allele accounts for 75-93% of PM alleles, whereas the *CYP2C19*3* allele is absent (73-76). There is a gene dose effect of defective alleles on CYP2C19 catalytic activity such that subjects carrying none, one or two defective *CYP2C19* alleles can be separated into EMs, IMs and PMs, respectively (77).

Together, CYP2C9 and CYP2C19 are estimated to account for 10% of drug metabolism (6). For CYP2C19 substrate drugs, they can be divided into categories based on the extent of CYP2C19 metabolism; drugs where CYP2C19 contributes to a) >80% in the clearance of the drug, examples of which are omeprazole and mephenytoin, b) 30-65% in the clearance, for example imipramine and clomipramine, and c) <30% in the clearance, such as phenytoin (20).

Omeprazole and similar drugs belong to the proton pump inhibitor class of drugs and are used in the treatment of gastrointestinal acid-related disorders. *H. pylori* infection is associated with upper gastrointestinal disorders such as gastritis or peptic ulcers (78). *H. pylori*-positive gastrointestinal disorders are treated by a PPI and antibacterial agents. Since as mentioned, the main metabolism of PPIs is carried out by CYP2C19, a higher cure rate of *H. pylori*-positive acid-related disorders is seen in CYP2C19 PMs compared to EMs, due to a higher PPI plasma concentration in PMs (13). Other important CYP2C19 substrates are within the class of antidepressants and the *CYP2C19* genotype is shown to affect drug levels of for example trimipramine, imipramine and citalopram (10). Kirchheiner *et al.* (10, 79) has estimated that several antidepressants would benefit from a *CYP2C19* genotype-adjusted drug dosage of on average 110% in EMs and 60% in PMs.

There is interindividual variation in CYP2C19 activity that can not be explained by the occurrence of defective *CYP2C19* alleles. The second aim of this study is thus to investigate genetic variation in the *CYP2C19* gene in relation to CYP2C19 catalytic activity.

THE PRESENT STUDY

AIMS

The present studies were undertaken in order to investigate the contribution of genetic components to interindividual variation in CYP3A and CYP2C19 expression and activity. For CYP3A, the investigation was exerted at the level of individual subfamily members' contribution to an activity collectively called CYP3A. For CYP2C19, the study aimed at investigating the genetic component of variability within one single gene. Specifically, the following projects were the focus of my thesis work:

1. Cloning and characterization of a novel human CYP3A isoform

2. Comparative analysis of CYP3A expression in adult human liver, with special emphasis on the contribution of polymorphic CYP3A5 to total CYP3A levels and the *CYP3A5* genotype as a determinant for CYP3A5 expression

3. Analysis of the contribution of the fetus-specific isoform CYP3A7 to CYP3A levels in adult human livers and the association of CYP3A7 protein expression with the *CYP3A7* genotype

4. Identification and functional characterization of a polymorphism in the 5'-flanking region of the *CYP2C19* gene, and the investigation of its effect on CYP2C19 activity *in vivo*

COMMENTS ON METHODOLOGY

Bioinformatics

Nucleotide sequences were obtained from The National Center for Biotechnology Information (NCBI, www.ncbi.nlm.nih.gov). The algorithm of NCBI's nucleotide-nucleotide BLAST program was used for homology searches. DNA sequencing results were compared with wildtype sequences using an alignment program, e.g. MultAlin (http://bioinfo.genopole-toulouse.prd.fr/multalin/multalin.html) or NCBI BLAST to display mismatches between sequences. Mismatches in sequences were investigated regarding their effect on the amino acid sequence using protein translation programs such as that of BCM Search Launcher (http://searchlauncher.bcm.tmc.edu/seq-util/seq-util.html). Translated sequences were then aligned with the wildtype protein sequence obtained from NCBI by using the protein alignment algorithm of the BCM Search Launcher. Transcription factor binding sites were predicted using the Transcription Element Search Software (TESS, www.cbil.upenn.edu/cgi-bin/tess/tess). Primer specificity was examined using NCBI BLAST.

EST-based cloning and RACE

The NCBI database of EST (expressed sequence tags) sequences contains sequenced parts of the nucleotide content in cDNA clones and thus can serve as a tool for identifying gene transcripts. The ESTs can be used for various purposes, i.e. chromosomal mapping or, as is the case of this thesis, for identification of novel (expressed) genes of a known gene family.

Rapid Amplification of cDNA Ends (RACE) is a method to isolate unknown sequences of a cDNA. RACE is aided by the access to EST sequences due to the necessity of knowing parts of the sequence of the cDNA to be cloned in order to design gene-specific primers. This method was employed to isolate the novel *CYP3A43* cDNA (paper I).

In vitro transfection

The forced introduction of DNA into cells is termed transfection. Transfection methods can be employed for a variety of purposes. There are several ways to transfect cells in culture of which DEAE-Dextran and lipofection has been used for the purposes of this thesis. **DEAE-Dextran** is a polysaccharide that facilitates the binding of DNA to cell membranes and the entry of the DNA into the cell via endocytosis. Today, this method would be considered quite old-fashioned and it

has been basically replaced by the more efficient method of lipofection. **Lipofection** uses liposomes as a mean to pack and deliver DNA to the cell by fusing with the outer membrane. Cationic liposomes, which are the mostly used type of liposomes today, are highly efficient in transfecting DNA due to the ability of the positive charge of the lipids to bind to the negatively charged DNA and to interact with the negative charge of the cell membrane. DNA can also be introduced into cells using viruses as a carrier, i.e. **viral transduction**. There are several commercially available cDNA-expressed P450s prepared this way. BD Biosciences offers microsomes from 20 different P450 enzymes expressed by baculovirus-infected insect cells. The microsomes also contain cDNA-expressed human P450 reductase and human cytochrome b5, and have been widely used to characterize P450 enzymes.

Cell lines and cell culture

Cell lines are easy to work with, relatively cheap, do not lead to the sacrifice of animal lives and is a time efficient way to produce results. However, they can not be considered to reflect the *in vivo* situation since the use of cell cultures is an artificial method to study living cells. Primary cell cultures, i.e. cells directly derived from tissue of a living organism, generally resemble the *in vivo* situation more than cell lines which have been immortalized by one way or the other, e.g. by induction of oncogenes or loss of tumor suppressor genes. Immortalization of cells generally lead to a change in the gene expression pattern, with an increased or decreased expression of some genes, a gain of expression of previously non-expressed genes, or the loss of expression of others. In a gene expression phenotype comparison of adult liver, B16A2 and HepG2 cells, Butura *et al.* (80) found that even under the best conditions, i.e. B16A2 cells let to differentiate for 5 weeks, gene expression reached less than 10% of the expression levels of that of human liver. Enzyme induction can help to retain the expression of certain genes (81, 82).

In the current study, several different cell lines have been used. The SV40-transformed African green monkey kidney cell line COS-1, the adenovirus 5-transformed human embryonic kidney cell line 293 (HEK293), the human hepatoma cell lines B16A2 and HepG2, the human cervical carcinoma cell line HeLa, and the SV40-transformed mouse hepatocytes H2.35. The background to the large number of cell lines used in this thesis is the above mentioned problem of a change in phenotype of cells in culture. Thus, transcription factors or other crucial factors needed for the expression of certain genes may be present in one cell line but not the other. For *CYP2C19* 5'-flank constructs where the aim is to assess the transcription regulated by a human promoter, the expression of the relevant transcription factors is of course relevant. For the expression of P450 enzymes from expression vectors, the presence of transcription factors is not as critical since the expression is triggered by a viral factor expressed by the immortalized cell line. From our experience, COS-1 and HEK293 were very efficient in expressing high

amounts of P450 enzyme from cDNA expression vectors. Despite originating from "non-classical" P450 expression tissues, they serve the purpose of producing high amounts of the desired protein. B16A2 cells are let to differentiate for 4 weeks at confluency in order to make them more hepatocyte-like and more prone to P450 expression. Still, B16A2 cells responded little to reporter constructs containing the *CYP2C19* 5'-regulatory region and could not show any difference between wildtype and mutated constructs as opposed to an *in vivo* system (see below). Thus, commonly used cell lines and cells in culture are far from the *in vivo* situation and data should also be interpreted as such and with caution.

In vivo transfection

As a part of this thesis, mice were transfected with reporter constructs containing the *CYP2C19* 5'-flanking region in its wildtype of mutated form. This *in vivo* transfection system was tested due to the improper phenotype of several cell lines that had been transfected with these constructs, giving a low expression in general and no difference between the constructs. The method used, TransIT-*In Vivo* Gene Delivery System (Mirus), is based on a polymer that complexes with DNA and together with a delivery solution is introduced to the mice through tail vein injections. The major organ where DNA is taken up is the liver, but some amounts are also transfected into spleen, lung, heart and kidney (83). The transfection is mediated by the polymer and by the **hydrodynamic effect** due to the comparatively large volume injected, which amounts to 1/10 of the mice's weight (i.e. 2.5 ml to a 25 g mouse). The transfection efficacy is highly dependent on the time taken to inject the volume; the lower the speed, the lesser the hydrodynamic effect and thus the degree of transfection. Typically, the solution should be injected in approximately 5 seconds. Due to the difficulty to insert the injection needle into the tail vein, the speed of injection may vary slightly between different mice. Thus, a mean of normalizing the transfection is crucial to be able to compare different mice. In this thesis, the firefly reporter enzyme was used as the construct reporter enzyme and the *Renilla* luciferase internal control vector was used for normalization.

For the experiments of this thesis, the tail veins of CD1 male mice were dilated by a heat lamp and the transfection solution injected in 3-5 seconds. The mice were sacrificed by carbon dioxide the following day, and the livers were dissected. A liver homogenate was prepared for every mouse and the luminescent signal of firefly and *Renilla* luciferase enzyme activities (Dual-Luciferase Reporter Assay, Promega) were measured for each sample (Fig. 3). Data from separate experiments were combined by normalizing individual measurements of each experiment with the mean ratio of the wildtype construct group of that experiment. This was done because of a shift among all samples within one experiment compared with the other, whereas the standard deviations were similar.

In order for an animal model to be successfully used for human studies, there should not be substantial species differences in transcriptional regulation or other relevant pathways. This requires sequence homologies and conserved functions among the nuclear factors involved. In the current study, the mice required a hepatic phenotype similar to that of the human liver with respect to the regulation of the transcription of human *CYP2C19*. Since in paper IV, CYP2C19 expression was regulated *in vivo* in mice in a similar manner as *in vivo* in humans (i.e. increased transcription/activity of *CYP2C19*17* compared to *CYP2C19*1*) the mice model was suitable for the purpose of the study and indicate that there are conserved transcription factors between man and mouse.

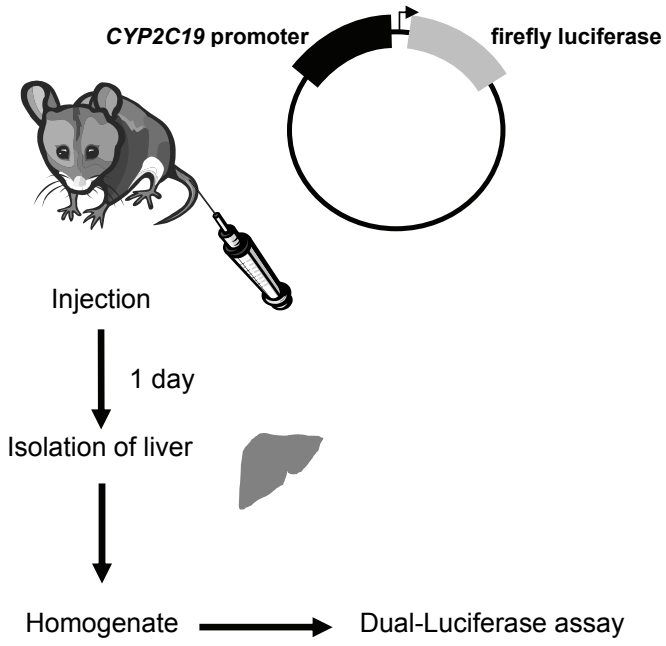

Figure 3. *In vivo* transfection protocol using Mirus TransIT®-*In Vivo* Gene Delivery System based on the hydrodynamic transfection of foreign DNA. Mice are injected via the tail vein with a polymer-DNA mix. Mice are sacrificed the following day and reporter activities are measured on liver homogenates.

Reporter gene assays

Reporter gene vectors are used for the purpose of monitoring levels of gene expression, for example expression over time, relative levels of expression or expression pattern. The most common example of reporter constructs, which has also been used in this thesis, are regions of the 5'-flank of the gene-of-interest cloned into the vector upstream of the reporter gene such that the gene-specific promoter and other regulatory elements will control the expression of the reporter gene. This set-up can be used for different types of experiments; promoter mutations can be characterized and regulatory elements may be mapped by deletion constructs.

Human liver samples

The access to human samples is a valuable source of information and an important tool to support data received from experimental samples. In addition, findings in human samples often trigger other experimental approaches to confirm or dissect molecular mechanisms of initial results. A tissue bank is also a valuable tool to look at several levels of regulation; genotype, transcription, translation, posttranscriptional and posttranslational modifications, and enzyme activity and is thus an important method to investigate genotype-phenotype correlations.

In this thesis, a bank of 46 human liver samples has been characterized at the level of DNA, mRNA, protein and catalytic activity (paper I and II). The samples had been collected from 1) Sahlgrenska Hospital (Gothenburg, Sweden) originating from patients undergoing liver resection, often due to malignant tumors, 2) Huddinge Hospital (Stockholm, Sweden) from organ donors dying in accidents, and 3) commercial samples from the International Institute for the Advancement of Medicine (IIAM; Exton, PA). As for the liver samples collected during excision of tumors, the samples collected were taken from regions without pathological alterations and are thus assumingly normal. The livers selected from IIAM were either high or low in CYP3A activity measured as the 6β-hydroxylation of testosterone for the purpose of CYP3A studies. All samples in the liver bank were reported to be of Caucasian origin.

Another liver bank of 59 samples, which in this study was characterized regarding *CYP3A7* genotype and CYP3A7 protein content (paper III), was derived from histologically normal human liver samples from transplant donors and was collected from Hammersmith Hospital, London, UK. Some samples were also obtained from Tissue Transformation Technologies, Inc. (Edison, New Jersey, USA).

One should keep in mind that despite an extensive record on some of these samples regarding drug intake etc, lack of such information does not necessarily mean that one can exclude the

possibility of drug intake and thus induction or inhibition of P450 activity. P450 levels and activity can also be affected by other factors such as those being of environmental or dietary origin, disease states etc. In addition, taking pieces from a seemingly normal area of a liver containing tumors does not mean that that part of the tissue is completely unaffected; local inflammation in the tumor can affect surrounding tissue or pathological signal molecules may have a low-grade effect on adjacent cells. Thus, parameters measured must always be interpreted with the risk of confounder effects kept in mind.

The quality of the liver bank can be considered good. It has been used for the study of several different P450s and has been studied at many levels. No mRNA degradation can be detected, and protein degradation has not been seen in any Western blotting experiment. Furthermore, CO-difference spectral analysis of the cytochrome P450 content has revealed no significant cytochrome P420, indicating only the presence of properly folded P450 enzyme. Data derived from the liver bank has also given good correlations between different parameters and successful phenotype-genotype correlations (paper II and III, (84, 85)), indicating a trustworthy and high-quality set of human liver samples.

Western blotting, protein standards and quenching effects

Quantitative Western blotting was conducted in two of the studies presented in this thesis (paper II and III). Compared to a qualitative way of measuring protein expression, i.e. protein is detected or not detected, quantitative Western blotting to determine exact amounts of proteins is more prone to errors and confounding factors.

The protein standard needs to be correctly determined regarding its content of the specific protein to be quantified. For P450 enzymes, the holoprotein levels of a sample can be quantified using a reduced CO difference spectrum detecting correctly folded P450 that has incorporated heme. However, for Western blotting purposes, this can lead to a great underestimation of the total immunoreactive amount of protein that is composed of the holo- and apoprotein levels. This is particularly relevant for cDNA expressed preparations where the relative amount of apoprotein could be expected to be high. This problem was circumvented in two separate ways in the two studies:

- In paper II concerning quantification of CYP3A4 and CYP3A5 in human liver microsomes using CYP3A4- and CYP3A5-specific antibodies (86), the respective standards to be used were of human liver origin and were quantified against the peptides that was initially used for immunization. This was achieved by determining the average number of peptides per carrier protein by separation of the conjugates on a gel followed by

Coomassie blue staining and measurement of the relative intensity of bands. The antiserum was then depleted from antibodies that recognized the group that links the peptide to the carrier. The antiserum did not need to be depleted from antibodies binding to the carrier protein since different carriers were used for the immunization of rabbits (KLH) and for the purpose of protein quantification (lysozyme). The depleted antiserum was used together with the peptide conjugates to determine the amount of CYP3A4 and CYP3A5 in samples of human liver microsomes by Western blotting. Thus, an exact quantification could be done using the same epitope present on the carrier protein and in the human liver samples, and these liver samples were then used as standards to quantify remaining samples in the liver bank.

- In paper III, where CYP3A7 levels was determined, a cDNA expressed CYP3A7 preparation that had been quantified by the manufacturer regarding its holoprotein content was used as a standard (BD Biosciences). The CYP3A7 preparation was quantified for the amount of immunodetectable CYP3A7 protein (holo- and apoprotein) using an antibody that reacts equally with the identical C-terminus of CYP3A4 and CYP3A7 (87) and a human liver microsomal sample from paper II with known CYP3A4 levels but undetectable CYP3A7 levels. In this way, it was found that the standard contained 4.4 times higher total CYP3A7 amounts as compared to the spectrally determined holoprotein levels. For subsequent CYP3A7 quantification of the liver bank, a CYP3A7-specific antibody was used.

As discovered in paper II, the amount of total protein loaded turned out to have an effect on the signal obtained by incubating with antibody:

- With increasing amounts of total protein loaded, the signal was increasingly suppressed, probably due to the high density of proteins around the specific protein. Thus, with high amounts of total protein, the lane will be packed with proteins that will not be separated enough, thereby quenching each other. This effect was investigated by loading cDNA expressed CYP3A5 together with different amounts of liver microsomes with undetectable CYP3A5 as determined by antibody screening. When higher amounts than 20 µg of total protein were used, the linearity was impaired (Fig. 4). Thus, no more than 20 µg of total protein of any standard or sample was used in any Western blot analysis within this thesis and the linearity was thus ensured. In paper II, 1-10 µg of the liver microsomal standards were loaded to produce standard curves of CYP3A4 and CYP3A5 (corresponding to approximately 0.1-1.5 pmol), and 10 µg was loaded for the samples to be quantified.

- In paper III concerning CYP3A7 levels, the handling of the quenching effect was improved by adding a control preparation for cDNA expressed CYPs (BD Gentest) lacking immunoreactivity with the antibody to make up for the total amount of protein loaded. Thus, when quantifying the CYP3A7 preparation, both the preparation and the liver sample to be used as a standard were loaded at a total amount of 10 µg. In the same way and for further analyses of the liver bank using the quantified CYP3A7 standard, the control preparation was used to make up for the total protein amount to 15 µg for all samples. One expects only very minor differences in quenching at these small amounts loaded, as had already been investigated in paper II, but controlling for protein amounts also at this range further ensures an accurately determined quantification.

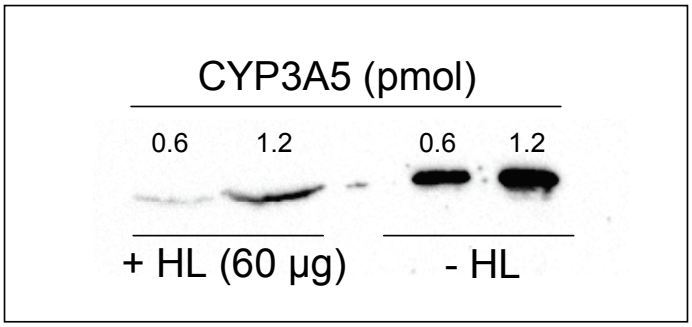

Figure 4. The effect of quenching by total protein amounts on the signal of specific protein by Western blotting. Two different amounts of cDNA expressed CYP3A5 (0.6 and 1.2 pmol, corresponding to 2.5 and 5 µg of total protein) was loaded with or without 60 µg of a human liver sample (HL) with non-detectable CYP3A5 levels.

Semi-quantitative PCR

In the literature, semi-quantitative PCR has often been performed by running a fixed number of PCR cycles after which the products are analyzed on a gel and their intensities compared. One major problem with this approach is that the PCR reactions may have reached plateau levels and that the comparison does not reflect amplification in the exponential phase. In addition, the relationship between band intensity (as measured by densitometry) and DNA amounts may not be linear and thus needs to be investigated. In paper I, an improved semi-quantitative PCR was performed to address the mRNA expression of CYP3A4 and CYP3A43 in different tissues. This was achieved by examining at which PCR cycle the product could be detected by running consecutive cycles of two and to analyze the reactions on gel. Since in every cycle a duplication of template occurs, the difference between one cycle and the next results in a large difference in product formed and the assay is thus quite insensitive. Also, the detection of expression was

divided into categories of 5 cycles due to a slight variation between the same sample and different runs, whereby a 32-fold difference in expression can be reached between one category and the other. Today, semi-quantitative PCR has been more or less replaced by the modern real-time techniques.

Real-time PCR

In general, real-time PCR measures the syntheis of product with time and is thus significantly more reliable and sensitive for comparing gene expression and for absolute quantification than semi-quantitative PCR. Real-time PCR measures the product as it is formed and at any given time point. Therefore, it can be decided exactly when a given PCR product reaches a threshold value (Ct) even if it is in the middle of a cycle. The detection can be achieved by fluorescent dyes that intercalate with double-stranded DNA or by the digestion of a probe due to elongation of a template by use of a primer (TaqMan) that releases a fluorescent dye from a quencher. By using DNA standards, quantification can be achieved by the given cycle at which the sample DNA reaches the threshold.

CYP2C19 *in vivo* phenotyping with omeprazole and mephenytoin

In paper IV, healthy volunteers were phenotyped using CYP2C19 probe drugs, i.e. drugs whose specific metabolism routes are reflecting CYP2C19 activity. This was achieved by the probe drugs mephenytoin, an anticonvulsant that is no longer being used in the clinics, and omeprazole, a proton pump inhibitor. Both drugs were given at dosages normally prescribed in the clinics but as single doses, thus avoiding possible adverse drug reactions. *In vivo* phenotyping is invaluable in pharmacogenetic studies since they can facilitate the identification of functional polymorphisms.

- 5-hydroxyomeprazole is the major metabolite formed by **omeprazole** metabolism and this hydroxylation is catalyzed by CYP2C19 (88). The second most common metabolite is the omeprazole sulphone, the formation of which is catalyzed by CYP3A4 (88, 89). 5-hydroxyomeprazole can be further metabolized to carboxyomeprazole and 5-hydroxyomeprazole sulphone, the latter being produced by CYP3A4 (88).

  CYP2C19 hydroxylates omeprazole in a stereoselective manner, with *R*-omeprazole being favored over *S*-omeprazole (89, 90). Furthermore, *R*-omeprazole is specifically hydroxylated by CYP2C19 whereas *S*-omeprazole is being hydroxylated by other P450s in addition to CYP2C19 (89, 90). The 5-hydroxylation of *R*-omeprazole constitutes the major route of elimination in human liver microsomes, whereas

S-omeprazole is similarly eliminated by three routes of which one leads to the formation of 5-hydroxyomeprazole (89). *R*-omeprazole is thus a more specific CYP2C19 probe drug than the racemate. However, *R*-omeprazole is not available as a drug and racemic omeprazole was thus used for phenotyping purposes in the current study.

The metabolic ratio (MR) of omeprazole is the plasma concentration ratio of the 5-hydroxy metabolite over the omeprazole substrate at a single time point and is thus inversely reflecting the CYP2C19 activity; a low ratio reflects a high CYP2C19-catalyzed metabolism and vice versa.

The omeprazole *in vivo* data used in this study was produced by giving healthy Swedish volunteers a single oral dose of 20 mg racemic omeprazole (Losec; AstraZeneca) and subsequently analyzing substrate and metabolite concentrations in plasma by high performance liquid chromatography (76). For calculation of the metabolic ratio, blood samples were drawn at 3 hours after drug intake, at which both the substrate and the metabolite concentrations can be determined in all individuals regardless of phenotype (76). AUC was calculated by blood samples taken at 0, 1, 2, 3, 3.5, 6, and 8 hours after drug intake (91).

Omeprazole gives a good separation between phenotypes and in this study a MR of higher than 5 was considered as the definition of a PM phenotype (76).

- The racemic drug **mephenytoin** has been the most frequently used CYP2C19 phenotypic marker in the past. The *S*-enantiomer is selectively 4-hydroxylated by CYP2C19 and the *S*/*R* ratio of mephenytoin can thus be used for monitoring CYP2C19 activity and to classify individuals as EMs or PMs (92).

  For the phenotype data used within this study, Ethiopians had been given 100 mg racemic mephenytoin orally and had collected urine for the following 8 hours, after which the total volume was measured and an aliquot determined with regard to its *S*- and *R*-mephenytoin content using stereoselective gas chromatography (72). Individuals with an *S*/*R*-mephenytoin ratio lower than the detection limit of 0.03 were set to 0.03 in the data analyses.

  Since the cysteine conjugate of *S*-mephenytoin, which is produced in extensive metabolizers, can be hydrolyzed back to *S*-mephenytoin during long-time storage of the urine, EMs encounter the risk of being classified as PMs if samples are not quantified early on (93). This problem can however be solved by acid treatment, whereby true PMs get the same ratio of *S*/*R*-mephenytoin as before acid treatment and false PMs get significantly increased ratios and can thus be truly identified as EMs (94). For the mephenytoin data within this thesis, all samples with an *S*/*R* ratio higher than 0.6 were reanalyzed after acid hydrolysis and all samples with a ratio of >0.9 that did not increase significantly after acid hydrolysis were defined as PMs (72). Even though samples with a high *S*/*R* ratio can be classified as EMs or PMs by means of acid treatment, one should

keep in mind that the pre-acid ratio, which is increased several folds in EMs after acid treatment, could have been increased by hydrolysis in the stored urine sample. Thus, when investigating parameters other than phenotype class, sample storage can lead to a confounding factor because the *S*/*R* value could be increased at the time of analysis compared to the time point when the samples were collected. Thus, for phenotype-genotype associations among EM individuals, as has been studied in this thesis, one should keep in mind that the *S*/*R* ratio in EMs may have been increased due to partial hydrolysis and was originally lower.

Due to sample instability and the necessity to collect large volumes of urine, probing CYP2C19 activity with mephenytoin is no longer common. In addition, mephenytoin generally gives a slightly lesser separation of phenotypes than does omeprazole (76), partially due to the inability to separate extensive metabolizer phenotypes with S/R ratios lower than the detection limit of 0.03.

Genotyping methods

There are many ways to determine sequence variations in DNA. Genotyping methods that have been used within this thesis are given below.

- **DNA sequencing** is the method of choice when searching for potentially new nucleotide variations but is not an efficient method to do routine or extensive genotyping analyses of single SNPs. Sequencing technologies has however developed extremely fast and are generally reliable and relatively cheap methods for addressing locus heterogeneity. DNA sequencing was used in all papers within this thesis.
- In **allele-specific PCR**, a first gene-specific fragment is amplified after which allele-specific primers are used to detect the mutation. Due to the necessity of two sequential PCR reactions, the risk of contamination is increased, i.e. amplification of unspecific bands or bands equivalent with the other genotype. In addition, allele-specific PCR can be considered laborious and time-consuming. Allele-specific PCR was used in paper II and IV. In paper IV, one set of the DNA samples was genotyped by allele-specific PCR and one set by Pyrosequencing. During the project, we were contacted by Biotage AB, a company interested in a collaboration where we would do genotyping analyses using their Pyrosequencing equipment. We thus changed to Pyrosequencing genotyping and found it to be a more robust and time efficient method (see below).
- In paper III and IV, **restriction fragment length polymorphism** (RFLP) analysis was proven as a robust and reliable genotyping method. A gene-specific fragment needs to be amplified, but instead of a second PCR reaction one uses the difference in restriction enzyme recognition sites between one allele and the other to detect the genotype. There

is a smaller risk for contamination than in allele-specific PCR, but the nucleotide variation needs to introduce or abolish a restriction site. Inhibition of the restriction enzyme could lead to inefficient digestion and thus false results. As for allele-specific PCR, RFLP analysis is laborious and time-consuming.

- **Pyrosequencing** is a form of minisequencing or sequencing by synthesis, where a primer close to the nucleotide variation is used for amplification using a gene-specific fragment generated by a first PCR reaction as a template. By adding one nucleotide at the time and by detecting the incorporation of the nucleotides by a chemiluminescent enzymatic reaction, the exact sequence can be determined. Pyrosequencing is an efficient and reliable method that can be automated and it was used in paper IV.

By comparing the distribution of genotype categories (wt/wt, wt/mut and mut/mut) with the expected relative distribution of the same categories one can address if the genotyped population of samples are in Hardy-Weinberg equilibrium. If not, assay problems in the genotyping analyses and/or in the random selection of the samples could be expected. If the Hardy-Weinberg equilibrium is fulfilled, the equation $p^2+2pq+q^2=1$ should be achieved, where p is the frequency of the common allele and q is the frequency of the rare allele.

Electrophoretic mobility shift assay

In paper IV, the electrophoretic mobility shift assay (EMSA) was used to examine a differential binding of hepatic nuclear proteins to polymorphic sites in the 5'-flank of the *CYP2C19*17* allele as compared to the *CYP2C19*1* allele. There are two polymorphic sites of the *CYP2C19*17* allele, and both were investigated regarding the binding of transcription factors to potential binding elements at these positions. Probes equivalent to both regions of both *CYP2C19*1* and *CYP2C19*17* DNA was labeled with [γ-^{32}P]-ATP. The radiolabeled probes where then incubated with hepatic nuclear proteins and run on a gel, after which the binding of nuclear proteins to the probe was analyzed as the retardation of probe mobility. The binding of proteins to probes is very sensitive to conditional changes and there are many parameters to control and to test for; amount of nuclear protein, salt concentration, type of salt, incubation time, incubation temperature etc. In the current study, the salt concentration and the type of salt turned out to be major determinants for the ability of nuclear proteins to bind to the probes. A slight change in one of these parameters may results in the appearance or loss of a specific band and once the conditions have been set, one should minimize the variations between one experiment and the other.

In this study, binding of nuclear proteins to a polymorphic element of the *CYP2C19*17* allele but not to the corresponding element of the *CYP2C19*1* allele was detected. In order to determine

the specificity of the binding to the *CYP2C19*17* allele, competition experiments were carried out in which unlabeled probes at different concentrations were used to compete with the labeled probe for protein binding.

Supershift analyses using antibodies targeted at specific transcription factors can assist in identifying the proteins being bound by inducing a further shift in probe retardation when the antibody binds its target transcription factor. In this study, three different antibodies were tested using different amounts of antibody, different incubation temperatures and by adding the antibody at different stages of the incubation procedures.

Statistical analyses

The 2-tailed **Mann-Whitney** test for non-parametric continuous data was used for assessing differences in age, phenotype markers and reporter construct activities. This test was chosen due to the apparent non-normal distribution of the data analyzed. Even if data could qualify as normally distributed, the Mann-Whitney test is much less likely, than for example the parametric *t* test, to give a false significant result because of outliers. The 2-tailed **Fisher's exact** test for categorical data was used for detecting sex differences and differences in allele frequencies. This test analyzes variables in a contingency table. By using **2-tailed** rather that 1-tailed statistical tests, one leaves the option to test for both directions of a difference rather than expecting one of them. A difference in phenotype parameters were analyzed by the **Kruskal-Wallis** non-parametric test for comparison of more than two groups and is an equivalent of the above mentioned Mann-Whitney test for two groups. Correlations between mRNA, protein and catalytic activity were measured by the **Spearman rank** correlation coefficient that measures the strength of the associations between variables. **Hardy-Weinberg** equilibrium was assessed for allele frequencies. A p-value of <0.05 was considered statistically significant in all analyses. The p-value can be interpreted as the probability of being wrong when concluding that there is statistical significance.

RESULTS AND DISCUSSION

Paper I - Cloning and tissue distribution of a novel human cytochrome P450 of the CYP3A subfamily, CYP3A43

It was hypothesized that novel CYP3A enzymes could contribute to activities attributed to CYP3A4 and/or CYP3A5 in adult human livers and thus be an explanation for interindividual differences in CYP3A expression and metabolism. In this study, a novel CYP3A isoform was identified, its expression level and tissue distribution determined and preparations after heterologous expression investigated.

Identification of the novel CYP3A43

By homology searches of CYP3A-related sequences in the NCBI dbEST database we identified a new CYP3A isoform, denoted CYP3A43. CYP3A43 shares 72-76% identity in amino acid sequence with the other CYP3A isoforms. In comparison, CYP3A5 and CYP3A7 show 84 and 88% identity with CYP3A4, respectively, thus emphasizing the apparent lower homology of CYP3A43 with the other CYP3A subfamily members. The basis for this could be a lower evolutionary pressure on the gene, thus giving rise to a higher mutation rate, or the evolution of a particular enzyme phenotype. The former theory would suggest a lack of function and/or importance of the *CYP3A43* gene, whereas the latter would implicate a different function and/or substrate specificity for the CYP3A43 enzyme. CYP3A43 carries amino acid substitutions in regions highly conserved in the other CYP3A enzymes, many of which are important for substrate recognition and thus could possibly alter substrate specificity compared to the other CYP3As. Many amino acid substitutions are however also found in regions not having been allocated a specific function and might instead affect protein folding.

CYP3A43 expression is insignificant in adult human liver

In order to investigate the hepatic expression level of the novel CYP3A43, we analyzed 11 human liver cDNA samples by quantitative real-time PCR and showed that CYP3A43 expression was much lower than both CYP3A4 and CYP3A5; 0.1 and 2% of the corresponding levels, respectively. To address the tissue specificity of CYP3A43 expression in relation to CYP3A4, gene-specific PCR was performed on a tissue cDNA panel of normal (n=17) and malignant (n=5) tissues that had been normalized to several different house-keeping genes by the manufacturer (Clontech). A cDNA produced from liver mRNA was also included. The results have been presented both as the band intensity observed at 40 cycles (Fig. 3 in paper I) and as a semiquantitative monitoring of the PCR product after every 2 cycles (Table 1 in paper I). It could

be concluded that the highest level of CYP3A43 is present in the liver, followed by that of the testis. Low or absent expression was detected in the remaining 20 tissue samples, examples of which are lung, kidney and brain. Relative comparison of levels can not be done at 40 cycles since plateau levels may have been reached for certain reactions. Thus, Figure 3 in paper I only provides with an overview of the expression pattern. However, a lack of detection of PCR product at 40 cycles is a strong indication of the absence of transcript in that particular tissue. Table 1 in paper I, describing the approximate cycle when a visible band is showing up on gel, provides with a somewhat better comparison between tissues and between isoforms. It should however be emphasized that the purpose of this part of the study was to screen different tissues for making a qualitative analysis.

Expression of recombinant CYP3A43 in mammalian cells yields no detectable protein

In order to analyze any CYP3A43 enzyme activity, the CYP3A43 and CYP3A4 cDNAs were heterologously expressed in different cell systems. In contrast to CYP3A4, no detectable CYP3A43 protein was produced as measured by Western blot analysis using four different antibodies in COS-1, H2.35 and HEK293 cells.

Since a quenching effect of total protein amounts on immunodetectable bands can be seen when loading >20 µg of total human liver protein (see paper II), the amounts of 30-40 µg that was used for Western blot analysis in paper I could be expected to exert a quenching effect on the immunosignal. However, since CYP3A4 expression was readily detected (Fig. 5), the total protein amounts loaded can not be considered a problem for this study, where the aim was not a quantitative but a qualitative analysis. In addition, quenching appears only to be a problem in Western blotting analysis using human liver microsomes but not when using transfected cell lines, probably due to a difference in the protein expression pattern. In fact, as apparent from staining gels with Coomassie blue or membranes with Ponceau S, human liver express many proteins with a molecular weight around 50 kD, which is the approximate molecular weight of P450s.

In total, four different antibodies were tested. The H2.35 cell line showed only low expression of the positive control transfected with CYP3A4 cDNA using one antibody (see Fig. 5) and this cell line was therefore not tested further due to its apparently lower efficiency for heterologous expression compared to the other cell lines. Three antibodies were directed to purified enzymes (human CYP3A4, rat CYP3A1 and rat CYP3A2) and were all binding to CYP3A4, CYP3A5 and CYP3A7, thus making cross reactivity with CYP3A43 very likely. Still, no CYP3A43 protein could be detected as evident from Figure 5.

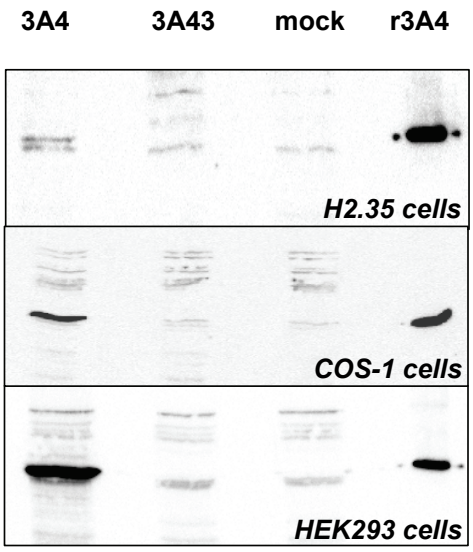

Figure 5. Lysate equivalent to 30-40 µg of protein from H2.35, COS-1 and HEK293 cells transfected with CYP3A4 cDNA, CYP3A43 cDNA or empty expression vector as analyzed by Western blotting using a CYP3A4/5/7 antibody (Gentest, A254) and cDNA expressed CYP3A4 as positive control (r3A4, Gentest, 0.07 pmol). As evident from the blots, CYP3A4 but not CYP3A43 is expressed. Another three antibodies were tested and gave similar results.

Expression of recombinant CYP3A43 in yeast yields no detectable protein

No CYP3A43 protein or activity could be detected when transfecting yeast cells with a CYP3A43 expression vector and assessing the expression and activity using CO spectra and testosterone incubations, respectively. By contrast, CYP3A4 was successfully expressed and displayed a high hydroxylation rate of testosterone. One could question whether testosterone is a suitable substrate to assess CYP3A43 activity since many of the protein sequence variations compared to the other CYP3A enzymes are present in substrate recognition sites. However, even though substrate specificity and regioselectivity varies slightly between CYP3A enzymes, there is normally a very large overlap between them (50, 95), thus making it likely that the CYP3A43 enzyme, if expressed, would metabolize testosterone at least to some extent.

In conclusion, CYP3A43 is expressed at low levels in human tissues and the lack of i) enzyme activity, ii) proper CO spectra, and iii) detection by Western blotting in cDNA transfected cell systems together suggest that CYP3A43 is expressed at levels too low to be detected and/or that the protein is not efficiently translated and/or that the protein product is unstable. Thus, the identification of CYP3A43 did not provide with an

explanation to interindividual variation in CYP3A expression and activity and the *CYP3A43* gene is likely to encode a defect gene product.

CYP3A43 as discussed by others

At about the same time as paper I was published, another two groups also presented their identification of the novel CYP3A43 enzyme (96, 97). Domanski *et al.* and Gellner *et al.* (96, 97) found that CYP3A43 was detected at the mRNA level in adult liver, kidney, pancreas and prostate, but not at levels high enough to be detected in dot blots of human tissue (96). In liver, the level of expression of *CYP3A43* mRNA was shown by Koch *et al.* (54) and Gellner *et al.* (97) to be <0.5% of that of total *CYP3A* transcripts, which is in agreement with our data. *CYP3A43* has also been shown to be subjected to extensive differential splicing (96, 97). A very high percentage (57%) of *CYP3A43* clones amplified from human liver are differentially spliced, the translation products of which are likely to be inactive (96). The low expression of CYP3A43 mRNA together with the apparently improper splicing indicates that there is no evolutionary pressure on the *CYP3A43* gene and that the gene product is not crucial for any physiological function.

Domanski *et al.* (96) expressed the N-terminally modified CYP3A43 enzyme in *E. coli* at a level of less than 10% of that of CYP3A4. Under standard procedures, the purified CYP3A43 protein showed no signs of catalytic activity towards testosterone or progesterone. Under modified assay conditions (type of buffer and molar ratios of P450 to NADPH-P450 reductase to cytochrome b_5 was modified), CYP3A43 showed a 6β-hydroxylation activity of testosterone that was 3% of that of CYP3A4 at the most. The rate of testosterone hydroxylation was however extremely low and data for mock transfected negative controls were not presented (96). Specifically, the 6β-hydroxylation activity of CYP3A4 varied from 10 to 45 nmol/min/nmol, whereas the corresponding activity for CYP3A43 was repeatedly either 0.2 or 0.3, thus possibly reflecting background activity. In contrast, we could not express the wildtype CYP3A43 cDNA in any of four expression systems, representing both yeast and mammalian cell lines. It is possible that the extensive modifications of the CYP3A43 N-terminus as carried out by Domanski *et al.*, and leading to a removal of ten amino acids and a substitution of another seven amino acids, had an impact on enzyme stability and/or activity that thus could not be considered to represent native CYP3A43 expression and/or activity. In fact, Finta *et al.* (98) has shown that the naturally occurring *trans*-spliced transcript of CYP3A43 exon 1 with CYP3A4 exon 2-13 significantly reduces the activity of the resulting protein towards testosterone as compared to the CYP3A4 full length transcript. The N-terminus has been shown to be important for the targeting, insertion and retention of P450s in membranes (99). In addition, modification of the N-terminus appears to affect the interaction between P450 and cytochrome P450 reductase (100).

By immunohistochemical analyses of normal and tumor colon samples by a range of different antibodies, CYP3A43 was found to associate with Dukes stage of tumors (101). In the paper, it is however neither described in which direction this association is present, i.e. increase or decrease of CYP3A43 expression with increasing severity of Dukes stage, nor is the specificity of the antibody described. The antibody could possibly cross-react with other CYP3A proteins or even completely unrelated proteins, thus giving erroneous results. CYP3A43 expression was also reported to be induced in ovarian cancers by the same group and using the same antibody (102).

Several papers have been published on different polymorphic *CYP3A43* alleles and their respective frequencies in different populations (103-105). Based on statistical analyses, two studies suggest an increased risk of prostate cancer among men carrying the *CYP3A43*3* allele (106, 107). However, true expression of the CYP3A43 enzyme is as mentioned questionable and the polymorphism is uncharacterized regarding its functional effect. These statistical associations can thus not be considered very meaningful.

Paper II - Comparative analysis of CYP3A expression in human liver suggests only a minor role for CYP3A5 in drug metabolism

To further investigate factors potentially contributing to CYP3A variability, we aimed at determining the relative contribution of the different CYP3A enzymes to the overall CYP3A expression and activity, with particular emphasis on the polymorphic CYP3A5 enzyme. At the time of initiating our study, polymorphic protein expression of CYP3A5 had been known for about a decade (61, 62, 66). However, the exact contribution of CYP3A5 to total CYP3A levels had been scarcely determined and most reports had quantified the sum of CYP3A rather than the exact level of individual CYP3A enzymes. The opinion was however that CYP3A5 expression could be significant and have a large impact on total CYP3A expression and activity in Caucasians. In particular, the paper by Kuehl *et al.* (65) claimed exceptionally high levels of hepatic CYP3A5. Our aim was thus to quantitatively determine the importance of CYP3A5 expression by use of isoform-specific antibodies and carefully determined standard preparations, and to relate the expression of CYP3A5 to the *CYP3A5* genotype and to CYP3A activity. Thus, our study contributed with an accurate determination of the hepatic CYP3A5 content in Caucasians.

CYP3A5 mRNA levels in adult human liver in relation to other CYP3A enzymes and to CYP3A5 genotype

The mRNA levels of CYP3A4, CYP3A5, CYP3A43 and PXR was determined in a human liver bank of 46 samples by quantitative real-time PCR. In paper I, quantification of CYP3A4, CYP3A5 and CYP3A43 had already been presented for 11 of the livers. In the extended set presented in paper II, CYP3A43 expression remained insignificant compared to the other CYP3A enzymes (0.1% of total CYP3A in both papers. CYP3A4 accounted for 96% and CYP3A5 for 4% of total CYP3A mRNA in both studies. CYP3A5 mRNA contributed to 10% of total CYP3A mRNA in livers carrying the *CYP3A5*1* allele (8% for all livers with quantifiable CYP3A expression) and the CYP3A5 mRNA levels were significantly higher in liver samples carrying the *CYP3A5*1* allele compared to those homozygous for the *CYP3A5*3* allele ($p<0.001$). The *CYP3A5*3* allele carries a polymorphism in intron 3 that creates a cryptic consensus splice site and leads to the production of aberrantly spliced CYP3A5 mRNA (65).

CYP3A5 protein levels in adult human liver in relation to other CYP3A enzymes and to CYP3A5 genotype

As has been discussed in the *Methodology* chapter, the CYP3A4 and CYP3A5 protein levels in human liver microsomes were quantified by Western blotting by use of a CYP3A4 and a CYP3A5 human liver standard sample that had been quantified against specific peptide-

conjugates and isoform-specific antibodies. The effect of quenching of the immunosignal by the amount of total protein was also taken into consideration (see *Methodology*).

We were able to quantify CYP3A5 protein in 11% of the livers based on the apparent detection limit of 6 pmol/mg calculated as the interception of the linear standard curve with the pmol axis. Very faint bands below the detection limit were occasionally visible but could not be quantified due to the low signal-to-noise ratio. The range of CYP3A5 protein levels was 7-48 pmol/mg in the five livers with quantifiable CYP3A5, thus a significantly more narrow expression range than that of CYP3A4 with a range of 15-600 pmol/mg in all samples with quantifiable CYP3A4 (mean 194 pmol/mg, detection limit of 15 pmol/mg). CYP3A5 was expressed on average at 31 pmol/mg in quantifiable samples, but only at 3 pmol/mg among all samples.

The only four livers that carried the *CYP3A5*1* allele in our sample set (allele frequency 4%) were all within the group of the five livers with quantifiable CYP3A5 protein amounts (18-48 pmol/mg). One liver was homozygous for the *CYP3A5*3* allele but had low but quantifiable CYP3A5 levels (7 pmol/mg). Thus, the *CYP3A5*3* allele can produce small amounts of correctly spliced mRNA (65) and subsequently also protein, as we have been able to show in this study. This is also in line with the trace amounts of CYP3A5 protein detected in some of the livers within our panel as mentioned above. CYP3A5 was calculated to contribute on average to 16% of total CYP3A protein (range 5-27%) in the subset of livers where CYP3A5 could be quantified, and to 17% of total CYP3A protein in the livers carrying the *CYP3A5*1* allele. Among all samples, CYP3A5 was only contributing to 2% of total CYP3A protein. Some papers have reported a higher contribution of CYP3A5 to total CYP3A protein in Caucasians, both in the aspect of a higher *CYP3A5*1* frequency and a higher level of CYP3A5 protein in *CYP3A5*1* carriers (65, 108). This discrepancy could be due to the composition of particular liver panels studied and/or differences in the quantification methods, such as differences in the specificity of the antibody or in the standards used for quantification.

In conclusion, our data indicate that the overall contribution of CYP3A5 protein to the hepatic Caucasian CYP3A pool is low. Based on our study, it appears that the polymorphic expression of CYP3A5 is not a major explanation for the extensive CYP3A variation observed in Caucasians.

CYP3A5 and drug metabolism

Considering the variability in *CYP3A5*1* allele frequency between populations, CYP3A5 expression could be considered significant in 10-90% of individuals depending on the population studied (109). Africans have a much higher frequency of the *CYP3A5*1*-mediated high expression phenotype as compared to Caucasians (109).

The occurrence of the *CYP3A5*1* allele has been associated with pharmacokinetics of the immunosuppressants **tacrolimus** and **cyclosporine**, which both have narrow therapeutic indexes and show high interindividual variation in their pharmacokinetics. The plasma levels of both drugs are markedly lower in patients carrying the *CYP3A5*1* allele, although the effect of *CYP3A5* genotype is larger for treatment with tacrolimus (110-112). In addition, tacrolimus plasma concentration in liver transplant recipients has been shown to be affected by the *CYP3A5* genotype of the donor liver (113, 114). A doubling of the tacrolimus dose has been suggested for *CYP3A5*1* carriers (115) and the *CYP3A5* genotype is predicted to explain up to 45% of the variability in dose requirement (112). Immunosuppressants are also substrates for the P-gp transporter. The gene encoding P-gp, *MDR-1*, lies in the same chromosomal region as CYP3A5 (7q21) and could thus be a confounding factor in CYP3A5 association analyses. However, linkage between the two genes is unlikely since there is an approximately 12 Mb distance between them, and lack of linkage has indeed been the result of linkage disequilibrium analyses (116). In addition, associations of *CYP3A5* genotype with for example tacrolimus pharmacokinetics has been widely reported, whereas P-gp associations are poorer (117). It seems however that the P-gp levels affect the bioavailability of tacrolimus during the first week after transplantation but not during subsequent weeks (113), possibly due to an increased saturation of the transporter during continuous treatment (57, 58). The opposite appears to be true for CYP3A5; i.e. the *CYP3A5* genotype does not affect tacrolimus levels during the first week, but during subsequent weeks (114). For cyclosporine, the *CYP3A5* genotype has been shown to have a relatively large effect on drug concentration also during the first week of treatment, in addition to the comparatively smaller effect exerted by the *MDR-1* genotype (110). Overall, it appears that CYP3A5 has a larger effect on immunosuppressant drug concentration during long-time treatment, whereas P-gp affects drug concentration only during the initial phase.

It seems apparent that subjects with a CYP3A5 high expression phenotype generally require higher doses of immunosuppressants to obtain sufficient plasma levels after organ transplantation. Due to the narrow therapeutic window of immunosuppressants and thus the necessity of therapeutic drug monitoring (118), it is however unlikely that information on the *CYP3A5* genotype would assist in individualizing drug dosing.

The pharmacokinetics of **alprazolam**, a drug used in the treatment of anxiety and panic disorders, has been shown to be affected by *CYP3A5* genotype. This is manifested in a lower alprazolam AUC after a single oral dose in carriers of the *CYP3A5*1* allele as compared to those not carrying the allele (119). Since the therapeutic window in alprazolam treatment is relatively narrow (120), it can be hypothesized that some patients would benefit from a *CYP3A5* genotype-based drug dosing. However, additional studies using continuous treatment protocols would be necessary in order to evaluate the validity of such a hypothesis.

The pharmacokinetics of the antimalaria drug **quinine** is influenced by the *CYP3A5* genotype, whereby *CYP3A5*1* confers an increased metabolism of quinine in particular in Africans but not in Caucasians. This is due to a 7 times higher *CYP3A5*1* frequency in Africans compared with Caucasians (121). *In vitro*, it has been shown that quinine causes a concentration-dependent inhibition of the infectivity of *Plasmodium falciparum* (122). Thus, *CYP3A5*1* has the potential to negatively influence the outcome of treatment of malaria.

In summary, CYP3A5 expression can be an important factor for drug pharmacokinetics in Caucasians on an individual basis, but is more important among Africans.

Paper III - CYP3A7 protein expression is high in a fraction of adult human livers and partially associated with the *CYP3A7*1C* allele

In the continuous search for factors explaining CYP3A interindividual variability and due to the finding that CYP3A7, which had previously been considered a fetal CYP3A isoform, was found to be expressed at the mRNA level at significant amounts in 10-15% of adult human liver samples (40, 54), we initiated a study to examine CYP3A7 protein expression in adult human liver to investigate the contribution of CYP3A7 to total CYP3A. Attempts to estimate the level of CYP3A7 based on enzyme activity had previously been carried out (123), as had poor Western blotting techniques using inadequately chosen standards and uncharacterized antibodies (124). The apparent lack of a reliable CYP3A7-specific antibody to determine the exact CYP3A7 levels in adult livers encouraged us to develop a new peptide-specific CYP3A7 antibody, by which we reliably could assess CYP3A7 expression in adult livers together with a carefully determined standard preparation.

CYP3A7 protein levels in adult human liver in relation to other CYP3A enzymes

A CYP3A7-specific antibody was developed that did not cross-react with CYP3A4 or CYP3A5. As has been described in the *Methodology* chapter, a recombinant CYP3A7 preparation (Gentest) was determined regarding its holo- and apoprotein amounts by use of a CYP3A4 antibody and a peptide-conjugate, and thereafter used as a standard. The CYP3A7 antibody was less sensitive than the CYP3A5 and CYP3A4 antibodies used in paper II, as reflected in a detection limit of 23 pmol/mg as compared to 6 and 15 pmol/mg for the CYP3A5 and the CYP3A4 antibody, respectively. A subset of 10% of livers (6 of 59) could be quantified regarding their CYP3A7 protein content and is thus in concordance with previous results on the frequency of a CYP3A7 high expression phenotype on the mRNA level (40, 54). The range of CYP3A7 expression among these six liver samples was 24-90 pmol/mg with a mean level of 42 pmol/mg (4 pmol/mg among all samples).

In order to relate CYP3A7 protein expression with the expression of total CYP3A, we quantified the CYP3A4 and CYP3A5 protein amount in a subset of 10 livers where CYP3A7 expression could be detected or was expected based on the *CYP3A7*1C* genotype. The *CYP3A7*1C* allele had previously been characterized and contains a partial *CYP3A4* promoter containing an PXR/CAR element that is associated with adult CYP3A7 mRNA expression (40, 65). As a result, we calculated that CYP3A7 contributed to 9-36% (average 24%) of total CYP3A protein content in livers where CYP3A7 could be quantified.

CYP3A5 was expressed in one of the livers that was heterozygous for the *CYP3A5*1* allele, and corresponds well with the expression frequency of 11% observed in paper II. However, the

CYP3A5 expression of 122 pmol/mg was higher in this liver sample compared to that found in paper II, where CYP3A5 was never expressed at a level higher than 48 pmol/mg. Since the liver panels in paper II and III are from different sources, this could be due to the different composition of the sample sets. As a matter of fact, four livers with quantifiable CYP3A5 in paper II and one liver in paper III are too few to make a comparison and to evaluate whether the CYP3A5 expression between the two panels are indeed different. Since we did not access data on drug intake, it is reasonable to assume that CYP3A5 expression could have been induced in this particular high-expression liver of paper III (125, 126).

CYP3A7 protein levels in adult human liver in relation to CYP3A7 genotype

All liver microsomal samples but four had available DNA samples. Promoter polymorphism in the *CYP3A7* gene was screened for by sequencing analysis of samples showing detectable CYP3A7 expression, but no other polymorphism than *CYP3A7*1C* was found (see below). All samples were genotyped for *CYP3A7*2* that was found at a frequency of 12% in our material but did not correlate with CYP3A7 expression, as was expected since the *CYP3A7*2* allele apparently confers increased enzyme activity but not increased CYP3A7 expression (127). The *CYP3A7*1C* allele was determined in all available DNA samples and was found heterozygously in seven livers, three of which could be quantified for CYP3A7 expression and another two showing trace amounts that could not be quantified. Thus, it appears that the *CYP3A7*1C* allele is associated with increased CYP3A7 expression, although the association could only be statistically proven when classifying samples as expressing or not expressing CYP3A7, and not when comparing the CYP3A7 levels (see paper III). *CYP3A7*1C* was however not the sole explanation for the high-expression phenotype of CYP3A7 protein.

CYP3A7 16α-hydroxylates DHEA more efficiently than CYP3A4 (123, 128), whereas CYP3A4 is mainly responsible for the 7β-hydroxylation of DHEA (123). Stevens *et al.* (123) used the regioselective difference in the CYP3A4- and CYP3A7-catalyzed hydroxylation of DHEA to produce a model and predict the CYP3A4 and CYP3A7 protein levels in adult human livers based on their respective DHEA metabolite pattern. The model predicted that two of a total of eight adult liver samples expressed significant amounts of CYP3A7 with levels of 32 and 41 pmol/mg, respectively. The estimated CYP3A7 amounts in livers where CYP3A7 could be quantified was thus very similar to our own data based on immunoquantification, whereas the frequency of livers with quantifiable CYP3A7 protein expression was 2-fold higher.

To our knowledge, only Tateishi *et al.* (124, 129) has claimed to specifically investigate CYP3A7-specific protein expression in adult human liver. However, data on the specificity of the antibody used has never been presented. In addition, the quantification of CYP3A7 is likely overestimated (range 1-15 pmol/mg in their Caucasian sample set), which was also stated by the authors. The

microsomal standard was originating from a 10-month old child that had been quantified using a CYP3A specific antibody. A large part of the detected immunoreactivity using the unspecific antibody supposedly comes from CYP3A4 and possibly CYP3A5 and thus does not mirror the actual CYP3A7 level in this sample (49, 60, 123). In addition, it appears that quantification was based on only one amount of standard loaded rather than a linear standard curve, which makes quantification practically impossible.

CYP3A7 in the metabolism of endogenous and exogenous compounds

CYP3A7 is involved in the metabolism of retinoic acids (RA, which are antiproliferative and differentiating agents. CYP3A7 has been shown to efficiently metabolize **all-*trans* retinoic acid** (atRA) to 4-hydroxy RA at a rate of almost three times faster than that of CYP3A5, and 15 times faster than that of CYP3A4 (130). atRA is also metabolized to its 4-oxo, 18-hydroxy and 5,6-epoxy derivatives by the contribution of several different P450s including CYP3A7, but at significantly lower efficiency than the 4-hydroxylation carried out by CYP3A7 (130). The 4-hydroxylation of the **9-*cis*** and **13-*cis* RA's** is also carried out by CYP3A7, whereby the 13-*cis* hydroxylation is mainly catalyzed by CYP3A7 whereas the 9-*cis* hydroxylation is more dependent on CYP2C8 and CYP2C9 (131). atRA (Tretinoin) is used to treat acute promyelocytic leukemia (APL) and can alone induce a very high frequency of haematological remission, but is however normally used together with chemotherapy to induce stable transmission (132). It can be hypothesized that an increased metabolism of atRA mediated by a CYP3A7 high-expression phenotype would impact on the outcome of APL treatment, due to the efficient metabolism of atRA by the CYP3A7 enzyme. Further investigation is however needed to deduce the relevance of such relationships. 13-*cis* RA (Isotretinoin) is an effective medication for the treatment of acne. Since adverse effects are significant for this acne therapy, individualized dosing based on drug effectiveness and side effects is often applied (133, 134). Despite the high metabolic activity of CYP3A7 towards *13*-cis RA, the high expression of CYP3A7 protein discovered in certain livers within this study would not be expected to have any major impact on the outcome of acne treatment due to the common application of individualized dosing. 9-*cis* RA (Alitretinoin) is used in the treatment of AIDS-related Kaposi's sarcoma (135). Due to the topical administration of Alitretinoin and the involvement of CYP2C enzymes in its metabolism, CYP3A7 expression is not expected to be of any relevance for this drug.

CYP3A7 16α-hydroxylates the endogenous steroid hormone **DHEA**, a precursor of androgens and estrogens, and its sulfated derivative DHEA-S, which is more stable but readily desulfated to DHEA (123, 128, 136). CYP3A7 is however 4 times more efficient in the metabolism of DHEA compared to DHEA-S (128). One study has found an association of the CYP3A7 high-expression phenotype conferred by the *CYP3A7*1C* allele with decreased endogenous levels of DHEA-S in two separate populations of elderly subjects (n=208, women and men; n=345, men)

(137). Beneficial effects of exogenous DHEA on disease states such as osteoporosis and systemic lupus erythematosus has been suggested (138, 139). Reduced DHEA-S levels have also been proposed to be associated with cardiovascular disease, depression and reduced immune response (140). Due to its suggested beneficial effect on a variety of physiological systems, DHEA has been widely used as a dietary supplement. However, the uncontrolled usage of DHEA is controversial and the long-term effects of DHEA supplementation are not known. Much of the DHEA-mediated effects and concentration-effect relationships remain to be investigated, thus it is too early to speculate on any effect of the CYP3A7 protein high-expression phenotype.

In summary, we confirmed a previously observed CYP3A7 high-expression phenotype on the mRNA level with that on the more relevant protein level, and found that CYP3A7 protein is expressed at levels similar to that of CYP3A5. In addition, an association of *CYP3A7* genotype with CYP3A7 protein expression was evident. CYP3A7 is however not a determining factor for CYP3A variability in an average Caucasian population. However, CYP3A7 expression could be important in a subset of 10% of Caucasians and for a subset of substrates.

Summary on CYP3A protein expression in Caucasian livers

Based on paper I-III, Figure 6 summarizes the contribution of individual CYP3A enzymes to total CYP3A protein in an average Caucasian liver. CYP3A43, CYP3A5 and CYP3A7 can not be considered to exert any major impact on CYP3A variability in a Caucasian population, although enzyme levels in specific subjects can be significant with regard to CYP3A5 and CYP3A7.

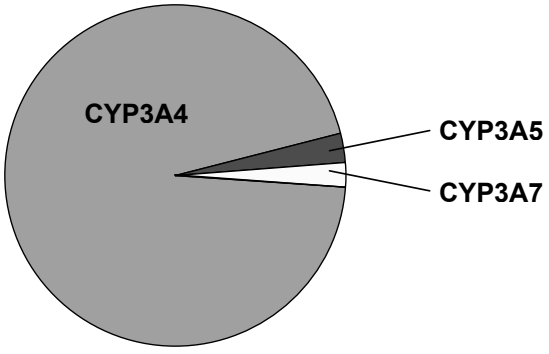

Figure 6. The relative contribution of CYP3A enzymes to total CYP3A protein levels in an average Caucasian liver as based on papers I, II and III. The specific contents are 173, 5 and 4 pmol/mg for CYP3A4, CYP3A5 and CYP3A7, respectively. CYP3A43 is not expressed.

Paper IV - A common novel *CYP2C19* gene variant causes ultrarapid drug metabolism relevant for the drug response to proton pump inhibitors and antidepressants

The interindividual variation in CYP2C19 activity *in vivo* among individuals with an EM phenotype is large (76, 141). Part of this variation can be attributed to known (see www.cypalleles.ki.se) or unknown rare defective alleles that generally are not genotyped for. However, since the *CYP2C19*2* allele accounts for 75-95% of all PM alleles in Caucasians (PM phenotype frequency of 3-5%), it can be calculated that in a population of Caucasian individuals defined as *CYP2C19*1/*1* based on *CYP2C19*2* genotyping, there will be only 2-8% that carry a defect *CYP2C19* allele other than *CYP2C19*2*. Thus, additional rare defective *CYP2C19* alleles do not fully explain the interindividual variability observed among individuals defined as *CYP2C19*1/*1*. Consequently, we aimed at investigating potential genetic factors that may affect CYP2C19 activity.

Identification of the CYP2C19*17 allele

In the search for novel genetic variations in the *CYP2C19* gene, we found a -806C>T polymorphism in the *CYP2C19* 5'-flanking region. In all cloned fragments of the *CYP2C19* 5'-flank, the -806C>T mutation co-existed with a mutation at -3402 (C>T), indicative of a linkage between the two mutations. This finding was supported by the finding of a complete linkage disequilibrium of the -806 and -3402 mutations in all genotyped populations, and the allele (*CYP2C19*17*) was found at a frequency of 18% in Ethiopians (n=190) and Swedes (n=314), and 4 % in Chinese (n=68).

CYP2C19*17 mediates a higher transcriptional rate compared to CYP2C19*1

In order to address the transcriptional activity of the *CYP2C19*17* in relation to the *CYP2C19*1* allele, the 5'-flanking regions were cloned in reporter vectors. Initially, transfection experiments were carried out in several cell lines (HEK293, HeLa, HepG2 and B16A2) using reporter constructs with -1570 to +70 bp of the *CYP2C19* gene, but no difference between the *CYP2C19*1* and *CYP2C19*17* alleles could be detected in any cell system. Instead, we employed an *in vivo* transfection method in mice with reporter constructs containing -0.9 or -3.5 kb to -30 bp of the *CYP2C19* 5'-flanking region. The plasmids were injected into the tail vein of the mice and the hepatic expression measured as described in the *Methodology* chapter. Using the -3.5 kb reporter constructs that encompass both polymorphic positions of the *CYP2C19*17* allele, there was a trend for higher reporter activity for the *CYP2C19*17* genotype compared to the *CYP2C19*1* genotype, but this difference did not reach statistical significance. However, with the -0.9 kb constructs that only contained the -806 polymorphism, the rate of hepatic reporter

expression was found to be 2-fold higher ($p<0.01$) for the *CYP2C19*17* as compared to the *CYP2C19*1* allele. Apparently, the mouse *in vivo* system was able to express the phenotype conferred by *CYP2C19* genotype, whereas the cell lines used in this study could not. This difference is probably due to the hepatic phenotype of the mouse liver similar to that of human liver, the expression of correct transcription factors and the conservation of transcription factors between species.

Prediction of transcription factor binding sites of relevance for the CYP2C19*17-mediated phenotype

Transcription factor binding sites possibly affected by the -806C>T and -3402C>T mutations of the *CYP2C19*17* allele were predicted using computer algorithms (see *Methodology*). For the -3402 position, there was no indication of loss or gain of relevant transcription factor binding sites between the *CYP2C19*1* and *CYP2C19*17* alleles. For the -806 site however, introduction of a T into the wildtype sequence (-806C>T; *CYP2C19*17*) introduces a transcription factor binding site that is highly similar to a GATA binding site. The sequence created by -806T is identical to a GATA-1 transcription factor binding site but also resembles binding sequences for other GATA transcription factors. There are six known GATA factors (GATA-1 to -6), all of which can bind to a consensus sequence. GATA-1 and GATA-2 are haematopoietic factors (142), GATA-3 is involved in T-cell development (143) and GATA-4, -5 and -6 are expressed in mesoderm- and endoderm-derived tissues, such as heart and gut. In mouse hepatocytes, GATA-6, but not GATA-4 or GATA-5, can be detected immunohistochemically (144). In adult human liver, GATA-4 and GATA-6 are both expressed at the mRNA level (145, 146). The GATA binding site of the *CYP2C19*17* allele is identical to a GATA site in the promoter of the liver-expressed epoxide hydrolase gene, which is shown to be transactivated by GATA-4, and to a lesser extent by GATA-6 (147). GATA-4 has also been implied in the regulation of the liver-enriched expression of the apolipoprotein AI, erythropoietin, serum albumin and Hex genes (146, 148-150). Based on binding specificity, expression pattern and regulation of key genes, GATA-1, GATA-4 and GATA-6 antibodies were chosen for supershift analyses in EMSA experiments (see below).

Nuclear transcription factors are binding specifically to the 5'-flank of the CYP2C19*17 allele

To investigate the physical binding of nuclear transcription factors to the polymorphic sites of the *CYP2C19*17* allele, EMSAs using hepatic nuclear extracts were conducted. No specific binding was detected at the -3402 site, neither using the *CYP2C19*1* nor the *CYP2C19*17* probe. However, two bands were detected with the -806T (*CYP2C19*17*) probe but not with the -806C (*CYP2C19*1*) probe, thus indicating a binding of nuclear proteins only to the *CYP2C19*17* allele.

In competition experiments with labeled -806T probe, unlabeled probes of the corresponding sequence of the *CYP2C19*17* and the *CYP2C19*1* alleles showed different efficiency in competing for protein binding; the *CYP2C19*17* probe was more efficient than the *CYP2C19*1* probe. This finding together with the lack of binding of nuclear proteins to the labeled *CYP2C19*1* probe indicates that the binding of nuclear proteins to the site at -806 is *CYP2C19*17* specific. In contradiction to the binding site predictions discussed above, we could neither detect competition by a GATA consensus sequence, nor produce any supershift using three different GATA antibodies. This could be due to the binding of transcription factors other than those belonging to the GATA family, or that the consensus probe was not bound efficiently by the particular GATA factor and that the conditions were not optimal for supershift analyses.

CYP2C19*17 is associated with increased enzyme activity in vivo

By phenotype-genotype association analyses, we found that individuals carrying the *CYP2C19*17* allele showed increased CYP2C19 probe drug activity compared with individuals lacking the allele. In Swedes, we observed a 50% lower median MR of omeprazole in individuals homozygous for the *CYP2C19*17* allele compared to those not carrying the allele (p=0.010). In Ethiopians, we found a 75% lower median S/R-mephenytoin ratio in individuals homozygous for the *CYP2C19*17* allele compared to those being homozygous for the *CYP2C19*1* allele (p=0.013). Based on the large variation in CYP2C19 activity observed within the *CYP2C19*1/*1* genotype group and compared to the apparent lack of variation in the *CYP2C19*17/*17* genotype group (Fig. 1 in paper IV), it can be postulated that the binding of nuclear protein to the *CYP2C19*17* allele that we have shown in the current study would sterically hinder the binding of other nuclear protein(s) with inhibitory function from exerting their effect. The inhibitory nuclear protein could be present at different levels or with different relative activities within the *CYP2C19*1/*1* group, thereby exerting a larger or smaller inhibitory effect, thus giving rise to the variation in enzyme expression and activity. Blocking of this effect would give rise to a high-activity characteristic, as could be the mechanism for the *CYP2C19*17*-mediated high activity phenotype.

Prediction of the effect of CYP2C19*17 on omeprazole AUC

Based on a correlation of omeprazole MR with AUC in 24 healthy Swedish volunteers, we were able to estimate that a 50% reduction in MR of omeprazole in *CYP2C19*17/*17* individuals mentioned above would result in a 37% lower omeprazole AUC compared to those of the *CYP2C19*1/*1* genotype when omeprazole is given as a single dose of 20 mg (Table 1).

Prediction of the effect of CYP2C19*17 on pH increase mediated by omeprazole

Based on our correlation of omeprazole MR with AUC, and the correlation of omeprazole AUC with pH presented by Tolman *et al.* (151), we predict a 0.7 unit difference in mean 24-hour intragastric pH between *CYP2C19*17/*17* and *CYP2C19*1/*1* subjects after omeprazole administration (Table 1, pH estimations have not been presented before). The data from Tolman *et al.* (151) is based on 20 mg of omeprazole administration, which is the same dose given to our Swedish study subjects. However, their AUC-pH correlation originates from data obtained after 5 days of repeated dosing, whereas our omeprazole MR and AUC predictions are based on a single dose of omeprazole. Even if it is tempting to make predictions related to the clinical use of drugs, one should keep in mind that the genotype to pH predictions are based on extrapolations at two levels; from omeprazole MR to AUC and from AUC to pH.

	****17/*17***	****1/*17***	****1/*1***	****1/*2***
n	4	32	71	39
Omeprazole MR	0.25±0.10	0.53±0.54	0.70±0.58	1.00±0.56
Predicted omeprazole AUC	742±93	1010±507	1171±545	1457±525
Predicted intragastric pH	3.45	3.90	4.10	4.45

Table 1. Prediction of mean omeprazole AUC (nmol*h/L) and intragastric pH from omeprazole MR in relation to *CYP2C19* genotype in Swedish subjects. Predictions were based on the correlation of omeprazole MR with AUC in 24 Swedish individuals (paper IV) and the correlation of omeprazole AUC with intragastric pH from Tolman *et al.* (151). Omeprazole MR and predicted omeprazole AUC are expressed as mean±s.d.

To summarize our findings, we have identified and characterized a *CYP2C19* allele that recruits binding of nuclear proteins, thereby increasing the level of transcription and conferring an increased CYP2C19 activity *in vivo*.

CYP2C19 and personality trait

Recently, Ishii *et al.* (152) found that *CYP2C19* genotype associates with particular phenotype traits in female Japanese subjects, but not in males. By assessing personality traits using the Temperament and Character Inventory (TCI), three of seven traits were scored differently in CYP2C19 PMs and EMs. Specifically, the scores of reward dependence (temperament trait), self-transcendence and cooperativeness (character traits) were lower in PM individuals (152), thus implying that PM subjects are more practical, tough-minded, socially insensitive, self-conscious, intolerant, critical and controlling (http://psychobiology.wustl.edu/TCI/whatIsTCI.htm).

Interestingly, the *CYP2D6* genotype has also been implicated in personality trait, whereby PMs are less prone to avoid harm and are less careful than EMs (153, 154). The difference in

personality trait between CYP2D6 phenotypes appears to be more pronounced in females than in males (154), as was also shown for CYP2C19 (152). Since CYP2D6 is expressed in brain and involved in the metabolism of neurotransmitter substances (155, 156), the link between CYP2D6 expression level, brain transmitter levels and personality trait is relatively apparent, although the exact mechanism by which CYP2D6 activity affects personality is not yet clear.

For CYP2C19, expression in brain remains to be determined and specific CYP2C19 substrates that may be involved in personality traits are not known. Ishii *et al.* (152) has suggested that sex hormones, which have been shown to be metabolized by CYP2C19, may be responsible for the impact of *CYP2C19* genotype on personality. However, future studies are needed to confirm the effect of *CYP2C19* genotype on personality and to investigate potential mechanisms whereby personality is affected by CYP2C19 phenotype. One may however speculate whether individuals carrying the *CYP2C19*17* allele would be more empathetic, supportive, compassionate, sensitive, sociable, spiritual and humble than individuals not carrying the allele.

CYP2C19 and antidepressants

CYP2C19 metabolizes several antidepressants. The influence of CYP2C19 activity on the pharmacokinetics and clinical effect of antidepressant drugs is likely dependent on the relative contribution of CYP2C19 metabolism compared with mainly other CYPs (particularly CYP2D6) and the outcome of CYP2C19 metabolism (pharmacologically active or inactive metabolites). The importance of CYP2C19 metabolism and the formation of active or inactive metabolites vary with different antidepressants.

For **moclobemide**, a 3-fold higher AUC has been observed in CYP2C19 PM compared to EM subjects after a single oral dose (157), and the CYP2C19 probe drug omeprazole and moclobemide inhibit each others metabolism in CYP2C19 EMs (157, 158). The plasma concentration of moclobemide has not been associated with therapeutic efficacy, instead there appears to be a correlation of plasma concentration with side effects (159). Since the *CYP2D6* genotype does not have the potential to affect moclobemide drug levels, *CYP2C19* genotype appears to be the main predictive factor in moclobemide metabolism (10, 79).

Amitriptyline is demethylated to nortriptyline by CYP2C19. The *CYP2C19* genotype affects the metabolic ratio of amitriptyline to nortriptyline as well as the AUC of them both (160-162). However, since the sum of amitriptyline and nortriptyline levels are used in therapeutic drug monitoring (163), CYP2C19 activity is probably not affecting treatment response and accordingly no relationship between *CYP2C19* genotype and response has been detected when investigated (17). In addition, there is no correlation between drug concentration and response for amitriptyline treatment (17). However, CYP2C19 can affect the risk of adverse events, since

nortriptyline levels correlate with side effects, as does also the number of active *CYP2C19* genes (17). Specifically, a combination of high CYP2C19 activity and low CYP2D6 activity confers the highest risk of side effects since CYP2C19 produces the active metabolite nortriptyline whereas CYP2D6 metabolizes nortriptyline into the inactive metabolite 4-hydroxynortriptyline (17).

The steady-state plasma concentration of **clomipramine** has been shown to be affected by the *CYP2C19* genotype; PMs have a 76 and 41% higher dose- and weight-adjusted plasma concentration than individuals carrying none or one defect *CYP2C19* allele, respectively (164). Despite being considered pharmacologically active, the plasma concentration of the metabolite desmethylclomipramine has been reported to be inversely correlated to the clinical effect (165).

Sertraline is demethylated to an almost inactive metabolite, and sertraline and desmethylsertraline AUC have been show to be 41% higher and 35% lower, respectively, in PMs compared to EMs when sertraline is given as a single oral dose (166). *In vitro* it appears however that several P450s are involved in the demethylation of sertraline, although CYP2C19 is representing the most important enzyme (167-169). In addition, the dose-response in sertraline treatment is poor as evident from a lack of improved therapeutic effect when increasing the dose in non-responders (170). Also, a concentration-response in sertraline treatment is not clear as evident from a lack of correlation between serum concentration and clinical effect (170).

Citalopram is metabolized by CYP2C19 into a demethylated metabolite with lower plasma concentration and potency than citalopram (171). CYP2C19 preferentially metabolizes the *S*-form of citalopram that is considered to mediate the antidepressant effect, and the AUC of citalopram and *S*-citalopram (escitalopram) has been shown to be significantly higher in PMs compared to EMs (172, 173). A gene-dose effect has also been shown for both citalopram and escitalopram with a difference in concentration/dose ratio, metabolic ratio (drug/metabolite) and serum concentration between individuals carrying one or no defective *CYP2C19* allele (174). Even though the escitalopram plasma concentration-response correlation is not clear, an increased dose from 10 to 20 mg appears to increase the response rates in severely depressed compared to moderately depressed patients (175). Since *CYP2D6* genotype appears not to affect citalopram drug levels, *CYP2C19* genotype is likely to be the main predictive factor in citalopram metabolism (10, 79).

A summary on the antidepressant drugs discussed in this section is found below in Table 2.

	CYP2C19 & concentration	Concentration & response	Concentration & side effects
Moclobemide	Yes	No	Yes
Amitriptyline	Yes	No	Yes
Clomipramine	Yes	-	Yes
Sertraline	Yes	No	-
Citalopram	Yes	?	-

Table 2. A summary on the relationships between drug concentration, CYP2C19 metabolism, response and side effects for antidepressant drugs discussed in this thesis.

Part of the lack of correlation between antidepressant dose and/or plasma concentration with clinical effect could be attributable to mechanistically different depression phenotypes, whereby one drug type can be successfully replaced by another, as has been shown for moclobemide (monoamine oxidase inhibitor) non-responders with sertraline (SSRI) replacement therapy (176).

Augmented CYP2C19 drug metabolism mediated by the *CYP2C19*17* allele can potentially be important for antidepressant treatment using drugs i) where CYP2C19 appears to serve as the major role in the clearance of the drug, ii) where the metabolites are pharmacologically irrelevant, and iii) where concentration-response relationships could be expected. At present, no antidepressant appears to completely fulfill all these criteria, but future studies are necessary to investigate the potential effect of the *CYP2C19*17* allele on treatment outcome.

CYP2C19 and proton pump inhibitors

CYP2C19 is the main enzyme involved in the metabolism of PPIs such as omeprazole and lansoprazole, and the pharmacokinetics and acid inhibitory effect of PPIs are well correlated with *CYP2C19* genotype *in vivo* (177-179). As a consequence, *CYP2C19* genotype is a strong determinant for the success rate in the treatment of acid-related disorders such as gastroesophageal reflux disease using PPI only, and *H. pylori*-positive gastric ulcers using today's triple or quadruple regimens of one PPI and two or three antibacterial agents (21, 179-182).

The effect of CYP2C19 is manifested in lower PPI plasma levels and decreased healing rates in subjects carrying none as compared to one or two defective *CYP2C19* alleles. Thus, it could be expected that the *CYP2C19*17* allele identified in the current study would confer a higher frequency and probability of non-response. So far, only one study has investigated the relationship of the treatment success rate of *H. pylori*-positive peptic ulcers and *CYP2C19*17*

genotype using triple therapy with pantoprazole and found no association (183). However, the effect of *CYP2C19* genotype on rabeprazole pharmacokinetics has been shown to be insignificant compared to other PPIs as evident from a 7.4-, 3.7- and 1.2-fold higher drug AUC in PMs compared to EMs for omeprazole, lansoprazole and rabeprazole, respectively (184). In addition, it has been shown that there is only a small or no effect of *CYP2C19* genotype on rabeprazole-mediated acid inhibition and eradication of *H. pylori* (177, 185, 186). Thus, any association of rabeprazole pharmacokinetics or pharmacodynamics with *CYP2C19* genotype is unlikely.

Since for PPIs there is a strong association of pharmacokinetics with the clinical effect, *CYP2C19*17* is likely to have an influence on treatment outcome for drug treatment using those PPI drugs being significantly metabolized by CYP2C19, such as omeprazole and lansoprazole. Due to our prediction of an almost 40% lower omeprazole AUC in individuals homozygous for *CYP2C19*17* compared to those homozygous for *CYP2C19*1*, one could expect that *CYP2C19*17* homozygotes would benefit from a 40 mg dose of omeprazole rather than the commonly prescribed dose of 20 mg.

In Japan, where 20% of the population are PMs, a prospective study of genotype-based (n=149) versus standard (n=144) therapy for *H. pylori* eradication was recently published by Furuta *et al.* (187). Patients assigned to the standard protocol were given 30 mg of lansoprazole twice daily together with clarithromycin (CLA, 400 mg) and amoxicillin (750 mg). The genotype-based therapy took both the *CYP2C19* genotype and the genetically based resistance of *H. pylori* to clarithromycin into account, and the lansoprazole treatment schedules are presented in Table 3.

		***CYP2C19*-genotype adjusted**	
	Standard	**CLA-sensitive**	**CLA-resistant**
EM	30 mg x 2	30 mg x 3	30 mg x 4
IM	30 mg x 2	15 mg x 3	15 mg x 4
PM	30 mg x 2	15 mg x 2	15 mg x 2
Eradication rate	70%	96%	

Table 3. Lansoprazole treatment regimens (dose and number of doses per day) and *H. pylori* eradication rates as presented by Furuta *et al.* (187).

The duration of the standard and CLA-sensitive treatment regimens were 1 week, whereas the CLA-resistant regimen was 2 weeks. In conclusion, it was found that genotype-based therapy lead to an eradication rate of 96%, whereas it was only 70% in those treated by the standard protocol. The cost per successful eradication was similar for both strategies, indicating that a higher frequency of eradication can be achieved without large costs. Since there is a more than four times higher frequency of PMs in the Japanese population as compared to Caucasians, it is

possible that a *CYP2C19* genotype test to identify carriers of defective alleles would be clinically relevant for the treatment of *H. pylori* infected acid-related ulcers in Japan but not in the Western world. Since increased PPI plasma levels in PM individuals do not lead to any significant side effects, it is possible that a general increase of the standard drug dose for all Caucasian individuals would be a better approach from an economical point of view.

Few studies have prospectively examined the cost-benefit of pharmacogenetic tests. There is however plenty of association studies that show the impact of genotype on drug levels. Such studies are of course very important to establish a relationship between genotype and pharmacokinetics. However, the most important studies are those evaluating the importance of pharmacogenetics in a clinical setting. Prospective studies that investigate the potential benefit of genotype-adjusted drug dosing on drug efficacy and therapy outcome are thus invaluable for the future implementation of pharmacogenetics in the clinics.

CYP2C19 and prodrugs

As opposed to the beneficial effect of carrying defective *CYP2C19* alleles in PPI treatment, defective alleles lead to reduced activation of the antithrombotic prodrug **clodipogrel**, whereby a higher platelet aggregation and thus higher thrombosis risk is observed in PM individuals compared to EMs (188). It can thus be hypothesized that carriers of the *CYP2C19*17* allele would have an advantage in clodipogrel treatment and suffer from a lower frequency of thrombotic events during antithrombotic treatment.

CYP2C19 and benzodiazepines

Benzodiazepines are CYP2C19 substrates. **Diazepam** has anxiolytic, sedative, and amnestic properties and can be used to treat preoperative anxiety. PMs of CYP2C19 have been shown to take almost double the time to emerge from general anesthesia than EM individuals, due to a 35% lower clearance of diazepam and thus prolonged sedation in these patients (189). The anesthetic sevoflurane and the analgesic fentanyl that were used in the study by Inomata *et al.* (189) are not metabolized by CYP2C19, whereby the observed effect is diazepam-mediated.

Etizolam is a related compound being used for anxiety disorders and the pharmacokinetics of single doses are affected by *CYP2C19* genotype. Thus, PMs demonstrate a higher etizolam AUC than EMs and a higher level of etizolam-induced sleepiness as determined by test scores (190).

Clobazam is used in the treatment of epilepsy and is mainly converted to *N*-desmethylclobazam that has a longer half-life than the parent drug and is considered to possess partial

pharmacodynamic effects, in addition to exerting much of the side effect characteristics of the drug (191). CYP2C19 catalyzes the further metabolism of N-desmethylclobazam to 4-hydroxydesmethylclobazam, a route that is drastically impaired in PM subjects, thereby potentially causing an increased risk of side effects such as drowsiness (192, 193).

*CYP2C19*17* might have the potential to reduce emergence time in general anesthesia due to a lower level of diazepam sedation, to reduce etizolam-induced sleepiness, and to reduce the risk of side effects in clobazam treatment of epilepsy.

In summary, the *CYP2C19*17* allele is most likely to influence treatment outcome for drugs that i) are mainly metabolized by CYP2C19, and ii) display a clear concentration-response relationship. Thus, the *CYP2C19*17* allele is more likely to be influencing on therapies using proton pump inhibitors than those using antidepressants. This is due to a strict plasma level-response relationship for antiulcer drug treatment, whereas this is not the case for antidepressant therapy.

Aspects and considerations in CYP nomenclature

The appendix (paper V) describes the submission criteria, procedures, and objectives regarding The Human Cytochrome P450 Allele Nomenclature (CYP-allele) Website (www.cypalleles.ki.se). This website summarizes P450 alleles and maintains a common P450 nomenclature system. It was initiated in 1999 as an attempt to limit the variety of nomenclature approaches that was already present within the field, in order to unify them into one nomenclature system. The aim was also to define alleles having an impact on enzyme function to create a better platform for future studies. I have been responsible for the website as its Webmaster since January 2004, thus reviewing all incoming errands, managing questions and suggestions, solving problems and reviewing submitted alleles for designation of allele names. For particularly problematic issues or questions regarding the content information of the website, the chairman of the Advisory Board Dr Ingelman-Sundberg has been consulted. The Advisory Board is responsible for overall nomenclature definitions and principles.

The CYP-allele website

On the CYP-allele website, all identified allelic variants of a specific P450 gene are listed. The summary of information on the website serves the purpose of introducing the visitor to known alleles, their sequence content, their effect on the gene products, and what could be expected regarding their influence on activity both *in vivo* and *in vitro*.

For functional polymorphisms, i.e. those giving rise to amino acid changes, splice defects or other types of measurable phenotypes, links to the NCBI dbSNP database has recently been introduced when present in this database. Only functional variations have been linked, since those are the important ones both for the resulting phenotype and normally for the purpose of genotype analyses. Thus, intronic SNPs with unknown function or conservative exonic SNPs have not been linked at this stage. Accordingly, the dbSNP links helps to highlight functional SNPs on the website.

A cDNA and/or genomic reference sequence is presented that corresponds to the **1* allele and also the wildtype protein sequence is provided. This makes it easy for the visitor to check exact positions and surrounding sequence context in addition to the obvious necessity of defining the wildtype allele. In the past, the reference sequences were chosen based on the first sequenced allele, which did not necessarily correspond to the most common one (although this may vary from population to population). Due to the genome project, genomic sequences are widely accessible and for incorporation of new P450 enzymes today, reference sequences are normally chosen from the respective reference sequence of the NCBI database. However, the NCBI reference is not necessarily the most frequent one, neither is it assured that it confers normal

activity of the respective protein, whereby choice of reference sequence for the website must be carefully considered.

Allele frequency links has recently been introduced on the CYP-allele website. It was discussed whether allele frequency data should be included as a column in the table along with the other parameters. However, due to varying allele frequencies among different populations this data would be too extensive and overwhelming and was instead put as reference links under the table.

A polymorphism is generally defined as a nucleotide variation occurring at a frequency higher or equal to 1%. One should remember that several alleles that are present on the CYP-allele website can not be considered to fulfill this criterion; some are very rare and thus not very important to consider for future genotype studies.

Haplotype considerations

The CYP-allele website attempts to serve as a form of quality control by demanding a thorough characterization of new alleles. The haplotype generally needs to be proven experimentally, although haplotype predictions produced by computer algorithms have been included. Such alleles are indicated as “predicted haplotypes”. Preferentially, the haplotype of novel alleles needs to be confirmed and not predicted. Examples of methods to sequence and characterize the haplotype are to amplify and clone the whole gene when possible (can be done for e.g. CYP2D6 with a gene size of only about 4.5 kb but is basically impossible for CYP2C19 with a gene size of approximately 90 kb), or to clone parts of the gene that contains more than one polymorphism, thus making it possible to build genotype maps. Another possibility is to run allele-specific PCR for one polymorphism and amplify over a region that contains other polymorphism(s) to characterize the linkage between them, thus making cloning procedures unnecessary.

Allele definitions

For assignment of a novel allelic number (for a definition see section *CYP nomenclature and definitions* in the *Introduction* chapter), any effect on enzyme activity does not need to be proven when polymorphisms yield apparent effects such as amino acid changes or frame shifts. However, for variations predicted to affect for example splicing, translation or gene expression, the effect needs to be experimentally confirmed. The majority of alleles are however based on amino acid substitutions.

In general, in order to define an allele as mediating a decreased, abolished or increased activity, experimental data must be convincing and should preferably be presented together with statistical analyses supporting the data. *In vitro* and *in vivo* characterizations are both presented on the website, of which *in vivo* data from human studies are naturally more reliable than *in vitro* studies due to the artificial way by which *in vitro* studies are carried out. However, one should also differentiate between the reliability of different *in vitro* methods; human liver microsomes may for example serve as a better tool than P450s expressed in bacteria where folding and post-transcriptional modifications can be affected. On the other hand, associations of genotype with *in vivo* phenotype could be impaired by for example a sample population showing an uneven distribution of another allele not being genotyped for, or by other factors that by one way or the other will make individuals within specific genotype groups heterogeneous. Therefore, there will always be a larger magnitude of variation in a partly uncontrolled *in vivo* system than in an *in vitro* system such as expression systems where the starting point is homogenous (e.g. the cells used are identical for all samples). Consequently, *in vivo* and *in vitro* studies complement each other and both should be taken into consideration when possible.

Gene size considerations

When defining how much of a gene should be sequenced in order to be considered well characterized and included as an allele, there is always a balance between the amount of work needed and the possible benefit of that work. As stated in the Inclusion criteria on the website (www.cypalleles.ki.se/criteria); “a gene is considered as 5 kb upstream from the transcription start site to 500 bp downstream of the last exon, unless a regulatory element has been characterised at a more distant part of the gene and thus will be considered as part of the gene”. Most often, this complete gene region would be considered too large for sequence analysis in relation to the potential profit. Thus, the minimum parts to be sequenced are the exons. Preferably, intron/exon junctions and at least some hundred base pairs upstream of the first exon should be sequenced. This way it can be shown that there are no additional polymorphisms likely to affect splicing or the basic transcription machinery. Since the 3’ end has increasingly found to be important for RNA stability, microRNA binding and RNA processing (85), sequencing the transcribed region downstream of the last exon would be advised. One should keep in mind that the current definitions of which parts of a gene should be considered important for sequencing analysis are just recommendations.

Suballeles

Lately, I have been forced to be more restrictive in providing novel suballele (letter) names such as **1B*, **1C* etc, i.e. alleles containing additional nucleotide changes with unclear function besides the functional one that gives rise to the allele number. This is due to the increasing

amount of sequence information submitted to the website and the apparent lack of additional function/information of these new suballeles. In addition, many suballeles may not be true novel alleles but only variations in the size of the gene studied and/or sequence resolution differences between different research groups/studies. The current guideline is not to assign new letter alleles unless they are described together with the novel finding of a functional variant.

For many colleagues in the field, it has become a standard procedure to submit new alleles to the website in order to receive an allelic name before submission to a journal. This has been encouraged by the committee and helps in unifying allele nomenclature and may also assist the authors since the allele composition and the way it has been characterized is reviewed beforehand. In addition, correct choice of reference sequence is ensured and inconsistencies may be cleared.

In the very near future, 27 allelic P450s are presented on the CYP-allele website. At the time of defence of this thesis, close to 180 000 visits have been registered since the website got started 6 years ago. There are currently 700-1000 visits per week from all parts of the world and from academic research institutions as well as from the industry. The possibility to easily access a summary of characterized alleles and their functional consequences is an invaluable tool that saves much time for individual researchers and aids in defining relevant genotype targets in future studies.

SUMMARY AND CONCLUSION

The current study has examined genetic factors potentially explaining the variability in enzyme expression and activity in the CYP2C and CYP3A subfamilies of drug metabolizing enzymes.

1. We have investigated genetic factors that may contribute to CYP3A interindividual variability in Caucasian adults at three levels:

   A. By the identification and characterization of possible novel CYP3A members

   B. By the evaluation of the importance of polymorphic CYP3A5 expression

   C. By determining the contribution of polymorphic CYP3A7 expression

   We were able to conclude that:

   - A yet unidentified member of the CYP3A subfamily, CYP3A43, is present in the human genome
   - The mRNA expression of the novel CYP3A43 was insignificant compared to CYP3A4 in adult human liver
   - The CYP3A43 protein was not properly expressed in heterologous expression systems
   - The CYP3A43 enzyme does not contribute to variation in CYP3A activity due to its very low expression and apparent lack of translation to protein

   - CYP3A5 is polymorphically expressed in approximately 10% of livers and its expression is almost completely explained by the *CYP3A5*1* allele
   - The expression of both CYP3A5 mRNA and protein is much lower than that of CYP3A4
   - CYP3A5 enzyme expression does not contribute to CYP3A levels to a significant extent in the general Caucasian population

   - CYP3A7 protein is expressed in adult liver at surprisingly high levels, contrary to the previous view of CYP3A7 as a fetus-specific isoform
   - CYP3A7 is expressed in approximately 10% of adult human livers and the expression is partially associated with the *CYP3A7* genotype
   - The expression of CYP3A7 protein is considerably less than that of CYP3A4, but equal to that of CYP3A5

- CYP3A7, although being expressed at unexpectedly high levels in adult liver, can not be considered as a major determinant for variation in CYP3A activity

In summary, part of the variation in CYP3A expression and activity can be explained by polymorphic CYP3A5 and CYP3A7 expression. However, variation in CYP3A activity appears not to be explained by any single major factor, but is rather reflected in a complex network of variability affecting CYP3A expression and activity. This effect is probably exerted at several levels; for example variable CYP3A expression due to *CYP3A* polymorphism, enzyme induction and inhibition events, or polymorphism in genes other than those studied here.

Since a large part of the interindividual variation in CYP3A activity has been suggested to be of genetic origin, other yet unidentified genetic factors may be exerting an effect on CYP3A activity. The *CYP3A4* gene has been extensively studied regarding genetic polymorphism, but no major findings on allelic variation affecting drug metabolism has been found. Thus, genetic variation elsewhere in the genome may show to be important in the future, such as those found in microRNA sequences or in genes encoding other products regulating CYP3A expression. Since a vast number of studies have tried to elucidate the genetic basis for CYP3A variation without any major success, it would be valuable to confirm the general impact of genetic factors on CYP3A activity by for example twin studies, which has not yet been carried out.

2. By investigating polymorphism behind variability in CYP2C19 extensive metabolism we could conclude that:

    - We identified a novel *CYP2C19* allele, *CYP2C19*17*, with a -806C>T and a -3402C>T single nucleotide polymorphism
    - The -806 polymorphism introduces a binding site for hepatic nuclear protein that is specific for the *CYP2C19*17* allele
    - The *CYP2C19*17* allele confers increased transcriptional activity of the *CYP2C19* gene
    - Carriers of the *CYP2C19*17* allele have an increased CYP2C19 enzyme activity as measured by two different probe drugs in two racially different populations
    - Based on correlation studies, we expect an almost 40% lower omeprazole AUC after a single oral dose in subjects of the *CYP2C19*17/17* genotype compared to those of the *CYP2C19*1/*1* genotype

In summary, we have identified a novel *CYP2C19* allele conferring increased CYP2C19 enzyme activity. Carriers of the *CYP2C19*17* allele are expected to experience a higher risk of non-response in the treatment of gastric ulcer due to increased metabolism of antiulcer drugs, a beneficial effect in treatment with certain antithrombotic drugs due to increased bioactivation of prodrug to active drug, and a potentially increased risk of non-response in antidepressant treatment due to increased metabolism of certain antidepressants.

In the future, it would be valuable to further evaluate the effect of the *CYP2C19*17* allele on the pharmacokinetics and also treatment response to CYP2C19 substrate drugs. Specifically, the direct impact of *CYP2C19*17* on drug AUC both after single and continuous drug intake would be necessary in order to validate our predictions on omeprazole AUC after a single dose. In fact, in collaboration with the Department of Clinical Pharmacology, Karolinska Institutet, Huddinge, we are currently running *in vivo* studies to investigate the effect of the *CYP2C19*17* allele on the AUC of CYP2C19 substrate drugs, both after single doses and during steady-state. Investigations on the relevance of a clinical *CYP2C19*17* genotype test to adjust drug dosage would be appropriate, especially with regard to antiulcer treatment, where the *CYP2C19* genotype is of unquestionable importance for treatment outcome. It is possible that the identification of individuals with a rapid CYP2C19 metabolism could lead to more efficient individualized treatment regimens in Caucasian and African populations, where the *CYP2C19*17* allele is common. Thus, future studies evaluating the impact of *CYP2C19*17* on drug levels and treatment outcome should be considered very relevant. To investigate the effect of the *CYP2C19*17* allele on escitalopram plasma levels and response, we are currently collaborating with Dr Aitchison who is in charge of a multi-center pharmacogenetics and pharmacogenomics study of response to antidepressants (GENDEP; GENome-based therapeutic drugs for DEPression).

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REFERENCES

1. Walsh, B. T., Seidman, S. N., Sysko, R., and Gould, M. (2002) *Jama* **287**, 1840-1847
2. Nemeroff, C. B., and Owens, M. J. (2004) *CNS Spectr* **9**, 23-31
3. Ingelman-Sundberg, M. (2004) *Trends Pharmacol Sci* **25**, 193-200
4. Eichelbaum, M., Ingelman-Sundberg, M., and Evans, W. E. (2006) *Annu Rev Med* **57**, 119-137
5. Nelson, D. R., Zeldin, D. C., Hoffman, S. M., Maltais, L. J., Wain, H. M., and Nebert, D. W. (2004) *Pharmacogenetics* **14**, 1-18
6. Gardiner, S. J., and Begg, E. J. (2006) *Pharmacol Rev* **58**, 521-590
7. Ingelman-Sundberg, M. (2005) *Pharmacogenomics J* **5**, 6-13
8. Kawanishi, C., Lundgren, S., Agren, H., and Bertilsson, L. (2004) *Eur J Clin Pharmacol* **59**, 803-807
9. Rau, T., Wohlleben, G., Wuttke, H., Thuerauf, N., Lunkenheimer, J., Lanczik, M., and Eschenhagen, T. (2004) *Clin Pharmacol Ther* **75**, 386-393
10. Kirchheiner, J., Nickchen, K., Bauer, M., Wong, M. L., Licinio, J., Roots, I., and Brockmoller, J. (2004) *Mol Psychiatry* **9**, 442-473
11. Koren, G., Cairns, J., Chitayat, D., Gaedigk, A., and Leeder, S. J. (2006) *Lancet* **368**, 704
12. Wojnowski, L., and Kamdem, L. K. (2006) *Expert Opin Drug Metab Toxicol* **2**, 171-182
13. Klotz, U., Schwab, M., and Treiber, G. (2004) *Basic Clin Pharmacol Toxicol* **95**, 2-8
14. Faber, M. S., Jetter, A., and Fuhr, U. (2005) *Basic Clin Pharmacol Toxicol* **97**, 125-134
15. Sellers, E. M., Tyndale, R. F., and Fernandes, L. C. (2003) *Drug Discov Today* **8**, 487-493
16. Binder, E. B., and Holsboer, F. (2006) *Ann Med* **38**, 82-94
17. Steimer, W., Zopf, K., von Amelunxen, S., Pfeiffer, H., Bachofer, J., Popp, J., Messner, B., Kissling, W., and Leucht, S. (2005) *Clin Chem* **51**, 376-385
18. Suzuki, Y., Sawamura, K., and Someya, T. (2006) *Neuropsychopharmacology* **31**, 825-831
19. Neurath, M. F., Kiesslich, R., Teichgraber, U., Fischer, C., Hofmann, U., Eichelbaum, M., Galle, P. R., and Schwab, M. (2005) *Clin Gastroenterol Hepatol* **3**, 1007-1014
20. Desta, Z., Zhao, X., Shin, J. G., and Flockhart, D. A. (2002) *Clin Pharmacokinet* **41**, 913-958
21. Furuta, T., Shirai, N., Sugimoto, M., Nakamura, A., Hishida, A., and Ishizaki, T. (2005) *Drug Metab Pharmacokinet* **20**, 153-167
22. Sanderson, S., Emery, J., and Higgins, J. (2005) *Genet Med* **7**, 97-104
23. Kamali, F. (2006) *Curr Opin Hematol* **13**, 357-361
24. Lehmann, D. F., Medicis, J. J., and Franklin, P. D. (2003) *J Clin Pharmacol* **43**, 1316-1323
25. de Leon, J. (2006) *Expert Rev Mol Diagn* **6**, 277-286
26. Xie, H. G., Kim, R. B., Wood, A. J., and Stein, C. M. (2001) *Annu Rev Pharmacol Toxicol* **41**, 815-850
27. Ingelman-Sundberg, M., Oscarson, M., and McLellan, R. A. (1999) *Trends Pharmacol Sci* **20**, 342-349
28. Gerbal-Chaloin, S., Pascussi, J. M., Pichard-Garcia, L., Daujat, M., Waechter, F., Fabre, J. M., Carrere, N., and Maurel, P. (2001) *Drug Metab Dispos* **29**, 242-251

29. Zineh, I., Pebanco, G. D., Aquilante, C. L., Gerhard, T., Beitelshees, A. L., Beasley, B. N., and Hartzema, A. G. (2006) *Ann Pharmacother*
30. Zhang, W., Gordon, M., Press, O. A., Rhodes, K., Vallbohmer, D., Yang, D. Y., Park, D., Fazzone, W., Schultheis, A., Sherrod, A. E., Iqbal, S., Groshen, S., and Lenz, H. J. (2006) *Pharmacogenet Genomics* **16**, 475-483
31. Mehra, R., and Burtness, B. (2006) *Expert Opin Biol Ther* **6**, 951-962
32. Chan, Y. C., Valenti, D., Mansfield, A. O., and Stansby, G. (2000) *Br J Surg* **87**, 266-272
33. Nagar, S., and Blanchard, R. L. (2006) *Drug Metab Rev* **38**, 393-409
34. Bakker, R. C., and Brandjes, D. P. (1997) *Pharm World Sci* **19**, 126-132
35. Pascussi, J. M., Gerbal-Chaloin, S., Drocourt, L., Maurel, P., and Vilarem, M. J. (2003) *Biochim Biophys Acta* **1619**, 243-253
36. Goodwin, B., Hodgson, E., and Liddle, C. (1999) *Mol Pharmacol* **56**, 1329-1339
37. Goodwin, B., Moore, L. B., Stoltz, C. M., McKee, D. D., and Kliewer, S. A. (2001) *Mol Pharmacol* **60**, 427-431
38. Xie, W., Barwick, J. L., Simon, C. M., Pierce, A. M., Safe, S., Blumberg, B., Guzelian, P. S., and Evans, R. M. (2000) *Genes Dev* **14**, 3014-3023
39. Honkakoski, P., Zelko, I., Sueyoshi, T., and Negishi, M. (1998) *Mol Cell Biol* **18**, 5652-5658
40. Burk, O., Tegude, H., Koch, I., Hustert, E., Wolbold, R., Glaeser, H., Klein, K., Fromm, M. F., Nuessler, A. K., Neuhaus, P., Zanger, U. M., Eichelbaum, M., and Wojnowski, L. (2002) *J Biol Chem* **277**, 24280-24288
41. Drocourt, L., Ourlin, J. C., Pascussi, J. M., Maurel, P., and Vilarem, M. J. (2002) *J Biol Chem* **277**, 25125-25132
42. Rushmore, T. H., and Kong, A. N. (2002) *Curr Drug Metab* **3**, 481-490
43. Paine, M. F., Hart, H. L., Ludington, S. S., Haining, R. L., Rettie, A. E., and Zeldin, D. C. (2006) *Drug Metab Dispos* **34**, 880-886
44. Shimada, T., Yamazaki, H., Mimura, M., Inui, Y., and Guengerich, F. P. (1994) *J Pharmacol Exp Ther* **270**, 414-423
45. Rawden, H. C., Carlile, D. J., Tindall, A., Hallifax, D., Galetin, A., Ito, K., and Houston, J. B. (2005) *Xenobiotica* **35**, 603-625
46. Greenblatt, D. J., Harmatz, J. S., von Moltke, L. L., Wright, C. E., Durol, A. L., Harrel-Joseph, L. M., and Shader, R. I. (2000) *J Pharmacol Exp Ther* **293**, 435-443
47. Ozdemir, V., Kalowa, W., Tang, B. K., Paterson, A. D., Walker, S. E., Endrenyi, L., and Kashuba, A. D. (2000) *Pharmacogenetics* **10**, 373-388
48. Gorski, J. C., Hall, S. D., Jones, D. R., VandenBranden, M., and Wrighton, S. A. (1994) *Biochem Pharmacol* **47**, 1643-1653
49. Lacroix, D., Sonnier, M., Moncion, A., Cheron, G., and Cresteil, T. (1997) *Eur J Biochem* **247**, 625-634
50. Williams, J. A., Ring, B. J., Cantrell, V. E., Jones, D. R., Eckstein, J., Ruterbories, K., Hamman, M. A., Hall, S. D., and Wrighton, S. A. (2002) *Drug Metab Dispos* **30**, 883-891
51. Ohmori, S., Nakasa, H., Asanome, K., Kurose, Y., Ishii, I., Hosokawa, M., and Kitada, M. (1998) *Biochim Biophys Acta* **1380**, 297-304
52. Gibbs, M. A., Thummel, K. E., Shen, D. D., and Kunze, K. L. (1999) *Drug Metab Dispos* **27**, 180-187
53. Daly, A. K. (2006) *Clin Pharmacokinet* **45**, 13-31

54. Koch, I., Weil, R., Wolbold, R., Brockmoller, J., Hustert, E., Burk, O., Nuessler, A., Neuhaus, P., Eichelbaum, M., Zanger, U., and Wojnowski, L. (2002) *Drug Metab Dispos* **30**, 1108-1114
55. Lamba, J. K., Lin, Y. S., Schuetz, E. G., and Thummel, K. E. (2002) *Adv Drug Deliv Rev* **54**, 1271-1294
56. Schuetz, E. G., Beck, W. T., and Schuetz, J. D. (1996) *Mol Pharmacol* **49**, 311-318
57. Ghauharali, R. I., Westerhoff, H. V., Dekker, H., and Lankelma, J. (1996) *Biochim Biophys Acta* **1278**, 213-222
58. Tubic, M., Wagner, D., Spahn-Langguth, H., Bolger, M. B., and Langguth, P. (2006) *Pharm Res* **23**, 1712-1720
59. Lin, J. H., and Yamazaki, M. (2003) *Clin Pharmacokinet* **42**, 59-98
60. Hakkola, J., Raunio, H., Purkunen, R., Saarikoski, S., Vahakangas, K., Pelkonen, O., Edwards, R. J., Boobis, A. R., and Pasanen, M. (2001) *Biol Neonate* **80**, 193-201
61. Wrighton, S. A., Brian, W. R., Sari, M. A., Iwasaki, M., Guengerich, F. P., Raucy, J. L., Molowa, D. T., and Vandenbranden, M. (1990) *Mol Pharmacol* **38**, 207-213
62. Aoyama, T., Yamano, S., Waxman, D. J., Lapenson, D. P., Meyer, U. A., Fischer, V., Tyndale, R., Inaba, T., Kalow, W., Gelboin, H. V., and et al. (1989) *J Biol Chem* **264**, 10388-10395
63. Hustert, E., Haberl, M., Burk, O., Wolbold, R., He, Y. Q., Klein, K., Nuessler, A. C., Neuhaus, P., Klattig, J., Eiselt, R., Koch, I., Zibat, A., Brockmoller, J., Halpert, J. R., Zanger, U. M., and Wojnowski, L. (2001) *Pharmacogenetics* **11**, 773-779
64. Paulussen, A., Lavrijsen, K., Bohets, H., Hendrickx, J., Verhasselt, P., Luyten, W., Konings, F., and Armstrong, M. (2000) *Pharmacogenetics* **10**, 415-424
65. Kuehl, P., Zhang, J., Lin, Y., Lamba, J., Assem, M., Schuetz, J., Watkins, P. B., Daly, A., Wrighton, S. A., Hall, S. D., Maurel, P., Relling, M., Brimer, C., Yasuda, K., Venkataramanan, R., Strom, S., Thummel, K., Boguski, M. S., and Schuetz, E. (2001) *Nat Genet* **27**, 383-391
66. Wrighton, S. A., and Vandenbranden, M. (1989) *Arch Biochem Biophys* **268**, 144-151
67. Greuet, J., Pichard, L., Bonfils, C., Domergue, J., and Maurel, P. (1996) *Biochem Biophys Res Commun* **225**, 689-694
68. Goldstein, J. A., Faletto, M. B., Romkes-Sparks, M., Sullivan, T., Kitareewan, S., Raucy, J. L., Lasker, J. M., and Ghanayem, B. I. (1994) *Biochemistry* **33**, 1743-1752
69. Nakamura, K., Goto, F., Ray, W. A., McAllister, C. B., Jacqz, E., Wilkinson, G. R., and Branch, R. A. (1985) *Clin Pharmacol Ther* **38**, 402-408
70. Kupfer, A., and Preisig, R. (1984) *Eur J Clin Pharmacol* **26**, 753-759
71. Masimirembwa, C., Bertilsson, L., Johansson, I., Hasler, J. A., and Ingelman-Sundberg, M. (1995) *Clin Pharmacol Ther* **57**, 656-661
72. Persson, I., Aklillu, E., Rodrigues, F., Bertilsson, L., and Ingelman-Sundberg, M. (1996) *Pharmacogenetics* **6**, 521-526
73. de Morais, S. M., Wilkinson, G. R., Blaisdell, J., Nakamura, K., Meyer, U. A., and Goldstein, J. A. (1994) *J Biol Chem* **269**, 15419-15422
74. De Morais, S. M., Wilkinson, G. R., Blaisdell, J., Meyer, U. A., Nakamura, K., and Goldstein, J. A. (1994) *Mol Pharmacol* **46**, 594-598
75. Ibeanu, G. C., Blaisdell, J., Ghanayem, B. I., Beyeler, C., Benhamou, S., Bouchardy, C., Wilkinson, G. R., Dayer, P., Daly, A. K., and Goldstein, J. A. (1998) *Pharmacogenetics* **8**, 129-135

76. Chang, M., Dahl, M. L., Tybring, G., Gotharson, E., and Bertilsson, L. (1995) *Pharmacogenetics* **5**, 358-363
77. He, N., Yan, F. X., Huang, S. L., Wang, W., Xiao, Z. S., Liu, Z. Q., and Zhou, H. H. (2002) *Eur J Clin Pharmacol* **58**, 15-18
78. Goodwin, C. S., Mendall, M. M., and Northfield, T. C. (1997) *Lancet* **349**, 265-269
79. Kirchheiner, J., Brosen, K., Dahl, M. L., Gram, L. F., Kasper, S., Roots, I., Sjoqvist, F., Spina, E., and Brockmoller, J. (2001) *Acta Psychiatr Scand* **104**, 173-192
80. Butura, A., Johansson, I., Nilsson, K., Warngard, L., Ingelman-Sundberg, M., and Schuppe-Koistinen, I. (2004) *Biochem Pharmacol* **67**, 1249-1258
81. Krusekopf, S., Roots, I., and Kleeberg, U. (2003) *Eur J Pharmacol* **466**, 7-12
82. Drocourt, L., Pascussi, J. M., Assenat, E., Fabre, J. M., Maurel, P., and Vilarem, M. J. (2001) *Drug Metab Dispos* **29**, 1325-1331
83. Liu, F., Song, Y., and Liu, D. (1999) *Gene Ther* **6**, 1258-1266
84. Rodriguez-Antona, C., Sayi, J. G., Gustafsson, L. L., Bertilsson, L., and Ingelman-Sundberg, M. (2005) *Biochem Biophys Res Commun* **338**, 299-305
85. Wang, J., Pitarque, M., and Ingelman-Sundberg, M. (2006) *Biochem Biophys Res Commun* **340**, 491-497
86. Westlind-Johnsson, A., Malmebo, S., Johansson, A., Otter, C., Andersson, T. B., Johansson, I., Edwards, R. J., Boobis, A. R., and Ingelman-Sundberg, M. (2003) *Drug Metab Dispos* **31**, 755-761
87. Edwards, R. J., Adams, D. A., Watts, P. S., Davies, D. S., and Boobis, A. R. (1998) *Biochem Pharmacol* **56**, 377-387
88. Andersson, T., Miners, J. O., Veronese, M. E., Tassaneeyakul, W., Meyer, U. A., and Birkett, D. J. (1993) *Br J Clin Pharmacol* **36**, 521-530
89. Abelo, A., Andersson, T. B., Antonsson, M., Naudot, A. K., Skanberg, I., and Weidolf, L. (2000) *Drug Metab Dispos* **28**, 966-972
90. Tybring, G., Bottiger, Y., Widen, J., and Bertilsson, L. (1997) *Clin Pharmacol Ther* **62**, 129-137
91. Christensen, M., Andersson, K., Dalen, P., Mirghani, R. A., Muirhead, G. J., Nordmark, A., Tybring, G., Wahlberg, A., Yasar, U., and Bertilsson, L. (2003) *Clin Pharmacol Ther* **73**, 517-528
92. Daniel, H. I., and Edeki, T. I. (1996) *Psychopharmacol Bull* **32**, 219-230
93. Tybring, G., Nordin, J., Bergman, T., and Bertilsson, L. (1997) *Pharmacogenetics* **7**, 355-360
94. Tybring, G., and Bertilsson, L. (1992) *Pharmacogenetics* **2**, 241-243
95. Emoto, C., and Iwasaki, K. (2006) *Xenobiotica* **36**, 219-233
96. Domanski, T. L., Finta, C., Halpert, J. R., and Zaphiropoulos, P. G. (2001) *Mol Pharmacol* **59**, 386-392
97. Gellner, K., Eiselt, R., Hustert, E., Arnold, H., Koch, I., Haberl, M., Deglmann, C. J., Burk, O., Buntefuss, D., Escher, S., Bishop, C., Koebe, H. G., Brinkmann, U., Klenk, H. P., Kleine, K., Meyer, U. A., and Wojnowski, L. (2001) *Pharmacogenetics* **11**, 111-121
98. Finta, C., and Zaphiropoulos, P. G. (2002) *J Biol Chem* **277**, 5882-5890
99. Neve, E. P., and Ingelman-Sundberg, M. (2000) *J Biol Chem* **275**, 17130-17135
100. Muller-Enoch, D., and Gruler, H. (2000) *Z Naturforsch [C]* **55**, 747-752
101. Kumarakulasingham, M., Rooney, P. H., Dundas, S. R., Telfer, C., Melvin, W. T., Curran, S., and Murray, G. I. (2005) *Clin Cancer Res* **11**, 3758-3765

102. Downie, D., McFadyen, M. C., Rooney, P. H., Cruickshank, M. E., Parkin, D. E., Miller, I. D., Telfer, C., Melvin, W. T., and Murray, G. I. (2005) *Clin Cancer Res* **11**, 7369-7375
103. Cauffiez, C., Lo-Guidice, J. M., Chevalier, D., Allorge, D., Hamdan, R., Lhermitte, M., Lafitte, J. J., Colombel, J. F., Libersa, C., and Broly, F. (2004) *Hum Mutat* **23**, 101
104. Shchepotina, E. G., Nikishina, M. V., Vavilin, V. A., and Lyakhovich, V. V. (2005) *Bull Exp Biol Med* **140**, 726-728
105. Thompson, E. E., Kuttab-Boulos, H., Yang, L., Roe, B. A., and Di Rienzo, A. (2006) *Pharmacogenomics J* **6**, 105-114
106. Stone, A., Ratnasinghe, L. D., Emerson, G. L., Modali, R., Lehman, T., Runnells, G., Carroll, A., Carter, W., Barnhart, S., Rasheed, A. A., Greene, G., Johnson, D. E., Ambrosone, C. B., Kadlubar, F. F., and Lang, N. P. (2005) *Cancer Epidemiol Biomarkers Prev* **14**, 1257-1261
107. Zeigler-Johnson, C., Friebel, T., Walker, A. H., Wang, Y., Spangler, E., Panossian, S., Patacsil, M., Aplenc, R., Wein, A. J., Malkowicz, S. B., and Rebbeck, T. R. (2004) *Cancer Res* **64**, 8461-8467
108. Lin, Y. S., Dowling, A. L., Quigley, S. D., Farin, F. M., Zhang, J., Lamba, J., Schuetz, E. G., and Thummel, K. E. (2002) *Mol Pharmacol* **62**, 162-172
109. Quaranta, S., Chevalier, D., Allorge, D., Lo-Guidice, J. M., Migot-Nabias, F., Kenani, A., Imbenotte, M., Broly, F., Lacarelle, B., and Lhermitte, M. (2006) *Xenobiotica* **36**, 1191-1200
110. Hu, Y. F., Qiu, W., Liu, Z. Q., Zhu, L. J., Tu, J. H., Wang, D., Li, Z., He, J., Zhong, G. P., Zhou, G., and Zhou, H. H. (2006) *Clin Exp Pharmacol Physiol* **33**, 1093-1098
111. Mourad, M., Wallemacq, P., De Meyer, M., Brandt, D., Van Kerkhove, V., Malaise, J., Chaib Eddour, D., Lison, D., and Haufroid, V. (2006) *Clin Chem Lab Med* **44**, 1192-1198
112. Haufroid, V., Mourad, M., Van Kerckhove, V., Wawrzyniak, J., De Meyer, M., Eddour, D. C., Malaise, J., Lison, D., Squifflet, J. P., and Wallemacq, P. (2004) *Pharmacogenetics* **14**, 147-154
113. Goto, M., Masuda, S., Kiuchi, T., Ogura, Y., Oike, F., Okuda, M., Tanaka, K., and Inui, K. (2004) *Pharmacogenetics* **14**, 471-478
114. Yu, S., Wu, L., Jin, J., Yan, S., Jiang, G., Xie, H., and Zheng, S. (2006) *Transplantation* **81**, 46-51
115. Burckart, G. J., Hutchinson, I. V., and Zeevi, A. (2006) *Pharmacogenomics J* **6**, 301-310
116. Bosch, T. M., Doodeman, V. D., Smits, P. H., Meijerman, I., Schellens, J. H., and Beijnen, J. H. (2006) *Mol Diagn Ther* **10**, 175-185
117. Fredericks, S., Moreton, M., Reboux, S., Carter, N. D., Goldberg, L., Holt, D. W., and MacPhee, I. A. (2006) *Transplantation* **82**, 705-708
118. Masuda, S., and Inui, K. (2006) *Pharmacol Ther* **112**, 184-198
119. Park, J. Y., Kim, K. A., Park, P. W., Lee, O. J., Kang, D. K., Shon, J. H., Liu, K. H., and Shin, J. G. (2006) *Clin Pharmacol Ther* **79**, 590-599
120. Greenblatt, D. J., von Moltke, L. L., Harmatz, J. S., Ciraulo, D. A., and Shader, R. I. (1993) *J Clin Psychiatry* **54 Suppl**, 4-11; discussion 12-14
121. Mirghani, R. A., Sayi, J., Aklillu, E., Allqvist, A., Jande, M., Wennerholm, A., Eriksen, J., Herben, V. M., Jones, B. C., Gustafsson, L. L., and Bertilsson, L. (2006) *Pharmacogenet Genomics* **16**, 637-645
122. Chotivanich, K., Sattabongkot, J., Udomsangpetch, R., Looareesuwan, S., Day, N. P., Coleman, R. E., and White, N. J. (2006) *Antimicrob Agents Chemother* **50**, 1927-1930

123. Stevens, J. C., Hines, R. N., Gu, C., Koukouritaki, S. B., Manro, J. R., Tandler, P. J., and Zaya, M. J. (2003) *J Pharmacol Exp Ther* **307**, 573-582
124. Tateishi, T., Watanabe, M., Moriya, H., Yamaguchi, S., Sato, T., and Kobayashi, S. (1999) *Biochem Pharmacol* **57**, 935-939
125. Schuetz, J. D., Schuetz, E. G., Thottassery, J. V., Guzelian, P. S., Strom, S., and Sun, D. (1996) *Mol Pharmacol* **49**, 63-72
126. Usui, T., Saitoh, Y., and Komada, F. (2003) *Biol Pharm Bull* **26**, 510-517
127. Rodriguez-Antona, C., Jande, M., Rane, A., and Ingelman-Sundberg, M. (2005) *Clin Pharmacol Ther* **77**, 259-270
128. Torimoto, N., Ishii, I., Toyama, K. I., Hata, M., Tanaka, K., Shimomura, H., Nakamura, H., Ariyoshi, N., Ohmori, S., and Kitada, M. (2006) *Drug Metab Dispos*
129. Tateishi, T., Nakura, H., Asoh, M., Watanabe, M., Tanaka, M., Kumai, T., Takashima, S., Imaoka, S., Funae, Y., Yabusaki, Y., Kamataki, T., and Kobayashi, S. (1997) *Life Sci* **61**, 2567-2574
130. Marill, J., Cresteil, T., Lanotte, M., and Chabot, G. G. (2000) *Mol Pharmacol* **58**, 1341-1348
131. Marill, J., Capron, C. C., Idres, N., and Chabot, G. G. (2002) *Biochem Pharmacol* **63**, 933-943
132. Lengfelder, E., Saussele, S., Weisser, A., Buchner, T., and Hehlmann, R. (2005) *Crit Rev Oncol Hematol* **56**, 261-274
133. Cooper, A. J. (2003) *Australas J Dermatol* **44**, 97-105
134. van de Kerkhof, P. C., Kleinpenning, M. M., de Jong, E. M., Gerritsen, M. J., van Dooren-Greebe, R. J., and Alkemade, H. A. (2006) *J Dermatolog Treat* **17**, 198-204
135. Cheer, S. M., and Foster, R. H. (2000) *Am J Clin Dermatol* **1**, 307-314; discussion 315-306
136. Miller, K. K., Cai, J., Ripp, S. L., Pierce, W. M., Jr., Rushmore, T. H., and Prough, R. A. (2004) *Drug Metab Dispos* **32**, 305-313
137. Smit, P., van Schaik, R. H., van der Werf, M., van den Beld, A. W., Koper, J. W., Lindemans, J., Pols, H. A., Brinkmann, A. O., de Jong, F. H., and Lamberts, S. W. (2005) *J Clin Endocrinol Metab* **90**, 5313-5316
138. Whelan, A. M., Jurgens, T. M., and Bowles, S. K. (2006) *Ann Pharmacother* **40**, 836-849
139. Kocis, P. (2006) *Am J Health Syst Pharm* **63**, 2201-2210
140. Leowattana, W. (2004) *Clin Chim Acta* **341**, 1-15
141. Kim, M. J., Bertino, J. S., Jr., Gaedigk, A., Zhang, Y., Sellers, E. M., and Nafziger, A. N. (2002) *Clin Pharmacol Ther* **72**, 192-199
142. Ohneda, K., and Yamamoto, M. (2002) *Acta Haematol* **108**, 237-245
143. Zhou, M., and Ouyang, W. (2003) *Immunol Res* **28**, 25-37
144. Divine, J. K., Staloch, L. J., Haveri, H., Jacobsen, C. M., Wilson, D. B., Heikinheimo, M., and Simon, T. C. (2004) *Am J Physiol Gastrointest Liver Physiol* **287**, G1086-G1099
145. Suzuki, E., Evans, T., Lowry, J., Truong, L., Bell, D. W., Testa, J. R., and Walsh, K. (1996) *Genomics* **38**, 283-290
146. Dame, C., Sola, M. C., Lim, K. C., Leach, K. M., Fandrey, J., Ma, Y., Knopfle, G., Engel, J. D., and Bungert, J. (2004) *J Biol Chem* **279**, 2955-2961
147. Zhu, Q. S., Qian, B., and Levy, D. (2004) *Biochim Biophys Acta* **1676**, 251-260
148. Ivanov, G. S., Kater, J. M., Jha, S. H., Stutius, E. A., Sabharwal, R., Tricarico, M. D., Ginsburg, G. S., and Ozer, J. S. (2003) *Gene* **323**, 31-42
149. Bossard, P., and Zaret, K. S. (1998) *Development* **125**, 4909-4917

150. Denson, L. A., McClure, M. H., Bogue, C. W., Karpen, S. J., and Jacobs, H. C. (2000) *Gene* **246**, 311-320
151. Tolman, K. G., Sanders, S. W., Buchi, K. N., Karol, M. D., Jennings, D. E., and Ringham, G. L. (1997) *J Clin Gastroenterol* **24**, 65-70
152. Ishii, G., Suzuki, A., Oshino, S., Shiraishi, H., and Otani, K. (2007) *Neurosci Lett* **411**, 77-80
153. Roberts, R. L., Luty, S. E., Mulder, R. T., Joyce, P. R., and Kennedy, M. A. (2004) *Am J Med Genet B Neuropsychiatr Genet* **127**, 90-93
154. Kirchheiner, J., Lang, U., Stamm, T., Sander, T., and Gallinat, J. (2006) *J Clin Psychopharmacol* **26**, 440-442
155. Yu, A. M., Idle, J. R., Herraiz, T., Kupfer, A., and Gonzalez, F. J. (2003) *Pharmacogenetics* **13**, 307-319
156. Yu, A. M., Idle, J. R., Byrd, L. G., Krausz, K. W., Kupfer, A., and Gonzalez, F. J. (2003) *Pharmacogenetics* **13**, 173-181
157. Yu, K. S., Yim, D. S., Cho, J. Y., Park, S. S., Park, J. Y., Lee, K. H., Jang, I. J., Yi, S. Y., Bae, K. S., and Shin, S. G. (2001) *Clin Pharmacol Ther* **69**, 266-273
158. Cho, J. Y., Yu, K. S., Jang, I. J., Yang, B. H., Shin, S. G., and Yim, D. S. (2002) *Br J Clin Pharmacol* **53**, 393-397
159. Bonnet, U. (2003) *CNS Drug Rev* **9**, 97-140
160. Jiang, Z. P., Shu, Y., Chen, X. P., Huang, S. L., Zhu, R. H., Wang, W., He, N., and Zhou, H. H. (2002) *Eur J Clin Pharmacol* **58**, 109-113
161. Shimoda, K., Someya, T., Yokono, A., Morita, S., Hirokane, G., Takahashi, S., and Okawa, M. (2002) *J Clin Psychopharmacol* **22**, 371-378
162. van der Weide, J., van Baalen-Benedek, E. H., and Kootstra-Ros, J. E. (2005) *Ther Drug Monit* **27**, 478-483
163. Perry, P. J., Zeilmann, C., and Arndt, S. (1994) *J Clin Psychopharmacol* **14**, 230-240
164. Yokono, A., Morita, S., Someya, T., Hirokane, G., Okawa, M., and Shimoda, K. (2001) *J Clin Psychopharmacol* **21**, 549-555
165. Noguchi, T., Shimoda, K., and Takahashi, S. (1993) *J Affect Disord* **29**, 267-279
166. Wang, J. H., Liu, Z. Q., Wang, W., Chen, X. P., Shu, Y., He, N., and Zhou, H. H. (2001) *Clin Pharmacol Ther* **70**, 42-47
167. Xu, Z. H., Wang, W., Zhao, X. J., Huang, S. L., Zhu, B., He, N., Shu, Y., Liu, Z. Q., and Zhou, H. H. (1999) *Br J Clin Pharmacol* **48**, 416-423
168. Obach, R. S., Cox, L. M., and Tremaine, L. M. (2005) *Drug Metab Dispos* **33**, 262-270
169. Kobayashi, K., Ishizuka, T., Shimada, N., Yoshimura, Y., Kamijima, K., and Chiba, K. (1999) *Drug Metab Dispos* **27**, 763-766
170. Schweizer, E., Rynn, M., Mandos, L. A., Demartinis, N., Garcia-Espana, F., and Rickels, K. (2001) *Int Clin Psychopharmacol* **16**, 137-143
171. Hyttel, J. (1982) *Prog Neuropsychopharmacol Biol Psychiatry* **6**, 277-295
172. Yu, B. N., Chen, G. L., He, N., Ouyang, D. S., Chen, X. P., Liu, Z. Q., and Zhou, H. H. (2003) *Drug Metab Dispos* **31**, 1255-1259
173. Herrlin, K., Yasui-Furukori, N., Tybring, G., Widen, J., Gustafsson, L. L., and Bertilsson, L. (2003) *Br J Clin Pharmacol* **56**, 415-421
174. Rudberg, I., Hendset, M., Uthus, L. H., Molden, E., and Refsum, H. (2006) *Ther Drug Monit* **28**, 102-105
175. Bech, P., Andersen, H. F., and Wade, A. (2006) *Pharmacopsychiatry* **39**, 128-134

176. George, T., Theodoros, M. T., Chiu, E., Krapivensky, N., Hokin, A., and Tiller, J. W. (1999) *Aust N Z J Psychiatry* **33**, 889-895
177. Shirai, N., Furuta, T., Moriyama, Y., Okochi, H., Kobayashi, K., Takashima, M., Xiao, F., Kosuge, K., Nakagawa, K., Hanai, H., Chiba, K., Ohashi, K., and Ishizaki, T. (2001) *Aliment Pharmacol Ther* **15**, 1929-1937
178. Furuta, T., Ohashi, K., Kosuge, K., Zhao, X. J., Takashima, M., Kimura, M., Nishimoto, M., Hanai, H., Kaneko, E., and Ishizaki, T. (1999) *Clin Pharmacol Ther* **65**, 552-561
179. Schwab, M., Schaeffeler, E., Klotz, U., and Treiber, G. (2004) *Clin Pharmacol Ther* **76**, 201-209
180. Take, S., Mizuno, M., Ishiki, K., Nagahara, Y., Yoshida, T., Inaba, T., Yamamoto, K., Okada, H., Yokota, K., Oguma, K., and Shiratori, Y. (2003) *Am J Gastroenterol* **98**, 2403-2408
181. Furuta, T., Shirai, N., Watanabe, F., Honda, S., Takeuchi, K., Iida, T., Sato, Y., Kajimura, M., Futami, H., Takayanagi, S., Yamada, M., Ohashi, K., Ishizaki, T., and Hanai, H. (2002) *Clin Pharmacol Ther* **72**, 453-460
182. Kawamura, M., Ohara, S., Koike, T., Iijima, K., Suzuki, J., Kayaba, S., Noguchi, K., Hamada, S., Noguchi, M., and Shimosegawa, T. (2003) *Aliment Pharmacol Ther* **17**, 965-973
183. Kurzawski, M., Gawronska-Szklarz, B., Wrzesniewska, J., Siuda, A., Starzynska, T., and Drozdzik, M. (2006) *Eur J Clin Pharmacol* **62**, 877-880
184. Sakai, T., Aoyama, N., Kita, T., Sakaeda, T., Nishiguchi, K., Nishitora, Y., Hohda, T., Sirasaka, D., Tamura, T., Tanigawara, Y., Kasuga, M., and Okumura, K. (2001) *Pharm Res* **18**, 721-727
185. Hokari, K., Sugiyama, T., Kato, M., Saito, M., Miyagishima, T., Kudo, M., Nishikawa, K., Ishizuka, J., Komatsu, Y., Mizushima, T., Kagaya, H., Hige, S., Takeda, H., and Asaka, M. (2001) *Aliment Pharmacol Ther* **15**, 1479-1484
186. Shimatani, T., Inoue, M., Kuroiwa, T., and Horikawa, Y. (2004) *Aliment Pharmacol Ther* **19**, 113-122
187. Furuta, T., Shirai, N., Kodaira, M., Sugimoto, M., Nogaki, A., Kuriyama, S., Iwaizumi, M., Yamade, M., Terakawa, I., Ohashi, K., Ishizaki, T., and Hishida, A. (2007) *Clin Pharmacol Ther*
188. Hulot, J. S., Bura, A., Villard, E., Azizi, M., Remones, V., Goyenvalle, C., Aiach, M., Lechat, P., and Gaussem, P. (2006) *Blood* **108**, 2244-2247
189. Inomata, S., Nagashima, A., Itagaki, F., Homma, M., Nishimura, M., Osaka, Y., Okuyama, K., Tanaka, E., Nakamura, T., Kohda, Y., Naito, S., Miyabe, M., and Toyooka, H. (2005) *Clin Pharmacol Ther* **78**, 647-655
190. Fukasawa, T., Yasui-Furukori, N., Suzuki, A., Inoue, Y., Tateishi, T., and Otani, K. (2005) *Eur J Clin Pharmacol* **61**, 791-795
191. Bun, H., Coassolo, P., Gouezo, F., Serradimigni, A., and Cano, J. P. (1986) *Int J Clin Pharmacol Ther Toxicol* **24**, 287-293
192. Contin, M., Sangiorgi, S., Riva, R., Parmeggiani, A., Albani, F., and Baruzzi, A. (2002) *Ther Drug Monit* **24**, 737-741
193. Kosaki, K., Tamura, K., Sato, R., Samejima, H., Tanigawara, Y., and Takahashi, T. (2004) *Brain Dev* **26**, 530-534