Clinical Pharmacogenetics of Olanzapine

with Focus on FMO Gene Polymorphisms

MAO MAO SÖDERBERG
Pharmacogenetics is the study of variability in drug response attributed to genetic variation. Olanzapine (OLA) is a widely used antipsychotic drug for schizophrenia treatment. The pharmacokinetics of OLA display large inter-individual variation leading to multiple-fold differences in drug exposure between patients at a given dose. This variation in turn gives rise to the need of individualized dosing in order to avoid concentration-dependent adverse effects and therapeutic failure. The observed variability has been partially explained by environmental and physiological factors. Genetically determined differences in drug metabolism represent a less studied source of variability. Precluded contribution by cytochrome P450 (CYP) 2D6 calls for evaluation of the other major OLA metabolizing enzymes. The objective of this thesis was to study pharmacogenetic influence of flavin-containing monoxygenase (FMO) 1 and 3, CYP1A2 and uridine diphosphate-glucuronosyltransferase (UGT) 1A4 on therapeutic OLA exposure. We conducted genetic association studies applying gene re-sequencing and genotyping of candidate and tagging SNPs.

Patients carrying the *FMO1* *6* allele displayed increased dose-adjusted concentrations (C/Ds) of OLA, in serum as well as cerebrospinal fluid. Patients who were homozygous for the FMO3 K158-G308 compound variant showed reduced C/Ds of OLA N-oxide metabolite, but no alteration in OLA exposure. This compound variant is expected to have clinical relevance primarily for non-African populations, since low frequencies were detected among native Africans. Deviation in OLA exposure was observed in carrier of a rare FMO3 mutation, predicted in silico to affect gene splicing. Reduced OLA exposure was observed in UGT1A4*3 carriers. The CYP1A2 -163(A) (CYP1A2*1F) variant was not associated with increase in CYP1A2-catalyzed OLA metabolism or reduction in OLA exposure. Correlations were detected for two cis-acting variants within the inter-genetic region of the CYP1A cluster and a trans-acting variant located upstream the locus encoding aryl hydrocarbon receptor. The inconsistent data reported for CYP1A2*1F could be explained by presence of ethnic specific haplotype structures incorporating the -163(A) variant.

A continuously improved understanding of the wide range of factors that can influence pharmacokinetics and pharmacodynamics will increase the likelihood of achieving optimal treatment response for individual patients.

**Keywords:** olanzapine, pharmacogenetics, drug metabolism, schizophrenia, therapeutic drug monitoring, FMO1, FMO3, CYP1A2, UGT1A4

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Grant me the serenity
to accept the things I
cannot change,
courage to change the things I can,
and wisdom to know the difference.

-Reinhold Niebuhr
To my mother
List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


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N.B. The authors Mao Mao Söderberg and Mao Mao refer to the same person after name change due to marriage.
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Abbreviations

AHR  aryl hydrocarbon receptor
ANCOVA  analysis of covariance
ANOVA  analysis of variance
C/D  dose-adjusted concentration
CSF  cerebrospinal fluid
CYP  cytochrome P450 monooxygenase
DMO  4'-desmethyl olanzapine
DNA  deoxyribonucleic acid
EPS  extra pyramidal symptom
FGA  first-generation (or typical) antipsychotics
FMO  flavin-containing monooxygenase
FMO1  flavin-containing monooxygenase isoform 1
FMO3  flavin-containing monooxygenase isoform 3
HWE  Hardy-Weinberg equilibrium
LD  linkage disequilibrium
MAF  minor allele frequency
OLA  olanzapine
PD  pharmacodynamics
PK  pharmacokinetics
SGA  second-generation (or atypical) antipsychotics
SNP  single nucleotide polymorphism
TDM  therapeutic drug monitoring
UGT  uridine diphosphate-glucuronosyltransferase
Introduction

Metabolism as determinant in drug response

Drug response is a result of interaction between an administrated drug and its target site (such as receptors and enzymes) in the body. Two concepts are central while studying this drug-target interaction, pharmacodynamics (PD) and pharmacokinetics (PK). The former describes the mechanisms of drug action, i.e. interactions with drug target(s) in the body and the events elicited by these interactions, whereas the latter displays drug disposition in the body including absorption, distribution, metabolism and excretion (Fig 1).

Sufficient drug exposure at target site is a prerequisite for successful pharmacotherapy. Lipophilicity of a drug enables passage through biological membranes, e.g. the blood-brain-barrier for psychotropic agents before reaching the target sites in the brain. Prior to final excretion from the body, lipophilic drugs need to undergo, metabolism, structural modification to become more water-soluble. Compared to the parent compound, the products generated under metabolic processes are commonly inactive, but may also be pharmacologically active (e.g. morphine as metabolite of codeine), show altered property (e.g. salicylic acid lacking the antiplatelet activity of aspirin), or even induce toxicity (e.g. hepatotoxicity of paracetamol due to its alkylating metabolite).1

Biochemical reactions during metabolism are often classified as Phase I (oxidation, reduction and hydrolysis) and Phase II (conjugation with polar endogenous molecules) reactions (Fig 1). They occur mainly in the liver but also in other organs such as intestine, lungs and kidney. Although Phase I reaction usually precedes Phase II reactions, it is not necessarily so in all cases. The cytochrome P450 monooxygenase (CYP) system is most extensively studied for its role in Phase I reactions and the enzyme family of uridine diphosphate-glucuronosyltransferases (UGT) in Phase II reactions. However, not all metabolic reactions involve CYPs and UGTs, examples of other enzyme groups are the flavin-containing monooxygenase (FMO) system and monoamine oxidases for Phase I reactions and N-acetyltransferases and glutathione S-transferases for Phase II reactions.1

For drugs undergoing extensive metabolism, the activity of the enzymes involved will influence the systemic drug exposure. For individual treatments, systemic exposure that exceeds or falls below the therapeutic window will lead to suboptimal drug response or induce adverse effects in patients.
Metabolic capacity varies in a population and is influenced by factors of various origins, i.e. environmental (e.g. smoking habits, diet), physiological (gender, age), epigenetic (histone modification and DNA methylation) and genetic (missense mutation, gene deletion and duplications). The observation of inherited traits in drug metabolism was the first example showing the importance of genetics for drug response, a field known today as "pharmacogenetics".

Figure 1. An overview of drug response and the major processes involved. PD, pharmacodynamics; PK, pharmacokinetics

Concept of pharmacogenetics

Pharmacogenetic studies aim to identify and quantify the effect of genetic markers that are associated with or are direct causes of the observed drug response differences between individuals, upon receiving the same treatment. In the long run, a continuously improved understanding on the wide range of factors, that can influence PK and PD, will increase the likelihood of achieving optimal treatment response for individual patients.

Inherited differences in individuals’ capacity to conduct chemical transformations were understood long before the discovery of the DNA double helix. In his book “Inborn error of metabolism” from 1909, Sir Archibald Garrod, an English physician, described alcaptonuria as a result of “chemical individuality” and concluded that this was due to inherited conditions. Being ahead of his time, Garrod also proposed the idea that toxic manifestation is caused by deviation from normal metabolism due to overproduction, an incomplete chain of catalytic reactions or formation of abnormal product. In 1950s, Arno Motulsky refined these ideas on the role of genetics in drug
response⁵ and the term “pharmacogenetics” was first introduced by Friedrich Vogel⁶. Genetic influence on metabolic traits can be demonstrated with studies in twins or family pedigrees. To study variability in a population at large, frequency distribution of a phenotypic parameter (e.g. a metabolic ratio, see Fig 2) in a population of biologically unrelated individuals can be plotted. If multimodal distribution of the data is obtained, individuals with deviating response can be identified at the far right or left of the frequency distribution (Fig 2). With the emergence of molecular genetics and decades of clinical observations on inherited differences in drug response, pharmacogenetics became a recognized science in the period spanning from the 1950s to the 1990s.⁷

From phenotype to genotype

Early pharmacogenetic research was characterized by phenotype-driven assessment of variation in drug metabolizing enzymes. Discovery of glucose-6-phosphate dehydrogenase deficiency was made in American black soldiers suffering from primaquine-induced hemolysis.⁸ Prolonged apnea and paralysis observed in some individuals treated with the muscle relaxant suxamethonium resulted in detection of serum cholinesterase deficiency.⁹,¹⁰ Polymorphic acetylation was demonstrated by bimodal frequency distribution of plasma isoniazid concentrations among treated subjects, and slow acetylators were determined to be carriers of an autosomal recessive trait.¹¹

Debrisoquine and CYP2D6

First indication of polymorphic metabolism by CYPs was reported in the late 60s for nortriptyline and imipramine, later known as substrates of CYP2D6.¹² The observed difference in steady-state plasma concentrations between two groups of patients after a fixed oral dose was subsequently showed to be genetic in nature.¹³ In the mid-1970s, the urinary ratio of the antihypertensive drug debrisoquine to its 4-hydroxy metabolite, termed “metabolic ratio” (Fig 2) was found bimodally distributed in Caucasian populations.¹⁴,¹⁵ The subjects with highest metabolic ratios, above the antimode of 12.6, were classified as poor metabolizers (PM, Fig 2) of debrisoquine and displayed intensified effect of the drug. The majority of individuals, with metabolic ratios below the antimode, were classified as extensive metabolizers (EM, Fig 2). Subsequent studies revealed that the PMs of debrisoquine were also deficient in metabolizing other compounds such as sparteine, metoprolol, codeine, tricyclic antidepressants and dextromethorphan.¹⁶-¹⁸

Prior to elucidation of the underlying molecular mechanisms, these compounds, all being substrates of CYP2D6, were used as “probe drugs” to clas-
sify subjects regarding their metabolic capacity, so called phenotyping. The metabolic ratio, based on concentration measurement in urinary or plasma samples, was used to assess absolute or partial deficiency of the enzyme. The genetic basis of the two metabolizer phenotypes was revealed 10-15 years after the initial clinical observations, with cloning of the human CYP2D6 cDNA\textsuperscript{19} and characterization of the altered restriction fragment length polymorphism (RFLP) patterns in PMs\textsuperscript{20-22}. In the 1990s, multiplication of the CYP2D6 genes was detected in individuals with extremely high metabolic capacity, termed ultra-rapid metabolizers (UM, Fig 2).\textsuperscript{23,24} Identification of gene variants associated with various levels of CYP2D6 activities enabled prediction of the phenotype based on genetic constitution of an individual.\textsuperscript{25,26} The inter-individual variation in CYP2D6 activity was also found to differ between populations.\textsuperscript{27,28} Caucasians metabolize debrisoquine on average faster than Orientals do, while the incidence of PM is higher among Caucasians than in other major ethnicities.\textsuperscript{29}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{metabolic_ratio.png}
\caption{Concept illustration of metabolic ratio, its distribution in two different populations and three categories of metabolizers; UM= ultra-rapid metabolizer, EM=extensive metabolizer, PM=poor metabolizer. The average level of metabolic activity may differ between populations (Population 2 > Population 1). (Adapted from Bertilsson et al. 1992 \textsuperscript{28})}
\end{figure}
Trimethylamine and FMOs

Trimethylamine (TMA), a volatile tertiary amine, is naturally present at high concentrations in marine fishes, but can also be produced through intestinal bacterial degradation from dietary choline. Trimethylamine was early shown to undergo hepatic oxidation that forms the N-oxide metabolite prior to urinary excretion. Case reports on deficiency in converting the malodorous trimethylamine (TMA) to its odorless N-oxide have been documented since 1970. This rare condition, termed trimethylaminuria (TMAuria), was shown to be genetic in nature. As direct elimination of TMA in urine, sweat and breath gives the characteristic fishy odor, TMAuria is also known as the fish-odor syndrome. TMA N-oxidation ability was subsequently characterized to be polymorphic in a British population. The finding has been verified in various populations of different ethnic origins, e.g. Caucasian Canadians, Oriental groups as Thai and Chinese, and also in Jordanian, Ecuadorian and New Guinean populations. Reduced, but not absent, capacity was recognized in a subject if proportion of TMA excreted as its N-oxide fell within the range of 50 - 80%, compared to more than 90% normally excreted. Occurrence of mild deficiency was reported to vary from 0.6% up to 11% among the studied populations.

Prior to the 1960s, oxidation of the NADPH-dependent heteroatom-containing compounds was thought to be mediated essentially by microsomal CYPs. FMO was first described in 1964, a novel monooxygenase containing flavin adenine dinucleotide (FAD) as redox cofactor, not heme as CYPs do. But like CYPs, FMO also utilizes molecular oxygen and the reducing agent NADPH while catalyzing oxidation. After its purification from pig liver in 1971, identification and characterization of multiple forms of FMO were carried out from the mid 1980s to early 1990s. Of the five active human FMO isoforms (FMO1 – 5) identified (Table 1), FMO3 was the major hepatic form and also exhibited a clear substrate preference for tertiary amines. In 1997, defective FMO3 was established as underlying cause of TMAuria with identification of nonsense and missense mutations in affected subjects.

Although FMOs are capable of metabolizing an exceptionally wide range of xenobiotics with very little structural features in common, they are considered to play a minor role compared to CYPs in drug metabolism. This view is rooted in the problems experienced while studying FMOs. FMOs and CYPs share a number of overlapping characteristics including expression pattern in tissues and cells, substrate specificity as well as type of catalytic reaction. Specific inhibitors of FMOs are not available. Together with thermal instability of FMO activity shown in absence of NADPH and the frequent inter-conversion between the parent amines and the N-oxide metabolites, these features have contributed to the difficulty and uncertainty in interpreting the relative contribution of FMO from CYP in drug metabolism.
Less therapeutic compounds are known FMO substrates compared to those of CYPs.\textsuperscript{56} The FMO-mediated oxidation of nitrogen (N) or sulfur (S) is seldom recognized as the predominant metabolic reaction for a substrate.\textsuperscript{56} Phenotyping for a given FMO is therefore difficult to perform.

Table 1. \textit{Features of human FMO enzyme family}

<table>
<thead>
<tr>
<th>Isoform</th>
<th>Major sites of expression</th>
<th>Clinical relevance</th>
</tr>
</thead>
<tbody>
<tr>
<td>FMO1</td>
<td>Kidney, intestine, liver (fetal)</td>
<td>Alteration in expression associated with amyotrophic lateral sclerosis\textsuperscript{60,61} Exaggerated drug response in FMO1 knockout mouse\textsuperscript{62} SNPs as risk factors for nicotine dependence\textsuperscript{63}</td>
</tr>
<tr>
<td>FMO2</td>
<td>Lung</td>
<td>Functional protein expressed mainly in Africans Ethnic difference in response to tuberculosis treatment\textsuperscript{64,65}</td>
</tr>
<tr>
<td>FMO3</td>
<td>Liver (adult)</td>
<td>Deleterious variants cause trimethylaminuria\textsuperscript{66,67}</td>
</tr>
<tr>
<td>FMO4</td>
<td>low expression levels in all studied tissues</td>
<td>Poorly understood</td>
</tr>
<tr>
<td>FMO5</td>
<td>Liver (adult), intestine</td>
<td>Poorly understood</td>
</tr>
</tbody>
</table>

However, FMOs do possess features that are distinct from CYPs and desirable for drug development.\textsuperscript{57} The enzymes are not known to be as easily induced by xenobiotics as the CYPs are. FMOs are unusual for not having the substrate binding as the rate-limiting step in its catalytic cycle. Due to the stability of the oxidant, i.e. 4a-hydroperoxyflavin of FAD, the enzyme is present in an activated form. FMO operates on full speed once its substrate has gained access to the catalytic site.\textsuperscript{55,58} In general, FMOs produce inactive and water-soluble metabolites, but a few exceptions to this have also been reported.\textsuperscript{55,56} Among the known substrates, many are active in the central nervous system (CNS), including chlorpromazine, clozapine, OLA, perazine, ziprasidone, amphetamine, methamphetamine and nicotine.\textsuperscript{56}

Results from in vitro studies support the observation of large variability between individuals, at expression levels, for several FMOs.\textsuperscript{68,69} Expression of FMO1 is highest in adult kidney (47±9 pmol/mg protein), and is detected to a less extent also in intestines.\textsuperscript{70} The expression level of FMO3 in adult liver (60±43 pmol/mg protein) is comparable to that of CYP3A4 and CYP2C.\textsuperscript{68} On the other hand, similar gene expression levels of all five FMO isoforms have been reported in human brain tissue.\textsuperscript{49} FMO activity has also been characterized in mammalian brain microsomes including human\textsuperscript{71-73} and detected in various brain regions in animal studies.\textsuperscript{74,75} FMO1 gene deletion in mice treated with imipramine, a FMO1 isoform-specific substrate, was shown to result in elevated systemic concentrations of the parent compound and increased cerebral levels of CYP-mediated metabolite, desipramine.\textsuperscript{62} Exaggerated adverse pharmacologic response, as body tremor and
spasm, was also observed in the knockout mice.\textsuperscript{62} On the contrary, the sedative effect of imipramine was abolished in the knockout mice, suggesting a pharmacological role of the N-oxide metabolite.\textsuperscript{62}

Genetics

Genetic polymorphisms

The hereditary information of any human being is coded by 3.2 billion nucleotides base pairs and stored on 23 chromosome pairs.\textsuperscript{76} Although only monozygotic twins have completely identical genomes, the difference is not large between individuals (estimated 99.9\% identical between two genomes).\textsuperscript{77} Still, this small fraction contributes to the observed genetic heritability among individuals. DNA sequence changes, when located in sequence elements of functional relevance, can alter expression, translocation or functionality of the encoded gene products, proteins, which are essential for all biological process.\textsuperscript{76}

When a DNA mutation occurs with a frequency greater than 1\% in the general population, it is a genetic polymorphism. Categories of polymorphism include single nucleotide polymorphism (SNP) as variation of a single base pair at a fixed position (Fig 3), insertion/deletion, duplication, inversion and copy number variation. SNP is shown to be an exceedingly common form of polymorphism and observed once every 1000-2000 base pairs in the genome.\textsuperscript{78} For CYP2D6 described above, genetic variants causing the wide spectrum of enzyme activity include, in PMs, SNPs causing frame shift, SNPs affecting gene splicing, insertion, and deletion of the whole gene whereas gene duplications and multi-duplications are identified in UMs.\textsuperscript{29} The frequencies of these alleles vary between populations, explaining the previously reported inter-ethnic differences in CYP2D6 activity.\textsuperscript{29}
Genetic association studies

The SNPs that influence drug response and clinical outcome make up only a tiny fraction of all the SNPs existing in the human genome (Fig 4). To identify this group of SNPs without having to assess all the SNPs, patterns of genetic structure are applied in designing genetic association studies. When a group of adjacent SNPs on the same chromosome tend to be inherited together, they form a haplotype (Fig 3). The size of a haplotype depends on the extent of recombination that have occurred between the loci. Non-random association between SNPs is commonly observed in the human genome and is expressed by degree of linkage disequilibrium (LD). The LD structure of one specific genomic region varies between regions as well as ethnic populations. Measure of LD is under the assumption of Hardy-Weinberg equilibrium (HWE). For a SNP in HWE, it states a stable distribution of genotypes (AA, Aa and aa) and their constant association with frequencies of the two alleles, p and q=(1-p) (AA=p^2, Aa=2pq and aa=q^2) through generations within a large population. The correlation assumes random mating and lack of genetic influences including natural selection, mutations and random drift. Assessment of HWE services also as a quality control for potential genotyping errors.
Figure 4. Hierarchy of SNPs based on function and clinical relevance.

LD is assessed in its simplest form between two SNP loci and expressed by two parameters, D’ and $r^2$, with different properties. D’ shows the probability for historical recombination between the loci in a given population whereas $r^2$ is used to determine the sample size required to detect association between a trait and the causal locus by using an associated SNP. Two SNP loci are in complete LD with each other if $r^2=1$. They are totally independent of each other when $r^2=0$. The lower the value of $r^2$ is, the larger the sample size that is required for association studies. Instead of assessing individual markers, a subset of the associated SNPs, called tagSNPs (Fig 3), can be selected to represent other SNPs nearby that are in strong LD with them. Compared to single marker analysis, a small number of tagSNPs can capture haplotype variation within a larger genomic region and increase statistical power.

Pharmacogenetic studies can be designed as hypothesis generating or hypothesis testing. Hypothesis generating studies, genome-wide association studies (GWAS), are assumption free and assess genetic variants on genome scale, with aim to detect novel correlations to clinical parameters of interest. In hypothesis testing studies, candidate gene association studies, one or more gene(s) and genetic variant(s) are selected with presumed involvement in the predefined endpoint, based on an acceptable level of biological understanding (i.e. annotated functionality or previous implicated association). For both study designs, the ability to detect variant-endpoint association is influenced by the factors described in above sections including LD, sample size, SNP frequency and the effect size of the gene product in generating the drug response.
Antipsychotics for schizophrenia

The term schizophrenia was coined around 1911 by Eugen Bleuler (1857-1939), a Swiss psychiatrist, and defined with four main symptom descriptions: affective disturbance, autism, associative disturbances and ambivalence (Bleuler’s 4A). They are now considered so called negative symptoms, one of two categories of symptoms often used to describe schizophrenia today. The other category is so called positive symptoms, resembling manifestations of psychosis, i.e. hallucinations and delusions. Current diagnostic criteria for schizophrenia are described thoroughly in The Diagnostic and Statistical Manual of Mental Disorders 4th Edition (DSM-IV-TR).

Schizophrenia has near 1% prevalence with an early onset in life (teens, early adulthood). Sustained recovery is reported in less than 14% of patients within the first five years after a psychotic episode. The disorder is often chronic with high risk for relapse, more frequently observed in patients with poor compliance than in those showing good drug adherence. It impairs social functioning and reduces lifespan of affected patients, as much as 15-25 years.

Two generations of drugs

Antipsychotics, the cornerstone of modern treatment for schizophrenia, started with the introduction of chlorpromazine (Fig 5) in the early 1950s. As the first antipsychotic medication, it revolutionized pharmacotherapy of schizophrenia by greatly improving quality of life for patients and contributing to a decrease in institutionalization, the then standard treatment. This was followed by intensive research and development of new structurally related compounds over the past 60 years (Table 2). Treatment with chlorpromazine and its successors, however, was complicated by high rates of neurologic side effects of involuntary movements, such as tardive dyskinesia and Parkinsonism, broadly termed extra pyramidal symptoms (EPS).

A new line of research started with the development of clozapine (Fig 5) in the 1960s, an effective compound with low propensity for EPS. Instead clozapine was challenged with a different side effect, agranulocytosis, caus-
ing acute decrease in white blood cell account, which restricted its clinical use. Clozapine was re-introduced in the 1990s as a result of documented superiority over other antipsychotic agents in treatment resistant patients and markedly reduced risk for the potentially fatal idiosyncratic side effect through safety routine of monitoring white blood cells. It too became a prototype for development of a new class of antipsychotics, often termed as second-generation or atypical antipsychotics (SGA), the earlier ones being referred to as first-generation or typical antipsychotics (FGA) (Table 2). Over time, FGAs have been gradually replaced by the growing number of SGAs.88,89

Table 2. Two classes of antipsychotic drugs

<table>
<thead>
<tr>
<th>First generation antipsychotics (typical)</th>
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<tbody>
<tr>
<td>1950s Chlorpromazine</td>
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</tr>
<tr>
<td>1960s Haloperidol Fluphenazine Perphenazine Trifluoperazine Loxapine</td>
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</tr>
<tr>
<td>1970s Molindone Pimozide</td>
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</table>

<table>
<thead>
<tr>
<th>Second generation antipsychotics (atypical)</th>
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</tr>
</thead>
<tbody>
<tr>
<td>1990s Clozapine Risperidone Olanzapine Quetiapine</td>
<td></td>
</tr>
<tr>
<td>2000s Ziprasidone Aripiprazole Paliperidone Iloperidone Asenapine</td>
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</table>

The need for treatment individualization

Antipsychotics are generally effective in treating positive symptoms and reduce risk of relapse, but are weaker in managing negative symptoms.89 Outcome of schizophrenia treatment, however, is highly variable and individual, with 30-40% of patients showing no initial response.90 Results from large pragmatic non-industry-sponsored clinical trials (CATIE, CUtLASS, EUFEST studies) in recent years failed to provide clear support for SGAs being more effective than FGAs.90-92 Two exceptions were observed. The first was clozapine having increased efficacy in patient who were resistant to other treatments and the other was olanzapine (OLA), a successor of clozapine, having the lowest rate in treatment discontinuation.90 On the other hand, a meta-analysis based on 150 double-blind studies concluded better overall efficacy with small to medium effect size and lower incidence of EPS for four SGAs (clozapine, OLA, amisulpride and risperidone) compared to FGAs.93

Although the superiority of SGAs in drug efficacy remains a topic of debate, views on their side-effect profile are rather aligned. Instead of having EPS as the primary concern, metabolic disturbance (i.e. weight gain, hyperlipidemia, and diabetes) are commonly seen in patients treated with SGAs.94,95 Having cardiovascular disease as long term consequence, the
metabolic adverse effects may thus increase both morbidity and mortality for schizophrenia.\textsuperscript{94,95} Treatment with clozapine and OLA are burdened with highest incidence/risk for developing metabolic disturbances.\textsuperscript{90-92}

Results from clinical trial and meta-analyses define an average level of observed treatment outcome in each patient population studied but generally overlook individual differences between patients. To achieve better clinical use of available drugs, it is desirable to establish sub-group specific treatments and doses and, thus, identify determinants of individual variation in drug disposition and response.

Olanzapine, an atypical antipsychotic

Olanzapine (OLA), (2-methyl-4-(4-methyl-1-piperazinyl)-10H-thieno[2,3-b][1,5]benzodiazepine) is a SGA (atypical) compound launched as Zyprexa by Eli Lilly and Company (Indianapolis, USA) in 1996 \textsuperscript{96} (Fig 6). It is now a commonly prescribed antipsychotic for acute and maintenance treatment of schizophrenia with estimated sales of 4.6 billion dollar in total at the end of 2011 (Lilly 2011 Annual Report, http://investor.lilly.com/annuals.cfm). OLA resembles the precursor compound, clozapine, both structurally and pharmacologically, but is not associated with agranulocytosis.\textsuperscript{97,98}

PD and PK

Meta-analysis studies assessing dose-response correlation in OLA treatment have suggested a near-maximal effective dose to be >16mg daily.\textsuperscript{99} Additional therapeutic advantage in dosing >20mg daily is reported only for treatment resistant or markedly ill patients, not apparent in non-treatment-resistant patients with diagnose chronic schizophrenia.\textsuperscript{100} In a clinical study with fixed-doses, increased adverse events were reported for a dose at 40mg daily.\textsuperscript{101}

Through neuroimaging techniques such as PET (positron emission tomography), OLA has shown binding affinity for a range of neuron receptors, dopamine, serotonin, cholinergic muscarinic, \(\alpha\)-adrenergic and histamine receptors. Although the precise mechanism of drug action is still unclear for antipsychotic drugs, blockade of dopamine- (D\textsubscript{2}) receptors is a shared feature and believed to control positive symptoms of schizophrenia.\textsuperscript{102} A range of 60-80\% striatal D\textsubscript{2} receptors occupancy is regarded as optimal to gain satisfactory response and reduce risk for EPS.\textsuperscript{103} In patients which have reached steady-state, OLA induced 43%-80\% D\textsubscript{2} blockade within the recommended clinical dose range of 5-20mg daily, 83%-88\% within 30-40mg daily as well as a near complete occupancy of serotonin receptors at low dose of 5mg daily.\textsuperscript{104}
OLA is well absorbed orally as > 65% of the dose is detected in urine and fecal samples.\textsuperscript{105} No data on absolute oral bioavailability based on intravenous administration is available. When given orally, plasma concentration increases proportionally with dose, (also known as linear or first-order kinetics) throughout the clinical dosage range.\textsuperscript{106} Pharmacokinetic characteristics of OLA at steady-state are reported to be consistent with those seen after a single dose.\textsuperscript{106} The maximum plasma concentration is reached within 5-8 hours and the average time required for the plasma concentration to fall by half (half-life, $t_{1/2}$) is estimated to 33 hours (90% within 21-54 hours) in adult healthy individuals.\textsuperscript{106} The steady-state concentration can thus be reached in about a week.\textsuperscript{106}

**Concentration-effect relationship**

Based on the 60-80% D\textsuperscript{2} receptor occupancy, a plasma concentration interval between 20 to 40ng/mL for 12-hour post-dose sampling has been estimated as an optimal therapeutic range for OLA.\textsuperscript{104,107} A threshold at 23ng/mL 10-16 hours post dose (or 9ng/mL predose or trough) has been suggested as the lower end of the therapeutic window.\textsuperscript{107,108} However, the predictive strength of the breakpoint of 23ng/mL is only moderate and even at a given serum concentration, the antipsychotic effect is largely individual. High median plasma OLA concentrations have been shown in patients with adverse effects compared to those without.\textsuperscript{101,109}

Drug concentrations in the central nervous system would be expected to be more closely related to therapeutic effects of centrally acting drugs than serum concentrations are. A strong linear correlation between serum and cerebrospinal fluid (CSF) concentrations of OLA has been reported in patients on long-term treatment with OLA.\textsuperscript{110} However, a significantly faster decline of serum OLA concentration has been observed as compared to reduction of D\textsuperscript{2} receptor occupancy in healthy volunteers, suggesting dissociation in kinetic profile between the two compartments.\textsuperscript{111}

**Metabolic pathways of olanzapine**

Metabolite characterization in urine samples identified 9 compounds.\textsuperscript{105} Direct glucuronidation is the primary route of OLA biotransformation and also a to humans unique metabolic pathway.\textsuperscript{112,113} OLA also undergoes hepatic oxidative metabolism through N-demethylation, N-oxidation and 2-alkyl hydroxylation\textsuperscript{105} (Fig 6). The metabolites identified are considerably less active than the parent compound in vivo.\textsuperscript{114} Approximately 50-60% of an orally administered dose is eliminated via urine and about 30% via feces.\textsuperscript{105}
Figure 6. Metabolic pathways of olanzapine in humans (adapted from Kassahun et al., 1997)
N-glucuronidation and UGT1A4/2B10

The attachment of glucuronic acid to the target compound is catalyzed by UGTs. The subfamilies 1A and 2B are most important for drug metabolism. Two N-glucuronide conjugates have been identified, the tertiary OLA 10-N-glucuronide (exists in two isomers) being the main circulating metabolite in plasma and the quaternary OLA 4’-N-glucuronide produced in minor amounts (Fig 6). In humans, N-glucuronidation is a major route of metabolism for tertiary amines, including many clinically used psychoactive drugs such as tricyclic antidepressants and antipsychotics, and usually resulting in the formation of quaternary N-glucuronides. OLA is an exception, additionally having a rare tertiary N-glucuronide metabolite. By quantifying the metabolites detected in urine and fecal samples, about 21-25% of a single dose is estimated to be eliminated as OLA 10-N-glucuronide. This metabolite is thus a quantitatively more important N-glucuronide.

To date, all known human UGT1A and 2B enzymes except UGT1A5 and 2B28 have been screened for their ability to glucuronidate OLA. The observed catalytic activity was first attributed to UGT1A4 and later also to UGT2B10, an orphan isoenzyme with high N-glucuronation activity discovered in 2007. However, UGT2B10 is shown to be most active in the formation of the minor OLA 4’-N-glucuronide, in contrast to UGT1A4 producing both N-glucuronides.

N-demethylation and CYP1A2

The formation of N-desmethyl OLA (DMO) was best correlated with the catalytic activity of CYP1A2 in vitro (Fig 6), although small amounts formed by CYP3A4 and CYP2D6 were also detected. The plasma ratio of DMO to OLA is significantly correlated to OLA clearance ($r^2=0.35$, $P<0.0002$). Hepatic CYP1A2 activity can be estimated in vivo using caffeine as probe drug given that this enzyme catalyzes more than 90% of its systemic clearance. Indeed, significant correlations have been reported between caffeine metabolic ratios and OLA clearance in healthy volunteers, as well as between caffeine metabolic ratios and dose-adjusted concentrations (C/Ds) of plasma OLA in psychiatric patients (Table 3).

Hydroxylation and CYP2D6

The formation of 2-hydroxy OLA was attributed to the polymorphic CYP2D6 activity in vitro (Fig 6). CYP2D6 has earlier been shown to be of importance for the metabolism of most FGAs. However, it appears to be of minor importance for OLA disposition in vivo (Table 3). CYP2D6 PMs
showed no deviating pharmacokinetic characteristics compared to individuals displaying normal CYP2D6 activity\textsuperscript{110,124,126} (Table 3).

N-oxidation and FMOs
The formation of OLA N-oxide is primarily a product of FMO activity (Fig 6).\textsuperscript{119} Limited formation at low rate has also been shown for CYP450 enzymes such as CYP3A4, 1A2, 2D6, 2E1 and 2C9.\textsuperscript{119} Although the catalytic activity was correlated to immunoquantified levels of the FMO isoform 3 (FMO3) in human liver microsome as well as to the FMO3-mediated formation of nicotine N-oxide in vitro\textsuperscript{119}, OLA is not known to be an isoform-specific substrate for FMO3 over other known FMO isoenzymes. The role of FMO in OLA disposition is insufficiently studied, and warrants in vivo evaluation.

Inter-individual variation in olanzapine metabolism
Elimination of OLA has been reported to vary nearly 10-fold within studied populations and exhibits larger variability between individuals than within.\textsuperscript{106,127} Data from various TDM (therapeutic drug monitoring service) studies revealed larger than 25 fold difference in plasma OLA C/Ds.\textsuperscript{109,128-130} Inter-individual variation in OLA exposure is attributed to individual characteristics of the patients.

Non-genetic sources
Smoking
Schizophrenic patients are prone to smoke, a behavior significantly more prevalent than in the general population.\textsuperscript{131} This is of clinical significance as smoking is consistently identified as strong predictor of reduced OLA exposure in vivo, independent of dose levels and other factors.\textsuperscript{109,127,130,132-136} This has been attributed to increased metabolic clearance following enzyme induction caused by polycyclic aromatic hydrocarbons in tobacco smoke.\textsuperscript{137} As much as 50\% lower median plasma OLA C/D as well as higher prescribed dose has been reported for smokers while compared to non-smokers.\textsuperscript{109,132,133} Population kinetic modeling has estimated that 26\% of observed variability in elimination of OLA can be explained by smoking.\textsuperscript{127} Adverse clinical outcomes related to increased drug concentrations following smoking cessation have also been documented.\textsuperscript{138}
**Gender**
OLA clearance is lower in females than in males, a difference estimated to account for 12% of the overall variability by population kinetic modeling.\(^{106,127}\) Women display higher median/mean OLA C/Ds than men at a given dose (30% to 60%), a difference remaining significant after adjustment for body-weight and/or smoking status.\(^{109,130,132,139}\) Hence, the female non-smoker group is predisposed to elevated OLA exposure whereas the male smoker group to suboptimal treatment. Oral contraceptives have been assessed as a potential underlying factor for the observed gender difference. They showed no clinically relevant influence on serum OLA concentrations despite an inhibitory effect on CYP1A2-mediated OLA N-demethylation by ethinyl estradiol.\(^{140}\)

**Concomitant medication**
Inhibitors or inducers of CYP1A2 or UGT1A4 can cause significant, though not necessarily clinically relevant, changes in OLA exposure in vivo. Co-administration of the CYP1A2-inhibitor, fluvoxamine resulted in 2-3 fold increase in plasma OLA C/Ds.\(^{129,141}\) Carbamazepine is known as a strong inducer of CYP1A2. Data from TDM studies reveal about 40 to 70% reduction in median OLA C/Ds in carbamazepine treated patients compared to patients on OLA monotherapy.\(^{109,128,142}\) Lamotrigine may exert inhibitory effect on OLA metabolism as both undergo UGT1A4-mediated glucuronidation. However, impact on OLA pharmacokinetics has been inconsistent, from no effect over the range of 50mg to 200mg lamotrigine daily in healthy volunteers\(^{143}\), significant but mild (16%) increase in mean plasma OLA concentration at 200mg daily in patients\(^{144}\), to a finding of 35% increase in plasma OLA C/Ds observed in smoking patients\(^{145}\). Significant decrease in plasma OLA C/Ds has been reported with co-administration of valproic acid.\(^{146,147}\) Studies based on TDM data, however, have so far shown no influence of valproic acid on plasma OLA.\(^{129,132}\)

**Age, weight and ethnicity**
Age and body weight have been identified as significant factors for plasma OLA concentrations. However, the contribution of age and body weight to increased OLA exposure has proven to be small in the overall concentration variability.\(^{109,129,130,132}\) In two recent studies, increased clearance and reduced plasma concentrations have been reported for patients of African origin compared to other major ethnic groups.\(^{127,135}\)
Pharmacogenetics of olanzapine metabolizing enzymes

UGT1A4/2B10

Both UGT1A4 and 2B10 are highly abundant in human liver but not detected in brain tissue. The UGT1A subfamily is encoded by a single gene locus on chromosome 2q37 with multiple first exons but shared exon 2-5. Of the 13 first exons exist in man, each is spliced to exon 2-5 to produces a unique UGT1A gene/pseudogene. UGT1A4 is one of the nine functional UGT1A isoforms.

Of the ten non-synonymous amino acid changes reported for UGT1A4 (www.pharmacogenomics.pha.ulaval.ca/cms/ugt_alleles/), two common polymorphisms, p.P24T and p.L48V, are most studied. Although, differential effect on in-vitro-glucuronidation have been reported for the two variants depending on substrates tested, increased efficiency is most often seen with the V48 variant and reduced/no activity alteration by the T24 variant. In addition, upstream promoter variants of UGT1A4 (g.-219T, g.-204A and g.-163A) have been shown to modulate transcriptional activity. In clinical studies with OLA treated patients, UGT1A4 has been included as candidate gene in few studies in which only the V48 variant was assessed (Table 3). Significant correlation with reduced serum OLA concentrations is reported in Caucasians, but not in Japanese patients.

UGT2B family is encoded by separated genes located on chromosome 4q13. The UGT2B10 gene consists of 6 exons separated by 5 introns. Few non-synonymous changes are reported with one missense variant, p.D67Y identified to confer reduced level of N-glucuronides of several substrates including OLA (Table 3).

CYP1A2

About 13% of total hepatic CYP content can be accounted by CYP1A2. Hepatic mRNA level of CYP1A2 is reported to vary 40-fold among individuals. The encoded gene, CYP1A2, together with CYP1A1 which is not constitutively expressed in human liver, forms the CYP1A cluster on human chromosome 15q24. The two genes are located in the head-to-head orientation and thus share a common region scattered with regulatory elements. The CYP1A2 gene comprises 7 exons separated by 6 introns. Although numerous polymorphisms are reported for CYP1A2 (http://www.cypalleles.ki.se/cyp1a2.htm), only a few of them have been targets of extensive studies and displayed considerable variation in population distribution.
### Table 3. Data summary on variability in olanzapine pharmacokinetics influenced by genotype and phenotype of olanzapine metabolizing enzymes

<table>
<thead>
<tr>
<th>SNP / Phenotyping marker</th>
<th>Effect</th>
<th>Parameters assessed</th>
<th>Study design</th>
<th>Sample size (M/F)</th>
<th>Smoker Y/N</th>
<th>Ethnic origin</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1A2</td>
<td>*1F, -163C&gt;A</td>
<td>no influence</td>
<td>OLA, DMO, OLA-10-NG, OLA/DMO, OLA-10-NG</td>
<td>51(34/17)</td>
<td>16/35</td>
<td>Japanese</td>
<td>Nozawa et al. 2008</td>
</tr>
<tr>
<td></td>
<td></td>
<td>reduction</td>
<td>OLA C/D</td>
<td>psychiatric patients</td>
<td>121(77/44)</td>
<td>50/71</td>
<td>Swedish</td>
</tr>
<tr>
<td></td>
<td></td>
<td>increase for both glucuronides</td>
<td>Vmax/Km for formation of OLA-10-NG and OLA-4'-NG</td>
<td>in vitro</td>
<td>Eriksson-Ridout et al. 2011</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>no influence on OLA C/D, decrease in OLA-10-NG C/D, increase in Vmax/Km</td>
<td>OLA C/D, OLA-10-NG C/D, Vmax/Km for formation of OLA-10-NG</td>
<td>TDM data from psychiatric patients and in vitro</td>
<td>Haslemo et al. 2012</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP2B10</td>
<td>D67Y, 199G&gt;T</td>
<td>Decrease for both glucuronides</td>
<td>Vmax/Km for formation of OLA-10-NG and OLA-4'-NG</td>
<td>in vitro</td>
<td>Ericksson-Ridout et al. 2011</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>no influence</td>
<td>OLA, DMO, OLA-10-NG, OLA/DMO, OLA-10-NG</td>
<td>51(34/17)</td>
<td>16/35</td>
<td>Japanese</td>
<td>Nozawa et al. 2008</td>
</tr>
<tr>
<td></td>
<td></td>
<td>reduction</td>
<td>OLA C/D, bodyweight</td>
<td>psychiatric patients</td>
<td>73(36/37)</td>
<td>30/43</td>
<td>German</td>
</tr>
<tr>
<td></td>
<td></td>
<td>no influence</td>
<td>OLA C/D</td>
<td>psychiatric patients</td>
<td>121(77/44)</td>
<td>50/71</td>
<td>Swedish</td>
</tr>
<tr>
<td></td>
<td></td>
<td>no influence</td>
<td>OLA, DMO</td>
<td>psychiatric patients</td>
<td>37(25/12)</td>
<td>10/27</td>
<td>Swedish</td>
</tr>
<tr>
<td></td>
<td>*1C, -3860G&gt;A</td>
<td>no influence</td>
<td>OLA, DMO, OLA-10-NG, OLA/DMO, OLA-10-NG</td>
<td>51(34/17)</td>
<td>16/35</td>
<td>Japanese</td>
<td>Nozawa et al. 2008</td>
</tr>
<tr>
<td></td>
<td>*1D, -2467delT</td>
<td>no influence</td>
<td>OLA C/D</td>
<td>psychiatric patients</td>
<td>121(77/44)</td>
<td>50/71</td>
<td>Swedish</td>
</tr>
<tr>
<td>PM, EM by dextromethorphan (saliva)</td>
<td>no influence</td>
<td>AUC and CL(oral)=dose/AUC adjusted for weight</td>
<td>healthy volunteers</td>
<td>17(13/4)</td>
<td>no info</td>
<td>American</td>
<td>Shirley et al. 2003</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4-OH-debrisoquine/debrisoquine (urine, 8hr)</td>
<td>OLA C/D</td>
<td>psychiatric patients</td>
<td>17(13/4)</td>
<td>no info</td>
<td>American</td>
</tr>
</tbody>
</table>

a) steady state obtained in psychiatric patients whereas a single dose given in healthy volunteers
b) no statistic p-values were provided for kinetic analysis in this study

OLAs-10-NG, OLA 10-N-glucuronide; OLA-4'-NG, OLA 4'-N-glucuronide

(Nozawa et al., 2008 134; Ghotbi et al., 2010 136; Erickson-Ridout et al., 2011 117; Haslemo et al., 2012 158; Laika et al., 2009 166; Skogh et al., 2011 110, Hägg et al., 2001 126; Carrillo et al., 2003 124; Shirley et al., 2003 123; Cashman et al., 2008 167)
Significant association with OLA disposition is known for merely one variant, g.-163A (also known as CYP1A2*1F, rs762551C>A, Table 3).\textsuperscript{110,166} None of the other CYP1A2 alleles studied for influence on OLA disposition was found as a significant contributor to inter-individual variation (Table 3). In Caucasian psychiatric patients, the -163A/A genotype carriers displayed on average 22% lower serum OLA concentrations (dose and body weight adjusted), independent of inducing factors.\textsuperscript{166} This C to A nucleotide change in intron 1 is recognized to confer a higher inducibility as well as an elevated basal enzyme activity, mainly in Caucasian cohorts.\textsuperscript{168-171} Replications of the finding in patient cohorts of Asian origin repeatedly failed, despite the high population frequencies of this variant among both ethnicities (60-70%).\textsuperscript{134,169,172-174} Worth noting is the fact that CYP1A2 g.-163C>A was not identified as a representative haplotype tagging SNP across major ethnic groups in CYP1A1/1A2 haplotype construction.\textsuperscript{175}

GWAS studies on searching genetic determinants for habitual caffeine consumption have identified several SNPs located within the inter-genic spacer region of the CYP1A cluster (CYP1A1/CYP1A2, rs2470893C>T and rs2472297C>T), at CYP1A2 locus (rs2472304A>G) as well as within the region upstream the aryl hydrocarbon receptor (AHR) coding locus (rs6968865T>A and rs4410790C>T).\textsuperscript{176-178} In addition to the well-established use of caffeine for CYP1A2-phenotyping, the key role of AHR in regulation of CYP1A2 gene expression\textsuperscript{179} further supports the biological plausibility of the association reported. These SNPs represent new candidate markers for assessment of the CYP1A2 genotype-phenotype relationship.

**FMO3 and FMO1**

Each FMO isoform is encoded by a single gene. The regional location of FMO genes has been refined to chromosome 1q23-25 for FMO1, 2, 3, 4 and 6, which form a gene cluster, whereas FMO5 is located on chromosome 1q21.1.\textsuperscript{47} The sixth isoform, FMO6, is a pseudogene.\textsuperscript{180} In addition to these 6 genes, a second FMO gene cluster, located between FMO5 and the cluster with FMO1 to 6 has also been described in humans. This cluster however contains five pseudogenes, FMO7P, 8P, 9P, 10P and 11P.\textsuperscript{47} They do not encode functional proteins.

In general, clinical pharmacogenetic studies including FMO polymorphisms are rare. Prior to our studies, none of the FMO SNPs have been assessed for association with variability in OLA disposition in vivo. Due to the pattern of tissue distribution relevant for drug metabolism and existing knowledge on clinical importance of each FMO isoform (Table 1), genetic variants of FMO3 and FMO1 are the main points of interest for association studies in this thesis.

Three common FMO3 coding variants, p.E158K, p.V257M, and p.E308G, as well as the K158-G308 compound allele have been best characterized for functional impact on enzyme activity. Variable degrees of re-
duced enzyme activity in a substrate dependent manner were observed.\textsuperscript{181-184} In subjects homozygous for K158 and G308, a transient or mild form of TMAuria can be triggered under exposure to TMA and hormonal influence.\textsuperscript{185,186} In vivo correlation between carriage of both variant alleles and reduced catalytic efficiency has been demonstrated for ranitidine and sulindac.\textsuperscript{187-190} Regarding psychoactive substrates, altered metabolic efficiency associated with K158 has been shown in vitro for amphetamine and methamphetamine.\textsuperscript{191} Reduction in OLA N-oxidation was reported in vitro for the protein variant expressing the K158-G308 compound allele, though supported only by raw data with no data on statistical assessment\textsuperscript{167} (Table 3). On the contrary, the common variants were not associated with inter-individual variation in clozapine metabolism in German patients.\textsuperscript{192}

Additional known coding variants affecting enzyme activity are ethnic specific and/or TMAuria-causing mutations.\textsuperscript{193-196} FMO3 p.D132H and p.L360P were only found in African Americans with reduced in vitro catalytic efficiency associated for the former and enhanced efficiency for the latter.\textsuperscript{197} Among the non-coding gene variants identified, two common upstream variants have been associated in vitro with alteration in promoter activity affecting \textit{FMO3} gene expression, g.-2177G>C causing an activity increase and g.-2106G>A causing an activity reduction.\textsuperscript{198}

The \textit{FMO1} gene locus is more conserved than other \textit{FMO} s.\textsuperscript{193} Four of the five non-synonymous \textit{FMO1} variants detected in gene re-sequencing studies have been studied, p.H97Q, p.I303V, p.I303T and p.R502X. These genetic variants have been characterized with modest activity impact and are rare alleles in general populations.\textsuperscript{193,199,200} Of the common upstream variants identified, only \textit{FMO1} g.-9536C>A, termed \textit{FMO1*6}, is considered to have potential functional impact as it eliminates the binding site of a transcription factor, leading to reduced gene expression in vitro.\textsuperscript{199}
Aim of the thesis

The overall aim of this thesis was to study genetic influence of the drug metabolizing enzymes on inter-individual variability in therapeutic olanzapine exposure, by exploring the significance of FMOs and further evaluating the polymorphic effect of UGT1A4 and CYP1A2.

Specific aims were:

Paper I
- To assess inter- and intra-ethnic variation in distribution of five non-synonymous FMO3 SNPs and their haplotypes in 13 defined ethnic groups from Europe, East Asia and sub-Saharan Africa
- To assess the appropriateness of extrapolating frequency data to corresponding ethnic populations from other continents

Paper II:
- To investigate potential influence of FMO1, FMO3 and UGT1A4 polymorphisms on steady-state concentrations of olanzapine and desmethyl olanzapine in serum and cerebrospinal fluid

Paper III:
- To quantify the steady-state concentrations of olanzapine N-oxide in patients treated with olanzapine
- To further evaluate the correlation between olanzapine concentrations and the two FMO SNPs observed in Paper II
- To search for additional SNP markers within the FMO3 genomic region

Paper IV:
- To validate the reported association of CYP1A2*1F with reduced systemic olanzapine exposure
- To investigate the potential impact of four additional candidate markers at CYP1A1/CYP1A2 and AHR loci on systemic olanzapine exposure
Material and Methods

Subjects

The ethnicity of all study subjects (healthy volunteers and patients) was defined by self-reporting. Informed consent was obtained from the subjects included in Paper I and II. The study presented in Paper I was approved by the local ethics committees of the countries from which the samples were collected. The study presented in Paper II was approved by the Ethics Committee of the Medical Faculty of Linköping University, Sweden, the Swedish Medical Products Agency, and the Swedish Data Inspection Board. The studies presented in Paper III and Paper IV were approved by the following Norwegian authorities: the Regional Committee for Medical and Health Research Ethics, the Privacy Ombudsman and the Investigational Review Board at Diakonhjemmet Hospital.

In Paper I, a total of 2152 unrelated adult subjects (healthy volunteers) were included. They represented the three major ethnicities (Caucasian, Asian and African) and formed 13 defined ethnic subgroups (Table 4). The Caucasian population consisted of Swedes, Italians and Turks, and the Asian population of Japanese. From the African continent, nine ethnic groups from three regions of Sub-Saharan Africa were included comprising in total 863 subjects. The Swedish volunteers were anonymous blood donors. Volunteers were recruited among university students and employees for the Italian, Turkish and Japanese groups. The African samples were retrieved from the biobank at African Institute of Biomedical Science and Technology, Zimbabwe.

Table 4. Sample size and country origin of the study subjects included in the 13 ethnic groups in Paper I.

<table>
<thead>
<tr>
<th>Caucasian</th>
<th>Asian</th>
<th>African</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Northern Europe</strong></td>
<td><strong>East Asia</strong></td>
<td><strong>North-Western Africa</strong></td>
</tr>
<tr>
<td>Sweden</td>
<td>Japan</td>
<td>Nigeria</td>
</tr>
<tr>
<td>410</td>
<td>300</td>
<td>100 Hausa</td>
</tr>
<tr>
<td><strong>Southern Europe</strong></td>
<td><strong>Central-Eastern Africa</strong></td>
<td><strong>Kenya</strong></td>
</tr>
<tr>
<td>Italy</td>
<td>North-Western Africa</td>
<td>Nigeria</td>
</tr>
<tr>
<td>279</td>
<td>100 Hausa</td>
<td>Kenya</td>
</tr>
<tr>
<td><strong>Eurasia</strong></td>
<td><strong>Southern Africa</strong></td>
<td><strong>143 Masaa</strong></td>
</tr>
<tr>
<td>Turkey</td>
<td><strong>Zimbabwe</strong></td>
<td><strong>99 Shona</strong></td>
</tr>
<tr>
<td>300</td>
<td>Zimbabwe</td>
<td>99 Shona</td>
</tr>
<tr>
<td></td>
<td><strong>63 San</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>South Africa</strong></td>
<td><strong>63 Venda</strong></td>
</tr>
</tbody>
</table>
In Paper II, a group of 37 Caucasian outpatients (25 males and 12 females; age range, 23-50 years) at Linköping University Hospital, with diagnosis of schizophrenia or schizoaffective disorder according to DSM-IV criteria was included. These patients were initially recruited for investigation of the relationship between steady-state serum and CSF concentration of OLA and the DMO metabolite. They were not first-episode patients and all but three had been medicated earlier with an antipsychotic drug other than OLA. At the time of study, all patients were on OLA as the only antipsychotic drug treatment with stable daily dose (dose range, 2.5 to 25mg/day) for at least 14 days. The length of OLA treatment varied between 0.2 and 11 years (median, 2 years). The concentration ranges for OLA were 3.5-102 ng/mL in serum, 0.56-9.43ng/mL in CSF and for DMO was 2.6-17.4ng/mL in serum, 0.12-2.79ng/mL in CSF.

For Paper III and Paper IV, serum OLA concentrations in 379 psychiatric patients (198 males and 177 females, no data on gender for 4 patients, Table 5), were obtained from routine TDM service conducted at the Center for Psychopharmacology in Diakonhjemmet Hospital, Oslo, Norway, during the period July 2007 - December 2010. One sample from each patient was included for analysis.

Table 5. Characteristics of patients included in Paper III and IV.1

<table>
<thead>
<tr>
<th></th>
<th>OLA²</th>
<th>OLA N-oxide</th>
<th>DMO³</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient number</td>
<td>379</td>
<td>123</td>
<td>342</td>
</tr>
<tr>
<td>Age (years)</td>
<td>45 (16 - 89)</td>
<td>39 (19 - 89)</td>
<td>45(16-89)</td>
</tr>
<tr>
<td>Gender/Smoking status</td>
<td>Non-smoker</td>
<td>Smoker</td>
<td>Non-smoker</td>
</tr>
<tr>
<td>Female</td>
<td>81 (22%)</td>
<td>96 (26%)</td>
<td>22 (18%)</td>
</tr>
<tr>
<td>Male</td>
<td>80 (21%)</td>
<td>118 (31%)</td>
<td>25 (20%)</td>
</tr>
<tr>
<td>Valproic acid co-medication (Yes/No)</td>
<td>29/350</td>
<td>12/111</td>
<td>26/316</td>
</tr>
<tr>
<td>Dose (mg/day)</td>
<td>15 (2.5 - 60)</td>
<td>20 (5 - 40)</td>
<td>15 (2.5 - 60)</td>
</tr>
<tr>
<td>Serum conc. (nmol/L)</td>
<td>101 (6 - 481)</td>
<td>5.8 (0.8 - 21)</td>
<td>23 (2 - 97)</td>
</tr>
<tr>
<td>Serum C/Ds (nmol/L/mg)</td>
<td>7.6 (2.4 - 38.6)</td>
<td>0.31 (0.08 - 1.8)</td>
<td>1.7 (0.25 - 7.0)</td>
</tr>
<tr>
<td>OLA N-oxide/OLA ratio (%)</td>
<td>4.2 (1.1 - 11.1)</td>
<td>23.6(4.0 - 70.3)</td>
<td></td>
</tr>
</tbody>
</table>

1) Data are median (range) or n (%), 2) No data on gender for 4 patients, 3) No data on gender for 1 patient

Criteria for inclusion were: a) the time between the last drug intake and serum sampling was 10-30 hours, b) measured OLA serum concentrations above the lower limit of quantification, and c) no concurrent use of CYP1A2
or UGT1A4 inducers/inhibitors except for tobacco smoking and co-medication with lamotrigine or valproic acid. The patients included were of Caucasian origin. Due to biobank regulation, serum samples for drug concentration determination can only be stored for 3 months post analysis. Re-analysis to measure the metabolites was therefore only possible for subgroups of the study population. Of the 379 patients, quantification of DMO could be done for 342 patients and OLA N-oxide for 123 patients (Table 5).

Methodological overview

Analysis of drug and metabolite concentrations

The molecular mass of OLA is 312.4g/mol. For unit conversion to ng/mL, multiply the concentration value in nmol/L with a factor of 0.3124. For unit conversion to nmol/L, multiply the concentration value in ng/mL with a factor of 3.2.

In Paper II, fasting blood samples for the analysis of OLA and DMO were collected at 9 to 14.5 hours (median 12 hours) after the evening dose. Lumbar puncture was carried out at close connection to blood sampling at the minimum of eight hours in the fasting state. The procedure was, however, unsuccessful in 8 patients (dose range, 7.5-15mg/day; median, 10mg/day) and CSF samples were thus available for 29 of the 37 patients. OLA and DMO concentrations in serum and CFS were analyzed using a liquid chromatography/tandem mass spectrometry (LC-MS/MS) method developed by Josefsson et al.202

In Paper III and Paper IV, serum concentrations of OLA, DMO and OLA N-oxide were analyzed by a validated and certified UPLC-MS/MS method developed for routine TDM analyses at the Center for Psychopharmacology in Diakonhjemmet Hospital, Oslo, Norway. The experimental procedure is described in details in the papers included.

DNA extraction

In Paper I and II, genomic DNA was purified from peripheral leukocytes and stored at -20°C, with the exception of the Swedish blood donors, from whom buffy coat was available. Standard extraction was performed using QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany), according to the guidelines of the manufacturer. In Paper III and IV, DNA was extracted from peripheral blood leukocytes using E.Z.N.A® Blood DNA Mini Kit (Omega Bio-tek, USA). DNA quantitation of study samples was evaluated using Nanodrop® in Paper I and II, and Picogreen® in Paper III and IV.
SNP genotyping

Candidate SNPs was genotyped using 1) TaqMan® allelic discrimination with Fluorogenic 5’ nuclease assays (Paper I, II and III) and 2) Illumina GoldenGate® genotyping assays with discriminatory DNA polymerase and ligase (Paper III and IV). The candidate SNPs included and the method(s) used in each paper are listed in Table 6. For both methods, pre-designed and validated assays for the SNP loci of choice were purchased, from Applied Biosystems for TaqMan® assays and from Illumina® for GoldenGate® assays. TaqMan® assay was custom designed for one novel FMO3 mutation identified in the gene re-sequencing study (Paper II), FMO3 g.18129T>C.

Forward primer: GTCTTCTGACACCACCTTTCTGC
Reverse primer: TGTTTTGGGCCTTACAGGACA

The TaqMan® assays were performed with ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, USA) and conducted at the department of clinical chemistry and pharmacology, Uppsala University Hospital. The Illumina® assays were performed with Illumina BeadXpress and conducted at The SNP&SEQ Technology Platform, Uppsala University Hospital. All analysis was carried out according to the guidelines of the manufacturer.

Table 6. The candidate SNPs genotyped in the thesis

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Gene re-sequencing

Detection of \textit{FMO3} and \textit{UGT1A4} polymorphisms in Paper II was done using gene re-sequencing analysis. The gene regions of interest were amplified by PCR reaction. The nucleotide sequences were determined by sequencing the purified PCR products using BigDye® Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, USA) and analyzed on ABI 3730XL DNA Sequencer (Applied Biosystems, Foster City, USA). The same primer pairs were used for PCR amplification as for gene sequencing. Amplicons were sequenced on both forward and reverse strands (Table 7).

The PCR reaction was carried out on a Peltier thermal cycler with a final reaction volume of 50 µl containing a) DNA template (60 ng – 120 ng), b) 1x PCR Buffer (20mM Tris-HCL and 50mM KCl), c) 1.5 mM MgCl2 d) 0.2 mM dNTP, e) 1 unit Platinum Taq polymerase and f) 0.2 µM each primer. Primer sequences and specific annealing temperatures and durations are summarized in Table 7. The cycling protocol started with an initial denaturation at 94°C for 2 minutes followed by 31 cycles for \textit{FMO3} and 29 cycles for \textit{UGT1A4} of denaturation at 94°C for 30 seconds, annealing with specific temperature and time duration, and extension at 72°C for 1 minute, completed with final extension at 72°C for 7 minutes. The PCR products were then purified using QIAquick PCR purification kit (QIAGEN Ltd, Hilden, Germany), sequenced using BigDye® Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, USA) and analyzed on ABI 3730XL DNA Sequencer (Applied Biosystems, Foster City, USA).

<table>
<thead>
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<th>Gene</th>
<th>Region</th>
<th>Sequence (5' to 3')</th>
<th>Product size (bp)</th>
<th>Annealing °C</th>
<th>Time (s)</th>
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</table>

Table 7. Primer pairs and PCR conditions used in \textit{FMO3} and \textit{UGT1A4} sequence analysis.
**Bioinformatics**

**Primer design**

In Paper II, reference nucleotide sequences were obtained from the online database of the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/) (AY895830.1 for *FMO3* and AF297093.1 for *UGT1A4*). Primer design and sequence specificity check for gene re-sequencing of *FMO3* and *UGT1A4* were carried out using the online tool, Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Primer and probe design for the custom designed TaqMan® genotype assay (AH20TVW, Table 6) were done using the software program ABI PRISM Primer Express (Applied Biosystems).

**tagSNP selection**

In Paper III, genotype data was retrieved from the International HapMap Project (http://hapmap.ncbi.nlm.nih.gov/cgi-perl/gbrowse/hapmap28_B36/, release #28) for the SNPs located within the genomic region of *FMO3* plus 10kb in 5’ and 5kb in 3’ flanking regions (chr1:169316660 to 169358581). The data was obtained for the CEPH group (Utah residents with ancestry from Northern and Western Europe). Selection and evaluation of the tagSNPs were done using the Tagger function implemented in Haploview v4.2 based on the empirical patterns of linkage disequilibrium obtained from the International HapMap Project.

**Sequence variant analysis**

Post-sequencing analysis in Paper II included fragment assembling, base calling as well as SNP and insertion/deletion detection, using the software package novoSNP. Reference sequences for the sequenced regions were downloaded from the GRCh37 (Genome Reference Consortium Human genome build 37) assembly via ENSEMBL (http://www.ensembl.org). All identified SNPs were compared with reported SNPs for *FMO3* and *UGT1A4* listed in NCBI Single Nucleotide Polymorphism database (dbSNP build 131, http://www.ncbi.nlm.nih.gov/projects/SNP/) at the time of the study.

The Haploview v4 (or higher) software was used for calculating MAF and probability of deviation from HWE due to chance for each polymorphism genotyped or detected in a defined study population. It was also used for assessment of pair-wise LD, characterized by D’ and r² values and visualized by LD plots (Paper I, II, III and IV).
Haplotype inference and frequency determination were carried out by the software package PHASE v2.1 in Paper I, Haplovew v4.1 in Paper II, III and IV. Population differentiation in haplotype distribution was evaluated with Arlequin v3.1\(^{205}\) in Paper I. The UNPHASED v3.1.5 software\(^{206}\) was used for haplotype association analysis in Paper IV.

**Putative effect prediction**

For the novel intronic *FMO3* g.18129T>C detected in Paper II, the online tool, Automated Splice Site Analysis (https://splice.uwo.ca/\(^{207}\) was used to assess possible disruption of consensus sequences for splicing and detect potential formation of cryptic splice sites (i.e. acceptor site, donor site and branch point). The prediction is based on the fact that sequences immediately adjacent to natural splice sites contain information that dictate the strength of recognition for spliceosome to utilize the splice site. Mutations located within such regions may alter strength of existing natural sites or putative cryptic sites as well as form new cryptic splice sites.

**Statistics**

Two-tailed \(P \leq 0.05\) was considered statistically significant. For comparison between ethnic populations in Paper I, allele distribution was compared using two tailed Chi-square test or Fisher’s exact test (GraphPad Prism v 4, Graphpad Software, CA). For genotype association studies in Paper II, III and IV, statistical analysis was performed using STATISTICA 9.1 or higher (StatSoft. Inc, Tulsa, OK). All variables (C/Ds and the DMO/OLA ratio) were tested for normality by Shapiro-Wilk test (Paper II, III and IV). When significant deviation was observed, log-transformation was applied to attain normality (Paper III and IV). Degree of correlation between the concentration variables was assessed using Spearman rank correlation test (coefficient: \(\rho\)) (Paper III and IV).

In Paper II and IV, concentration comparisons between genotypes were carried out using ANOVA (analysis of variance) or ANCOVA (analysis of covariance). Planned comparisons of least squares means (contrast analysis) were used to test the statistical significance of predicted specific differences associated with the variant alleles. The combined effect of SNPs and non-genetic covariates on OLA C/Ds was assessed by multiple linear regression analysis. Best-subset regression with Mallow’s cp as criterion was applied in Paper II whereas backward stepwise regression was used in Paper IV. Due to the small sample size in Paper II, no more than four variables for serum concentrations and three variables for CSF concentrations were included in each analysis. In Paper IV, evaluation of *CYP1A1/CYP1A2* haplotype association to DMO/OLA ratios and OLA C/Ds were carried out using UNPHASED (v3.1.5).
In Paper III, multiple linear regression analysis applying best-subset method (Mallow’s cp as criterion) was used to identify the subset of FMO3 SNPs that best describe concentration variability. ANCOVA was used to assess the two FMO1 polymorphisms. Bonferroni test was used as post hoc analysis. The combined effect of the significantly associated SNPs and non-genetic covariates was further assessed in the final regression models applying backward stepwise regression.
Results and Discussion

Paper I

Considerable variability in distribution of non-synonymous FMO3 SNPs across 13 ethnic populations

Majority of the reported population studies were based on cohorts of individuals from Northern America. Due to the long immigration history and large admixture in the region, the genetic backgrounds of the socially defined ethnic groups in these countries are not always as clear as they appear to be. Variability in distribution of FMO3 polymorphisms among subpopulations within the same ethnicity has too been overlooked. In this study, we investigated population distribution of five functionally relevant FMO3 SNPs: three common variants E158K, V257M and E308G; two African specific variants D132H and L360P.

Inter- and intra-ethnic variation in FMO3 SNP distribution

We confirmed inter-ethnic variability in allele distribution (detailed frequency data summarized in Table 1 in Paper I). Population comparison revealed the highest frequency of K158 variant to be found in native Africans (42 – 52% except the San group, 33%), followed by European Caucasians (34 – 44%) and East Asians (Japanese, 23%). A reversed order was shown for the M257 variant with highest frequency in East Asians (Japanese, 15%), followed by European Caucasians (6 – 7%) and native Africans (0 – 5%). The G308 variant was also rare among native Africans (0 – 1.6%) but much more prevalent in Japanese (21%) and European Caucasians (6 – 22%).

Concerning the two variants originally identified in African-Americans, occurrence of H132 was confirmed whereas P360 variant was not observed in any of the native Africans samples. This may be due to the P360 variant being region specific or less common than previously reported.

The published data on FMO3 polymorphisms in African populations is largely based on African Americans. Here, only the K158 variant was common in all native African groups whereas the other variant alleles were rare. The ancestral history of African Americans is complex as they are not only descendants of many different populations within Africa but also have on
average 20% European ancestry. Estimates of allele frequencies in African Americans should therefore not be uncritically taken as representative of African populations in other parts of the world.

Intra-ethnic differences were detected for E158K and E308G among the three European Caucasian populations (Swedish, Italian and Turkish). The Swedes (K158, G308; 44%, 22%) had higher allele frequencies of both K158 and G308 than the Italians (34%, 11%), and the Turks (36%, 6%, P<0.005 in all group comparisons). The reported studies with Northern American cohorts displayed frequency values, for all three sites, either between the Swedes and the Italians/Turks or higher than our groups (data summarized in Table 1 in Paper I). The differences between European and American studies can be due to the heterogeneous ancestral background of American Caucasians from different parts of Europe.

Among the native African groups, regional differences were detected for H132 with higher frequencies in Western Africa groups (5.0 – 8.2%) compared to the groups from Eastern and Southern Africa (1.1 – 3.5%, p<0.01 in both cases) with no difference between the latter two regions. The intra-ethnic difference observed for K158 variant (P=0.03) and M257 variant (P=0.02), was on the other hand entirely due to deviating low frequencies in single groups, the San group in particular.

The *FMO3* K158-G308 compound variant prevalent among non-Africans

We further confirmed the cis-linkage between K158 and G308 variants, and observed no LD for additional pair-wise combinations of the polymorphisms. The $r^2$ values were consistently close to zero for all combinations across all groups, except for the combination of K158 and G308 variants in the non-African groups. As the $r^2$ value denotes the power to predict prevalence of one variant by measuring another variant, the group order of increase in the $r^2$ value went hand in hand with the decreasing difference in allele frequencies between the K158 and G308 variants in these groups. The $r^2$ values varied from being low for the Turkish ($r^2=0.1$) and the Italian ($r^2=0.2$) groups, to being moderate for the Swedish ($r^2=0.4$) and to high for the Japanese ($r^2=0.9$).

The variant G308 was inferred to co-occur always with K158 variant in haplotype construction (haplotype A-G-G as K158-V257-G308 in Table 2 in Paper I). The proportion of individuals carrying this construction was higher in non-African groups (12 – 38%) compared to the Africans (1.3%, Figure 1). Since $r^2=0.9$ between E158K and E308G among Japanese, the Japanese carrying the K158-G308 compound variant were either heterozygous or homozygous for both sites (158-308: GA-AG and AA-GG) (Fig 7). A third
type of carrier (AA-AG) was identified in the Caucasian groups being homozygous mutated for site 158 but heterozygous for site 308. Substantial proportions of all three types of carriers (GA-AG, 23.4%; AA-AG, 8.3%; AA-GG, 6.6%) were observed in the Swedish group compared to in the other populations (Fig 7). Given the impaired FMO3 activity reported for the K158-G308 compound variant and the frequency variability shown here, inter- and intra-ethnic differences in drug metabolism catalyzed by FMO3 can be expected. The Swedes are detected here with relatively high frequencies of the variant compound genotypes and might thus in theory be more prone to decreased activity of the enzyme.

Figure 7. Genotype distribution of the FMO3 K158-G308 compound variant in ethnic populations (carrier fractions colored in purple, green and red).
Paper II.

*Inter-patient variation in OLA exposure is correlated to UGT1A4 in serum and FMO in cerebrospinal fluid*

To investigate the potential polymorphic influence of FMO1, FMO3 and UGT1A4 on inter-patient variation observed for OLA exposure, we performed a pilot study consisting of 37 Swedish Caucasian patients treated with OLA as the only antipsychotic drug. Steady-state concentrations of OLA and the metabolite DMO were available for both serum and CSF, providing estimations on systemic and local drug exposure, respectively. From the previous evaluation of the same patient cohort, smoking and age were identified as significant covariates for C/Ds of OLA and DMO, respectively, whereas functional polymorphisms of \( \text{CYP1A2} \) and \( \text{CYP2D6} \) displayed no major influence other than in association with smoking.\(^{110}\)

**FMO and UGT1A4 SNP detection and haplotype construction**

One novel intronic mutation and 25 known variants were identified within the \( \text{FMO3} \) gene region and 11 known variants for \( \text{UGT1A4} \) by sequencing analysis (detailed SNP data summarized in Supplemental Table A in Paper II).

Four \( \text{FMO3} \) haplotype structures were deduced in addition to the wild type haplotype (Supplemental Figure A in Paper II). Each of them incorporated one of the four variants with reported functional impact, g.-2177G>C, E158K, V257M and E308G. For \( \text{FMO1} \), we chose to genotype one candidate SNP, \( \text{FMO1*6} \), and identified five carriers who were all heterozygous mutated.

Of the two non-synonymous \( \text{UGT1A4} \) SNPs identified, P24T was not in LD to any of other \( \text{UGT1A4} \) SNPs detected whereas L48V was in complete LD (\( r^2=1.0 \)) with 4 upstream variants (Supplemental Figure A in Paper II). Our data is in agreement with the earlier reported linkage pattern for \( \text{UGT1A4} \) polymorphisms. Six \( \text{UGT1A4} \) haplotypes were inferred and further grouped into \( \text{UGT1A4*1} \), \( \text{UGT1A4*2} \) carrying T24, and \( \text{UGT1A4*3} \) carrying V48 (Supplemental Figure A in Paper II). Patients identified carrying \( \text{UGT1A4*2} \) and \( \text{UGT1A4*3} \) were all heterozygous carriers.

**Serum concentration variation and \( \text{UGT1A4*3} \)**

No significant correlation with C/Ds of OLA was found for any of the \( \text{FMO3} \) haplotypes or \( \text{FMO1*6} \) in serum. The influence of homozygosity for the K158-G308 compound variant was however not possible to assess since only one carrier was identified. On the other hand, we observed lower serum
OLA C/Ds in carriers of *UGT1A4*3 suggesting increased OLA glucuronidation activity (Table 8). The effect of reduced *UGT1A4* gene expression reported in vitro for two of the linked upstream variants g.-219(T) and g.-163(A)\(^{157}\) seems to be limited in vivo. The impact associated with this haplotype is suggested to be primarily attributed to the V48 variant.

Mean OLA C/D in the non-smoking *UGT1A4*3 carriers were 35% lower than those of wild type non-smokers (P=0.06), comparable to the mean C/D in wild type smokers (Table 8). Although the association suffered in statistical power due to the small sample size, the observation was in line with findings in earlier studies assessing this variant.\(^{117,152-156}\) The hypothesis of increased OLA clearance by UGT1A4-mediated glucuronidation was further supported by a 37% lower serum DMO C/Ds observed in the non-smoking *UGT1A4*3 carriers compared to wild type non-smokers (Table 8). The effect of *UGT1A4*3 on serum DMO C/D remained significant even after adjustment of age (age, P=0.002; *UGT1A4*, P=0.005). Smoking alone had no significant influence on DMO C/D (Table 8). However, in a recently published study with 407 TDM patients, the effect of *UGT1A4* L48V on OLA disposition was shown to be evident only on the formation of OLA N-10-glucuronide, but not on systemic OLA exposure.\(^{158}\) Whether there is any pharmacogenetic influence of *UGT1A4*3 on OLA exposure in vivo will need to be demonstrated in future studies.

Table 8. Serum OLA and DMO concentrations (ng/mL/mg) in relation to UGT1A4 genotype and smoking habit

<table>
<thead>
<tr>
<th>Smoker</th>
<th><em>UGT1A4</em> genotype</th>
<th>No.</th>
<th>OLA C/D P value(^2)</th>
<th>DMO C/D P value(^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No</td>
<td>*1/*1 or *1/*2</td>
<td>24</td>
<td>3.45 ± 1.09</td>
<td>0.75 ± 0.19</td>
</tr>
<tr>
<td>Yes</td>
<td>*1/*1 or *1/*2</td>
<td>9</td>
<td>2.28 ± 0.96</td>
<td>0.006</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.68 ± 0.25</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>&gt;0.1</td>
</tr>
<tr>
<td>No</td>
<td>*1/*3</td>
<td>3</td>
<td>2.24 ± 0.43</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.47 ± 0.16</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.04</td>
</tr>
<tr>
<td>Yes</td>
<td>*1/*3</td>
<td>1</td>
<td>1.81</td>
<td></td>
</tr>
</tbody>
</table>

1) The data are given as mean ± SD. 2) The P values refer to comparisons to the non-smoking *1/*1 or *1/*2 group

The *UGT1A4*2 was not associated with any significant change in C/Ds of OLA or DMO, which is in line with data from in vitro studies. The *UGT1A4* g.-204A variant (*UGT1A4*1k, Supplemental Figure A in Paper II) was previously associated with reduced glucuronide formation in vitro. The effect of this variant could, however, not be statistically assessed in the current study due to limited number of carriers identified, two heterozygous patients with one of them also being a *UGT1A4*3 carrier.
CSF concentration variation and upstream FMO SNPs

Carriers of UGT1A4*3 did not show deviating OLA C/D in CSF. Given the lack of data supporting UGT1A4-specific expression in brain tissue, our findings in serum and CSF seem to support the UGT1A4-mediated glucuronidation of OLA to be restricted to the liver.

On the other hand, a two-fold difference in OLA C/Ds was observed between carriers of the FMO3 g.-2177C variant and carriers of FMO1*6 (Fig 8). The comparatively high and low OLA C/Ds observed for the carriers of these two upstream SNPs, respectively, is in line with in vitro function data reported for them.198,199 Given that comparable expression levels for all FMO isoforms have been reported in human brain, our data suggest that FMOs might mediate OLA metabolism locally in the central nervous system.

Figure 8. Boxplot with median and interquartile range of CSF OLA C/D, in relation to combined FMO1/FMO3 genotypes and smoking habits (○ non-smoker; ● smoker).

Elevated OLA exposure in the carrier of a novel FMO3 mutation

The novel FMO3 intronic mutation (g.18129T>C) was located at 11 nucleotides upstream exon 6 and thus adjacent to the intron 5 splice acceptor site (Fig 9A). Assessing the individual information content of natural splice sites in FMO3, intron 5 has the lowest \( R_i \) value for its splice acceptor site (3.3 bits) and is the only intron having low \( R_i \) values at both splice donor and acceptor sites compared to the other introns. The nucleotide change, g.18129T>C, caused a decrease in \( R_i \) value of the intron 5 splice acceptor site from 3.3 to 2.6 bits. As the information content remains above the lower threshold of recognition, 1.6 bits, the impact of the mutation was inferred to
weaken the natural splice acceptor site for intron 5 and cause leaky pre-mRNA splicing.

Interestingly, the one patient carrying the novel variant displayed the highest serum OLA C/D within the study group, together with one of the highest OLA C/Ds in CSF (Fig 9B). Whether there is a cause-effect association warrants further studies. However, when population distribution of the variant allele was assessed in a group of 300 Swedish blood donors, no additional carrier was identified. Hence, this nucleotide change seems to be a rare mutation among Swedes.

Figure 9. A) A chromatogram showing the position of the novel FMO3 g.18129T>C mutation in intron 5. The splice acceptor site at the end of intron 5 is underlined. B) Boxplot with median and interquartile range of serum and CSF OLA C/Ds in 37 schizophrenic patients on long-term olanzapine treatment. The arrows indicate OLA C/Ds of the patient heterozygous for the g.18129T>C mutation.
Paper III.

*FMO3 and FMO1 SNPs as genetic factors influencing OLA N-oxidation and systemic OLA exposure in vivo*

In the following study, statistical power was improved with a larger patient group (n=379) compared to the cohort in Paper II. The subgroup with available concentration data on OLA N-oxide did not differ from the study population regarding OLA C/Ds and patient characteristics such as gender, smoking status and co-medication with valproic acid (Table 5). We analyzed two candidate SNPs for *FMO1* (*FMO1*6 and rs7877) and 15 tagSNPs for *FMO3* (detailed SNP data summarized in Table 2 in Paper III). The *FMO1* SNPs displayed little pair-wise LD with each other, or to any of the *FMO3* SNPs (for LD plot see Supplementary Figure 1 in Paper III).

**Quantification of OLA N-oxide**

The levels of OLA N-oxide detected in patient serum ranged from 1% to 11% of those of OLA, with an average of 4% (Table 5, Fig 10). This is in accordance with a minor quantitative role of this metabolic pathway reported for the elimination of OLA in human. We observed however a relatively high correlation between the serum concentrations of OLA N-oxide and the parent compound ($\rho=0.72$, $P<0.001$, Fig 10) which could suggest an equilibrium between them.

*Figure 10.* Serum concentrations of OLA and the metabolite, OLA N-oxide, in 123 patients on long term treatment.
Reduced serum OLA N-oxide concentrations in the homozygous FMO3 G308 carriers

We found FMO3 E308G (MAF = 0.23) to be the only significant genetic factor for OLA N-oxide C/D (P = 0.0005). The association remained significant (P = 0.001) after further adjustment for the non-genetic covariates, smoking and gender. The finding supports the in vitro data on FMO3 catalyzing N-oxidation of OLA. Homozygous carriers of the minor G-allele (n=6) had a median C/D OLA N-oxide approximately 50% lower compared to both heterozygous carriers and non-carriers (Fig 11). All the homozygous carriers of FMO3 E308G in this study were also homozygous mutated for FMO3 E158K, in accordance with the observation from Paper I. Our data support the impact of this variant on FMO3 enzyme activity, but no effect on the C/Ds of OLA was found.

Figure 11. Serum OLA N-oxide C/Ds in 123 OLA-treated patients stratified by FMO3 rs2266780A>G (p.E308G). Genotype composition and sample size are given for each subgroup. The median concentrations with interquartile range are shown both numerically (raw data) and graphically (log-transformed). Non-outlier range is also displayed in the boxplot. Comparisons to G/G genotype are denoted by P values (Bonferroni test).

No additional FMO3 polymorphisms were identified as genetic markers for variability in C/Ds, despite the large genomic region studied. Up to now, the majority of reported FMO3 variants with functional impact are rare nonsense and missense mutations detected in patients affected with TMAuria. Few markers have been associated with polymorphic variation of enzyme activity within the general population. Inter-individual variability in FMO3 gene expression and protein content has been reported to be correlated in vitro to the levels of transcription regulatory factors, not affected by FMO3 haplotypes. Although our data indirectly support the direction of incorporating
trans-acting factors into future studies, this approach is challenged as FMO3 regulation is exceptionally complex and poorly characterized at the current stage.

**FMO1 SNPs associated with increased serum OLA concentrations**

We observed no significant association of the two *FMO1* SNPs with C/Ds of OLA N-oxide. However, *FMO1*<sup>6</sup> and rs7877T correlated significantly (P<0.05 for both) to serum OLA C/Ds after correction for smoking. The smokers carrying both variants had 45% higher median OLA C/D than wild type smokers (P<0.05 for both) (Fig 12). Among carriers of both variant alleles, no significant effect of smoking on C/Ds of OLA was found (P<0.05 for both). This is in contrast to the other groups where smokers consistently had lower OLA C/Ds than non-smokers (P<0.001 for all three group comparisons) (Fig 12).

![Figure 12](image_url)

*Figure 12.* Serum OLA C/Ds in 379 OLA-treated patients stratified by *FMO1*<sup>6</sup>C>A, *FMO1*rs7877C>T and smoking status. Genotype composition and sample size are given for each subgroup. The median concentrations with interquartile range are shown both numerically (raw data) and graphically (log-transformed). Non-outlier range is also displayed in the boxplot. Comparisons between smokers and non-smokers carrying the same genotype composition are denoted by P(smoker vs non-smoker) values. For multiple pair-wise comparisons post hoc analysis with Bonferroni test was performed.

These findings suggest a reduced influence of smoking on OLA clearance in patients carrying both *FMO1*<sup>6</sup> and rs7877T. FMO1 has been shown to be
an efficient catalyst of nicotine N-oxidation and rs7877C>T was originally identified as a determinant for maintenance of nicotine addiction. The reported odds ratio of 0.77 for heavy smoking associated with rs7877C>T suggests a prolonged duration of nicotine effect. A hypothetical explanation for our observations could be that if carriers of this polymorphism smoke less heavily, there would also be lower induction of drug metabolic enzymes such as CYP1A2, resulting in higher than expected OLA C/Ds.

Assessing impact on the overall concentration variability, the non-genetic factors (age, gender, smoking status and co-medication with valproic acid) and FMO1*6 (P=0.02) remained significant factors whereas rs7877C>T did not (P=0.08). Approximately 37% (adjusted R²=0.37) of the variability in serum OLA C/Ds in the study cohort (n=379) could be explained by the five factors, FMO1*6 accounting for 2%. The significant correlation between FMO1*6 and elevated serum C/Ds of OLA is in line with the increased OLA C/Ds in CSF of treated patients carrying FMO1*6 observed in Paper II. The influence of the SNP observed supports the reported in vitro data on the reduced promoter activity (2- to 3-fold) associated with this variant. ¹⁹⁹

Paper IV

Candidate SNPs of CYP1A1/2 and AHR loci are novel genetic factors influencing desmethylation of OLA and systemic OLA exposure

The plasma DMO/OLA ratio has been shown to be significantly correlated to OLA clearance. This ratio was chosen as a pathway-specific marker to study the effect of 5 candidate markers on the CYP1A2-mediated OLA metabolism (Table 9).

Table 9. Characteristics of the candidate SNPs studied in Paper IV

<table>
<thead>
<tr>
<th>SNP</th>
<th>rs2470893</th>
<th>rs2472297</th>
<th>rs762551</th>
<th>rs2472304</th>
<th>rs4410790</th>
</tr>
</thead>
<tbody>
<tr>
<td>Allele</td>
<td>C&gt;T</td>
<td>C&gt;T</td>
<td>C&gt;A</td>
<td>A&gt;G</td>
<td>C&gt;T</td>
</tr>
<tr>
<td>Chromosome</td>
<td>Chr15:</td>
<td>Chr15:</td>
<td>Chr15:</td>
<td>Chr15:</td>
<td>Chr13:</td>
</tr>
<tr>
<td>Genomic position</td>
<td>75019449</td>
<td>75027880</td>
<td>75041917</td>
<td>75044238</td>
<td>17284577</td>
</tr>
<tr>
<td>Gene</td>
<td>CYP1A1/1A2</td>
<td>CYP1A1/1A2</td>
<td>CYP1A2</td>
<td>CYP1A2</td>
<td>AHR</td>
</tr>
<tr>
<td>SNP type</td>
<td>inter-gene</td>
<td>inter-gene</td>
<td>intron 1</td>
<td>intron 4</td>
<td>upstream</td>
</tr>
<tr>
<td>MAF from HapMap database</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CEU (n=226)</td>
<td>0.323</td>
<td>0.248</td>
<td>0.721</td>
<td>0.336</td>
<td>0.420</td>
</tr>
<tr>
<td>JPT (n=172)</td>
<td>0</td>
<td>0</td>
<td>0.605</td>
<td>0.797</td>
<td>0.680</td>
</tr>
<tr>
<td>YRI (n=226)</td>
<td>0</td>
<td>0</td>
<td>0.566</td>
<td>0.991</td>
<td>0.500</td>
</tr>
<tr>
<td>MAF in this study</td>
<td>0.278</td>
<td>0.225</td>
<td>0.674</td>
<td>0.433</td>
<td>0.387</td>
</tr>
</tbody>
</table>

Abbreviation: n, sample size. Data retrieved from HapMap database is sorted by population descriptor. CEU: Utah residents with Northern and Western European ancestry from the CEPH collection. JPT: Japanese in Tokyo, Japan. YRI: Yoruban in Ibadan, Nigeria.
No influence by \textit{CYP1A2*1F} on OLA disposition in vivo

We found no influence of \textit{CYP1A2-163}(A) (\textit{CYP1A2*1F}, rs762551A) alone on either the DMO/OLA ratio or OLA C/Ds. Our data contradict the reported finding of a 22% reduction of serum OLA concentrations in Caucasian patients carrying \textit{CYP1A2-163}(A/A) genotype, independent of inducing factors.\textsuperscript{166} The allele frequency of 67% in our study cohort was similar to that reported for Caucasians in the HapMap database (72.1%, Table 9). As our sample size is much larger than that in the previous study (n=342 vs. n=73), our analysis should not suffer in power to validate the effect of this highly frequent allele.

However, \textit{CYP1A2-163}(A) was deduced in four (H2 to 5) of the five major haplotype constructions (Table 10). Haplotypes H3 and H4 were significantly associated with variability in DMO/OLA ratio and/or OLA C/D (Table 10). This finding is consistent with the fact that \textit{CYP1A2-163}(A) is present in a number of reported \textit{CYP1A2} haplotype constructions (http://www.cypalleles.ki.se/cyp1a2.htm) with different functional impact, but displays lack of mechanistic impact in functional evaluation in vitro.\textsuperscript{210}

Table 10. \textit{CYP1A1/1A2} haplotype inference (>1%) and effect association

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>%</th>
<th>rs2470893</th>
<th>rs2472297</th>
<th>rs762551</th>
<th>rs2472304</th>
<th>DMO/OLA</th>
<th>OLA C/D</th>
</tr>
</thead>
<tbody>
<tr>
<td>H1</td>
<td>32.4</td>
<td>C</td>
<td>C</td>
<td>C</td>
<td>G</td>
<td>Ref</td>
<td>Ref</td>
</tr>
<tr>
<td>H2</td>
<td>28.6</td>
<td>C</td>
<td>C</td>
<td>A</td>
<td>A</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>H3</td>
<td>21.9</td>
<td>T</td>
<td>T</td>
<td>A</td>
<td>A</td>
<td>↑ 0.004</td>
<td>↓ 0.04</td>
</tr>
<tr>
<td>H4</td>
<td>10.7</td>
<td>C</td>
<td>C</td>
<td>A</td>
<td>G</td>
<td>↓ 0.001</td>
<td>ns</td>
</tr>
<tr>
<td>H5</td>
<td>5.7</td>
<td>T</td>
<td>C</td>
<td>A</td>
<td>A</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>rare</td>
<td>0.7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{1)} Effect displayed for patients who were non-smokers and without co-medication with valproic acid (n=135)

ns: not significant. The direction of impact displayed by the markers

Decreased systemic OLA exposure in carriers of a Caucasian specific \textit{CYP1A1/1A2} haplotype

The H3 carriers (tagged by rs2472297C>T), compared to non-carriers, displayed 20-30% increase in median DMO/OLA ratio independent of smoking habit (Fig 13A) and 17% reduction in median C/D of OLA, though among non-smokers only (Fig 13B).

This haplotype was further confirmed as a significant contributor (P=0.01) to the overall variability in OLA exposure together with the non-genetic factors (smoking, gender, co-medication with valproic acid and age). In total, 36% of the variability (adjusted R\textsuperscript{2}=0.36) could be explained by the factors. As the two \textit{CYP1A1/1A2} variants included in the haplotype H3 are Caucasian specific according to population diversity data publically avail-
able (Table 9), they may thus, at least partly, explain the conflicting findings on the impact of CYP1A2-163(A) between ethnicities.

Figure 13. Serum DMO/OLA ratios (A) and OLA C/Ds (B) in relation to CYP1A1/1A2 rs2472297C>T genotypes (tagging the H3 haplotype). Data are categorized by smoking status in (A) and by smoking status and co-medication with valproic acid (Co-med) in (B). Group sizes are displayed (n). The medians with interquartile range are shown graphically and numerically. Non-outlier range is also displayed in the boxplots. P-values were determined by Kruskal-Wallis tests. ns, not significant.
Indication of trans-acting effect on OLA desmethylation by a SNP upstream \textit{AHR}

The effect association between DMO/OLA ratio and \textit{CYP1A2rs2472304(G)} (H4 in Table 10) did not remain after taking \textit{AHRrs4410790C>T} into account. The influence of \textit{AHRrs4410790C>T} was apparent only among non-smokers with carriers showing a 20\% decrease in DMO/OLA ratios (Fig 14A) and a 17\% increase in OLA C/Ds (Fig 14B). As AHR holds a central role in regulating the induction of CYP1A enzymes, the association observed suggests potential trans-acting influence.

\textbf{Figure 14.} Serum DMO/OLA ratios (A) and OLA C/Ds (B) in relation to \textit{AHRrs4410790C>T} genotypes, categorized by smoking status. Group sizes are displayed (n). The medians with interquartile range are shown both graphically and numerically. Non-outlier range is also displayed in the boxplots. P-values were determined by Kruskal-Wallis tests. ns, not significant.
General discussion

The importance of pharmacogenetics in drug metabolism is best characterized for the enzymes catalyzing the oxidative metabolism of a large number of drugs in clinical use, e.g. CYP2D6, CYP2C9 and CYP2C19. When contribution by any of these polymorphic enzymes is limited in metabolism of a drug compound, potential polymorphic influence of the enzymes responsible for the remaining metabolic routes should not be disregarded. With respect to OLA, CYP2D6 was early precluded as major predictor of variability in OLA exposure whereas influence by polymorphic CYP1A2 and UGT1A4 has been suggested. The main objective in the current thesis was to assess pharmacogenetic influence of FMO in OLA disposition. It had its rationale in the FMO-mediated OLA N-oxidation pathway being overlooked and the functional polymorphisms of FMO3 (hepatic isoform) and FMO1 (extra-hepatic isoform) being poorly evaluated for their potential effects in vivo.

Relevance of the findings

Catalytic activity of FMO3 towards OLA is identified in vitro using liver microsomes, but its effect in vivo is unknown. Our data provided, for the first time, in vivo support for the role of FMO3 in the formation of OLA N-oxide. The median C/D of OLA N-oxide was shown approximately 50% lower in homozygous carriers of the FMO3 K158-G308 compound allele compared to those of heterozygous carriers and non-carriers (Figure 11, Paper III). The role of FMO1 in metabolism of many psychoactive compounds is well supported by existing literature, but its role in OLA metabolism is unknown as there is no assessment available on FMO isoform specificity towards OLA N-oxidation. Since FMO1 is known to be the FMO isoform with broadest substrate range and efficient towards tertiary amines, we hypothesized that it could also contribute to the metabolism of OLA. In Paper III, the FMO1*6 allele was identified as a significant factor, together with the non-genetic covariates, influencing the overall variability in systemic OLA exposure. Patients carrying FMO1*6 displayed increased C/Ds of OLA, in serum (Fig. 12, Paper III) as well as cerebrospinal fluid (Fig. 8, Paper II). The effect in serum was further enhanced in smokers carrying an additional FMO1 SNP, rs7877 (Fig. 12, Paper III). Our data suggests an
increased systemic (and central nervous system) exposure of OLA in FMO1*6 carriers, possibly due to decreased intestinal first-pass metabolism catalyzed by FMO1. As FMO1 is highly expressed in kidney, potential role of renal metabolism in systemic elimination of OLA is yet another intriguing aspect for future studies.

Based on published data and our findings, CYP1A2 seems to be the most important enzyme for systemic exposure of OLA. Hepatic CYP1A2 activity estimated by caffeine metabolic ratios has been correlated to OLA clearance as well as plasma C/Ds of OLA.\textsuperscript{123,124} Significant correlation between plasma DMO/OLA ratio and OLA clearance has too been documented.\textsuperscript{106} Although the reported influence by CYP1A2*1F (CYP1A2-163(A))\textsuperscript{166} could not be verified in Paper IV, we made a novel observation. Significant influence on both DMO/OLA ratio and OLA C/Ds was detected for a Caucasian specific CYP1A1/1A2 haplotype structure incorporating the -163(A) variant (Table 10, Paper IV). Considering the role of AHR in regulating the induction of CYP1A enzymes and the well characterized effect of smoking on OLA exposure, our finding of a significant association between the SNP located upstream AHR locus and the DMO/OLA ratio (Fig. 14A, Paper IV) further support importance of CYP1A2 mediated OLA desmethylation.

Although direct glucuronidation by UGTs is considered to be the primary route of OLA biotransformation due to the large amount of OLA N-glucuronides detected in plasma, fecal and urinary samples\textsuperscript{105,112}, inconsistent results have been reported for the effect of UGT1A4*3 on systemic OLA exposure.\textsuperscript{134,136} We observed lower serum C/Ds of OLA in UGT1A4*3 carriers compared to non-smoking non-carriers (Table 8, Paper II), but our study suffered from having a small sample size. A recently published large study, with assessments both in vitro and in vivo, found the SNP effect to be only apparent on formation of the N-glucuronide metabolite, but cause no alteration in serum C/Ds of OLA.\textsuperscript{158} Verification of this finding and/or identification of novel genetic markers of UGT1A4 are warranted in future studies for clarifying the importance of UGT1A4 polymorphism in OLA metabolism.

Obstacles and limitations

For any pharmacogenetic study, characteristics of the SNPs and the clinical parameters chosen are the core. Multiple factors determine the study power including effect size of the causative locus on the clinical parameter, accuracy in measurement of phenotype and genotype, the degree of LD between the causative locus and the SNP marker, MAFs and sample size. A number of these aspects can be discussed in relation to the studies included in this thesis.

To estimate the role of FMO enzyme activity in OLA disposition in vivo is rather a difficult task. In contrast to CYP2D6 and CYP1A2 having de-
brisoquine, respective, caffeine as well characterized probe drug, phenotyping of FMO is underdeveloped. Although TMA and ranitidine have been used to assess FMO3 activity in few clinical studies\textsuperscript{35,187,190}, their suitability as probe drugs for FMO3 has not been systematically evaluated. Phenotyping of the other FMO isoforms has not been reported.

The steady-state serum levels of OLA N-oxide were found to be low in relation to those of the parent compound (Table 5, Figure 10, Paper III). As cyclic inter-conversion is known to occur between tertiary amines and their N-oxide metabolites\textsuperscript{59,212,213}, it is unclear whether the OLA N-oxide quantified reflects the activity of the oxidative pathway, the reduction or a combination of both. Whether this inter-conversion actually occurs between OLA and OLA N-oxide has not been shown so far. The quantitative contribution of N-oxidation in OLA metabolism is thus difficult to determine on the basis of current knowledge. As OLA biotransformation involves multiple pathways, catalyzed by a number of different enzymes (Fig. 6), multiple sources of variability in drug exposure can be expected. The influence of any specific pathway on the overall OLA exposure could thus be expected to have limited effect size.

Our SNP selection was thorough ranging from known functional SNPs, to the SNPs that can help to capture pattern of inheritance among the SNPs scattered within the genomic region of interest:

- candidate SNPs with reported functional impact in vitro and/or in vivo
- candidate SNPs identified as hits in GWAS studies assessing clinical parameters relevant for xenobiotic metabolism
- SNPs detected by gene re-sequencing analysis
- SNPs tagging haplotype structure in the genomic region of interest

The \textit{FMO} SNP hits identified by GWAS studies have only been reported for \textit{FMO1} at the time the current studies were designed. Sequencing and tagSNP based analysis was only carried out for \textit{FMO3} given that \textit{FMO1} was reported to be a more conserved gene compared to \textit{FMO3} \textsuperscript{214}, hepatic metabolism was considered more important than extra-hepatic clearance for OLA disposition\textsuperscript{105} as well as the matter of cost to conduct analysis for both genes.

MAFs set the start point for the sample size. MAF of one SNP might display population variation related to both ethnicity and geography, as demonstrated in Paper I. If the effect of the SNP is autosomal recessive, rather than being dominant, and the MAF is low, a large sample size will be needed in order to find an adequate number of homozygous carriers of the minor allele. The number of SNPs included as variables in association studies further influences the samples sizes required, due to the need to reduce the likelihood of false positive results. Large sample size is, thus, always desirable in order to identify sufficient carriers and obtain confidence in statistical significance. However, the challenges for collecting large data sets from well-characterized patient populations are considerable, especially in psychiatry. The study design in Paper II can be criticized for being underpowered. The
The study was designed primarily to assess the relationship between serum and CSF concentrations of OLA and its metabolites. For this purpose, it was an adequate number of patients.

Phenotype-driven assessment is vital in clinical pharmacogenetic research, with observation of outliers in clinic foregoing elucidation of genotype. When in vivo phenotype data is not available, in vitro characterization of genetic variants will provide indication of functional relevance. Significant associations obtained here support the functional impact of *FMO1*/*6 and the *FMO3* K158-G308 compound allele, earlier reported in vitro. However, when functional characterization of genetic variants has been conducted in limited extent, such as for *FMO* SNPs, an alternative approach to select candidate SNPs has to be applied. In Paper III and IV, our hypothesis was generated by correlations detected in exploratory GWAS studies assessing clinical parameters relevant for xenobiotic metabolism. In Paper III, the candidate SNP *FMO1*rs7877 was identified as determinant for nicotine dependence. In paper IV, genetic determinants for caffeine consumption were chosen for evaluation of the role of the CYP1A2 mediated metabolic pathway. As CYP1A2 is better characterized than FMO1 regarding variation in enzyme activity and its molecular mechanism, the hypothesis was biologically plausible and able to assess both cis- and trans-acting influence. Hence, taking the exploratory data from GWAS studies into consideration can be helpful in selecting potential SNP markers for genes that are short of SNPs with characterized functional impact.

Implementation of personalized drug prescribing requires adequate knowledge on factors contributing to variability in drug response. The magnitude of effect by variables of physiological, environmental or genetic origin varies between patients. Each category of factors needs to be evaluated for a complete evidence base. Improved knowledge on the clinical relevance of less well characterized drug metabolic pathways and the polymorphic characteristics of the responsible enzymes will help in the process of improving the clinical use of existing drug treatment, as well as be valuable in future drug development.
Conclusions

The pharmacokinetics of olanzapine display large inter-individual variation leading to multiple-fold differences in drug exposure between patients at a given dose. This variation in turn gives rise to the need of individualized dosing in order to avoid concentration-dependent adverse effects and therapeutic failure. In addition to environmental factors (smoking habit, co-medications) and physiological characteristics (gender, age and body weight), genetic variants of drug metabolizing enzymes are a potential source of variability in OLA exposure. The current thesis has evaluated the pharmacogenetic effect of OLA metabolizing enzymes FMO3, FMO1, CYP1A2 and UGT1A4, and their impact on OLA and metabolite kinetics in patients. The major conclusions are the following,

- The metabolite OLA N-oxide is presented in low concentrations in serum, but displays high correlation to OLA concentrations.

- The role of FMO3 in the formation of OLA N-oxide is supported. The reduced enzyme activity associated with the $FMO3$ K158-G308 compound variant in vitro is too supported by our data in vivo. The effect seems to be autosomal recessive.

- The $FMO3$ K158-G308 compound variant can be attributed to the fact that G308 variant co-occurs always with K158 variant, but not vice versa. The compound variant is expected to have potential clinical importance primarily in non-African populations due to its low prevalence in Africa.

- No additional $FMO3$ SNP marker was associated with variation in C/Ds of OLA N-oxide, despite the large genomic region studied. Common $FMO3$ polymorphisms do not seem to influence serum C/Ds of OLA. Rare point mutations with potential functional impact on gene expression might contribute to altered OLA exposure.

- The upstream polymorphism $FMO1^6$ (g.-9536C>A) is a novel factor associated with increased C/Ds of OLA in serum and CSF. The effect in serum might be further enhanced among smokers carrying $FMO1$
rs7877C>T located in 3’UTR of the gene. The role of FMO1 in OLA metabolic clearance, concerning the first-pass metabolism in the intestine as well as local metabolism in the brain, need to be clarified further.

- The CYP1A2-catalyzed OLA demethylation is influenced by both cis- and trans-acting variants; the CYP1A2 haplotype [rs2470893(T)-rs2472297(T)-rs762551(A)] with increased OLA demethylation and AHR rs4410790C>T with decreased activity.

- The inconsistent data on the impact of CYP1A2*1F (-163A) in different populations might be explained by ethnic specific CYP1A2 haplotype structures incorporating the -163(A) variant.

- The UGT1A4 L48V (UGT1A4*3) polymorphism was correlated with increased systemic OLA clearance but showed no influence on the cerebral exposure. No additional UGT1A4 SNP marker was identified with significant impact. Due to low statistic power, further examination is needed to verify the finding.

In summary, novel observations on polymorphic influence of FMOs on variability in OLA exposure are presented. The importance of CYP1A2-mediated OLA demethylation on systemic OLA exposure is further supported whereas the effect of UGT1A4 SNPs cannot be concluded. Although identified as significant genetic factors, the SNPs described in the current studies displayed minor contribution to the overall variability in OLA metabolism at population level. For individuals who harbor undesirable combinations of multiple determinants, they are nerveless expected to be relevant.
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