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Pharmacogenetic screening of psychiatric inpatients:
Associations between clinical outcome and selected polymorphisms in drug metabolism, drug transport and drug target structures

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If it were not for the great variability among individuals, medicine might be a science not an art.

(Sir William Osler, 1892)

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Abbreviations

Acc.No.	accession number
ACE	angiotensin converting enzyme
AD	antidepressant(s)
ADR	adrenoceptor
AGNP	Arbeitsgemeinschaft für Neuropsychopharmakologie und Pharmakopsychiatrie
Ala	alanine
Anc(h)	anchor probe
AP	antipsychotic(s)
ARMS	amplification refractory mutation system
ATC	anatomic therapeutic chemical classification system
BDNF	brain derived neurotropic factor
BHQ	black hole quencher
BLAST	basic local alignment search tool
BMI	body mass index
bp	base pair(s)
BSA	bovine serum albumin
C	cytosine
cAMP	cyclic adenosine mono phosphate
CGI	clinical global impression
CNS	central nervous system
COMT	catechol-O-methyl transferase
CYP	cytochrome P450
Cys	cysteine
DAT	dopamine transporter
DBH	dopamine beta hydroxylase
del	deletion
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DOTES	dosage record and treatment emergent symptom (side effects) scale
DRD	dopamine receptor
EDTA	ethylenediamine tetraacetic acid
EM	extensive metabolizer
EPS	extrapyramidal-motoric symptoms
F20-F29	schizophrenia, schizotypal and delusional disorders
F30-39	mood (affective) disorders
FAM	fluoresceine
FKBP5	FK506 binding protein 51
FRET	fluorescence resonance energy transfer
G	guanine
Gly	glycine
GNB	guanine nucleotide binding protein, G-protein
HEX	hexachlorofluorescein
His	histidine
HPA	hypothalamic-pituitary-adrenal
HPLC	high performance liquid chromatography
5-HT	5-hydroxy-tryptamine = serotonin
5-HTT	serotonin transporter
5-HTTLPR	serotonin transporter length polymorphism
HWE	Hardy-Weinberg equilibrium
ICD-10	international classification of diseases by World Health Organization
IM	intermediate metabolizer (= with impaired metabolism)
ins	insertion
kbp	kilo base pair(s)
KCl	potassium chloride
KWT	Kruskal-Wallis test
LC	LightCycler
LCRed610	LightCycler Red 610 Fluorescent Dye (emits at 610 nm)

LCRed640	LightCycler Red 640 Fluorescent Dye (emits at 640 nm)
LCRed705	LightCycler Red 705 Fluorescent Dye (emits at 705 nm)
LED	light-emitting diode
MAO-A	monoamine oxidase A
MDR1	multi drug resistance gene 1
Met	methionine
$MgCl_2$	magnesium chloride
mL	milliliter
mM	millimolar
mRNA	messenger ribonucleic acid
MS/MS	tandem mass spectrometry
MWU	Mann-Whitney U test
µL	microliter
µM	micromolar
n	number
NA	noradrenaline = norepinephrine
NaCl	sodium chloride
NEB	New England Biolabs
NCBI	National Center for Biotechnology Information
NET	norepinephrine transporter
NMDA	N-methyl-D-aspartate
NRI	noradrenaline reuptake inhibitor
OR	odds ratio
Ph	phosphate
PCR	polymerase chain reaction
PD	pharmacodynamics
PDS	paranoid depression scale
PDS-P	paranoid depression scale, paranoid subscale
PDS-D	paranoid depression scale, depression subscale
PG	pharmacogenetics
P-gp	P-glycoprotein
PK	pharmacokinetics
PM	poor metabolizer
RFLP	restriction fragment length polymorphism
r	correlation coefficient
SD	standard deviation
sen(s)	sensor probe
Ser	serine
SNP	single nucleotide polymorphism
SNRI	selective noradrenaline reuptake inhibitor
SSNRI	selective serotonin noradrenaline reuptake inhibitor
SSRI	selective serotonin reuptake inhibitor
T	thymine
Taq	Thermophilus aquaticus
TaqMan probe	exonuclease probe
TBE	Tris-Borate-EDTA buffer
TCA	tricyclic antidepressant(s)
TDM	therapeutic drug monitoring
T_m (°C)	melting temperature (or point)
TPH	tryptophan hydroxylase
Tyr	tyrosine
UM	ultrarapid metabolizer
U/µL	units/microliter
UV	ultraviolet
V	volt
Val	valine
VNTR	variable number of tandem repeats
WHO	World Health Organization

Terms

adapted from Roden et al. (2006) [1]:

Allele	one of alternative forms at a genetic locus on a single chromosome, one inherited from each parent.
Candidate gene	a gene in which variants could plausibly explain a given phenotype, such as severity of disease or variable response to drug.
Genome	the collection of all DNA in an organism. Only a small proportion (probably <3%) of human genomes encodes proteins.
Genetic variant	a difference in DNA sequence compared with a reference sequence.
Genotype	the genetic makeup of an individual, which may refer to the whole genome or to specific genes or regions of genes.
Haplotype	a set of genetic variants which are inherited together. Polymorphisms that are co-inherited more often than by chance alone are in linkage disequilibrium. Haplotype blocks may include many individual polymorphisms in high linkage disequilibrium.
Homozygous	the same alleles in a specific region of DNA.
Heterozygous	different alleles in a specific region of DNA.
Mutation	rare variants, most often in coding regions, which are often associated with genetic diseases, such as cystic fibrosis or sickle cell anemia.
Phenotype	measurable characteristics of an organism. These may derive from the genotype, the environment, or the combination of both. Organisms with the same phenotype can have different genotypes.
Polymorphism	mutation with a frequency greater than 1% in a given population.
Wildtype	(in most cases more frequent) genetic variant of the reference sequence.

Contents

1 Introduction

What is Pharmacogenetics?

Pharmacogenetics studies the genetic basis of an individual's ability to respond to a specific pharmacotherapy. The individual's genetic variations might have an impact on the disposition, safety, tolerability, and efficacy of a drug [2,3]. While the term "pharmacogenetics" was introduced to describe inherited differences in drug response [3], the - more recent - term "pharmacogenomics" grasps much wider and subsumes the analysis of the whole genome (DNA) and its products (RNA and proteins) as they relate to drug response [4,5].

The DNA sequence of any two people is 99.9% identical. Gene variations or mutations, however, are largely responsible for the differences in the way humans respond to drugs. Several types of mutations can be responsible for these variations [6]. The most common type of polymorphism is a change in one nucleotide (base pair) in the DNA sequence, referred to as single nucleotide polymorphism (SNP). Sets of nearby SNPs on the same chromosome are inherited in blocks. This pattern of SNPs on a block is a haplotype. Blocks may contain a large number of SNPs, but a few SNPs maybe enough to uniquely identify the haplotypes in a block [6,7]. Other polymorphisms are insertion and deletion of multiple sequential nucleotides ("ins/del"), variable numbers of repeats (such as doublets or triplets) or large-scale duplications or deletions up to the whole gene or chromosome.

Polymorphisms can be in coding regions of a gene (where they may be synonymous, resulting in a change of the amino acid sequence, or nonsynonymous/silent, resulting in no change of the amino acid sequence) or, more commonly, in noncoding regions. The possible functional consequences of most polymorphisms are unknown. Some genetic variants are known to alter amino acid sequence and thus protein abundance or function. Mutations can also affect gene expression when they occur in regulatory or promoter sequences or in the exon/intron boundary [2,8]. Even if the exact causal relationship has not been established so far, the haploblock structure of the DNA sequence - as defined by representative SNPs - can be utilized to examine the influence of genetic variation on protein function or abundance [9-12].

These different kinds of genetic variations can affect [2,3,5,13]:

- the biotransformation of the drug in gut and liver by enzymes such as the large family of cytochrome P450 isoenzymes.
- the adsorption of the drug from gut and distribution of the drug through the blood-brain barrier by transport proteins such as the efflux pump P-glycoprotein (P-gp).
- the target structure of the drug (= site-of-action, receptor) or
- structures in the subsequent cellular and physiological pathways mediating long-term adaptive response. A drug may have an effect on the receptor itself thereby changing e.g., the neurotransmission. But polymorphisms in neurotransmitter transporters, neurotransmitter biosynthesis or neurotransmitter metabolizing enzymes can also lead to a variation in neurotransmission affecting the efficacy of a drug. In the long-term, the changed neurotransmission leads - accomplished by protein signaling cascades, which can also be affected by genetic variants - to the desired or adverse effects [14,15].

Pharmacogenetics (PG) thus comprises (see Figure 1)[2]:

a) genetic differences in drug metabolism and disposition, i.e. pharmacokinetics
b) genetic influences on drug response, such as drug-receptor interactions, i.e. pharmacodynamics

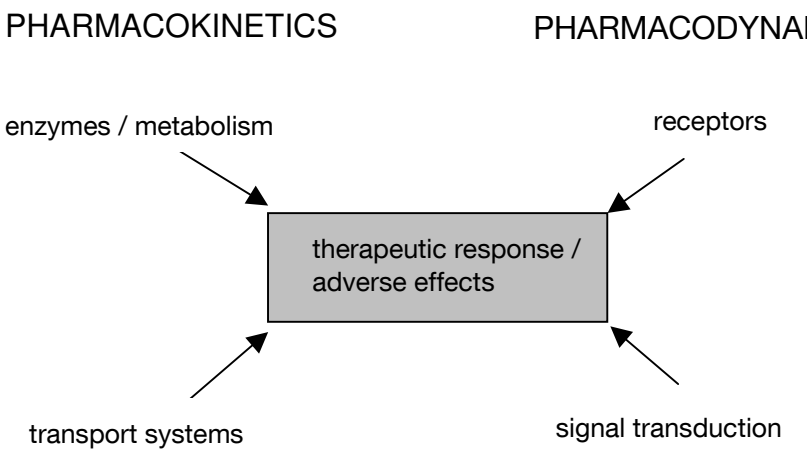

Figure 1: Genetic influence on pharmacotherapy

Note: Figure adapted from Steimer et al. (2002) [2]

Pharmacokinetics (PK) studies the impact of the organism on the drug i.e., the dissemination of a drug in an organism, which depends upon the processes of absorption, distribution, metabolism, and excretion. The main aim is to describe the relationship between the administered drug dose and drug concentrations in plasma or tissue and to answer questions such as: Are there genetic variations in the metabolic enzymes that affect the amount of drug at its site of action? Is the drug present in sufficient concentration for appropriate duration in the body to produce the desired effect? Or is the medication at a concentration that increases the risk of adverse effects?

Pharmacodynamics (PD) studies the impact of the drug on the organism, which depends upon the interaction of drugs with their receptors and the subsequent cellular and physiological changes. The main aim is to describe the relationship between drug concentrations and drug effects and to answer questions such as: Are there genetic variations in transporters or receptors that affect drug efficacy (binding or target expression)? Are there genetic variations in targets downstream from the primary site that may modulate efficacy and tolerability?

Pharmacogenetics - what is it good for? - Aims of Pharmacogenetics in Modern Medicine

Today, most major drugs are effective in only 25 - 60% of patients, and more than 2 million cases of adverse drug reactions occur annually in the United States, including 100,000 deaths [16]. Fatal adverse drug reactions have been reported to be the fourth leading cause of death in the USA [17].

One major aim of pharmacogenetics is to improve therapy with existing drugs i.e., to maximize the clinical outcome and minimalize risks. The individual genetic information could be used to guide pharmacotherapy by providing individualized and biologically based treatment decisions [18]. The ultimate aim would be to identify risk patients by pharmacogenetic screening of the relevant polymorphisms before start of therapy. By choosing the optimal dose and drug, toxic overdoses or ineffective drugs resulting in unsuccessful therapy could be avoided thus lowering therapy duration and costs [2]. Thus, much of the research today is being driven by a desire to use genetics to predict which patients deviate from the norm in terms of drug response or side effects. By identifying these people, it would be able to direct clinical practice so that therapies for these disorders can be individualized [19].

Pharmacogenetic research - what has been done so far? - Use and Relevance of Pharmacogenetics in Modern Medicine and Pharmacogenetic Research Today

Much knowledge has been gathered throughout the last 3 decades about the genetic basis. Most early research in pharmacogenetics has focused on pharmacokinetics. This is not altogether surprising since drug levels are easily measured and correlated to clinical response and side effects (= 'therapeutic drug monitoring'). It was at the level of drug metabolizing enzymes at which genetic factors were first found to influence drug response. It is now evident that most drug metabolizing enzymes are expressed in genetically variant forms with altered functional properties. Genotyping tests for drug metabolizing enzymes for the routine laboratory are already available. Nowadays, the possible use of these genetic tests in clinical practice is widely discussed and advantages/disadvantages are compared to older phenotyping methods and therapeutic drug monitoring [2,3,5,13].

Mutations in metabolizing enzymes causing different drug blood levels cannot fully account for the heterogeneity observed in the response of an individual to drug treatment. Until now, the genetic basis of pharmacodynamic variability has been less well studied than the genetics of pharmacokinetics [17]. More recently, the focus has shifted to pharmacodynamics and the downstream mediators of drug effects. Pharmacodynamic variability often even exceeds pharmacokinetic variability [20]. This should not be surprising as the genetic control of an enzyme occurs most usually via a single locus. The target structures of the drugs are often much more complex involving multiple subunits and proteins. Additionally, possible subsequent cellular and physiological pathways prior to expression of response will of necessity involve multiple genes thus multiplying the potential for polymorphisms [2]. The genetic basis of pharmacodynamic variability has been much less studied than that of pharmacokinetics and routine testing is still far away. The clinical relevance of PD genetic variants has - with just a few exceptions - not been established so far.

There are, however, limitations of pharmacogenetic testing. As a result of many non-genetic environmental influences on drug response such as non-compliance and due to population/ethnic differences with regard to frequencies of gene variants, it is likely that pharmacogenetic testing will be neither completely predictive nor universally applicable [1].

1.1 Study Outline

The variability of treatment response is a major problem in psychiatry. In depression and schizophrenia, 30 - 50% of all patients do not sufficiently respond to the initial treatment. Due to the delayed effect onset of antidepressants and antipsychotics, it can take up to 6 weeks for these therapy-resistant patients to be identified. No prediction of the response is possible up to now, time- and cost-consuming therapy over at least weeks is necessary until it can be seen whether a drug is effective. Additionally, many patients suffer from substantial adverse effects caused by antidepressants and neuroleptics making switches to other drugs necessary thus extending therapy duration as well [2]. The intention of this study was to replicate previous findings from studies with controlled, selected monotherapeutic study populations in a naturalistic clinical setting. The aim was to evaluate whether genotyping maybe helpful in everyday clinical practice and to evaluate which polymorphisms have such an impact on clinical outcome that an examination maybe useful as additional assistance for future treatment decisions.

CANDIDATE GENE APPROACH

Neuropsychiatric diseases arise from a combination of genetic and environmental factors. First of all, depression and schizophrenia - the two neuropsychiatric disorders with the highest prevalence in the population - are presented and their etiopathogenesis, symptoms and therapy in consideration of the possible pharmacogenetic influence are discussed in § 1.2.

A substantial fraction of the non-responders maybe due to genetic variabilities in metabolism and drug target structures (receptors, transporters etc.) [18]. Twin studies showed that 60 - 90% of interindividual variability in antidepressant treatment response is due to heritable traits [21]. Antipsychotic response and side effects also showed concordance between twins or relatives [22-26]. Pharmacogenetic research in the area of neuropsychiatric illnesses is rapidly evolving. Due to the complexity of the human brain, however, it is not surprising that our knowledge about the interaction between genetics and the treatment of these illnesses is still very small [19]. Thus, candidate genes known to be linked to drug metabolism, drug transport and drug target structures of antidepressants and antipsychotics were chosen (see Figure 2). Polymorphisms possibly linked to treatment response or side effects were selected within the candidate genes on the basis of known association studies and in order to find predictors of the response or side effects to drugs that are relevant within a naturalistic clinical setting. The examined genes or polymorphisms and their link to pharmacogenetics of antidepressants and antipsychotics are discussed in § 1.3.

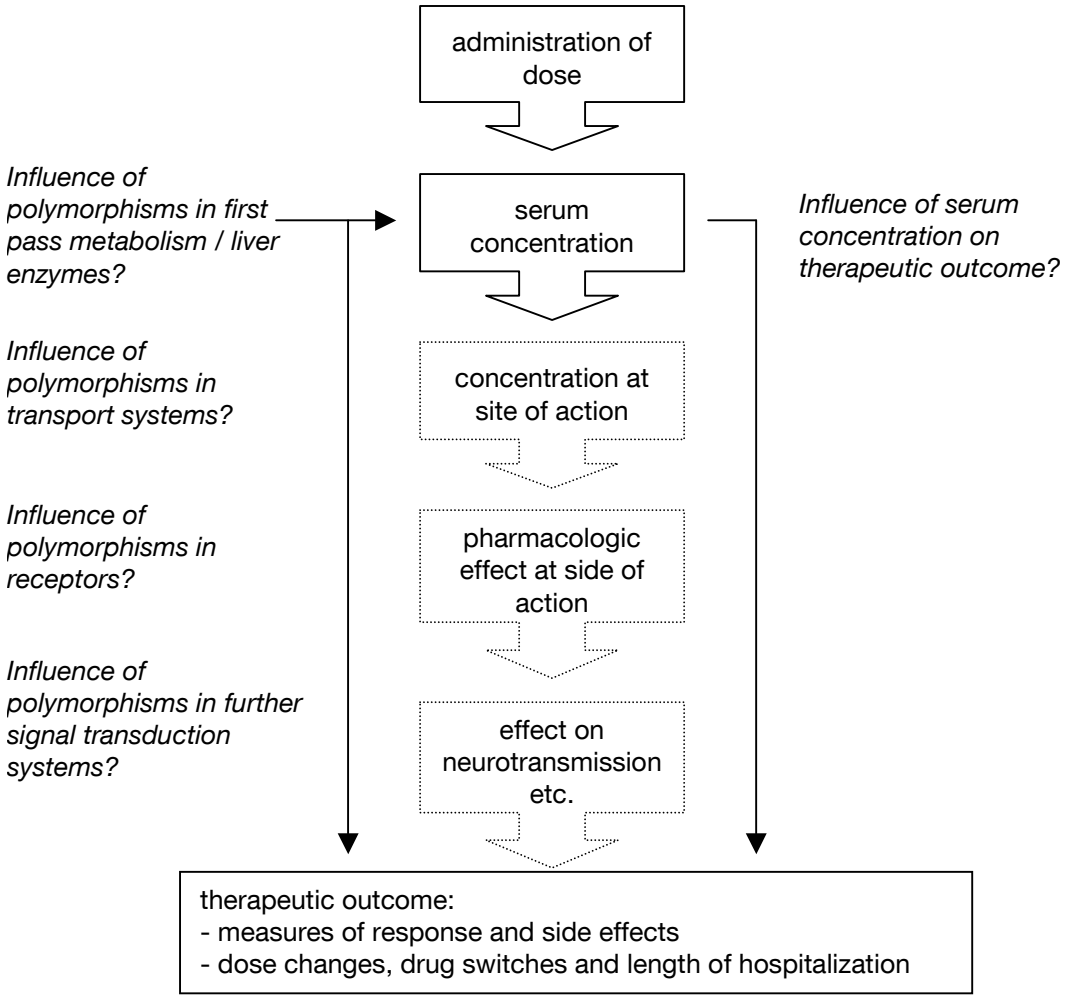

Figure 2: Study outline - genetic influence on pharmacotherapy

The scheme summarizes the possible genetic influence of candidate genes in drug metabolism, drug transport and drug target structures on pharmacotherapy of antidepressants or antipsychotics.

IMPLEMENTATION OF THE PHARMACOGENETIC PROJECT

A prospective, double-blind with respect to genotype, diagnostic study was conducted in the psychiatric department at the Klinikum rechts der Isar. The study design is presented in § 2.1.

GENOTYPING AND SERUM MEASUREMENT

For the selected candidate polymorphisms, several genotyping methods had been already routinely conducted at the PCR laboratory of the Institute for Clinical Chemistry and Pathobiochemistry [27,28]. For the other candidate polymorphisms, fast, sensitive and reliable methods suitable for routine testing in clinical practice were established and validated (see § 2.2). Moreover, serum concentrations of widely used drugs (olanzapine, mirtazapine, citalopram) were measured by HPLC-MS/MS (see § 2.3). The subsequent statistical analysis of the data was conducted as given in § 2.4.

1.2 Therapy of Depression and Schizophrenia

In the following chapters, the two neuropsychiatric disorders with the highest prevalence in the population - depression and schizophrenia - are presented and their etiopathogenesis, symptoms and therapy in consideration of the possible pharmacogenetic influence are discussed.

1.2.1 Depression

1.2.1.1 Symptoms and Diagnosis

Clinical depression is currently the leading cause of disability as measured by YLDs (Years Lived with A Disability), and it is expected to become the second leading cause of disability as measured in DALYs (Disability Adjusted Life-Years) worldwide (after heart disease) by the year 2020, according to the World Health Organization (WHO) [29].

Depression as defined by the ICD-10 (International Statistical Classification of Diseases and Related Health Problems, 10th version of the World Health Organization) is an acute or chronic clinically relevant condition severe enough to need treatment. It is characterized by severely depressed mood, loss of interest or pleasure in combination with other features such as feelings of guilt or low self-worth, disturbed sleep or appetite, low energy and poor concentration that persists for at least two weeks [29].

Mood disorders (F3 according to the ICD-10) are further subclassified by severity, additional somatic and psychotic features, recurrence or persistence of symptoms. Depressive episodes in conjunction with episodes of mania or markedly elevated mood are classified as bipolar disorder [29].

1.2.1.2 Etiopathogenesis

Clinical depression is a complex illness with multifactorial origin, which arises from a combination of sociopsychological and biological influences. Environmental risk factors may be e.g., stressful life events. Family, twin and adoption studies showed that depressive disorders are partly due to genetic predisposition, though no definite risk genes for depression could be identified so far [30,31].

The most prominent theory on the etiopathogenesis of depression is the monoamine deficiency hypothesis where deficiency of the neurotransmitters serotonin and noradrenaline in certain CNS regions causes depression. This theory is mainly supported by the successful therapy with antidepressants, which increase the serotonin and noradrenaline levels in the synaptic cleft [32]. The monoamine hypothesis of depression, however, explains the mechanisms of the illness only in part. In the last years, it has been widened to a dysbalance of different (e.g., serotonergic, noradrenergic, dopaminergic, glutaminergic and peptide neurotransmitter systems in combination with trophic factors such as brain-derived neurotrophic factor (BDNF) [14,15,33,34].

Certain neuroendocrinal changes such as changes in thyroid function and increased cortisol levels leading to hippocampal atrophy were also postulated to be involved in the etiopathogenesis of depression. The neuroplasticity hypothesis combines some aspects mentioned so far: stress and increased cortisol levels lead to a dysfunction in the hypothalamic-pituitary-adrenal (HPA) axis. In conjunction with genetic predisposition, this results in a decrease of transcription factors such as the cAMP-responsive-element-binding protein (pCREB) and transcription of neuroprotective factors such as the neurotrophin BDNF. Loss of neurons in the hippocampus is found in depression and correlates with impaired memory and dysthymic mood. Treatment resulting in a long-term increase of brain serotonin levels stimulates neurogenesis of the hippocampus, the center for both mood and memory [35].

1.2.1.3 Therapy

There are two primary modes of treatment that are typically used in conjunction: psychotherapy and somatic therapy. Somatic therapy includes light therapy, exercise, sleep deprivation, electroconvulsive therapy, repetitive transcranial magnetic stimulation and - most important - medication with antidepressants [31,36].

The acute effect of antidepressants on neurotransmission, however, cannot fully explain the effects on mood, which are only apparent after 2 - 6 weeks. This late onset of antidepressant efficacy is explained by long-term adaptive changes in monoamine neurotransmission (altered sensitivity and abundance of receptors), which arise from chronic inhibition of reuptake. Moreover, resultant changes in the subsequent intracellular signaling cascades and altered transcription of neuroprotective factors such as the neurotrophin BDNF [32] are discussed to play a role for the long-term effects of antidepressants on the mood.

Classes of antidepressants are defined by their chemical structure and mode of action. The main groups are [31,37]:

- inhibitors of monoamine oxidase enzymes, which are responsible for the degradation of monoamines such as serotonin and dopamine (MAO inhibitors e.g., tranylcypromine, moclobemide).
- tri- and tetracyclic antidepressants (TCAs e.g., amitriptyline, imipramine, doxepin, maprotiline).

  The major pharmacological effect of TCAs is their ability to block the transporters responsible for the presynaptic neuronal uptake of the neurotransmitters noradrenaline and serotonin following their release into the synaptic cleft.
- selective serotonin reuptake inhibitors (SSRIs e.g., fluoxetine, citalopram).

  SSRIs bind rather specifically to the serotonin transporter (5-HTT) and block the serotonin (5-hydroxytryptamine = 5-HT) reuptake from the synaptic cleft into the presynaptic neuron.
- selective serotonin and noradrenaline reuptake inhibitor (SSNRI, venlafaxine).
- selective noradrenaline reuptake inhibitors (SNRI e.g., reboxetine, milnacipram).

  Similar to SSRIs, the SNRI block rather selectively only the noradrenaline transporter (NET), but show no significant affinity to other receptors.
- adrenoceptor (ADR2) and serotonin (5-HTR2 and 5-HTR3) receptor antagonist mirtazapine, which increases the dopaminergic, serotonergic and noradrenergic neurotransmission [38,39].

Side effects of antidepressants are partly due to the - desired - reuptake inhibition and activation of postsynaptic receptors resulting in serotonergic side effects (e.g., initially decreased appetite and weight gain later, nausea, vomiting, diarrhea, headache, sleep disorder, sexual dysfunction) and noradrenergic side effects (e.g., tremor, tachycardia, anxiety, headache) [40].

Especially TCAs also bind to other postsynaptic receptors leading to a typical adverse effects profile characterized by

- anticholinergic (M1) side effects (e.g., blurred vision, memory and cognitive impairment, dry mouth, constipation, micturition problems)
- antihistaminergic (H1) side effects (e.g., drowsiness, sedation, weight gain)
- antiadrenergic (ADR1) side effects, (e.g., hypotension, dizziness, reflex tachycardia)
- antiserotonergic (5-HTR2A) side effects (e.g., weight gain, sedation, anxiolysis)
- dopaminergic (DRD2) side effects (e.g., prolactin elevation, extrapyramidal-motoric side effects) and
- cardiotoxic side effects by blockade of cardiac sodium and calcium channels.

1.2.2 Schizophrenia

1.2.2.1 Symptoms and Diagnosis

Schizophrenia is a chronic, debilitating psychotic disorder that affects about 1% of people worldwide. It is characterized in general by fundamental and characteristic distortions of thinking and perception, and affects that are inappropriate or blunted [29]. The symptoms are classified as either psychotic or positive symptoms such as hallucinations and delusions or as negative symptoms such as severely inappropriate emotional responses, disordered thinking and concentration, erratic behavior, as well as social and occupational deterioration [41]. The course of schizophrenic disorders can be either continuous or episodic, with progressive or stable cognitive deficits, or there can be one or more episodes with complete or incomplete remission [29].

Due to several forms and subforms with many possible combinations of symptoms, there is debate about whether the diagnosis represents a single disorder or a number of discrete syndromes since Eugen Bleuler raised 1911 the term “group of schizophrenias” or “the schizophrenias” [42]. The ICD-10 F2 block (F20 – F29) includes besides the most important member schizophrenia other disorders with similar features (schizotypal disorder, persistent delusional disorders, and a larger group of acute and transient psychotic disorders), but leaves cases out where the symptoms result from medication, psychoactive substance abuse or other brain diseases such as epilepsy or autism. A special case are schizoaffective disorders where affective and schizophrenic symptoms are prominent, but which do not justify a diagnosis of either schizophrenia or depressive or manic episodes [29].

1.2.2.2 Etiopathogenesis

Schizophrenia is complex in origin. Epidemiological findings from family, twin and adoption studies estimate that the heritable component accounts for about 63-85% of the risk [43]. However, the exact mode of inheritance is still unclear, though several putative susceptibility genes e.g., BDNF (brain derived neurotrophic factor), COMT (catechol-O-methyltransferase), and DISC1 (disrupted in schizophrenia 1) were recently identified [44]. Altered function or abundance of these susceptibility genes may - as well as environmental risk factors (e.g., perinatal and childhood brain injury, psychosocial stress) - contribute to disturbances in brain development and function.

The consequence is a brain that is hypersensitive to stimuli and unable to regulate its response to the incoming information through normal inhibitory mechanisms and to respond properly [41]. Disturbed dopaminergic signal transduction seems to play a prominent role. Increased dopaminergic activity in the mesolimbic pathway of the brain is thought to be responsible for the positive symptoms of schizophrenia. Problems with adequate dopamine function in the mesocortical pathway are thought to contribute to the negative symptoms. The dopamine hypothesis of schizophrenia is supported by the observation that antipsychotics, which block the dopamine (DRD2) receptor, suppress the increased dopaminergic activity in the mesolimbic pathway thereby reducing psychotic symptoms [45-47]. Additional evidence is drawn from the observation that - particularly after large doses or prolonged use - drugs such as amphetamine or cocaine, which increase dopamine neurotransmission, can cause psychosis. Moreover, quite small amounts of these drugs can exacerbate positive symptoms in people predisposed to developing schizophrenia [48,49]. The dopamine hypothesis of schizophrenia is, however, not without controversy. Blockade of dopamine neurotransmission does not fully alleviate symptoms of schizophrenia, especially negative symptoms. Dopamine levels and receptors in schizophrenics measured before and after treatment are similar to healthy subjects. Dopamine inhibiting medications modify dopamine levels within minutes, but the associated improvement develops over the course of weeks [41].

Thus, the theory of disturbed dopaminergic signal transduction is thought to be overly simplistic as a complete explanation and led to the hypothesis that other neurotransmitter circuits are involved or are even the underlying cause. Atypical antipsychotics also block serotonin (5-HTR2) receptors. This and the observation that the hallucinogen effect of lysergic acid diethylamide (LSD) is due to its partial agonism at 5-HTR2A receptors led to the hypothesis that altered serotonin function is involved in schizophrenia [50]. Later on, the excitatory neurotransmitter glutamate and the reduced function of the NMDA glutamate receptor were discussed to be involved in schizophrenia. This has largely been suggested by abnormally low levels of glutamate receptors found in postmortem brains of people previously diagnosed with schizophrenia and the discovery that the glutamate (NMDA) receptors blocking drugs such as phencyclidine and ketamine cause psychosis closely resembling

schizophrenia [51,52]. The fact that glutamate can affect dopamine function has suggested an important mediating role of glutamate pathways in schizophrenia [53].

Recently, it was shown in a variety of animal models of psychosis that a marked behavioral supersensitivity to dopamine and a marked rise in the number of dopamine DRD2 receptors in the high-affinity state for dopamine could be induced [54] (e.g., sensitization of animal behavior by the dopamine releasing amphetamine, or the glutamate blocker phencyclidine, or excess steroids, or by removing various genes - e.g., COMT, DBH, DRD4 - , or by making brain lesions in newborn animals, or by delivering animals abnormally by Caesarian section). As this dopamine supersensitivity is a common feature of schizophrenia [55], these results suggest that there are multiple genes and neuronal pathways that can lead to psychosis and that all these multiple psychosis pathways converge via the high-affinity state of the DRD2 receptor, the common target for all antipsychotics [46,56].

1.2.2.3 Therapy

Besides psychological care and social intervention, antipsychotics have been a mainstay of therapy, in the treatment of acute psychotic episodes and for preventing relapse. Most antipsychotics take around 7 - 14 days to reach their main effect [41]. Antipsychotics are broadly divided into two groups, the typical or first generation antipsychotics (e.g., chlorpromazine and haloperidol) and the atypical or second generation antipsychotics (such as clozapine, olanzapine, risperidone, amisulpride, aripiprazole, quetiapine, zotepine, ziprasidone, sulpiride).

All currently available antipsychotic drugs - typical or atypical - block central dopamine receptors. Reduction of the excess release of dopamine in the mesolimbic pathway, which has been linked to positive symptoms, is thought to have the desired effect. Antipsychotics, however, are not particularly selective and also block dopamine receptors all over the brain. Blocking DRD2 receptors in the tuberoinfundibular pathway and the nigrostriatal pathway is thought to produce elevation of prolactin (leading to gynecomastia in men and galactorrhea and amenorrhea in women) and extrapyramidal-motoric side effects (such as rigor, tremor, akathisia, tardive dyskinesia) [46]. A further severe side effect of dopamine receptor blockade in the hypothalamus is temperature dysregulation [57].

Typical antipsychotics are further subclassified on a spectrum of low potency to high potency, where potency refers to the ability of the drug to bind to dopamine receptors, and not to the effectiveness of the drug. High potency antipsychotics such as haloperidol typically have doses of a few milligrams and cause more extrapyramidal-motoric side effects (EPS) than low potency antipsychotics such as chlorpromazine, which have dosages of several hundred milligrams and a greater degree of antihistaminergic activity leading to sleepiness and calming effects [41].

Atypical antipsychotic drugs have a similar blocking effect on DRD2 receptors and are similarly effective for the treatment of positive symptoms [58]. But atypical drugs are less tightly bound than typical antipsychotics to the DRD2 receptor (hypothesis of fast dissociation) [45,50,59,60]. Most atypical drugs also block serotonin receptors (particularly 5-HTR2A, 5-HTR2C and 5-HTR1A receptors): ranging from risperidone, which acts overwhelmingly on serotonin receptors, to amisulpride, which has no serotonergic activity but blocks DRD3 and DRD4 receptors [61]. Clozapine, the first atypical drug, exhibits additionally to DRD2 and 5-HTR2 blockade also antagonism on other receptors, such as dopamine (DRD1, DRD4) receptors, adrenergic (ADR), cholinergic (mACh) receptors, histaminergic (H1) receptors [50,62].

These additional features are discussed to contribute to the atypicality of the drugs with beneficial effects on the negative symptoms and less side effects. Newer atypical antipsychotic drugs are often better tolerated and were associated with lower rates of extrapyramidal-motoric side effects, although they seem to be more likely to induce weight gain and obesity-related diseases such as diabetes [63]. A recent review has, however, refuted the claim that all of the atypical antipsychotics have fewer extrapyramidal side effects than typical antipsychotics, especially when the latter are used in low doses or when low potency antipsychotics are chosen [64].

Side effects are varying between the different atypical antipsychotics due to the different binding profile e.g., weight gain is more (clozapine, olanzapine) or less (ziprasidone, amisulpride) pronounced and may be due to antagonism at different receptors such as histamine (H1) or serotonin (5-HTR2C) receptors [65,66].

1.3 Candidate Genes and Relevant Polymorphisms

In this chapter, the polymorphisms selected on basis of known association studies and their possible link to treatment response or side effects of antidepressants or antipsychotics are discussed.

1.3.1 Drug Metabolism

The family of cytochrome P450 (CYP) is an important oxidative enzyme system mainly located in the liver, but also in the intestinal epithelium. Over 100 isoenzymes have been found in mammals, with at least 57 isoenzymes described so far in humans (current status available from the homepage of the Human Cytochrome P450 (CYP) Allele Nomenclature Committee [67]). Some of these isoenzymes are involved in the metabolism of xenobiotics. Cytochrome P450 enzymes are responsible for roughly 70 -80% of the phase I metabolism of commonly used medications [13]. The vast majority of drugs used clinically are metabolized by CYP2D6, CYP3A4, CYP2C19 and CYP2C9 [68]. The highly polymorphic CYP2D6 and CYP2C19, taken together, roughly influence 25 - 30% of prescribed medications [13,69]. Other isoforms involved less frequently are CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9 and CYP2E1 [13,19].

Polymorphisms can influence the functionality or abundance of key enzymes and can lead to severe changes in the metabolism of drugs resulting in higher or lower drug concentrations and may explain part of the variable drug response or side effects. The genetic variations form the basis for the phenotypes, which were defined long before the genetic variations were identified in detail: poor, intermediate, extensive (i.e., the wild-type) and ultrarapid metabolizers. A test substance was used to probe the activity of the enzyme e.g., debrisoquine for CYP2D6 [3,70]. In general, four classes of metabolizers based on the extent of drug metabolism are distinguished [71,72]:

- extensive (EM) with two functional alleles of the respective enzyme representing the normal metabolism in the majority of the population.
- poor (PM) with no functional allele of the respective enzyme resulting from different genetic abnormalities such as gene deletion, incomplete transcript, splicing error, or inactive enzyme transcript.
- intermediate (IM) with one functional and one deficient allele or two partially defective alleles leading to impaired metabolism.
- ultrarapid (UM) with extremely high metabolic capacity compared to the extensive metabolizers either due to enhanced transcriptional activity or gene duplication.

1.3.1.1 CYP1A2

CYP1A2 is in part responsible for the metabolism of several antidepressants (amitriptyline, clomipramine, fluvoxamine, imipramine) and antipsychotics (clozapine, haloperidol, olanzapine) [69]. The metabolic activity of CYP1A2 shows a high interindividual variability that may result in different serum concentrations and clinical outcome. CYP1A2 is inducible by tobacco smoke, certain foods such as broccoli, brussels sprouts and char-grilled meat or co-medication with e.g., omeprazole. CYP1A2 activity can be inhibited by several drugs such as fluoroquinolones or fluvoxamine [69].

Several gene variants with decreased *in vivo* activity are known, but are too rare among Caucasians for statistical evaluation in the study population [73-77] (< 1% for *C, *1K, *3, *4, *6 and *7 in Caucasians). Furthermore, previous studies explained part of the variability of CYP1A2 enzyme activity by the CYP1A2*1F (rs762551) polymorphism. It is a common C to A transition in intron 1 of the CYP1A2 gene at position 734 downstream of the first transcribed nucleotide and termed C-163A according to the Human Cytochrome P450 (CYP) Allele Nomenclature Committee [67]. The CYP1A2*1F homozygote variant (A/A) genotype was associated with higher enzyme inducibility and higher clearance of CYP1A2 substrates like melatonin [78] or caffeine in Caucasian smokers [79,80]. The effect of this polymorphism on CYP1A2 substrates such as olanzapine, however, has not been examined yet.

1.3.1.2 CYP2C19

CYP2C19 is responsible for the metabolism of several antidepressants (amitriptyline, citalopram clomipramine, fluoxetine, moclobemide, imipramine) and antipsychotics (clozapine, haloperidol, olanzapine). CYP2C19 is induced by e.g., carbamazepine and inhibited by proton pump inhibitors such as omeprazole [69].

An estimated 2 - 5% of Caucasians are CYP2C19 poor metabolizers with a lack of functional enzyme. Mainly responsible are three SNPs leading to aberrant splicing (*2), a premature stop codon (*3) or a mutation in the initiation codon (*4) and account for approx. 87% of Caucasians poor metabolizers [81-84]. Recently, a new allele (CYP2C19*17), which causes increased transcriptional activity and leads to an ultrarapid metabolism in approx. 18% of Caucasians, was identified in the promoter region of the CYP2C19 gene [85].

Poor metabolizers (PMs) of CYP2C19 are well known to have significantly reduced clearance and elevated serum concentrations compared to extensive metabolizers (EMs), but less is known about the intermediate metabolizer (IM) status. The clinical significance of intermediate metabolism (heterozygosity with one inactive allele) on clinical outcome (response and side effects) has not yet clearly been demonstrated for most CYP2C19 substrates [86,87].

1.3.1.3 CYP2D6

CYP2D6 metabolizes about 25% of the clinically used drugs including several antidepressants (amitriptyline, clomipramine, fluoxetine, fluvoxamine, nortriptyline, moclobemide, desipramine, imipramine, paroxetine) and antipsychotics (aripiprazole, chlorpromazine, haloperidol, perphenazine, risperidone, thioridazine, zuclopenthixol) [13,69]. CYP2D6 is induced by fluoxetine, paroxetine and weakly by sertraline. It is inhibited by dexamethasone and rifampin [69].

The CYP2D6 gene (GenBank Acc. No. M33388) resides in the CYP2D6 cluster on chromosome 22 in association with the CYP2D7P and CYP2D8P pseudogenes [13]. It exhibits much more diversity than many other cytochrome P450 enzymes and the allele frequencies vary widely between different ethnicities. At least 50 variations have been described that determine the level of activity of the enzyme - and thus the effects of drugs that are metabolized by the CYP2D6 pathway (see the homepage of the Human Cytochrome P450 (CYP) Allele Nomenclature Committee [67]).

The most relevant mutations are gene amplification, gene deletion *5, non-functional alleles such as *3, *4, *6, *7, *8 and impaired alleles such as *9, *10, *41. In Central Europe, 2 - 5% of the population are UMs and 5 - 10% are PMs. Recent estimations for IMs reach - due to inclusion of the new CYP2D6*41 allele - up to approx. 30 - 40% IMs [72,88,89]. It must be noted that the frequent CYP2D6*41 allele was not included in the genotyping of most former studies leading to inaccuracies in gene dose assignment. This might have prevented the detection of significant associations for the IM status. Thus, new studies on the relevance of the CYP2D6 IM status are necessary.

1.3.2 Drug Transport and Disposition

Genetic variations in drug metabolizing enzymes are not sufficient in explaining the wide interindividual variation observed in drug concentrations and clinical outcome. The disposition of many drugs is influenced not only by the drug metabolizing enzymes but also by transporters such as P-glycoprotein.

1.3.2.1 MDR1

The multidrug resistant (MDR1) gene encodes the P-glycoprotein, which functions as an efflux transporter in different cells and which is widely localized in normal tissues including the gastrointestinal tract, blood cells, biliary tract, kidney and brain. It plays a major role in absorption, distribution and elimination of various xenobiotics [90].

As part of the blood-brain barrier, P-glycoprotein (P-gp) actively exports significant amounts of drugs from the brain. Recent studies showed that brain penetration of several antidepressants and antipsychotics such as olanzapine, risperidone and its active metabolite 9-OH-risperidone [91,92], amitriptyline, nortriptyline, citalopram, trimipramine, doxepin, venlafaxine, and paroxetine, but not mirtazapine, is enhanced in mice lacking P-gp [93-95]. Therefore, dysfunctional polymorphisms of the MDR1 gene might lead to a reduced activity of P-gp and higher bioavailability of P-gp substrates in the CNS. Increased antipsychotic penetration to the primary site of activity may result in greater symptom improvement or occurrence of side effects.

One polymorphism has been identified, C3435T, in which T/T homozygotes show lower expression of the transporter and, in theory, higher drug concentrations in the brain and more pronounced drug effects. This synonymous SNP, however, seems not to be causative for the phenotype, but serves as marker for the so-far unidentified changes in and around the highly polymorphic MDR1 gene (so called linkage effect) [96]. C3435T in exon 26 is frequently linked with the synonymous SNP C1236T in exon 12 and with a nonsynonymous, triallelic SNP, G2677TA (Ala893Ser/Thr) in exon 21. Recently, the 3435T-2677T-1236T haplotype variant was shown to have a markedly different expression and function compared to the wild type [97].

Recently, better response to risperidone [98] and olanzapine [99,100] treatment was partly associated with the T-allele of one of these three common polymorphisms, C1236T, G2677TA or C3435T, respectively. Postural hypotension, an adverse drug effect of nortriptyline was linked to MDR 3435T/T genotype [101]. In a Japanese study, the wildtype 2677G was associated with poor paroxetine treatment response [102]. These positive results, however, were not found in all studies conducted so far [90,103-106] and a final decision on the influence of these three MDR1 polymorphisms on therapeutic outcome has not emerged yet.

1.3.3 Drug Targets (Site of Action)

It is now becoming increasingly evident that polymorphisms of pharmacological targets (pharmacodynamic polymorphisms) may in fact be more important and clinically relevant than polymorphisms of drug disposition (pharmacokinetic polymorphisms). In a pharmacogenetic study that compared paroxetine and mirtazapine, paroxetine-induced side effects were strongly associated with the 5-HTR2A genotype rather than with the CYP2D6 genotype [107]. Thus, although paroxetine is metabolized by CYP2D6, the 5-HTR2A polymorphism might be a more important determinant of paroxetine-induced side effects.

Most pharmacogenetic studies exist for the primary drug targets i.e., the serotonergic, dopaminergic and noradrenergic transporters and receptors. Further pharmacogenetic investigation into histaminergic and glutaminergic receptors - which are also partially antagonized by antipsychotic drugs - is still at its infancy and no conclusive data has emerged yet. Targets in the subsequent signaling pathways or several other genes of the monoaminergic system are discussed in the following chapter § 1.3.4.

1.3.3.1 Serotonergic System

1.3.3.1.1 Serotonin Transporter (5-HTT)

Just as CYP2D6 represents a prototypical pharmacokinetic gene, the serotonin transporter (5-HTT or SLC6A4) has played a similar role as a pharmacodynamic gene in psychiatry, particularly for antidepressant response. As the site of action of the selective serotonin reuptake inhibitors (SSRIs), the serotonin transporter represents a logical site at which genetic variation could influence treatment response.

A common variation in the promoter region of this gene is the 5-HTT length polymorphism (5-HTTLPR) with a 44-base-pair region either being present or absent. The resulting alleles are described as either long (L) or short (S). The L-allele has been associated with increased transcription of the gene and higher biological activity of 5-HTT [108]. This polymorphism has been examined in several association studies with SSRIs yielding inconsistent results [109]. A recent meta-analysis on 15 studies, with data from 1435 subjects including studies in non-Caucasian populations, showed significant evidence of association of the L-allele of the 5-HTTLPR with treatment response [110]. However, this was not found in a large clinical sample with 1914 subjects [111].

Recently, a new SNP in the 5-HTT gene (rs25531 A/G) nearby the 5-HTTLPR was reported to have additional influence on transcription activity [112-114]. The high expression variant L_A (i.e., 5-HTTLPR L-allele in combination with rs25531 A-allele) was associated with better treatment response and less adverse effects in SSRI therapy [115-117]. Thus, the combined effect of both polymorphisms on treatment response and side effects were examined herein.

1.3.3.1.2 5-HTR1A Receptor

Antidepressants, such as serotonin or noradrenaline reuptake inhibitors, desensitize the 5-HTR1A autoreceptors by increased synaptic 5-HT levels, which may contribute to their clinical efficacy [118,119]. The functional 5-HTR1A C-1019G polymorphism lies within a palindromic sequence that contains discrete DNA-binding sites for at least two repressors. Binding of the transcription factors and repression was highly specific for the -1019C-allele, and blocked for the -1019G-allele. It was postulated that the -1019G-allele results in impaired repression of the 5-HTR1A receptor gene, leading to elevated levels of 5-HTR1A autoreceptors and inhibition of basal raphe neuronal activity [118]. Correspondingly, better treatment response for the 5-HTR1A -1019C-allele was reported in some studies on SSRIs and TCAs in depressive patients [119-125].

1.3.3.1.3 5-HTR2A Receptor

The serotonin receptor 5-HTR2A is one of the two major 5-HTR2 receptor subtypes. Serotonin reuptake inhibitors increase the availability of serotonin (5-HT) in the synaptic cleft. The efficacy of these antidepressants and related side effects could be due to the effect of the released serotonin on postsynaptic 5-HTR2A receptors, although other receptors such as 5-HTR3 may also be involved [107,126]. Thus, the 5-HTR2A gene is a candidate gene for clinical outcome to antidepressants, especially SSRIs. Moreover, especially atypical antipsychotics also target serotonin receptors (5-HTR1-7) in particular the 5-HTR2 subtypes [61].

Several 5-HTR2A polymorphisms were examined for associations with clinical outcome under antidepressant or antipsychotic therapy. Only few results, however, could be replicated at all [127]. Some significant associations were reported for the silent 5-HTR2A T102C polymorphism and/or the 5-HTR2A A-1438G, which are almost in complete linkage. The association between the 102 C-allele and better treatment response/more side effects to the SSRI paroxetine could not be replicated in all studies [107,109,128-130]. The results published so far for atypical antipsychotics are also inconsistent, although some positive results were reported for the silent T102C polymorphism, especially for clozapine treatment response in a metaanalysis, but also for typical antipsychotics [131-135].

In a recent study with 1953 depressive patients, who were treated with the antidepressant citalopram, a highly significant and reproducible association between treatment outcome and a marker in intron 2 of 5-HTR2A (rs7997012) was found. Participants that were homozygous for the A-allele had an 18% reduction in absolute risk of having no response to treatment, compared with those homozygous for the other allele [126]. Thus, the effect of this new polymorphism on clinical outcome was examined herein and compared to results for the well known 5-HTR2A polymorphism.

1.3.3.1.4 5-HTR2C

The 5-HTR2C receptor gene is found on the X-chromosome and codes for one of the two major 5-HTR2 receptor subtypes, which are targeted by antipsychotics [136]. *In vitro* data suggested that the 5-HTR2C C-759T promoter polymorphism is functional. The -759C-allele showed less transcriptional activity and thus underexpression of 5-HTR2C compared to haplotypes containing the -759T-allele [137,138]. Promoter polymorphisms near the major transcription initiation site of the 5-HTR2C receptor at -703 were associated with obesity and diabetes in Japanese [139]. Accordingly, several studies showed a significant association between the 5-HTR2C -759C-allele and higher weight gain under antipsychotic treatment [140-144] except for one study [145].

1.3.3.1.5 Further Serotonin Receptors

Though antipsychotics, especially atypical ones, target serotonin receptors (5-HTR1-7), studies on other 5-HTR receptors have yielded conflicting results or suggest only a minor contributing role in clinical outcome of antipsychotics. Moreover, the role of these receptors in the clinical action of antipsychotics remains unclear [146,147]. For example, many antipsychotics have demonstrated high affinity for the 5-HTR6 receptor [148]. In addition, this receptor exhibits high affinity for a number of tricyclic antidepressant drugs, including amitriptyline [149]. Significant associations between a silent 5-HTR6 T267C polymorphism and treatment response to atypical antipsychotics (clozapine, risperidone) or antidepressants were, however, only found in some of the studies conducted so far [150-154].

1.3.3.2 Dopaminergic System

1.3.3.2.1 Dopamine Receptors

All currently available antipsychotic drugs - typical or atypical - act on central dopamine receptors. Of the 5 known dopamine receptor subtypes, DRD1 to DRD5, especially polymorphisms in the DRD2 to DRD4 have been examined extensively in pharmacogenetic studies whether they have an impact on antipsychotic treatment response. The occupancy of the dopamine DRD2 receptor plays a major role in the mechanism of action of both typical and atypical antipsychotics, but also other dopamine receptors such as DRD3 are involved [133]. Polymorphisms within these receptors may have an impact on treatment response.

Despite contradictory results, altogether a trend toward an association with favorable antipsychotic response seems to exist for the -141C ins-allele of the DRD2 -141C ins/del polymorphism and the A1-allele of the DRD2 Taq1A polymorphism [155,156]. In the case of the DRD3 receptor, the examination of the DRD3 Ser9Gly polymorphism yielded conflicting results. The Ser9-allele seem to be associated with better response to typical antipsychotics, and the 9Gly-allele with response to atypical antipsychotics [155,156]. The 9Gly-allele was also reported to be connected with extrapyramidal-motoric side effects under antipsychotic therapy [157].

1.3.3.2.2 Dopamine Transporter (DAT1)

As dopamine pathways play an important role in psychiatric diseases, the high affinity dopamine transporter DAT1 is another promising candidate gene, which could influence clinical outcome. The DAT1 VNTR is localized in the 3'-non-coding region. Higher - *in vitro* as well as *in vivo* - DAT1 expression in carriers of the 10-repeat allele compared with carriers of the 9-repeat allele were reported in some of the previous studies [158-162]. The reason for these differences might be an incomplete linkage of the VNTR with transcriptionally relevant polymorphisms [161]. Correspondingly, studies on extrapyramidal-motoric side effects [158,163] and response [164] under antipsychotic therapy led also to conflicting results.

Dopamine may also play an important role in depression [165] as well as in the response to antidepressant drugs. Dopamine transporter (DAT1) activities were found to be modulated during treatment with the SSRI citalopram. Reduced extracellular dopamine concentrations have been measured in striatum during medication with SSRIs [166,167]. In patients with a major depressive episode, a significantly lower number of rapid responders (i.e., within three weeks) was seen among homozygous carriers of the DAT1 9-repeat allele compared to heterozygous and homozygous carriers of the 10-repeat allele. Interestingly, the effect was seen independently from the type of antidepressive medication (SSRIs, tricyclic antidepressants, mirtazapine, venlafaxine) [168].

1.3.3.3 (Nor)adrenergic System

1.3.3.3.1 Norepinephrine Transporter (NET)

Antidepressant efficacy is mediated not only by serotonin reuptake inhibition but also by norepinephrine (= noradrenaline) reuptake inhibition. The norepinephrine transporter (NET) modulates noradrenergic signaling by reuptake of norepinephrine that has been secreted in the synaptic cleft. Norepinephrine reuptake inhibitors (NRIs) include e.g., the TCAs nortriptyline, desipramine and maprotiline, as well as milnacipram, reboxetine and venlafaxine (see § 1.2.1.3).

A clinical study on antidepressant response to the SNRI milnacipram revealed an influence of the NET T-182C and NET G1287A polymorphisms with superior response in carriers of the -182T-allele and slower response in carriers of the 1287 A/A genotype. No influence of the 5-HTTLPR polymorphism on response to milnacipram was detected in the same study [169]. A further study in Koreans revealed that the G/G genotype of the non-functional NET G1287A polymorphism plays a major role in better response to nortriptyline treatment [170].

Neither of NET polymorphisms examined so far alter the amino acid sequence. The NET T-182C variant, however, is localized within the NET promoter. The NET1 G1287A variant is a silent mutation in exon 9 [167], but higher cerebrospinal fluid (CSF) concentrations of 3-methoxy-4-hydrophenylglycol, a major norepinephrine metabolite [171], were found in 1287 G/G homozygote patients. Because patients with the A/A genotype have less active reuptake of norepinephrine, the effect of reuptake inhibition might occur more slowly [169].

1.3.3.3.2 ADR2A

Different subtypes of alpha adrenergic (ADR) receptors have been identified (ADR1A, 1B, 1D and ADR2A, 2B, 2C). Two of them, ADR1A and 2A, have been implicated in mediating antipsychotic response. Antagonism of adrenergic receptors has been shown to support antipsychotic action when coupled to neuroleptic treatment in patients with schizophrenia [172]. Most new antipsychotic drugs such as clozapine, risperidone, olanzapine, quetiapine and sertindole include adrenergic antagonism amongst their mechanism of action [172,173].

The adrenergic system has also been reported to play a key role in regulating energy balance through the stimulation of both thermogenesis and lipid mobilization in adipose tissue. Human body fat lipolysis is controlled by several hormones, among which catecholamines are the most important lipolytic ones. They act via G-protein-coupled adrenergic receptors and have a dual effect on lipolysis by stimulating via beta 1, beta 2, beta 3-adrenoceptors and inhibiting via alpha-2 adrenergic receptors. There is evidence that adrenergic alpha 2 receptors are related to weight change [174,175].

In contrast to other adrenergic genes, variability of the ADR2A is primarily due to SNPs in the promoter or in the 5' and 3' untranslated regions (UTR). Substantial differences in transcript and cell-surface protein expression, by as much as approx. 5-fold, was observed between haplotypes, including those with common frequencies [176]. In Caucasians, the most frequent polymorphism is the ADR2A C-1291G SNP. Recently, clozapine and olanzapine-induced weight gain was related to the -1291G-allele [177,178]. Moreover, mirtazapine shows a high affinity to ADR2A receptors, therefore this polymorphism may play a role in therapeutic outcome or side effects, especially in weight gain under mirtazapine therapy.

1.3.4 Novel Pharmacodynamic Candidates

The term pharmacodynamics encompasses all processes influencing the relationship between the drug concentration and the resulting effect [179]. The mechanism of therapeutic effect (and in some cases even the initial mechanism of action) for many psychotropics is poorly understood, which limits selection of candidate genes.

The primary drug targets such as the serotonin transporter and other neurotransmitter receptors were extensively studied in the recent years, whereas novel candidates more indirectly involved in drug action downstream of monoaminergic activation or indirectly influenced by monoaminergic modulation received much less attention. This focus has begun to broaden somewhat and in several previous studies associations between neurotransmitter synthesis (TPH1 and TPH2) and metabolism (COMT, MAO-A, DBH, ACE) and response or side effects were observed. Moreover, genes involved in signal transduction (G-protein-coupled receptors), genes modulating the hypothalamic-pituitary-adrenal (HPA) axis activity or genes involved in the neurodevelopment of neurotransmitter-related systems (BDNF) were associated in some studies with therapeutic outcome.

1.3.4.1 Synthesis and Metabolism of Neurotransmitters

1.3.4.1.1 TPH1 and TPH2

The tryptophan hydroxylase (TPH1) converts L-tryptophan into the serotonin precursor L-5-hydroxytryptophan, which is the rate-limiting step in serotonin synthesis. No common functional polymorphisms of TPH1 are yet known. Two relatively common polymorphisms in the TPH1 intron 7, C779A and A218C, which are in strong linkage disequilibrium [180,181], have been studied intensively in psychiatric disorders and treatment outcome but with mixed results [182-187].

Tryptophan hydroxylase (TPH1) has long been considered as the sole rate-limiting enzyme for the synthesis of 5-HT. However, the recently identified TPH2 is preferentially expressed in the brain. This form is an extremely plausible candidate for an association with depression. A functional variation, TPH2 G1463A, was associated, in a very small patient sample, with treatment resistance, but the SNP examined was not found in other study populations [188-193].

1.3.4.1.2 ACE

Angiotensin-converting enzyme (ACE) is well known as the key enzyme catalyzing the synthesis of angiotensin II. It is now emerging that the endopeptidase has pleiotropic effects cleaving many other substrates [194]. ACE is also expressed in the central nervous system where it is co-localized with substance P, and it is postulated that one of its important functions in the CNS is the degradation of neuropeptides including substance P. Besides the hypothalamic-pituitary-adrenal (HPA) axis, the substance P system is a candidate system for antidepressant efficacy. Enhanced substance P signaling via the neurokinin 1 (NK1) receptor has also been implicated in the pathophysiology of depression [179]. The role of ACE for antidepressant efficacy is supported by reports of an antidepressant-like effect of ACE inhibitors and neurokinin 1 (NK1) receptor antagonists [195-199]. Moreover, there is body of evidence supporting an interaction between brain angiotensin and central catecholamine systems [200].

The fact that the intraindividual ACE concentration is on a relatively constant level suggests a genetically driven component in the regulation of this peptide [194]. An ins/del polymorphism of the ACE gene, characterized by a 287 bp Alu repeat sequence within intron 16 of the gene, determines functional variants of the ACE gene and the del-allele was associated with higher ACE plasma levels [201], and also with higher CNS substance P levels [202]. The ACE ins/del polymorphism was also discussed to influence hypothalamic-pituitary-adrenal (HPA) axis reactivity in depressed patients and faster response to antidepressant treatments [203-205]. This finding could not be replicated in Chinese patients [206].

1.3.4.1.3 MAO-A

Monoamine oxidase A (MAO-A) and tryptophan hydroxylase (TPH) are the staple enzymes in the metabolism of serotonin (5-HT). The genetic polymorphisms of these two enzymes might individually alter the production, release, reuptake or degradation of 5-HT during the treatment of selective serotonin reuptake inhibitors (SSRIs), leading to the individual differences in the antidepressant effects of SSRIs. The MAO-A uVNTR (upstream Variable Number Tandem Repeats) polymorphism, which is located 1.2 kbp upstream of the MAOA coding sequence in the X-chromosome, consists of a 30 bp sequence present in 2, 3, 3.5, 4, or 5 repeats. The polymorphism has been shown to affect the transcriptional activity of the MAO-A gene promoter. Alleles with 3.5 or 4 repeats are transcribed 2-10 times more efficiently than those with 3 repeats, suggesting an optimal length for the regulatory region [207,208].

The clinical studies conducted so far on the possible role of the MAO-A uVNTR did not detect significant influence on therapeutic outcome [187,209,210] except for one study in Chinese depressive patients with an increased frequency of 4-repeat allele in female patients. Female patients that were homozygote for the 3-repeat allele showed a significantly better response to 4-week fluoxetine treatment when compared to 4-repeat carriers [211].

1.3.4.1.4 COMT

The catechol-O-methyltransferase (COMT) is a major degrading enzyme in the metabolic pathways of catecholaminergic neurotransmitters such as dopamine and norepinephrine. The involvement of the COMT gene in the metabolic pathway of these neurotransmitters and the fact that deletion of 22q11 (containing COMT) is associated with schizophrenia has made it an attractive candidate for many psychiatric disorders [212].

A functional polymorphism results in the substitution of the amino acid valine with methionine (codon 158 for the membrane-bound form and codon 108 for the soluble form). It has been shown that the high activity genotype (Val/Val) catabolizes dopamine at up to four times faster than the low activity COMT genotype (Met/Met). This results in a significant reduction of synaptic dopamine after neurotransmitter release and a significant reduction of dopaminergic stimulation of the post-synaptic neuron [212].

The results of several studies indicate that the low activity COMT genotype (Met/Met) is associated with unsatisfactory response to conventional neuroleptics [200,213]. There have been contradictory results regarding the influence of COMT Val158Met on antidepressant therapeutic response [214-216].

1.3.4.1.5 DBH

Dopamine beta-hydroxylase (DBH) is the enzyme that catalyses the conversion of dopamine to norepinephrine (noradrenaline). The involvement of the DBH gene in the metabolic pathway of the neurotransmitters dopamine and noradrenaline and the fact that there are well known polymorphisms affecting enzyme activity has made it an attractive candidate for many psychiatric disorders [217].

Recently, two polymorphisms (DBH 5'-ins/del and DBH G444A) of the dopamine beta-hydroxylase (DBH) gene, which are in linkage disequilibrium, were found to be associated with low DBH activity [217,218]. The DBH 5´ins/del polymorphism consists of a 19 bp insertion or deletion located approx. 3 kb upstream of the transcriptional start codon. The 5'-del / 444A haplotype was also found to be significantly more common in patients non-responding to antipsychotic treatment in one study suggesting that the DBH gene may be a modulator of psychotic symptoms, severity of the disorder or the therapeutic response to antipsychotic drugs [219].

1.3.4.2 Signal Transduction

1.3.4.2.1 GNB3

As most monoaminergic receptors belong to the class of G-protein-coupled receptors, G-protein subunits, such as the subunit b3, are candidate genes for the pharmacogenetics of antidepressant and antipsychotic drugs [179]. The cytosine to thymidine change at coding position 825 in exon 10 of the G-protein subunit b3 gene (GNB3 C825T) results in alternative splicing and was linked to increased signal transduction [220-222]. The underlying mechanism, however, is not fully understood. The polymorphism has also been associated with an inactive G-protein and with decreased function in expression studies in rat neurons and isolated subcutaneous fat cells. This may indicate cell-specific effects [223,224].

The 825T-allele was found to be associated with better response to antidepressant treatment in several independent studies [225-228], but could not be replicated in some studies [129,229]. Moreover, the 825T-allele was associated with better olanzapine response, and olanzapine or clozapine weight gain [230,231], whereas in studies on typical antipsychotics the 825C-allele was associated with better response [134,232].

1.3.4.2.2 FKBP5

Since abnormal activity of the hypothalamic-pituitary-adrenal (HPA) axis has been repeatedly associated with causality as well as with the treatment of depression, one study examined various genes regulating the HPA axis [233]. A significant association between a variation in the gene for FKBP5 (a glucocorticoid receptor-regulating co-chaperone of hsp-90) and faster antidepressant response (to TCAs, SSRIs, mirtazapine) was identified. The SNP (rs1360780) was also associated with increased intracellular FKBP5 protein expression, which triggers adaptive changes in glucocorticoid receptors and, thereby, hypothalamic-pituitary-adrenal (HPA) axis regulation. Individuals carrying the associated genotype had less HPA axis hyperactivity during the depressive episode [233].

Another study could not replicate this association between the FKBP5 polymorphism and response to antidepressant treatment [234]. This polymorphism, however, was also associated with greater rate of recurrence of major depressive episodes. Thus, the FKBP5 variant-dependent alterations in HPA axis regulation could simply represent a marker of greater cyclicity rather than specific drug response [234].

1.3.4.2.3 BDNF

Brain-derived neurotrophic factor (BDNF) is a member of the neurotrophin family of growth factors, abundant in the brain and periphery, which plays an important role in the survival, differentiation, and outgrowth of neurons during development and in adulthood. There is accumulating evidence that brain-derived neurotrophic factor (BDNF) is involved in the pathophysiology of mental disorders as it is involved in the neurodevelopment of neurotransmitter-related systems. Researchers have reported that serum BDNF levels are lower in depressive and schizophrenic patients, which might reflect a failure of neuronal plasticity. Moreover, BDNF serves as a modulator of neurotransmitter activity [235]. In addition, BDNF promotes and maintains dopamine DRD3 receptor expression [236]. There are several lines of evidence that the expression of BDNF is the downstream target of a variety of antidepressants. Antidepressants, but not antipsychotics, were reported to increase serum BDNF levels [237-240]. Therefore, differences in BDNF expression or functionality may be important for the therapeutic response to antidepressant drugs. For antipsychotics, altered serum BDNF levels were reported to be associated with weight gain in females [238].

In several studies in Asian patients on a variety of antidepressants and in one study on antipsychotics, the BDNF Val66Met (rs6265) heterozygote genotype was reported to be associated with better treatment response [235,241-243]. No association with treatment response was found in Caucasians treated with antipsychotics [244]. The BDNF Val66Met polymorphism is a G to A change at nucleotide position 196, which results in an amino acid change at codon 66 of the BDNF precursor protein. The amino acid change is cleaved away, rendering the change absent from mature BDNF.

2 Material and Methods

2.1 Clinics

2.1.1 Study Design

The study presented herein was a prospective, observational, one-center, diagnostic study. It was conducted with psychiatric in-patients at the Klinikum rechts der Isar (MRI), Technische Universität München (TUM), Munich, Germany. The study was a collaboration of the department of psychiatry and psychotherapy (head: Prof. Dr. med. H. Förstl) with the Institute for Clinical Chemistry and Pathobiochemistry (head: Prof. Dr. med. D. Neumeier). Responsible project leader was PD Dr. W. Steimer, senior physician at the Institute for Clinical Chemistry and Pathobiochemistry. The study was supervised by Dr. W. Kissling and PD Dr. S. Leucht, senior physicians, and Dr. S. Heres, resident physician of the Department of Psychiatry and Psychotherapy.

The study was approved by the Institutional Review Boards (IRB) of the Technische Universität München and followed the principles of the Helsinki declaration. Patients were informed of the aims of the study and gave written consent, which could be withdrawn at any time. The patients were treated according to local clinical practice with regard to the relevant guidelines. The treating physician and the patients were blind to genotype.

2.1.2 Inclusion and Exclusion Criteria for Study Population

Over a period of 3.5 years (May 2002 - November 2005), all new psychiatric in-patients at the department of psychiatry and psychotherapy, who met the inclusion criteria and gave written consent, were included in the study. Included were male and female adults suffering from mood disorders (ICD-10: F3 diagnosis), schizophrenic disorders (ICD-10: F2 diagnosis) and other disorders making treatment with neuroleptics or antidepressants necessary. Patients younger than 18 years, patients suffering from organic disorders (ICD-10: F0 e.g., dementia) or patients diagnosed as drug addicts (ICD-10: F1) were excluded from the study evaluation. Further exclusion criteria were breast-feeding or pregnancy. No patients admitted to hospital by law or authority direction were included in the study.

2.1.3 Clinical Data Collection

Patient data including demographic data, diagnosis according to ICD-10, medical anamnesis and case history, drug treatment and length of hospitalization was determined in a structured interview and collected from medical charts. Changes in drug therapy as well as concomitant medication were recorded at least during the first four weeks. As alcohol and nicotine may also influence drug metabolism, alcohol consumption and smoking habits were recorded as well.

EDTA blood was taken during routine blood collection for genotyping. If possible, serum was collected 12 - 14 hours after the last evening dose after three to four weeks of therapy and stored at -20°C to conduct serum concentration analysis. The design of the study was open and drug dosage could be adjusted individually as clinically indicated. Patients had to be treated with a fixed dose for at least one week (10 days for citalopram) before blood samples were collected to attain steady-state concentrations.

By use of the Paranoid Depression Scale (PDS) and the Clinical Global Impression (CGI) [245], the course of illness for each patient was assessed on admission, after 4 weeks and before dismissal. Adverse drug events were investigated using a varied version of the Dosage Record and Treatment Emergent Symptoms Scale (DOTES) [245] and with an overall rating of the side effects as “none”, “mild”, “moderate”, “marked” or “not assessable”.

2.1.3.1 Paranoid Depression Scale (PDS)

The Paranoid Depression Scale (PDS) is a self-assessment tool containing a P-scale for the measurement of paranoid and a D-scale for depressive symptoms each with 16 items developed by Zerssen [245]. For assessment of treatment response, an improvement of the PDS-D or PDS-P scores of more than 50% was chosen. This is due to the high rate of placebo response as observed in placebo-controlled studies e.g., for the depression scale (PDS-D), a placebo response of 41% was reported previously [246].

2.1.3.2 Dosage Record and Treatment Emergent Symptom Scale (DOTES)

The treating psychiatrist assessed the adverse drug events after 4 weeks of therapy according to a varied version of the Dosage Record and Treatment Emergent Symptoms Scale (DOTES) [247] and documented whether the reported side effect(s) were due to a specific drug. The DOTES scale includes 30 single items with a rating of “mild” (score value of 1), “moderate” (score value of 2), or “severe” effect (score value of 3) and is organized in five clusters: cluster a (mental side effects), cluster b (neuromuscular symptoms), cluster c (anticholinergic/gastrointestinal symptoms), cluster d (cardiovascular symptoms) and cluster e (other symptoms).

Moreover, a global assessment of the severity of all side effects from which the patients suffered altogether was done. The overall undesirable effects could be rated as “none”, “mild”, “moderate”, “marked” or “not assessable”. Side effects were evaluated as “positive” for an overall side effects rating of moderate/marked; and as “negative” for an overall rating of none or mild.

2.1.3.3 Clinical Global Impressions (CGI)

The CGI scale is robust, simple to understand and carry out and is sensitive to changes of mental illness. The assessment, however, is based on the rater's subjective views of symptom severity, which can vary between raters and make consistent interpretation of CGI scores problematic in practice. CGI is a three-item scale used to assess treatment response in psychiatric patients [247].

They are:

- CGI(1) = severity of mental illness is rated on a seven-point scale (normal, borderline mentally ill, mildly ill, moderately ill, markedly ill, severely ill, extremely ill or not assessable).
- CGI(2) = global improvement is rated on a seven-point scale (very much improved, much improved, minimally improved, no change, minimally worse, much worse, or very much worse or not assessable).
- CGI(3) = efficacy index is rated on a four-point scale (from 'none' to 'outweighs therapeutic effect').

The CGI was assessed by the treating psychiatrist. All patients with a CGI(2) score of 1 or 2 (very much or much improved) were evaluated as CGI responders within this study. In case of treatment response, the onset of the response i.e., in which week and day after hospitalization the response became visible was also documented by the treating psychiatrist.

2.2 Genotyping

2.2.1 Materials

Table 1: Reagents

Agarose MetaPhor® (for high resolution separation of 20 - 800 bp DNA)	Cambrex Bio Science Rockland Inc., Rockland, ME USA (distributor: Biozym Scientific GmbH, Hessisch Oldendorf)
Agarose PeqGold Universal Agarose	PeqLab Biotechnologie GmbH, Erlangen
Bovine Serum Albumin (for PCR), 20 mg/mL	Roche Diagnostics GmbH, Mannheim
Bovine Serum Albumin, purified 100x (for enzymatic restriction), 10 mg/mL	New England Biolabs GmbH, Frankfurt am Main
DNA ladder 1 kbp plus (100 bp - 12 kbp)	Invitrogen GmbH, Karlsruhe
DNA ladder Quantitas 2 (50 bp - 2 kbp)	Biozym Scientific GmbH, Hessisch Oldendorf
DNA ladder Quantitas 5 (25 bp - 500 bp)	Biozym Scientific GmbH, Hessisch Oldendorf
Dimethyl sulfoxide (DMSO) for 'Long Range' PCR System	PeqLab - Biotechnologie GmbH, Erlangen
dNTP-Mix (10 mM each)	PeqLab - Biotechnologie GmbH, Erlangen
dNTP-Mix 'Long Range' (10 mM each)	PeqLab - Biotechnologie GmbH, Erlangen
Ethidium bromide (1 µg/mL in deionized water)	Merck KGaA, Darmstadt
Expand Long Template PCR System DNA Polymerase Mix (5 U/µL)	Roche Diagnostics GmbH, Mannheim
Expand Long Template PCR System Buffer 1, 10x conc. (17.5 mM $MgCl_2$)	Roche Diagnostics GmbH, Mannheim
Gel Loading Buffer 10x (50% glycerol, 0.25% bromophenol blue, 0.25% xylene cyanol FF in 1x TBE buffer)	Ingredients by Sigma-Aldrich Chemie GmbH, Steinheim and Merck KGaA, Darmstadt
Gel Loading Solution, Type 1, 6 x (bromphenol blue (0.25% w/v), xylene cyanol FF (0.25% w/v), sucrose (40% w/v))	Sigma-Aldrich Chemie GmbH, Steinheim
LightCycler® Color Compensation Set	Roche Diagnostics GmbH, Mannheim
LightCycler® DNA Master HybProbe (contains enzyme and reaction mix 10x, 10 mM $MgCl_2$)	Roche Diagnostics GmbH, Mannheim
LightCycler® FastStart DNA Master HybProbe (enzyme and reaction mix 10x, 10 mM $MgCl_2$)	Roche Diagnostics GmbH, Mannheim
LightCycler® TaqMan Master (enzyme and reaction mix 5x)	Roche Diagnostics GmbH, Mannheim
Mae III restriction endonuclease	Roche Diagnostics GmbH, Mannheim
NEB restriction endonucleases, div.	New England Biolabs GmbH, Frankfurt am Main
NEBuffer 1, 10x 100 mM Bis-Tris-Propane-HCl, 100 mM $MgCl_2$, 10 mM dithiothreitol	New England Biolabs GmbH, Frankfurt am Main

Table 1 (ctd.): Reagents

NEBuffer 2, 10x 500 mM NaCl, 100 mM Tris-HCl, 100 mM $MgCl_2$, 10 mM dithiothreitol	New England Biolabs GmbH, Frankfurt am Main
NEBuffer 3, 10x 1000 mM NaCl, 500 mM Tris-HCl, 100 mM $MgCl_2$, 10 mM dithiothreitol	New England Biolabs GmbH, Frankfurt am Main
NEBuffer 4, 10x 200 mM Tris-acetate, 500 mM K-acetate, 100 mM Mg-acetate, 10 mM dithiothreitol	New England Biolabs GmbH, Frankfurt am Main
MasterAmp™ 2x PCR PreMixes D / J / K 100 mM Tris-HCl (pH 8.3, 22° C), 100 mM KCl, 400 µM each dNTP and 3 / 3 / 5 mM $MgCl_2$ and 4x / 8x / 8x MasterAmp Enhancer (with betaine = trimethyl glycine)	EPICENTRE® Biotechnologies (distributor: Biozym Scientific GmbH, Hessisch Oldendorf)
$MgCl_2$ stock solution (25 mM)	Roche Diagnostics GmbH, Mannheim
Mineral Oil (for overlaying aqueous reactions), molecular biology tested	Sigma-Aldrich Chemie GmbH, Steinheim
PeqLab 'Long Range' PCR System (5 U/µL)	PeqLab - Biotechnologie GmbH, Erlangen
PeqLab Dilution buffer for DNA Polymerases 20 mM Tris-HCl, (pH 8.0 at 25°C), 100 mM KCl, 0.2 mM EDTA, 0.5% Tween 20, 0.5% Nonidet P40 and 55% (v/v) glycerol	PeqLab - Biotechnologie GmbH, Erlangen
PeqLab Reaction buffer for 'Long Range' PCR System 10x 500 mM Tris-HCl (pH 9.2 at 25°C), 140 mM $(NH_4)_2SO_4$ and 22.5 mM $MgCl_2$	PeqLab - Biotechnologie GmbH, Erlangen
Probes for fluorescence detection	TIB MOLBIOL Syntheselabor GmbH, Berlin
Primers (oligonucleotides)	Invitrogen GmbH, Karlsruhe
Qbiogene Taq DNA Polymerase (5 U/µL)	Qbiogene (distributor MP Biomedicals Germany, Heidelberg)
Qbiogene Incubation Buffer 10x (100 mM Tris-HCl pH 9.0, 500 mM KCl, 1% Triton X-100, 2 mg/mL BSA) with or without 15 mM $MgCl_2$	Qbiogene (distributor MP Biomedicals Germany, Heidelberg)
UltraPure™ 10x TBE Buffer 1 M Tris, 0.9 M Boric Acid, 0.01 M EDTA	Invitrogen GmbH, Karlsruhe
Water, PCR grade	Roche Diagnostics GmbH, Mannheim
Water, sterile (Aqua DeltaSelect Irrigation Solution)	DeltaSelect GmbH, Dreieich
Wizard Genomic DNA Purification Kit	Promega GmbH, Mannheim

Table 2: Laboratory equipment and disposables

Aerosol-resistant pipette tips (free of pyrogen, nucleic acid, RNAase/DNAase) 1 µL to 1000 µL	PeqLab, Erlangen and SLG Süd-Laborbedarf GmbH, Gauting
Analytical balance 1702 MP 8	Sartorius AG, Göttingen
BioPhotometer	Eppendorf AG, Hamburg
Circomix vortex mixer	B. Braun Melsungen AG
Eppendorf cups (1.5 mL), sterile	Eppendorf AG, Hamburg
GeneAmp® PCR System 9700	PE Applied Biosystems GmbH, Weiterstadt
LightCycler® Capillaries	Roche Diagnostics GmbH, Mannheim
LightCycler® Instrument (Version 1.5 and 2.0)	Roche Diagnostics GmbH, Mannheim
LightCycler® Carousel Centrifuge	Roche Diagnostics GmbH, Mannheim
Microcentrifuge: Centrifuge 5415 C	Eppendorf AG, Hamburg
Mupid® - 2 electrophoresis system	Eurogentec Deutschland GmbH, Köln
Mupid® - exU electrophoresis system	Eurogentec Deutschland GmbH, Köln
Mupid® gel maker set (trays and combs)	Eurogentec Deutschland GmbH, Köln
PCR soft tubes 0.2 and 0.5 mL	Biozym Scientific GmbH, Hessisch Oldendorf
Photographic gel documentation system: UV transilluminator TCP-20.M Video copy processor P91 (Sony) Black and White Monitor SSM-930CE (Mitsubishi Digital Electronics) Photo print OO-IP-010.SD Thermal Printer DPU-414 Seiko Instruments GmbH, Neu-Isenburg	LTF Labortechnik, Wasserburg/Bodensee
Pipettes: SL-Pette autoclavable (range 0.1 µL to 1000 µL)	SLG Süd-Laborbedarf GmbH, Gauting
Pipettes: Eppendorf Research (range 1 µL to 1000 µL)	Eppendorf AG, Hamburg
Thermomixer 5433	Eppendorf AG, Hamburg
Thermostat 5320	Eppendorf AG, Hamburg
UVette®	Eppendorf AG, Hamburg
Water bath WNB 7	Memmert GmbH & Co.KG, Schwabach

Table 3: Software

Primer3 software	Primer Design Software by Whitehead Institute for Biomedical Research [248]
MeltCalc program	Microsoft EXCEL add-in [249]
NEB cutter	Restriction analysis [249]
LightCycler® Software (Version 3.5 and 4.05)	Idaho Technology Inc. (distributor: Roche Diagnostics GmbH, Mannheim)
LightCycler® Probe Design Software (Version 1.0)	Roche Diagnostics GmbH, Mannheim

2.2.2 Overview

One of the main aims of this work was to establish genotyping methods for routine analysis. Direct sequencing is the gold standard for initially characterizing polymorphisms, but it is time- and cost-intensive and thus not practical for routine analysis.

Polymerase Chain Reaction (PCR) is a relatively quick, specific (after optimization of primers as well as temperature and buffer conditions) and sensitive (3 to 5 DNA double strands are sufficient for amplification) method. The major problem is the contamination risk by carry-over. Thus, special separation of the PCR preparation and post-PCR-analytics as well as the use of filter tips (to avoid micro aerosols), the use of sterile, PCR-grade reagents and use of sterile gloves was of utmost importance. If possible, disposables were used. Glass equipment was decontaminated by autoclavation, which destroys DNA.

If possible and useful for further application in routine analysis, fluorescence-based methods were developed. The advantages of fluorescence-based detection methods are that they allow a faster performance and higher throughput due to the faster preparation, faster heating and cooling speed during amplification and the subsequent fluorescence detection in the same apparatus without further manual steps. For this purpose it is only necessary to mix fluorescence labeled probes together with reagents, which are also needed for a traditional PCR reaction. The closed single-tube approach with no post-amplification processing minimizes also the risk of contamination and potential problems with sample tracking. Since the preparation is simpler, performance failures are reduced compared to traditional methods. Furthermore, fluorescence detection is more sensitive compared to the traditional UV detection of DNA stained with ethidium bromide. This offers an advantage in case of sparse or low quality DNA material.

The patients' blood samples underwent the following work steps and described in detail in the following chapters:

A) sample collection in EDTA collection tubes and DNA extraction

B) amplification of the DNA by conventional or real-time polymerase chain reaction (PCR)

C) detection of the genotype by one of the following methods:
    - length polymorphisms or allele-specific amplification of SNPs
        - agarose gel electrophoresis
    - single nucleotide polymorphisms SNPs
        - restriction fragment length polymorphism (RFLP), agarose gel electrophoresis and UV detection of DNA bands stained with ethidium bromide
        - with specific fluorescent labeled probes during real-time PCR

2.2.3 DNA Isolation

DNA was isolated from human blood (anticoagulant: EDTA). In general, freshly drawn blood was available, although blood stored for 1 week in the cold (refrigerator) was - in most cases - equally good for DNA extraction and amplification. Each patient gave 2.7 mL of EDTA blood. DNA was extracted from 2 x 300 µL of blood using the Wizard Genomic DNA Purification Kit from Promega in accordance with the manufacturer's recommendations [250] and eluted in 2 x 100 µL buffer. Depending upon the number of leukocytes, approx. 5 - 15 µg of DNA per 300 µL of fresh whole blood are obtained with an average size ≥ 50 kbp according to the manufacturer's manual [250]. Hence, the average DNA concentration in the rehydration buffer was about 50 - 150 ng/µL. One aliquot was stored at 4°C and one was frozen at -70°C. Additionally, the remaining EDTA blood sample was frozen at -70°C in a 1.5 mL centrifuge tube.

2.2.4 Genotyping by Use of Conventional PCR Methods

2.2.4.1 Principle of Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR) is an *in vitro* technique used to amplify specific regions of a DNA strand and requires following basic components [251,252]:

- DNA template that contains the region of the DNA fragment to be amplified
- Oligonucleotides (= primers) that consist of about 16 to 30 base pairs and are complementary to the DNA regions at the 5' and 3' ends of the DNA region to be amplified
- DNA polymerase (e.g., Taq polymerase or another DNA polymerase with a temperature optimum at around 70°C), used to synthesize a DNA copy of the region to be amplified
- the four different deoxynucleotide triphosphates (dNTPs) from which the DNA polymerase builds the new DNA
- buffer solution, which provides a suitable chemical environment for optimum activity and stability of the DNA polymerase with divalent cations (generally magnesium ion Mg^{2+} is used) and monovalent cations (potassium K^{+} ion)

  A standard PCR buffer contains 50 mM KCl and 1.5 mM Mg^{2+}. Mg^{2+} cations have a 140 times higher stabilizing effect on duplexes compared to Na^{+} [253].

The PCR procedure itself consists of the following steps [8]:

1) denaturation step prior to the first cycle, the PCR reaction is heated to 93 - 98°C and held for up to 10 minutes:
   a. to ensure that the double stranded DNA template is melted by disrupting the hydrogen bonds between complementary bases of the DNA strands.
   b. in case of performing hot start PCR, polymerases require this step for activation.
2) 20 - 45 amplification cycles:
   a. denaturation step at 94 - 98°C for 20 - 30 seconds to ensure that DNA is denaturated to single strands.
   b. annealing step at a temperature suitable for the primers to anneal to their complementary sequence on the single-stranded DNA template. Stable bonds are only formed when the primer sequence very closely matches the template sequence. The temperature at this step depends on the melting temperature of the primers, and is usually between 50 - 65°C for 20 - 40 seconds.
   c. extension/elongation step during which the DNA polymerase attaches to the short section of double-stranded DNA formed by the primer-oligonucleotide and one single-stranded DNA template. The DNA polymerase condenses the 5'-phosphate group of the dNTPs with the
   3'-hydroxyl group at the end of the nascent (extending) DNA strand i.e., the polymerase adds dNTPs that are complementary to the template in 5' to 3' direction, thus reading the template in 3' to 5' direction. The temperature at this step depends upon the DNA polymerase used. The Taq polymerase has a temperature optimum of 70 - 74°C. Thus, in most cases a temperature of 72°C is used. The extension time depends both upon the DNA polymerase used and upon the length of the DNA fragment to be amplified. As a rule-of-thumb, at its optimum temperature, the DNA polymerase will polymerize a thousand bases in one minute.
3) A final elongation step of 3 - 15 minutes (depending on the length of the DNA template) after the last cycle is used to ensure that any remaining single-stranded DNA is fully extended.
4) A final hold of 4°C for an indefinite time is employed for short-term storage of the reaction e.g., if reactions are run overnight.

The amount of PCR product is theoretically amplified to 2^n (n = number of cycles), the actual efficiency is lower (about 70 - 80% of the theoretical value). This is due to thermolabile reagents (nucleotides, polymerase), decrease of reaction components and the occurrence of unspecific products such as primer dimers. The number of cycles suitable for amplification is therefore limited. Nested PCR with a second pair of specific primers (located within the first set) can be used to provide new reagents and to avoid unspecific products.

2.2.4.2 Optimization of Conventional PCR Protocols

The aim of the optimization of the polymerase chain reaction was to maximize the amount of the target product and to minimize the occurrence of unwanted unspecific products, which may interfere with the detection of the polymorphism and decrease the yield of the target product. Therefore, optimization of a) structure and length of primers (see § 2.2.6.1) and b) temperature and buffer conditions was undertaken.

Many cycling parameters influence both the specificity and efficiency of amplification including the temperature and duration of denaturation, annealing and elongation as well as the ramp speed and the total cycle number. The annealing temperature was adjusted to be low enough and the annealing time to be long enough to make annealing and an efficient amplification possible, but not too low and too long to avoid unspecific binding of primers [254].

Different buffers and salt concentration especially of the divalent cation Mg^{2+} were tested for every PCR assay. Optimization especially of the Mg^{2+} concentration is particularly critical as it affects primer annealing, product specificity and DNA polymerase activity. The normal cell concentration is 1.5 mM Mg^{2+}. A titration up to 4 mM was done. Too high concentrations, however, lead to higher rate of mismatches and to inaccuracies in the duplicated DNA. Moreover, a too high salt concentration stabilizes the DNA double strand making DNA synthesis more difficult [254].

If helpful, enhancer molecules such as betaine (trimethyl glycine), dimethyl sulfoxide (DMSO) or bovine serum albumin (BSA) were used. The zwitterion betaine improves yield and specificity of amplification of many target sequences, especially those containing high GC content or secondary structures. It lowers the melting temperature of GC rich regions to a temperature more similar to AT rich regions. This results in destabilization of double strand DNA. This limits polymerase pausing and increases the yield of full-length product. In addition, betaine may also enhance PCR by protecting DNA polymerases from thermal denaturation. Similarly, dimethyl sulfoxide (DMSO) destabilizes the base pairing and improves the PCR efficiency. Bovine serum albumin stabilizes the polymerase and prevents its denaturation by adsorption to the surface of tubes or capillaries [255-258].

Moreover, the efficiency of a PCR reaction depends on the quality of DNA template, the type of DNA polymerase (e.g., with proofreading activity), sufficient concentration of primers and deoxynucleotide triphosphates (dNTPs). The ratio of primer to template is important for controlling the specificity and efficiency of amplification. An excess of primer ensures that the denatured template molecules bind to primers instead of binding to the complementary strand. Nevertheless, too much primer may lead to the formation of non-specific products or primer dimers. Therefore, the concentration of primers was optimized as well [254]. The (optimized) PCR protocols used for the amplification of the studied polymorphisms are given together with the PCR temperature programs in § 2.2.7.

2.2.4.3 Principle of Restriction Fragment Length Polymorphism (RFLP)

Restriction fragment length polymorphism (RFLP) uses the different length of fragments after digestion by restriction endonucleases to detect different genotypes. Restriction endonucleases cut double strand DNA at characteristic motifs (often palindromes of 4 to 6 bases). In case of an underlying SNP either the wildtype sequence is cut by the enzyme and the variant not, or vice versa.

RFLP is confined to polymorphisms in sequence motifs that contain either a natural restriction enzyme cutting site or allow the introduction of such sites via modified PCR primers. These amplification-created restriction sites are generated by use of so-called trick primers with a deliberate mismatch, which is immediately adjacent to, but not encompassing the polymorphism [8].

The unambiguous genotype identification is dependent upon complete restriction digestion. Therefore - if possible - the time-saver™ qualified restriction enzymes of New England Biolabs with certified digestion power and purity were used in consideration of the manufacturer's instructions. For one control assay (5-HTR2A rs7997012), the MaeIII enzyme (Roche Diagnostics GmbH, Mannheim) was used. To check the restriction sites given in published methods or to obtain the length of restriction products for control assays, the NEBcutter internet tool (see Table 3 in § 2.2.1) was used. The restriction enzymes used for the detection of the studied polymorphisms (see § 2.2.7) are summarized in Table 4.

Table 4: Restriction endonucleases

Abbreviation	Source of the gene	Restriction site	Polymorphisms
MaeIII	*Methanococcus aeolicus*	/ G T N A C	FKBP5 rs1360780*
NheI	*Neisseria mucosa heidelbergensis*	G / C T A G C	MDR1 G2677TA* TPH1 A218C
XbaI	*Xanthomonas badrii*	T / C T A G A	MDR1 G2677TA*
RsaI	*Rhodopseudomonas sphaeroides*	G T / A C	MDR1 G2677TA* 5-HTR6 T267C
HaeIII	*Haemophilus aegypticus*	G G / C C	MDR C1236T*
MspI	*Moraxella* species	C / C G G	5-HTT rs25531* 5-HTR2A T102C, ADR2A C-1291G*
HpyCH4IV	*Helicobacter pylori* CH4	A / C G T	5-HTR_{1A} C-1019G*
PacI	*Pseudomonas alcaligenes*	T T A A T / T A A	5-HTR2A rs7997012*
AciI	*Arthrobacter citreus*	C / C G C	5-HTR2C C-759T*
Sau96I	*Staphylococcus aureus* PS96	G / G N C C	NET G1287A*
BslI	*Bacillus* species	C C N N N N N / N N G G	NET T-182C*
BsaAI	*Bacillus stearothermophilus* G668	Y A C / G T R	BDNF Val66Met*
NlaIII	*Neisseria lactamica*	C A T G /	COMT Val158Met*
BsaJI	*Bacillus stearothermophilus* J695	C / C N N G G	GNB3 C825T

Note: N = A or C or G or T, R = A or G, Y = C or T
Note: * RFLP used as control for validation of fluorescence-based methods

2.2.4.4 Principle of Allele-Specific PCR

If RFLP is not possible, allele-specific PCR can be used instead to detect SNPs. Primers are designed to differ at the nucleotide that occurs *at the extreme 3' terminus* and to match either the wildtype or the variant base on the DNA strand. The DNA synthesis step in a PCR reaction is crucially dependent upon correct base-pairing at the 3' end. The allele-specific oligonucleotide only leads to a PCR product when it is perfectly complementary to the SNP at 3´end. In case of a mismatch, no amplification by the polymerase is possible. Therefore, two tubes are necessary for detection of one SNP with a forward primer matching either the wildtype or the variant and the (unchanged) reverse primer. In the subsequent electrophoresis, a PCR product is seen only if the wildtype (or variant) primer matches with the SNP base in the sample DNA [8]. It is of utmost importance to keep the annealing temperature high enough to avoid unspecific binding of primers despite a mismatch. The conditions for the two allele-specific PCRs used are given in § 2.2.7 (see CYP2D6*10 control assay and TPH2 G1463A polymorphism).

2.2.4.5 Principle of Electrophoresis

Agarose gel electrophoresis was used to separate DNA by size. This is achieved by moving negatively charged nucleic acid molecules through an agarose matrix with an electric field (electrophoresis). Shorter molecules move faster and migrate further than longer ones. The separated DNA bands were stained with the DNA intercalating ethidium bromide, which emits fluorescence under UV light. The sizes of PCR products were determined by comparison with a suitable DNA ladder, which contains DNA fragments of known size. Moreover, a negative control (without template) and positive controls were included in every experiment. The exact agarose concentration and amount pipetted into the slots for every polymorphism are given in § 2.2.7.

2.2.5 Genotyping by Use of Fluorescence-Based Detection

2.2.5.1 Principle of LightCycler Instrument

The LightCycler Instrument provides a platform not only for performing the PCR amplification itself, but also for monitoring fluorescence during temperature transitions. This allows the annealing and denaturation of nucleic acid to be followed in real-time, using either unspecific double strand DNA dyes (e.g., SYBR Green I, which intercalates in the minor groove of duplex DNA and emits fluorescence at 530 nm) or fluorescence-labeled oligonucleotides, which bind to the complementary DNA strand and form a short DNA heteroduplex [259].

The instrument consists basically of:

- a thermal chamber, which can be heated and cooled
- a sample carousel, which can be placed into the thermal chamber and which contains up to 32 glass capillaries
- a fluorimeter with a blue 470 nm LED light source and different filters/photohybrids to measure the samples' emitted fluorescence (530 nm, 640 nm, 710 nm, additionally 555 nm, 610 nm and 670 nm in the LightCycler 2.0 system)

Due to faster thermal transfer (heating or cooling rate up to 20°C/sec) compared to traditional thermocyclers, amplification cycles can be reduced to 15 - 20 seconds with the entire amplification being completed within 30 minutes. During amplification, the emitted fluorescence, which is proportional to the number of the DNA strands, can be measured in every cycle. Depending upon the number of DNA copies at the beginning, the fluorescence rises sooner or later above the background noise. This principle is used for TaqMan assays.

After amplification, continuous monitoring during slow heating provides qualitative information about the target. The temperature at which a DNA duplex separates (or "melts") provides information about the particular sequence. Each probe/target duplex has a characteristic thermal stability that depends upon such factors as length, GC content, sequence order and Watson-Crick pairing [260]. Base pair mismatches decrease the stability of a duplex by varying amounts depending on the type, position and number of mismatches as well as upon the nearest neighbors [261]. When a probe hybridizes over a sequence variant, a mismatch is formed, which destabilizes the duplex and lowers the temperature needed for the duplex separation (or melting) compared to the completely complementary duplex. This principle is used for hybridization probe assays where during slow heating (melting curve) single base alterations can be detected by fluorescence resonance energy transfer (FRET).

2.2.5.2 Principle of Hybridization Probes and Fluorescence Resonance Energy Transfer

For genotyping, a hybridization probes assay involves an unlabeled primer set for amplification of the target sequence and a pair of adjacent hybridization probes labeled either with fluorescein at the 3' end (donor probe) or with an acceptor dye at the 5' end (acceptor probe). If the probe is not annealed to the target sequence, fluorescein is excited by the 470 nm LED light and emits green fluorescence detected by the 530 nm photohybride. The second dye is not excited by 470 nm, but only the FRET partner of fluoresceine.

If the temperature is low enough, both probes anneal at the target DNA and a fluorescence resonance energy transfer (FRET) takes place. Fluorescein transfers the energy to the second dye (e.g., LCRed640) and its emitted red fluorescence can be measured at 640 nm (see Figure 3).

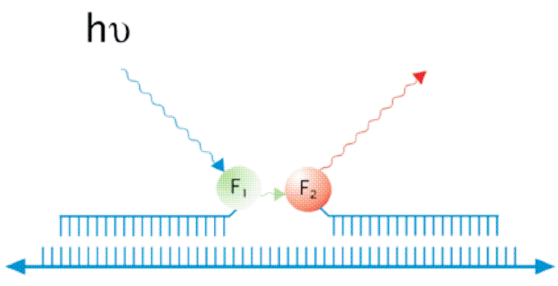

Figure 3: Fluorescence resonance energy transfer (FRET)

Figure adapted from LightCycler Manual (Roche) [262]

Scheme of FRET: On the left, donor probe annealed to the complementary DNA strand and linked at its 3' end to fluorescein. On the right, acceptor probe with an acceptor dye coupled to its 5' end. Fluorescein is excited by the 470 nm LED light and transfers the energy to the second dye e.g., LCRed640, which emits red light at 640 nm.

For genotyping, one of the probes (the sensor) is spanning the variant and has a lower melting temperature (T_m) than its adjacent probe (the anchor). After amplification of the target DNA sequence, the reaction mixture containing the DNA amplificate and the hybridization probes is slowly heated (so-called melting curve analysis). The anchor probe remains annealed to the template while the sensor probe is slowly separating from the target DNA (see A in Figure 4). The higher the temperature (see B in Figure 4), the less of the sensor probe is annealed to the target DNA, and the lower is the FRET and the fluorescence measured at e.g., 640 nm (see C in Figure 4).

The mismatch underlying the sensor probe leads to a lower thermal stability of the sensor-DNA heteroduplex and a lower melting temperature (T_m). This shift is used for detection of single base alterations as shown in Figure 4. The melting temperature T_m is the temperature where, at equilibrium, and containing equal amounts of probe and target, 50% of the probe is hybridized to the target [263]. The T_m can be estimated from the inflection point of the melting curve. The mathematical transformation of the data - the negative first derivative of fluorescence with respect to temperature (-dF/dT) vs. T - allows for an easier interpretation of the results were the maximum represents the T_m (see D in Figure 4). Heterozygotes show bimodal melting curves due to the presence of two alleles with different melting points [264,265].

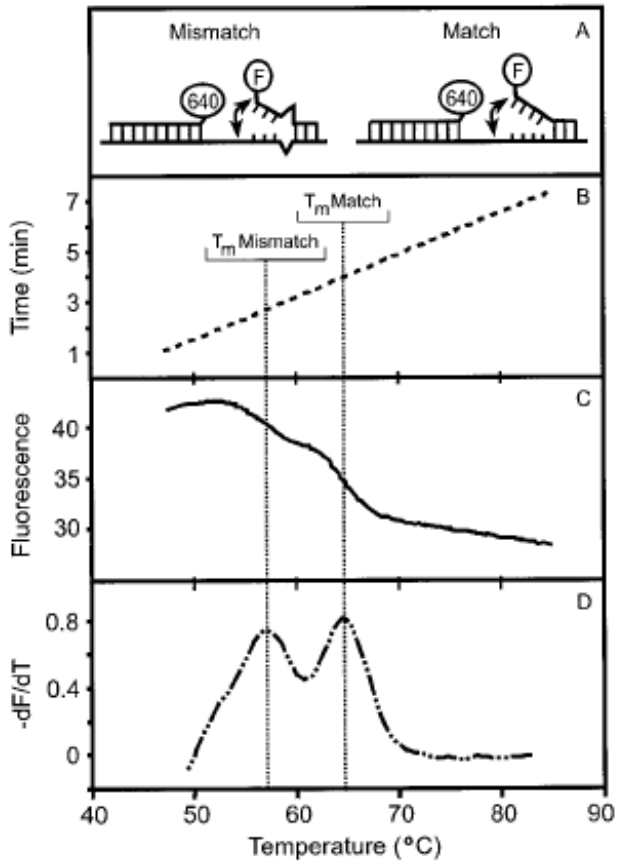

Figure 4: Schematic display of fluorescent melting curve for genotyping

Figure adapted from LightCycler Manual (Roche) [262]

(A) portrays the equilibrium between annealing and melting at the T_m of matched and mismatched duplexes.

(B) plots time vs. sample temperature during the melting phase of the reaction. As the solution is heated slowly, the fluorescein probe spanning the polymorphic site is thermally denatured away from the adjacent LCRed640 acceptor probe.

(C) the point of inflection on a plot of fluorescence vs. temperature identifies the temperature at which 50% of the fluorescein probe has melted i.e., the melting temperature T_m.

(D) melting temperatures are more easily discerned by plotting a derivative melting curve where the center of the melting peak corresponds to the point of inflection. The ordinate of D is the negative derivative of fluorescence (F) with respect to the temperature (T) or -dF/dT.

For genotyping, fluorescein-labeled probes were used in combination with LCRed610, LCRed640 or LCRed705-labelled ones. The PCR protocols including primer and probe concentrations and the derived melting curves are given in § 2.2.7 with the experimentally derived melting temperatures (T_m) for wildtype and variant, respectively. Negative controls (without template) were included in every experiment to check for carry-over contamination and are visible as flat lines without any fluorescence peaks. To detect more than one SNP in a single tube, it is possible to use different fluorescent dyes (i.e., more than one hybridization probe pair, for an example see § 2.2.7.6 MDR1 multiplex assay) [264,266].

2.2.5.3 Principle of TaqMan Probes and 5' Exonuclease Assay

The Taq polymerase possesses a 5' → 3' exonuclease activity. This property can be exploited for detection of specific alleles. For genotyping, a TaqMan reaction involves two PCR primers for amplification of the target sequence and two allele-specific TaqMan probes. Each probe is complementary to one of the alleles of a SNP. Both probes are labeled with (different) reporter fluorophores at the 5' end and quencher dyes attached at the 3' end. If both the dye and the quencher are bound to the oligonucleotide, the reporter dye emits no fluorescence as it is in close proximity to the quencher. Because its 3' end is blocked, the TaqMan probe cannot by itself prime any new DNA synthesis [8,267].

When the *Taq* DNA polymerase approaches the annealed TaqMan probe during PCR amplification, its 5' → 3' exonuclease activity cleaves the reporter dye from its 5' end, which is no longer quenched and starts emitting fluorescence, when it is excited by the 470 nm LED light (see Figure 5).

The presence of a mismatch between probe and target reduces the efficiency of probe binding and subsequent cleavage leading to a discrimination of the alleles. The exponential amplification of the template during the PCR is accompanied by an increasing signal from the released fluorophore. This indicates the presence of the corresponding allele in the DNA sample. The increase of fluorescence is less intensive in heterozygote than in homozygote samples. No fluorescence is seen for no-template controls and negligible fluorescence for incongruous homozygotes [8,267].

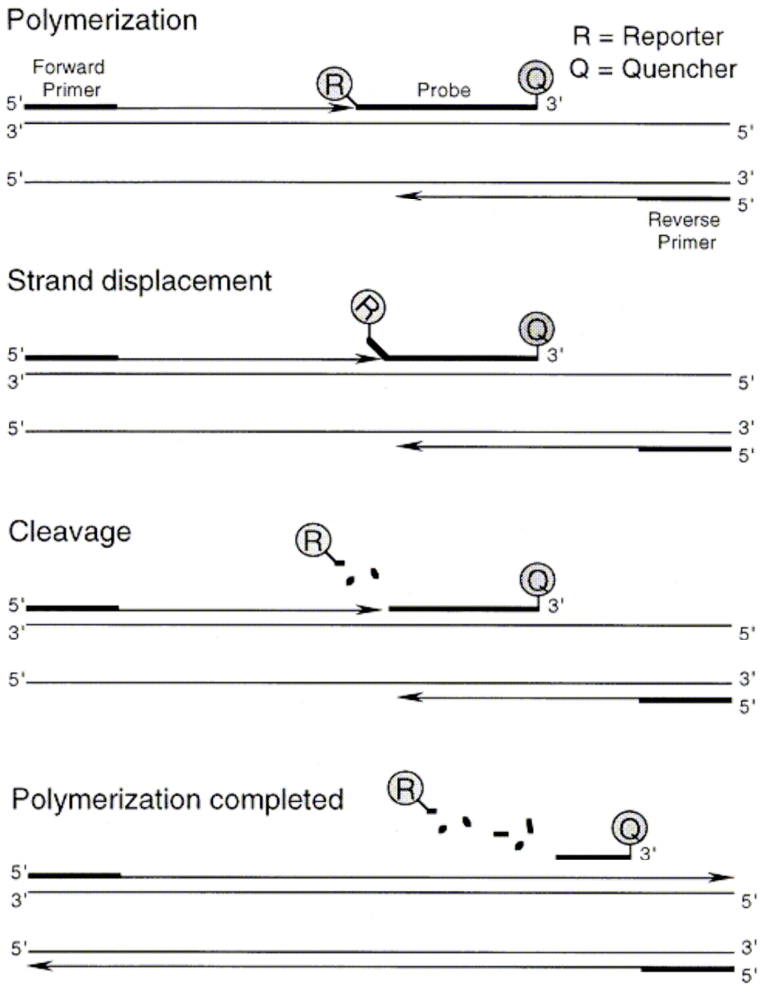

Figure 5: Schematic display of TaqMan reaction

Note: Figure adapted from Livak et al. (1999) [267]

By careful design of the probe sequences, both probes may be combined within the same PCR to detect the presence of the corresponding allele in a DNA sample without cross-reacting with the alternate allele and generating an unspecific signal [268]. Reporter dyes with high fluorescence levels such as fluorescein (fluorescence emission at 530 nm) and hexachlorofluorescein (HEX) or LCRed610 (fluorescence emission at 560 nm or 610 nm, respectively) in combination with a non-fluorescent quencher (Black Hole Quencher ™ BHQ1 or BHQ2) were used to minimize the background fluorescence and maximize the sensitivity. The (optimized) PCR protocols used for the TaqMan assays are given in § 2.2.7.7, § 2.2.7.14 and § 2.2.7.16.

2.2.5.4 Optimization of Real-Time PCR Conditions

Optimization of a) structure and length of primers (see § 2.2.6.1) and b) temperature and buffer conditions was carried out. The basic requirements for real-time PCR optimization on the LightCycler are generally the same as for traditional thermocyclers and protocols (see § 2.2.4.2). Additional optimization features used for real-time PCR are given below:

Hot Start PCR

The higher stringency (more exact heating, higher ramping rate, short annealing and extension time) in the temperature conditions may result in lower PCR efficiency [269]. If necessary, hot start PCR was performed to improve the specificity, sensitivity, and yield of PCR.

Commonly used DNA polymerases show a very small, but measurable polymerase activity at room temperature during PCR set-up and prior to the initial denaturation step. This inefficient DNA polymerase activity catalyzes the extension of any annealed 3' ends resulting in non-specific products. Moreover, the 5' - 3' exonuclease activity of the DNA polymerase degrades any free 5' ends of partially annealed nucleic acids, destroying the primer and template substrates of the polymerase reaction. This results in a lower yield of the desired PCR product.

The LightCycler® FastStart DNA Master HybProbe is an easy-to-use hot start reaction mix for PCR, containing FastStart Taq DNA Polymerase. The addition of heat-labile blocking groups on some of the amino acid residues of FastStart Taq DNA Polymerase makes the modified enzyme inactive at room temperature. Therefore, there is no elongation during the period when primers can nonspecifically bind. The FastStart Taq DNA Polymerase is “activated” by removing the blocking groups at a high temperature (i.e., a pre-incubation step at 95°C for 10 min).

Optimization of Multiplex PCR

The color compensation feature of the LightCycler software can be used to eliminate the cross-talk of fluorescence emission into channels other than the color specific detection channel. This is due to the broad emission spectra of the fluorescence dyes and the bandwidth of the photometer filters. Due to fluorophore emission overlap, each channel contains an emission composite of all probes. Individual color signals can be obtained by correcting for the amount of overlap in each channel for each fluorophore [264].

Thus, color compensation was performed for each assay where more than one dye was used. To correct signal overflow between the different channels, the Color Compensation Kit provided by Roche or the assay probes were used to create a color-compensation file in accordance with the instructions provided in the manual and LightCycler software. The (optimized) PCR protocols used for the amplification of the studied polymorphisms are given together with the PCR temperature programs in § 2.2.7.

2.2.6 Design of Primers and Probes

2.2.6.1 Primer Design

The establishment of new genotyping methods was partly done by adapting published genotyping methods. Then the published primer sequences were checked by the Basic Local Alignment Search Tool (BLAST) [270] and compared to the reference nucleotide sequences available from the NCBI database. In cases of inconsistencies (oligo sequence, length of product), these were corrected. If no suitable primers were available, new primers were designed using the Primer3 software program from the Whitehead Institute for Biomedical Research [248] and the genomic reference DNA sequence flanking the polymorphism (available from the NCBI database [271]).

In order to avoid problems during amplification with primer specificity, formation of primer dimers or low performance, the program features were adjusted to minimize homopolymeric stretches, highly repetitive regions, low-complexity regions, and secondary structures (hairpin loops within a primer). It is important to ensure that each primer does not form hairpins or dimers with itself or the other PCR primer. Complementary primers can cause PCR artifacts if a 3' overhang is formed, especially in control lanes (without template). In this case, the primers anneal to each other and are extended during the PCR, sometimes creating large products.

The search for optimal primers was, however, limited by the given DNA sequence. If possible, the length of primers was at least 16 bp to be long enough to be specific for its target region, but not too long (less than 25 bp) since longer primers are less efficient when annealing. An optimal GC content of 50 - 60% could not always be reached. The (theoretical) T_m difference between the primers was less than 5°C to avoid mispriming of the higher melting primer and unspecific products at low annealing temperatures as well as inefficient annealing and amplification of the low melting primer at high annealing temperatures.

For design of primers of hybridization assays, the LightCycler® Probe Design Software was used. This software is specifically designed to find the best combination of primers and probes for the entered target sequence and target SNP (see § 2.2.6.2). If possible, one pair of primers was designed suitable for fluorescence detection as well as restriction analysis. Finally, the primers were tested versus the BLAST algorithm to ensure that when combined, these primers would only amplify the desired DNA template.

Special diligence was necessary in the case of CYP2D6*10 and *41 polymorphisms due to the existence of the CYP2D7 and CYP2D8 pseudogenes with a high sequence homology. No direct amplification of the target sequences was possible. Specific intergenetic primer-sites were used to produce a long PCR amplificate spanning the whole CYP2D6 gene as published and routinely used at the PCR laboratory [28,272]. Subsequently, nested PCR was carried out to avoid amplification of unwanted products. Within the MDR1 Multiplex PCR (see § 2.2.7.6), two primer pairs are used for amplification. The T_ms of all four primers were all within 62 - 65°C to avoid preferable amplification of one of the products.

The sequence and location of the primers are shown together with the probes in Table 65 - Table 70 in appendix A3. Changes of published primers are discussed in § 2.2.7 (see § 2.2.7.8, § 2.2.7.10, § 2.2.7.20).

2.2.6.2 Probe Design

For design of primers and probes for hybridization assays, the LightCycler® Probe Design Software was used. This software is specifically designed to find the best combination of primers and probes for the entered target sequence and target SNP as given below (see also Roche Manual [262]).

Design Guidelines for Hybridization Probes and Primers for FRET

The T_m of the anchor probe is approx. 5 - 10°C higher than that of the sensor probe to assure annealing when the sensor probe is slowly heated through its matched and mismatched template T_ms.

A gap of 1-3 base pairs between the adjacent probes was complied with as recommended by the Roche manual and the LC software tool to assure an efficient FRET. The upstream probe is linked at its 3' end to fluorescein and the downstream probe is coupled at the 5' end to a fluorescent dye (such as LCRed 640) and at the 3' end to phosphate to prevent extension during amplification.

Probes have to compete with primer extension (favored during amplification as extension makes primer binding irreversible) and product re-annealing (typically prominent in late cycles with high product concentration, maybe seen as decrease of fluorescence after the logarithmic phase i.e., hook effect). Probes should therefore have a T_m about 5 - 10°C higher than the primers, but not more than 75 - 80°C (otherwise extension may be inhibited as the polymerase is not able to displace the probes). If the probe T_m is higher than the annealing temperature, then the fluorescence can be measured during each amplification cycle and its increase - indicating the increase of PCR product - during amplification can be monitored.

The T_m of the matched and mismatched template should be well separated to make classification as wildtype, heterozygous or variant reliable. For a SNP, four basically different probes are possible (sense and antisense, and for each a wildtype and mutation compatible probe). When designing a probe, the more destabilizing mismatches should be chosen for the best resolution of heterozygous samples [265]. Beneath the - empirically determined - general ranking of base pair stabilities from DNA duplexes (G:C > A:T > G:T ≥ G:A > T:T ≥ G:G > A:A ≥C:C > C:T > C:A), the nearest neighbors influence the stability of a mismatch [273-277].

Probes should not contain strong binding GC clusters (which may bind to undesired sites) and should not contain complementary structures a) within the probe leading to stem loops (lowering the amount of effective probe), b) with the other probe (maybe leading to fluorescence independent of target binding) or c) with a primer leading to primer-probe dimers.

Design of Hybridization Probes and Primers for FRET

Not all design rules, however, can be followed strictly as the target sequence cannot be changed. Moreover, a single nucleotide mismatch may lead to stem loop conformation of the target at the binding site. Consequently, the probes bind with different efficiency to different alleles making a reliable detection impossible. In such cases, RFLP analysis was chosen instead.

The obtained probes were checked with the BLAST algorithm to ensure that they only bind to the desired DNA target. Mutations at the primer site may inhibit the amplification of one allele and thereby suggest a (wrong) homozygous result. Therefore, the 3' terminus of the primer sequences was checked for underlying SNPs. The sequence was checked for neighboring SNPs (available from the NCBI SNP database) as to whether other variants were known near the polymorphism of interest that might cause similar T_m shifts and a false positive result.

When designing the FRET probes for the COMT Val158Met, it was unavoidable that the anchor probe spanned a rare variant (rs8192488 C to T) with an allele frequency of 0.6% for variant according to the NCBI SNP database. To avoid problems with FRET detection due to a poorly binding anchor probe, the theoretical T_m shifts of the nearby SNP were calculated with the MeltCalc program (Microsoft EXCEL add-in), which is based on the thermodynamic nearest-neighbor model [277]. The theoretical T_m of the anchor was with 67.7°C still above the T_m of the sensor probe.

Design Guidelines for Dual-Labeled Probes and Primers for 5' Nuclease Assay

The TaqMan probe should anneal near the center of the amplicon, and the amplicon should be 50 to 150 bases long. The probe's melting temperature should be 68°C to 70°C. The probe should be at least 20 bases long to prevent nonspecific annealing. The TaqMan probes and primers used were adapted from literature or public data bases [278]. The sequence and location of the primers and probes in the reference DNA sequence are shown in Table 65 - Table 70 in appendix A3. The variable nucleotides in the sensor probes are given in bold. The MeltCalc program was used to calculate the theoretical T_ms of the primers and probes in accordance with a thermodynamic model [261] assuming a concentration of 0.5 µM for primers, 0.2 µM for probes and a buffer concentration of 300 sodium equivalents.

2.2.7 Performance of Genotyping Methods and Verification of Results

2.2.7.1 Method Overview

Genotyping was carried out either with traditional genotyping methods such as RFLP analysis, allele-specific PCR and subsequent gel electrophoresis or with non-gel based high-throughput fluorescence-based detection methods. Design of suitable primers and probes and optimization of reaction conditions was conducted as described above in § 2.2.4.2, § 2.2.5.4 and § 2.2.6. The sequences of primers and probes that were finally chosen are given in Table 65 - Table 70 in appendix A3.

Conventional PCR methods were established for the detection of five length polymorphisms (5-HTTLPR, DAT1 VNTR, MAO-A uVNTR, DBH ins/del, ACE ins/del). The genotypes were assigned by the different lengths of the amplified PCR fragments by use of suitable DNA ladders and were in accordance with the theoretical lengths of the amplificates.

Similarly, the PCR products of the allele-specific methods (cf. § 2.2.4.4 for principle, CYP2D6*10 control assay, TPH2 G1463A assay) were checked by comparison with suitable DNA ladders whether they showed the expected lengths. The genotypes were assigned based on the occurrence of amplificates in the wildtype and/or the variant assay.

For RFLP methods (cf. § 2.2.4.3 for principle, 5-HTR2A T102C, 5-HTR6 T267C, GNB3 C825T, TPH1 A218C and control assays) the theoretical length of both the PCR products and the digestion fragments was determined and checked. The 5-HTR2A T102C PCR product (342 bp) and one of the digested fragments was found to be 30 bp shorter than published [279]. The theoretical check was confirmed by the result of the gel electrophoresis (see Figure 20 in § 2.2.7.10). The completeness of restriction digestion was checked by inclusion of control samples in every experiment. DNA contamination was controlled by using a sample without genomic DNA.

Moreover, RFLP methods, as described in the literature, were used to check the performance of fluorescence-based assays and to screen for controls for every genotype (wildtype, heterozygote, variant). Controls were subsequently included as known standards for each informative genotype to verify assay performance. If possible, the same set of primers was used for both fluorescence detection and RFLP digestion. Typically, 60 samples had to show a concordance rate of 100% for each polymorphic site with both methods before application of the new methods for routine measurement. In some of the control assays permanent restriction sites were present (MDR1 C1236T, 5-HTR2C C-759T, NET G1287A, NET T-182C, ADRA2A C-1291G and COMT Val158Met), which made an additional monitoring of partial digestion possible.

New hybridization probes assays (for principle see § 2.2.5.2) were developed for ten polymorphisms (CYP2D6*10 and *41, MDR1 G2677TA and C1236T, 5-HTR1A C-1019G, 5-HTR2A intron 2, 5-HTR2C C-759T, BDNF Val66Met, COMT Val158Met and FKBP5 intron 2. A published FRET assay was adapted for NET T-182C [280]. TaqMan assays (for principle see § 2.2.5.3) were adapted for 5-HTT rs25531 [114], NET G1287A [281], and ADR2A C-1291G [282].

Some of the polymorphisms evaluated were routinely performed at the PCR laboratory of the Institute for Clinical Chemistry and Pathobiochemistry as described elsewhere [27,28,88,272,283-285]: CYP1A2*1F, CYP2C19*2, *3, and *4, MDR1 C3435T, DRD2 -141C ins/del, DRD2 Taq1A, DRD3 Ser9Gly, CYP2D6 gene duplication and deletion, CYP2D6 null alleles as well as CYP2D6*9, and *2 (-1584G/C) and differentiation of gene duplication as CYP2D6*1xN, *2xN, *4xN.

2.2.7.2 Description of Assay Procedures

In the following subchapters, the complete laboratory procedure is listed schematically for every polymorphism including information on:

1. Temperature Program

The temperature program includes an initial denaturation step at 95°C (LightCycler) or 94°C (thermocycler), followed by the amplification cycles each consisting of denaturation at 95 / 94°C, annealing and elongation at 72°C. For thermocycler PCRs, a final elongation step was performed. For hot start PCR, the initial denaturation was extended to 10 min to remove the blocking groups and activate the polymerase. For LightCycler Hybridization Probes assays, melting analysis was performed directly after amplification. The temperature transition was programmed at 0.05 - 0.2°C/s with continuous fluorescence acquisition from approx. 40°C to 80°C. For TaqMan assays, the fluorescence signal was acquired once per cycle at the end of the annealing period during the amplification.

2. PCR Protocol

DNA was stored at 4°C while the other reagents were stored at -20°C, aliquots were thawed and thoroughly vortex-mixed (except solutions containing polymerases, albumin or restriction enzymes, which were only flipped lightly) before use. The lyophilized primers and probes were centrifuged, dissolved in PCR grade water to a typical stock concentration of 25 µM for primers and 10 or 3 µM for probes according to the manufacturer's instructions, stored frozen at -20°C in small aliquots to avoid frequent freezing and thawing, and protected from light. If published by the manufacturer, the exact composition of used buffers and reagents is given in § 2.2.1.

The stock was prepared in Eppendorf cups for n + 2 samples according to the preparation schemes given in the following subchapters. The conventional PCR was carried out in small 0.2 mL reaction tubes, containing a reaction volume of typically 20 µL. The tubes were inserted into a thermocycler, which heated and cooled the reaction tubes within it to the precise temperature required for each step of the reaction. Additionally to the heated lid to prevent condensation on the inside of the reaction tube caps, a layer of mineral oil was placed on the reaction mixture to prevent water evaporation. For allele-specific PCR, a two-tube approach was performed for every DNA sample. For LightCycler methods, the stock mixtures were prepared according to the protocol, then 9 or 19 µL of the reaction mixture and 1 µL of DNA template were added to each of the capillaries, which were placed in a LightCycler carousel. Then the capillaries were sealed with plastic lids. The LightCycler carousel with the capillaries was centrifuged and inserted in the LightCycler unit for amplification and detection. Moreover, a negative control (without template) and positive controls (wildtype, heterozygote or variant DNA) were included in every experiment.

3. Detection

a) Conventional Methods (RFLP and/or Electrophoresis):

For RFLP, reagents were stored at -20°C, aliquots were thawed and thoroughly vortex-mixed (except solutions containing polymerases, albumin or restriction enzymes, which were only flipped lightly). The stock was prepared for n + 2 samples, pipetted into Eppendorf tubes and mixed with the PCR product by pipetting the solution up and down. A layer of mineral oil was placed on the reaction mixture to prevent water evaporation during incubation.

For electrophoresis, agarose gels were prepared according to the manufacturer's instructions with PeqGold Universal agarose or with MetaPhor® agarose (for high resolution separation of 20 - 800 bp DNA) in a concentration up to 3% (m/V) in 1x TBE buffer prepared from UltraPure™ 10X TBE Buffer. Under air flue, with lab clothing and nitrile gloves to protect against the carcinogenic ethidium bromide, 3 µL of the ethidium bromide solution (1 µg/mL in deionized water) was added to a 30 mL hot agarose gel solution and mixed carefully. After gelling, up to 10 µL of a mixture of the PCR or RFLP product with gel loading buffer (10:1 or 5:1) was applied into the slots. The exact agarose concentration and amount pipetted into the slots for every polymorphism are given in the following subchapters.

After the separation of DNA fragments in the Mupid® electrophoresis system, the gel was visualized and photo-documented by the photographic gel documentation system. The sizes of PCR or RFLP products were determined by comparison with a suitable DNA ladder, which contains DNA fragments of known size. In the case of allele-specific PCR a PCR product is only seen if the wildtype (or variant) primer matches with the SNP base in the sample DNA.

b) Hybridization Probes Assays

For genotyping, a FRET (fluorescence resonance energy transfer) reaction involves two PCR primers and a pair of adjacent hybridization probes labeled either with fluorescein at its 3' end or with an acceptor dye at its 5' end. After amplification of the target DNA sequence, the reaction mixture containing the DNA amplificate and the hybridization probes is slowly heated (so-called melting curve analysis, for principle see § 2.2.5.2). The mismatch underlying the sensor probe leads to a lower thermal stability of the sensor-DNA heteroduplex and a lower melting temperature (T_m). This shift is used for detection of single base alterations. The specific parameters - probe pair, the wavelength used for FRET detection as well as the PCR product size and the melting temperatures for wildtype and variant alleles - are presented in the following subchapters for each assay in the PCR assay protocols.

c) TaqMan Assays

For genotyping, a TaqMan reaction involves two PCR primers and two allele-specific probes. The TaqMan wildtype probe was labeled with fluorescein and detected at 530 nm. The TaqMan variant probe was labeled with LCRed610 dye or hexachlorofluorescein and detected at 610 or 560 nm. Both allele-specific TaqMan probes were used within the same PCR (single-tube approach). When the *Taq* DNA polymerase approaches the annealed TaqMan probe during PCR amplification, its 5' → 3' exonuclease activity cleaves the reporter dye from its 5' end, which is no longer quenched and starts emitting fluorescence (for principle see § 2.2.5.3). The increase of fluorescence is less intensive in heterozygote than in homozygote samples. No fluorescence is seen for no-template controls and negligible fluorescence for incongruous homozygotes. The specific parameters - probe sequences, dyes, wavelength used for detection and PCR product size - are presented in the following subchapters for each assay in the PCR assay protocols.

2.2.7.3 CYP2D6*10

CYP2D6*10 (rs1065852) is one of the alleles of the highly polymorphic CYP2D6 gene responsible for reduced metabolic capacity with an allele frequency of approx. 2% in Central Europe [72,89]. The CYP2D6*10 polymorphism is a C to T change at nucleotide position 100 (relatively to the start codon), causing a Pro34 → Ser amino acid substitution and a more unstable gene product [286,287] . A new fluorescence-based LightCycler hybridization probes assay was developed and carried out as described in § 2.2.7.2 and specified in Table 5. The sequences of the primers and probes used as well as their location in the reference sequence are given in appendix A3 for both methods (see Table 65).

Table 5: CYP2D6*10 assay procedure

Temperature Program			
95°C/90 s; 30 x (95°C/10 s, 58°C/10 s, 72°C/30 s); 45°C → 75°C @ 0.1°C/s			
Reagents	Amount (µL)	Concentration	Final concentration[1]
H_2O, sterile	15.5		
$MgCl_2$	0.4	25 mM	1.5 mM[2]
LC 2D6*10 F (primer)	0.4	25 µM	500 nM
LC 2D6*10 R (primer)	0.4	25 µM	500 nM
2D6*10 sen (probe)	0.4	10 µM	200 nM
2D6*10 anc (probe)	0.4	10 µM	200 nM
LightCycler DNA Master HybProbe	2.0	10 x	1 x
DNA (1:10 diluted preamplicon)[3]	0.5		
FRET Detection (640 nm) of the 346 bp product			
Wildtype peak (100C) 55°C Mutation peak (100T) 60°C			

[1] related to total volume of 20 µL (19.5 µL stock solution and 0.5 µL DNA)
[2] total $MgCl_2$-concentration of the LightCycler DNA Master HybProbe and the additional 25mM $MgCl_2$ -solution
[3] nested PCR as published previously [272]

The CYP2D6*10 anchor probe was labeled with fluorescein (GGCAGTGGCAGGGGGCC-fluorescein, cf. Table 65), while the FRET partner, the sensor probe, was labeled with LCRed640 dye (LCRed640-GTG**A**GTAGCGTGCAGCC-phosphate). The sensor probe is spanning the C100T SNP and was designed to be completely complementary to the sense strand and the variant T-allele. Thus, the melting temperature of the wildtype C-allele was 55°C and that of the variant T-allele 60°C. The derived melting curves for the different CYP2D6*10 genotypes are shown in Figure 6.

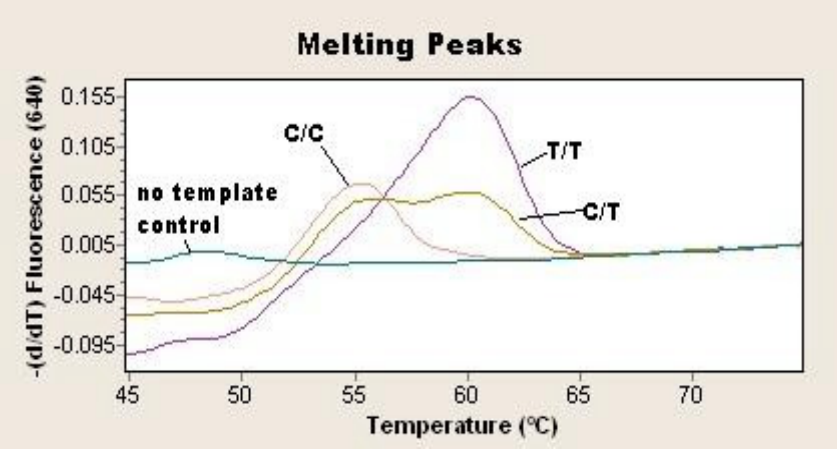

Figure 6: CYP2D6*10 melting curves

Melting curve plots negative derivative of fluorescence with respect to the temperature (-dF/dT) vs. temperature (for principle see § 2.2.5.2).

Note: fluorescence detection of the different CYP2D6*10 genotypes (SNP C100T) at 640 nm as follows: wildtype homozygote C/C - one peak at 55°C, variant homozygote T/T - one peak at 60°C and heterozygote C/T – two peaks at 55 and 60°C. No fluorescence peak observed for no-template control.

The allele-specific thermocycler assay (two-tube approach) published by Ji et al. (2002) [288] was used to check the assay performance of the newly developed fluorescence-based CYP2D6*10 assay. Both methods showed a 100% concordance in more than 100 DNA samples. A typical electrophoresis result is given in Figure 7 (on the top wildtype assay and on the bottom variant assay). The forward primer and one of two allele-specific reverse primers produced a 137 bp band only if the matching allele is present in the DNA sample. No interfering unspecific bands were present.

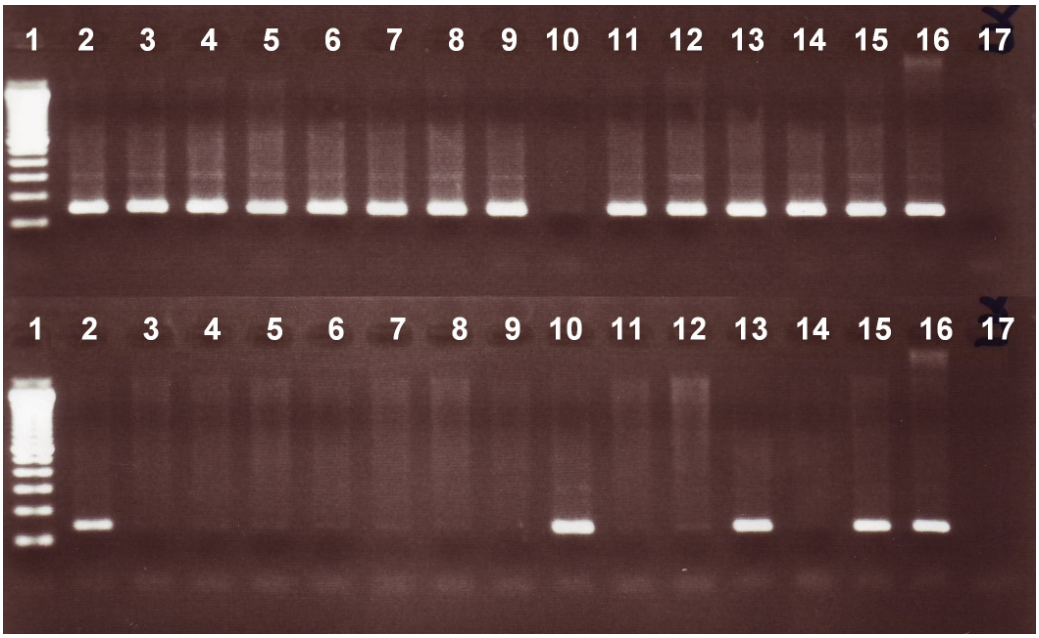

Figure 7: CYP2D6*10 gel electrophoresis

Allele-specific wildtype control assay with reverse primer specific for 100C-allele on the top; allele-specific variant control assay with reverse primer specific for 100T-allele on the bottom with the same set of samples:

Lane 1:	length marker (100 bp DNA ladder)
Lane 2, 13, 15, 16:	C/T heterozygotes (137 bp amplificate for both wildtype and variant assay)
Lane 3 - 9, 11, 12, 14:	C/C homozygotes (137 bp amplificate only for wildtype assay)
Lane 10:	T/T homozygotes (137 bp amplificate only for variant assay)
Lane 17:	no-template control

For electrophoresis, 7 µL of the PCR product was mixed with 1 µL loading buffer and analyzed on a 1.5% agarose gel using a 100 bp DNA-ladder (15 min at 100 V).

2.2.7.4 CYP2D6 *41

Approximately 30 - 40% [72,88,89] of the individuals in Central Europe have a CYP2D6 intermediate metabolizer (IM) phenotype characterized by low residual enzyme activity. The identification remained unsatisfactory because of incomplete characterization of the major allele involved, termed CYP2D6*41. Initial sequence analysis of the exons and promoter of the CYP2D6*41 allele revealed an additional change in the promoter (-1584C) compared with the *2 allele. The *2 (-1584G) allele has a normal function but, compared to the wildtype *1 allele, two additional amino acid substitutions [89,289].

The recently identified novel polymorphism 2988G>A within intron 6, which is in linkage to the -1584C>G change, was shown to cause the low expression phenotype of the impaired CYP2D6*41 allele by aberrant splicing [89,290]. Therefore, this SNP was chosen as basis for the new developed assay. The fluorescence-based LightCycler hybridization probes assay was carried out as described in § 2.2.7.2 and specified in Table 6. The sequences of the primers and probes used as well as their locations in the reference sequence are given in appendix A3 (see Table 65).

The existing routine assay for the CYP2D6*41 allele was based on the absence of the -1584C polymorphism on a genetic *2 background. The control assay was routinely performed as published previously [272]. In this assay, a PCR amplificate and subsequent fluorescence detection of the C-1584G polymorphism was only present for the *2 allele due to the use of *2 specific primers. The results of both assays are compared in § 3.2.

Table 6: CYP2D6*41 (G2988A) assay procedure

Temperature Program			
95°C/90 s; 30 x (95°C/3 s, 65°C/10 s, 72°C/12 s); 40°C → 75°C @ 0.1°C/s			
Reagents	Amount (µL)	Concentration	Final concentration[1]
H_2O, sterile	14.0		
$MgCl_2$	1.2	25 mM	2.5 mM[2]
2D6*41 F (primer)	0.5	25 µM	625 nM
2D6*41 R (primer)	0.5	25 µM	625 nM
2D6*41 sen (probe)	0.4	10 µM	200 nM
2D6*41 anc (probe)	0.4	10 µM	200 nM
LightCycler DNA Master HybProbe	2.0	10 x	1 x
DNA (1:10 diluted preamplicon)[3]	1.0		
FRET Detection (640 nm) of the 186 bp product			
Wildtype peak (2988G) 62°C Mutation peak (2988A) 53°C			

[1] related to total volume of 20 µL (19 µL stock solution and 1 µL DNA)
[2] total $MgCl_2$-concentration of the LightCycler DNA Master HybProbe and the additional 25mM $MgCl_2$ -solution
[3] nested PCR as published previously [272]

The sensor probe is spanning the G2988A SNP and was designed to be completely complementary to the sense strand and the wildtype G-allele. It was labeled with fluorescein (CCCTT**C**CTCCCTCGGC-fluorescein), while the FRET partner, the anchor probe, was labeled with LCRed640 dye (LCRed640-CTGCACTGTTTCCCAGATGGGC-phosphate, cf. Table 65). Thus, the melting temperature of the wildtype G-allele was 62°C and that of the variant A-allele 53°C. The melting curves for the different genotypes are shown in Figure 8.

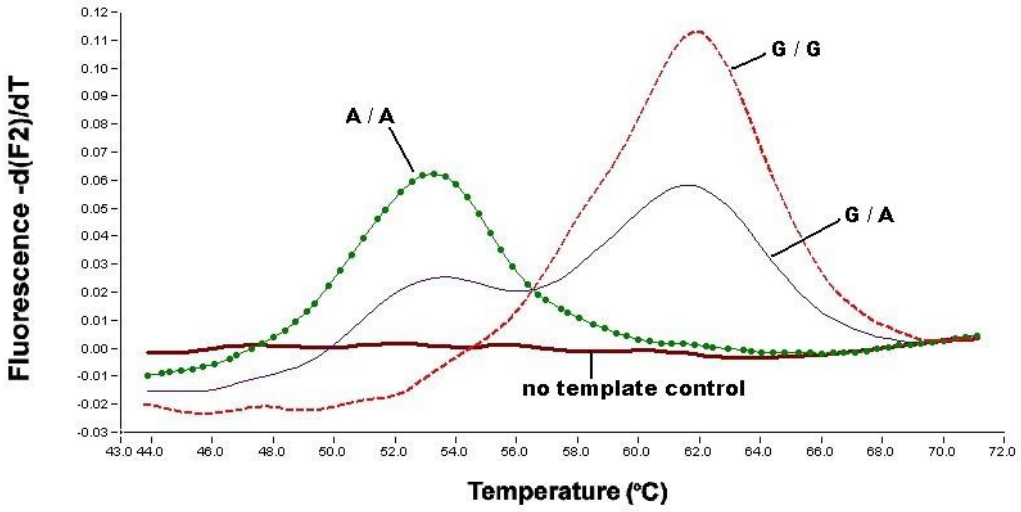

Figure 8: CYP2D6*41 melting curves

Melting curve plots negative derivative of fluorescence with respect to the temperature (-dF/dT) vs. temperature (for principle see § 2.2.5.2). Note: fluorescence detection of the different CYP2D6*41 genotypes (SNP G2988A) at 640 nm as follows: wildtype homozygote G/G - one peak at 62°C, variant homozygote A/A - one peak at 62°C and heterozygote G/A – two peaks at 62 and 53°C, respectively. No fluorescence peak observed for no-template control.

2.2.7.5 MDR1 G2677TA

A triallelic SNP (rs2032582) in exon 21 of the highly polymorphic MDR1 gene with a G to T or A change at nucleotide position 2677 (relatively to the start codon) causes an Ala893→Ser or Thr amino acid substitution. A new fluorescence-based LightCycler hybridization probes assay was developed for this triallelic SNP and carried out as described in § 2.2.7.2 and specified in Table 7. The sequences of the primers and probes used as well as their locations in the reference sequence are given in appendix A3 (see Table 65).

Table 7: MDR1 G2677TA assay procedure

Temperature Program			
95°C/60 s; 30 x (95°C/10 s, 55°C/10 s, 72°C/10 s); 50°C → 80°C @ 0.1°C/s			
Reagents	Amount (µL)	Concentration	Final concentration[1]
H_2O, sterile	6.3		
$MgCl_2$	1.0	25 mM	3.5 mM[2]
MDR 2677 F (primer)	0.2	25 µM	500 nM
MDR 2677 R (primer)	0.2	25 µM	500 nM
MDR 2677 sen (probe)	0.15	10 µM	150 nM
MDR 2677 anc (probe)	0.15	10 µM	150 nM
LC DNA Master HybProbe	1.0	10 x	1 x
Genomic DNA	1.0		
FRET Detection (640 nm) of the 309 bp product			
Wildtype peak (2677G) 57°C Mutation peak (2677T) 61°C Mutation peak (2677A) 55°C			

[1] related to total volume of 10 µL (9 µL stock solution and 1 µL DNA)
[2] total $MgCl_2$-concentration of the LightCycler DNA Master HybProbe and the additional 25 mM $MgCl_2$ -solution

The fluorescein-labeled sensor probe spanning the G2677TA SNP was designed to be completely complementary to the sense strand and the variant T-allele (ACCTTCCCAG**A**ACCTTCTAG-fluorescein, cf. Table 65). The FRET partner, the anchor probe, was labeled with LCRed640 dye (LCRed640-CTTTCTTATCTTTCAGTGCTTGTCCAGAC-phosphate). The sensor probe allows detecting the three alleles within one run. The melting temperature of the wildtype G-allele was 57°C, that of the variant T-allele 61°C and that of the variant A-allele 55°C. The melting curves for the different genotypes are shown in Figure 9. Due to the small difference between the melting temperatures of the G and the A-allele, however, only one broad peak at 55 - 57°C is detected for the G/A-genotype (cf. Figure 9). Thus, this genotype was verified by a re-analysis of the respective samples with the RFLP reference method published by Tanabe et al. (2001) [291] which was also used for validation of the new LightCycler method.

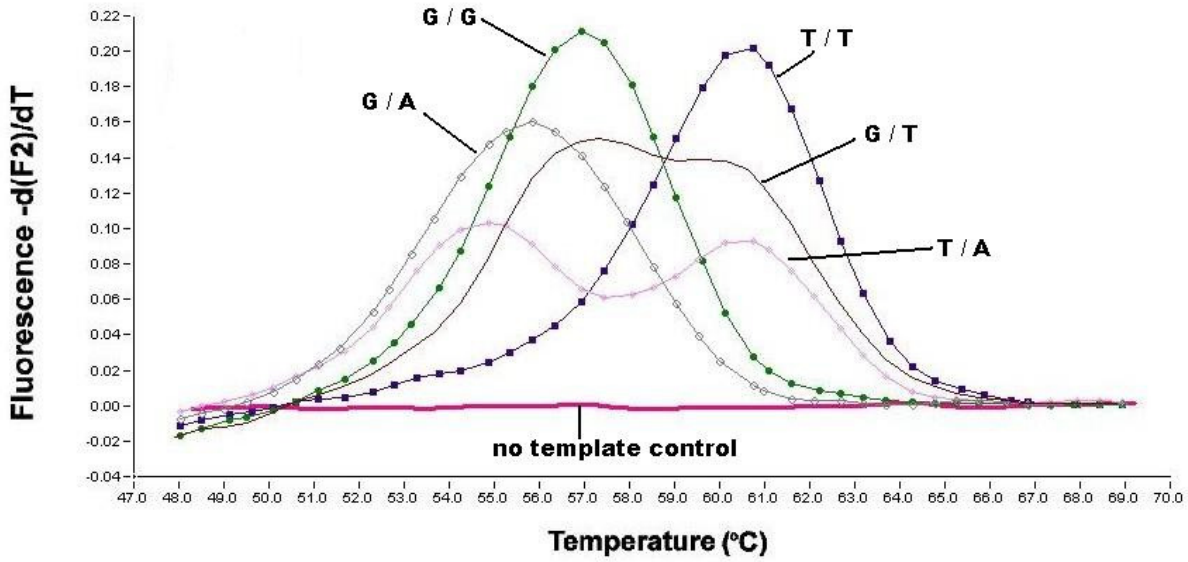

Figure 9: MDR1 G2677TA melting curves

Melting curve plots negative derivative of fluorescence with respect to the temperature (-dF/dT) vs. temperature (for principle see § 2.2.5.2). Note: fluorescence detection of the different MDR1 G2677TA genotypes at 640 nm as follows: wildtype homozygote G/G - one peak at 57°C, variant homozygote T/T - one peak at 61°C, variant heterozygote T/A two peaks at 61 and 55°C, heterozygote G/T – two peaks at 57 and 61 °C and heterozygote G/A – one broad peak at 55 - 57°C. Variant homozygote A/A (theoretically one peak at 55°C) not present in study population. No fluorescence peak observed for no-template control.

This thermocycler assay is an allele-specific three-tube method using a forward primer and one of three reverse primers with a subsequent RFLP digestion [291]. The allele-specific reverse primers for the G- and the T-allele were additionally "trick" primers with additional mismatches (underlined in the sequences below) to create restriction sites for the detection of the G- and the T-allele. The three 107 bp PCR products obtained for each DNA sample were digested with the appropriate restriction enzymes (cf. Table 4 in § 2.2.4.3).

Product size:		
3´G-allele/Nhe I-product:	107 bp for G;	84 + 23 bp for T or A
3´T-allele/Xba I-product:	107 bp for T;	84 + 23 bp for G or A
3´A-allele/Rsa I-product:	107 bp for A;	83 + 24 bp for G or T

The NheI enzyme cuts specifically the G-allele with the recognition site G / C T A G C (framed C on the reverse strand, site introduced by the MDR 2677G reverse primer TTTAGTTTGACTCACC TTGCTAG). The XbaI enzyme cuts specifically the T-allele with the recognition site T / C T A G A (framed A on the reverse strand, site introduced by the MDR 2677T reverse primer TTTAGTTTGACTCACCTTTCTAG). The RsaI enzyme cuts specifically the A-allele with the recognition site G T / A C (framed T on the reverse strand, amplified with the MDR 2677A reverse primer TTTAGTTTGACTCACCTTCCC). The genotype was determined by combination of the absence or presence of digested products in the three tubes, as shown exemplarily in Figure 10.

The 107 and 83/84 bp DNA fragments were visualized by gel electrophoresis and no interfering unspecific PCR products were observed. The 24/23 bp fragments were too short to be visible in the agarose gel. The method showed a 100% concordance with the newly developed fluorescence-based assay more than 60 DNA samples.

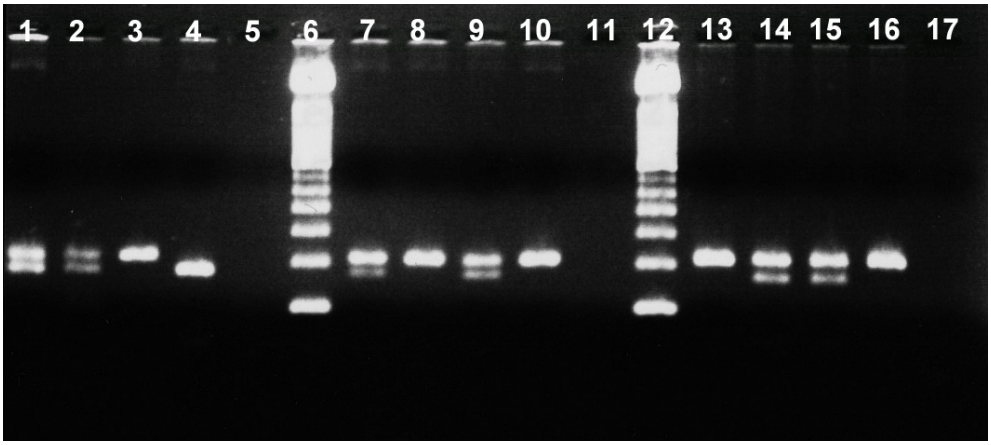

Figure 10: MDR1 G2677TA gel electrophoresis

Three-tube approach visualized for four genotypes G/T (Lane 1, 7, 13), G/A (Lanes 2, 8, 14), T/A (Lanes 3, 9, 15) and G/G (Lanes 4, 10, 16) and the three different RFLP digestions.

Lane 1- 5: RFLP with NheI (cuts G-allele) unambiguously identifies in Lane 1 and 2: G/T or A (83 bp / 107 bp); Lane 3: T or A/T or A (107 bp) and in Lane 4: G/G (83 bp) with no-template control in Lane 5

Lane 7-11: RFLP with XbaI (cuts T-allele) unambiguously identifies in Lane 7 and 9: T/G or A (83 bp / 107 bp); Lane 8 and 10: G or A/G or A (107 bp) with no-template control in Lane 11

Lane 13-17: RFLP with RsaI (cuts A-allele) unambiguously identifies in Lane 13 and 16: G or T/G or T (107 bp); Lane 14 and 15: A/ G or T (83 bp / 107 bp) with no-template control in Lane 17

Lane 6 and 12: 100 bp DNA ladders

For electrophoresis, 9 µL of the PCR product was mixed with 1 µL loading buffer and analyzed on a 2.5% agarose gel using a 100 bp DNA-ladder (20 min at 100 V).

2.2.7.6 MDR1 C3435T and C1236T

The two synonymous SNPs C3435T in exon 26 and C1236T in exon 12 of the MDR1 gene are frequently linked. Lower expression of the P-gp transporter was shown for MDR1 3435 T/T homozygotes [96]. Thus, this polymorphism may serve as marker for the so-far unidentified changes in and around the highly polymorphic MDR1 gene.

A published fluorescence-based LightCycler hybridization probes method was routinely performed at the PCR laboratory of the Institute for Clinical Chemistry and Pathobiochemistry for the C3435T polymorphism [284]. A multiplex assay was developed for the simultaneous detection of C3435T in exon 26 and C1236T in exon 12 of the MDR1 gene with two sets of primers and two sets of probes with different fluorescent dyes and carried out as described in § 2.2.7.2 and specified in Table 8.

The sequence of the primers and probes well as their locations in the reference sequence are given in appendix A3 (see Table 66). The second set of primers was designed with similar melting temperature (T_m of all four primers within 62 - 65°C) to avoid preferable amplification of one of the products. All four primers were checked for primer dimerisation against each other. Due to fluorophore emission overlap, each channel contains an emission composite of all probes. Color-compensation was used to correct for the amount of overlap in each channel for each fluorophore (see § 2.2.5.4) and to optimize the assay performance.

The anchor probe of the C3435T SNP was labeled with fluorescein (GACAACAGCCGGGTGGTGTCA-fluorescein, cf. Table 66), while the FRET partner, the sensor probe, was labeled with LCRed640 dye (LCRed640-GGAAGAGAT**C**GTGAGGGCAG-phosphate). The sensor probe spanning the SNP was completely complementary to the antisense strand and the wildtype C-allele. The melting peaks derived at 640 nm were 65°C for the wildtype C-allele and 56°C for the variant T-allele (see Figure 11) and similar to those published previously [284].

Table 8: MDR1 C3435T and C1236T assay procedure

Temperature Program			
95°C/60 s; 30 x (95°C/10 s, 55°C/10 s, 72°C/10 s); 50°C → 80°C @ 0.1°C/s			
Reagents	Amount (µL)	Concentration	Final concentration[1]
H_2O, sterile	4.2		
$MgCl_2$	0.8	25 mM	2.5 mM[2]
MDR 1236 F3 (primer)	0.5	25 µM	625 nM
MDR 1236 R3 (primer)	0.5	25 µM	625 nM
MDR 1236 anc (probe)	0.5	10 µM	250 nM
MDR 1236 sen (probe)	0.5	10 µM	250 nM
MDR 3435 F (primer)	0.3	25 µM	375 nM
MDR 3435 R (primer)	0.3	25 µM	375 nM
MDR 3435 S3´ (probe)	0.3	10 µM	150 nM
MDR 3435 S5´(probe)	0.3	10 µM	150 nM
Bovine Serum Albumin	0.5	20 mg/mL	0.5 mg/mL
Premix D	10.0	2 x	1x
Taq-Polymerase Qbiogene	0.3	5 U/µL	1.5 U/µL
Genomic DNA	1.0		
FRET Detection (640 nm) of the 197 bp product: Wildtype peak (3435C) 65°C Mutation peak (3435T) 56°C			
FRET Detection (705 nm) of the 286 bp product: Wildtype peak (1236C) 56°C Mutation peak (1236T) 42°C			

[1] related to total volume of 20 µL (19 µL stock solution and 1 µL DNA)

[2] total $MgCl_2$-concentration of the Premix D and the additional 25mM $MgCl_2$ –solution

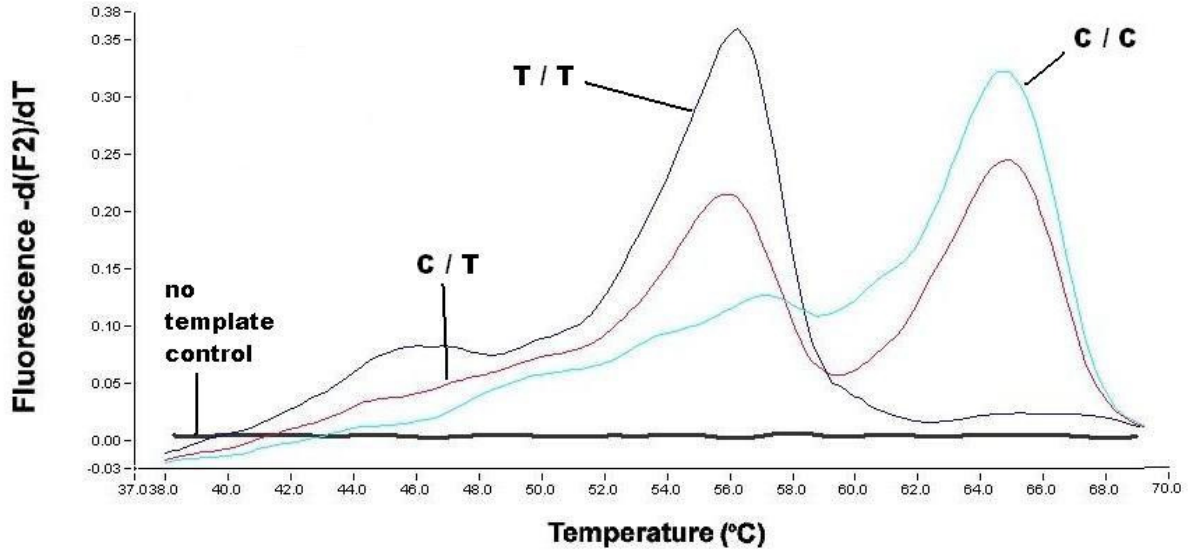

Figure 11: MDR1 C3435T melting curves at 640 nm

Melting curve plots negative derivative of fluorescence with respect to the temperature (-dF/dT) vs. temperature (for principle see § 2.2.5.2). Note: fluorescence detection of the different MDR1 C3435T genotypes at 640 nm as follows: wildtype homozygote C/C - one peak at 65°C, variant homozygote T/T - one peak at 56°C, heterozygote C/T – two peaks at 65 and 56°C, respectively. No fluorescence peak observed for no-template control.

The sensor probe spanning the C1236T SNP was designed to be completely complementary to the antisense strand and the wildtype C-allele. It was labeled with fluorescein (AGGG**C**CTGAACCTGA-fluorescein), while the FRET partner, the anchor probe, was labeled with LCRed705 dye (LCRed705-GTGCAGAGTGGGCAGACG-phosphate, cf. Table 66). Thus, the following melting peaks were derived at 705 nm: 56°C for the wildtype C-allele and 42°C for the variant T-allele (see Figure 12). The primers chosen also allowed a (subsequent) RFLP analysis for control and validation of assay performance. For this purpose, the MDR C1236T PCR product was digested with HaeIII (cf. Goreva et al. 2004 [292] and Table 4 in § 2.2.4.3). The C-allele was cut to fragments of 106 + 35 + 145 bp, while the T-allele was cut to 106 + 180 bp.

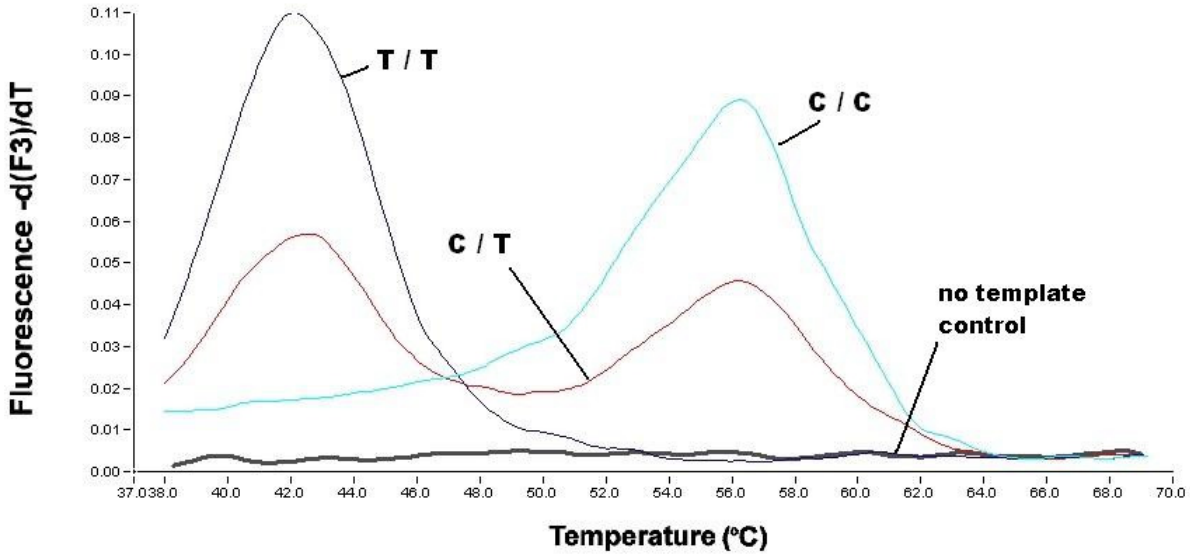

Figure 12: MDR1 C1236T melting curves at 705 nm

Melting curve plots negative derivative of fluorescence with respect to the temperature (-dF/dT) vs. temperature (for principle see § 2.2.5.2). Note: fluorescence detection of the different MDR1 C1236T genotypes at 705 nm as follows: wildtype homozygote C/C - one peak at 56°C, variant homozygote T/T - one peak at 42°C, heterozygote C/T – two peaks at 56 and 42°C, respectively. No fluorescence peak observed for no-template control.

2.2.7.7 5-HTTLPR and A/G (rs25531)

The 5-HTT length polymorphism (5-HTTLPR) is a common variation in the promoter of the serotonin transporter gene SLC6A4 with a 44 bp region either being present (L-allele) or absent (S-allele). Recently, a new SNP (rs25531 A/G) nearby was reported to have additional influence on transcription activity and clinical outcome with the high expression variant L_A (i.e., 5-HTTLPR L-allele in combination with rs25531 A-allele).

A published TaqMan assay [114] for the rs25531 A/G polymorphism was adapted to the equipment at hand. The assay was carried out as described in § 2.2.7.2 and specified in Table 9. The sequences of the primers and probes well as their location in the reference sequence are given in appendix A3 (see Table 66).

For the fluorescence detection of the rs25531 A/G SNP, two allele-specific TaqMan probes were used within the same PCR (single-tube approach). The wildtype TaqMan probe is complementary to the antisense strand of the A-allele. It was labeled with fluorescein as reporter dye (FAM-CCCCCCTGCACCCCC**A**GCATCCC-BHQ1) and was detected at 530 nm (see Figure 13). The variant probe is complementary to the antisense strand of the G-allele. It was labeled with LCRed610 (LCRed610-CCCCTGCACCCCC**G**GCATCCCC-BHQ2) and was detected at 610 nm (see Figure 14). Both probes were additionally labeled with a non-fluorescent quencher at the 3´end to minimize the background fluorescence and maximize the sensitivity. Moreover, color-compensation was used to correct for the fluorophore emission overlap in each channel for each fluorophore (see § 2.2.5.4) to optimize the assay performance.

Table 9: 5-HTTLPR and rs25531 A/G assay procedure

Temperature Program			
95°C/10 min; 80 x (95°C/3 s, 74°C/55 s)			
Reagents	Amount (µL)	Concentration	Final concentration[1]
H_2O, sterile	7.6		
$MgCl_2$	0.4	25 mM	2.0 mM[2]
HTT L_AL_G F (primer)	0.2	25 µM	500 nM
HTT L_AL_G R (primer)	0.2	25 µM	500 nM
HTT A (probe)	0.05	10 µM	50 nM
HTT G (probe)	0.05	10 µM	50 nM
LC Fast Start DNA Master HybProbe	1.0	10 x	1 x
Genomic DNA	1.0		
Fluorescence Detection of the 138 bp (S-allele) or 181 bp (L-allele) product			
Wildtype (rs25531 A-allele) at 530 nm Mutation (rs25531 G-allele) at 610 nm			

[1] related to total volume of 10 µL (9.0 µL stock solution and 1.0 µL DNA)

[2] total $MgCl_2$-concentration of LC Fast Start DNA Master HybProbe and the additional 25mM $MgCl_2$ -solution

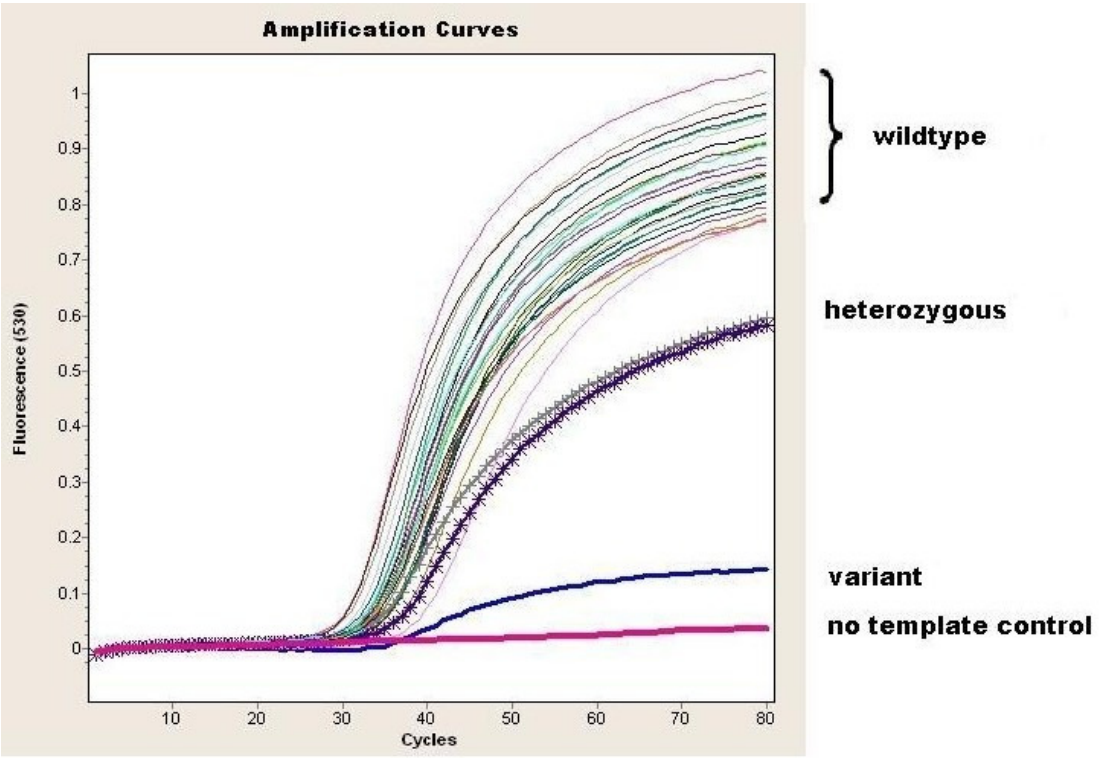

Figure 13: 5-HTT rs25531 A/G amplification curves at 530 nm

Amplification curve plots increase of fluorescence vs. cycle number (for principle see § 2.2.5.3). Note: fluorescence detection of the A-allele of the rs25531 SNP in the 5-HTT promoter at 530 nm as follows: increase of fluorescence with increasing PCR product during amplification of the wildtype homozygote A/A and – less intensive – for the A/G heterozygote. No increase for no-template control and negligible increase of fluorescence for the variant homozygote G/G.

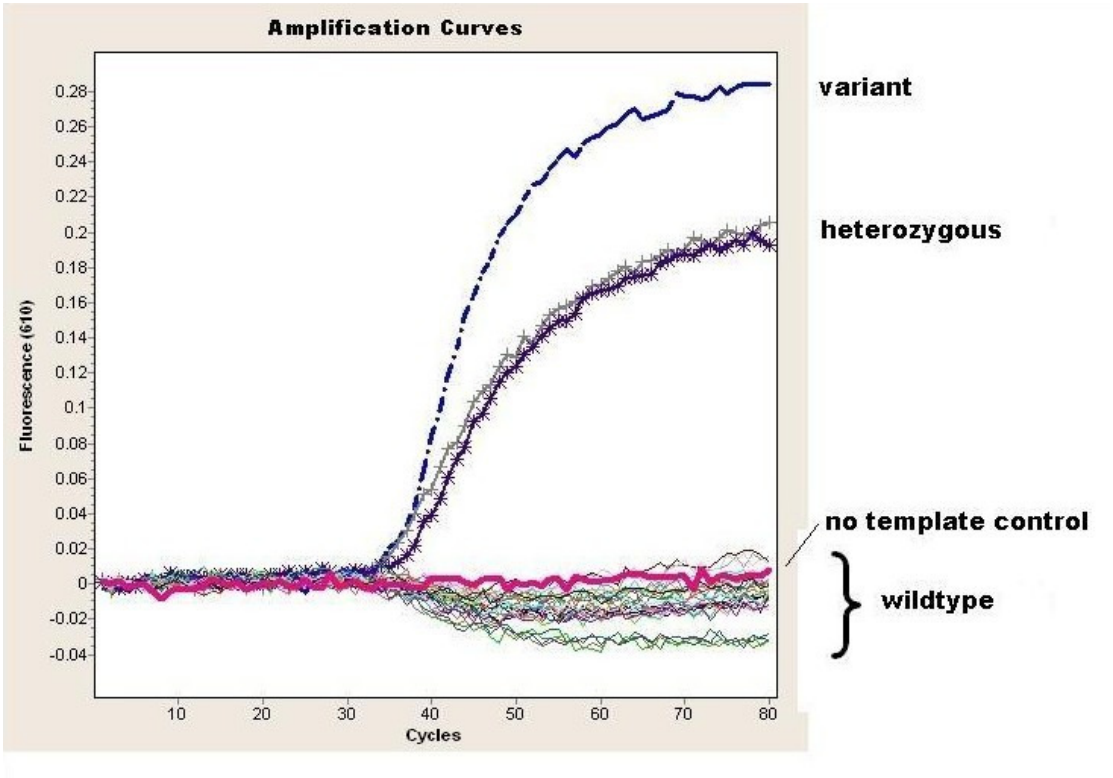

Figure 14: 5-HTT rs25531 A/G amplification curves at 610 nm

Amplification curve plots increase of fluorescence vs. cycle number (for principle see § 2.2.5.3). Note: fluorescence detection of the G-allele of the rs25531 SNP in the 5-HTT promoter at 610 nm as follows: increase of fluorescence with increasing PCR product during amplification of the variant homozygote G/G and – less intensive – for the A/G heterozygote. No increase of fluorescence for no-template control and for the wildtype homozygote A/A.

Moreover, the primers chosen for the rs25531 A/G polymorphism allowed a detection of the 5-HTT length polymorphism in a subsequent gel electrophoresis. The 5-HTTLPR S-allele was detected with a length of 138 bp and the L-allele with 181 bp. No unspecific fragments were observed (see Figure 15). The chosen primers also allowed a subsequent RFLP analysis for control and validation of the rs25531 A/G assay. For this purpose, the TaqMan PCR product was digested with MspI (cf. Wendland et al. 2006 [293] and Table 4 in § 2.2.4.3). MspI cuts the G-allele of the rs25531, but not the A-allele. For the 5-HTTLPR L-allele, the rs25531 A-allele remained with 181 bp uncut (cf. L_A in Figure 16), whereas the G-allele was cut to fragments of 96 and 85 bp (cf. L_G in Figure 16). All 5-HTTLPR S-alleles remained uncut and were visualized with 138 bp (cf. S_A in Figure 16). The rare S_G-allele was not present in the study population.

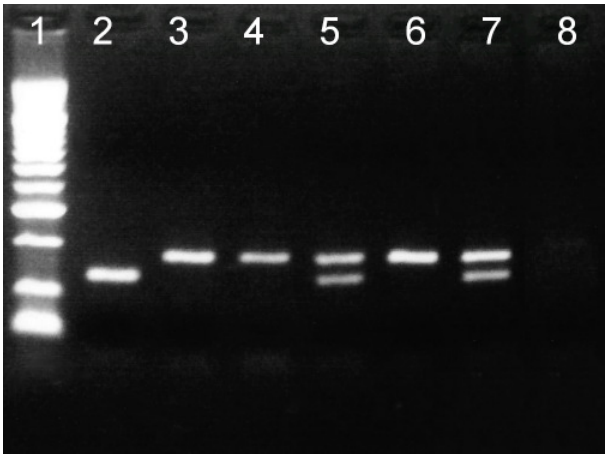

Figure 15: 5-HTTLPR gel electrophoresis

PCR product of TaqMan assay:

Lane 1: length marker (100 bp DNA ladder with additional 50 bp lane)

Lane 2: homozygous S/S (138 bp)

Lane 3 and 4: homozygous L/L (181 bp)

Lane 5: heterozygous S/L (138 bp / 181 bp)

Lane 6: homozygous L/L (181 bp)

Lane 7: heterozygous S/L (138 bp / 181 bp)

Lane 8: no-template control

For electrophoresis, 9 µL of the PCR product was mixed with 1 µL loading buffer and analyzed on a 3% agarose gel (25 min at 100 V).

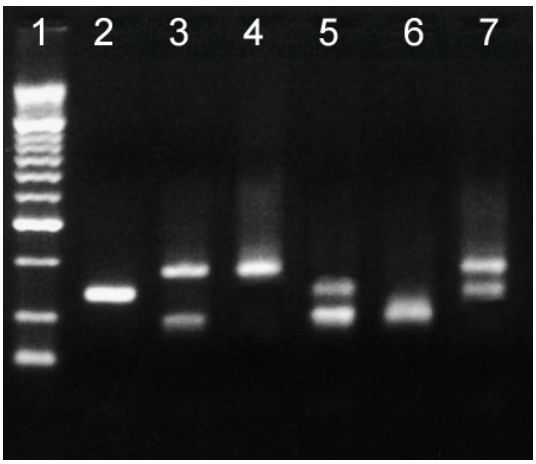

Figure 16: 5-HTTLPR and rs25531 A/G gel electrophoresis

RFLP of PCR product with Msp I:

Lane 1: length marker (100 bp DNA ladder with additional 50 bp lane)

Lane 2: S_A /S_A (138 bp)

Lane 3: L_A /L_G (181 bp / 96 and 85 bp)

Lane 4: L_A /L_A (181 bp)

Lane 5: S_A /L_G (138 bp / 96 and 85 bp)

Lane 6: L_G /L_G (96 and 85 bp)

Lane 7: S_A /L_A (138 bp / 181 bp)

2.2.7.8 5-HTR1A C-1019G

The C-1019G polymorphism (rs6295) is part of a palindromic sequence in the promoter region of the 5-HTR1A gene that contains DNA-binding sites for transcription factors. A new fluorescence-based LightCycler hybridization probes assay was developed and carried out as described in § 2.2.7.2 and specified in Table 10. The sequences of the primers and probes used as well as their locations in the reference sequence are given in appendix A3 (see Table 66).

The anchor probe was labeled with fluorescein (CGCGAGAACGGAGGTAGCTTTT-fluorescein, cf. Table 66), while the FRET partner, the sensor probe, was labeled with LCRed640 dye (LCRed640-AAAA**C**GAAGACACACTCGGTC-phosphate). The sensor probe is spanning the C-1019G SNP and was designed to be completely complementary to the antisense strand of the wildtype C-allele. Thus, the melting temperature of the wildtype C-allele was 63°C and that of the variant G-allele 56.5°C. The melting curves for the different genotypes are shown in Figure 17.

Table 10: 5-HTR1A C- 1019G assay procedure

Temperature Program			
95°C/2 min; 50 x (95°C/3 s, 55°C/10 s, 72°C/10 s); 45°C → 72°C @ 0.1°C/s			
Reagents	Amount (µL)	Concentration	Final concentration[1]
H_2O, sterile	6.8		
$MgCl_2$	0.4	25 mM	2.0 mM[2]
HT_{1A} -1019 F LC (primer)	0.15	25 µM	375 nM
HT_{1A} -1019 R LC (primer)	0.15	25 µM	375 nM
HT_{1A} -1019 sen (probe)	0.5	3 µM	150 nM
HT_{1A} -1019 anc (probe)	0.5	3 µM	150 nM
LightCycler DNA Master HybProbe	1.0	10x	1x
Genomic DNA	0.5		
FRET Detection (640 nm) of the 188 bp product			
Wildtype peak (-1019 C) 63°C Mutation peak (-1019 G) 56.5°C			

[1] related to total volume of 10 µL (9.5 µL stock solution and 0.5 µL DNA)

[2] total $MgCl_2$-concentration of the LightCycler DNA Master HybProbe and the additional 25mM $MgCl_2$ -solution

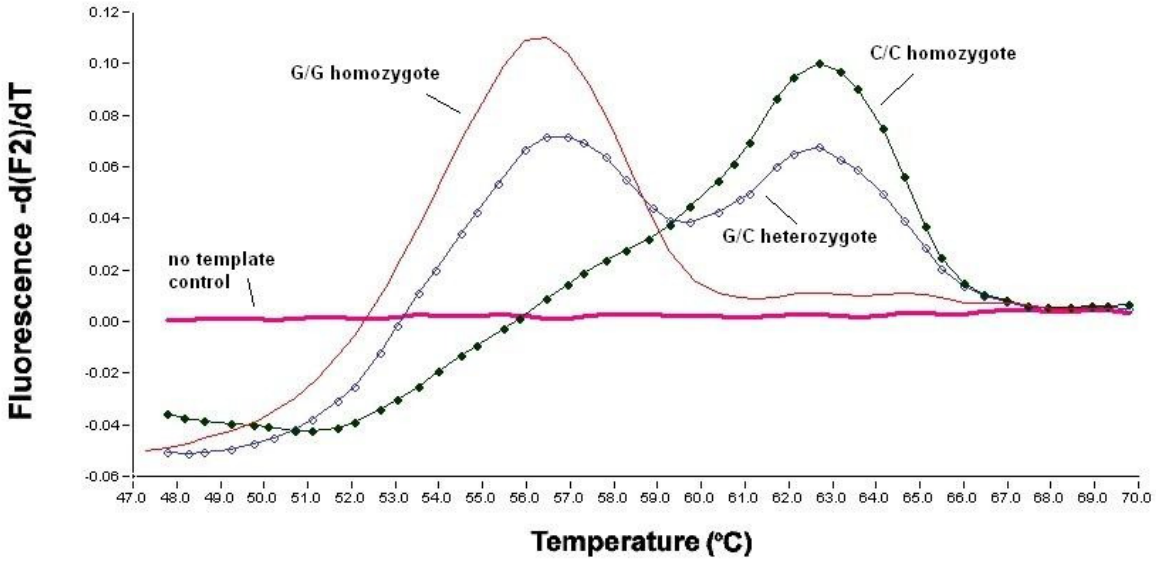

Figure 17: 5-HTR1A C-1019G melting curves

Melting curve plots negative derivative of fluorescence with respect to the temperature (-dF/dT) vs. temperature (for principle see § 2.2.5.2). Note: fluorescence detection of the different 5-HTR1A C-1019G genotypes at 640 nm as follows: wildtype homozygote C/C - one peak at 63°C, variant homozygote G/G - one peak at 56.5°C, heterozygote C/G – two peaks at 63 and 56.5°C, respectively. No fluorescence peak observed for no-template control.

The method was validated by an adapted RFLP thermocycler assay (single-tube approach with modified forward primer for an amplification-created restriction site) [294]. The primers used are given in Table 66. The reverse primer was corrected to completely match the NCBI reference sequence (TTCTCCCTGAGGGAGTAAGGCTGG instead of TTCTCCCTGGGAGAGTAAGGCTGG). The PCR product was digested with HpyCH4IV (cf. Table 4 in § 2.2.4.3). As the GenBank sequence used herein is complementary to that used in the published method [294], the G-allele remained uncut (182 bp), while C-allele was cut to fragments of 157 + 25 bp. Only the 157 bp band was visible on the agarose gel, the 25 bp fragment was too short (< 50 bp). No interfering unspecific PCR products were observed for the PCR product, the digested samples or the no-template control (see Figure 18).

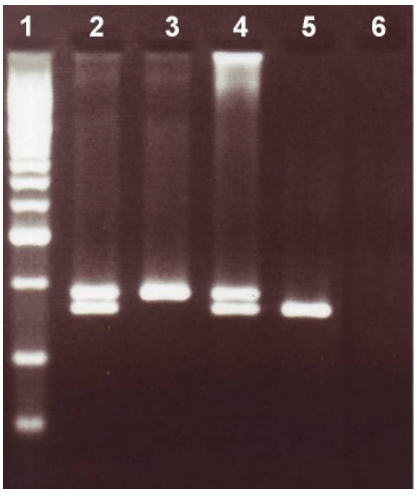

Figure 18: 5-HTR1A C- 1019G gel electrophoresis

RFLP with HpyCH4IV:
Lane 1: length marker (100 bp DNA ladder with additional 50 bp lane)
Lane 2 and 4: heterozygous C/G (157 bp / 182 bp)
Lane 3: homozygous G/G (182 bp)
Lane 4: homozygous C/C (157 bp)
Lane 6: no-template control

For electrophoresis, 9 µL of the PCR product was mixed with 1 µL loading buffer and analyzed on a 2% agarose gel (20 min at 100 V).

2.2.7.9 5-HTR2A rs7997012

The SNP rs7997012 resides in intron 2 of the 5-HTR2A gene. A new fluorescence-based LightCycler hybridization probes assay was developed and carried out as described in § 2.2.7.2 and specified in Table 11. The sequences of the primers and probes used as well as their locations in the reference sequence are given in appendix A3 (see Table 67).

Table 11: 5-HTR2A rs7997012 assay procedure

Temperature Program			
95°C/10 min; 40 x (95°C/3 s, 55°C/10 s, 72°C/10 s); 45°C → 65°C @ 0.1°C/s			
Reagents	Amount (µL)	Concentration	Final concentration[1]
H_2O, sterile	6.7		
$MgCl_2$	0.4	25 mM	2.0 mM[2]
HT_{2A} in2 F (primer)	0.15	25 µM	375 nM
HT_{2A} in2 R (primer)	0.15	25 µM	375 nM
HT_{2A} in2 sen (probe)	0.3	3 µM	90 nM
HT_{2A} in2 anc (probe)	0.3	3 µM	90 nM
LC Fast Start DNA Master HybProbe	1.0	10x	1x
Genomic DNA	1.0		
FRET Detection (640 nm) of the 194 bp product			
Wildtype peak (rs7997012 G) 55.5°C Mutation peak (rs7997012 A) 59°C			

[1] related to total volume of 10 µL (9.0 µL stock solution and 1.0 µL DNA)

[2] total $MgCl_2$-concentration of the LC Fast Start DNA Master HybProbe and the additional 25mM $MgCl_2$ -solution

The sensor probe spanning the rs7997012 G/A SNP was designed to be completely complementary to the antisense strand and the variant A-allele. It was labeled with fluorescein (GCCATTATCTTCAAAGACTTAATT**A**ACAA-fluorescein), while the FRET partner, the anchor probe, was labeled with LCRed640 dye (LCRed640-TTTGTCACTTGCCTATGCAAGCCC-phosphate, cf. Table 67). Thus, the following melting peaks were derived at 640 nm: 55.5°C for the G-allele and 59°C for the variant A-allele (see Figure 19). The primers chosen also allowed a (subsequent) RFLP analysis for control and validation of assay performance. For this purpose, the 194 bp PCR product was digested with PacI (cf. Table 4 in § 2.2.4.3). The A-allele was cut to fragments of 105 + 99 bp, while the T-allele remained uncut (194 bp).

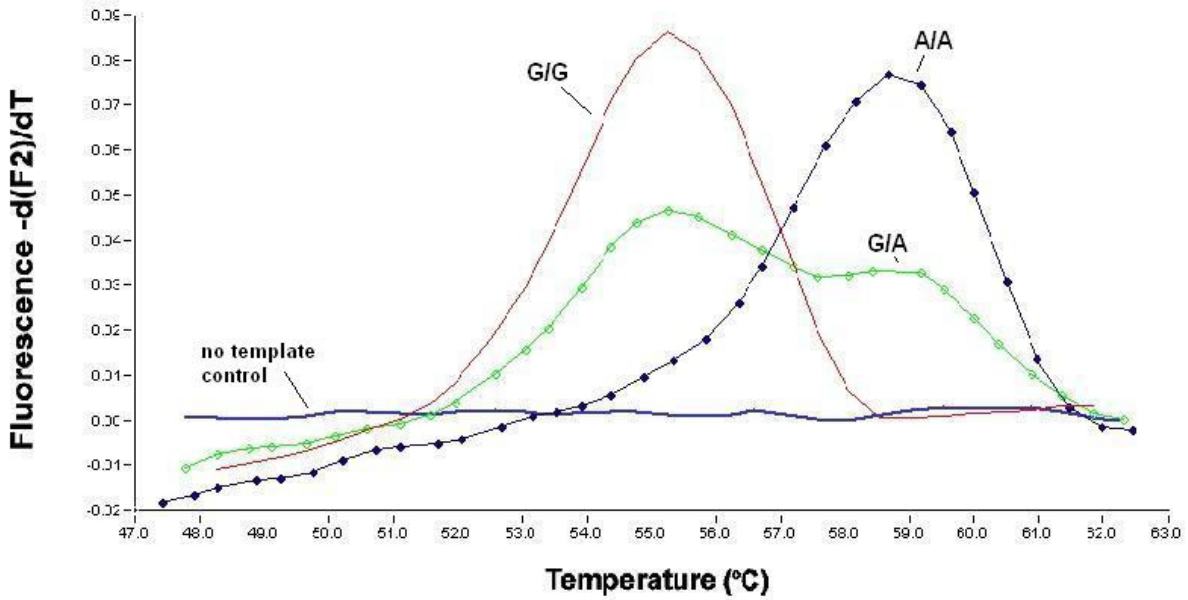

Figure 19: 5-HTR2A rs7997012 melting curves

Melting curve plots negative derivative of fluorescence with respect to the temperature (-dF/dT) vs. temperature (for principle see § 2.2.5.2). Note: fluorescence detection of the different 5-HTR2A rs7997012 G/A genotypes at 640 nm as follows: wildtype homozygote G/G - one peak at 55.5°C, variant homozygote A/A - one peak at 59°C, heterozygote G/A – two peaks at 55.5 and 59°C, respectively. No fluorescence peak observed for no-template control.

2.2.7.10 5-HTR2A T102C

A published RFLP assay [279] for the silent T102C (rs6313) polymorphism in the 5-HTR2A gene was adapted to the equipment at hand. The assay was carried out as described in § 2.2.7.2 and specified in Table 12. The sequences of the primers used as well as their locations in the reference sequence are given in appendix A3 (see Table 67). The theoretical length of the PCR product obtained by the adapted primers was checked by BLAST search. According to the reference nucleotide sequences available from the NCBI database, the PCR product and one of the digested fragments was found to be 30 bp shorter than published. This was confirmed by the result of the gel electrophoresis (see Figure 20).

Table 12: 5-HTR2A T102C RFLP assay procedure

Temperature Program			
94°C/3 min; 40 x (94°C/30 s, 55°C/30 s, 72°C/30 s); 72°C/3 min			
Reagents	Amount (µL)	Concentration	Final concentration[1]
H_2O, sterile	8.1		
HT_{2A} T102C F (primer)	0.3	25 µM	375 nM
HT_{2A} T102C R (primer)	0.3	25 µM	375 nM
Premix D	10.0	2 x	1x (1.5 mM $MgCl_2$)
Taq-Polymerase Qbiogene	0.3	5.0 U/µL	1.5 U
DNA	1.0		

[1] related to total volume of 20 µL (19 µL stock solution and 1 µL DNA)

The 342 bp PCR product was digested with the MspI enzyme with the recognition site C / C G G (SNP framed, cf. Table 4 in § 2.2.4.3). The C-allele was cut to fragments of 217 + 125 bp, while the T-allele remained uncut (342 bp). No interfering unspecific PCR products were observed for the PCR product, the digested samples or the no-template control (see Figure 20).

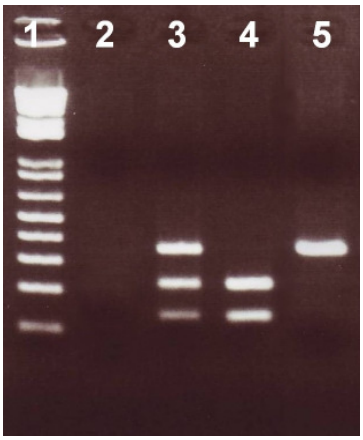

Figure 20: 5-HTR2A T102C gel electrophoresis

RFLP with MspI:
Lane 1: length marker (100 bp DNA ladder)
Lane 2: no-template control
Lane 3: heterozygous C/T (217 + 125 bp / 342 bp)
Lane 3: homozygous C/C (217 + 125 bp)
Lane 4: homozygous T/T (342 bp)

For electrophoresis, 7.5 µL of the PCR product was mixed with 1 µL loading buffer and analyzed on a 2% agarose gel using a 100 bp DNA-ladder (20 min at 100 V).

2.2.7.11 5-HTR2C C-759T

The functional C-759T (rs3813929) polymorphism lies near the major transcription initiation site in the promoter of the 5-HTR2C receptor gene, which is located on the X-chromosome. A new fluorescence-based LightCycler hybridization probes assay was developed and carried out as described in § 2.2.7.2 and specified in Table 13. The sequences of the primers and probes used as well as their locations in the reference sequence are given in appendix A3 (see Table 67). The primers used for the hybridization probes assay were adapted from a published literature RFLP method [139] and allowed also a RFLP analysis of the 252 bp PCR product with AciI (cf. Table 4 in § 2.2.4.3). The

C-allele was cut to fragments of 126 + 34 + 27 + 54 + 11 bp, while the T-allele was cut to 160 + 27 + 54 + 11 bp.

Table 13: 5-HTR2C C-759T assay procedure

Temperature Program			
95°C/60 s; 40 x (95°C/3 s, 57°C/10 s, 72°C/10 s); 40°C → 75°C @ 0.1°C/s			
Reagents	Amount (µL)	Concentration	Final concentration[1]
H_2O, sterile	7.5		
$MgCl_2$	0.6	25 mM	2.5 mM[2]
HT_{2C} -759 F (primer)	0.1	25 µM	250 nM
HT_{2C} -759 R (primer)	0.1	25 µM	250 nM
HT_{2C} -759 sen (probe)	0.1	10 µM	100 nM
HT_{2C} -759 anc (probe)	0.1	10 µM	100 nM
LC DNA Master HybProbe	1.0	10x	1x
Genomic DNA	0.5		
FRET Detection (640 nm) of the 252 bp product			
Wildtype peak (-759 C) 53°C Mutation peak (-759 T) 60°C			

[1] related to total volume of 10 µL (9.5 µL stock solution and 0.5 µL DNA)
[2] total $MgCl_2$-concentration of the LightCycler DNA Master HybProbe and the additional 25mM $MgCl_2$ -solution

The anchor probe was labeled with fluorescein (GCACCACGCTCTTGGGCCA-fluorescein, cf. Table 67), while the FRET partner, the sensor probe, was labeled with LCRed640 dye (LCRed640-AGC**A**GGATGAGGGGAGG- phosphate). The sensor probe is spanning the C-759T SNP and was designed to be completely complementary to the variant T-allele. Thus, the melting temperature of the wildtype C-allele was 53°C and that of the variant T-allele 60°C.The melting curves for the different genotypes are shown in Figure 21.

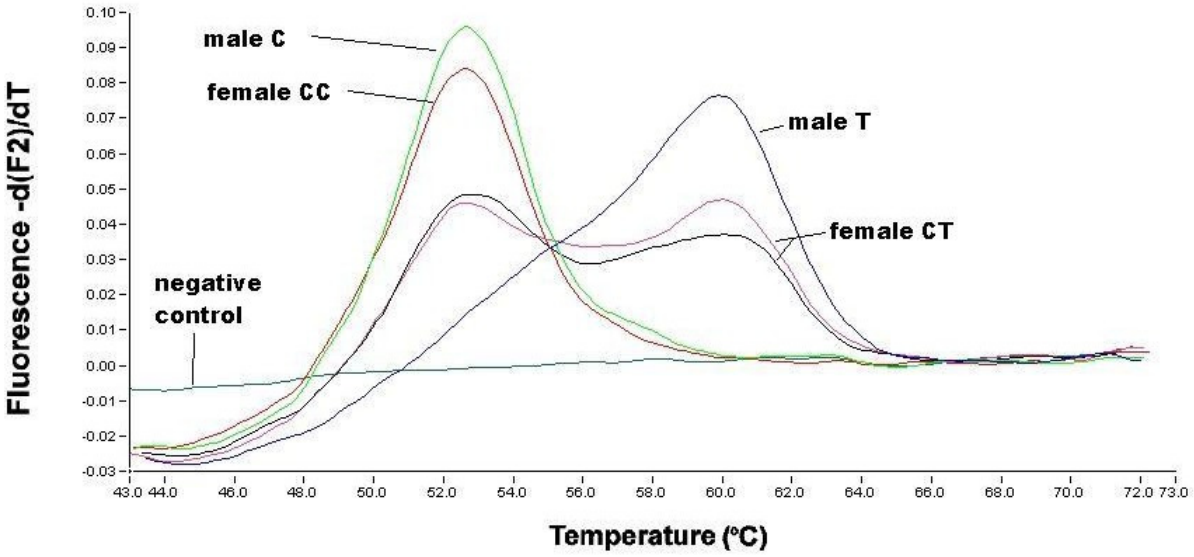

Figure 21: 5-HTR2C C-759T melting curves

Melting curve plots negative derivative of fluorescence with respect to the temperature (-dF/dT) vs. temperature (for principle see § 2.2.5.2). Note: fluorescence detection of the different 5-HTR2C C-759T genotypes at 640 nm as follows (X-chromosomal inheritance): female homozygote C/C or male hemizygote C - one peak at 53°C, female homozygote T/T (not shown) or male hemizygote T - one peak at 60°C, female heterozygote C/T – two peaks at 53 and 60°C, respectively. No fluorescence peak observed for no-template (=negative) control.

2.2.7.12 5-HTR6 T267C

A silent T to C polymorphism at position 267 lies within the first exon of 5-HTR6 (rs1805054). A published RFLP assay [152] was adapted to the equipment at hand. The assay was carried out as described in § 2.2.7.2 and specified in Table 14. The sequences of the primers used as well as their locations in the reference sequence are given in appendix A3 (see Table 67).

Table 14: 5-HTR6 T267C assay procedure

Temperature Program			
94°C/3 min; 30 x (94°C/30 s, 60°C/30 s, 72°C/30 s); 72°C/3 min			
Reagents	Amount (µL)	Concentration	Final concentration[1]
H_2O, sterile	8.1		
HT_6 T267C F (primer)	0.3	25 µM	375 nM
HT_6 T267C R (primer)	0.3	25 µM	375 nM
Premix D	10.0	2x	1x (1.5 mM $MgCl_2$)
Taq-Polymerase Qbiogene	0.3	5.0 U/µL	1.5 U
DNA	1.0		

[1] related to total volume of 20 µL (19 µL stock solution and 1 µL DNA)

The 578 bp PCR product was digested with RsaI (cf. Table 4 in § 2.2.4.3). The RsaI enzyme cuts specifically the C-allele with the recognition site G T / A C (SNP framed) to fragments of 129 + 449 bp, while the T-allele remained uncut (578 bp). No interfering unspecific PCR products were observed for the PCR product, the digested samples or the no-template control. Primer dimers were visible as very weak, diffuse fragments < 100 bp especially in the no-template control but did not interfere with the determination of genotypes (see Figure 22).

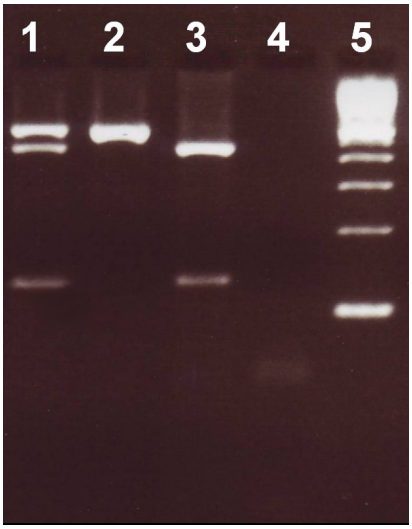

Figure 22: 5-HTR6 T267C gel electrophoresis

RFLP with RsaI:
Lane 1: heterozygous C/T (129 + 449 bp / 578 bp)
Lane 2: homozygous T/T (578 bp)
Lane 3: homozygous C/C (129 + 449 bp)
Lane 4: no-template control
Lane 5: length marker (100 bp DNA ladder)

For electrophoresis, 9 µL of the PCR product was mixed with 1 µL loading buffer and analyzed on a 2.5% agarose gel using a 100 bp DNA-ladder (30 min at 100 V).

2.2.7.13 DAT1 VNTR

The DAT1 VNTR polymorphism (rs28363170) lies in the 3'-untranslated region of the dopamine transporter gene SLC6A3. Analysis of the 40 bp repeat element revealed variable numbers of the repeats ranging from 3 to 11 copies. Most frequent copy numbers were 9 or 10 repeats [295]. A published thermocycler assay [296] was adapted to the equipment at hand. The assay was carried out as described in § 2.2.7.2 and specified in Table 15. The sequences of the primers used as well as their locations in the reference sequence are given in appendix A3 (see Table 67).

Table 15: DAT1 VNTR assay procedure

Temperature Program			
94°C/3 min; 40 x (94°C/30 s, 68°C/30 s, 72°C/45 s); 72/3 min			
Reagents	Amount (µL)	Concentration	Final concentration[1]
H_2O, sterile	8.6		
DAT1 VNTR F (primer)	0.3	25 µM	375 nM
DAT1 VNTR R (primer)	0.3	25 µM	375 nM
Premix D 2x	10.0	2x	1x (1.5 mM $MgCl_2$)
Taq-Polymerase	0.3	5 U/µL	1.5 U
Genomic DNA	0.5		

[1] related to total volume of 20 µL (19.5 µL stock solution and 0.5 µL DNA)

The published forward primer was corrected to completely match the NCBI reference sequence (TGCGGTGTAGGGAACGGCCTGAGA instead of TGTGGTGTAGGGAACGGCCTGAGA). The theoretical product sizes for the different 40 bp repeat copy numbers were checked by BLAST search and confirmed by the result of the gel electrophoresis (see Figure 23). In the study population, only 9 repeats (443 bp), 10 repeats (483 bp) or 11 repeats (523 bp) were present. No interfering unspecific PCR products were observed for the PCR product or the no-template control (see Figure 23).

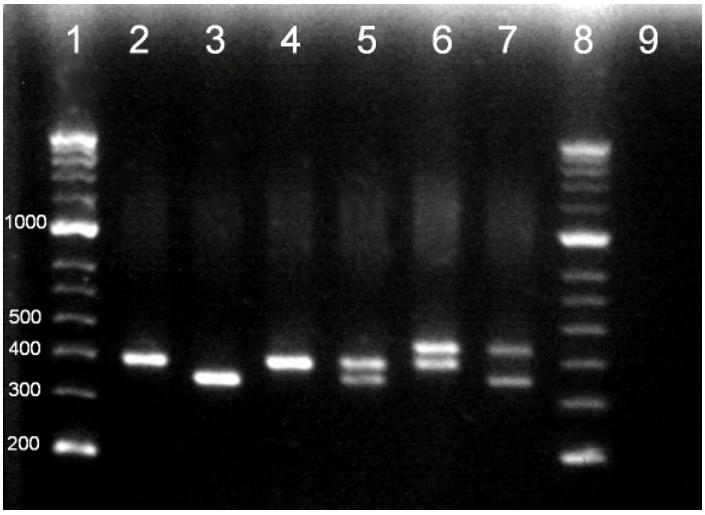

Figure 23: DAT1 VNTR gel electrophoresis

Lane 1/8: length marker (DNA ladder)
Lane 2/4: 10/10 repeats (483 bp)
Lane 3: 9/9 repeats (443 bp)
Lane 5: 9/10 repeats (443 / 483 bp)
Lane 6: 10/11 repeats (483 / 523 bp)
Lane 7: 9/11 repeats (443 / 523 bp)
Lane 9: no-template control

For electrophoresis, 7 µL of the PCR product was mixed with 1 µL loading buffer and analyzed on a 2% agarose gel (45 min at 100 V).

2.2.7.14 NET G1287A

A published TaqMan assay [281] for the NET G1287A (rs5569) polymorphism was adapted to the equipment at hand as specified in § 2.2.7.2 and in Table 16. The sequences of the primers and probes used as well as their locations in the reference sequence are given in appendix A3 (see Table 68).

Table 16: NET G1287A assay procedure

Temperature Program			
95°C/10 min; 60 x (95°C/3 s, 60°C/20 s); 40°C/0 s			
Reagents	Amount (µL)	Concentration	Final concentration[1]
H_2O, sterile	6.0		
NET G1287A F2 (primer)	0.2	25 µM	500 nM
NET G1287A R2 (primer)	0.2	25 µM	500 nM
NET Taq G1287 (probe)	0.1	10 µM	100 nM
NET Taq 1287A (probe)	0.1	10 µM	100 nM
LightCycler TaqMan Master	2.0	5 x	1 x[2]
Genomic DNA	1.0		
Fluorescence Detection of the 75 bp product			
Wildtype (1287G) at 530 nm Mutation (1287A) at 560 nm			

[1] related to total volume of 10 µL (9.0 µL stock solution and 1.0 µL DNA)
[2] exact composition and $MgCl_2$-concentration of the LightCycler TaqMan Master not given by the manufacturer

For the fluorescence detection of the G1287A SNP, two allele-specific TaqMan probes were used within the same PCR (single-tube approach). The wildtype TaqMan probe complementary to the G-allele was labeled with fluorescein as reporter dye (FAM-CAGGCC**C**GTGATGA-BHQ1) and detected at 530 nm (see Figure 24). The variant probe complementary to the A-allele was labeled with hexachlorofluorescein (HEX-AGGCC**T**GTGATGACA-BHQ1) and detected at 560 nm (see Figure 25). Both probes were additionally labeled with a non-fluorescent quencher at the 3´end to minimize the background fluorescence and maximize the sensitivity. Moreover, color-compensation was used to correct for the fluorophore emission overlap in each channel for each fluorophore (see § 2.2.5.4) to optimize the assay performance.

The method was checked by RFLP digestion. Published primers were used [280] and are given in appendix A3. The 241 bp PCR product was digested with Sau96I (cf. Table 4 in § 2.2.4.3). The G-allele was cut to fragments of 76 + 21+ 113 + 31 bp, while the A-allele was cut to 97 + 113 + 31 bp. No interfering unspecific PCR products were observed for the PCR product, the digested samples or the no-template control (see Figure 26). The G-allele was identified by the 76 bp band and the A-allele by the 97 bp band. The constant 113 bp band had to be visible for all genotypes and was used to check for completeness of the digestion. The 21 bp and 31 bp fragments were only visible as very diffuse, weak bands below the 50 bp marker together with the primer dimers (see Figure 26).

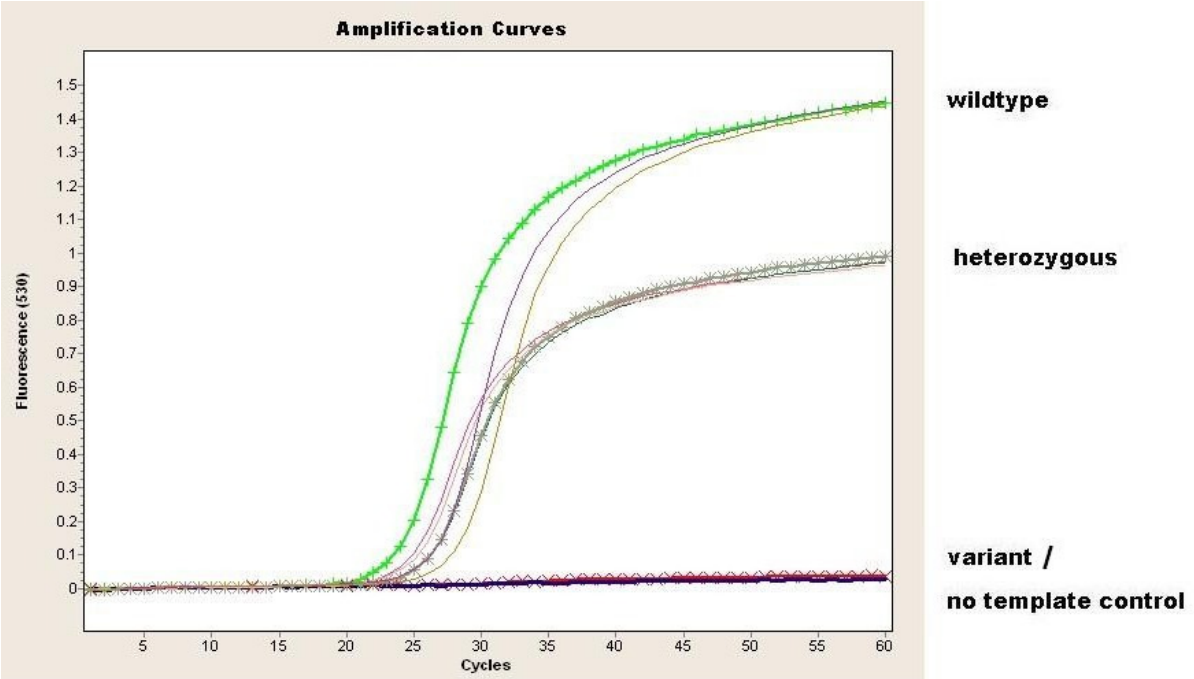

Figure 24: NET G1287A amplification curves at 530 nm

Amplification curve plots increase of fluorescence vs. cycle number (for principle see § 2.2.5.3). Note: fluorescence detection of the G-allele of the G1287A SNP in the NET gene at 530 nm as follows: increase of fluorescence with increasing PCR product during amplification of the wildtype homozygote G/G and – less intensive – for the G/A heterozygote. No increase of fluorescence for no-template control and for the variant homozygote A/A.

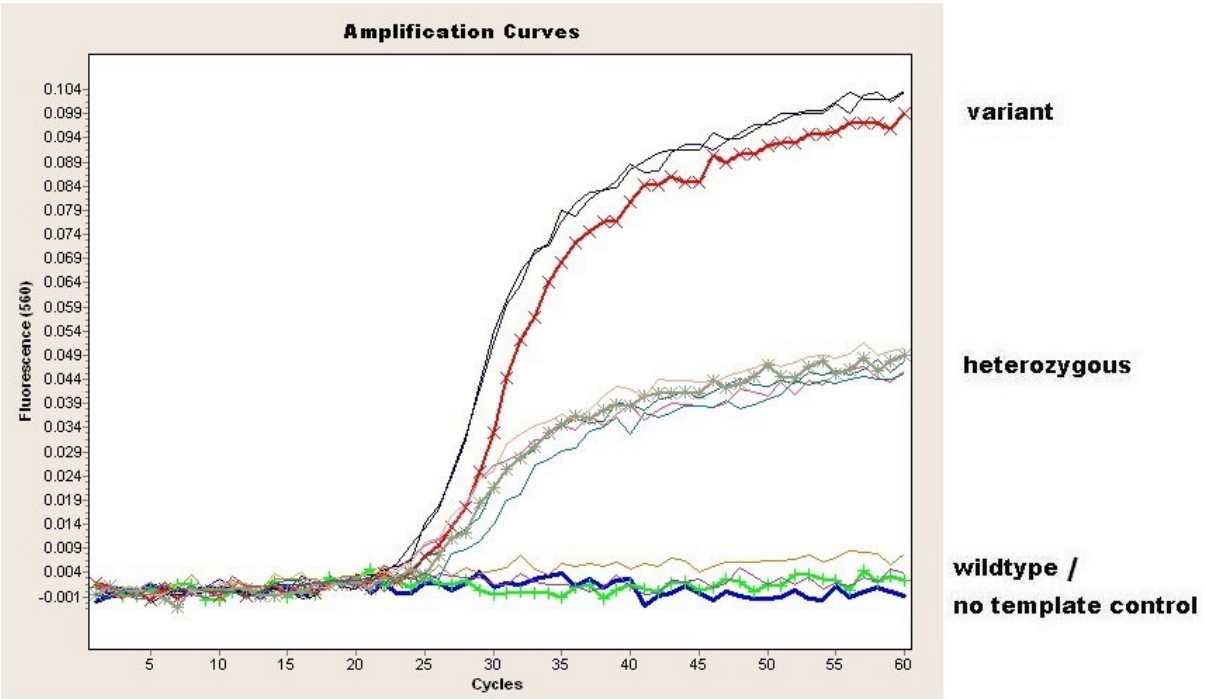

Figure 25: NET G1287A amplification curves at 560 nm

Amplification curve plots increase of fluorescence vs. cycle number (for principle see § 2.2.5.3). Note: fluorescence detection of the A-allele of the G1287A SNP in the NET gene at 560 nm as follows: increase of fluorescence with increasing PCR product during amplification of the variant homozygote A/A and – less intensive – for the G/A heterozygote. No increase of fluorescence for no-template control and for the wildtype homozygote G/G.

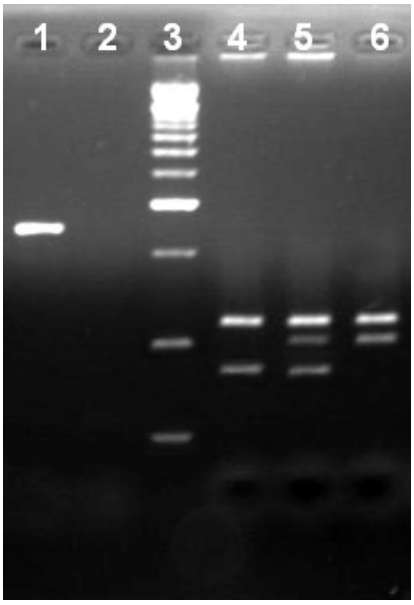

Figure 26: NET G1287A gel electrophoresis

RFLP with Sau96I:
Lane 1: PCR product (241 bp)
Lane 2: no-template control
Lane 3: length marker (100 bp DNA ladder with additional 50 bp marker)
Lane 4-6: Constant band at 113 bp
Lane 4: homozygous G/G (76 bp)
Lane 5: heterozygous G/A (76 bp / 97 bp)
Lane 6: homozygous A/A (97 bp)

For electrophoresis, 9 µL of the PCR product was mixed with 1 µL loading buffer and analyzed on a 3% agarose gel (35 min at 100 V).

2.2.7.15 NET T-182C

A published LightCycler hybridization probes assay [280] for the NET T-182C (rs2242446) polymorphism was adapted to the equipment at hand as specified in § 2.2.7.2 and in Table 17. The sequences of the primers and probes used as well as their locations in the reference sequence are given in appendix A3 (see Table 68).

Table 17: NET T-182C assay procedure

Temperature Program			
95°C/10 min; 70 x (95°C/0 s, 60°C/10 s, 72°C/10 s); 55°C → 80°C @ 0.1°C/s			
Reagents	Amount (µL)	Concentration	Final concentration[1]
H_2O, sterile	6.0		
$MgCl_2$	0.6	25 mM	2.5 mM[2]
NET T182C F (primer)	0.2	25 µM	500 nM
NET T182C R (primer)	0.2	25 µM	500 nM
NET T182C sen (probe)	0.5	3 µM	150 nM
NET T182C anc (probe)	0.5	3 µM	150 nM
LC Fast Start DNA Master HybProbe	1.0	10 x	1 x
Genomic DNA	1.0		
FRET Detection (640 nm) of the 257 bp product			
Wildtype peak (-182T) 70.5°C Mutation peak (-182C) 67°C			

[1] related to total volume of 10 µL (9.0 µL stock solution and 1.0 µL DNA)
[2] total $MgCl_2$-concentration of the LC Fast Start DNA Master HybProbe and the additional 25mM $MgCl_2$ -solution

The sensor probe spanning the T-182C SNP was designed to be completely complementary to the antisense strand of the variant T-allele. It was labeled with fluorescein (GACGCGCGC**T**CTTTTCTGGGA-fluorescein, cf. appendix A3), while the FRET partner, the anchor probe, was labeled with LCRed640 dye (LCRed640-CCCTGCGTCCGCTCAGCGCGCGCTCATCCC-phosphate). Thus, the melting temperature of the wildtype T-allele was 70.5°C and that of the variant C-allele 67°C. The melting curves for the different genotypes are shown in Figure 27.

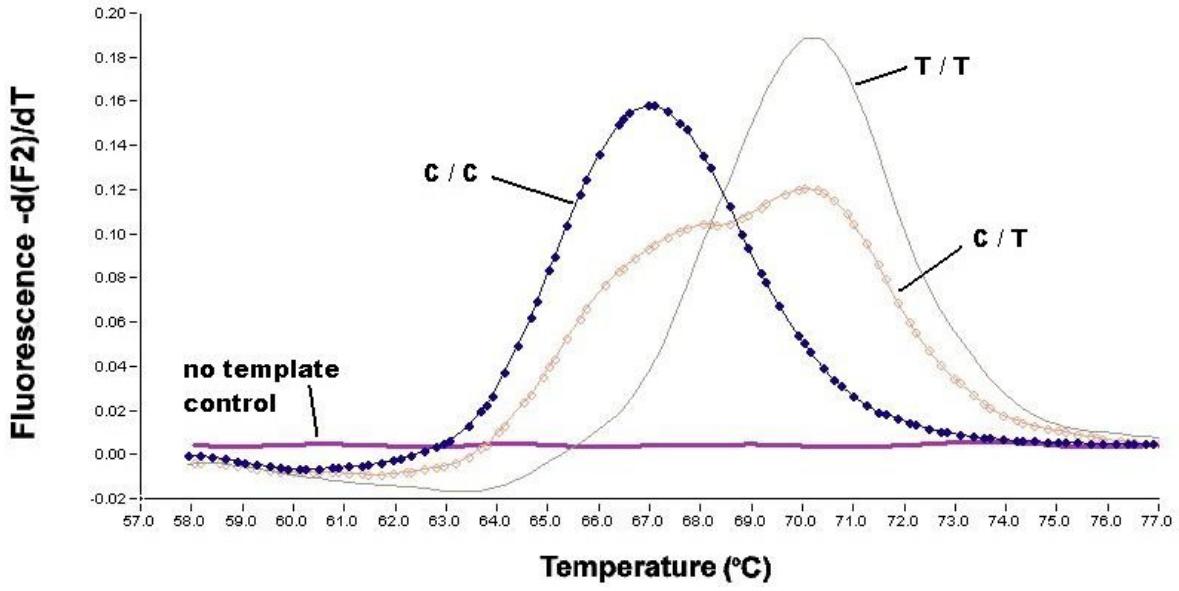

Figure 27: NET T-182C melting curves

Melting curve plots negative derivative of fluorescence with respect to the temperature (-dF/dT) vs. temperature (for principle see § 2.2.5.2). Note: fluorescence detection of the different NET T-182C genotypes at 640 nm as follows: homozygote T/T - one peak at 70.5°C, homozygote C/C - one peak at 67°C, heterozygote T/C - two peaks at 70.5 and 67°C, respectively. No fluorescence peak observed for no-template control.

The primers chosen also allowed a (subsequent) RFLP analysis for control and validation of assay performance. For this purpose, the 257 bp PCR product was digested with BslI (cf. Table 4 in § 2.2.4.3). The C-allele was cut to fragments of 40 + 85 + 132 bp, while the T-allele was cut to 40 + 217 bp. No interfering unspecific PCR products were observed for the PCR product, the digested samples or the no-template control (see Figure 28). The C-allele was identified by the 132 bp band and the T-allele by the 217 bp band. The 40 and 85 bp fragments were only visible as relatively weak and diffuse bands (see Figure 28).

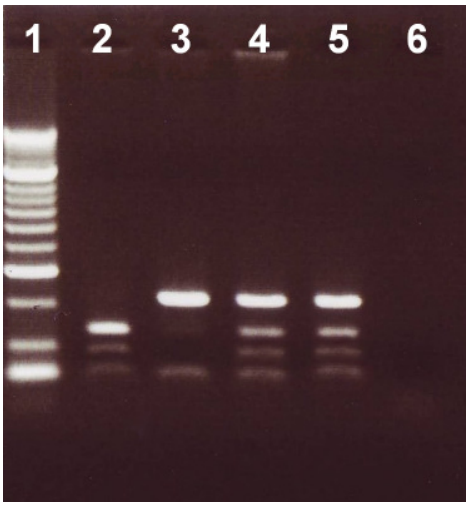

Figure 28: NET T-182C gel electrophoresis

RFLP with BslI

Lane 1: length marker (100 bp DNA ladder with additional 50 bp lane)

Lane 2 - 5: constant band at 40 bp

Lane 2: homozygous C/C (85 + 132 bp)

Lane 3: homozygous T/T (217 bp)

Lane 4/5: heterozygous C/T (85 + 132 bp/ 217 bp)

Lane 6: no-template control

For electrophoresis, 9 µL of the PCR product was mixed with 1 µL loading buffer and analyzed on a 2% agarose gel (25 min at 100 V).

2.2.7.16 ADR2A C-1291G

A published TaqMan assay [282] for the ADR2A C-1291G (rs1800544) polymorphism was adapted to the equipment at hand as specified in § 2.2.7.2 and in Table 18. The sequences of the primers and probes used as well as their locations in the reference sequence are given in appendix A3 (see Table 68).

Table 18: ADR2A C-1291G assay procedure

Temperature Program			
95°C/10 min; 40 x (95°C/3 s, 60°C/20 s); 40°C/0 s			
Reagents	Amount (µL)	Concentration	Final concentration[1]
H_2O, sterile	6.4		
A_{2A} -1291 F (primer)	0.2	25 µM	500 nM
A_{2A} -1291 R (primer)	0.2	25 µM	500 nM
A_{2A} -1291 C (probe)	0.1	10 µM	100 nM
A_{2A} -1291 G (probe)	0.1	10 µM	100 nM
LightCycler TaqMan Master	2.0	5 x	1 x[2]
Genomic DNA	1.0		
Fluorescence Detection of the 51 bp product			
Wildtype peak (-1291C) at 530 nm Mutation peak (-1291G) at 560 nm			

[1] related to total volume of 10 µL (9 µL stock solution and 1 µL DNA)
[2] exact composition and $MgCl_2$-concentration of the LightCycler TaqMan Master not given by the manufacturer

For the fluorescence detection of the C-1291G SNP, two allele-specific TaqMan probes were used within the same PCR (single-tube approach). The TaqMan wildtype probe is complementary to the antisense strand of the C-allele. It was labeled with fluorescein as reporter dye (FAM-CCGTCGGCCC**C**GAG-BHQ1) and was detected at 530 nm (see Figure 29). The TaqMan variant probe is complementary to the antisense strand of the G-allele. It was labeled with hexachlorofluorescein (HEX-CCGTCGGCCC**G**GAG-BHQ1) and was detected at 560 nm (see Figure 30). Both probes were additionally labeled with a non-fluorescent quencher at the 3´end to minimize the background fluorescence and maximize the sensitivity. Moreover, color-compensation was used to correct for the fluorophore emission overlap in each channel for each fluorophore (see § 2.2.5.4) to optimize the assay performance.

The method was validated by RFLP digestion. The primers used are given in Table 68. The 236 bp PCR product was digested with MspI (cf. Table 4 in § 2.2.4.3). The G-allele was cut to fragments of 4 + 120 + 55 + 57 bp, while the C-allele was cut to 4 + 175 + 57 bp. No interfering unspecific PCR products were observed for the PCR product, the digested samples or the no-template control (see Figure 31). The G-allele was identified by the 120 bp band and the C-allele by the 175 bp band. The 57 bp band visible for all genotypes overlapped with the 55 bp band of the G-allele. For the C/G heterozygotes, an approx. 100 bp heteroduplex formed by fragments of different lengths is visible as weak band (see Lane 3 in Figure 31).

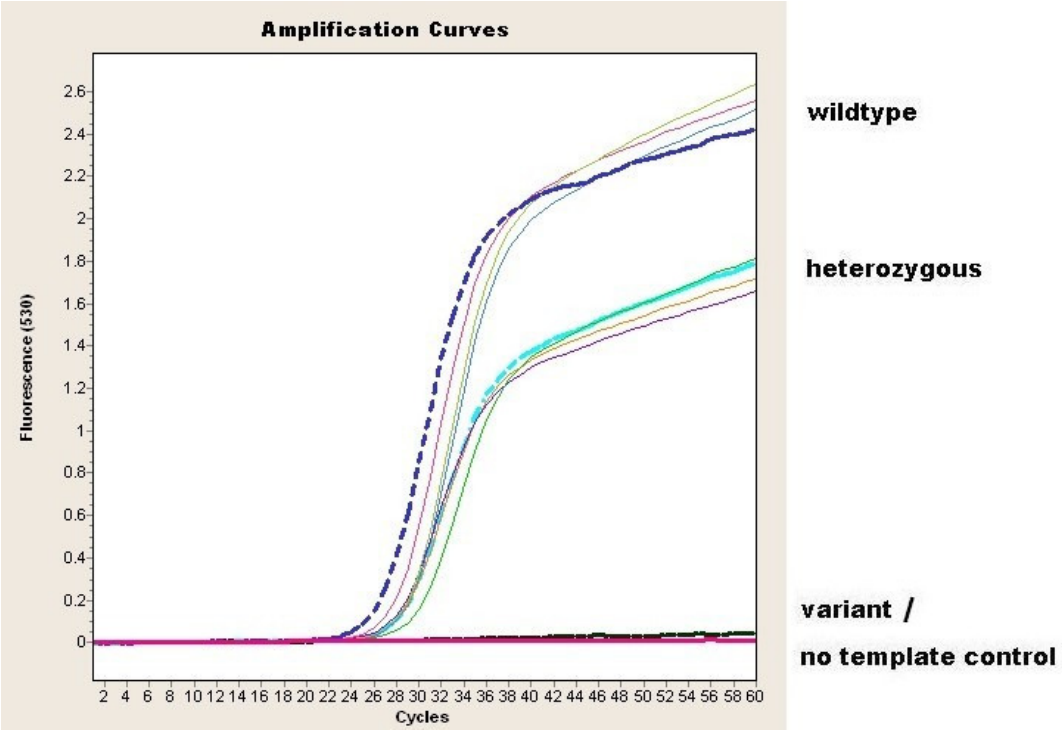

Figure 29: ADR2A C-1291G amplification curves at 530 nm

Amplification curve plots increase of fluorescence vs. cycle number (for principle see § 2.2.5.3). Note: fluorescence detection of the C-allele of the C-1291G SNP in the ADR2A gene at 530 nm as follows: increase of fluorescence with increasing PCR product during amplification of the wildtype homozygote C/C and – less intensive – for the C/G heterozygote. No increase of fluorescence for no-template control and for the variant homozygote G/G.

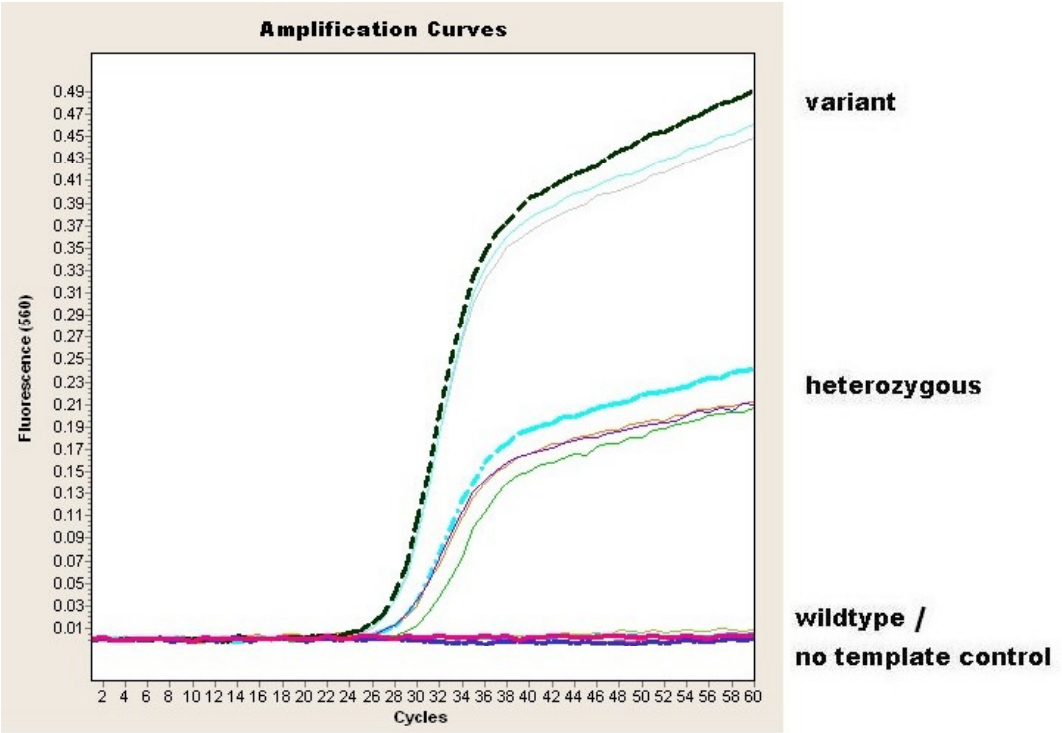

Figure 30: ADR2A C-1291G amplification curves at 560 nm

Amplification curve plots increase of fluorescence vs. cycle number (for principle see § 2.2.5.3). Note: fluorescence detection of the G-allele of the C-1291G SNP in the ADR2A gene at 560 nm as follows: increase of fluorescence with increasing PCR product during amplification of the variant homozygote G/G and – less intensive – for the C/G heterozygote. No increase of fluorescence for no-template control and for the wildtype homozygote C/C.

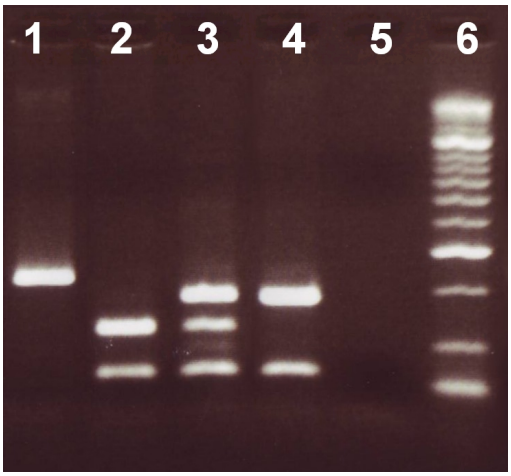

Figure 31: ADR2A C-1291G gel electrophoresis

RFLP with MspI:
Lane 1: PCR product (236 bp)
Lane 2 - 4: constant band at 57 bp
(overlaps with 55 bp band of G-allele)
Lane 2: homozygous G/G (120 bp)
Lane 3: heterozygous G/C (120 bp / 175 bp)
Lane 4: homozygous C/C (175 bp)
Lane 5: no-template control
Lane 6: 100 bp DNA ladder with additional 50 bp lane

For electrophoresis, 9 µL of the PCR product was mixed with 1 µL loading buffer and analyzed on a 3% agarose gel (25 min at 100 V).

2.2.7.17 BDNF Val66Met

The BDNF Val66Met polymorphism (rs6265) is a G to A change at nucleotide position 196, which results in an amino acid change at codon 66 of the BDNF precursor protein that is cleaved away, rendering the amino acid change absent from mature BDNF. A new fluorescence-based LightCycler hybridization probes assay was developed and carried out as described in § 2.2.7.2 and specified in Table 19. The sequences of the primers and probes used as well as their locations in the reference sequence are given in appendix A3 (see Table 70).

Table 19: BDNF Val66Met assay procedure

Temperature Program			
95°C/3 min; 40 x (95°C/3 s, 52°C/10 s, 72°C/10 s); 40°C → 67°C @ 0.1°C/s			
Reagents	Amount (µL)	Concentration	Final concentration[1]
H_2O, sterile	7.4		
$MgCl_2$	0.4	25 mM	2.0 mM[2]
BDNF F (primer)	0.2	25 µM	500 nM
BDNF R (primer)	0.2	25 µM	500 nM
BDNF anc (probe)	0.15	10 µM	150 nM
BDNF sen (probe)	0.15	10 µM	150 nM
LC DNA Master HybProbe	1.0	10 x	1x
Genomic DNA	0.5		
FRET Detection (640 nm) of the 177 bp product			
Wildtype peak (196G) 50.5°C Mutation peak (196A) 57°C			

[1] related to total volume of 10 µL (9.5 µL stock solution and 0.5 µL DNA)
[2] total $MgCl_2$-concentration of the LightCycler DNA Master HybProbe and the additional 25mM $MgCl_2$ -solution

The sensor probe spanning the G196A SNP was designed to be completely complementary to the antisense strand of the variant A-allele. It was labeled with fluorescein (AACAC**A**TGATAGAAGAGCTGT-fluorescein, cf. Table 70), while the FRET partner, the anchor probe, was labeled with LCRed640 dye (LCRed640-GATGAGGACCAGAAAGTTCGGCC-phosphate). Thus, the melting temperature of the wildtype G-allele was 50.5°C and that of the variant A-allele 57°C. The melting curves for the different genotypes are shown in Figure 32.

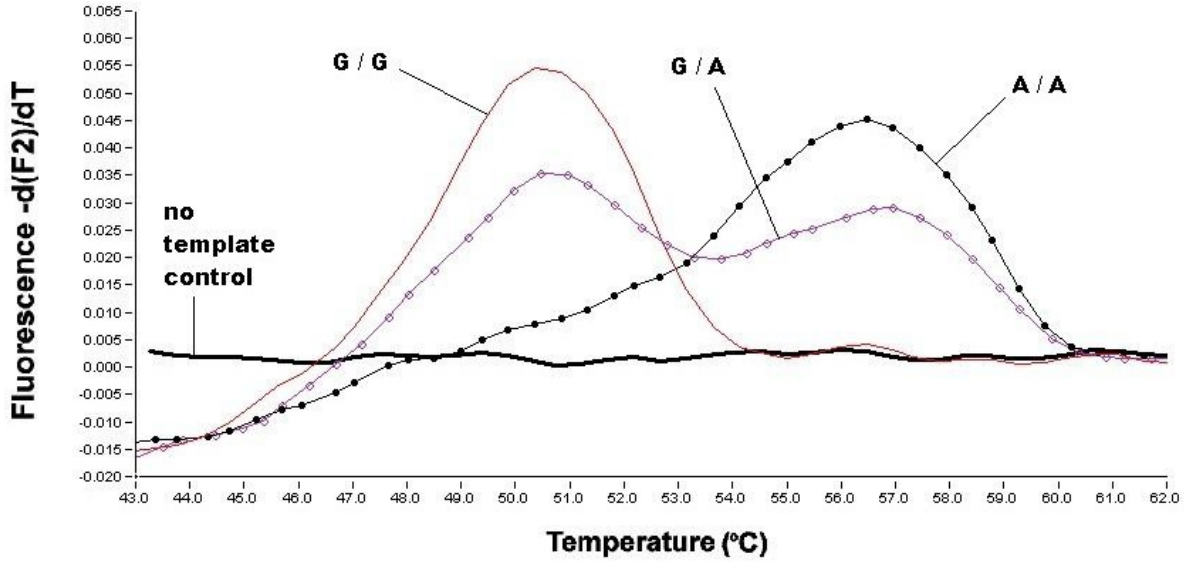

Figure 32: BDNF Val66Met melting curves

Melting curve plots negative derivative of fluorescence with respect to the temperature (-dF/dT) vs. temperature (for principle see § 2.2.5.2). Note: fluorescence detection of the different BDNF G196A genotypes at 640 nm as follows: homozygote G/G - one peak at 50.5°C, homozygote A/A - one peak at 57°C, heterozygote G/A - two peaks at 50.5 and 57°C, respectively. No fluorescence peak observed for no-template control.

The primers chosen also allowed a (subsequent) RFLP analysis for control and validation of assay performance. For this purpose, the 177 bp PCR product was digested with BsaAI (cf. Table 4 in § 2.2.4.3). The G-allele coding for valine was cut to fragments of 71 + 106 bp, while the A-allele coding for methionine remained uncut (177 bp). No interfering unspecific PCR products were observed for the PCR product, the digested samples or the no-template control (see Figure 33).

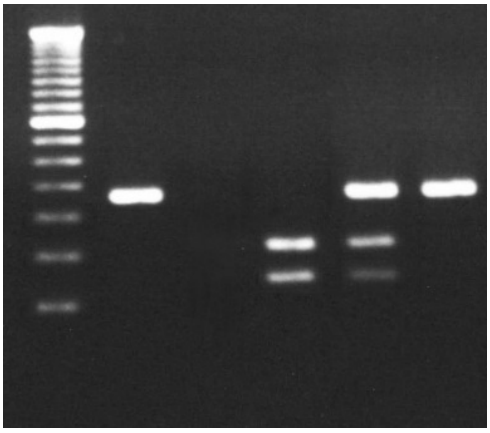

Figure 33: BDNF Val66Met gel electrophoresis

RFLP with BsaAI:
Lane 1: length marker (50 bp ladder)
Lane 2: PCR product (177 bp)
Lane 3: no-template control
Lane 4: homozygous G/ G = Val/Val (106 + 71 bp)
Lane 5: heterozygous G/ A = Val/Met (106 + 71 / 177 bp)
Lane 6: homozygous A/ A = Met/Met (177 bp uncut)

For electrophoresis, 9 µL of the PCR product was mixed with 1 µL loading buffer and analyzed on a 2% agarose gel using a 50 bp DNA-ladder (25 min at 100 V).

2.2.7.18 COMT Val158Met

The COMT Val158Met polymorphism (rs4680) is a G to A change at nucleotide position 472, which results in the substitution of the amino acid valine with methionine (at codon 158 for the membrane-bound form and at codon 108 for the soluble form) and a decrease in the dopamine catabolizing activity of the COMT enzyme. A new fluorescence-based LightCycler hybridization probes assay was developed and carried out as described in § 2.2.7.2 and specified in Table 20. The sequences of the primers and probes used as well as their locations in the reference sequence are given in appendix A3 (see Table 69).

Table 20: COMT Val158Met assay procedure

Temperature Program			
95°C/10 min; 50 x (95°C/3 s, 57°C/10 s, 72°C/10 s); 50°C → 65°C @ 0.1°C/			
Reagents	Amount (µL)	Concentration	Final concentration[1]
H_2O, sterile	4.0		
$MgCl_2$	0.2	25 mM	1.5 mM[2]
COMT F (primer)	0.15	25 µM	375 nM
COMT R (primer)	0.15	25 µM	375 nM
COMT anc3 (probe)	2.0	3 µM	600 nM
COMT sen3 (probe)	2.0	3 µM	600 nM
LC Fast Start DNA Master HybProbe	1.0	10 x	1.0x
Genomic DNA	0.5		
FRET Detection (640 nm) of the 177 bp product			
Wildtype peak (472G = 158Val) 59.5°C Mutation peak (472A = 158Met) 55.5°C			

[1] related to total volume of 10 µL (9.5 µL stock solution and 0.5 µL DNA)

[2] total $MgCl_2$-concentration of the LC Fast Start DNA Master HybProbe and the additional 25mM $MgCl_2$ -solution

The sensor probe spanning the G472A SNP was designed to be completely complementary to the wildtype G-allele. It was labeled with fluorescein (TCA**C**GCCAGCGAAATCCAC-fluorescein, cf. Table 69), while the FRET partner, the anchor probe, was labeled with LCRed640 dye (LCRed640-TCCGCTGGGTGATGGCGGC-phosphate). Thus, the melting temperature of the wildtype G-allele was 59.5°C and that of the variant A-allele 55.5°C. The melting curves for the different genotypes are shown in Figure 34.

The primers chosen also allowed a (subsequent) RFLP analysis for control and validation of assay performance. For this purpose, the 177 bp PCR product was digested with NlaIII (cf. Table 4 in § 2.2.4.3). The A-allele (Met) was cut to fragments of 96 + 33 + 31 + 18 bp, while the G-allele (Val) was cut to 114 + 33 + 31 bp. The G-allele was identified by the 114 bp band and the C-allele by the 96 bp band. No interfering unspecific PCR products were observed for the PCR product or the no-template control (see Figure 35). After digestion, the 31 bp and 33 bp DNA fragments were visible as diffuse bands < 50 bp (see Lane 4 to 6 in Figure 35), whereas the primers and 18 bp fragments were barely visible.

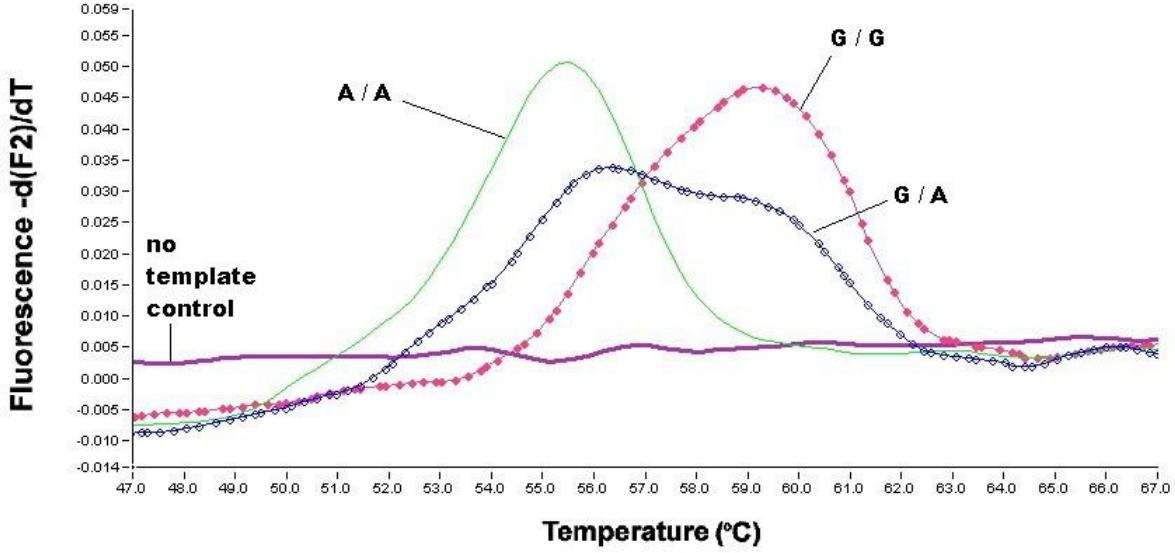

Figure 34: COMT Val158Met melting curves

Melting curve plots negative derivative of fluorescence with respect to the temperature (-dF/dT) vs. temperature (for principle see § 2.2.5.2). Note: fluorescence detection of the different COMT G472A genotypes at 640 nm as follows: homozygote G/G - one peak at 59.5°C, homozygote A/A - one peak at 55.5°C, heterozygote G/A – two peaks at 59.5 and 55.5°C, respectively. No fluorescence peak observed for no-template control.

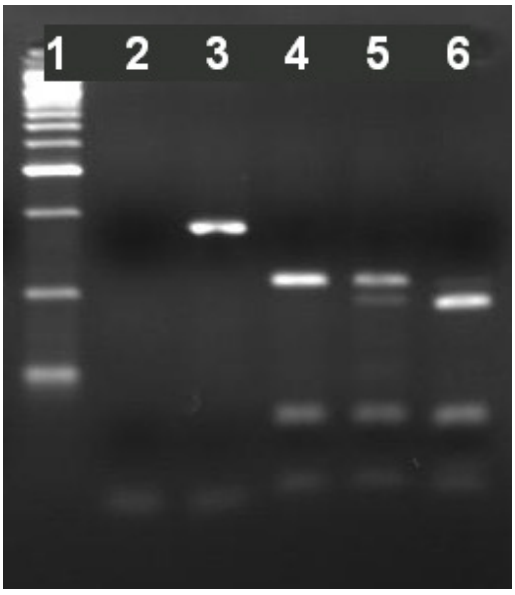

Figure 35: COMT Val158Met gel electrophoresis

RFLP with NlaIII:

Lane 1: length marker (50 bp ladder)

Lane 2: no-template control

Lane 3: PCR product (177 bp)

Lane 4: homozygous G/G = Val/Val (114 bp)

Lane 5: heterozygous G/A = Val/Met (96 / 114 bp)

Lane 6: homozygous A/A = Met/Met (96 bp)

Lane 4 - 6: 31 / 33 bp fragments

For electrophoresis, 9 µL of the PCR product was mixed with 1 µL loading buffer and analyzed on a 2% agarose gel using a 50 bp DNA-ladder (25 min at 100 V).

2.2.7.19 FKBP5 rs1360780

The SNP rs1360780 resides in intron 2 of the FKBP5 gene. A new fluorescence-based LightCycler hybridization probes assay was developed and carried out as described in § 2.2.7.2 and specified in Table 21. The sequences of the primers and probes used as well as their locations in the reference sequence are given in appendix A3 (see Table 70).

Table 21: FKBP5 rs1360780 assay procedure

Temperature Program			
95°C/60 s; 40 x (95°C/10 s, 48°C/10 s, 72°C/10 s); 40°C → 75°C @ 0.1°C/s			
Reagents	Amount (µL)	Concentration	Final concentration[1]
H_2O, sterile	6.4		
$MgCl_2$	0.8	25 mM	3.0 mM[2]
FKBP5 F2 (primer)	0.25	25 µM	625 nM
FKBP5 R2 (primer)	0.25	25 µM	625 nM
FKBP5 sen2 (probe)	0.15	10 µM	150 nM
FKBP5 anc2 (probe)	0.15	10 µM	150 nM
LC DNA Master HybProbe	1.0	10x	1 x
Genomic DNA	1.0		
FRET Detection (640 nm) of the 197 bp product: Wildtype peak (rs1360780 T-allele) 53°C Mutation peak (rs1360780 C-allele) 60°C			

[1] related to total volume of 10 µL (9 µL stock solution and 1 µL DNA)
[2] total $MgCl_2$-concentration of the LC DNA Master HybProbe and the additional 25mM $MgCl_2$ –solution

The anchor probe of the rs1360780 T/C SNP was labeled with fluorescein (AGATCCAGGCACAGAAGG-fluorescein, cf. Table 70), while the FRET partner, the sensor probe, was labeled with LCRed640 dye (LCRed640-TTCACATAAGCAAAGTTA**C**ACAAA--phosphate). The sensor probe spanning the SNP was completely complementary to the antisense strand of the variant C-allele. The melting peaks derived at 640 nm were 53°C for the wildtype T-allele and 60°C for the variant C-allele. The melting curves for the different genotypes are shown in Figure 36.

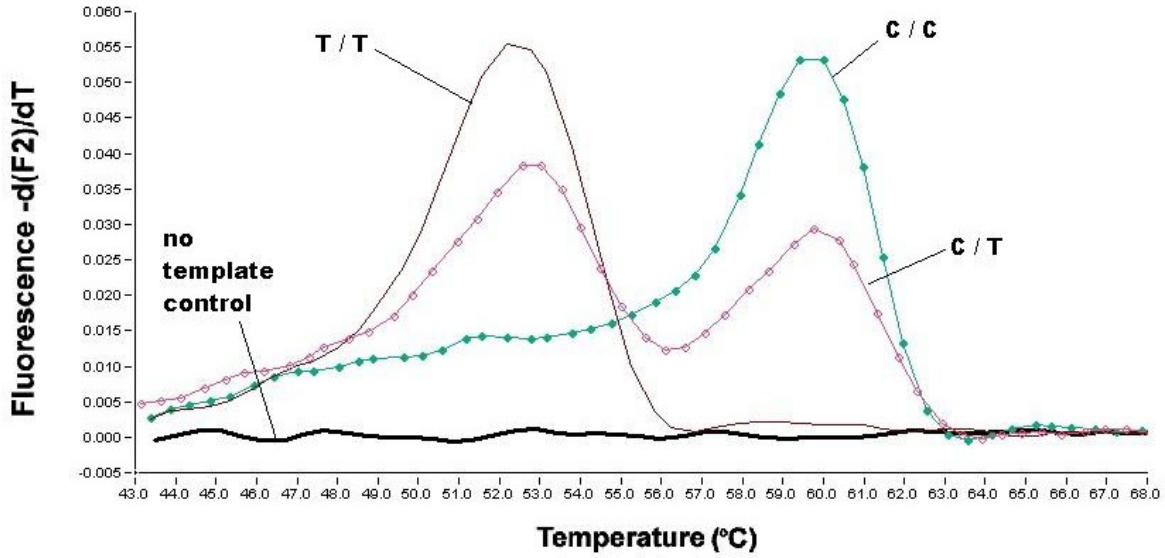

Figure 36: FKBP5 rs1360780 melting curves

Melting curve plots negative derivative of fluorescence with respect to the temperature (-dF/dT) vs. temperature (for principle see § 2.2.5.2). Note: fluorescence detection of the different FKBP5 T/C genotypes at 640 nm as follows: homozygote T/T - one peak at 53°C, homozygote C/C - one peak at 60°C, heterozygote T/C – two peaks at 53 and 60°C, respectively. No fluorescence peak observed for no-template control.

The primers chosen also allowed a (subsequent) RFLP analysis for control and validation of assay performance. For this purpose, the 197 bp PCR product was digested with MaeIII (cf. Table 4 in § 2.2.4.3). The C-allele was cut to fragments of 96 + 101 bp, while the T-allele remained uncut (197 bp). No interfering unspecific PCR products were observed for the PCR product or the no-template control. Primers dimers with a length of approx. 50 bp, however, were visible as diffuse

bands (see Figure 37). The T-allele was identified by the 197 bp band and the C-allele by a band containing the 96 + 101 bp fragments (see Figure 37).

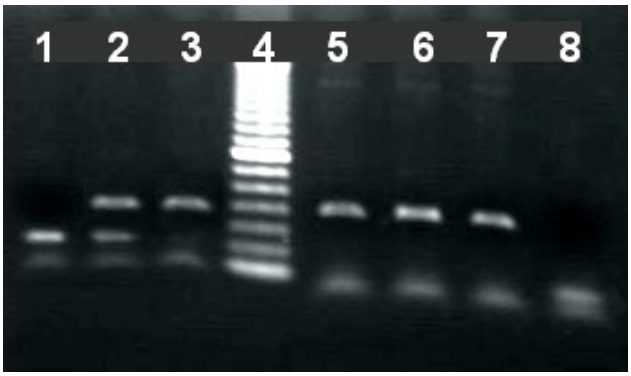

Figure 37: FKBP5 rs1360780 gel electrophoresis

RFLP with MaeIII:

Lane 1: homozygous C/C (96 + 101 bp)

Lane 2: heterozygous C/T (96 + 101 / 197 bp)

Lane 3: homozygous T/T (197 bp)

Lane 4: length marker (50 bp ladder)

Lane 5-7: PCR product

Lane 8: no-template control

For electrophoresis, 9 µL of the PCR product was mixed with 1 µL loading buffer and analyzed on a 1.5% agarose gel (15 min at 100 V).

2.2.7.20 GNB3 C825T

The GNB3 C825T polymorphism (rs5443) is a C to T change at coding position 825 in exon 10 of the G-protein subunit b3 gene and results in alternative splicing and increased signal transduction. A RFLP assay similar to a published one [222] was performed. To avoid a second cut position leading to a 13 bp fragment, the reverse primer was moved upstream. The forward primer was changed to have a suitable T_m compared to the new reverse primer. The sequences of the primers used as well as their locations in the reference sequence are given in appendix A3 (see Table 70). The assay was carried out as described in § 2.2.7.2 and specified in Table 22.

Table 22: GNB3 C825T assay procedure

Temperature Program			
94°C/3 min; 35 x (94°C/30 s, 50°C/30 s, 72°C/30 s); 72°C/7 min			
Reagents	Amount (µL)	Concentration	Final concentration[1]
H_2O, sterile	7.8		
GNB3 C825T F (primer)	0.4	25 µM	500 nM
GNB3 C825T R (primer)	0.4	25 µM	500 nM
Premix J	10.0	2x	1x (1.5 mM $MgCl_2$)
Taq-Polymerase Qbiogene	0.4	5.0 U/µL	2 U
DNA	1.0		

[1] related to total volume of 20 µL (19 µL stock solution and 1 µL DNA)

The 221 bp PCR product was digested with BsaJI (cf. Table 4 in § 2.2.4.3) which recognizes C / C N N G G (here: C / C G T G G, SNP framed). The T-allele remained uncut (221 bp), while the C-allele was cut to fragments of 130 + 91 bp. No interfering unspecific PCR products were observed for the PCR product, the digested samples or the no-template control (see Figure 38).

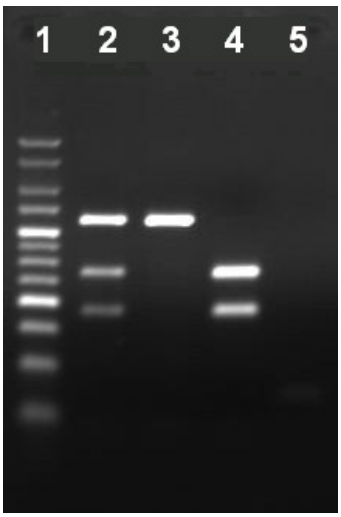

Figure 38: GNB3 C825T gel electrophoresis

RFLP with BsaJI:
Lane 1: length marker (25 bp ladder)
Lane 2: heterozygous C/T (130 + 91/ 221 bp)
Lane 3: homozygous T/T (221 bp)
Lane 4: homozygous C/C (130 + 91 bp)
Lane 5: no-template control

For electrophoresis, 8 µL of the PCR product was mixed with 1 µL loading buffer and analyzed on a 1.5% agarose gel using a 25 bp DNA-ladder (30 min at 100 V).

2.2.7.21 ACE Ins/Del, MAO-A uVNTR, DBH Ins/Del

Methods for the following three length polymorphisms were developed and afterwards combined to a multiplex thermocycler assay. The ins/del polymorphism of the ACE gene, characterized by a 287 bp Alu repeat sequence within intron 16 of the gene, determines putative functional variants [203-205]. A uVNTR (upstream Variable Number Tandem Repeats) polymorphism, which is located 1.2 kbp upstream of the MAO-A coding sequence in the X-chromosome, consists of a 30 bp repeated sequence present in 2, 3, 3.5, 4, or 5 copies and was shown to affect the transcriptional activity of the MAO-A gene promoter [207,208]. The DBH 5'-ins/del of the dopamine beta hydroxylase (DBH) gene was found to be associated with low DBH activity [217,218].

1. For ACE ins/del the forward primers of published thermocycler assays [297] were shifted somewhat to avoid SNPs underlying the original forward primer sequence. The product sizes were therefore somewhat different compared to the published ones with 469 bp for the ins-allele and 180 bp for the del-allele. The performance was checked by a control assay [297]. The ins-allele, which contained the 288 bp insertion, comprised 323 bp. For the del-allele, no band was visible as no fragment was amplified (see Figure 39). As published previously [297], unique bands were not generated until adjustment of the Mg^{2+} concentration and addition of dimethyl sulfoxide which was, due to the high GC content of the target DNA, of utmost importance for successful and correct genotyping. After optimization, both primer sets resulted in specific PCR products.

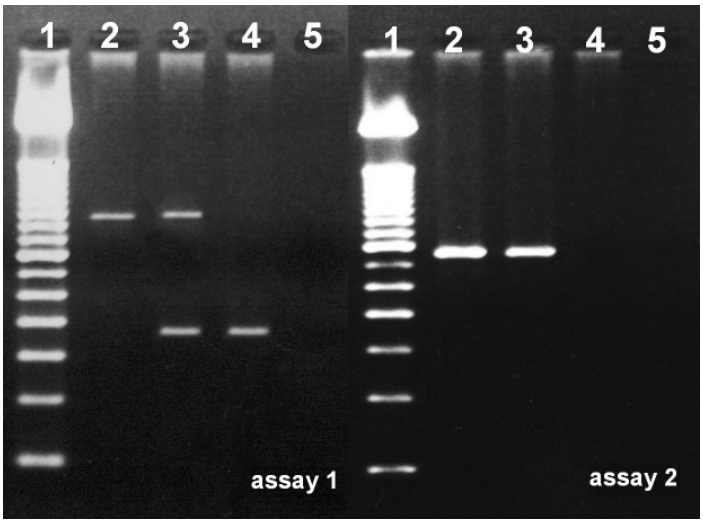

Figure 39: ACE ins/del assays

Lane 1: 50 bp DNA ladder
Lane 2: homozygote ins/ins
assay 1: 469 bp
assay 2: 323 bp
Lane 3: heterozygote ins/del
assay 1: 469 / 180 bp
assay 2: 323 bp
Lane 4: homozygote del/del
assay 1: 180 bp
assay 2: no band
Lane 5: no-template control

2. For the MAO-A uVNTR new primers were designed. The choice of primers was restricted by the desire to obtain products of an appropriate size for the multiplex format and to obtain similar annealing temperatures. PCR products were assigned the alleles based on the mobility in agarose gels as follows: allele 2 containing two repeats of the 30 bp sequence (301 bp), allele 3 containing three repeats of the 30bp sequence (331 bp), allele 3.5 containing 3.5 repeats (349 bp), allele 4 containing four repeats (361 bp) and allele 5 containing five repeats (391 bp). No interfering unspecific PCR products were observed for the amplification conditions used (see Figure 40).

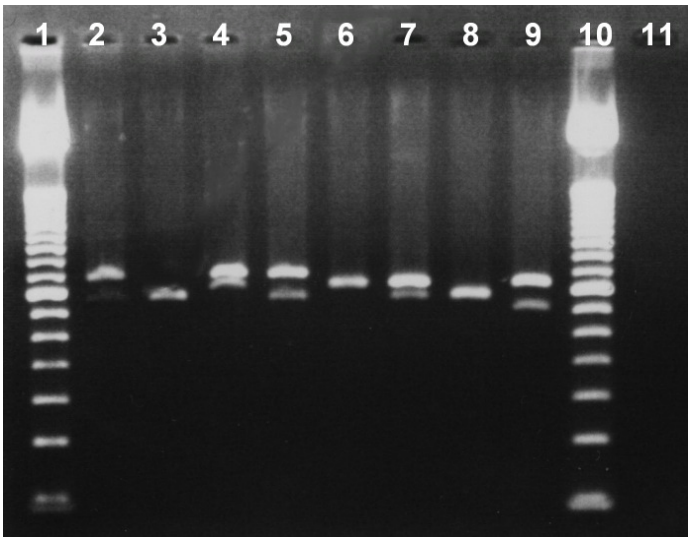

Figure 40: MAO-A uVNTR assay

Lane 1/10: 50 bp DNA ladder
Lane 2: 5/5 (391 bp)
Lane 3: 3/3 (331 bp)
Lane 4: 5/4 (391 / 361 bp
Lane 5: 5/3 (391 / 331 bp)
Lane 6: 4/4 (361 bp)
Lane 7: 4/3 (361 / 331 bp)
Lane 8: 3/3 (331 bp)
Lane 9: 4/2 (361 / 301 bp)
Lane 11: no-template control

3. For the detection of the DBH ins/del polymorphism a primer pair used in previous studies [219] was modified due to the low 3´stability and self complementarity of the original primers and to obtain products of an appropriate size for the multiplex format and to obtain similar annealing temperatures. The ins-allele, which contained the 19 bp insertion, comprised 168 bp and the del-allele 148 bp. PCR products were assigned the alleles based on the mobility in agarose gels as seen in Figure 41. Primer dimers with a length of approx. 50 bp were visible as diffuse bands, but did not interfere with the determination of genotypes.

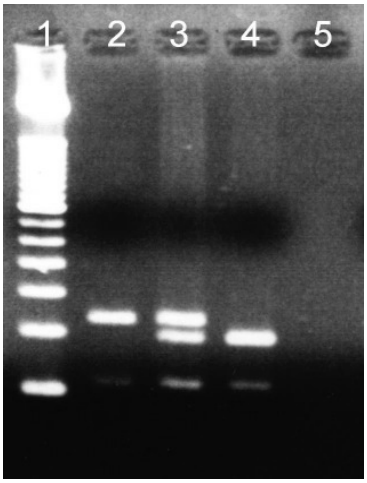

Figure 41: DBH ins/del assay

Lane 1: 50 bp DNA ladder
Lane 2: homozygote ins/ins (168 bp)
Lane 3: heterozygote ins/del (168 / 148 bp)
Lane 4: homozygote del/del (148 bp)
Lane 5: no-template control

4. Each PCR reaction was optimized separately for $MgCl_2$ and primer concentrations. The multiplex assay was established by choosing the best compromise for all reactions e.g., the extension time was increased to 25 sec to ensure proper amplification of the larger fragments and the number of cycles was reduced to 30 to ensure specificity. It was necessary to increase the relative yield of the DBH 5´ins/del products by increasing the concentration of the DBH primers. The assay was carried out as described in § 2.2.7.2 and specified in Table 23. The sequences of the primers used as well as their locations in the reference sequences are given in appendix A3 (see Table 69 and Table 70).

Table 23: ACE ins/del, MAO-A uVNTR, DBH ins/del - multiplex assay procedure

Temperature Program			
94°C/3 min; 40 x (94°C/20 s, 60°C/25 s, 72°C/20 s); 72/3 min			
Reagents	Amount (µL)	Concentration	Final concentration[1]
H_2O, sterile	3.7		
MAO-A F (primer)	0.3	25 µM	375 nM
MAO-A R (primer)	0.3	25 µM	375 nM
DBH F (primer)	0.4	25 µM	500 nM
DBH R (primer)	0.4	25 µM	500 nM
ACE 1F (primer)	0.3	25 µM	375 nM
ACE 2R (primer)	0.3	25 µM	375 nM
dimethyl sulfoxide (DMSO)	1.0		
Premix D 2x	12.0	2x	1.2x (1.8 mM $MgCl_2$)
Taq-Polymerase	0.3	5 U/µL	1.5 U
Genomic DNA	1.0		

[1] related to total volume of 20 µL (19 µL stock solution and 1µL DNA)

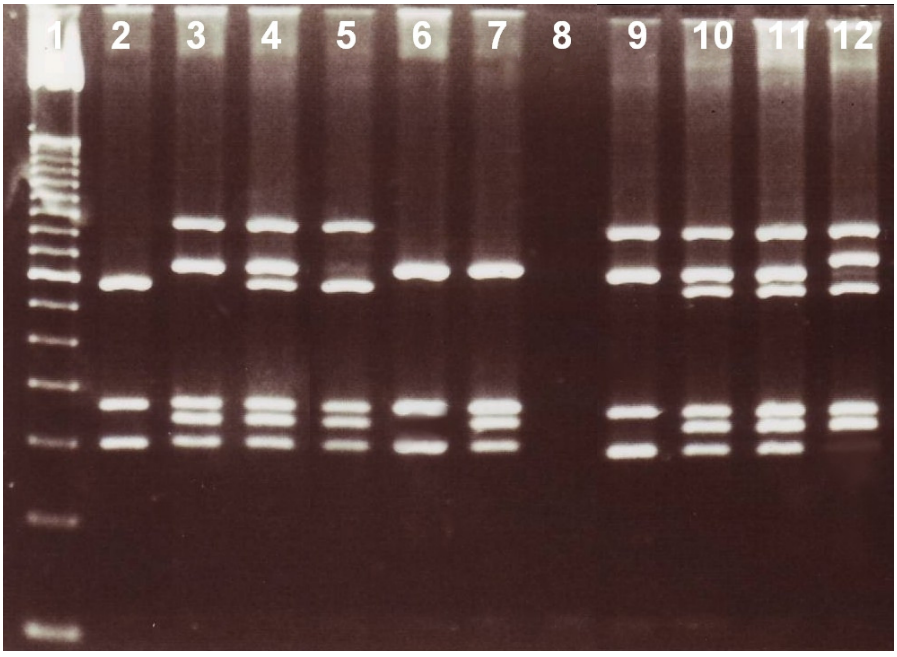

Figure 42: ACE ins/del, MAO-A uVNTR, DBH ins/del multiplex thermocycler assay

Note: for classification of PCR fragments, see also single-tube assays above.

Lane 1	50 bp DNA ladder		
Lane 2:	ACE del/del (180 bp)	MAOA 3/3 (331 bp)	DBH del/del (148 bp)
Lane 3:	ACE ins/del (469/180 bp)	MAOA 4/4 (361 bp)	DBH ins/del (168/148 bp)
Lane 4:	ACE ins/del (469/180 bp)	MAOA 3/4 (331/361 bp)	DBH ins/del (168/148 bp)
Lane 5:	ACE ins/del (469/180 bp)	MAOA 3/3 (331 bp)	DBH ins/del (168/148 bp)
Lane 6:	ACE del/del (180 bp)	MAOA 4/4 (361 bp)	DBH del/del (148 bp)
Lane 7:	ACE del/del (180 bp)	MAOA 4/4 (361 bp)	DBH ins/del (168/148 bp)
Lane 8:	no-template control		
Lane 9:	ACE ins/del (469/180 bp)	MAOA 4/4 (361 bp)	DBH del/del (148 bp)
Lane 10:	ACE ins/del (469/180 bp)	MAOA 3/4 (331/361 bp)	DBH ins/del (168/148 bp)
Lane 11:	ACE ins/del (469/180 bp)	MAOA 3/4 (331/361 bp)	DBH ins/del (168/148 bp)
Lane 12:	ACE ins/del (469/180 bp)	MAOA 5/3 (391/331 bp)	DBH ins/ins (168 bp)

For electrophoresis, 9 µL of the PCR product was mixed with 1 µL loading buffer and analyzed on a 2.5% agarose gel using a 50 bp DNA-ladder (40 min at 100 V).

2.2.7.22 TPH2 G1463A

The recently identified tryptophan hydroxylase TPH2 is preferentially expressed in the brain. The rare functional variation G1463A was associated with treatment resistance [189]. As there was no suitable restriction site, a published allele-specific assay was modified and adapted to the equipment at hand. It is a two-tube approach using a forward primer, a reverse primer (producing the 492 bp internal control band for the check of assay performance in every single tube) and one of two allele-specific reverse primers (producing a 294 bp band only if the matching allele is present).

The allele-specific primers used by Zang et al [189] were optimized with regard to the rules published by Little (1997) and Ye (2001) for allele-specific PCR [298]. An extra destabilizing mismatch at position -2 from the 3´-terminus was encompassed to increase the specificity of the assay. The weak mismatch at the 3´-terminus (G/T or C/A) required a strong mismatch at the position -2 from the 3´-terminus (C/T). Therefore, the following modified allele-specific primers were used: TAGGGATTGAAGTATACTGAGAAGGTA**C** (G-allele) and TAGGGATTGAAGTATACT GAGAAGGTA**T** (A-allele). The sequences of the primers used as well as their locations in the reference sequence are given in appendix A3 (see Table 69). The assay was carried out as described in § 2.2.7.2 and specified in Table 24.

Unable to obtain DNA containing the rare A-allele, the specificity of the assay was tested by using „mock“ templates. This DNA template was generated by carrying out PCR on a wildtype DNA with the forward and reverse primer for the internal control and with the mutation-specific primer under conditions that encouraged mispriming on the wildtype DNA by the mutation-specific primer. An annealing temperature of 52°C for 45 cycles was used to produce templates that mimicked the heterozygote condition. The resulting products were diluted in rehydration buffer (1:100 as 1:10 dilution produced even more unspecific products) and used as templates in the ARMS assay.

Table 24: TPH2 G1463A assay procedure

Temperature Program			
94°C/5 min; 30 x (94°C/20 s, 60°C/20 s, 72°C/20 s); 72°C/3 min			
Reagents	Amount (µL)	Concentration	Final concentration[1]
H_2O, sterile	15.0		
Qbiogene Incubation Buffer	1.6	10x	0.8x (1.2 mM $MgCl_2$)
$MgCl_2$	1.0	25 mM	2.45 mM[2]
dNTP	0.3	5 mM	100 µM
TPH2 hOut F (primer)	0.1	25 µM	125 nM
TPH2 hOut R (primer)	0.1	25 µM	125 nM
TPH2 1463 G or TPH2 1463 A (primer)	0.3	25 µM	375 nM
Bovine Serum Albumin	0.3	20 mg/mL	0.66 mg/mL[3]
Taq-Polymerase Qbiogene	0.3	5 U/µl	1.5 U
Genomic DNA	1.0		

[1] related to total volume of 20 µL (19 µL stock solution and 1 µL DNA)
[2] total $MgCl_2$-concentration of the Qbiogene Buffer and the additional 25 mM $MgCl_2$ -solution
[3] total albumin concentration of the Qbiogene Buffer and the additional BSA solution

The 294 bp allele-specific bands were used to determine the genotype (see Figure 43). The 492 bp internal control band was used to check if amplification was sufficient in every single reaction tube. Background smear bands were visible when the “mock” templates were used (see Lanes 3 - 4 and 8 - 9 in Figure 43). Nevertheless, a reliable determination of the genotype was possible. No unspecific bands were observed for the no-template controls (Lane 2 and 7 in Figure 43).

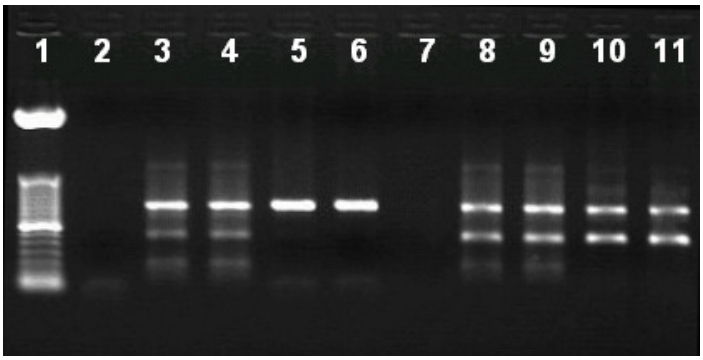

Figure 43: TPH2 G1463A gel electrophoresis

Lane 1: length marker (50 bp DNA ladder)
Lane 2 - 6: variant assay with primer specific for A-allele
Lane 2: no-template control
Lane 3 - 4: G/A heterozygote (492 bp and 294 bp) – mock template
Lane 5 - 6: G/G homozygote (only 492 bp control band visible)
Lane 7-11: wildtype assay with primer specific for G-allele
Lane 7: no-template control
Lane 8 - 9: G/A heterozygote (492 bp and 294 bp) – mock template
Lane 10 - 11: G/G homozygote (492 bp and 294 bp)

For electrophoresis, 7 µL of the PCR product was mixed with 1 µL loading buffer and analyzed on a 1.5% agarose gel using a 50 bp DNA-ladder (15 min at 100 V).

2.2.7.23 TPH1 A218C

The TPH1 A218C polymorphism (rs1800532) is an A to C change at coding position 218 in intron 2 of the tryptophan hydroxylase (TPH1) gene, which catalyzes the rate-limiting enzyme step in serotonin synthesis. A published RFLP assay [184] was adapted to the equipment at hand. The assay was carried out as described in § 2.2.7.2 and specified in Table 25. The sequences of the primers used as well as their locations in the reference sequence are given in appendix A3 (see Table 69).

Table 25: TPH1 A218C assay procedure

Temperature Program			
94°C/3 min; 35 x (94°C/30 s, 60°C/30 s, 72°C/30 s); 72°C/3 min			
Reagents	Amount (µL)	Concentration	Final concentration[1]
H_2O, sterile	7.8		
TPH 218 F (primer)	0.4	25 µM	500 nM
TPH 218 R (primer)	0.4	25 µM	500 nM
Premix D	10.0	2 x	1x (1.5 mM $MgCl_2$)
Taq Polymerase Qbiogene	0.4	5.0 U/µL	2 U
DNA	1.0		

[1] related to total volume of 20 µL (19 µL stock solution and 1 µL DNA)

The 847 bp PCR product was digested with NheI enzyme (cf. Table 4 in § 2.2.4.3) that recognizes the G / C T A G C site. The A-allele remained uncut (847 bp), while the C-allele was cut to fragments of 250 + 597 bp. No interfering unspecific PCR products were observed for the PCR product, the digested samples or the no-template control (see Figure 44).

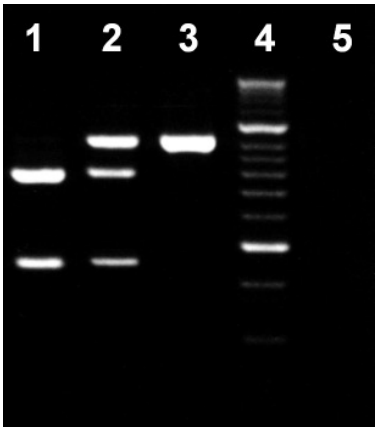

Figure 44: TPH1 A218C gel electrophoresis

RFLP with NheI:
Lane 1: homozygote C/C (250 + 597 bp)
Lane 2: heterozygote A/C (847 / 250 + 597 bp)
Lane 3: homozygote A/A (847 bp)
Lane 4: length marker (100 bp DNA ladder)
Lane 5: no-template control

For electrophoresis, 7 µL of the PCR product was mixed with 1 µL loading buffer and analyzed on a 2% agarose gel using a 100 bp DNA-ladder (15 min at 100 V).

2.3 HPLC-MS/MS Quantification for Serum Concentrations

Table 26: HPLC-MS/MS Materials

API3000 (LC/MS/MS)	Applied Biosystems, Foster City, CA, USA
Cyclone® HTLC™ Column (1.0*50 mm)	Cohesive, Franklin, MA, USA
Onyx Monolytic C18 (4.6*100 mm)	Phenomenex Ltd. Deutschland, Aschaffenburg
olanzapine serum control samples	Chromsystems GmbH, München
Milli-Q water	Millipore GmbH, Eschborn
acetonitrile, methanol, isopropanol HPLC grade, acetic acid (100%), sodium acetate	Sigma, Taufkirchen and VWR/Merck, Darmstadt
methylrisperidone	Janssen Pharmaceutica, Beerse, Belgium
citalopram	Sigma, Taufkirchen
mirtazapine	Sigma, Taufkirchen
olanzapine	Toronto Research Chemicals Inc., Downsview, Ontario, Canada (via Fischer Chemical Ag, Zürich)
fluoxetine-D5 solution	Sigma, Taufkirchen
Analyse-it + Clinical Laboratory for EXCEL (Version 1.61)	Analyse-it Software Ltd., Leeds, UK

The quantification of olanzapine, mirtazapine and citalopram was carried out by tandem mass spectrometry (LC/MS/MS) according to Kirchherr et al. [299]. In addition, a two-dimensional liquid chromatography was carried out before HPLC and mass analysis. 50 µL of serum was diluted with 50 µL water, precipitated by 150 µL acetonitrile:methanol (10:1, v/v) containing the internal standards fluoxetine-D5 or methylrisperidone, respectively, and centrifuged at 10 000 x g. Twenty µL of the supernatant was injected. The analytes were diluted by water, trapped on a Cyclone® HTLC™ Column (1.0*50 mm) and transferred by methanol:isopropanol (70:30) onto the analytical column (Onyx Monolytic C18; 4.6*100 mm). Linear gradient separation was made by starting with 60% water containing 1 mL acetic acid (HOAc) and 0.8 g sodium acetate (NaOAc) per liter and ending with 100% methanol containing 1 mL acetic acid (HOAc) and 0.8 g sodium acetate (NaOAc) per liter. The total turnaround time for the LC/MS/MS method was 6 min. Calibration samples, patient samples and control samples for olanzapine were measured in duplicate. Linear regression was applied for quantification. The mass transitions (m/z) recorded by an API3000 for quantification are given in Table 27.

Table 27: HPLC-MS/MS Parameters

drug	formula	molecular weight (g/moL)	m/z	retention time (min)
citalopram hydrobromide	$C_{20}H_{21}FN_2OHBr$	405.3	325/109	3.0
mirtazapine	$C_{17}H_{19}N_3$	265.4	266/195	2.9
methylrisperidone (IS)	$C_{24}H_{25}FN_4O_2$	420.5	421/201	3.1
olanzapine	$C_{17}H_{20}N_4S$	312.4	313/256	2.8
fluoxetine-D5 (IS)	$C_{17}D_5H_{13}F_3NO$	314.4	315/153	3.8

2.4 Statistics

Statistical analysis was performed using SPSS, version 12.0 for Windows (SPSS Inc., Chicago, USA) und Excel 2000 (Microsoft Corporation, Seattle, USA). A two-tailed p-value of $p<0.05$ was considered to be of statistical significance.

Quantitative variables that showed no normal distribution according to the Kolmogorov-Smirnov test or Shapiro-Wilk test were compared by nonparametric tests for continuous data divided into categories (Mann-Whitney U test for comparison of two groups or Kruskal-Wallis test for comparison of more than two groups with regard to a continuous variable). Parameters with normal distribution were compared by two-tailed t-test (two groups) or analysis of variance (more than two groups) with post-hoc Bonferroni testing for multiple comparisons, if applicable. The results were checked by box-and-whisker plots to for distribution of data and outliers. The center box shows median and interquartile range of data. When outliers and/or extremes exist, the whiskers range is defined as 1.5 times the interval from the 25th to 75th percentile i.e., the box length. Outliers are between 1.5 and 3 box lengths away, extremes are more than 3 box lengths away. Otherwise the whiskers give the full range of data.

Spearman rank correlation (coefficient and p-value) was used to detect the strength and significance of the (linear) relationship between two continuous variables (without normal distribution). Scatter plots were used to check the linearity of the relationship and to search for outliers. The following interpretations as suggested by Cohen (1988) [300] for correlations were used: ± 0.1 to 0.3 small, ± 0.3 to 0.5 medium and ± 0.5 to 1.0 large.

The combined effects of possible covariates/factors (such as dose, diagnosis, height, weight, age, gender, smoking, duration of illness, genotypes and co-medication) on the variables were investigated using multiple linear regression analysis. The possible covariates were entered stepwise into the model. A parameter was included in the model when the 95% confident interval of its standard error did not include zero and the objective function value was decreased for more than 3.84. This corresponds to a significance niveau (probability) of the F value < 0.05.

For differences between nominal or ordinal variables, the Fisher exact test was used for prevalence comparisons among 2x2 cross tabulations, otherwise Pearson's Chi square test was performed. The Odds Ratio provides an estimate (with 95% confidence interval) for the relationship between binary variables. It is the probability that the event of interest occurs to the probability that it does not. Thus, it is an indicator of the strength of the relationship.

The genotype frequencies of bi- and triallelic polymorphisms were checked for deviation from the Hardy-Weinberg-Equilibrium (HWE) [301] by Pearson-Chi-square-test for goodness of fit. For expected frequencies also the Fisher's exact test was used. The frequencies were compared to published allele frequencies. A p-value > 0.05 indicated that the null hypothesis that the population is in Hardy-Weinberg proportions is not rejected.

3 Results

3.1 Outline of Study Data Evaluation

In total, 489 patients eligible for inclusion in the study gave informed consent and were initially included in the study. No DNA was available for 58 patients. Sixty-five patients were hospitalized too short to provide enough data for further statistical evaluation. Thus, genotyping and statistical analysis was performed for 365 patients.

Complete data on exact dosage within the first four weeks was available for 353 patients of the 365 patients, data on adverse effects for 328 patients, data on the Paranoid Depression Scale (PDS scores) for 230 patients and on the Clinical Global Impression improvement rating CGI(2) for 338 patients. Steady-state serum concentrations were available for 73 patients treated with olanzapine, 50 with mirtazapine and 39 with (es)citalopram.

The performance and verification of genotyping results and serum concentrations is given in § 3.2 and in § 3.3. The statistical evaluation of all obtained data is described in detail in § 3.4. The evaluation of the genetic variants of the different candidate genes as described in the introduction and their relation with the clinical data is given in the subsequent chapters as depicted below:

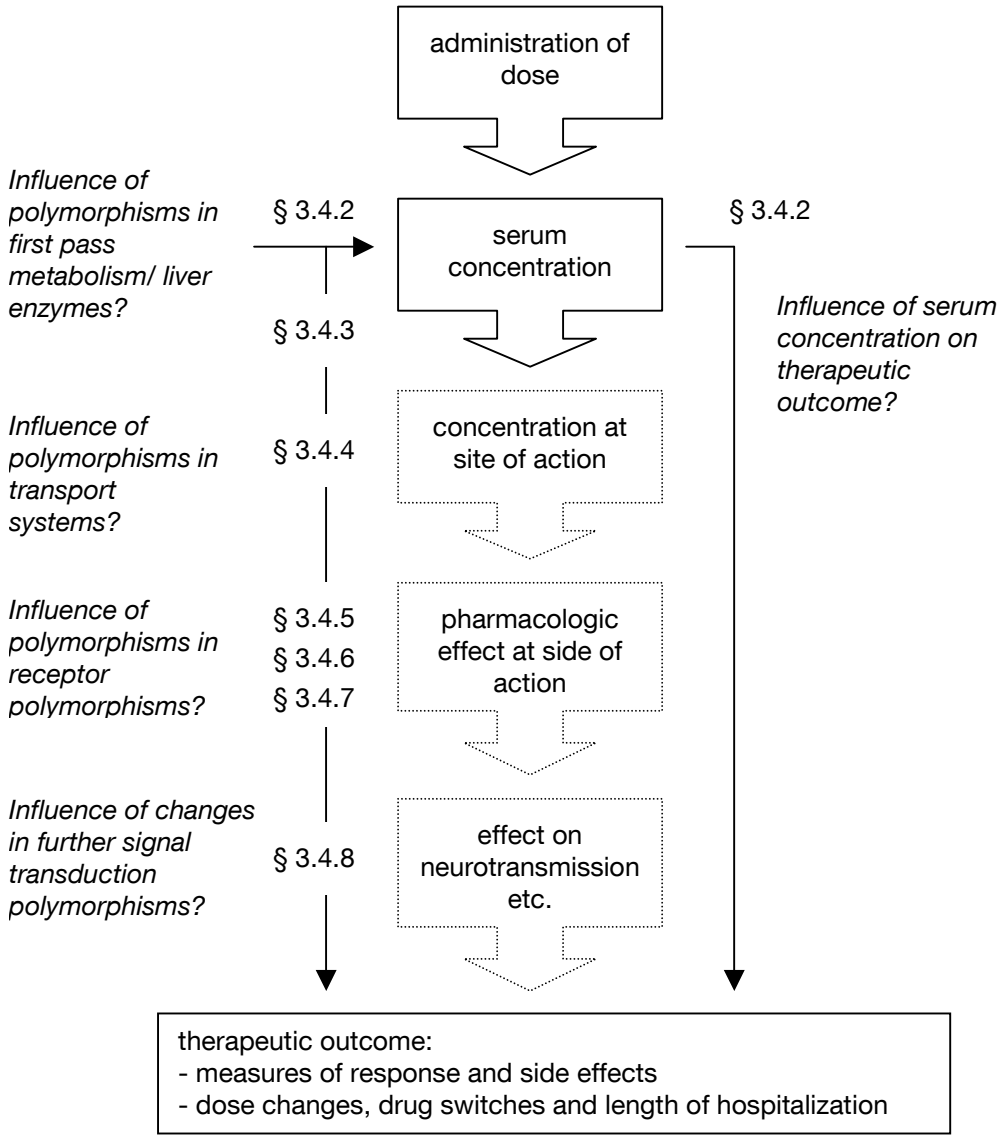

Figure 45: Overview - evaluation of clinical data, serum concentrations and genotyping results

3.2 Genotyping Methods

3.2.1 Performance and Verification of Genotyping Results

3.2.1.1 Length Polymorphisms, Allele-specific and RFLP Assays - Determination of Genotypes

Conventional PCR methods were established for the detection of length polymorphisms (5-HTTLPR, DAT1 VNTR, MAO-A uVNTR, DBH ins/del, ACE ins/del), allele-specific methods (CYP2D6*10 and TPH2 G1463A) and RFLP methods (5-HTR2A T102C, 5-HTR6 T267C, GNB3 C825T, TPH1 A218C). The genotypes were assigned by the different lengths of the PCR fragments by use of suitable DNA ladders and were in accordance with the theoretical lengths. The completeness of restriction digestion was checked by inclusion of control samples in every experiment. DNA contamination was controlled by using a sample without genomic DNA in each batch of the experiment. The methods are described in detail in § 2.2.7. Moreover, RFLP assays were used to screen for controls and to check the performance of fluorescence-based methods.

3.2.1.2 FRET Assays - Determination of Genotypes

New hybridization probes assays were developed for CYP2D6*10 and *41, MDR1 G2677TA and C1236T, 5-HTR1A C-1019G, 5-HTR2A intron 2, 5-HTR2C C-759T, BDNF Val66Met, COMT Val158Met and FKBP5 intron 2 and a published assay was adapted for NET T-182C [280] (for principle see § 2.2.5.2, the methods are described in detail in § 2.2.7). The sensor probes were designed either to match the wildtype or the variant allele (depending upon the more destabilizing mismatch). This had to be kept in mind when assigning the melting peaks to wildtype or variant. The melting curves for every assay are also depicted in § 2.2.7.

The fluorescence assays were robust and reliable as documented by the complete concordance of at least 60 samples determined with both the conventional RFLP and LightCycler protocol. Except for the triallelic MDR1 G2677TA polymorphism, successful genotyping with clearly separated melting peaks was possible for all newly designed assays (delta T between wildtype and variant ranging from 3.5 to 14°C). As the difference of melting temperature difference between the G- and the A-allele of the MDR1 G2677TA polymorphism is very small (delta T 2°C), no separation of the two peaks was possible in G/A heterozygotes and only one broad peak at 55 - 57°C was detected for the G/A-genotype. Thus, the correctness of genotyping of the G/A genotype was verified by a re-analysis of the respective samples with the reference method by Tanabe et al. (2001) (see § 2.2.7.5).

Moreover, the melting temperatures (T_m) should be highly reproducible under constant reaction conditions including heating rate, salt concentrations and probe-target concentrations [265] with typical within-run variation < 0.1°C and between run variation < 0.5°C. Shifts > 1°C are causes for suspicion of new mutations [302,303]. During genotyping, no atypical melting peaks with deviating T_m or T_m shifts > 1°C were detected when compared to the control samples included in every run, except for two samples in the routine CYP2D6*9 assay and two samples in the CYP2D6*41 assay.

Both samples in the routine CYP2D6*9 assay had a deviating peak with 46.5°C. Both samples were genotyped *1*2 and one of the deviating melting peaks is depicted in Figure 46. Recently, a novel CYP2D7 gene conversion in exon 5 (2470-2610) was found that includes two novel SNPs within the sensor probe (Tandon *et al.*, manuscript in prep., see homepage of the Human Cytochrome P450 Allele Nomenclature Committee [67]). One of these other SNPs underlying the sensor probe could have led to the deviating peak.

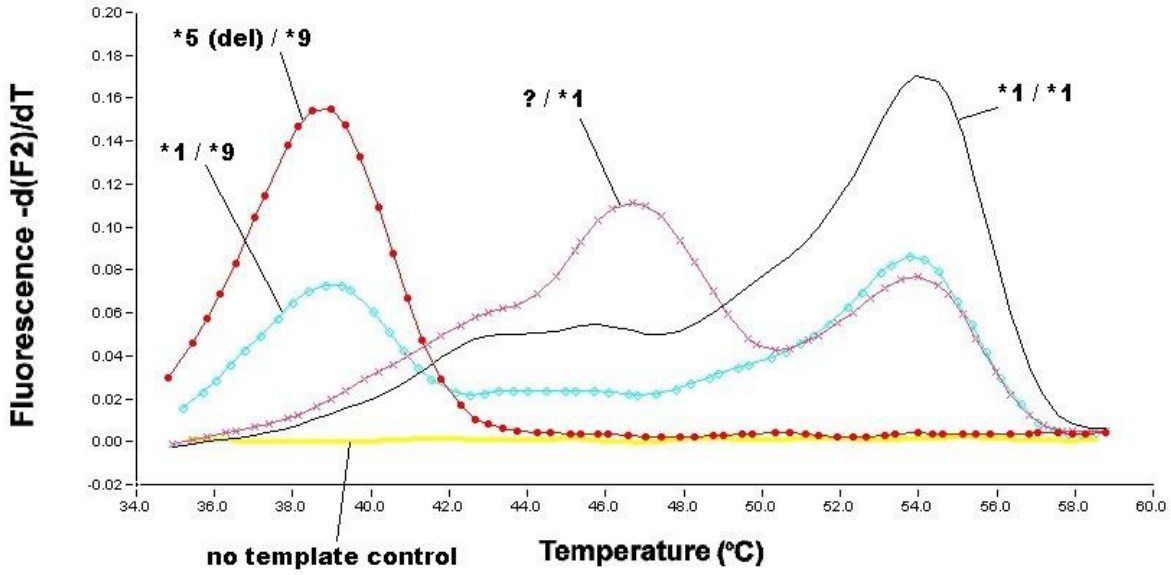

Figure 46: CYP2D6*9 assay - additional peak at 46.5°C

Note: fluorescence detection of the different CYP2D6*9 genotypes at 640 nm as follows: wildtype peak at 54°C and variant peak at 39°C. No fluorescence peak observed for no-template control. Additional peak at 46.5°C observed in two study samples.

Moreover, two samples in the CYP2D6*41 (G2988A) assay had a deviating peak with 56°C. The samples were genotyped *1*1 and *1*5 and their deviating melting peaks are depicted in Figure 47 and Figure 48 below. CYP2D6*2 (-1584C) was present in neither of the samples. An unknown intronic SNP within the sensor sequence (4612 - 4597 according to M33388 with G4607A as *41) in intron 6 of the CYP2D6 gene may have been the reason.

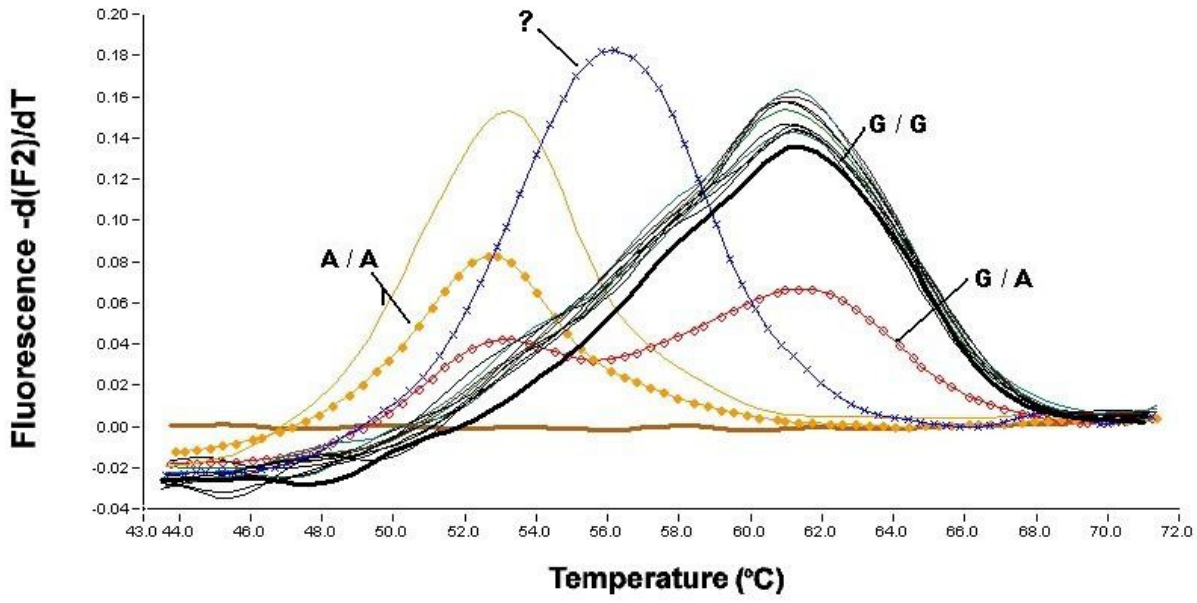

Figure 47: CYP2D6*41 assay - additional peak at 56°C for sample genotyped *1*5

Note: fluorescence detection of the different CYP2D6*41 genotypes (SNP G2988A) at 640 nm as follows: wildtype homozygote G/G - one peak at 62°C, variant homozygote A/A - one peak at 62°C and heterozygote G/A – two peaks at 62 and 53 °C, respectively. No fluorescence peak observed for no-template control. Additional peak at 56°C observed in sample genotyped *1*5 (hemizygote for the putative unknown SNP in intron 6).

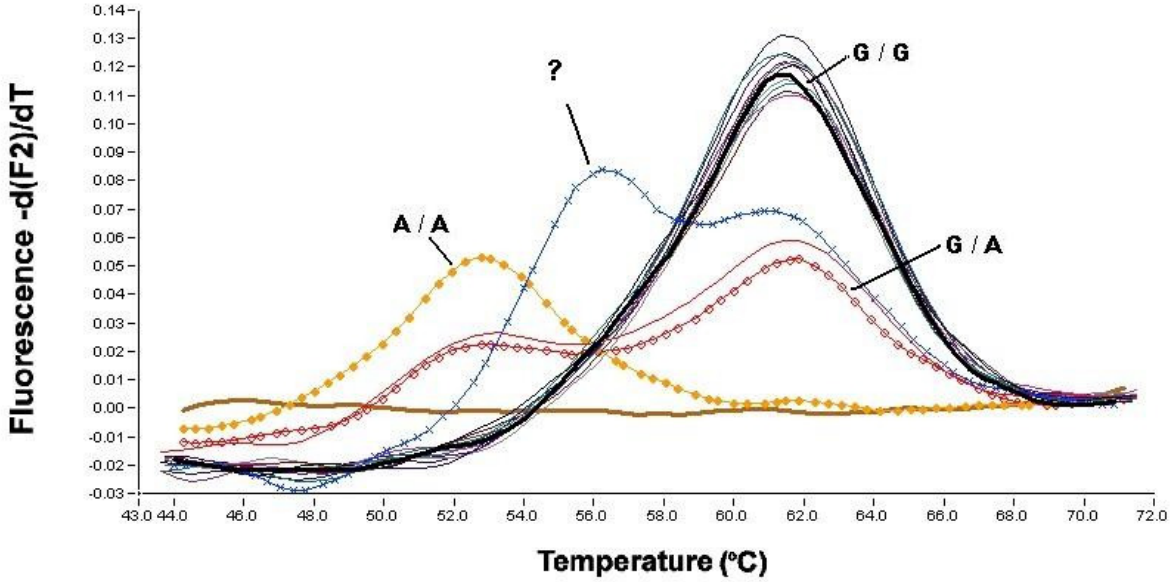

Figure 48: CYP2D6*41 assay - additional peak at 56°C for sample genotyped *1*1

Note: fluorescence detection of the different CYP2D6*41 genotypes (SNP G2988A) at 640 nm as follows: wildtype homozygote G/G - one peak at 62°C, variant homozygote A/A - one peak at 62°C and heterozygote G/A - two peaks at 62 and 53 °C, respectively. No fluorescence peak observed for no-template control. Additional peak at 56°C observed in sample genotyped *1*1 (heterozygote for the putative unknown SNP in intron 6).

3.2.1.3 TaqMan Assays - Determination of Genotypes

Fluorescence-based TaqMan assays (for principle see § 2.2.5.3, the methods are described in detail in § 2.2.7) were adapted for 5-HTT rs25531 [114], NET G1287A [281], and ADR2A C-1291G [282]. The TaqMan wildtype probes were labeled with fluorescein and used to detect the wildtype alleles at 530 nm (see fluorescence curves in Figure 13, Figure 24, and Figure 29 in § 2.2.7). The TaqMan variant probes were labeled with LCRed610 dye or hexachlorofluorescein and the variant alleles were detected at 610 or 560 nm (see Figure 14, Figure 25, and Figure 30 in § 2.2.7). All probes were additionally labeled with a non-fluorescent quencher at the 3´end to minimize the background fluorescence and maximize the sensitivity. Moreover, color-compensation was used to correct for the fluorophore emission overlap in each channel for each fluorophore (see § 2.2.5.4) and thus to optimize the assay performance.

The performance of the adapted TaqMan methods was controlled by plotting the maximum fluorescence intensities of both channels/probes/alleles against each other. As depicted in Figure 49 for 5-HTT rs25531 A/G, in Figure 50 for NET G1287A and in Figure 51 for ADR2A C-1291G below, distinct clusters for the three different genotypes and the no-template control were observed. As the fluorescence intensities showed no overlapping, an unambiguous allele discrimination and genotyping is possible.

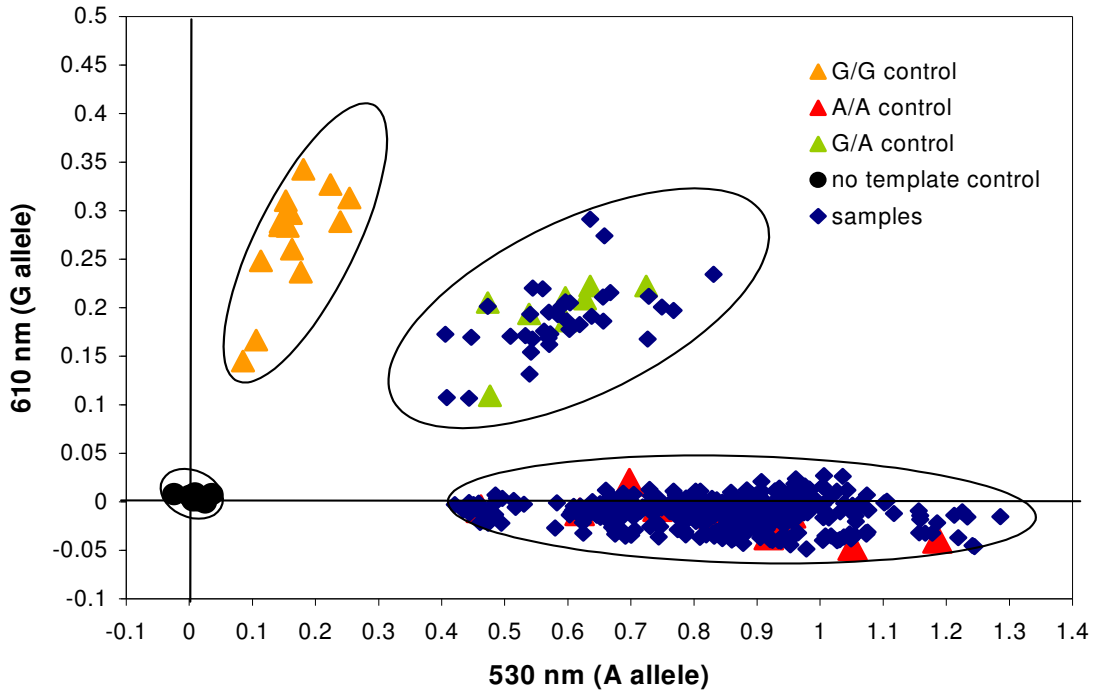

Figure 49: 5-HTT rs25531 A/G TaqMan assay - fluorescence intensity plot

Note: The maximum fluorescence intensities at the end of the amplification (after 80 cycles) were plotted against each other (see also example amplification curves of the A-allele at 530 nm shown in Figure 13 and of the G-allele at 610 nm shown in Figure 14 in § 2.2.7.7). Each point represents either a study sample or a control sample. Four distinct clusters of samples were observed: the wildtype A/A homozygotes, the variant G/G homozygotes, the A/G heterozygotes, and the no-template controls where no fluorescence was observed.

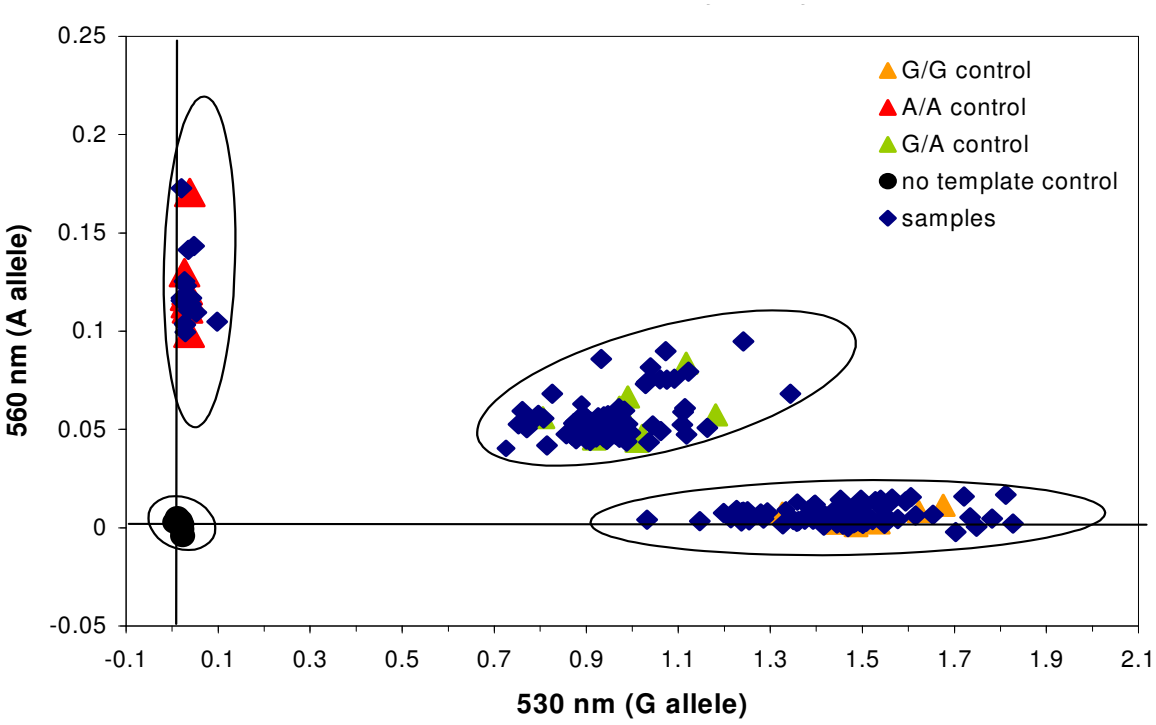

Figure 50: NET G1287A TaqMan assay - fluorescence intensity plot

Note: The maximum fluorescence intensities at the end of the amplification after 80 cycles were plotted against each other used (cf. end point of the amplification curve of the G-allele at 530 nm shown in Figure 24 and end point of the A-allele at 560 nm shown in Figure 25 in § 2.2.7.14). Each point represents either a study sample or a control sample. Four distinct clusters of samples were observed: the wildtype G/G homozygotes, the variant A/A homozygotes, the G/A heterozygotes, and the no-template controls where no fluorescence was observed.

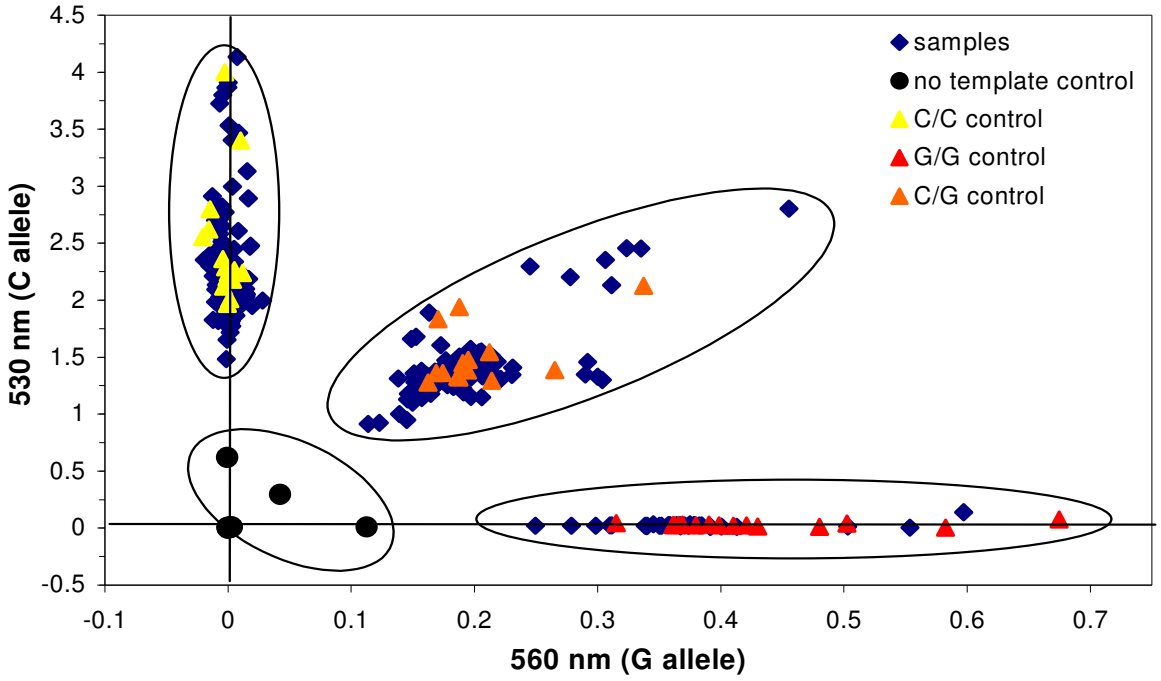

Figure 51: ADR2A C-1291G TaqMan assay - fluorescence intensity plot

Note: The maximum fluorescence intensities at the end of the amplification after 40 cycles were plotted against each other used (cf. end point of the amplification curve of the C-allele at 530 nm shown in Figure 29 and end point of the G-allele at 560 nm shown in Figure 30 in § 2.2.7.16). Each point represents either a study sample or a control sample. Four distinct clusters of samples were observed: the wildtype C/C homozygotes, the variant G/G homozygotes, the C/G heterozygotes, and the no-template controls where negligible fluorescence was observed.

3.2.2 Allele Frequencies

The allele and genotype frequencies of all analyzed polymorphisms are given in appendix A2 for the total study population (n = 365). The genotype frequencies of all bi- and triallelic polymorphisms were compatible with the Hardy-Weinberg equilibrium ($p > 0.05$, exact results given in appendix A2) and the genotype and allele frequencies were similar to published allele frequencies of Caucasian populations (for detailed comparison see also appendix A2). No variant allele of the TPH2 G1463A polymorphism was found in the study population as reported meanwhile in some other studies [188-193].

An estimated 2 - 5% of Caucasians are CYP2C19 poor metabolizers with a lack of functional enzyme mainly due to the CYP2C19*2, *3, and *4 null alleles. Accordingly, the observed prevalence in the study population was comparable to the published ones (2.5% poor metabolizers and 27.7% intermediate metabolizers). Similarly, the frequencies for the combination of 5-HTTLPR and the SNP nearby (rs25531 A/G) were compared to those published in literature for Caucasians [115,304]. The observed prevalences did not differ from the expected ones. Detailed data on these genotypes and allele frequencies in the study population is given in appendix A2.

For the highly polymorphic CYP2D6 gene, gene duplication, gene deletion *5, the non-functional alleles *3, *4, *6, *7, *8 and the impaired alleles *9, *10, *41 were examined. The different polymorphisms were rated according to their functionality with semi-quantitative gene doses as described previously by Steimer et al. (2004) [305]. The non-functional alleles *3, *4, *5, *6 were evaluated as null alleles, the impaired alleles *9, *10, *41 (2988A) with gene dose 0.5, the functional alleles *1 and *2 with gene dose 1 and for duplicated genes the respective gene dose was doubled. In a second step, the gene dose of 0 was rated as poor metabolizer, 0.5 and 1 as intermediate metabolizer, 1.5 and 2 as extensive metabolizer and > 2 as ultrarapid metabolizers to have sufficiently large groups for statistical evaluation. The frequency of CYP2D6 PMs, UMs and IMs was with 8.5%, 3% and 38% similar to frequencies given in literature (5 - 10%, 2 - 5% and up to 40%, respectively) [72,88,89].

When comparing the results of both CYP2D6*41 methods, 64 patients were found to be carriers of the *2(-1584C) in the promoter and 60 of these patients (93.8%) were also carriers of the novel intronic 2988A polymorphism. The frequency is in accordance with the results of Raimundo et al. (2004) [89] for another Caucasian population, where the novel 2988A polymorphism was present in 52 of 56 CYP2D6 *2 (-1584C) alleles (frequency of 92.9%). The classification, based on the semi-quantitative gene doses [305] and the derived metabolizer status as described above, is tabulated in Table 28. The metabolizer status was unchanged in three of the four patients. As the 2988G>A polymorphism was shown to cause the low expression phenotype of the impaired *41 allele [89,290] by aberrant splicing, this SNP was chosen as basis for the classification of the gene dose groups.

Table 28: Comparison of CYP2D6*41 methods

	gene dose according to:		Metabolizer
2988G with *2(-1584C)	old method	new method	Status
*2xDup/*2(-1584C)	2.5	3	UM
*2xDup/*2(-1584C)	2.5	3	UM
*2 (-1584C)xDup /*3	1	2	IM → EM
*2 (-1584C)/*1	1.5	2	EM

3.3 Performance of HPLC-MS/MS Quantification

The serum samples were measured in a total of 11 runs. The applied method was reliable, precise and accurate during sample measurement, as demonstrated by the results obtained for accuracy and precision (see Table 29). For olanzapine, the calibration range was 2.5 - 1000 ng/mL. Commercial control samples (Chromsystems GmbH) containing 29 or 101 ng/mL were found to be measured accurately with a recovery of 100 ± 8%. Within the calibration range, average coefficients of variation were 5.0% for intra-assay and 5.7% for inter-assay comparisons, respectively, while average deviations from spiked concentrations ranged from - 3.1% to 4.7%. For mirtazapine, the calibration range was 5 - 1000 ng/mL with average coefficients of variation of 3.7% for intra-assay and 5.1% for inter-assay comparisons, respectively. Average deviations from spiked concentrations ranged from - 9.8% to 14.4%. For citalopram, the calibration range was 5 - 1000 ng/mL with average coefficients of variation of 4.8% for intra-assay and 5.6% for inter-assay comparisons, respectively. Average deviations from spiked concentrations ranged from - 6.6% to 8.3%. Example chromatograms are depicted in Figure 52 - Figure 54.

Table 29: Accuracy and precision of HPLC-MS/MS quantification

	Olanzapine			Mirtazapine			Citalopram		
Conc. (ng/mL)	Intra-run precision (%)	Inter-run precision (%)	accuracy (%)	Intra-run precision (%)	Inter-run precision (%)	accuracy (%)	Intra-run precision (%)	Inter-run precision (%)	accuracy (%)
2.5	7.3	8.8	1.6	-	-	-	-	-	-
5	3.6	8.9	2.3	2.7	5.3	10.1	9.4	7.6	8.3
10	5.7	6.4	3.4	2.5	6.9	14.4	6.1	8.2	-4.6
25	3.8	5.2	2.7	3.3	5.2	6.1	3.9	7.4	0.6
50	5.7	4.1	3.6	5.0	5.2	5.8	4.8	5.6	0.6
100	5.5	5.5	2.8	5.0	3.6	3.6	3.5	5.0	5.7
500	4.1	5.0	4.7	2.9	3.4	-1.8	2.5	1.3	0.2
1000	4.3	1.5	-3.1	4.2	6.0	-9.8	3.7	4.0	-6.6
Mean	5.0	5.7	2.3	3.7	5.1	4.1	4.8	5.6	0.6
Min	3.6	1.5	-3.1	2.5	3.4	-9.8	2.5	1.3	-6.6
Max	7.3	8.9	4.7	5.0	6.9	14.4	9.4	8.2	8.3

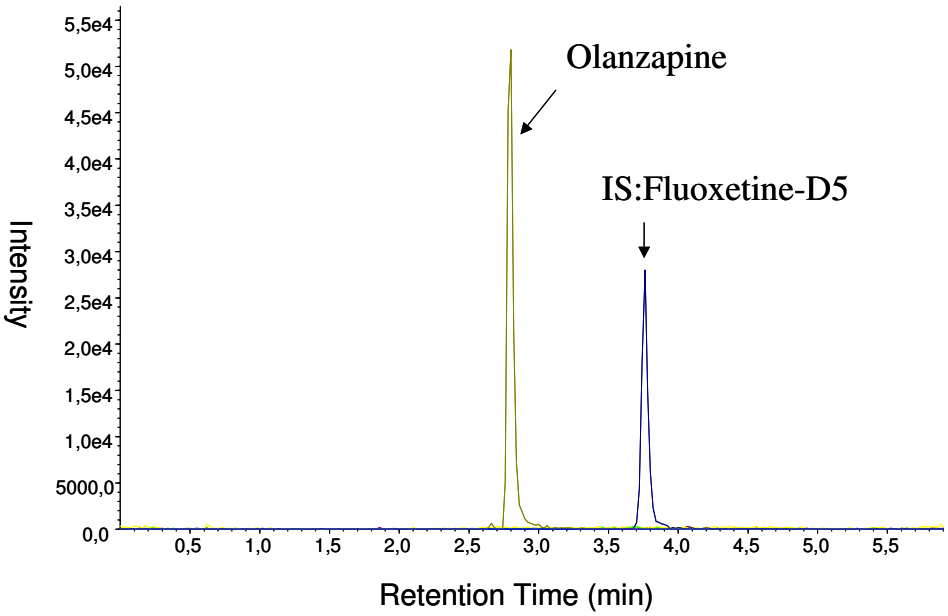

Figure 52: Example LC-MS/MS chromatogram of olanzapine

Mass transition (m/z) olanzapine 313/256 and Internal Standard fluoxetine-d5 315/153.

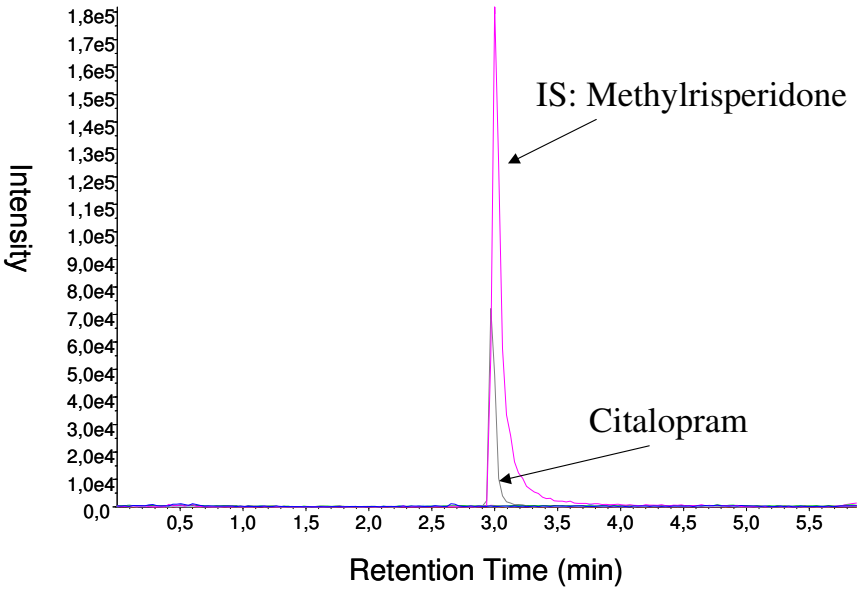

Figure 53: Example LC-MS/MS chromatogram of citalopram

Mass transition (m/z) citalopram 325/109 and Internal Standard methylrisperiodone 421/201.

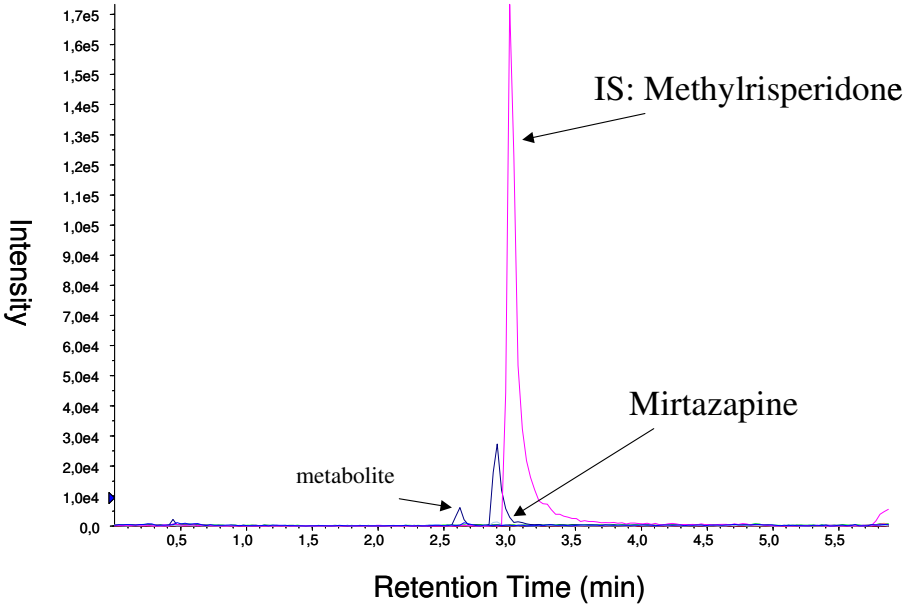

Figure 54: Example LC-MS/MS chromatogram of mirtazapine

Mass transition (m/z) mirtazapine 266/195, Internal Standard methylrisperiodone 421/201 and additional mass transition(m/z) 266/195 for a metabolite of mirtazapine with a shorter retention time in reverse phase column and thus higher polarity than mirtazapine (most likely mirtazapine-N-oxide [306]).

Note: Mirtazapine is mainly metabolized by CYP2D6 (and less by CYP1A2) to 8-hydroxy-mirtazapine and by CYP3A4 to N-demethyl-mirtazapine and mirtazapine-N-oxide [307].

3.4 Statistical Evaluation of Clinical Data and Genotyping Results

3.4.1 Demographics and Baseline Characteristics of Study Population

Of the 365 patients included in the evaluation, 162 (44.4%) were male and 153 (41.9%) were smokers. Mean (± standard deviation) age was 44 years (± 16 years), 70 (19.2%) were older than 60 years. Fifty-six (16.6% of the available 338 ratings) patients had a CGI(2) improvement rating of 1 or 2 and were therefore evaluated as CGI responders.

One hundred twenty-seven (34.8%) of the patients did not suffer from adverse effects according to the rating of the treating psychiatrist, 105 (28.8%) suffered from mild and 96 patients (26.3%) from moderate or marked adverse effects. For 37 (10.1%) patients no rating was reported.

Fifty-two (15.8%, complete weight data available for 330) patients had a weight gain of more than 7% after 4 weeks compared to baseline. All patients included were Caucasians with exception of three patients of African, Jamaican and Hispanic descent, respectively. Baseline demographics are given in Table 30.

Table 30: Baseline demographics

Baseline Characteristics of Patients						
Variable	**n**	**Mean**	**SD**	**Range**		**Median**
age (years)	365	43.6	16.0	18	89	41
height (cm)	365	171.7	9.1	154	196	171
weight (kg)	365	72.4	14.1	40	125	70
BMI (kg/m^2)	365	24.5	4.2	14.2	43	24
length of hospitalization (days)	365	58.8	38.2	3	243	51
onset of treatment response (days)	323	31.3	29.5	0	236	21
CGI(1) severity baseline	360	5.9	0.9	2	8	6
CGI(1) severity 4 weeks	325	4.9	1.2	2	9	5
CGI(2) improvement 4 weeks	338	3.4	1.0	1	6	3
PDS-P at baseline	230	8.3	8.5	0	41	6
PDS-P after 4 weeks	230	5.7	6.6	0	29	3
PDS-P (baseline-4 weeks)	230	2.6	6.6	-17	34	1
PDS-D at baseline	230	18.5	10.0	0	44	18
PDS-D after 4 weeks	230	13.4	10.1	0	45	12
PDS-D (baseline-4 weeks)	230	5.1	9.1	-28	32	4
Adverse effects - DOTES score	328	2.3	3.3	0	30	1
cluster a (mental side effects)	328	0.7	1.4	0	9	0
cluster b (neuromuscular symptoms)	328	0.5	1.3	0	8	0
cluster c (anticholinergic symptoms)	328	0.4	1.1	0	9	0
cluster d (cardiovascular symptoms)	328	0.2	0.7	0	6	0
cluster e (other symptoms)	328	0.4	0.9	0	5	0
weight at baseline (kg)	330	72.7	14.5	43.6	124.0	70.0
weight after 4 weeks (kg)	330	74.2	14.2	44.0	124.5	70.8
weight gain (kg) after 4 weeks	330	1.5	3.1	-11.3	11.4	1.1
BMI gain after 4 weeks	330	0.5	1.0	-3.7	4.2	0.4
weight gain after 4 weeks (%)	330	2.3	4.4	-12.0	19.0	1.4

Note: n = number of available data, SD = standard deviation, BMI = body mass index

CGI = Clinical Global Impression, PDS = Paranoid Depression Scale, DOTES = Dosage Record and Treatment Emergent Symptoms Scale (see § 2.1.3 for measurement of clinical variables)

3.4.1.1 Diagnosis

One hundred sixty-four patients (44.9% of all 365 patients) were diagnosed with mood disorders (F3 according to the ICD-10). Two had a manic episode (F30), 26 (7.1%) a bipolar affective disorder (F31), 62 (16.9%) a depressive episode (F32), 73 (20%) a recurrent depressive episode and one a persistent mood disorder (F34.1 dysthymia).

Of the 174 (47.7%) patients with schizophrenia and other psychotic disorders (F2), 118 (32.3%) were diagnosed with schizophrenia (F20) and 45 (12.3%) with schizoaffective disorders. The remaining F2 patients were assessed with schizotypal disorder (F21, n = 1), persistent delusional disorders (F22, n = 3) and acute or transient psychotic disorders (F23, n = 7).

Twenty-seven (7.4%) patients had other mental and behavioral diagnoses according to the ICD-10 such as F60 (specific personality disorders that may be characterized by paranoid, schizoid, anxious or emotional unstable features), F40 (phobic anxiety disorders), F41 (other anxiety disorders), F42 (obsessive-compulsive disorders) and F43 (reaction to severe stress, and adjustment disorders) making treatment with antidepressants or neuroleptics necessary.

3.4.1.2 Medication

Two hundred thirty-four (64.1%) of the 365 patients were treated with antidepressants. As depicted in Figure 55 and specified in Table 62 in appendix A1, the most common antidepressants were mirtazapine (n = 122/365, 33.4%) and citalopram/escitalopram (n = 72/365, 19.7%). Overall, 114 (31.2%) of the patients were treated with selective serotonin reuptake inhibitors (SSRI) and 41 (11.2%) with tricyclic antidepressant drugs (TCA).

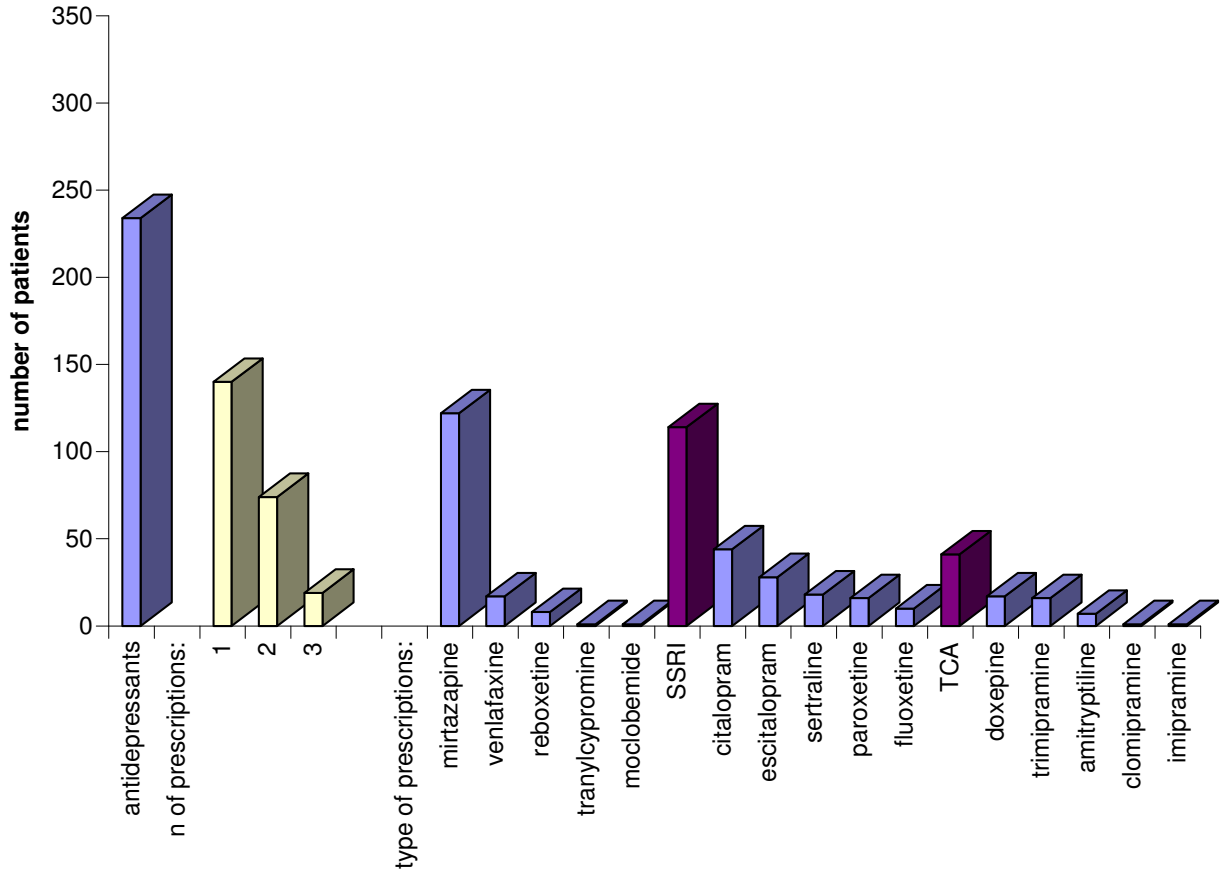

Figure 55: Prescribed antidepressant medication

Note: column 1: total number of patients treated with antidepressants during hospitalization (234 of 365 patients, 64.1%)
columns 2 - 4: number of prescribed antidepressants per patient: one (n = 171, 46.8%), two (n = 53, 14.5%) or three (n = 10, 2.7%) antidepressants during hospitalization
columns 5 - 21: type of prescribed antidepressants (detailed numbers are tabulated in Table 62 in appendix A1)

Two hundred ninety-two (80.0%) of all patients were treated with antipsychotic drugs. Of these, 187 (51.2%) received atypical, 37 (10.1%) typical and 68 (18.6%) both antipsychotics. As depicted in Figure 56 and specified Table 63 in appendix A1, the most common antipsychotics were olanzapine (n = 130/365, 35.6%) and risperidone (n = 61/365, 16.7%).

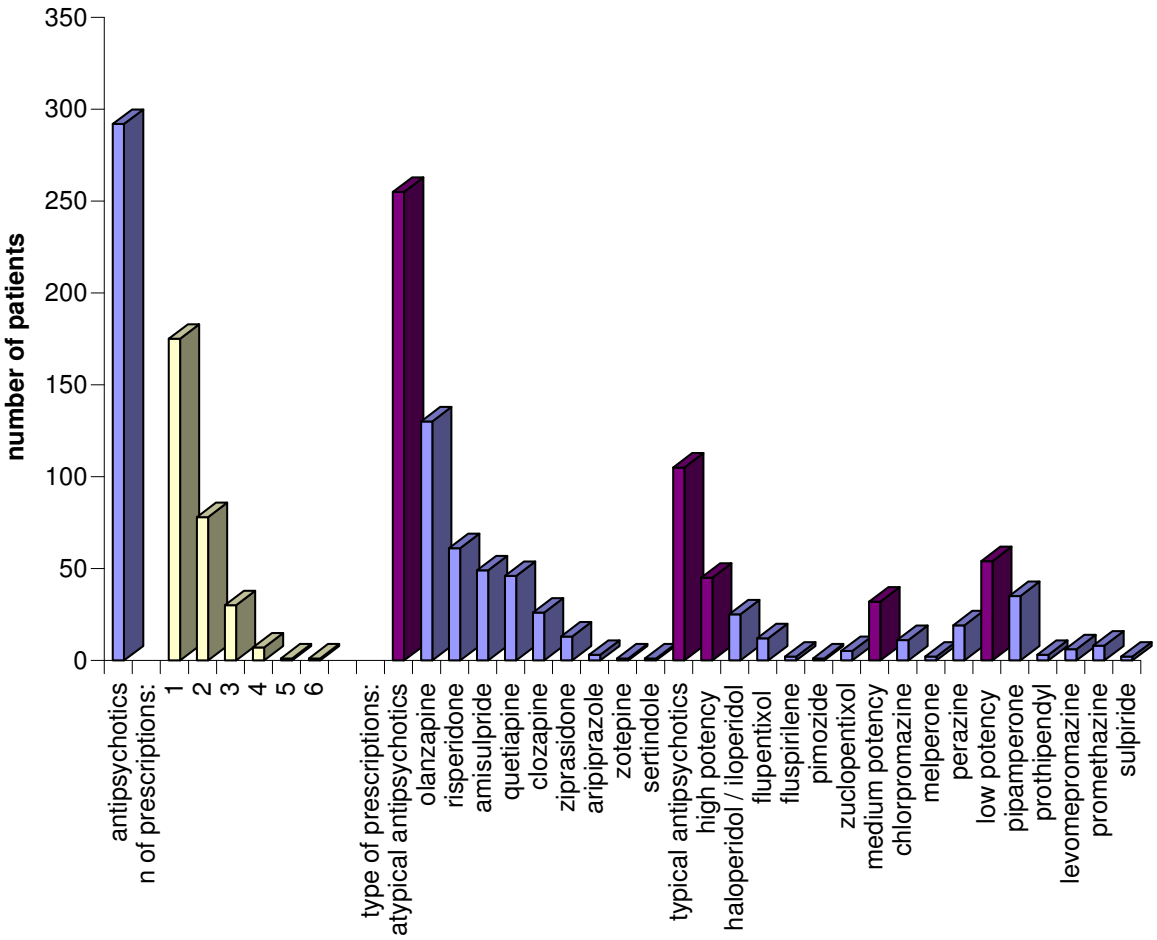

Figure 56: Prescribed antipsychotic medication

Note: column 1: total number of patients treated with antipsychotics during hospitalization (292 of 365 patients, 80.0%)
columns 2 - 7: number of prescribed antipsychotics per patient: one (n = 175, 47.9%), two (n = 77, 21.1%), three (n = 31, 8.5%), four (n = 7, 1.9%), five (n = 1, 0.3%) or six (n = 1, 0.3%) different antidepressants during hospitalization
columns 8 - 34: type of prescribed antidepressants further subdivided in typical and atypical ones (detailed numbers are tabulated in Table 63 in appendix A1)

92 (25.2%) of the patients received mood stabilizers. The most common mood stabilizers were lithium (n = 40/365, 11.0%) and carbamazepine (n = 29/365, 7.9%). Biperiden, which is used to treat parkinsonian side effects in antipsychotic drug therapy, was administered to 52 (14.2%) patients. Two hundred and sixteen (59.2%) received one or more sedative/anxiolytic drugs mainly lorazepam (n = 157/365, 43%) and lormetazepam (n = 37/365, 10.1%). Other frequent concomitant drugs were lipid-lowering drugs, antihypertensives, antidiabetic drugs, iodine, thyroxine, anticoagulants and proton pump inhibitors. The complete concomitant medication is tabulated in appendix A1 (Table 64).

3.4.1.3 Anamnestic Data

Former drug abuse was present in 72 patients (21 alcohol, 36 cannabinoids and 15 other substances or mixed abuse). Thirty-eight patients reported non-response to former antidepressant or antipsychotic medication. One hundred seven patients reported side effects due to antidepressant or antipsychotic medication. One hundred forty-nine (40.8%) patients reported to have at least one parent or sibling suffering also from mood or psychotic disorders, especially in patients with schizoaffective disorder (n = 26 of 45, 57.8%) and in bipolar patients (F31, n = 19 of 26, 73.1%).

Data on the course of disease was available for 351 patients. For 120 patients, the present episode was the first one. Of these, 61 received no antipsychotic or antidepressant treatment before hospitalization. The other patients had a median of 2 episodes before the present episode. The average duration of illness was 10.1 years with an average age of onset of 34.1 (± 13.9) years. For 188 patients, more detailed data on illness history was available with an average of 2.4 months for duration of a previous episode and an overall sum of 5.2 months of illness.

3.4.1.4 Response and Length of Hospitalization

Response was assessed at admission to hospital and after 4 weeks as described in § 2.1.3. The available response data is listed in Table 30 above. A small but significant reduction in PDS-Paranoid scale ($p < 0.001$, Wilcoxon-Signed-Rank-Test for two related samples), PDS-Depression scale ($p = 0.003$) and CGI(1) severity ratings ($p < 0.001$) was present in the study population showing an improvement of illness within the observation period.

The CGI(1) severity ratings by the treating psychiatrists and the self-assessments by the patients (PDS scales) were significantly correlated ($p < 0.001$). The correlations were of small size [300] at the admission to hospital (range $r = 0.1$ - 0.3) and of medium size [300] after 4 weeks (range $r = 0.3$ - 0.5). The correlation was most pronounced in the F2 subgroup (schizophrenic disorders) between CGI severity ratings and the PDS-Paranoid scores.

The correlation between CGI(2) improvement ratings after 4 weeks by the treating psychiatrists and the corresponding PDS-Depression scores was more pronounced ($r = 0.4$, $p < 0.001$, $n = 230$) than for the corresponding PDS-Paranoid scores ($r = 0.2$, $p = 0.003$, $n = 230$). Examination of the diagnosis subgroups gives an explanation for this. The correlation to the PDS-Paranoid scores was non-existent for the F3 subgroup (mood disorders).

The length of hospitalization was strongly correlated with the onset of treatment response ($r = 0.71$, $p < 0.001$, $n = 323$), although the patients were free to leave the hospital any time they wished. Length of hospitalization or onset of treatment response were only weakly correlated with the PDS-P and PDS-D scores or score changes ($r = 0.2$ - 0.3). But both showed a medium correlation with the CGI(1) severity rating after 4 weeks (both $r = 0.4$, $p < 0.001$, $n = 324$ and $n = 287$).

3.4.2 Serum Concentrations: Influence of Cytochrome P450 Polymorphisms

The available steady-state through levels (at least 10 days of drug intake for citalopram and at least 7 days for mirtazapine and olanzapine) were evaluated to examine the effect of CYP1A2, CYP2C19 and CYP2D6 polymorphisms on olanzapine, citalopram and/or mirtazapine serum concentrations. Moreover, the relationship to the clinical outcome (DOTES score - side effects and PDS / CGI - treatment response, see § 2.1.3) was assessed.

The following items were evaluated (cf. also § 2.4 Statistics):

- baseline characteristics of the respective subset
- strength and significance of the (linear) relationship between dose and serum concentrations (Spearman's rank correlation coefficient and p-value). This non-parametric method was used as the serum data did not fulfill the normal distribution assumption. The further possible influence variables on serum concentrations or dose-normalized concentrations were tested by Spearman's rank correlation (e.g., age, height, weight, duration of illness). Scatter plots were used to check the linearity of the relationship and to search for outliers.
- comparison of different groups to detect significant influence on (dose-normalized) serum concentrations e.g., between the genders, between genotypes, between smokers and non-smokers and between patients receiving different kinds of co-medication. This was tested by nonparametric tests for continuous data divided into categories i.e., Mann-Whitney U test (2 groups) or Kruskal-Wallis test (> 2 groups) if the data were not normal distributed. The dose-normalized, normal distributed olanzapine concentrations were tested by parametric tests (ANOVA, t-test). Box-and-whisker plots were used to examine the distribution of data and to search for outliers. Multiple linear regression analysis was used to calculate the combined effect of all relevant influence variables/factors together.
- in a second step, the relationships between dose-normalized serum concentrations and outcome variables (side effects, response, weight gain) were examined under consideration of possible other influence factors if the data set was large enough.
- in a third step, the relationships between genotypes and outcome variables were examined under consideration of possible other influence factors (e.g., co-medication, diagnosis) if the data set was large enough. The Chi-square test, the Fisher-Exact-test (for 2 x 2 groups comparison) and the Odds Ratio were used for comparison of categorical data. The Odds Ratio is the probability that the event of interest occurs to the probability that it does not. Thus, it is an indicator of the strength of the relationship between categorical data.

3.4.2.1 Citalopram

CYP2C19 plays an important role in the metabolism of the selective serotonin reuptake inhibitor (SSRI) citalopram that is a racemic drug consisting of a 1:1 mixture of the *R*(–)- and *S*(+)-enantiomers [308-310]. The pharmacological antidepressive effect resides mainly in the *S*(+)-enantiomer, which is also marketed alone as escitalopram [311]. Moreover, the two enatiomers have different clearance rates as the eutomer S(+)-enantiomer is preferentially metabolized by CYP2C19 [312].

It is well known from many studies that CYP2C19 poor metabolizers (two inactive alleles) have a significant slower citalopram metabolism with elevated serum concentrations [313-317]. The clinical significance of heterozygosity with one inactive allele and partially impaired metabolic activity, however, has not yet been clearly demonstrated.

3.4.2.1.1 Study Subpopulation – Baseline Characteristics

Of the 39 patients included, 13 were treated with escitalopram doses ranging from 5 to 30 mg/day (median 10 mg/day) and 26 were treated with citalopram doses ranging from 10 to 60 mg/day (median 20 mg/day). Twenty-two patients were diagnosed with mood disorders (F3 diagnosis), 14 patients with schizophrenic disorders (F2 diagnosis) and 3 patients with other disorders (F4 diagnosis) according to ICD-10 [29].

The obtained demographic data, baseline characteristics and serum concentrations are summarized in Table 31. Moreover, the co-medication is summarized in Table 31. The study was not designed as an interaction study. For single drugs, there was insufficient data to support a statistical evaluation of correlations with dose-corrected citalopram concentration. Therefore, drugs were rated as substrates, inhibitors and inducers of cytochrome P450 isoenzymes CYP1A2, CYP2C19, CYP2D6 and CYP3A4 according to the Cytochrome P450 Drug Interaction Table [69] and the dose-corrected concentrations were compared for each cytochrome P450 isoenzyme.

There were no significant differences in baseline characteristics and demographic data between the escitalopram and the racemic citalopram group or the different CYP2C19 genotypes. In Table 33, the baseline characteristics and (dose-normalized) serum concentrations are presented separately for CYP2C19 extensive and intermediate/poor metabolizers.

The treating psychiatrist assessed the clinical adverse effects according to the DOTES rating scale. If clearly attributable, the drug(s) causing the reported side effect(s) were documented as well. It has to be considered in a naturalistic setting that co-medication may also lead to side effects. Thus, patients suffering from side effects due to co-medication were evaluated in an extra group ("adverse effects due to co-medication", see Table 32). Moreover, only DOTES items known to be associated with citalopram intake were evaluated together as (possibly) citalopram-related side effects: nausea/vomiting, sweating, diarrhea, dry mouth, anxiety/agitation, trembling, insomnolence/sleep disturbance, headache, dizziness, fatigue/drowsiness, cardiac arrhythmia and blood pressure changes. Sexual dysfunction was not reported within the study population. Other DOTES items such as extrapyramidal-motoric side effects were not considered. Due to the small number of patients and low number of side effects reported, only the overall incidence of side effects and not the scores were evaluated.

Clinical outcome measures were assessed after 4 weeks of therapy and included the PDS rating scale and the CGI(1) severity and CGI(2) improvement ratings. A small but significant reduction in the PDS-Depression subscale ($p = 0.001$, Wilcoxon-Signed-Rank-Test for two related samples) and CGI(1) severity ratings ($p = 0.004$) was observed after 4 weeks indicating improvement of illness within the observation period. Clinical response was defined as CGI(2) score of 1 or 2 after 4 weeks of therapy or a PDS-D score improvement of more than 50%.

Table 31: Baseline characteristics of citalopram subpopulation

	R,S-citalopram (n= 26)		S-citalopram (n= 13)	
	Mean	**SD**	**Mean**	**SD**
age (years)	44.4	15.2	44.1	14.8
height (cm)	172.7	9.6	167.5	10.3
weight (kg)	74.2	14.3	71.5	11.4
dose (mg/day)	28.1	12.0	15.4	7.5
serum drug concentration (ng/mL)	58.5	39.3	19.9	9.2
dose-normalized conc.(ng/mL/mg)	2.02	0.83	1.43	0.77
dose- and body weight normalized conc. (ng/mL per mg/kg)	148.0	66.2	98.5	45.6
CGI(1) severity baseline	5.9	0.9	6.0	0.7
CGI(1) severity 4 weeks	5.3	1.2	5.2	1.2
CGI(2) improvement 4 weeks	3.7	1.2	3.6	1.0
	n	**%**	**n**	**%**
CGI responder (CGI(2) < 3 after 4 weeks)	3	11.5	3	23.1
PDS responder (PDS-D improvement > 50%)	6	23.1	5	38.5
citalopram-specific adverse effects	7	26.9	2	15.4
male	14	53.8	4	30.8
CYP2C19 genotype				
poor (gene dose 0)	1	3.8	0	0.0
intermediate (gene dose 1.0)	7	26.9	4	30.8
extensive (gene dose 2.0)	18	69.2	9	69.2
Relevant co-medication				
CYP3A4 inducer carbamazepine	1	3.8	1	7.7
CYP2C19 substrates*	3	11.5	1	7.7
no co-medication	4	15.4	0	0.0
other co-medication**	18	69.2	10	76.9
CYP2C19 inhibitor omeprazole	0	0	1	7.7

* amitriptyline, diazepam, pantoprazole, trimipramine [318], flunitrazepam [319]
** olanzapine, clozapine, risperidone, mirtazapine, doxepin, metoprolol, venlafaxine, medroxyprogesterone

Table 32: Citalopram-related adverse effects

	citalopram (ng/mL)			dose (mg)	
	n	Mean	SD	Mean	SD
	R,S-citalopram (n = 26)				
adverse effects due to co-medication	2	40.1	13.0	20.0	0.0
no adverse effects	17	53.3	30.4	27.1	12.1
adverse effects due to citalopram	7	76.6	58.1	32.9	12.5
	S-citalopram (n = 13)				
adverse effects due to co-medication	7	15.6	7.8	14.3	7.3
no adverse effects	4	20.9	7.4	17.5	9.6
adverse effects due to citalopram	2	33.0	2.4	15.0	7.1

Table 33: Baseline characteristics for CYP2C19 intermediate and extensive metabolizers

	R,S-citalopram (n = 26)				S-citalopram (n = 13)			
	EM (n = 18)		IM/PM (n = 7/1)		EM (n = 9)		IM (n = 4)	
Variable	Mean	SD	Mean	SD	Mean	SD	Mean	SD
age (years)	42.4	16.1	48.9	13.1	39.8	14.9	53.8	10.0
height (cm)	174.4	9.5	168.8	9.1	169.0	12.2	164.8	6.7
weight (kg)	77.7	13.5	66.2	13.4	74.9	12.1	63.9	4.2
dose (mg/day)	27.8	13.1	28.8	9.9	15.6	8.5	15.0	5.8
serum drug concentration (ng/mL)	52.1	43.7	73.0	23.4	15.7	7.1	29.3	5.6
dose-normalized conc. (ng/mL/mg)	1.76	0.80	2.61	0.59	1.11	0.44	2.16	0.92
dose- and body weight normalized conc. (ng/mL per mg/kg)	138.7	75.3	168.9	34.3	81.6	30.5	136.8	54.9
	n	%	n	%	n	%	n	%
citalopram-specific adverse effects	4/18	22	3/8	38	0/9	0	2/4	50

EM = extensive metabolizer, IM = intermediate metabolizer, PM = poor metabolizer

3.4.2.1.2 Racemic Citalopram

There was a strong and significant relationship between daily dose and serum concentrations (Spearman's r = 0.8, $p < 0.001$). Age, height, weight did not show any significant correlations with absolute or normalized serum concentrations or administered dose.

Patients with impaired metabolism (CYP2C19 IM/PMs) had significant higher serum concentrations, dose-normalized serum concentrations as well as dose/body weight-normalized concentrations compared to patients with normal metabolism (CYP2C19 EMs, Mann-Whitney U test, p = 0.017, p = 0.003 and p = 0.023, respectively, mean ± SD concentrations are given in Table 33). The only poor metabolizer (PM) showed a similar dose-normalized citalopram concentration compared with the 7 intermediate metabolizers (IMs, 2.72 ng/mL/mg, 197.8 ng/mL per mg/kg compared to overall median of 2.81 ng/mL/mg, 180.5 ng/mL per mg/kg) and was therefore evaluated together with the IMs in one group (see Table 33).

Administered doses or dose/body weight-normalized concentrations were not significantly different between male and female patients or between patients who received CYP2D6, CYP3A4 or CYP2C19 relevant co-medication and those who did not (all p > 0.05). One EM patient received the CYP3A4 inducer carbamazepine (200 mg/day) and showed similar normalized concentrations (1.65 ng/mL/mg, 90.5 ng/mL per mg/kg) compared with the 17 other EM patients (overall median 1.54 ng/mL/mg, 107.6 ng/mL per mg/kg). One EM patient with measurable concentrations of norfluoxetine had a 2.6-fold higher serum concentration of citalopram (4.07 ng/mL/mg, 329.7 ng/mL per mg/kg) compared to the other EM patients despite discontinuation of the CYP2C19, CYP3A4 and CYP2D6 inhibitor fluoxetine four weeks ago (Note: half-life of fluoxetine 4 - 6 days, half-life of norfluoxetine 4 - 16 days [320]). None of the patients received other CYP2C19, CYP2D6 or CYP3A4 inhibitors such as omeprazole, fluvoxamine or cimetidine.

Citalopram concentrations did not differ between those patients with a subsequent switch to another antidepressant and those without. Dose reduction was not significantly more common in patients with higher serum concentrations or vice versa. Patients with citalopram-related side effects (n = 7, 76.6 ng/mL, see Table 32) had higher citalopram concentrations compared to those without side effects or those where co-medication was responsible for the side effects (n = 17, 53.3 ng/mL and n = 2, 40.1 ng/mL). This was not statistically significant (Mann-Whitney U test, p > 0.2). Citalopram concentrations did not significantly differ between responder vs. non-responder (p > 0.2).

3.4.2.1.3 Escitalopram

The correlation between daily dose and escitalopram serum concentrations was not statistically significant (Spearman's r = 0.45, p = 0.122) due to the small number of patients and outliers (e.g., due co-medication with esomeprazole). Among the 13 patients treated with escitalopram, there were 4 IMs (CYP2C19*1/*2, *1/*3 or *1/*4) and 9 EMs (CYP2C19*1/*1).

In the IM group, co-medication with the CYP2C19 inhibitor esompeprazole (20 mg/day) led to a more than doubled escitalopram concentration (3.47 ng/mL/mg, 214.8 ng/mL per mg/kg) compared with the patients without relevant co-medication (1.56 and 2.13 ng/mL/mg, 93.8 and 134.5 ng/mL per mg/kg), while the patient receiving the CYP3A4 inducer carbamazepine (600 mg/day) showed a similar normalized concentration (1.49 ng/mL/mg, 104.0 ng/mL per mg/kg)

CYP2C19 IMs had significantly higher absolute concentrations, dose-normalized concentrations as well as dose/body weight-normalized concentrations compared to the EMs (Mann-Whitney U test, p = 0.009, p = 0.031 and p = 0.045, respectively, mean ± SD concentrations are given in Table 33). Apart from the CYP2C19 genotype and esomeprazole, no significant correlations or differences were observed for age, height, weight, gender, co-medication vs. dose/body weight-normalized concentrations, absolute serum concentrations or administered dose.

In the treatment period following the assessment of serum concentrations until release from hospital, the escitalopram concentrations did not significantly differ between patients with switch to another antidepressant and those without switch. Dose changes showed a trend towards an inverse relationship with the serum concentrations especially for dose reduction in patients with higher concentration, but this was not statistically significant. Citalopram concentrations did not significantly differ between responder vs. non-responder (p > 0.1).

Patients with (possibly) citalopram-related side effects (33.0 ng/mL, n = 2, see Table 32) were compared to those without side effects (20.9 ng/mL, n = 4) plus to those where co-medication was responsible for the side effects (15.6 ng/mL, n = 7). As shown in Figure 57, citalopram-related adverse effects were associated with higher escitalopram concentrations (Mann Whitney U test, p = 0.026). A trend towards more side effects was also seen for the CYP2C19 IMs compared to EMs (2/4 vs. 0/9, see Table 33, Fisher-Exact-test p = 0.077).

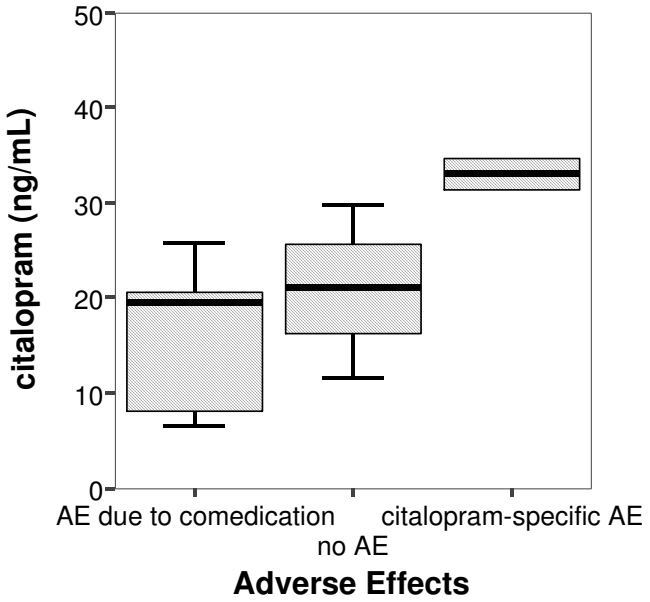

Figure 57: Escitalopram: serum concentrations and side effects

Box-and-whisker plot: The center box shows median and interquartile range of data with whiskers indicating total range of data.
Group "AE due to co-medication" = side effects are due to co-medication (median 20 ng/mL, range 7.5 - 26 ng/mL, n = 7)
Group "no AE" = no side effects (median 21 ng/mL, range 12.6 – 30 ng/mL, n = 4)
Group "citalopram-specific AE" = side effects are due to escitalopram (median 33 ng/mL, range 31 – 35 ng/mL, n = 2)

3.4.2.1.4 Comparison of Racemic Citalopram vs. Escitalopram

As shown in Figure 58, serum concentrations were significantly higher in patients with impaired metabolism compared to extensive metabolizers both for racemic citalopram and for escitalopram (also after normalization for dose or dose and body weight, see previous chapters for p-values and Table 33 for concentrations).

Concisely, approx. two-fold higher dose-normalized serum concentrations of escitalopram were measured in IMs compared to EMs. Moreover, more side effects were observed for higher escitalopram serum concentrations. This effect was less pronounced in patients treated with racemic citalopram where an increase of only 1.5-fold for IMs compared to EMs was observed. Serum concentrations were not significantly associated with side effects.

Comparing side effects between the genotypes (CYP2C19 IMs vs. EMs), a trend towards more citalopram-specific side effects was found for patients receiving escitalopram (2/4 vs. 0/9, Fisher-Exact-test $p = 0.077$) but not for the racemate (3/8 = 38% vs. 4/18 = 22%, $p > 0.1$). The study subset was too small to detect significant differences in side effects between racemic citalopram (7/26, 27%) and escitalopram (2/13, 15%, Fisher-Exact-test $p > 0.1$).

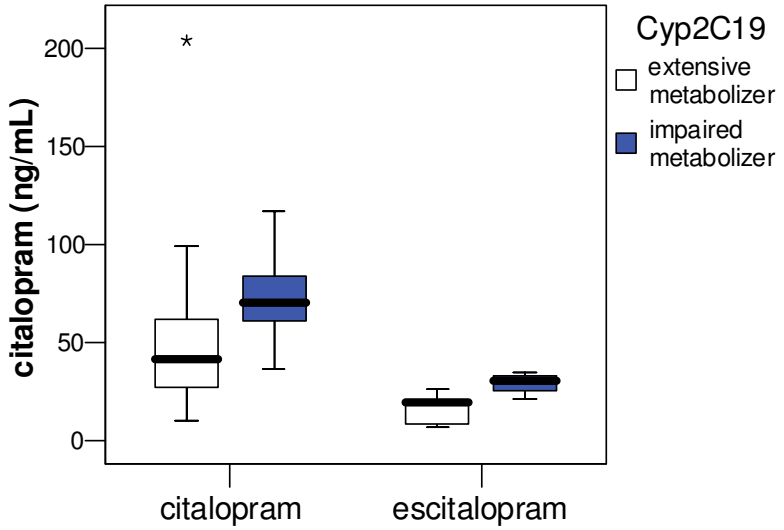

Figure 58: Comparison of racemic citalopram and escitalopram concentrations in patients with extensive and impaired CYP2C19 metabolism

Box-and-whisker plot: The center box shows median and interquartile range of data. Outliers and extremes are marked individually (here one extreme value: EM, 204 ng/mL racemic citalopram, former intake of fluoxetine). As outliers and/or extremes exist, the whiskers range is defined as 1.5 times the interval from the 25th to 75th percentile i.e., the box length. Extremes are more than 3 box lengths away (cross).

Significant difference (Mann-Whitney U test, $p = 0.017$) between racemic citalopram / EM (median 41 ng/mL, range 10 - 99 ng/mL, n = 18, outlier 204 ng/mL) and racemic citalopram / IM+PM (median 71 ng/mL, range 36 - 117 ng/mL, n = 8). Significant difference (Mann-Whitney U test, $p = 0.009$) between escitalopram / EM (median 20 ng/mL, range 7 - 26 ng/mL, n = 9) and escitalopram / IM (median 31 ng/mL, range 21 - 35 ng/mL, n = 4). Significant differences between escitalopram and racemic citalopram for EM (Mann-Whitney U test, $p = 0.0002$) and IM/PM (Mann-Whitney U test, $p = 0.007$).

3.4.2.2 Mirtazapine

The antidepressant mirtazapine is a racemic drug with both enantiomers exhibiting pharmacological activity [321]. The adrenoceptor (ADR2) and serotonin (5-HTR2) receptor antagonist is metabolized by CYP3A4, by CYP2D6 and to a lesser extent by CYP1A2. CYP2D6 contributes especially to the metabolism of the S-enantiomer. Therefore, the clinical relevance of the CYP2D6 metabolizer status for the pharmacokinetics of the racemate is under discussion [307,322-324].

3.4.2.2.1 Study Subpopulation

Mirtazapine steady-state serum concentrations were available for 50 patients, who received mirtazapine as part of their clinical treatment. Of these, 40 patients had mood disorders (F3 diagnosis), 7 schizophrenic disorders (F2 diagnosis) and 3 other disorders (F4 diagnosis) according to ICD-10 [29]. The obtained demographic data, baseline characteristics and serum concentrations are summarized in Table 34. Moreover, the CYP2D6 and CYP1A2*1F genotypes are given in Table 34. Of the 50 participants, 3 were CYP2D6 poor metabolizers, 20 were intermediate metabolizers, 24 were extensive metabolizers and 3 were ultrarapid metabolizers.

Table 34: Baseline characteristics of mirtazapine subpopulation

Mirtazapine (n = 50)	**Mean**	**SD**
age (years)	51.6	15.0
height (cm)	171.8	9.3
weight (kg)	73.4	12.6
dose (mg/day)	35.5	11.2
serum drug concentration (ng/mL)	74.8	49.2
dose-normalized conc. (ng/mL/kg)	2.08	1.11
dose- and body weight normalized conc. (ng/mL per mg/kg)	150.1	77.3
CGI(1) severity baseline	6.0	0.8
CGI(1) severity 4 weeks	5.1	1.1
CGI(2) improvement 4 weeks	3.5	0.8
	n	**%**
CGI responder (CGI(2) < 3 after 4 weeks)	5	10.0
PDS responder (PDS-D improvement > 50% after 4 weeks)	13 of 43 available	30.2
adverse events rated as moderate/marked	16	32.0
male	26	52.0
smoker	12	24.0
CYP2D6 genotype		
poor (gene dose 0)	3	6.0
intermediate (gene dose 0.5 / 1.0)	4 / 16	8.0 / 32.0
extensive (gene dose 1.5 / 2.0)	7 / 17	14.0 / 34.0
ultrarapid (gene dose > 2.0)	3	6.0
CYP1A2*1F genotype		
wildtype	5	10.0
heterozygote	23	46.0
mutant	22	44.0

Note: PDS-response was defined as improvement of > 50% after 4 weeks compared to baseline depression score in patients with an at least moderate depression score at baseline

The subjects participating in this study received co-medication as given in Table 35. The study was not designed as an interaction study. For single drugs, there was insufficient data to support a statistical evaluation. Therefore, drugs were rated as substrates, inhibitors and inducers of cytochrome P450 isoenzymes CYP1A2, CYP2C19, CYP2D6 and CYP3A4 [69].

Table 35: Co-medication of mirtazapine subpopulation

	Substrates	Inhibitors	Inducers
CYP1A2	clozapine, estradiol, haloperidol, olanzapine, propranolol, verapamil	-	omeprazole, carbamazepine [325,326]
CYP2C19	amitriptyline, citalopram, clopidogrel, doxepin [327], medroxyprogesterone, pantoprazole, propranolol, omeprazole	pantoprazole, omeprazole	carbamazepine
CYP2D6	amitriptyline, doxepin [327], haloperidol, metoprolol, propranolol, metoclopramide, risperidone	sertraline (*very weak inhibitors: citalopram, doxepin, haloperidol, metoclopramide)*	
CYP3A4	alprazolam, amlodipine, atorvastatin, felodipine, haloperidol, medroxyprogesterone/estradiol, pioglitazone [328], propranolol, quetiapine, risperidone, simvastatin, verapamil, ziprasidone, zolpidem	verapamil	carbamazepine, pioglitazone

Note 1: If not listed in Cytochrome P450 Drug Interaction Table [69], the literature reference is noted.

Note 2: no major contribution of cytochrome P450 isoenzymes given above to the metabolism of the following drugs: allopurinol, amisulpride, bisoprolol, bromazepam, candesartan, enalapril, flupentixol, furosemide, hydrochlorothiazide, iodine, lithium, lorazepam, lamotrigin, maprotiline, melperone, metformin, pravastatin, pipamperone, piretanide, ramipril, rofecoxib, reboxetine, T4, tamsulosine, torasemide, triamteren, zopiclon and CYP2C9 substrates losartan, torsemide, glimepiride

Clinical outcome measures were assessed after 4 weeks of therapy and included the PDS rating scale and the CGI(1) severity and CGI(2) improvement ratings. A small but significant reduction in the PDS-Depression subscale ($p < 0.001$, Wilcoxon-Signed-Rank-Test for two related samples) and CGI(1) severity ratings ($p < 0.001$) was observed after 4 weeks indicating improvement of illness within the observation period. Clinical response was defined as CGI(2) score of 1 or 2 after 4 weeks of therapy or a PDS-D score improvement of more than 50% with a baseline score > 6.

Mirtazapine is known to induce weight gain [329]. Thus, side effects and weight gain were assessed after 4 weeks. It has to be considered in a naturalistic setting that co-medication may also lead to side effects. Patients suffering from side effects or weight gain and receiving relevant co-medication were evaluated in an extra group.

3.4.2.2.2 Serum Concentrations - Influence of Age, Gender, Co-medication and Genotype

There was a strong and significant relationship between daily dose (range 15 to 60 mg/day) and the serum concentrations (range 14.5 - 227 ng/mL, Spearman's $r = 0.6$, $p < 0.001$).

Women had on average 1.6-fold higher absolute and dose-corrected concentrations than men (mean ± SD: 93 ± 56 vs. 58 ± 35 ng/mL; Mann-Whitney U test p= 0.020 and 2.6 ± 1.3 vs. 1.6 ± 0.7 ng/mL/mg; $p = 0.002$) despite comparable doses (36 ± 13 mg vs. 35 ± 10 mg in men, $p > 0.8$). This is partly due to the weight differences. The difference in dose- and body weight-normalized concentrations was not significant (172 ± 90 vs. 130 ± 59 ng/mL per mg/kg, $p = 0.111$). Similar statistical results were obtained after exclusion of the three patients receiving carbamazepine.

Moreover, a clear age-related difference was found. Elderly patients (over 60 years, n = 17) had on average 1.6-fold higher absolute mirtazapine concentrations (101 ± 61 vs. 61 ± 36 ng/mL; Mann-Whitney U test, p = 0.023) than younger ones (n = 33, 44 ± 12 years). Despite the trend to somewhat higher doses in elderly patients (39 ± 11 mg vs. 33 ± 11 mg, p = 0.076), the dose- as well as the dose- and body weight-corrected concentrations were on average 1.3-fold higher in elderly patients (2.5 ± 1.2 vs. 1.9 ± 1.0 ng/mL/mg; p = 0.036 and 180 ± 84 vs. 135 ± 70 ng/mL per mg/kg, p = 0.050). Similar statistical significant results were obtained after exclusion of the three patients receiving carbamazepine. Notably, the variability of concentrations was in elderly patients much higher than in younger ones, especially in females (compare concentration ranges in Figure 59).

Dose- and body weight normalized serum concentrations - in consideration of age and gender as given in Figure 59 - were compared for each of the relevant cytochrome P450 enzymes (CYP3A4, CYP1A2 and CYP2D6 substrates, inducers and inhibitors). CYP3A4 inducer carbamazepine significantly decreased serum levels in three patients to about a third (n = 3, 51 ± 8 ng/mL per mg/kg) compared to all other patients (n = 46, 154 ± 75 ng/mL per mg/kg). The patient receiving the CYP3A4 inhibitor verapamil seem to have an elevated mirtazapine level (252 ng/mL per mg/kg), but the level was within the range of the age/gender subgroup (compare column females > 60 years in Figure 59). Mirtazapine concentrations were similar for patients receiving CYP3A4 substrates compared to those receiving no CYP3A4 substrates. Similar comparisons for CYP1A2 and CYP2D6 showed no differences beyond the “background” noise of age and gender. Neither the coadministration of CYP1A2 inducer omeprazole in 4 patients nor the coadministration of CYP2D6 inhibitors such as sertraline (n = 3) led to any outlier deviations.

The combined influence of co-medication, gender and age on mirtazapine dose- and body weight normalized concentrations is depicted in Figure 59. Notably, two outliers (female, < 60 years, > 320 ng/mL) could not be explained by age, gender or co-medication. However, the exceptional high number of co-medications (losartan, hydrochlorothiazide, glimepirid, metformin, pioglitazone) and the CYP2D6 poor metabolizer status may provide an explanation.

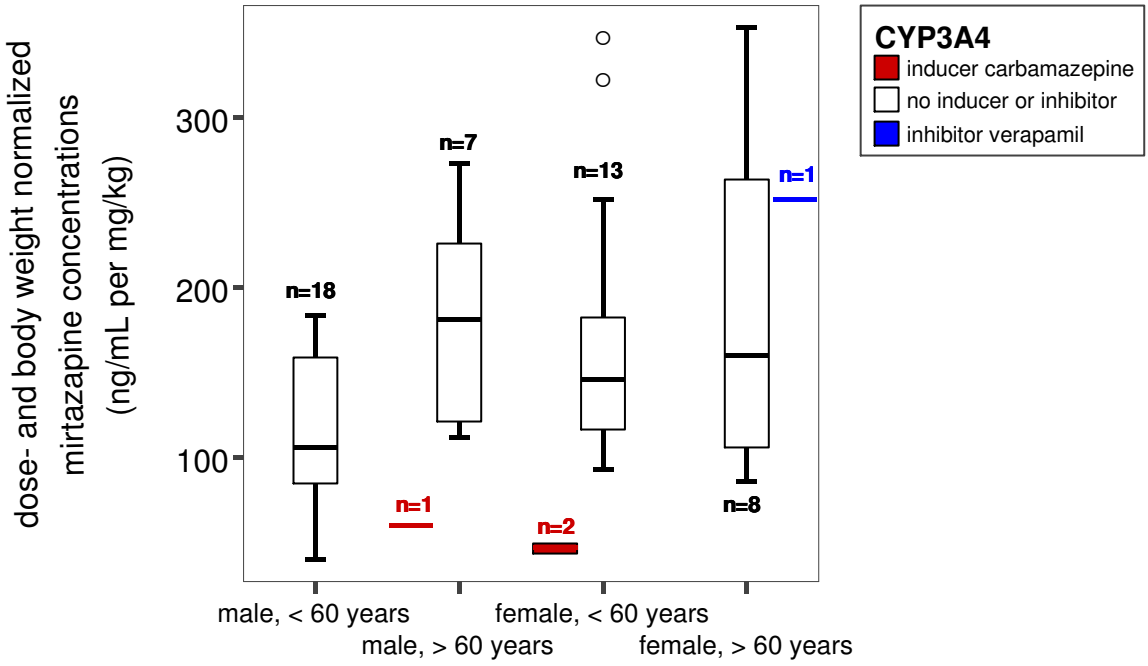

Figure 59: Influence of age and gender on mirtazapine concentrations

Box-and-whisker plot: The center box shows median and interquartile range of data. Outliers are marked individually as open circles (female, < 60 years, 322 and 347 ng/mL per mg/kg). As outliers exist, the whiskers range is defined as 1.5 times the interval from the 25th to 75th percentile i.e., the box length. Outliers are between 1.5 and 3 box lengths away.
male < 60 years (n = 18, median 105, range 39 - 183 ng/mL per mg/kg)
male > 60 years (n = 7, median 180, range 112 - 273 ng/mL per mg/kg, and 1 with carbamazepine 60 ng/mL per mg/kg)
female < 60 years (n = 13, median 146, range 93 - 347 ng/mL per mg/kg and 2 with carbamazepine 44 / 49 ng/mL per mg/kg)
female > 60 years (n = 8, median 160, range 86 - 353 ng/mL per mg/kg, and 1 with verapamil 252 ng/mL per mg/kg)

A significant difference was detected when all four groups were tested without the 3 patients receiving carbamazepine (Kruskal-Wallis test, p = 0.031). This difference remained significant in the one-to-one test for male < 60 years vs. all other groups (Mann-Whitney U tests, p = 0.021, p = 0.020, p = 0.023).

No significant differences in absolute and normalized serum concentrations were seen for CYP2D6 and CYP1A2*1F genotypes. A trend for the different CYP2D6 metabolizers was visible within the age/gender subgroups despite the small sample size (see Figure 60). In general, higher dose- and body weight normalized concentrations were observed for PMs in all subgroups. Except for male > 60 years, IMs had the second highest concentrations, whereas EMs and UMs had similar low concentrations.

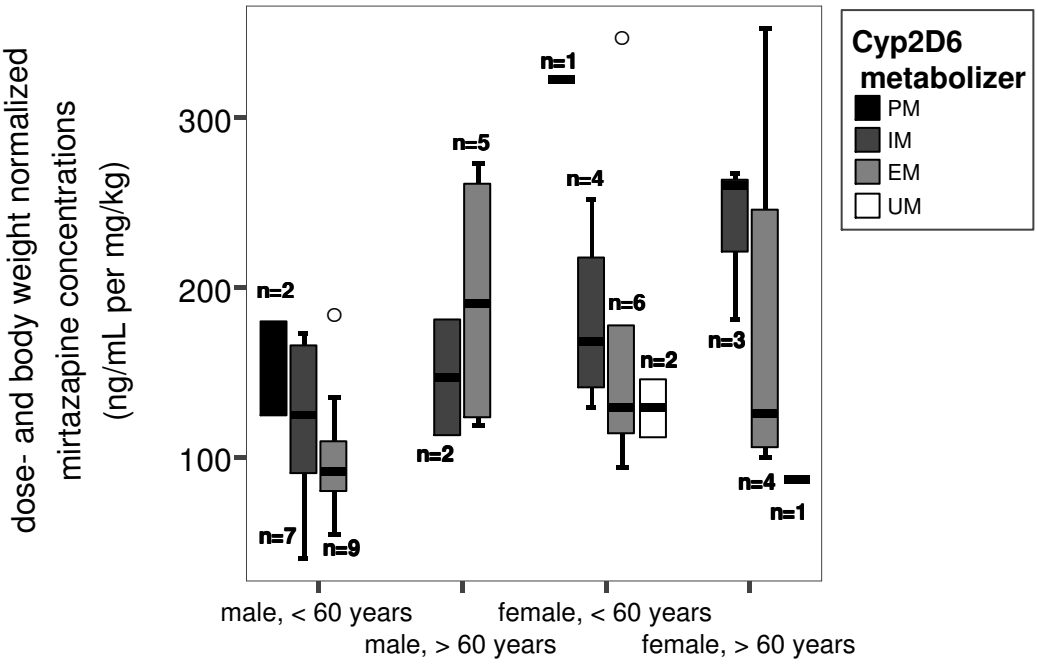

Figure 60: Influence of genotype on mirtazapine concentrations

Box-and-whisker plot: The center box shows median and interquartile range of data (data of patients receiving the CYP3A4 inducer carbamazepine not included). The two outliers are marked individually with open circles. As outliers exist, the whiskers range is defined as 1.5 times the interval from the 25th to 75th percentile i.e., the box length. Outliers are between 1.5 and 3 box lengths away.
PM = poor metabolizers (gene dose 0), IM = intermediate metabolizers, gene dose 0.5 - 1), EM = extensive metabolizers (gene dose 1.5 - 2) and UM = ultrarapid metabolizers (gene dose > 2)
Male < 60 years: higher average concentrations were observed for 2 PM > 7 IM > 9 EM (no UM)
Male > 60 years: a deviating trend was observed with 5 EM > 2 IM
Female < 60 years: higher average concentrations were observed for 1 PM > 4 IM > 6 EM ~ 2 UM
Female > 60 years: higher average concentrations were observed for 3 IM > 4 EM ~1 UM.

3.4.2.2.3 Serum Concentrations and Clinical Outcome

Better therapeutic improvement tended to be associated with higher mirtazapine concentrations (CGI responders, n = 5, 109 ± 68 ng/mL vs. non-responders, n = 45, 71 ± 46 ng/mL, Mann-Whitney U test, p = 0.081). No such relationship was observed for PDS-D scores. No significant differences in administered doses, age, gender, baseline weight, height or baseline severity of illness CGI(1) were present for non-responders vs. responders.

Serum concentrations were not associated with side effects (16 patients with side effects, 75 ± 40 ng/mL, compared to 34 without, 74 ± 53 ng/mL, Mann-Whitney U test, p > 0.6). Patients with and without moderate/marked side effects received comparable doses (35 ± 10 mg vs. 35 ± 12 mg, p > 0.9).

Weight data was available for 49 patients. No significant correlation between weight gain and mirtazapine concentrations was observed. Of the 12 patients with clinically significant weight gain after 4 weeks (> 7% of baseline), 9 patients received co-medication leading also to weight gain (risperidone 3x, olanzapine 4x, clozapine 2x) and that had similar concentrations to those without relevant weight gain (see Figure 61). The three patients receiving no relevant co-medication tended to higher concentrations than the rest of the patients (Mann-Whitney U test p = 0.080).

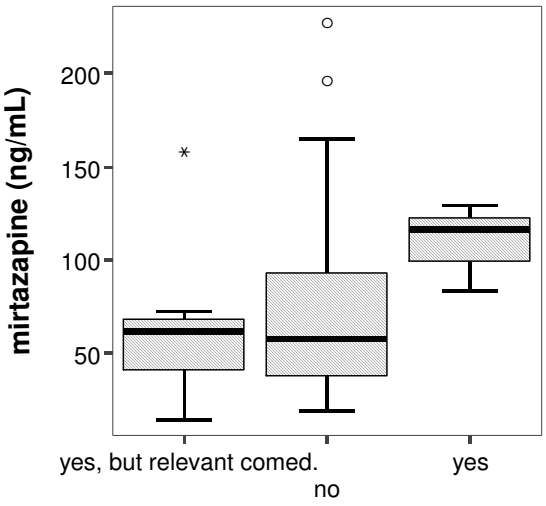

Figure 61: Mirtazapine serum concentrations and weight gain

Box-and-whisker plot: The center box shows median and interquartile range of data with whiskers indicating total range of data. Outliers and extremes are marked individually, and as outliers and extremes exist, the whiskers range is defined as 1.5 times the interval from the 25th to 75th percentile (the box length). Outliers are between 1.5 and 3 box lengths away (open circle), extremes are more than 3 box lengths away (cross). One extreme and two outliers were detected.

patients with relevant co-medication (n = 9, median 62, range 15 - 72 ng/mL with one extreme at 158 ng/mL)
patients without weight gain (n = 37, median 58, range 19 - 165 ng/mL with two outliers at 227 and 195 ng/mL)
patients with weight gain (n = 3, 84, 116 and 129 ng/mL)

The treating psychiatrist was blind to the result of serum concentrations. In the treatment period following the assessment of serum concentrations until release from hospital, dose changes (assessed as higher, unchanged or lower, see Table 36 below) and switches from mirtazapine to other antidepressant drugs were recorded.

The dose changes showed an inverse relationship (dose reduction in patients with higher levels and vice versa) with serum concentrations, the dose itself and dose-normalized concentrations ($p = 0.002$, $p = 0.034$ and 0.041) but not for dose- and body weight normalized results ($p = 0.119$, see Table 36 below). Dose decrease was more common in patients with marked/moderate side effects (5/7, 71%), whereas side effects were reported for only 33% (3/9) or 24% (8/34) of the patients with dose increase or stable dose, respectively (Pearson Chi-square test, $p = 0.047$). Mirtazapine concentrations did not differ between those patients with a subsequent switch to another antidepressant and those without (n = 12, 83 ± 48 ng/mL compared to 72 ± 50 in all other patients, $p > 0.8$). All 12 switched patients were CGI non-responders and 7 had marked or moderate side effects and received significantly higher doses (43 ± 9 mg compared to 33 ± 11 mg in other patients, Mann-Whitney U test, $p = 0.011$).

Table 36: Mirtazapine concentrations and dose changes

dose change after day of serum measurement		mirtazapine (ng/mL)	mirtazapine dose (mg/day)	dose-normalized mirtazapine (ng/mL/mg)	dose- and body weight normalized mirtazapine (ng/mL per mg/kg)
lower dose	Mean	109.6	42.9	2.7	198.1
n = 7	SD	54.6	10.4	1.5	99.9
unchanged dose	Mean	78.1	36.0	2.1	148.7
n = 34	SD	48.4	10.7	1.0	73.8
higher dose	Mean	35.1	27.8	1.4	118.1
n = 9	SD	7.3	9.7	0.6	59.4
total	Mean	74.8	35.5	2.1	150.1
n = 50	SD	49.2	11.2	1.1	77.3
Kruskal-Wallis test (p)		0.002	0.034	0.043	0.119

3.4.2.2.4 Genotype and Clinical Outcome

There were no significant differences in baseline characteristics (age, weight, height) and demographic data between the different CYP2D6 and CYP1A2 genotypes except for an overrepresentation of females in the wildtype (C/C) genotype of the CYP1A2*1F (C-163A) polymorphism. Absolute and dose- (and body weight) normalized serum concentrations or response data were similar for the different CYP1A2*1F genotypes or CYP2D6 genotype groups.

All CYP2D6 poor metabolizers (3/3 = 100%, none receiving CYP2D6 relevant co-medication), however, showed marked/moderate side effects compared to only 28% of the other genotypes (13/47, Fischer-Exact test, p = 0.029). The side effects (DOTES) sum score was more than doubled for the PMs compared to the other genotypes (Mann-Whitney U test, p = 0.048, see Table 37 part a).

Similar results were obtained when regarding all patients receiving mirtazapine (n = 122, side effect data available for 110). Six of eleven (55%) PMs had moderate/marked side effects compared to only 13/45 = 29% IMs, 8/50 = 16% EMs and 1/4 = 25% UMs (Fischer-Exact-test p = 0.030). CYP2D6 PMs showed also a trend to a higher side effects (DOTES) sum score (Mann-Whitney U test, p = 0.078). The baseline demographics (age, height, weight, baseline CGI(1) and PDS scores, duration of illness, if available) and the doses of mirtazapine as well as of other antidepressants or antipsychotic drugs did not significantly differ between the different CYP2D6 genotype groups.

Table 37: Side effects (DOTES) sum score

CYP2D6 metabolizer status	a) mirtazapine serum subset (n = 50)			b) all study patients receiving mirtazapine (n = 110)		
	Mean	SD	n	Mean	SD	n
PM poor	5.33	3.055	3	4.00	3.82	11
IM intermediate	2.55	2.417	20	1.98	2.42	45
EM extensive	1.54	1.978	24	1.38	1.78	50
UM ultrarapid	2.00	2.000	3	1.50	1.91	4
Total	2.20	2.347	50	1.89	2.41	110

3.4.2.3 Olanzapine

Olanzapine is an atypical antipsychotic, which is effective in treating schizophrenia and acute manic episodes, and in preventing the recurrence of bipolar disorders [330]. CYP1A2 and - as a minor pathway - CYP2D6 are involved in the metabolism of olanzapine [326,331]. Induction (e.g., tobacco smoke, drugs) or inhibition of the CYP1A2 gene leads to a high interindividual variability in enzyme activity. The CYP1A2*1F homozygote variant (A/A) genotype was associated with higher enzyme inducibility and higher clearance of CYP1A2 substrates like melatonin [78] or caffeine in Caucasian smokers [79,80]. The impact of this genotype with respect to olanzapine serum concentrations, response and side effects in a clinical setting has not yet been demonstrated until now.

3.4.2.3.1 Study Subpopulation

Patients were treated with a fixed olanzapine dose for at least one week before blood samples were collected to attain apparent steady-state concentrations. Sample collection was conducted 12 - 14 hours after the evening dose. Steady-state serum samples were available for 73 patients (36 males, 37 females, age 19 - 76 years), who had been treated with olanzapine doses ranging from 2.5 to 30 mg/day according to local clinical practice for at least 4 weeks. Thirty-four patients had depressive disorders (F3 diagnosis), 32 schizophrenic disorders (F2 diagnosis) and 7 another F diagnosis according to ICD-10 [29].

The baseline characteristics of the patient cohort are given in Table 38. Overall, 30 (41%) were smokers. In the week of olanzapine serum measurement, the patients were taking a mean daily dose of 15 mg olanzapine (SD 7.5 mg) and had on average an olanzapine concentration of 21 ng/mL. Complete response data was available for 70 patients (for 31 with F2 and for 33 with F3 diagnosis). Data on body weight was available for 68 patients (for 29 with F2 and for 31 with F3 diagnosis).

Table 38: Baseline characteristics of olanzapine subpopulation

Olanzapine (n = 73)	**Mean**	**SD**
age (years)	41.7	14.7
height (cm)	172.3	10.1
weight (kg)	74.2	14.6
BMI (kg/m²)	24.7	4.4
dose (mg/day)	14.6	7.5
serum drug concentration (ng/mL)	20.6	15.2
dose-normalized conc. (ng/mL/kg)	1.39	0.68
dose- and body weight normalized conc. (ng/mL per mg/kg)	100.6	49.5
CGI(1) severity baseline (n = 70)	6.19	0.78
CGI(1) severity 4 weeks (n = 70)	5.40	0.86
CGI(2) improvement 4 weeks (n = 70)	3.52	0.99
	n	**%**
CGI responder (CGI(2) < 3 after 4 weeks, n = 70 available)	7 / 70	10.0
PDS responder (PDS-P improvement > 50%, n = 70)	16 / 70	22.8
male	36 / 73	49.3
smoker	30 / 73	41.1
CYP1A2*1F wildtype (C/C)	5 / 73	6.9
CYP1A2*1F heterozygote (C/A)	27 / 73	37.0
CYP1A2*1F mutant (A/A)	41 / 73	56.2

Note: PDS-response was defined as improvement of > 50% after 4 weeks compared to baseline PDS-Paranoid score in patients with an at least moderate PDS-P score at baseline

The subjects participating in this study received co-medication as given in Table 39. Only five patients received no co-medication. The study was not designed as an interaction study. For most single drugs, there was insufficient data to support statistical evaluation. Thus, drugs were rated as substrates, inhibitors and inducers of cytochrome P450 isoenzymes CYP1A2, CYP2D6 and CYP3A4 according to the Cytochrome P450 Drug Interaction Table [69]. The dose-corrected concentrations were compared for each cytochrome P450 isoenzyme in consideration of CYP1A2*1F (A/A) genotype and smoking status. A check for outlier concentrations (compare § 2.4 for definition) was done for the different types of co-medications by means of box-and-whisker plots again under consideration of CYP1A2*1F (A/A) genotype and smoking status.

Additionally, a screening test scrutinized possible drug-drug interactions by comparing "signal-to-noise" as used previously [332,333]. Drugs with sufficient data were also compared as single groups such as lorazepam, mirtazapine, citalopram, lithium, diazepam and carbamazepine. SSRIs (fluoxetine, paroxetine, sertraline), tricyclic antidepressants (amitriptyline, doxepin) and beta blockers (bisoprolol, propanolol, metoprolol) were evaluated together. Moreover, drugs were defined according to the anatomic therapeutic chemical classification (ATC) system of the WHO as either CNS drugs (included in the N section of the ATC) or as somatic drugs (included in any other section) and both groups were compared to each other.

Table 39: Co-medication - substrates, inducers and inhibitors of CYP P450 isoenzymes

	Substrates	**Inhibitors**	**Inducers**
CYP1A2	clozapine (1), mirtazapine (21) [334], propanolol (2)	-	carbamazepine (6) [325,326]
CYP2C19	amitriptyline (5), citalopram (5), diazepam (7), doxepin (2) [327], clopidpgrel (1), flunitrazepam (1)[319], pantoprazole (1), propanolol (2)	pantoprazole (1)	carbamazepine (6)
CYP2D6	amitriptyline (5), doxepin (2) [327], fluoxetine(4), metoprolol (2), mirtazapine (21) *[334]*, paroxetine (5), propanolol (2), risperidone (3), venlafaxine (3), zuclopenthixol (2)	fluoxetine (4), paroxetine (5), sertraline (6)	
CYP3A4	alprazolam (2), atorvastatin (2), risperidone (3), diazepam (7), flunitrazepam (1) [319], mirtazapine (21*) [334]*, nitrendipine (1), propanolol (2), simvastatin (1), zolpidem (1)	-	carbamazepine (6), oxcarbazepine (1)

Note 1: If not listed in Cytochrome P450 Drug Interaction Table [69], the literature reference is noted.

Note 2: number of patients with co-medication is given in brackets

Note 3: no major contribution of cytochrome P450 isoenzymes given above to the metabolism of the following drugs: acetyl salicylic acid (1), amisulpride (2), biperiden/flupentixol/opipramol (1), bisoprolol (4), bromazepam (1), hydrochlorothiazide (3), iodine (3), fenofibrate (1), furosemide (1), lithium (9), lormetazepam (2), lorazepam (22), ramipril (1), ranitidine (1), reboxetine (1), T4 (1), pipamperone (5), valproic acid (2), prothipendyl (1), zopiclon (1) and losartan (1)

3.4.2.3.2 Serum Concentrations - Influence of Dose, Co-medication, Genotype and Smoking

Within the patient cohort, serum concentrations obtained 12 - 14 hours after the last dose ranged from below the lower limit of quantification (LLOQ 2.5 ng/mL) to 87.4 ng/mL with a mean ± SD concentration of 20.6 ± 15.2 ng/mL. Dose-normalized as well as dose- and body weight normalized olanzapine concentrations showed normal distribution according to the Kolmogorov-Smirnov test and Shapiro-Wilk test and were tested by parametric tests (ANOVA, t-test). Dose and absolute concentrations showed no normal distribution and were compared by nonparametric tests (see also § 2.4).

Olanzapine concentrations were found to increase linearly with the daily dose (Spearman's $r = 0.75$, $p < 0.001$). Neither serum concentrations nor administered dose nor normalized concentrations showed significant correlations with age, height, weight or differences for gender ($p > 0.1$, data not shown). Elderly women (> 60 years) showed a trend to lower doses (11 ± 7 vs. others 15 ± 8 mg

olanzapine, p = 0.079), but serum concentrations and dose-normalized serum concentrations were not different compared to all other patients.

Patients with the CYP1A2*1F homozygote (A/A) genotype had significantly lower absolute, dose-normalized as well as dose- and body weight-normalized serum concentrations (see Table 40). No significant baseline differences (gender, age, height, weight, diagnosis, smoking or CYP-rated co-medication, p > 0.1) were present for the different CYP1A2 genotypes.

Smokers received not only significantly higher doses of olanzapine, but had also lower dose- and body weight-normalized olanzapine concentrations (see Table 40). This is explainable by CYP1A2 induction. Moreover, schizophrenic patients receiving the highest antipsychotic doses of all patients (F2 diagnosis) were overrepresented and patients receiving CYP1A2 relevant co-medication (mainly the antidepressant mirtazapine) were underrepresented in the smoker group, though both trends were not significant.

In the six patients receiving carbamazepine, the normalized serum concentrations were significantly decreased (on average only half of those without carbamazepine co-medication, see Table 40). Moreover, a trend towards lower absolute concentrations for patients treated with carbamazepine was evident (Mann-Whitney U test, p = 0.057). Three patients were smokers (two CYP1A2*1F (A/A) genotyped) and three non-smokers (two CYP1A2*1F (A/A) genotyped).

Except carbamazepine, no other drug or drug group was associated with deviating olanzapine levels. Patients receiving additional (possibly) CYP1A2 relevant co-medication (mostly mirtazapine and amitriptyline) had on average somewhat (+29%) elevated dose- and body weight normalized olanzapine concentrations compared to those receiving no CYP1A2 substrates. They also received significantly lower doses. This effect was however, mainly due to the overrepresentation of non-smokers in this subgroup (see Table 40). Coadministration of CYP3A4 or CYP2D6 substrates did not show a significant effect on olanzapine concentrations (ng/mL per mg/kg) when compared to those without such co-medication in the sample set. No significant effect on olanzapine levels was also visible for other inducers such as omeprazole and oxcarbazepine or inhibitors such as fluoxetine, paroxetine or sertraline.

Table 40: Influence factors on olanzapine concentrations and dose

	Mean	SD	Mean	SD	Mean	SD	p-value
CYP1A2*1F genotype	**C/C (n = 5)**		**C/A (n = 27)**		**A/A (n = 41)**		
administered dose (mg)	17.4	9.3	15.0	7.5	14.1	7.4	0.636[a]
concentration per dose/body weight (ng/mL per mg/kg)	120.9	37.1	117.4	44.7	87.1	50.5	0.028[b+]
CYP1A2 relevant Co-medication	**carbamazepine (n = 6)**		**none (n = 44)**		**CYP1A2 substrate (n = 26)**		
administered dose (mg)	16.7	8.2	16.7	7.3	10.2	6.0	0.003[a]
concentration per dose/body weight (ng/mL per mg/kg)	46.8	16.1	96.0	46.1	123.5	49.4	0.001[b#]
Smoking	**non-smoker (n = 43)**		**smoker (n = 30)**		**total (n = 73)**		
administered dose (mg)	12.8	6.6	17.3	8.1	14.6	7.5	0.022[a]
concentration per dose/body weight (ng/mL per mg/kg)	111.5	46.5	85.1	50.3	100.6	49.5	0.024[b]

[a] non-parametric testing (Kruskal-Wallis test or Mann-Whitney U test)

[b] two-tailed t-test or univariate Analysis of Variance ANOVA

[#] post-hoc Bonferroni testing for multiple comparisons: significant for carbamazepine - no CYP1A2 co-medication (p = 0.046) and carbamazepine - CYP1A2 substrate (p = 0.001), trend for no co-medication - CYP1A2 substrate (p = 0.067).

[+] post-hoc Bonferroni test for multiple comparisons: significant for A/A – C/A (p = 0.038).

3.4.2.3.3 Serum Concentrations - Combined Influence of Co-medication, Genotype and Smoking

Carriers of the highly inducible A/A genotype who were smoking or taking carbamazepine (n = 22, 75 ± 52 ng/mL / mg/kg) had significantly lower (on average -42%) dose- and body weight normalized serum concentrations compared to C-allele carriers without induction (n = 21, 130 ± 37 ng/mL per mg/kg; see Figure 62). For C-allele carriers with induction or A/A carriers without induction, the concentrations were almost equal (n = 11, 94 ± 52 and n = 19, 101 ± 46 ng/mL per mg/kg) and reached on average 72% and 78% of the C-allele carriers without induction.

In 4 of 73 serum samples, olanzapine was below the lower limit of quantification (LLOQ <2.5 ng/mL). For evaluation, these results were set to 1/2 LLOQ. Of these, two were smokers and CYP1A*1F A/A homozygote, one non-smoker and CYP1A*1F A/A homozygote and one smoker and CYP1A*1F C/A heterozygote. To exclude the possibility that non-compliance influences the relationship between genotype/smoking status and serum concentrations, a second examination was done without these patients, but it did not change the significance of the results. The relationship between genotype and dose or dose- and body weight normalized concentrations remained significant. The same was true for the combined influence of CYP1A2*1F and tobacco or carbamazepine.

Multiple linear regression analysis was used to detect correlations between dose-normalized and dose- and body weight normalized olanzapine concentrations and several factors including age, baseline height and weight, gender, smoking, F diagnosis, CYP1A2*1F genotype, duration of illness, MDR1 genotypes and CYP1A2 relevant drugs.

Only CYP1A2*1F A/A genotype (standardized partial correlation coefficients beta = -0.29; p = 0.005) and co-medication with carbamazepine (beta = -0.31; p = 0.009) were significantly correlated with dose- and body weight normalized serum concentrations. Smoking missed statistical significance (p = 0.063). The coefficient of determination r^2 = 0.192 indicates that 19% of the variation of the dose/body weight normalized olanzapine concentrations can be explained by genotype and co-medication. For dose-normalized olanzapine concentrations, similar results were obtained, but smoking reached statistical significance and was included in the regression model.

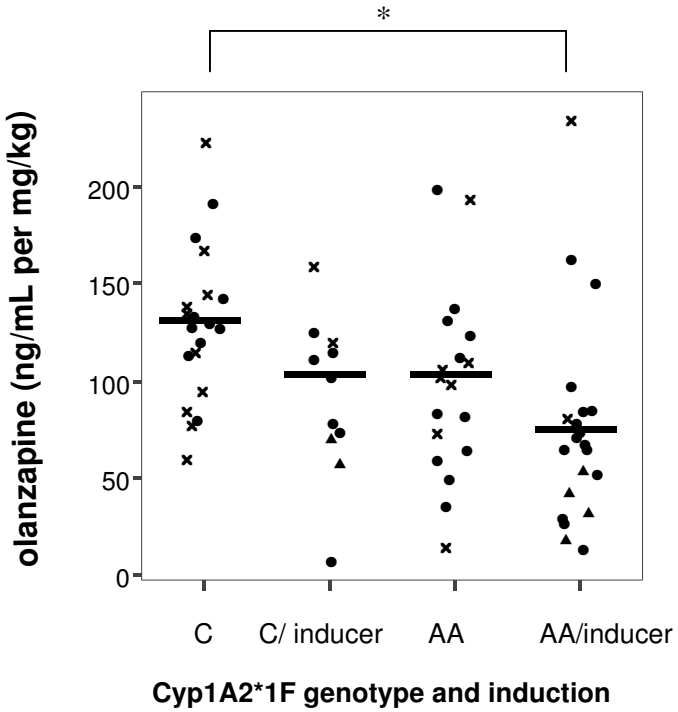

Figure 62: Influence of CYP1A2*1F and inducers on olanzapine concentrations

A or C = CYP1A2*1F alleles; inducers = tobacco smoke and carbamazepine. Lines denote the median values (C-allele without induction 126 ng/mL per mg/kg; C-allele with induction 101 ng/mL per mg/kg; AA-carriers 102 ng/mL per mg/kg and AA-carriers with induction 69 ng/mL per mg/kg). Cross denotes CYP1A2 relevant co-medication, circle denotes no CYP1A2 relevant co-medication, triangle denotes co-medication with carbamazepine. Significant difference were observed between groups (ANOVA, p = 0.002), which remained significant after Bonferroni correction for C vs. A/A + inducer (p = 0.001).

3.4.2.3.4 Response - Influence of Serum Concentration, Co-medication and Genotype

Clinical outcome measures were assessed after 4 weeks of therapy and included the PDS rating scale and the CGI(1) severity and CGI(2) improvement ratings (see Table 38). Complete response data was available for 70 patients (n = 31 with F2 diagnosis and n = 33 with F3 diagnosis). A small but significant reduction in PDS-Paranoid scale ($p < 0.001$, Wilcoxon-Signed-Rank-Test for two related samples), PDS-Depression scale ($p = 0.003$) and CGI(1) ($p < 0.001$) ratings was observed after 4 weeks compared to hospital admission indicating improvement of illness within the observation period.

Not all patients, however, had an acute episode reflected in high baseline scores of PDS-paranoid scale. Olanzapine is not only used to treat acute psychopathological symptoms of psychosis, but is also used in acute manic episodes, and in preventing the recurrence of bipolar disorders [330]. In the cohort examined, schizophrenic patients had higher PDS-P baseline scores, but lower PDS-D scores at baseline and after 4 weeks compared to patients with affective disorders. They showed better CGI(2) improvement and a trend to a higher PDS-P score reduction. They received higher doses and had higher olanzapine concentrations and showed also a trend to higher dose- and body weight normalized serum concentrations. They were younger and received less co-medication, especially somatic drugs. Thus, the response data were evaluated separately for both groups.

No correlations were observed in patients with mood or affective disorders (F3 diagnosis, n = 33, see Table 41). Schizophrenic patients with higher olanzapine concentrations showed better improvement of paranoid and depressive symptoms after 4 weeks of treatment according to the PDS self-rating (score differences baseline – 4 weeks for 31 patients available, see Table 41). Similarly, higher olanzapine concentrations were correlated with lower severity of illness after 4 weeks according to the CGI(1) rating by the treating psychiatrist. The % PDS score changes showed similar trends. No significant relationship was observed for the baseline scores or ratings. As dose changes during the 4 weeks of treatment might have an impact on response, a recalculation was done according to the formula: serum concentration / dose x average dose of all 4 weeks of treatment, but the evaluation led to similar results. The mean dose change was less than 4% in the total population and less than 1.6% in schizophrenic patients.

Similar results were obtained when only the schizophrenic patients without (possibly interfering) antipsychotic co-medication were examined (n = 21, see Table 41). Antipsychotic co-medication was significantly higher in patients with higher PDS-P scores on admission to hospital. Moreover, co-medication with typical antipsychotics (n = 4), but not atypical ones (n = 6) was related to a higher reduction in PDS-P scores after 4 weeks (Mann-Whitney U test, $p = 0.023$).

The CGI(2) improvement rating after 4 weeks, however, showed no relationship to serum levels in schizophrenics ($r = -0.09$, $p > 0.6$). It seems to represent rather a relative change of symptoms depending on the severity of symptoms at baseline ($r = -0.46$, $p = 0.008$) than an absolute symptom reduction. No relationships were present between serum concentrations and side effects (DOTES score) or weight gain.

Table 41: Correlation between olanzapine concentration and response

Spearman correlation for serum concentrations vs. response data		CGI(1) baseline		CGI(1) after 4 weeks		PDS-P difference		PDS-D difference	
diagnosis	n	r	p	r	p	r	p	r	p
F2 (schizophrenic)	31	0.22	0.234	0.36	0.049	0.48	0.026	0.48	0.006
F2 without antipsychotic co-medication	21	0.34	0.127	0.46	0.041	0.49	0.029	0.49	0.026
F3 (mood disorders)	33	-0.01	0.954	0.22	0.218	0.09	0.652	0.09	0.636

Spearman's rank correlation gives strength (coefficient r) and significance (p-value) of the linear relationship between serum concentrations and response measures: CGI(1) = Global Clinical Impression Severity Rating, PDS-P = Paranoid Depression Scale, Paranoid Subscale and PDS-D = Paranoid Depression Scale, Depression Subscale (differences baseline to 4 weeks)

Non-parametric testing and multiple linear regression analyses were used to detect relationships between treatment response (i.e., PDS-P score difference) and genetic variants under consideration of olanzapine serum levels, co-medication, and illness at baseline (PDS-P score or CGI(1) score). No further significant associations were detectable for response in the patient cohort for the F2 and F3

subgroups. Similar results were obtained, when all patients receiving olanzapine in the whole study population were examined.

Similar to Bergemann et al. (2004) [335], there were no differences in olanzapine concentrations in schizophrenic patients in whom olanzapine was discontinued because of non-response or adverse effects and those who were discharged while still on olanzapine. In the treatment period following the assessment of serum concentrations until release from hospital, switches from olanzapine to other drugs were not more common in the patients outside the therapeutical range (20 - 50 ng/mL). Dose changes showed a trend towards an inverse relationship with the serum concentrations (dose reduction in patients with higher levels and vice versa), but this did not reach statistical significance. Neither switches nor dose changes were related to the response status of the patients.

3.4.2.3.5 Side Effects - Influence of Co-medication, Genotype and Serum Concentration

Multiple linear regression analyses were used to detect correlations between the DOTES score as a measure of side effects and several factors including olanzapine serum levels, height, weight, age, gender, diagnosis, dosage, treatment naïveness, co-medication, treatment response, and genetic variants. No significant associations were detectable for the DOTES score in the patient cohort (also for F2 and F3 subgroups). Observed trends were due to an outlier patient with high DOTES score, treatment naïveness, and co-medication paroxetine.

3.4.2.3.6 Weight Gain - Baseline Characteristics, Influence of Serum Concentration, Genotype and Co-medication

Olanzapine is known to induce weight gain [329]. Body weight data was available for 68 patients (see Table 42). Of those, 19.1% (13 of 68) had clinically relevant weight gain after 4 weeks (≥ 7% increase versus baseline weight). Weight gain (in % of baseline) was correlated with better response after 4 weeks (CGI(2) improvement rating, $r = -0.36$, $p = 0.003$) and lower baseline weight or BMI ($r = -0.33$, $p = 0.007$ and $r = -0.31$, $p = 0.011$, respectively). The risk of weight gain was not higher in women (Mann-Whitney U test $p > 0.1$). The relationship between olanzapine concentration and weight gain (%) was not significant. Similar results were obtained for the absolute weight or BMI difference to baseline.

Table 42: Weight data

n = 68	Mean	SD
weight baseline (kg)	74.16	14.61
weight 4 weeks (kg)	76.07	13.91
weight 4 weeks-baseline (kg)	1.91	3.21
BMI baseline (kg/m²)	25.03	4.45
BMI 4 weeks (kg/m²)	25.68	4.19
BMI 4 weeks-baseline (kg/m²)	0.65	1.07
weight gain after 4 weeks (%)	2.89	4.33

In a previous study, a threshold concentration of 20.6 ng/mL associated with an increased likelihood of clinically significant weight gain (≥ 7% baseline weight) after 6 weeks of olanzapine treatment [336]. Additionally, the therapeutic range is 20 - 80 ng/mL according to the AGNP-TDM consensus group [337].

Weight gain (% of baseline) was on average more than doubled for the 32 patients with concentrations above the threshold of 20 ng/mL, but this was non-significant (Mann-Whitney U test, $p = 0.120$, see Table 43). However, patients with concentrations above the threshold of 20 ng/mL had a significantly higher risk of clinically relevant weight gain (> 7% of baseline, 10 of 32 = 31.3% vs. 3 of 36 = 0.8%; Odds Ratio 5, confidence interval 1.2 - 20.2, Fischer-Exact test, $p = 0.018$).

Genetic variants across the following candidate genes: ADRA2A, 5-HTR2A, 5-HTR2C, 5-HTR6, DRD2, DRD3, MDR1, CYP1A2 and CYP2D6 were examined for relationship with weight gain under

olanzapine therapy. Significantly higher weight gain was detected for the 5-HTR2A -759 C/C or C vs. C/T, T/T or T carriers and DRD2-141 ins vs. del (p = 0.012 and p = 0.012, see Table 43), while baseline characteristics such as age, gender, baseline weight/BMI were not significantly different. All 13 patients with substantial weight gain > 7% within the first weeks of treatment were carriers of 5-HTR2C -759 C/C. Trends towards higher weight gain were observed for the 5-HTR2A 102 C-allele carriers (p = 0.068), and MDR1 3435 T/T carriers (p = 0.098).

Table 43: Weight gain after 4 weeks (% of baseline)

	n	Mean	SD	p-value
Total	68	2.89	4.33	
DRD2-141 ins/del genotype				0.012⁺
wildtype (ins/ins)	59	3.35	4.15	
heterozygote (ins/del)	9	-0.15	4.49	
5-HTR2C C-759T				0.012⁺
T/T, C/T or T	16	0.79	2.58	
CC or C	52	3.54	4.56	
Olanzapine concentration				0.120⁺
< 20 ng/mL	36	1.90	3.34	
≥ 20 ng/mL	32	4.00	5.05	

⁺ Mann-Whitney U test
Note: 5-HTR2C lies on the X-chromosome

Co-medication may also lead to weight gain. Lithium, valproic acid and amitriptyline are known to lead to marked weight gain; mirtazapine and risperidone to intermediate weight gain [329]. A greater number of co-medications per patient, and coprescription of antidepressants significantly and independently increased antipsychotic-associated weight gain in a previous study [338].

No significant influence of number or kind of co-medication was observed. A trend was detected for lithium co-medication (n = 8 of 68 patients, mean ± SD weight gain 5.2 ± 3.8% with lithium vs. 2.6 ± 4.3% without lithium co-medication, Mann-Whitney U test, p = 0.075, see Figure 63), which could not be explained by baseline differences in age, weight, BMI, olanzapine dose or serum concentrations, or overrepresentation of genotypes. However, more male (n = 7) patients received lithium, and a better CGI(2) improvement was observed for this subgroup. A negative trend detected for mirtazapine was mostly due to one outlier with approx. -10% weight change under mirtazapine co-medication (p = 0.088) and not regarded as underlying reason.

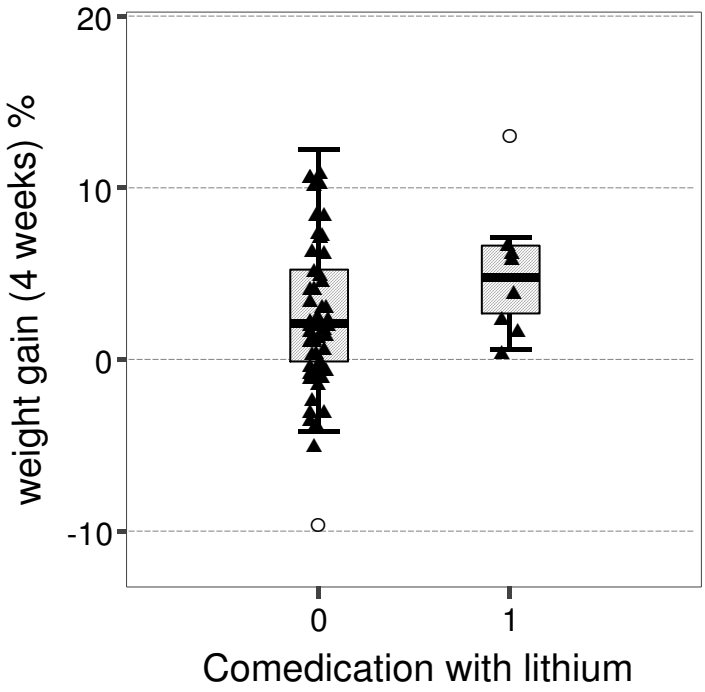

Figure 63: The impact of lithium co-medication on weight gain

Box-and-whisker plot: The center box shows median and interquartile range of data. Two outliers are marked individually (open circle), while all other values are marked as triangle. When outliers exist, the whiskers range is defined as 1.5 times the interval from the 25th to 75th percentile i.e., the box length. Note: Group 0 received no lithium (n = 60, median weight gain 2.1% range -9.6 to 12.2%). Group 1 received lithium (n = 8, median 4.8%, range 0.6 to 13.0).

3.4.2.3.7 Weight Gain - Combined Influence of Baseline Characteristics, Serum Concentration and Genotype

Multiple linear regression analyses were used to detect correlations between weight gain and several factors including baseline weight or BMI, age, gender, diagnosis, dosage, treatment naïveness, co-medication with antidepressants and lithium, treatment response, and genetic variants. Significant effects of CGI(2) rating (standardized r coefficient beta = -0.276; p = 0.012), baseline BMI (beta = -0.366; p = 0.001), DRD2 -141C del (beta = -0.230; p = 0.035) on weight gain could be detected (r^2 = 30%). 5-HTR2C -759 C/C genotype and olanzapine concentrations above the threshold of 20 ng/mL, however, were not included in the model as they did not significantly improve the fit. For the 5-HTR2C -759 C/C genotype, however, a trend was visible (beta = 0.2, p = 0.063).

Similar results were obtained, when all patients receiving olanzapine in the whole study population were examined. The influence of 5- HTR2C -759 C/C, however, was significant and an additional trend was seen for ADR2A -1291C/G (see § 3.4.9.1 and § 3.4.9.2). In exchange, the effect of the DRD2-141ins/del polymorphism was not significant - only the CGI(2) was included as a measure of treatment response.

3.4.3 Influence of Cytochrome P450 Polymorphisms on Therapeutic Outcome

Subsequently to the examination of the relationship between genotypes and serum concentrations in the serum subsets in the previous chapters, the direct influence of CYP1A2, CYP2C19 and CYP2D6 polymorphisms on clinical outcome variables was evaluated in the whole study population. Therefore, all patients treated with antidepressants/antipsychotics that are substrates of these enzymes were evaluated. Only drugs where the enzyme is significantly involved in the metabolism were included (e.g., mirtazapine was not rated as CYP1A2 substrate due to the small fraction metabolized by this enzyme). The classification was done according to published literature [69,87,307,311,318,327,330,339-341].

One hundred sixty-two (44%) of the 365 patients received CYP1A2 substrates such as olanzapine, clozapine and haloperidol. Ninety-eight (27%) received CYP2C19 substrates (citalopram or the tricyclic antidepressants amitriptyline, clomipramine, imipramine, doxepin, and trimipramine). One hundred eight (30%) received CYP2D6 substrates according to a narrow definition (amitriptyline, clomipramine, imipramine, doxepin, trimipramine, fluoxetine, paroxetine, aripiprazole, chlorpromazine, promethazine, haloperidol and zuclopenthixol. Additionally for evaluation of anamnestic data: chlorprothixene, desipramine). A second, wider definition of CYP2D6 substrates included additionally mirtazapine, venlafaxine and risperidone. Mirtazapine has a stereoselective CYP2D6 metabolism with a preference for the S-enantiomer. In case of venlafaxine and risperidone, equally active metabolites result from the CYP2D6 metabolism. According to this definition, 254 (70%) of the 365 patients received CYP2D6 substrates.

3.4.3.1 CYP2D6 and Baseline Characteristics

To allow an evaluation of the highly polymorphic CYP2D6 gene, the different polymorphisms were rated according to their functionality with semi-quantitative gene doses as described by Steimer et al. (2004) [305]. The non-functional alleles *3, *4, *5, *6 were evaluated as null alleles, the impaired alleles *9, *10, *41 (2988A) with gene dose 0.5, the functional alleles *1 and *2 with gene dose 1 and for duplicated genes the respective gene dose was doubled. In a second step, the gene dose of 0 was rated as poor metabolizer, 0.5 and 1 as intermediate metabolizer, 1.5 and 2 as extensive metabolizer and > 2 as ultrarapid metabolizer to obtain sufficiently large groups for statistical evaluation (see also Appendix A2.1 Allele Frequencies). Thus, 8.5% poor metabolizers (31 PM), 37.8% intermediate metabolizers (138 IM), 50.7% extensive metabolizers (185 EM) and 3.0% ultrarapid metabolizers (11 UM) were present in the whole study population (n = 365).

The different CYP2D6 gene doses as well as metabolizer groups were compared with regard to baseline demographics, diagnosis and anamnestic data (number of previous episodes, duration of previous hospital stays, all available data). Ultrarapid metabolizers showed a trend to a higher rate of switches due to non-response in medical history (3/11 = 27% compared to 35/293 = 12% for all others, n = 304 data available, Odds Ratio OR = 2.8, confidence interval 0.7 - 10.9, Fischer-Exact test p = 0.15). No such differences were reported for adverse effects. No further significant differences were observed.

3.4.3.2 CYP2D6 Gene Dose and Pharmacotherapy

Patients with CYP2D6 dependent medication were hospitalized on average 6 days longer (63 ± 37 days, n = 108) than those treated without CYP2D6 dependent medication (57 ± 38 days, n = 257; Mann-Whitney U test , p = 0.075) and they responded on average 4 days later to pharmacotherapy (n = 88, 34 ± 27 days vs. n = 235, 30 ± 30 days, p = 0.034, data of 323 patients available). Of the 42 patients who did not respond to pharmacotherapy during hospitalization, 20 were treated with CYP2D6 dependent drugs. No such trends were observed for the wider definition of CYP2D6 substrates. Within the patients receiving CYP2D6 dependent medication, the length of hospitalization was similar between the different gene dose groups (52, 64, 63, and 63 days for 4 PMs, 45 IMs, 55 EMs and 4 UMs, respectively, p > 0.8): The response onset was similar, too (28, 34, 35, and 34 days for 4 PMs, 33 IMs, 48 EMs and 3 UMs, respectively, p > 0.6).

3.4.3.2.1 CYP2D6 Gene Dose and Prescription Frequencies of CYP2D6 Substrates

Patients with a CYP2D6 gene dose of 0 or 0.5 had significantly lower prescription frequencies of CYP2D6 substrates (narrow definition) within the first four weeks of hospitalization compared to patients with higher gene doses. Eight of 51 (16%) of the patients with gene dose 0 or 0.5 received at least one CYP2D6 substrate, whereas 100 of 314 (32%) of the patients with gene doses of 1 to 3 received at least one CYP2D6 substrate (Fisher-Exact test p = 0.020; Odds Ratio OR 2.5 with a 95% confidence interval of 1.1 - 5.5, see Figure 64). For the poor metabolizers with gene dose 0, the frequency of CYP2D6 dependent medication was even somewhat lower (4/31 = 13%) than for patients with gene dose 0.5 (4/20 = 20%). For the gene doses 1, 1.5 and 2, similar prescription frequencies were observed (35, 32 and 29%) and a somewhat higher frequency of 36% in ultrarapid metabolizers with gene doses > 2.

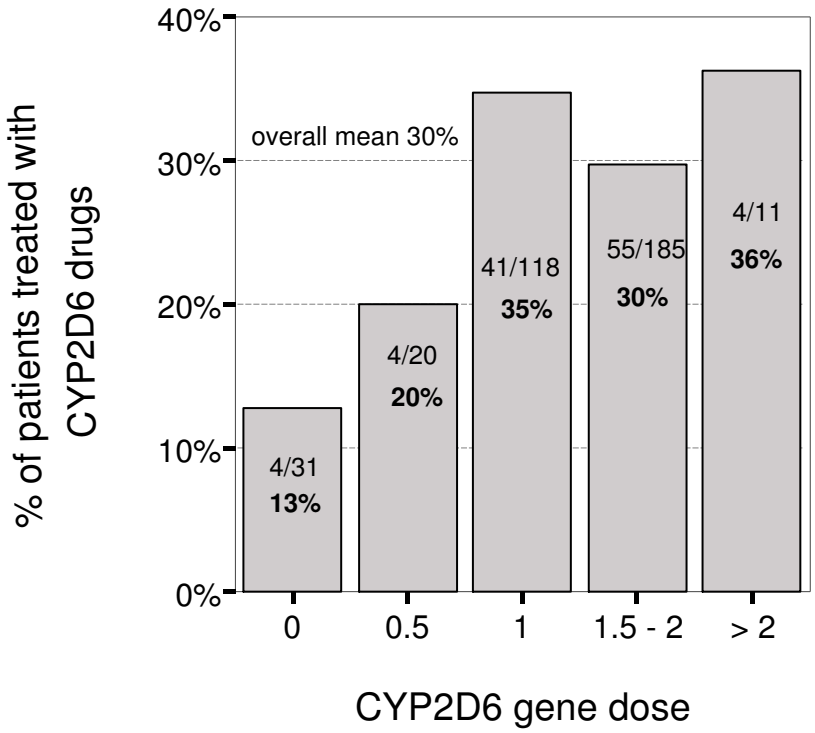

Figure 64: Prescription frequencies of CYP2D6 substrates for different CYP2D6 gene doses

Bar chart depicts number of prescriptions of CYP2D6 substrates within each CYP2D6 genotype group. Overall, 30% (108 of 365) patients received CYP2D6 dependent medication according to the narrow definition (cf. page 116).

Gene dose 0 (poor metabolizers): 4 of the 31 = 13% PM within the whole study population were treated with CYP2D6 substrates. Gene dose 0.5: 4 of 20 = 20% received CYP2D6 substrates. Gene dose 1: 41 of 118 =35% received CYP2D6 substrates. Gene dose 1.5 - 2: 55 of 185 = 30% received CYP2D6 substrates. Gene dose >2 (ultrarapid metabolizers) : 4 of 11 = 36% received CYP2D6 substrates.

Comparison of CYP2D6 substrates according to the wider definition showed no such differences. For the gene doses 0, 0.5, 1, 1.5 - 2 and > 2, the prescription frequencies were similar with 65, 65, 75, 68 and 73%, respectively. Notably, 36% of the ultrarapid metabolizers received more than one CYP2D6 substrate, compared to only 6%, 15%, 18% and 14% of the patients with gene doses of 0, 0.5, 1, 1.5 - 2, respectively (see Figure 65). The trend missed statistical significance when comparing UMs with all others (Fisher-Exact-test p = 0.071; Odds Ratio OR 3.3 with a 95% confidence interval of 0.9 - 11.7).

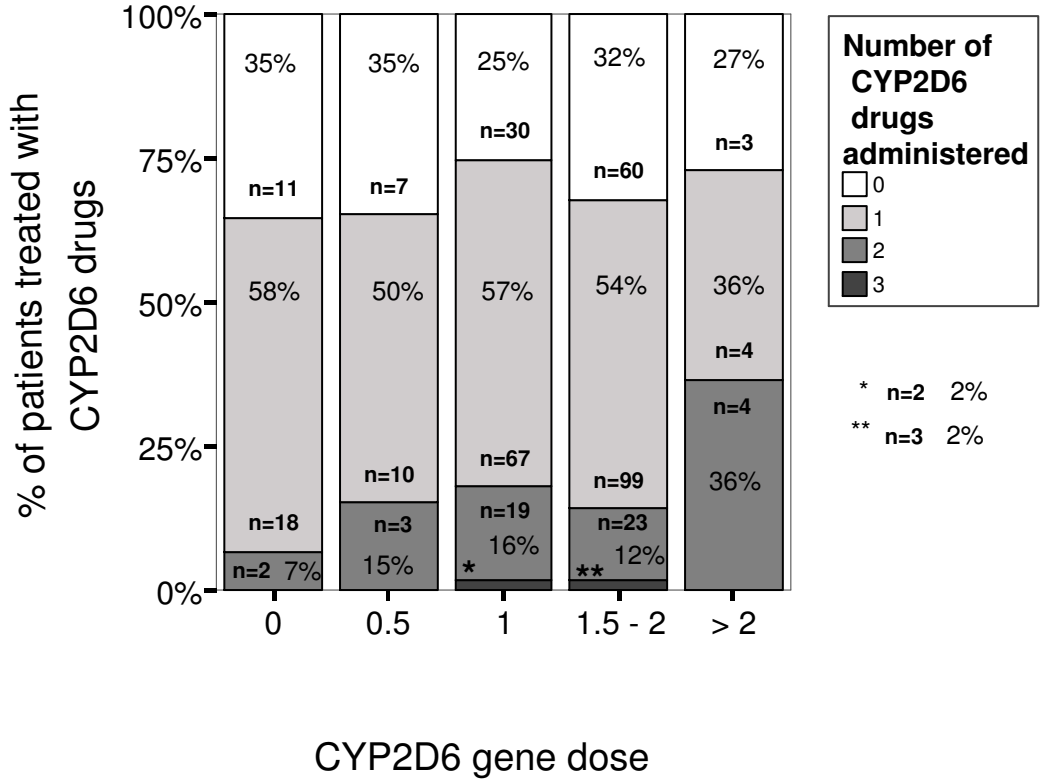

Figure 65: Prescription frequencies of CYP2D6 substrates for different CYP2D6 gene doses

Bar chart depicts number of prescriptions of CYP2D6 substrates within each CYP2D6 genotype group. Overall, 70% (254 of 365) patients received CYP2D6 dependent medication according to the wide definition (cf. page 116).

Gene dose 0: (poor metabolizers): 58% received one and 7% two CYP2D6 substrates. Gene dose 0.5: 50% received one and 15% two substrates. Gene dose 1: 57% received one, 16% two and 2% three CYP2D6 substrates. Gene dose 1.5 - 2: 54% received one, 12% two and 2% three substrates. Gene dose >2 (ultrarapid metabolizers) : 36% received one and another 36% two CYP2D6 substrates.

3.4.3.2.2 CYP2D6 Gene Dose and Drug Switches/Dose Changes during Hospitalization

A drug switch was defined as drug change within the drug class e.g., from one antidepressant to another due to non-response or side effects. Moreover, dose changes after more than two weeks of treatment (i.e., after the initial gradual increase) were evaluated. As it is difficult to define the exact time point when switching from or to an intramuscular (i.m.) drug, the i.m. applicated zuclopenthixol was not rated.

More switches due to non-response were reported for UMs in medical history (see above in § 3.4.3.1). A similar non-significant trend was observed during the current trial (PMs with one or more switches 2/4 = 50%, IMs 21/45 = 47%, EMs 32/55 = 58% and UMs 3/4 = 75%, n = 104, $p > 0.5$). With regard to the wider definition, a significant difference was only observed for the relative risk for more than two switches (PMs two or more switches 4/20 = 20%, IMs 21/101 = 21%, EMs 11/125 = 9% and UMs 1/8 = 13%, Fischer-Exact-test for PM/IM vs. EM/UM $p = 0.012$).

When the compound switches were divided into subgroups with regard to the metabolic pathway (i.e., switches from CYP2D6 drugs to non-CYP2D6 drugs, switches from non-CYP2D6 drugs to CYP2D6 drugs or switches without metabolic changes, switches to lower or higher doses) no differences could be observed between the different groups.

3.4.3.3 CYP2D6 Gene Dose and Administered Dose of CYP2D6 Substrates

Complete data on exact dosage within the first four weeks was available for 353 of the 365 patients. The relative doses per compound per patient based on the daily dose were calculated as defined by the WHO for each drug [342] (e.g., 30 mg for mirtazapine, 20 mg for citalopram, 10 mg for escitalopram, 10 mg for olanzapine and 5 mg for risperidone). The relative doses were summarized for each patient and compared for the CYP2D6 gene dose groups.

UMs and PMs tended to receive slightly lower doses of CYP2D6 dependent drugs than EMs and IMs. These observed differences were not significant ($p > 0.5$ for narrow definition and $p > 0.2$ for wide definition; Kruskal-Wallis test). The mean doses applied during the first four weeks - related to the defined daily doses - are given in Table 44.

A second evaluation with body weight adjusted doses was done to take different volumes of distribution into account. As this evaluation led to similar - rather worse than better - results, only the results of the first evaluation are described in the following chapters.

Table 44: Administered doses (relative to daily dose) of CYP2D6 drugs

CYP2D6 gene dose group		wide definition (side effects data available)	narrow definition (side effects data available)	wide definition (all patients)	narrow definition (all patients)
PM	Mean	1.05	0.73	1.04	0.92
gene dose 0	SD	0.27	0.44	0.30	0.51
	n	18	3	20	4
IM	Mean	1.41	1.37	1.39	1.32
gene dose	SD	0.92	1.16	0.90	1.11
0.5 - 1	n	88	35	98	41
EM	Mean	1.24	1.26	1.29	1.32
gene dose	SD	0.91	1.22	0.98	1.34
1.5 - 2	n	111	49	123	54
UM	Mean	1.27	0.80	1.27	0.80
gene dose	SD	0.60	0.57	0.60	0.57
>2	n	8	4	8	4
Total	Mean	1.29	1.27	1.30	1.28
	SD	0.88	1.16	0.90	1.20
	n	225	91	249	103

UM = ultrarapid metabolizer, EM = extensive metabolizer, IM = intermediate metabolizer, PM = poor metabolizer

3.4.3.4 CYP2D6 Gene Dose, Administered Dose and Adverse Drug Effects

Complete data on adverse effects within the first four weeks was available for 328 patients. Of these, 233 received non-CYP2D6 dependent medication and 95 CYP26 dependent medication according to the narrow definition (for 91 of the 95 were data on exact dosing available). The frequency of side effects was compared for the different CYP2D6 genotype groups and whether CYP2D6 dependent medication was prescribed.

In the patient group receiving CYP2D6 drugs, a pronounced increase of side effects as indicated by a higher DOTES sum score was observed for the PMs. The difference between PM (on average 7.7 score points) and non-PM (on average 2.0 score points) receiving CYP2D6 dependent medication was statistically significant (see Table 45, Mann-Whitney U test, p = 0.007). The side effects rating was similar between PMs receiving non-CYP2D6 dependent medication (on average 2.8 score points) and all others (on average 2.3 score points).

Table 45: Side effects for different CYP2D6 gene doses

Type of medication	CYP2D6 metabolizer status	CYPD6 gene dose	n	Side effects (DOTES) sum score		Moderate/marked side effects	
				Mean	SD	n	%
non-CYP2D6 dependent medication	PM	0	25	2.8	2.55	9	36
	IM	0.5	15	1.8	1.47	3	20
		1	69	2.7	4.77	21	30
	EM	1.5	36	1.6	1.86	6	17
		2	81	2.4	2.95	26	32
	UM	>2	7	1.3	1.11	1	14
	Total		**233**	**2.3**	**3.35**	**66**	**28**
CYP2D6 dependent medication*	PM	0	3	7.7	4.73	3	100
	IM	0.5	4	1.0	2.00	1	25
		1	34	2.2	3.50	14	41
	EM	1.5	17	1.9	3.29	4	24
		2	33	2.0	2.74	7	21
	UM	>2	4	1.3	1.89	1	25
	Total		**95***	**2.2**	**3.24**	**30**	**32**

* Exact dosing was missing for 3 patients with gene dose 1 (2 without and 1 with moderate/marked side effects) and for 1 patient with gene dose 2 (with moderate/marked side effects). For the remaining 91 patients, the difference in DOTES sum scores between PM (on average 7.7 score points) and non-PM (on average 2.0 score points) as well as the incidence of side effects was also statistically significant (p = 0.008 and p = 0.027).

All PMs receiving CYP2D6 substrates (3/3) had marked or moderate side effects (overall rating by the treating psychiatrist), while only 39% of the IMs (15/38), 22% of the EMs (11/50) and 25% of the UMs (1/4) suffered from at least moderate side effects (cf. Table 45). The difference between PMs and non-PMs receiving CYP2D6 medication (cf. solid line in Figure 66) was statistically significant (Fisher-Exact test, p = 0.029). A similar trend was observed for the wide definition (adverse effects rates were 44% for PMs, 33% for IMs, 24% for EMs and 25% for UMs). No significant differences were observed for the different gene dose groups of those patients receiving non-CYP2D6 dependent medication (cf. dashed line in Figure 66; PMs 9/25 = 36%, IMs 24/84 = 29%, EMs 32/117 = 27% and UMs 1/7 = 14%, Fisher-Exact test, p = 0.358). The difference in adverse effects in PMs with and without CYP2D6 dependent medication (100% vs. 36%) almost reached significance (Fischer-Exact-test, p = 0.067).

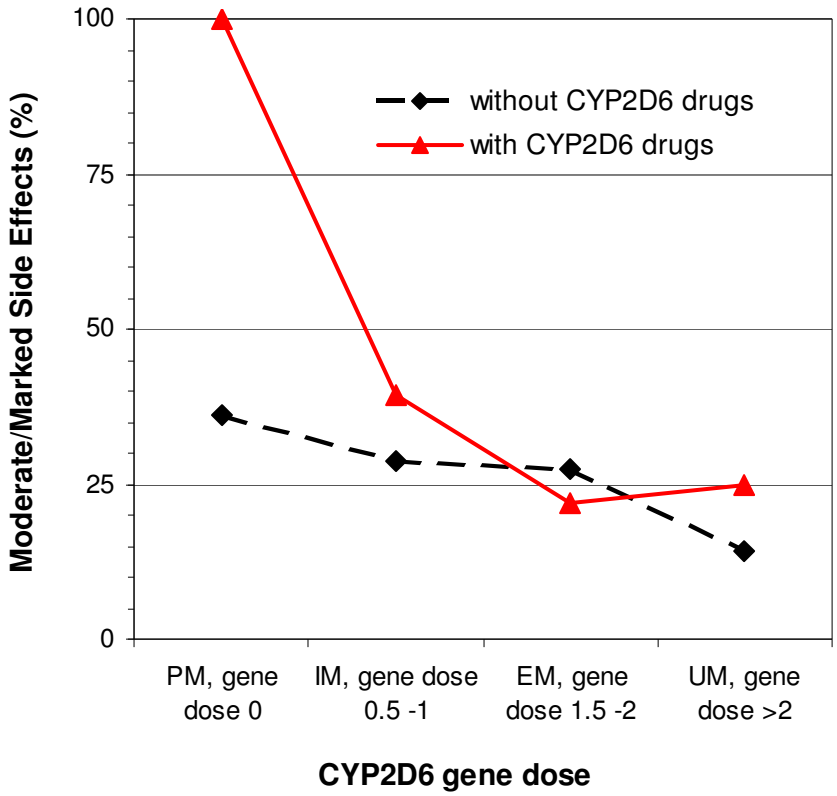

Figure 66: Frequency of adverse effects for different CYP2D6 gene doses

Line chart depicts number of marked/moderate side effects separately for the CYP2D6 genotype groups and according to whether CYP2D6 dependent medication was administered or not. Data available for 95 patients with CYP2D6 drugs according to the narrow definition (3 PMs, 38 IMs, 50 EMs and 4 UMs) and for 233 patients without CYP drugs (25 PMs, 84 IMs, 117 EMs and 7 UMs).

The difference between IMs and EMs receiving CYP2D6 medication did not reach statistical significance when the frequency of side effects was compared (39% vs. 22%, Fischer-Exact-test, p = 0.099, Odds Ratio 0.4 with a 95% confidence interval of 0.2 to 1.1, cf. solid line in Figure 66 and cf. Table 45). Similar results were observed when only patients with exact dosing data were evaluated (n = 91). The incidence of side effects tended to be higher in IMs than in EMs receiving CYP2D6 medication (14/35 = 40% vs. 10/49 = 20%, Fischer-Exact-test, p = 0.085, Odds Ratio 0.4 with a 95% confidence interval of 0.1 to 1.0, cf. Table 45).

When both groups were further subdivided into those receiving daily doses above the population median and those below or equal, however, a statistically significant difference was observed for those patients receiving doses above the population median (IMs 9/13 = 69% and EMs 4/23 = 17%, Fischer-Exact-test, p = 0.003), but not for those whose doses were below or equal the population median (IMs 5/22 = 23% and EMs 6/26 = 23%, p = 1.0, see Figure 67). The 3 PMs and 4 UMs were not included into evaluation because of the small sample size (Note: all PMs suffered from side effects). Within the IMs, the side effects frequency was rather lower for gene dose 0.5 (1/2 = 50% above median and 0/2 = 0% below median) than gene dose 1 (8/11 = 73% above median and 5/20 = 25% below median). Similar was true for gene dose 1.5 and 2 within the EM group (gene dose 1.5 with 0/5 = 0% above median and 4/12 = 33% below median vs. gene dose 2 with 4/18 = 22% above the median and 2/14 = 14% below the median).

The difference in the adverse effects frequency of IMs with doses below or equal (23%) and above the population median (69%) was also statistically significant. The Odds Ratio (OR) was 7.7 (95% confidence interval of 1.6 to 36, Fisher-Exact-test p = 0.012, see Figure 67). IMs treated with doses above the median also suffered from significantly more severe side effects (9/13 = 69%) when compared to IMs without CYP2D6 dependent medication (24/84 = 29%, Odds Ratio 5.6 with a 95% confidence interval of 1.64 to 20, Fischer-Exact test, p = 0.009).

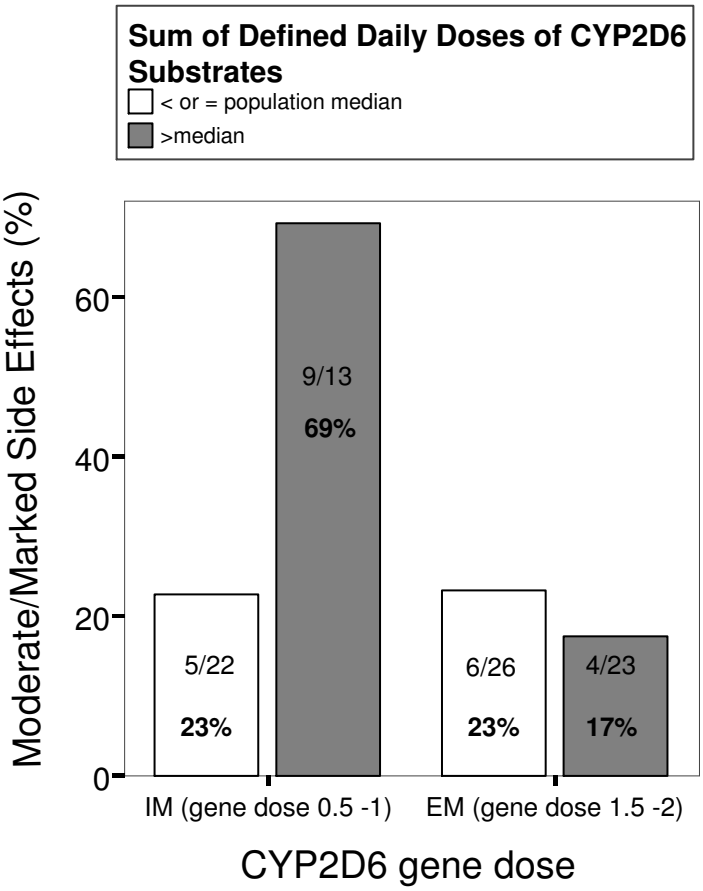

Figure 67: Frequency of adverse effects IMs and EMs in relation to dose

Bar chart depicts number of marked/moderate side effects separately for the IM and EM CYP2D6 genotype groups and according to the relative sum of administered CYP2D6 substrates (narrow definition). Dosing and side effects data was available for 91 patients (3 PMs, 35 IMs, 49 EMs and 4 UMs).

3.4.3.5 CYP2D6 Gene Dose, Administered Dose and Treatment Response

Complete data on PDS scores within the first four weeks was available for 230 patients and CGI(2) improvement rating was available for 338 patients. As patients with different diagnoses (mood as well as psychotic disorders) were included, the CGI rating was used for evaluation. Patients with a CGI(2) rating of 1 or 2 after four weeks were regarded as responders. Only the results of the narrow definition are presented as the results of the wider definition showed similar but weaker trends. Of the 338 patients, 99 patients received CYP2D6 dependent medication (95 with data on exact dosing) and 239 non-Cyp2D6 dependent medication.

The CGI(2) responder rate to CYP2D6 dependent medication and non-CYP2D6 dependent medication was with 14% (n = 14/99) and 18% (n = 42/239) similar (overall n = 338, Fisher-Exact-test, $p > 0.5$, see Table 46).

By comparison of patients receiving CYP2D6 dependent medication with those who did not, a statistically significant difference was found within the IM group: only 7% (3/42) of patients receiving CYP2D6 dependent medication but 25% (21/84) of patients without CYP2D6 drugs responded (Fisher-Exact-test, $p = 0.017$, see Figure 68). The opposite trend for EMs (18% (9/49) with and only 10% (13/125) without, see Table 46) was not significant (Fischer-Exact test, $p = 0.203$). The sample sizes of the PM and UM genotype groups were too small for a meaningful conclusion.

The difference in the responder rate was not significant for IMs vs. EMs receiving CYP2D6 dependent medication (7% vs. 18%, Fisher-Exact-test, $p = 0.134$), while the difference was significant for non-CYP2D6 dependent medication (25% vs. 10%, $p = 0.007$, see Figure 68 and Table 46).

Table 46: Responder rates for different CYP2D6 gene doses (I)

CYP2D6 metabolizer status	CYPD6 gene dose	non-CYP2D6 dependent medication			CYP2D6 dependent medication		
		n	CGI(2) responder		n	CGI(2) responder	
			n	%		n	%
PM	0	23	5	**22**	4	1	**25**
IM	0.5	15	3	**20**	4	0	**0**
	1	69	18	**26**	38	3	**8**
EM	1.5	36	4	**11**	17	2	**12**
	2	89	9	**10**	32	7	**22**
UM	>2	7	3	**43**	4	1	**25**
Total		**239**	**42**	**18**	**99***	**14**	**14**

* Exact dosing was missing for 3 patients with gene dose 1 (3 non-responder) and for 1 patient with gene dose 2 (non-responder). For the remaining 95 patients, the responder rates were also statistically significant different between IMs with and without (p = 0.041).

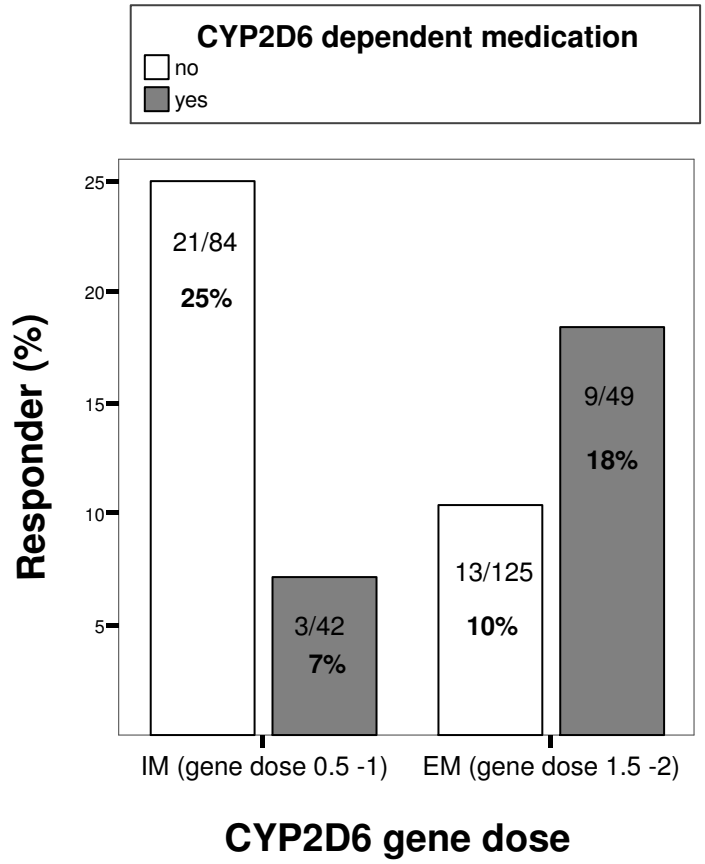

Figure 68: Responder rates for CYP2D6 genotype groups and different medication

Bar chart depicts number of responders i.e., CGI(2) improvement of 1 or 2 = (very) much improved, separately for the IM (gene dose 0.5 - 1) and EM (gene dose 1.5 - 2) CYP2D6 genotype groups and according to whether CYP2D6 dependent medication was administered or not. CGI(2) data available for 99 patients with CYP drugs (4 PMs, 42 IMs, 49 EMs and 4 UMs) and for 239 patients without CYP drugs (23 PMs, 84 IMs, 125 EMs and 7 UMs).

Differences in responder rates within CYP2D6 genotype groups were non-significant for both doses above and for doses below the study population median (IMs below median 2/25 = 8% and above the median 1/14 = 7%; p = 1.0; EMs below median 4/27 = 15% and above the median 5/21 = 24%, p = 0.477). Interestingly, a (non-significant) trend towards higher responder rates for higher gene doses was observed for doses above the population median (cf. Table 47, Pearson Chi, p = 0.091, Odds Ratio OR 6 with 95% confidence interval of 0.6 - 52).

Table 47: Responder rates for different CYP2D6 gene doses (II)

CYP2D6 metabolizer status	CYPD6 gene dose	CYP2D6 dependent medication / doses below population median			CYP2D6 dependent medication / doses above population median		
		n	CGI(2) responder		n	CGI(2) responder	
			n	%		n	%
PM	0	2	1	**50**	2	0	**0**
IM	0.5	2	0	**0**	2	0	**0**
	1	23	2	**9**	12	1	**8**
EM	1.5	12	1	**8**	5	1	**20**
	2	15	3	**20**	16	4	**25**
UM	>2	3	0	**0**	1	1	**100**
	Total	**57**	**7**	**12**	**38**	**7**	**18**

3.4.3.6 CYP1A2*1F Polymorphism and Therapeutic Outcome

One hundred sixty-two (44%) of the 365 patients received CYP1A2 substrates such as olanzapine, clozapine and haloperidol. The latter ones are - in contrast to olanzapine - also metabolized by other cytochrome P450 enzymes such as CYP3A4 [69,325]. Only the results obtained for olanzapine are presented below, since the evaluation of all CYP1A2 substrates together showed similar, but non-significant trends. Moreover, only patients with schizophrenic disorders (F2 diagnosis, n = 174) were included in the evaluation. Patients with mood disorders were also medicated with olanzapine, but they received lower doses and at the same time the baseline demographics were different (less smokers). This made a combined evaluation with regard to the influence of the induced CYP1A2*1F genotype impossible.

The inducible CYP1A2*1F genotype was evaluated in combination with CYP1A2 inducers (carbamazepine and tobacco smoke) as done in § 3.4.2.3. Thus, four groups were compared: the inducible CYP1A2*1F A/A genotype with (53/174 = 30%) and without inducers (38/174 = 22%) and the CYP1A2 wildtype C-allele in combination with (39/174 = 22%) and without inducers (44/174 = 25%; see Figure 69). No significant differences were observed for these four groups with regard to baseline demographics, diagnosis and anamnestic data (number of previous episodes, duration of previous hospital stays, all available data). No differences were also seen in frequency of adverse effects, response, response onset or length of hospitalization.

Non-smokers without the inducible form of CYP1A2 and without co-medication of carbamazepine are expected to reach higher olanzapine serum concentrations with the same dose (cf. § 3.4.2.3). As higher olanzapine concentrations are associated with better treatment response, this group should have the fewest number of switches from olanzapine due to non-response. Interestingly, in this group the prescription frequency of olanzapine was higher compared to all others (45% vs. 29% for all other groups, Fisher-Exact-test p = 0.064 for F2 patients, n = 174; see Figure 69).

Fifty-eight schizophrenic patients received olanzapine (dose data available). Patients with CYP1A2*1F A/A genotype who were smokers or received carbamazepine were treated with significantly higher doses of 19 ± 6 mg (mean ± SD) of olanzapine compared to all others with 14 ± 6 mg (Mann-Whitney U test , p = 0.011). The median doses applied during the first four weeks are depicted in Figure 70.

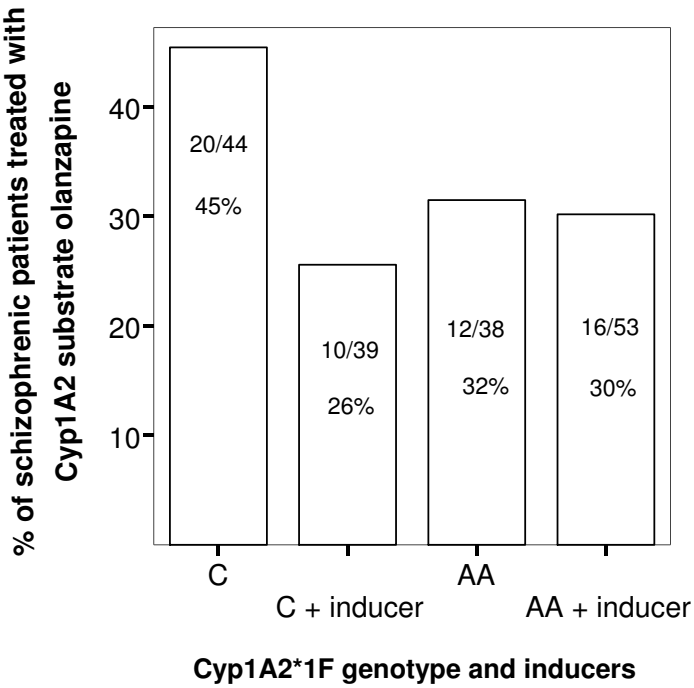

Figure 69: Prescription frequencies of olanzapine for induced CYP1A2

Bar chart depicts number of patients treated with olanzapine. Overall, 33% (58 of 174) schizophrenic patients received olanzapine. Wildtype C-allele/no inducer: 20/44 = 45% received olanzapine. Wildtype C-allele/inducer: 10/39 = 26% received olanzapine. Highly inducible AA genotype/no inducer: 12/38 = 32% received olanzapine. Highly inducible AA genotype/ inducer: 16/53 = 30% received olanzapine.

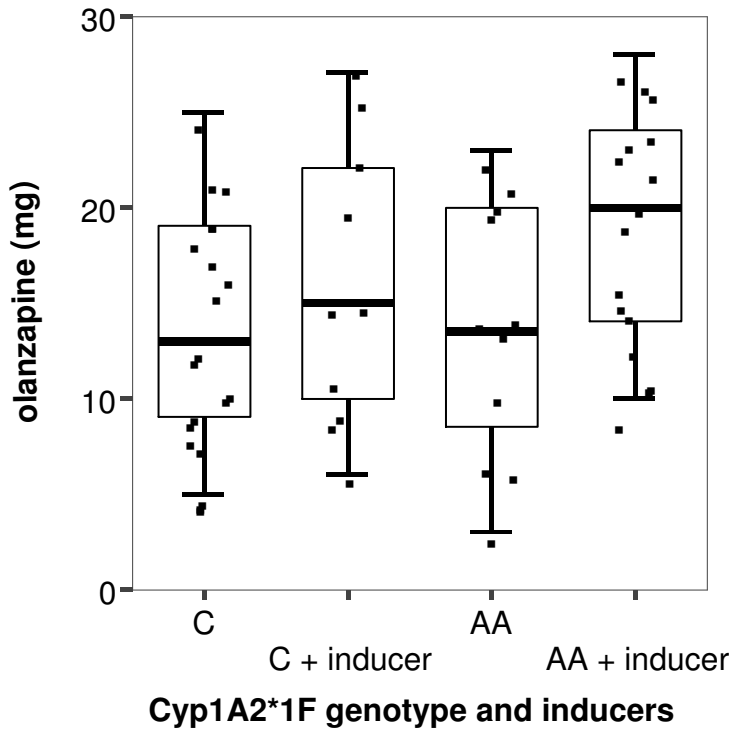

Figure 70: Administered doses of olanzapine for induced CYP1A2

A or C = CYP1A2*1F alleles; inducers = tobacco smoke and carbamazepine. Box-and-whisker plot: The center box shows median and interquartile range of data. The whiskers range is defined as 1.5 times the interval from the 25th to 75th percentile i.e., the box length. The two outliers in the C-allele/inducer group are between 1.5 and 3 box lengths away (open circle).

C-allele without inducer (median 13, mean ± SD = 13 ± 6, n = 20); C-allele with inducer (median 15, 16 ± 7, n = 10); AA genotype without inducer (median 14, 14 ± 6, n = 12); C-allele without inducer (median 20, 19 ± 6, n = 16).

3.4.3.7 CYP2C19 Gene Dose and Therapeutic Outcome

Nine (2.5%) of the patients were CYP2C19 poor metabolizers with two non-functional CYP2C19 alleles (gene dose 0), 101 (27.7%) intermediate or heterozygote metabolizers with one non-functional allele (gene dose 1) and 255 (69.9%) were extensive metabolizers with two functional alleles (gene dose 2). Ninety-eight of 365 patients (27%) received CYP2C19 substrates (citalopram or the tricyclic antidepressants amitriptyline, clomipramine, imipramine, doxepin or trimipramine). No significant results were observed for the evaluation of (es)citalopram or all CYP2C19 substrates together.

As some trends were visible despite the small number of patients treated with tricyclic antidepressants (n = 41), the results obtained for tricyclic antidepressants (TCAs) are presented herein. Higher prescription frequencies were observed for lower CYP2C19 gene doses (2/9 = 22% for PMs vs. 14/101 = 14% for IMs and 25/255 = 10% for EMs, Chi-square-test $p > 0.2$). No significant differences were observed for the different CYP2C19 genotypes with regard to baseline demographics, diagnosis and anamnestic data (number of previous episodes, duration of previous hospital stays, all available data).

To make the administered doses of the different TCAs comparable, the relative doses per compound per patient were calculated based on the daily dose as defined by the WHO for each drug [342] (i.e., 75 mg for amitriptyline, 100 mg for clomipramine, imipramine and doxepin and 150 mg for trimipramine were rated as a relative dose of 1). The relative doses were compared for the different CYP2C19 gene dose groups. Patients with lower CYP2C19 gene dose showed a trend to lower TCAs doses. The two CYP2C19 PMs received both only 50% of the defined daily dose, the 15 IMs on average 80% ± 40% and the 25 EMs received on average 106 ± 66% (Kruskal-Wallis test, $p = 0.15$).

Side effects data were available for 35 of the 41 patients (1 PM, 2 IM and 3 EM missing). In the patient group receiving TCAs, a pronounced increase in the rate of moderate or marked side effects (overall evaluation by the treating psychiatrist) was observed for the EM genotype group vs. the other gene doses (10/22 = 46% for EMs vs. 1/13 = 8% for IMs and PMs, Fischer-Exact-test, $p = 0.027$, see solid line in Figure 71). No significant differences were observed in the patients receiving medication that was not preferably metabolized by CYP2C19 (see dashed line in Figure 71). Of the 293 patients, 2/7 = 29% PMs, 24/81 = 30% IMs and 58/205 = 28% EMs had moderate/marked side effects ($p > 0.9$). No further differences in number of switches, response, response onset or length of hospitalization were observed.

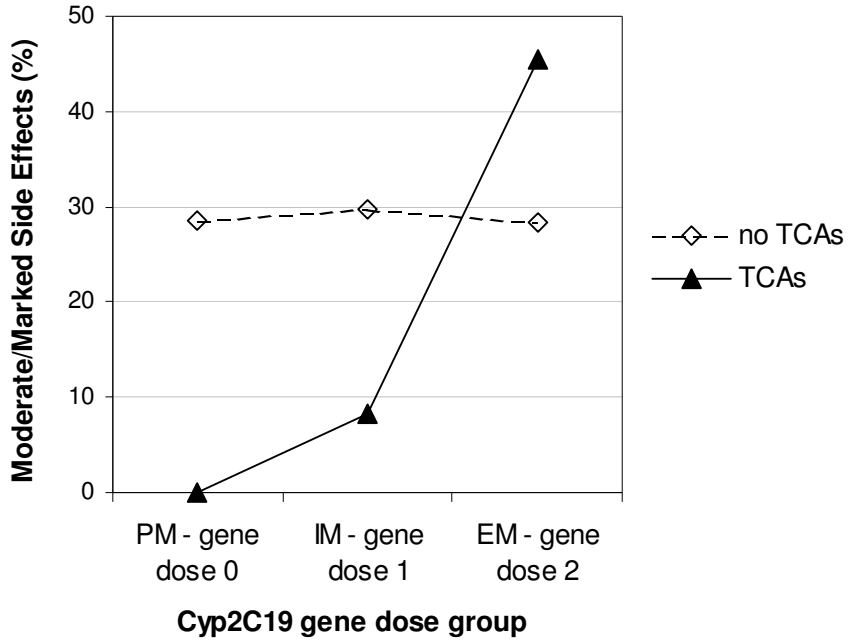

Figure 71: Frequency of adverse effects for different CYP2C19 gene doses under treatment with tricyclic antidepressants

Bar chart depicts number of marked/moderate side effects separately for the CYP2C19 genotype groups and according to whether tricyclic antidepressants were administered (35 patients; 1 PMs, 12 IMs, 22 EMs) or not (293 patients; 7 PMs, 81 IMs, 205 EMs).

3.4.4 Influence of MDR1 Polymorphisms on Therapeutic Outcome

Genetic variations in drug metabolizing enzymes such as the cytochrome P450 enzymes are not sufficient to explain the wide interindividual variation in drug concentrations and clinical outcome. The disposition of many drugs is influenced not only by the drug metabolizing enzymes but also by transporters such as P-glycoprotein.

The multidrug resistant (MDR1) gene encodes for the P-glycoprotein, which functions as an efflux transporter and is a part of the blood-brain barrier. Putative dysfunctional variants of the MDR1 gene (MDR1 C3435T, G2677TA and C1236T) may be associated - via higher bioavailability of P-gp substrates in the CNS - with better treatment response or occurrence of side effects. Olanzapine, risperidone, amitriptyline, citalopram, trimipramine, doxepin, venlafaxine and paroxetine, but not mirtazapine, are well known P-glycoprotein substrates.

Patients receiving P-glycoprotein substrates were evaluated whether there was an effect of the single MDR1 polymorphisms or the MDR1 haplotype (3435T-2677T-1236T) [97] on clinical outcome (such as prescription frequencies, administered daily doses, switches, frequency of adverse effects, improvement of symptoms, response onset or length of hospitalization).

Patients receiving antipsychotics, especially olanzapine, showed a significant difference for the baseline response parameters: lower CGI(1) for the heterozygote MDR1 haplotype TTT and MDR1 3435C/T genotype as well as lower PDS-P and PDS-D baseline score for the MDR1 3435T-allele. Performing multiple regression analysis taking the difference in baseline into account, no significant differences for response or side effects after four weeks were observed for the different MDR polymorphisms or haplotype.

3.4.5 Influence of Serotonergic Polymorphisms on Therapeutic Outcome

3.4.5.1 Antidepressants and 5-HTTLPR/rs25531 (A/G)

The serotonin transporter (5-HTT) is the primary site of action of the selective serotonin reuptake inhibitors (SSRIs) and plays also a role in therapeutic mechanism of venlafaxine and tricyclic antidepressants such as amitriptyline. A length polymorphism in the promoter region in combination with a SNP nearby was reported to have influence on transcription activity [112-114] and to be linked to better treatment response and less adverse effects. Thus, patients with the favorable high expression variant L_A (i.e., 5-HTTLPR L-allele in combination with rs25531 A-allele) were compared to patients with the unfavorable variants, the 5-HTTLPR S-allele and the L_G variant (i.e., 5-HTTLPR L-allele in combination with rs25531 G-allele).

Patients receiving only SSRIs as antidepressant medication (n = 71, complete response data not available for all patients, see Table 48 below) and homozygous for unfavorable 5-HTT promoter polymorphisms (SS, SL_G, L_GL_G) had higher CGI(1) severity ratings after 4 weeks of treatment than the other genotypes (Mann-Whitney U test, p = 0.040 for CGI(1) after 4 weeks, p-values ≥ 0.2 for CGI(1) at baseline, CGI(2) rating or CGI(1) difference). No significant associations were found PDS-D scores for all patients as well as for patients diagnosed with mood disorders only.

Patients with the unfavorable 5-HTT promoter polymorphisms and treated only with SSRIs stayed on average 110 ± 73 days in hospital, whereas the other genotypes stayed only 60 ± 34 days (p = 0.011). The hospital stay was also significant longer when compared to patients with the unfavorable 5-HTT promoter polymorphisms, but without SSRI therapy, which stayed on average for 53 ± 32 days (p = 0.003). For the response onset, the trends were not significant (comparison of genotypes: 74 days vs. 32 days, p = 0.120 and comparison of therapy with/without SSRI: 74 days vs. 29 days, p = 0.067).

Table 48: Response data for 5-HTT promoter polymorphisms

5-HTT		CGI(1) baseline	CGI(1) 4 weeks	CGI(2) 4 weeks	CGI(1) difference
L_AL_A	Mean	5.71	5.00	3.52	-0.65
	SD	1.12	1.03	0.98	0.93
	n	24	20	21	20
L_AS, L_AL_G	Mean	5.70	4.73	3.48	-0.97
	SD	0.88	1.20	1.18	1.22
	n	33	30	31	30
SS, SL_G, L_GL_G	Mean	6.08	5.90	3.91	-0.30
	SD	0.86	1.60	0.83	1.06
	n	13	10	11	10
Total	Mean	5.77	5.02	3.57	-0.75
	SD	0.97	1.27	1.06	1.11
	n	70	60	63	60

L_A = HTTLPR L-allele in combination with rs25531 A-allele; S = HTTLPR S-allele; L_G = HTTLPR L-allele plus rs25531G-allele
Note 1: Mann-Whitney U test (SS, SL_G, L_GL_G vs. other genotypes): CGI(1) severity baseline p = 0.26, CGI(1) severity after 4 weeks p = 0.04, CGI(2) improvement after 4 weeks p = 0.25, CGI(1) difference p = 0.20
Note 2: similar p-values were obtained when only patients with full CGI rating data were compared

More pronounced was the effect of the 5-HTT promoter polymorphisms under SSRI monotherapy on the adverse effects: patients that were homozygous for unfavorable 5-HTT promoter polymorphisms (SS, SL_G, L_GL_G) and that received only SSRIs as antidepressant medication (n = 71, side effects data available for 66 patients, see Table 49 below) suffered from more gastrointestinal side effects and dizziness than patients without these genotypes (Mann-Whitney U test vs. other genotypes p < 0.001 for cluster c, p = 0.084 for cluster d and p = 0.068 for total DOTES sum score).

With regard to the overall rating (incidence of moderate/marked side effects) by the treating psychiatrist, however, the differences were not significant. Baseline characteristics (age, gender, height, weight, smoking habits, diagnosis, defined daily dose as well as baseline CGI and PDS rating, age of onset and duration of illness if available) were comparable between the different 5-HTT genotype groups.

Table 49: Adverse effects for 5-HTT promoter polymorphisms under SSRI therapy

5-HTT		total score	cluster a	cluster b	cluster c	cluster d	cluster e
L_AL_A	Mean	2.10	1.14	0.29	0.10	0.05	0.52
(n = 21)	SD	2.79	2.08	0.78	0.44	0.22	1.12
L_AS, L_AL_G	Mean	1.33	0.36	0.30	0.15	0.03	0.48
(n = 33)	SD	1.59	0.70	1.16	0.51	0.17	1.15
SS, SL_G, L_GL_G	Mean	3.83	1.00	0.58	1.08	0.58	0.58
(n = 12)	SD	4.95	1.81	1.38	1.08	1.73	1.00
Total	Mean	2.03	0.73	0.35	0.30	0.14	0.52
(n = 66)	SD	2.94	1.50	1.09	0.72	0.76	1.10

L_A = HTTLPR L-allele in combination with rs25531 A-allele; S = HTTLPR S-allele; L_G = HTTLPR L-allele plus rs25531G-allele

Note: Mann-Whitney U test (SS, SL_G, L_GL_G vs. other genotypes): total DOTES/side effects score, p = 0.07; Cluster a (mental side effects: toxic confusion, agitation, depression, increased/decrease motoric activity, sleeplessness, somnolence), p = 0.70; Cluster b (neuromuscular symptoms: rigor, tremor, dystonia, akathisia, tardive dyskinesia), p = 0.54; Cluster c (anticholinergic and gastrointestinal symptoms: dry mouth, blocked nose, blurred vision, congestion, salivation, sweating, nausea/vomiting, diarrhea), p = 0.00002; Cluster d (cardiovascular symptoms: hypotonia, dizziness, tachykardia, hypertonia, abnormal ECG), p = 0.08; Cluster e (other symptoms: dermal symptoms, weight gain, weight loss, anorexia, headache, other), p = 0.47

When patients with the unfavorable 5-HTT promoter polymorphisms (SS, SL_G, L_GL_G) were compared, those receiving only SSRIs as antidepressant therapy, suffered from more gastrointestinal side effects than patients with other treatment (cluster c, p = 0.004). No significant associations were found for patients receiving (only) unselective serotonin reuptake inhibitors or other antidepressants.

3.4.5.2 Antidepressants/Atypical Antipsychotics and 5-HTR2A Polymorphisms

The efficacy of serotonin reuptake inhibitors is partly mediated by the effect of the serotonin that is released by antidepressants on the 5-HTR2A receptors. This was suggested to be an underlying mechanism for side effects, although other receptors such as 5-HTR3 may also be involved [107,126]. Thus, the 5-HTR2A gene is a candidate gene that may be linked to clinical outcome of antidepressants, especially SSRIs. Moreover, atypical antipsychotics also target serotonin receptors (particularly 5-HTR2A, 5-HTR2C and 5-HTR1A receptors) [61].

Olanzapine was the most prescribed atypical antipsychotic in the study population (n = 124). Patients that were homozygous for the A/A variant of the 5-HTR2A intron 2 (rs7997012) polymorphism and taking olanzapine suffered from more pronounced side effects especially from more gastrointestinal side effects compared carriers of the wildtype G-allele. The side effects total score and in particular the cluster c score was significantly higher in A/A than G-allele carriers (p = 0.012 and p = 0.009, see Table 50). With regard to the overall rating by the treating psychiatrist, only 28% (28/101) of G-allele carriers, but 50% (7/14) with A/A genotype had moderate/marked side effects (p = 0.121).

But the A/A genotype tended to have also a better response to olanzapine therapy within 4 weeks (mean CGI(2) improvement rating of 3.1 vs. 3.5 for G carriers, p = 0.010, data available for 118 patients) despite similar baseline CGI(1) severity of illness (p = 0.60). Moreover, patients with A/A genotype were on average only 48 days in hospital compared to 65 days for G-allele carriers (p = 0.034, see Table 50). The response onset was with 28 vs. 31 days similar.

As mirtazapine blocks the 5-HTR2 and 5-HTR3 serotonin receptors and as the efficacy of SSRIs is partly mediated via the 5-HTR2A receptor, three groups were compared within the olanzapine group in a second step: patients with mirtazapine co-medication (n = 48), patients with SSRI co-medication (n = 21) and patients without mirtazapine or SSRI co-medication (n = 55). In the group with

mirtazapine co-medication no association between 5-HTR2A intron 2 genotype and side effects was present for the overall rating and the total side effects score (n = 45 available, both p > 0.65).

In the group without mirtazapine co-medication the total side effects score was significantly higher in A/A than G-allele carriers (n = 50 available, p = 0.006 and with inclusion of patients with SSRI co-medication, n = 70, p = 0.003). Moreover, without mirtazapine/SSRI co-medication, only 25% (11/44) of G-allele carriers, but 83% (5/6) of the patients with A/A genotype had moderate/marked side effects (n = 50, Fisher-Exact-test, p = 0.0098, Odds Ratio of 15 with a 95% confidence interval of 1.6 – 143).

Table 50: Adverse effects for 5-HTR2A intron 2 polymorphism under olanzapine therapy

5-HTR2A intron 2		total score	cluster a	cluster b	cluster c	cluster d	cluster e	hospitalization (days)	mean daily dose (mg)
G/G	Mean	1.89	0.76	0.31	0.20	0.11	0.51	62	12.5
	SD	2.92	1.63	0.87	0.69	0.44	0.99	35	6.8
	n	45	45	45	45	45	45	49	49
G/A	Mean	2.27	0.82	0.39	0.30	0.23	0.52	67	11.8
	SD	2.50	1.16	1.07	0.69	0.79	0.89	35	7.2
	n	56	56	56	56	56	56	57	57
A/A	Mean	3.86	1.00	1.14	1.14	0.00	0.57	48	12.0
	SD	2.91	1.75	2.57	1.92	0.00	0.85	37	5.1
	n	14	14	14	14	14	14	18	18
Total	Mean	2.31	0.82	0.45	0.37	0.16	0.52	63	12.1
	SD	2.76	1.42	1.29	0.96	0.62	0.92	36	6.8
	n	115	115	115	115	115	115	124	124

Data are presented by mean ± SD. Complete DOTES score data were available for 115 of the 124 patients treated with olanzapine (45 GG, 56 GA and 14 AA). Mann-Whitney U test (AA vs. G-allele): total DOTES/side effects score p = 0.012; Cluster a (mental side effects) p = 0.91; Cluster b (neuromuscular symptoms) p = 0.56; Cluster c (anticholinergic/ gastrointestinal symptoms) p = 0.009; Cluster d (cardiovascular symptoms) p = 0.28; Cluster e (other symptoms) p = 0.64; length of hospitalization p = 0.034, mean daily dose p = 0.79

Furthermore, the influence of the 5-HTR2A intron 2 polymorphism on patients receiving SSRIs as antidepressant was examined. A significant effect was only observed when patients with co-medication of mirtazapine or antipsychotics, especially olanzapine were excluded from evaluation (n = 23, side effects data available for 19 patients). Patients with the 5-HTR2A intron 2 A/A genotype suffered from significantly more side effects compared to G-allele carriers (p = 0.0007, see Table 51). Only 7% (1/15) of G-allele carriers, but 75% (3/4) of the patients with A/A genotype had moderate/marked side effects (n = 19, Fisher-Exact-test, p = 0.016, Odds Ratio of 42 with a 95% confidence interval of 2 - 877).

Table 51: Adverse effects for 5-HTR2A intron 2 polymorphism under SSRI therapy

5-HTR2A intron 2		total score	cluster a	cluster b	cluster c	cluster d	cluster e
G/G	Mean	0.33	0.17	0.00	0.00	0.17	0.00
(n = 6)	SD	0.82	0.41	0.00	0.00	0.41	0.00
G/A	Mean	0.44	0.00	0.00	0.00	0.00	0.44
(n = 9)	SD	1.33	0.00	0.00	0.00	0.00	1.33
A/A	Mean	4.25	0.00	0.75	1.00	0.50	2.00
(n = 4)	SD	1.50	0.00	1.50	2.00	1.00	2.45
Total	Mean	1.21	0.05	0.16	0.21	0.16	0.63
(n = 19)	SD	1.99	0.23	0.69	0.92	0.50	1.54

Note: Mann-Whitney U test (AA vs. G-allele): total DOTES/side effects score, p = 0.0007; Cluster a (mental side effects), p = 0.611; Cluster b (neuromuscular symptoms), p = 0.05; Cluster c (anticholinergic/ gastrointestinal symptoms), p = 0.05; Cluster d (cardiovascular symptoms), p = 0.26; Cluster e (other symptoms), p = 0.04

There were no significant differences in length of hospitalization (p = 0.1) or CGI(2) response (p = 0.7). Baseline characteristics (age, gender, height, weight, smoking habits, daily olanzapine/ SSRI dose as well as baseline CGI rating, age of onset and duration of illness if available) were comparable for the different 5-HTR2A intron 2 genotypes in the study subpopulations examined. The negative effect of the 5-HTR2A intron 2 polymorphism was only seen in those patients receiving olanzapine or SSRI, but not in any other patient groups. Chi-square tests showed no association between 5-HTR2A intron 2 A/G genotype and 5-HTTLPR polymorphisms. No significant associations were found for patients receiving (only) unselective serotonin reuptake inhibitors or other antidepressants as well as for patients receiving various atypical antipsychotics. No significant associations were observed for the different 5-HTR2A T102C genotypes.

3.4.5.3 Antidepressants/Atypical Antipsychotics and Other 5-HTR Polymorphisms

Antidepressants desensitize the 5-HTR1A autoreceptors by increased synaptic serotonin levels, which may contribute to their clinical efficacy [118,119]. Less treatment response to SSRIs and TCAs was reported for the putative non-functional 5-HTR1A -1019G-allele in some previous studies [119-125].

Antipsychotics, especially atypical ones, also target serotonin receptors (5-HTR1-7) in addition to dopamine receptors. Many antipsychotics have high affinity for the 5-HTR6 receptor [148]. In addition, this receptor exhibits high affinity for a number of tricyclic antidepressant drugs including amitriptyline [149]. An association between the silent 5-HTR6 T267C polymorphism and treatment response to atypical antipsychotics (clozapine, risperidone) or antidepressants were reported in some previous studies [150-154].

Neither response nor side effects were significantly associated with the different 5-HTR2C C-759T, 5-HTR6 T267C or 5-HTR1A C-1019G genotypes in any of the different medication subgroups. For the association between 5-HTR2C C-759T and weight gain see § 3.4.9.

3.4.6 Influence of Dopaminergic Polymorphisms on Therapeutic Outcome

3.4.6.1 Antipsychotics and DRD Polymorphisms

All currently available antipsychotic drugs - typical or atypical - act on CNS dopamine receptors. Their major antipsychotic effect is mediated via the DRD2, but also DRD3 is involved. Polymorphisms within these receptors may have an impact on treatment response.

Homozygote carriers of the DRD2 -141C del-allele were very rare in the study population (only 1.6%, cf. also appendix A2.4 for allele frequencies). To obtain sufficiently large groups for statistical evaluation, the heterozygote and homozygote carriers of the DRD2 -141C del were compared with the wildtype patients (DRD2 -141C ins/ins). The same procedure was applied to the rare DRD2 Taq A1/A1 genotype (3.6%) and the DRD3 9Gly-allele (9.6%).

For all patients receiving antipsychotic treatment (n = 292), the DRD2 -141C ins/ins as well as the DRD2 TaqI A1-allele were both associated with better CGI(2) improvement after 4 weeks (data available for 271 patients, see Table 52 and Table 53). Similar trends were observed for subgroups (patients with F2 diagnosis only, patients receiving only typical antipsychotics). Pearson chi-square test revealed a significant association between the two examined DRD2 polymorphisms (Pearson Chi-Square p = 0.002).

Carriers of the DRD3 9Ser/9Ser wildtype suffered from less side effects (n = 130, DOTES sum score 2.5 ± 4.2) compared to carriers of the DRD3 9Gly-allele (n = 133, DOTES sum score 2.7 ± 2.8, Mann-Whitney U test p = 0.024; adverse effects data available for 263 of 292 patients). No significant baseline differences (age, gender, height, weight, smoking habits, diagnosis, daily antipsychotic dose as well as baseline CGI rating, age of onset and duration of illness if available) were observed for the different genotypes in the study subpopulation examined.

Table 52: Response for DRD2 -141C ins/del polymorphism under antipsychotic therapy

DRD2 -141C ins/del		CGI(1) baseline	CGI(1) 4 weeks	CGI(2) 4 weeks
del/del or del/ins	Mean	6.0	5.3	3.7
	SD	0.9	1.0	0.8
	n	47	42	44
ins/ins	Mean	6.0	5.0	3.4
	SD	0.9	1.3	1.0
	n	240	221	227
Total	Mean	6.0	5.0	3.5
	SD	0.9	1.2	1.0
	n	287	263	271
Mann-Whitney U test	p-value	0.617	0.160	0.038

Note: Mann-Whitney U test for comparison of del-allele vs. ins/ins genotype, CGI = Clinical Global Impression (see § 2.1.3.3) by the treating psychiatrist with the CGI(1) severity rating and CGI(2) improvement rating

Table 53: Response for DRD2 TaqI polymorphism under antipsychotic therapy

DRD2 TaqI		CGI(1) baseline	CGI(1) 4 weeks	CGI(2) 4 weeks
A1/A1, A1/A2	Mean	6.0	4.7	3.3
	SD	1.0	1.3	0.9
	n	101	93	97
A2/A2	Mean	6.0	5.2	3.6
	SD	0.9	1.2	1.0
	n	186	170	174
Total	Mean	6.0	5.0	3.5
	SD	0.9	1.2	1.0
	n	287	263	271
Mann-Whitney U test	p-value	0.929	0.003	0.050

Note: Mann-Whitney U test for comparison of A1 allele vs. A2/A2 genotype

3.4.6.2 Antipsychotics/Antidepressants and DAT1 VNTR Polymorphism

As dopamine pathways play an important role in psychiatric diseases, the high affinity dopamine transporter DAT1 is another candidate gene which may influence clinical outcome in both mood and schizophrenic disorders. Only 9- and 10-repeat allele carriers were evaluated. The number of 11-repeat allele carriers (6 of 365, see appendix A2.4 for allele frequencies) were too small for a conclusive evaluation. With respect to response or side effects to antipsychotic drugs, the DAT1 VNTR did not show any significant associations.

In patients with a major depressive episode (diagnosis F32 - 34, n = 136, response data according to the PDS-Scale available for n = 79) receiving any kind of antidepressant therapy, the PDS-D scores were compared between the different DAT1 VNTR genotypes. Baseline PDS-D scores were similar for all three genotypes (see Table 54). The % improvement after 4 weeks, however, increased from only 14% for 10/10-repeat carriers to 32% for heterozygote 9/10-repeat carriers and to 60% for the 9/9-repeat carriers. If the 9/9-repeat carriers were compared to the hetero- and homozygote 10-repeat allele carriers, the difference was statistical significant (Mann-Whitney U test, p = 0.039, see Table 54).

Correspondingly, the CGI(1) severity rating showed a non-significant trend after 4 weeks (p = 0.078) but not at baseline (p = 0.905). No significant baseline differences (age, gender, height, weight, smoking habits, diagnosis, daily antidepressant dose, age of onset and duration of illness if available) were observed for the different genotypes in the study subpopulation examined.

Table 54: Response for DAT1 VNTR polymorphism under antidepressant therapy

DAT1 VNTR		PDS-D score			
		baseline	4 weeks	difference	difference (%)
9/9	Mean	22.6	9.1	13.4	59.6
(n = 7)	SD	8.5	6.3	7.6	24.0
9/10	Mean	22.1	16.0	5.8	32.3
(n = 28)	SD	8.3	11.6	7.2	36.5
10/10	Mean	22.1	17.2	4.9	13.6
(n = 44)	SD	8.7	11.0	10.8	61.4
Total	Mean	22.1	16.1	6.0	24.3
(n = 79)	SD	8.4	11.0	9.7	52.8

Note: Mann-Whitney U test (comparison of 9/9-repeat genotype vs. 10-repeat allele): PDS-D score baseline p = 0.95, PDS-D score after 4 weeks 0.091, PDS-D score difference p = 0.023 and (%) difference p = 0.039

3.4.7 Influence of (Nor)adrenergic Polymorphisms on Therapeutic Outcome

3.4.7.1 Antidepressants and NET Polymorphisms

Antidepressant efficacy is mediated not only by serotonin reuptake inhibition, but also by norepinephrine (= noradrenaline) reuptake inhibition. The norepinephrine transporter (NET) modulates noradrenergic signaling by reuptake of norepinephrine that has been secreted in the synaptic cleft. Thus, the influence of the NET T-182C and G1287A polymorphisms on patients receiving norepinephrine reuptake inhibitors (NRIs) such as TCAs, venlafaxine or reboxetine as antidepressant was examined.

A significant effect was only observed when patients with co-medication of SSRIs or mirtazapine were excluded from evaluation. Of the remaining 31 patients, only two patients were NET -182 C/C homozygote and only for one of these NET -182 C/C homozygotes, full response data was available. Thus, only C/T carriers were compared to T/T carriers. The carriers of T/T genotype showed worse response according to the PDS-D score and CGI rating after 4 weeks (see Table 55).

Table 55: Response for NET T-182C polymorphism under NRI therapy

NET T-182C		PDS-D score				CGI rating		
		baseline	4 weeks	difference	% difference	CGI(1) baseline	CGI(1) after 4 weeks	CGI(2) after 4 weeks
C/C	Mean	22	19	3	14	5	5	4
	n	1	1	1	1	2	2	2
C/T	Mean	23.0	12.5	10.5	48.6	5.5	4.5	3.2
	SD	10.7	10.9	9.0	30.5	0.8	1.2	1.1
	n	8	8	8	8	10	10	10
T/T	Mean	22.2	22.6	-0.3	-6.2	6.1	5.6	4.1
	SD	11.1	12.0	5.6	49.7	1.0	1.2	1.0
	n	9	9	9	9	15	15	15
Total	Mean	22.6	17.9	4.7	19.3	5.8	5.1	3.7
	SD	10.3	11.9	8.8	47.9	0.9	1.2	1.1
	n	18	18	18	18	27	27	27
MWU	p-value	0.743	0.036	0.008	0.008	0.121	0.055	0.053

Note: MWU = Mann-Whitney U test for comparison of C/T vs. T/T genotype

Baseline characteristics (age, gender, height, weight, smoking habits, daily doses) were similar for the different genotypes in the study subpopulation examined. Unlike the 31 patients receiving only NRI as antidepressant therapy, the negative effect of the NET -182 T/T polymorphism on response was not seen in all other patients receiving antidepressants. No significant associations were observed for the different NET G-1287A genotypes. For the association between ADR2A C-1291G and weight gain see § 3.4.9.

3.4.8 Influence of Polymorphisms in Signal Transduction Cascades on Therapeutic Outcome

In addition to the primary drug targets such as the dopaminergic, serotonergic and noradrenergic transporters and receptors, novel candidate genes more indirectly involved in drug action downstream of primary monoaminergic activation were examined. These candidates included genes in the neurotransmitter synthesis (TPH) and metabolism (COMT, ACE, MAO-A, DBH), in signal transduction (G-protein), genes modulating hypothalamic-pituitary-adrenal axis activity that is linked to depression (FKBP5) or involved in the neurodevelopment of neurotransmitter-related systems (BDNF).

Significant associations were observed neither for response nor for side effects under antidepressant and/or antipsychotic therapy and the examined TPH1, COMT, MAO-A, DBH, COMT, ACE, FKBP5, BDNF or GNB genotypes. Carriers of the T-allele of the FKBP5 polymorphism, especially heterozygotes, with a major depressive episode (diagnosis: F32 to F34) had a significantly longer duration of illness in anamnesis and a trend to more previous episodes. Moreover, the CGI(1) severity rating at baseline was lower for T-carriers.

In patients with mood disorders (F3 diagnosis), the age of onset was significantly higher for the high activity COMT genotype (Kruskal-Wallis test, p = 0.002, see Table 56). This result was also true for all subgroups of mood disorders, except for those patients with F32 diagnosis experiencing their first depressive episode.

Table 56: Age of onset (years) for COMT Val 158Met polymorphism

diagnosis	COMT	Mean	SD	n
F3	low (Met / Met)	37.5	14.0	28
	medium (Met / Val)	40.7	16.2	54
	high (Val / Val)	52.7	13.3	16
	Total	41.7	15.8	98
all except F3	low (Met / Met)	30.3	10.6	45
	medium (Met / Val)	30.6	9.7	97
	high (Val / Val)	32.3	14.9	44
	Total	30.9	11.3	186

Patients treated with olanzapine and carrying the GNB 825T/T genotype had a significantly faster response onset (Kruskal-Wallis test, p = 0.010, see Table 57), though the total length of the hospital stay was comparable between the GNB genotypes (p = 0.75, see Table 57). Thirteen of these 124 patients left the hospital before onset of response. Notably, only two had the favorable T/T genotype, 4 were C/T and 7 C/C. No further differences in demographics, baseline or side effects data were observed.

Table 57: Response for GNB polymorphism under olanzapine therapy

GNB C825T		length of hospitalization (days)	response onset (days)
C/C	Mean ± SD	64 ± 36	33 ± 25
	n	58	55
C/T	Mean ± SD	61 ± 35	31 ± 23
	n	58	50
T/T	Mean ± SD	59 ± 47	10 ± 5
	n	8	6
Total	Mean ± SD	63 ± 36	31 ± 24
	n	124	111

3.4.9 Influence of Polymorphisms on Weight Gain during Therapy

Weight gain is a severe problem in therapy of mood and schizophrenic disorders. Of all antipsychotics, clozapine and olanzapine induce particularly profound weight gain [343]. Lithium, valproic acid and amitriptyline are known to lead to marked weight gain; mirtazapine and risperidone to intermediate weight gain [329]. The exact underlying pharmacodynamic mechanisms involved in antipsychotic-induced weight gain have not been fully elucidated. Candidate gene studies have produced significant findings in the 5HTR2C and ADR2A receptor genes, which were also examined herein [66,177,178,344].

3.4.9.1 Atypical Antipsychotics and 5-HTR2C C-759T

The 5-HTR2C receptor gene lies on the X-chromosome and codes for one of the two major 5-HTR2 receptor subtypes that are targeted by antipsychotics. The -759C-allele showed less transcriptional activity compared to the -759T-allele and was associated with higher weight gain under antipsychotic treatment.

Patients receiving olanzapine or clozapine as only antipsychotic treatment (n = 85) were compared for weight gain after four weeks. Patients homo- or hemizygous for the 5-HTR2C -759C-allele had a significantly higher risk of weight gain (weight data available for n = 81, see Table 58). The negative effect of the 5-HTR2C polymorphism on weight gain was not seen in all other patients. Patients without olanzapine or clozapine treatment had on average a weight gain of 2%, which was similar for both genotype groups (2.1% for 67 C/T, T/T or T vs. 2.0% for 182 C/C or C, $p > 0.5$).

Table 58: Weight gain for 5-HTR2C polymorphism under olanzapine/clozapine therapy

5-HTR2C C-759T		weight (kg)			BMI (kg/m²)			weight gain (%)
		baseline	4 weeks	difference	baseline	4 weeks	difference	
C/T, T/T or T	Mean	69.2	70.1	0.9	24.3	24.4	0.1	1.4
(n = 23)	SD	13.1	13.2	2.7	4.7	4.6	0.9	3.9
C/C or C	Mean	72.3	74.8	2.4	24.3	25.1	0.8	3.8
(n = 58)	SD	14.3	13.5	3.4	4.3	4.0	1.1	4.8
Total	Mean	71.5	73.5	2.0	24.3	24.9	0.7	3.1
(n = 81)	SD	14.0	13.5	3.3	4.4	4.2	1.1	4.7
MWU	p-value	0.448	0.226	0.004	0.753	0.241	0.006	0.007

Note: MWU = Mann-Whitney U test for comparison of T-allele carriers vs. C/C-homozygotes and C-hemizygotes

With regard to baseline and demographic data, the patients homo- or hemizygous for the 5-HTR2C -759C-allele were younger (38 ± 15 years vs. 48 ± 17 years, $p = 0.014$), had a higher PDS- P baseline score (9 ± 8 vs. 4 ± 6, $p = 0.016$) and were hospitalized longer (71 ± 36 vs. 56 ± 53 days, $p = 0.012$). This is due the fact that more male schizophrenic patients are present within the CC, C-allele group, which were on average younger, suffered from more psychotic symptoms and stayed longer in hospital. When the genders were compared separately, antipsychotic-induced weight gain was observed both in males (on average 0.5% in 7 T-hemizygotes and 4% in 31 C-carriers, $p = 0.036$) and females (on average 1.7% in 16 T-carriers and 3.5% in 27 CC-carriers, $p = 0.080$). Weight gain was also more pronounced in schizophrenic patients (with more male patients) and it seems to be dose-dependent as patients with schizophrenic disorders (F2 diagnosis) were treated with higher doses of olanzapine than patients with mood disorders (on average 15 mg vs. 9 mg olanzapine, none of the depressive patients was treated with clozapine).

Thus, multiple regression analysis was performed to take possible additional influence factors into account (baseline weight or BMI, age, gender, diagnosis, dose, smoking status, response, treatment naïveness/first episode, co-medication with antidepressants and lithium). This analysis identified baseline BMI (standardized partial correlation coefficients beta = -0.36; $p < 0.001$), CGI(2) improvement (standardized partial correlation coefficients beta = -0.27; $p = 0.007$) and the homo- or hemizygous 5-HTR2C C-759 genotype (beta = 0.23; $p = 0.019$) as significant influence factors. The

coefficient of determination $r^2 = 0.27$ indicates that 27% of the weight variation can be explained by genotype, response and baseline BMI. The ADR2A C-1291G (see § 3.4.9.2) genotype (beta = 0.18; p = 0.072) missed inclusion in the model.

3.4.9.2 Atypical Antipsychotics and ADR2A C-1291G

In addition to the 5-HT2C -759C-allele, the ADR2A -1291G-allele was previously associated with olanzapine- and clozapine-induced weight gain. The Pearson Chi-square test revealed no significant association between the ADR2A C-1291G and the 5-HTR2C C-759T polymorphism (p = 0.6) in the study population.

Patients receiving only clozapine or olanzapine as antipsychotic treatment were compared for weight gain after four weeks (weight data available for n = 81). A trend to higher weight gain after 4 weeks was observed in patients with the ADR2A -1291G-allele (see Table 59).

No influence of the ADR2A polymorphism on weight gain was seen all other patients except those receiving mirtazapine (see § 3.4.9.3). Patients without olanzapine or clozapine treatment had on average a weight gain of 2%, which was similar for the genotype groups (2.1% for 148 C/C, 1.7% for 82 C/G and 2.5% for 19 G/G, p > 0.4).

Table 59: Weight gain for ADR2A polymorphism under olanzapine/clozapine therapy (I)

ADR2A C-1291G		weight (kg)			BMI (kg/m²)			weight gain (%)
		baseline	4 weeks	difference	baseline	4 weeks	difference	
C/C	Mean	72.5	73.9	1.4	24.4	24.8	0.5	2.2
(n = 37)	SD	14.9	14.0	3.7	4.7	4.4	1.2	4.9
C/G	Mean	70.4	72.6	2.2	24.1	24.9	0.7	3.3
(n = 35)	SD	13.3	13.4	2.9	4.2	4.2	1.0	4.7
G/G	Mean	71.3	75.0	3.7	24.3	25.6	1.3	5.6
(n = 9)	SD	13.9	12.9	2.3	3.9	3.4	0.8	3.4
Total	Mean	71.5	73.5	2.0	24.3	24.9	0.7	3.1
(n = 81)	SD	14.0	13.5	3.3	4.4	4.2	1.1	4.7
KWT	p-value	0.893	0.839	0.120	0.995	0.753	0.084	0.093

Note: KWT = Kruskal-Wallis test for comparison of the three genotype groups (C/C, C/G, G/G)

With regard to baseline and demographic data, the 9 ADR2A -1291 G/G patients were ill for a significantly shorter period compared to the other ADR2A -1291 genotypes (Pearson Chi-square test, p = 0.015). For 8 of the 9 G/G patients, the present episode was the first one, whereas the present episode was the first one for only approximately a third of the patients with C/C (13/37) and only a half of the patients with C/G genotype (17/35).

Patients with and without first episode were compared separately for the effect of the ADR2A polymorphism on olanzapine/clozapine induced weight gain. For first episode patients, no definite and clear trends were observed. For the other patients, however, a trend to increased weight gain for carriers of the ADR2A -1291G-allele (18 C/G and 1 G/G) compared to the 24 C/C homozygotes was observed, despite the small sample size (p = 0.087, see Table 60).

Table 60: Weight gain for ADR2A polymorphism under olanzapine/clozapine therapy (II)

weight gain after 4 weeks (%)		first episode	not first episode
C/C	Mean ± SD	4.5 ± 5.2	1.0 ± 4.3
	n	13	24
C/G	Mean ± SD	2.8 ± 4.4	3.8 ± 5.0
	n	17	18
G/G	Mean ± SD	5.3 ± 3.6	7.6
	n	8	1
Total	Mean ± SD	3.9 ± 4.5	2.3 ± 4.7
	n	38	43
MWU	p-value	0.86	0.087

Note: MWU = Mann-Whitney U test for comparison of C/C genotype vs. G-allele

3.4.9.3 Mirtazapine and ADR2A C-1291G

Mirtazapine shows a high affinity to ADR2A receptors, therefore the C-1291G may play a role in therapeutic outcome or side effects, especially weight gain, under mirtazapine therapy.

Patients receiving only mirtazapine as treatment were compared for weight gain after four weeks (n = 23, weight data available for 19). Patients receiving antipsychotics were excluded due to the effect seen for olanzapine and clozapine (see § 3.4.9.2). Despite the small sample size, a higher risk of weight gain was observed for the carriers of the G-allele (see Table 61). This effect was less pronounced, if patients receiving also other antidepressants were included. Baseline and demographic data (age, gender, height, weight, smoking habits, diagnosis, daily mirtazapine dose as well as baseline CGI rating, and duration of illness if available) were similar for the different genotypes in the study subpopulation examined.

Table 61: Weight gain for ADR2A polymorphism under mirtazapine therapy

ADR2A C-1291G		weight (kg)			BMI (kg/m²)			weight gain (%)
		baseline	4 weeks	difference	baseline	4 weeks	difference	
C/C	Mean	67.9	68.2	0.3	22.6	22.7	0.1	0.5
(n = 10)	SD	10.0	10.0	1.7	2.6	2.6	0.6	2.8
C/G	Mean	76.7	79.7	3.0	25.3	26.3	1.0	4.0
(n = 7)	SD	12.1	12.4	2.0	3.8	4.0	0.7	2.8
G/G	Mean	72.5	75.3	2.8	27.3	28.3	1.0	3.8
(n = 2)	SD	3.5	3.9	0.4	1.8	2.0	0.1	0.3
Total	Mean	71.6	73.2	1.5	24.1	24.6	0.5	2.1
(n = 19)	SD	10.8	11.5	2.2	3.4	3.7	0.7	3.1
MWU	p-value	0.113	0.035	0.004	0.095	0.013	0.003	0.008

Note: MWU = Mann-Whitney U test for comparison of C/C vs. G-allele

4 Discussion and Conclusion

The intention of this study was to examine associations between genotypes, serum concentrations and response/side effects under antidepressant/antipsychotic treatment and under real-life conditions in a clinical setting. In the following chapters, the suitability of the study design, the methods and the results of the statistical evaluation are discussed.

4.1 Study design

The study presented herein was designed as a prospective, observational, one-center, diagnostic study. Included were all male and female adults suffering from mood disorders (ICD-10: F3 diagnosis), schizophrenic disorders (ICD-10: F2 diagnosis) and other disorders making treatment with neuroleptics or antidepressants necessary. Thus, the study population consisted of a very heterogeneous pool with treatment-naïve or refractory patients with different diagnoses, treated with a variety of antipsychotic and/or antidepressant drugs and concomitant medication. This kind of population may not be suitable for initial detection of associations; it was, however, the intention of this study to replicate previous findings from studies with controlled, selected, monotherapeutic study populations in a naturalistic clinical setting. The chosen study design reflects the *de facto* conditions of treating psychotic and depressive disorders in real-life practice very well.

When the expected effect size of each locus is small, as in common complex traits, large numbers of individuals are required to detect significant differences [345]. Only a small number of patients was available for some associations. Thus, some existing but small effects may have been missed in the study population; all the more as the observation period was with only four weeks comparatively short. But the aim was to find out which polymorphisms have such an impact on clinical outcome that their effect is also seen in a naturalistic setting so that a future examination maybe useful not only in theory, but in real life. The ultimate intention was to evaluate whether genotyping and of which polymorphisms maybe helpful in everyday clinical practice as additional assistance for treatment decisions [229].

Although the 4-week observation period was comparatively short, it was very intensive. One major advantage of the study described herein is that the psychiatric in-patients could be observed constantly, permitting a reliable rating of therapy compliance, response to therapy and the severity of side effects. The ratings were conducted by the treating psychiatrists, who were blind to genotype and serum concentration.

The clinical setting with the absence of a placebo-treated group made a differentiation between response to a specific drug and non-specific (placebo) response impossible. Ethic reasons do not allow for such a group. The genotype-clinical outcome relationships were not only examined for the matching subpopulation, but were also examined for patients receiving another kind of treatment to compensate for the lack of a comparator arm at least partly. This was a basic help to decide whether the observed effect was really contributable to the specific medication and not attributable to other e.g., non-genetic confounders.

4.2 Genotyping and Measurement of Serum Concentrations

It was the aim to establish and validate fast, sensitive and reliable methods suitable for routine testing in clinical practice (see § 2.2 and § 3.2). Several genotyping methods had been already routinely conducted at the PCR laboratory of the Institute for Clinical Chemistry and Pathobiochemistry [27,28]. Conventional PCR methods (gel electrophoresis, RFLP, allele-specific PCR) were established for the detection of five length polymorphisms and five SNPs. By making use of the LightCycler technology (cf. § 2.2.5.1 to § 2.2.5.3 for principle), fast and reliable methods were developed for genotyping 15 other candidate SNPs. Suitable primers and probes were checked or designed according to the basic principles defined in § 2.2.6.1 and § 2.2.6.2 and the conditions of the PCR reactions were optimized (cf. § 2.2.4.2 and § 2.2.5.4). The fluorescence assays were robust and reliable as documented by the complete concordance of at least 60 DNA samples determined with both the conventional control RFLP assays and fluorescence-based LightCycler assays. No unambiguous results were observed for the TaqMan assays or hybridization probes assays (see § 3.2.1.3 and § 3.2.1.2) except for the highly polymorphic CYP2D6 gene. Deviating peaks due to other SNPs underlying the sensor probes, which were unknown so far, were observed in two samples for the CYP2D6*9 routine method and in two samples for the new CYP2D6*41 assay. Unambiguous genotyping was nevertheless possible in these cases.

The number of polymorphisms examined was limited by the application of traditional PCR and LightCycler technology instead of newer DNA chip or MS/MS methods. It was, however, one of the aims of the work presented herein to provide validated and reliable methods for use in clinical practice for small sets of patients. Moreover, a candidate gene approach was followed instead of a genome-wide pharmacogenetic approach. Genome-wide genetic association studies have not yet been performed due to technical and cost limitations. Moreover, these techniques are limited to SNPs as markers and are hampered by the high number of false-positive associations due to the high degree of multiple testing [179]. Thus, in case of future whole genome scanning, it was suggested to consider weighting schemes that assign higher prior probabilities of variants in genes related to the mode of action of metabolism of medicines [346].

Genotyping for all patients included in this study was accomplished. The observed allele frequencies in the study population were comparable with other Caucasian populations and the bi- and tri-allelic polymorphisms were in Hardy-Weinberg equilibrium [301] (for detailed comparison see appendix A2). Furthermore, serum concentrations of three different drugs (mirtazapine, olanzapine, citalopram) were measured by application of a specific and sensitive LC-MS/MS method [299].

4.3 Serum Concentrations and Genotype

The effects of CYP1A2, CYP2C19 and CYP2D6 polymorphisms on olanzapine, citalopram and mirtazapine steady-state serum concentrations were evaluated after 4 weeks of treatment. The relationship with clinical outcome (treatment response and side effects were measured in terms of ratings, scales and scores as described in § 2.1.3) was examined despite the limited pharmacokinetic data (only one measurement per patient available).

4.3.1 Citalopram and CYP2C19

The selective reuptake inhibitor (SSRI) citalopram is a racemic drug consisting of a 1:1 mixture of the *R*(–)- and *S*(+)-enantiomers. It is metabolized by the cytochrome P450 (CYP) isoenzymes CYP2C19 and CYP3A4 with a minor contribution of CYP2D6 [308-310]. The pharmacological effect resides mainly in *S*(+)-enantiomer [311]. The two enatiomers have different clearance rates as the eutomer S(+)-enantiomer (also marketed alone as escitalopram) is preferentially metabolized by CYP2C19 to its N-desmethyl-metabolite [312]. CYP2C19 poor metabolizers (PMs) are well known to have significantly reduced clearance and elevated citalopram concentrations compared to extensive metabolizers (EMs) [313-317]. Less is known about the influence of intermediate metabolism (IM, with only one defective allele) on serum concentrations of racemic citalopram and escitalopram [86,87]. Thus, it was evaluated whether partially impaired metabolic activity has an impact on the serum concentrations of racemic citalopram or escitalopram against the “background noise” of a naturalistic clinical setting.

4.3.1.1 Influence of Co-medication on Serum Concentrations

It is well known from previous studies that co-medication with the CYP2C19 inhibitor omeprazole increases citalopram concentration approx. 1.5-fold [347] and co-medication with the CYP2C19, CYP3A4 and CYP2D6 inhibitor fluoxetine approx. 1.5 to 2-fold [348]. Interference of omeprazole and fluoxetine was also observed in this study. Both concentrations were more than doubled - compared within the treatment group (racemic drug/escitalopram) and thus somewhat more pronounced than reported previously, which may be due to the fact that patients on polypharmacy and not healthy subjects with citalopram monotherapy were examined herein.

Furthermore, co-medication with the CYP3A4 inhibitor carbamazepine led to a relatively small decrease of approx. 30% for both enantiomers in one previous study [349], while the CYP3A4 inhibitor ritonavir did not influence citalopram serum concentrations [311]. These and other results indicate that CYP3A4 is not the major enzyme in the *in vivo* N-demethylation of citalopram [313]. Moreover, a small but significant correlation with age (but not gender) was observed in one study, which accounted for only 18% of the variability of citalopram concentrations [350]. The influence was considered as not clinically relevant and no dose adjustment was proposed for elderly patients [311]. In accordance with this review [311], no significant influence of age, gender or co-medication with carbamazepine was observed in the clinical cohort examined herein. This is explainable by the small effect sizes and the “background noise” of a heterogeneous patient pool with different diagnoses and co-medications, and consequently a higher variability in the data measured.

4.3.1.2 Influence of CYP2C19 Genotype on Serum Concentrations

Recently [86], significantly higher non-dose-corrected serum concentrations were observed in Norwegian CYP2C19 intermediate metabolizers (IMs) compared with extensive metabolizers (EMs) both for escitalopram and racemic citalopram. Moreover, the dose-normalized concentrations were - with approx. 2-fold for escitalopram and approx. 1.6-fold for racemic citalopram - significantly higher in CYP2C19 IMs compared with EMs. The less pronounced increase for racemic citalopram can be explained by the fact that CYP2C19 preferentially metabolizes the S(+)-enantiomer.

In accordance with these recent results, CYP2C19 IMs had significantly higher non-dose-corrected serum concentrations than EMs in the patient cohort for both escitalopram (29 vs. 16 ng/mL, $p = 0.009$) and racemic citalopram (73 vs. 52 ng/mL, $p = 0.017$). Dose-normalized escitalopram

concentrations were approx. 2-fold higher in IMs (p = 0.031), while the increase of racemic citalopram was about 1.5-fold in IMs (p = 0.003). This shows that the metabolism of racemic citalopram and escitalopram is significantly impaired, not only in PMs, but also in IMs compared with EMs. Moreover, higher absolute serum concentrations indicate that this is not compensated for by dose reductions in clinical practice. The effect of the reduced N-demethylation capacity on serum concentrations was observable against the "background noise" of a naturalistic clinical setting and for a one point per patient measurement, which emphasizes the clinical relevance.

4.3.1.3 Influence of Serum Concentrations and Genotype on Clinical Outcome

There is only limited data on the relationship between serum concentrations of citalopram or escitalopram and therapeutic response. Therefore, the AGNP-TDM consensus group rated the therapeutic drug monitoring (TDM) of citalopram as 'useful' (recommendation level 3) and TDM of escitalopram as 'probably useful' (recommendation level 4 of 5 levels) [337,351] with wide proposed ranges of 30 - 130 ng/mL and 15 - 80 ng/mL, respectively. Accordingly, no relation between serum concentration and response was observed in the study population.

In a recent study with Chinese CYP2C19 PM and EM patients [317], a significant CYP2C19 genotype-citalopram concentration relationship was observed, but only a weak correlation between drug concentrations and adverse effects and only a trend towards higher side effects scores in PM than EM patients [317]. A similar pattern was found in the study cohort: a significant relationship between CYP2C19 genotype and serum concentration for both racemic citalopram and escitalopram (see above); an association between citalopram-related side effects and higher serum concentrations that was significant only for escitalopram; and a trend association between genotype and side effects for escitalopram. In this study, however, IMs (and not PMs, as previously [317]) were compared with EMs. This shows that one defective allele might be sufficient for a significant reduction of drug metabolism and increased incidence of side effects.

Patients with citalopram-related side effects in the study cohort had approx. 1.5 to 2-fold higher escitalopram concentrations compared with patients without citalopram-related side effects (p = 0.026). Thus, the observed 2-fold increase in serum concentrations caused by impaired CYP2C19 metabolism could possibly be important for the occurrence of side effects. Correspondingly, a trend towards more citalopram-specific side effects was found for the CYP2C19 IMs receiving escitalopram compared to EMs despite the small sample size (2/4 = 50% vs. 0/9 = 0%, p = 0.077).

For CYP2C19 IMs treated with racemic citalopram in the study cohort, the trend to more side effects was not significant (IMs 3/8 = 38% vs. EMs 4/18 = 22%, p > 0.1). Only a small number of observations was available within this study and an interference of co-medication cannot be excluded completely. Moreover, the *R*-enantiomer possibly interferes with the metabolism and pharmacological effectivity of S-citalopram. A series of recent studies compared escitalopram and citalopram to placebo and found that equivalent doses of these two drugs i.e., containing the same amount of the *S*-enantiomer, showed better effect for escitalopram. These results suggested that *R*-citalopram inhibits the effect of the *S*-enantiomer [352]. Recently, an attenuating effect of R-citalopram on the occupancy of S-citalopram binding at its target, the serotonin transporter, was proposed by two studies. Data from clinical trials indicated that the two drugs have similar overall adverse event profiles, but that the percentage of patients withdrawing due to adverse events was lower with escitalopram [352-355]. Though a trend towards less side effects in patients treated with escitalopram was visible in the study subset as postulated previously [352], the cohort was too small to detect significant differences in the incidence of side effects between racemic citalopram (7/26, 26.9%) and escitalopram (2/13, 15.4%).

4.3.2 Mirtazapine and CYP2D6

The adrenoceptor (ADR2) and serotonin receptor (5-HTR2 and 5-HTR3) antagonist mirtazapine is a racemic drug with both enantiomers exhibiting antidepressant activity [38,39,321]. Mirtazapine is metabolized by CYP2D6, especially at higher concentrations by CYP3A4 and - to a lesser extent - by CYP1A2 [307,334,356]. The pharmacokinetics of mirtazapine are enantioselective and only the S(+)-enantiomer is metabolized by CYP2D6. For S(+)-mirtazapine distinct differences in clearance and half-lives for the different CYP2D6 metabolizers were found in several previous studies [307,322,323], but the clinical relevance of the CYP2D6 metabolizer status for the pharmacokinetics of the racemate is under discussion [307,322-324,357].

4.3.2.1 Influence Factors for Serum Concentrations

Mirtazapine is known to show a marked interindividual variability in absolute and dose-adjusted serum concentrations [307,333]. Plasma concentrations were reported to range on average from 5 to 100 ng/mL for therapeutic dosages (15 to 45 mg/day). Accordingly, a high interindividual variability was observed in the cohort. The serum concentrations (median of 63 ng/mL, interquartile range 40 - 99 ng/mL, full range 14.5 – 227 ng/mL) as well as dose-normalized levels were on average higher than in studies with healthy young volunteers and monotherapy. It has to be considered that in the study cohort a high percentage of elderly patients (37% older than 60 years) and patients with different kinds of comorbidities and concomitant medications were present. Not surprisingly, the (dose-normalized) concentrations were similar to other clinical studies in depressed patients [333,358].

The pharmacokinetics of mirtazapine were reported to be dependent on gender and age: females and the elderly show higher dose-corrected concentrations [307,332,333]. Accordingly, women in the examined cohort had significantly higher (on average 1.6-fold) absolute and dose-corrected concentrations despite comparable doses. This is most probably caused by significant differences in body fat disposition, body weight and height leading to a lower volume of distribution in women. This cannot be the sole explanation as dose- and body weight-normalized concentrations were on average - yet non-significant - 1.3-fold higher in women. Differences in biotransformation such as different expression of hepatic enzymes may contribute to the lower clearance in women. Furthermore, significantly higher dose-normalized and dose- and body weight- normalized mirtazapine serum concentrations (on average 1.3-fold) were observed in patients older than 60 years. This may be attributable to a physiologically age-related lower clearance.

Furthermore, several previous studies on depressed patients reported an effect of co-medication on (dose-corrected) mirtazapine levels. Polypharmacy in elderly - regardless of the kind of concomitant drugs used - caused higher mirtazapine levels compared to monotherapy in one study [333]. But this result might be attributable to the older age of this patient group as well. No effect was observed in other studies where other antidepressants [358], diazepam or risperidone [39] were coadministered. Inhibitors of CYP2D6 and CYP3A4 isoenzymes, such as paroxetine and fluoxetine, were reported to cause modestly increased mirtazapine concentrations (17 and 32%, respectively) without leading to clinically relevant consequences. Enzyme induction by carbamazepine was reported to cause a considerable decrease (60%) in mirtazapine concentrations [307,359]. In the cohort examined, all patients (except one) received co-medication. Except for the CYP3A4 inducer carbamazepine with a decrease of about 67%, no further influence of co-medication was detectable against the background noise of a naturalistic setting. This study was not designed as an interaction study, but the serum concentrations of particular substance-mirtazapine combinations were compared to all others. If there is a deviation, it can easily be detected with this ‘‘signal-to-noise’’ screening method as proven previously [332,333].

CYP1A2, CYP2D6, and CYP3A4 each contribute approx. 25 to 45% to the *in vitro* mirtazapine clearance. Due to the involvement of multiple cytochrome P450 (CYP) isoforms in mirtazapine metabolism, it was postulated that even complete non-functionality of CYP2D6 is unlikely to result in a clinically significant increase in mirtazapine plasma concentration [39,307,334,356,357]. Consistently, no clinically relevant differences between CYP2D6 poor and extensive metabolizers (PMs and EMs) were found *in vivo* for the pharmacokinetics of the racemate after single dose [87,357]. Moreover, CYP1A2 seems to contribute to the mirtazapine metabolism only at higher concentrations or in individuals with CYP2D6 gene deletion [87]. Accordingly, absolute and dose- and body weight-normalized serum

concentrations were similar for the different CYP2D6 and CYP1A2*1F genotypes in the examined study cohort. Neither of them seems to be of clinical relevance for the serum concentrations unlike age, gender or co-medication with carbamazepine. Yet a trend to higher (dose- and body weight-normalized) concentrations was visible in the study cohort - under consideration of age and gender - for CYP2D6 PMs and - to a lesser extent - for the CYP2D6 IMs, whereas CYP2D6 EMs and UMs had similar concentrations. Interestingly, a significant reduced clearance of CYP2D6 IMs compared with EMs was found for mirtazapine steady-state concentrations in a recent study with similar design (naturalistic clinical setting, depressed patients with different kinds of co-medication) [324]. The effect of CYP2D6 gene duplications (UMs) was lower than expected in another study when compared to EMs and PMs [360] matching the trends observed within this study.

4.3.2.2 Influence of Serum Concentrations on Clinical Outcome

A linear mirtazapine concentration - response relationships could not be established until now, though a lower threshold (30 ng/mL) for response seems to exist [307,361]. Therapeutic drug monitoring is 'useful' (recommendation level 3) according to the AGNP-TDM consensus group [337,351] and a concentration range of 40 - 80 ng/mL was recommended. Moreover, mirtazapine treatment was found to be associated with acute and long-term weight gain in several studies [329,362]. Most patients in the study cohort were above the postulated threshold of 30 ng/mL (88%). Thus, insufficient mirtazapine concentrations did not seem to be the critical factor for non-response in the cohort examined herein. Nevertheless, the five CGI responders in the study cohort tended to have higher mirtazapine concentrations ($p = 0.081$). Similar, a trend towards higher mirtazapine levels was observed for those three patients with clinically relevant weight gain (> 7% of baseline) receiving no other co-medication leading also to weight gain ($p = 0.080$). No relationship was found between concentrations and side effects.

This was a naturalistic study with no restrictions regarding possible meaningful covariates (diagnosis, comorbidities, co-medication [338]), which may also influence treatment response, side effects or weight gain. Thus, the possible interference of cofounders - together with the small sample size - makes it difficult to detect clear relationships between mirtazapine concentrations and clinical outcome. Moreover, the determination of serum levels was not stereoselective and no metabolites were measured. In two previous studies [332,333], the desmethyl-metabolite and the ratio to mirtazapine was associated with side effects. Thus, the mere racemic serum concentration may not be sufficient to predict e.g., side effects clearly.

A more indirect measure of clinical outcome is the change of dose (here subdivided in higher, unchanged and lower) or switch to another drug either due to non-response or side effects. It can be assumed that these have to be strict measures as the non-response or side effects have to be severe enough to justify a change or switch. The treating psychiatrists were not aware of their patients' serum concentrations and hence a deliberate change or switch could not be expected. But dose is - under consideration of age, gender, body weight - a good indicator for the expected serum concentration. Dose changes seem not to be related to the response status of the patients, but dose decrease and switches were more common in patients with moderate/marked side effects ($p = 0.047$).

4.3.2.3 Influence of CYP2D6 Genotype on Clinical Outcome

Only a trend to higher dose- and body weight-normalized concentrations was visible - under consideration of age and gender - for CYP2D6 PMs and - to a lesser extent - for the CYP2D6 IMs. But all CYP2D6 PMs (3/3, none receiving CYP2D6 relevant co-medication) showed marked/moderate side effects compared to only 28% of the other genotypes (13/47, $p = 0.029$). The side effects score was more than doubled for the PMs compared to all others ($p = 0.048$). Similar results were obtained for all patients receiving mirtazapine in the whole study population. Only 29% IMs, 16% EMs and 25% UMs had side effects, but 55% (6/11) PMs had moderate/marked side effects ($p = 0.030$) and a 1.5 fold higher side effects score ($p = 0.078$, for $n = 110$ side effects data available).

The *S*(+)-enantiomer is responsible for 5-HTR2 and ADR2A-receptor antagonism, while the *R*(–)-enantiomer blocks 5-HTR3 receptors [39]. Thus, both enantiomers are pharmacological active, but not in the same way. Moreover, transport mechanisms at the blood-brain barrier [363] and in the gut [323]

seem to be different for both enantiomers. Side effects were associated with the concentration of the desmethyl-metabolite and its ratio to mirtazapine [332,333]. These findings may give a hint why the CYP2D6 PM status - leading to higher *S*(+)-enantiomer serum concentrations [307,322,323] and thus changing the S/R ratio - may be responsible for more side effects, although no significant relationship with racemic mirtazapine serum concentrations were found in the study cohort.

4.3.3 Olanzapine and CYP1A2

Olanzapine is an atypical antipsychotic, which is effective in treating schizophrenia and acute manic episodes, and in preventing the recurrence of bipolar disorders [330]. CYP1A2 and - as a minor pathway - CYP2D6 are involved in the metabolism of olanzapine [326,331]. The CYP1A2*1F (A/A) genotype was associated with higher enzyme inducibility and higher clearance of CYP1A2 substrates like melatonin [78] or caffeine in Caucasian smokers [79,80]. Whether this variant has an impact on olanzapine serum concentrations has not been demonstrated so far.

4.3.3.1 Influence of Genotype, Smoking and Co-medication on Serum Concentrations

The metabolic activity of CYP1A2 is known to exhibit a high interindividual variability (4 to 5-fold) [330]. Accordingly, the olanzapine concentrations in the study cohort showed a high interindividual range from <2.5 to 87 ng/mL. A linear dose-serum concentration relationship ($r = 0.75$, $p < 0.001$) with a comparable average concentration of 21 ± 15 ng/mL was found as reported previously [330,364].

CYP1A2 is inducible by tobacco smoke, certain dietary chemicals and certain drugs. Several studies reported that the CYP1A2 inducers tobacco smoke and carbamazepine significantly decreased olanzapine concentrations [326,364-367] while potent CYP1A2 inhibitors such as fluvoxamine increased olanzapine concentrations [325,341]. In accordance with previous studies, smokers in the study cohort received not only significantly higher doses of olanzapine (on average +35%, $p = 0.022$), but also had lower dose- and body weight normalized olanzapine concentrations (on average -24%, $p = 0.024$). The clearance among nonsmokers was estimated to be 37 - 48% lower in a previous study [326]. Study patients with coadministration of carbamazepine received similar olanzapine doses compared to all others, but had significantly lower normalized serum concentrations (on average only half of those without carbamazepine co-medication, $p = 0.001$). Moreover, a trend towards lower absolute concentrations was evident in the study cohort ($p = 0.057$). This is in accordance with previous findings, where decreases of approx 36 - 38% for dose-normalized olanzapine concentrations were reported [368-370] under carbamazepine co-medication. No other drug or drug group was associated with deviating olanzapine levels in the study cohort. Except carbamazepine and fluvoxamine, which was not present in the study cohort, the influence of concomitant medication is contradictory [326,335,364,371]. This study was not designed as an interaction study and the majority of patients were on polypharmacy. Thus, smaller effects of drugs may have been missed.

Some studies reported lower olanzapine clearance in females and elderly patients [326,365,371,372] whereas other studies did not find a decreased clearance [335,364]. Within the study cohort, no significant influence of gender and age on absolute and dose- (and body weight) normalized olanzapine concentrations was observed. Elderly women, however, received lower doses. This may compensate the lower clearance and result in similar serum levels.

The influence of the highly inducible CYP1A2*1F (A/A) genotype was examined in this study as it was associated with higher clearance of CYP1A2 substrates like melatonin [78] or caffeine in Caucasian smokers [79,80]. The CYP1A2 activity - measured by means of the CYP1A2 substrate caffeine - was approx. doubled in smokers with A/A genotype compared with smokers with C/A genotype. No differences were detected for non-smokers for whom, however, the less sensitive urinary concentration ratios were used and thus a smaller effect could have been missed. In another clinical trial, a close concordance between olanzapine and caffeine clearance was observed [331]. In the study cohort, patients with the CYP1A2*1F (A/A) genotype had significantly lower absolute, dose-normalized as well as dose- and body weight-normalized serum concentrations compared to C-allele carriers. The difference in olanzapine concentration for the different CYP1A2 genotypes was not explainable by dose, baseline differences (gender, age, height, weight, diagnosis) or CYP1A2 inducers alone (co-medication, smoking). Under consideration of CYP1A2 inducers, the mean dose- and body-weight normalized serum concentrations of carriers of the highly inducible A/A genotype

who were smoking or receiving carbamazepine were on average -42% lower compared to C-allele carriers without induction. Less decreased serum concentrations (-28%) were measured for patients without CYP1A2*1F but under influence of CYP1A2 inducing agents (tobacco smoke, carbamazepine).

Interestingly, the CYP1A2*1F genotype alone - without induction of carbamazepine or tobacco smoke - also led to decreased serum concentrations (-22%). Thus, the CYP1A2*1F may have an effect on substrate concentrations on its own, and not only in case of simultaneous intake of inducers. One might reply that the observed effect may be due to intake of other, unknown inducers. Indeed, it has been reported that certain dietary chemicals, which are contained in e.g., broccoli, Brussels-sprouts or char-grilled meat [69,373] have Cyp1A2 inducing features. Moreover, the CYP1A2 substrate caffeine might have an influence - there have been several reports that ingestion or removal of caffeine containing beverages influenced concentrations of the CY1A2 substrate clozapine [341]. However, the patients were hospitalized receiving a standard diet which did not contain major amounts of these foods. An extraordinary exposure to and influence of alimentary inducers is therefore unlikely (though the diet of the patients was not monitored within this study) and cannot explain the decreased concentrations.

An effect of the genotype alone was also detected in a previous study on the risk for tardive dyskinesia, a severe and potentially irreversible side effect, under treatment with typical antipsychotics such as haloperidol (which is also a CYP1A2 substrate). The risk was lower for CYP1A2*1F A-allele carriers in general. The risk reduction was most pronounced in A-allele carriers who were smoking [374].

According to the AGNP-TDM consensus group [337], therapeutic drug monitoring is ‘strongly recommended’ (recommendation level 1) with a proposed concentration range of 20 - 80 ng/mL. Therefore, genotyping of CYP1A2*1F may provide important additional information beyond the known influence factors on olanzapine clearance such as co-medication, smoking, age and gender. Studies with larger patient groups are needed to confirm the effect of this polymorphism on olanzapine concentrations. To date, only studies on the metabolism of clozapine, which is in contrast to olanzapine also metabolized by CYP3A4 [325], have been published reporting contradictory results . No effect of the CYP1A2*1F A/A genotype was detected on clozapine metabolism in one study [375], whereas in another study an effect in smokers was observed [376].

4.3.3.2 Influence of Serum Concentration on Response

The AGNP-TDM recommendation for olanzapine proposed a concentration range of 20 - 80 ng/mL to optimize therapeutic response and minimize side effects. In some previous studies, a lower threshold olanzapine concentration (approx. 23 ng/mL 12 hours postdose was associated with an increased likelihood for response [372,377]. Other studies showed even a linear relationship between olanzapine concentrations and clinical improvement in schizophrenic patients [378-380].

A significant olanzapine concentration - response relationship was found in the schizophrenic study patients. The absolute improvement of psychotic symptoms on the self-rating scale after 4 weeks of treatment correlated significantly with the olanzapine concentrations ($r = 0.5$, $p = 0.036$). The relationship between olanzapine concentrations and the CGI(1) severity rating by the treating psychiatrist confirmed this result ($r = 0.4$, $p = 0.049$). No or little relationship was observed with baseline scores or with CGI(2) improvement rating. The latter seems to rather represent a relative change of symptoms depending on the severity of symptoms at baseline than an absolute symptom reduction as published recently [381]. The relationship between olanzapine concentrations and % changes also showed only a trend. This is explainable by the fact that a heterogeneous patient pool with different baseline severity of illness was examined. The same % improvement means more absolute score reduction in more severe ill patients compared to only moderately ill patients. This makes a comparison between % changes and olanzapine concentrations for a (linear) relationship difficult and thus absolute differences were used. Nevertheless, by use of absolute score reduction, the olanzapine concentration - response relationship found previously [378-380] could be confirmed for the schizophrenic patients in a naturalistic clinical setting despite the heterogeneous patients cohort.

Olanzapine is known for its effect on affective and depressive symptomatology in acute psychotic patients [378,379]. Notably, a correlation between olanzapine concentration and improvement of depressive symptoms was found in schizophrenic study patients ($r = 0.5$, $p = 0.006$), but not in study

patients with affective disorders ($r < 0.22$, $p > 0.2$). This is probably due to the fact that olanzapine is only used as add-on in the therapy of mood disorders, mainly in manic episodes and for prevention of recurrence of bipolar disorders [330]. The main antidepressive effect in patients with affective disorders is probably due to medication with antidepressants.

4.3.3.3 Influence of Polymorphisms in Drug Target Structures on Response and Side Effects

A vast number of potentially relevant factors has been associated with response to antipsychotic treatment, side effects and antipsychotic-induced weight gain: environmental factors independent of therapy (gender, age, ethnicity, smoking, baseline weight, social factors), treatment dependent factors (length of exposure, dose/serum concentrations, treatment duration) and genetic factors [382,383].

Olanzapine has a broad receptor binding profile with high *in vitro* affinity for dopamine receptors, all of the 5-HTR2 receptor subtypes and the 5-HTR6 receptor, muscarinergic M1 receptors, adrenoceptors and histamine H1 receptors. *In vivo* studies have shown that olanzapine acts potently on DRD2 and 5-HTR2A receptors in the CNS accounting for its pharmacological effects [330,384,385]. Several polymorphisms in the DRD2 and 5-HTR receptors have been linked to treatment response (e.g., DRD2 -141ins/del, DRD2 Taq1A, 5-HTR2A 102T/C, 5-HTR6 267T/C) or weight gain (5-HTR2C -759C/T) of atypical antipsychotics [131,133,135,136,156,231].

In the serum subset ($n = 73$), response was not significantly related to any of the examined polymorphisms under consideration of olanzapine serum levels, age, gender, diagnosis, co-medication, and illness at baseline. Similarly, no influence of genetic variants on side effects - except on weight gain as given below - was detected. The patient group was very heterogeneous with regard to diagnosis. Only limited data for assessment of treatment response was available (only one rating time point after 4 weeks), which may not be exact enough to detect significant differences in the relatively small sample set. Most patients were treated with at least one further drug that could also lead - by other pharmacodynamic mechanisms - to response or side effects. Thus, a smaller effect may have been missed. Significant associations were, however, found between the 5-HT2A (rs7997012) and olanzapine side effects as well as DRD2 -141ins/del and antipsychotic response in the whole study population, where a larger number of patients was compared (see following chapters).

4.3.3.4 Influence Factors on Weight Gain

Clinically significant weight gain (> 7% of baseline) similar to clozapine has been noted with olanzapine in large clinical trials both acutely and long-term [329,386]. Potentially relevant environmental and genetic factors were examined for potential associations with antipsychotic induced weight gain (in % of baseline) in the study cohort.

One study found that a greater number of co-medications per patient and co-prescription of antidepressants significantly and independently increased antipsychotic-associated weight gain [338]. No influence of number or kind of co-medication was observed in the study cohort except for patients comedicated with lithium and a trend to higher weight gain ($n = 8$, on average 5.2% vs. 2.6% without lithium co-medication, $p = 0.075$). Other drugs known to induce weight gain [329] were comedicated too seldom ($n < 5$). Thus, weight effects may have been missed. Another study [387] reported an influence of gender, overweight or treatment ≤ 1 year. None of these risk factors was found in the study cohort. This may be due to the different study designs in these previous studies: schizophrenic outpatients on monotherapy for up to several years. Long-term weight gain may have other mechanisms than short-term weight gain. Moreover, because of the short observation period, the clinically relevant weight gain was with 19.1% (13 of 68 patients) in the study cohort less frequent than in the long-term trials (with up to 80 - 90%).

Data from short-term studies identified better clinical outcome and low baseline BMI - but not age, gender and dose - as predictive factors of acute weight gain [388,389]. Matching these results, weight gain was correlated with better response after 4 weeks (CGI(2) rating, $r = -0.4$, $p = 0.003$) and lower baseline weight/BMI ($r = -0.3$, $p < 0.011$) in the study cohort. Age, gender or dose showed no such influence.

The olanzapine serum concentration, another possible influence factor, was not directly correlated with the weight gain in the study cohort. However, study patients with concentrations above a previously reported threshold of 20 ng/mL [336] had a 5-fold higher risk of clinically relevant weight gain (Odds Ratio OR 5, p = 0.028). Matching this relatively indirect concentration-weight gain relationship, no significant association with the CYP1A2*1F (A/A) genotype was found in the study cohort. Similarly, in a previous study on weight gain after 6 weeks of clozapine treatment, no significant correlation between CYP1A2*1F genotype and weight gain was found [390]. Clozapine, however, is also metabolized by CYP3A4 and thus the expectable pharmacokinetic effect of the CYP1A2*1F genotype on concentrations is lower [391].

The pharmacodynamic mechanisms involved in antipsychotic -related weight gain have not been fully elucidated, although serotonergic and adrenergic affinities have been implicated along with other metabolic mechanisms [66,392]. While associations with serum or plasma concentrations are reported rarely, several previous studies reported associations of antipsychotic-induced weight gain with polymorphisms e.g., in the ARD2A and 5-HTR2C receptors [66,133,177,344]. Pharmacodynamic variants seem to be more important for weight gain under treatment with atypical antipsychotics than pharmacokinetic polymorphisms. This can be explained by the fact that, first of all, serum levels are only an indirect measure of the drug concentration at the site of action, the central nervous system. The blood-brain barrier prevents uncontrolled access. Interestingly, a trend to greater weight gain for carriers of a common polymorphism in P-glycoprotein (MDR1 3435 T/T, p = 0.098) was seen in the study cohort. As part of the blood-brain barrier, this transporter actively exports significant amounts of drugs including olanzapine from the brain. MDR1 3435 T/T homozygotes were associated with lower expression of the transporter [96] leading to higher CNS drug concentrations and more pronounced drug effects, including weight gain.

Secondly, gene variants affecting the drug target may be much more critical for the efficacy as well as side effects of a drug than the drug concentration itself. Olanzapine has a broad receptor binding profile including several DRD and 5-HTR receptor subtypes as well as adrenoceptors amongst others, which are possible candidates to explain part of the weight gain. In the study cohort, 5-HTR2C -759T-allele carriers showed significantly less weight gain (on average 0.8%, n = 16) compared to the homo- or hemizygous 5-HTR2C -759 C/C or C-allele carriers (on average 3.5%, n = 52, p = 0.012). All 13 patients with substantial weight gain > 7% within the first weeks of treatment were carriers of 5-HTR2C -759 C/C (6 females) or C (7 males). This is in accordance with the results of several previous studies, which have shown a significant influence of 5-HTR2C -759 C-allele on weight gain under olanzapine therapy [140,143,144,393].

Moreover, DRD2-141 wildtype carriers showed on average 3.4% weight gain compared with no weight gain for del-allele carriers (on average -0.2%, n = 9, p = 0.012). In several studies this polymorphism was associated with antipsychotic treatment response [155,156], but no association with weight gain was reported so far. On the one hand, this polymorphism was associated with antipsychotic treatment response in the whole study population, but not in the olanzapine serum cohort, probably due to the small sample size. On the other hand, better treatment response is - as discussed above - a predictive factor of acute weight gain [388,389]. Thus, this polymorphism might be more a measure of treatment response than of weight gain. This was also confirmed by the multiple linear regression analysis for weight gain under olanzapine/clozapine monotherapy in the whole study population (see also § 4.4.2). This analysis identified baseline weight, CGI(2) improvement and the homo- or hemizygous 5-HTR2C C-759 genotype as significant influence factors, but the DRD2 -141C ins/del polymorphism was not included - unlike the result in the olanzapine serum cohort.

4.4 Genotyping and Clinical Outcome

The influence of drug metabolism polymorphisms - notably of CYP1A2*1F on olanzapine concentrations and CYP2C19 on citalopram concentrations, less of the different CYP2D6 genotypes on mirtazapine concentrations - was shown in the previous chapter. Associations between serum concentrations and response (significant for olanzapine) or side effects (trend for escitalopram) were observed. The number of patients was, however, too small for a direct association between these genotypes and clinical outcome.

Thus, the next step was to evaluate whether and to what extent CYP1A2, CYP2C19 and CYP2D6 polymorphisms influence clinical outcome variables (such as prescription frequencies, drug switches, adverse events, response, response onset and length of hospitalization) in patients receiving substrates of these enzymes in the whole study population (see § 4.4.1).

After evaluation of the drug disposition polymorphisms the pharmacodynamic polymorphisms were evaluated that lie in the target structures of the drugs (= site-of-action) or that lie in the structures in the subsequent cellular and physiological pathways and mediate long-term adaptive response (see § 4.4.2).

4.4.1 Pharmacokinetic Polymorphisms and Clinical Outcome

The different polymorphisms of the highly polymorphic CYP2D6 gene were rated according to their functionality with gene doses as described by Steimer et al. (2004) [305] and in a second step the number of poor metabolizers (31 PM, 8.5%), intermediate metabolizers (138 IM, 37.8%), extensive metabolizers (185 EM, 50.6%) and ultrarapid metabolizers (11 UM, 3.0%) was determined to obtain sufficiently large groups for statistical evaluation.

4.4.1.1 CYP2D6

Pharmacotherapy with CYP2D6 drugs can hardly be avoided since CYP2D6 metabolizes about 25% to 30% of the clinically used drugs [13,69]. In the study population 108 (30%) of the patients received drugs that are predominantly metabolized by CYP2D6 (here: amitriptyline, clomipramine, imipramine, doxepin, trimipramine, fluoxetine, paroxetine, aripiprazole, chlorpromazine, promethazine, haloperidol, zuclopenthixol).

Only 4 of the 31 PMs (13%) were treated with CYP2D6 substrates, but 4 of 11 UMs (36%). Interestingly, only 4 of the 20 (20%) patients with the gene dose 0.5 were treated with CYP2D6 substrates, a prescription frequency similar to the poor metabolizers. For the gene doses 1, 1.5 and 2, similar prescription frequencies were observed (35, 32 and 29%), which approximate the overall mean of 30%. The difference between gene dose 0 and 0.5 on the one hand and gene doses ≥ 1 was significant with an Odds Ratio of 2.5 ($p = 0.020$). The lower prescription frequencies for the patients with poor or severely impaired CYP2D6 metabolism may reflect unfavorable results under CYP2D6-dependent medication in medical history. All of the patients with gene dose 0 or 0.5 were treated for more than one year before the present hospitalization making side effects and subsequent switch as explanation possible.

The hospital stay of patients with CYP2D6 dependent medication tended to be longer (on average 6 days longer than without CYP2D6 dependent medication, $p = 0.075$) than those treated without CYP2D6 dependent medication. Patients responded significantly later under CYP2D6 medication (on average 4 days, $p = 0.034$) which is in line with previous findings [394]. An overall increase in the length of hospitalization and response onset for patients receiving drugs primarily metabolized by the CYP2D6 enzyme seems to imply that treatment with CYP2D6 drugs is more protracted than drug treatment with other therapeutic agents that are not predominantly metabolized by the CYP2D6 enzyme. An explanation could be that the metabolic pathway mediated by CYP2D6 is easily inhibited or saturated by a multitude of psychoactive or cardiovascular co-medications [395-399]. The resulting drug-drug-interactions may delay response to therapeutic drug treatment.

The length of hospitalization and response onset was not significantly different for the CYP2D6 gene dose groups (for patients receiving CYP2D6 dependent medication as well as for those receiving non-CYP2D6 dependent medication). The number of PMs and UMs, however, may have been too

small to detect significant differences and thus for a meaningful conclusion. A prolonged hospital stay may be on the one hand due to unsatisfactory treatment response (expected especially for UMs) and on the other hand due to side effects (expected especially for PMs). Both may cause drug switches, too. In clinical practice, these cases cannot be assigned easily without therapeutic drug monitoring (TDM) and pharmacogenetic testing, as side effects may mimic the psychiatric illness itself and therapeutic failure due to ultrarapid metabolism may be mistaken for poor compliance with the prescription [400].

Accordingly, ultrarapid metabolizers as well as poor metabolizers were somewhat more prone to switches either as a consequence of non-response or side effects during the present treatment, but this did not reach statistical significance. In addition, UMs tended to more switches due to non-response in medical history (Odds Ratio OR = 2.8, p = 0.15). However, compound switches changing the metabolic pathways of the drugs involved were evenly distributed within the genotype groups during the present treatment. This may be due to the fact that the treating psychiatrists were not aware of their patients' genotypes and hence a deliberate switch of the metabolic pathway could not be expected.

The doses of CYP2D6-dependent drugs were similar for all genotype groups ($p > 0.5$). One would expect higher doses in UMs as suggested previously [87,339,401]. The treating psychiatrists, however, were unaware of the genotypes and thus dose titration matching the patients' metabolic capacity could not be expected. Therapeutic problems were probably rather solved by switching therapeutic agents.

PMs treated with CYP2D6 substrates suffered from significantly more moderate/marked adverse effects when compared to non-PMs with CYP2D6 medication (PMs 3/3 = 100% vs. IMs 15/38 = 39%, EMs 11/50 = 22% and UMs 1/4 = 25%, p = 0.029). The side effects were not elevated if the CYP2D6 PMs received non-CYP2D6 dependent medication (PMs 9/25 = 36% vs. IMs 24/84 = 29%, EMs 31/117 = 26% and UMs 1/7 = 14%, Fisher-Exact test, p = 0.351). The difference in adverse effects in PMs with and without CYP2D6 dependent medication (100% vs. 36%) was almost significant despite the low number of patients (p = 0.067). Thus, previous findings on CYP2D6 PMs suffering from more side effects than IMs, EMs or UMs under CYP2D6 dependent medication [70,87,394,402-412] could be confirmed for a naturalistic setting without restrictions with regard to co-medication or comorbidities. The study design made it also possible to compare patients receiving CYP2D6 substrates with those receiving non-CYP2D6 drugs and to show that CYP2D6 PMs might profit from a drug switch to non-CYP2D6 dependent medication.

IMs treated with CYP2D6 medication suffered from somewhat more moderate/marked side effects than EMs (39% vs. 22%, p = 0.099), but this did not reach statistical significance. Interestingly, IMs treated with daily doses of CYP2D6 substrates above the population median had more side effects compared to EMs receiving above-median doses (IMs 69% vs. EMs 17%, p = 0.003). They had also more side effects than IMs receiving doses below or equal to the population median (OR 7.7) or IMs without CYP2D6 dependent medication (OR 5.6, IMs above-median 69% vs. IMs below-median 23%, p = 0.012 and vs. without CYP2D6 drugs 29%, p = 0.009). The adverse effect rate was with 23% equal for IMs and EMs with doses below or equal the population median (p = 1.0).

The impact of CYP2D6 polymorphisms on plasma or serum concentrations of CYP2D6-dependent drugs has been examined in several clinical studies so far. Based on the results dose adjustment has been suggested for PMs, IMs and UMs [87]. Many studies on PMs suffering from more side effects were published so far (see above), but reports on the impact of IMs on clinical outcome are seldom and mostly on pharmacokinetics [87,413,414]. However, genotyping for the highly polymorphic CYP2D6 gene is just evolving and improving. The genetic basis of the most frequent polymorphism with impaired function, CYP2D6*41, was only discovered in the past few years [89,289,415]. It must be noted that the frequent CYP2D6*41 allele was not included in the genotyping of most former studies which led to inaccuracies in gene dose assignment. This might have prevented the detection of significant associations for the IMs. Thus, new studies on the relevance of the CYP2D6 IM status are important, all the more, as the number of IMs was estimated with up to 40% in Caucasians - compared to only 5 to 10% PMs [72,88,89]. These results implicate that identification not only of PM, but also IM status might help to avoid adverse effects by starting treatment with *a priori* lower doses for CYP2D6 drugs and keeping doses low throughout the treatment. Moreover, special diligence is necessary in case of non-response: switching to another drug might be a better option than increasing the dose for IMs.

One would expect lower response rates for PMs and IMs than EMs or UMs under CYP2D6 dependent medication as they suffer from more side effects which may interfere with the response. However, the responder rates were similar for patients with vs. without CYP2D6 medication (14% vs. 18%, $p > 0.5$). The response was not significantly different for IMs vs. EMs (if including only CYP2D6-dependent medication: 7% vs. 18%, $p > 0.1$). The sample sizes of the PMs and UMs in the population were too small for a meaningful conclusion. In accordance with these results, in several previous studies response rates were similar for the different CYP2D6 metabolizer groups [87,408,412,416-418].

IMs receiving CYP2D6-dependent medication, however, showed significantly less CGI(2) improvement than IMs receiving non-CYP2D6 drugs after 4 weeks of treatment (7% vs. 25%, $p = 0.017$). This may be due to the higher adverse effects frequency of IMs under CYP2D6 medication. Suffering from unpleasant side effects may on the one hand lead to underrating of the improvement. On the other hand drug switches might be necessary and might interfere with the treatment response within a 4 week period. Consistently, no significantly different responder rates were seen for the EMs with and without CYP2D6 dependent medication (18% vs. 10%, $p > 0.2$). Moreover, responder rates under CYP2D6 treatment were similar for the IMs treated with doses below or above the study population median (IMs 8% vs. 7%, $p = 1$). Lower doses seem not to be the limiting variable for treatment response in IMs.

Interestingly, treatment response increased continuously with the gene dose for doses above the population median (gene dose 0/0%, 0.5/0%, 1/8%, 1.5/20%, 2/25% and >2/100%) - though not significant due to the low number of patients per gene dose group - whereas no such trend was visible for below-median doses (gene dose 0/50%, 0.5/0%, 1/9%, 1.5/8%, 2/20% and >2/0%). Different CYP2D6 drugs with different therapeutic windows were evaluated together. Some CYP2D6 drugs such as the tricyclic antidepressants have narrow therapeutic windows, which led to relatively cautious dose recommendations to prevent side effects at higher doses. These recommended “normal” doses may be not high enough for sufficient treatment response in EMs and especially UMs. Accordingly, for some CYP2D6 substrates increased “normal” doses for EMs and/or UMs have been proposed earlier [87,419,420]. This response-limiting dose effect for EMs (and UMs) is matching the observations reported herein. In case of higher, above-median doses the response of EMs and UMs tended to be higher than for below-median doses. Thus, genotyping might help to forecast an appropriate initial dose in a patient [397]; not only to prevent side effects in PMs and IMs but also to ensure response in EMs and of course in UMs.

At first it seems strange that the responder rates were higher for IMs than EMs if the patients received non-CYP2D6 drugs (25% vs. 10%, $p = 0.007$). IMs are more prone to adverse effects under CYP2D6 substrates, which may mimic the illness itself or may have led to non-compliance in the past. These incompatibilities known from medical history may have led to drug switch at the very beginning of the present hospital stay and subsequent treatment success that was not achieved with the CYP2D6 drugs used before. This is also supported by the fact that the prescription frequency of CYP2D6 dependent drugs in the present treatment was lower than the overall mean for IMs (especially with gene dose 0.5).

There are also drugs, where CYP2D6 plays a minor role in the metabolism e.g., mirtazapine has a stereoselective CYP2D6 metabolism with a preference for the S-enantiomer. In case of venlafaxine and risperidone, equally active metabolites result from the CYP2D6 metabolism. There are only limited positive data on the effect of the CYP2D6 metabolizer status on clinical outcome for these drugs [324,407,421,422]. Most studies did not detect a significant relationship between genotype and pharmacokinetics or clinical outcome [39,307,334,356,357,423,424]. Inclusion of these drugs into the evaluation showed mostly trend associations - if at all - with clinical outcome. Thus, CYP2D6 seem not to have a major impact on clinical outcome of these drugs, at least within a naturalistic clinical setting.

4.4.1.2 CYP1A2

One hundred sixty-two (44%) of the 365 patients received CYP1A2 substrates such as olanzapine, clozapine and haloperidol. The inducible CYP1A2*1F genotype was evaluated in combination with CYP1A2 inducers (carbamazepine and tobacco smoke, see also § 3.4.2.3). Thus, four groups were compared: the inducible CYP1A2*1F A/A genotype with and without inducers and the CYP1A2 wildtype C-allele in combination with and without inducers.

The combined evaluation of all CYP1A2 substrates showed only non-significant trends. Olanzapine, however, is the only drug which was prescribed frequently and where CYP1A2 metabolism is a major pathway (see also § 4.3.3). Moreover, baseline demographics were different for patients with schizophrenic and mood disorders (lower doses, less smokers) making a combined evaluation with regard to the influence of the induced CYP1A2*1F genotype impossible. Thus only schizophrenic patients (n = 174) receiving olanzapine were compared.

Patients with the inducible CYP1A2*1F genotype in combination with CYP1A2 inducers (30% of 174 patients) received significantly (on average 36%) higher doses of olanzapine ($p = 0.011$). No further differences in the conduct of treatment (number of switches, frequency of adverse effects, response, response onset or length of hospitalization) were observed. This may be due to the fact that the higher dose compensates the higher metabolism rate in those patients and thus no further impact on treatment response or side effects was seen.

Non-smokers without the inducible form of CYP1A2 (and without co-medication of carbamazepine, 25% of 174 patients) are expected to reach higher olanzapine serum concentrations with the same dose. As higher olanzapine concentrations are associated with better treatment response, this group should have the fewest number of switches from olanzapine due to non-response. Accordingly, a trend towards a higher prescription frequency of olanzapine in this group was seen compared to all others (45% vs. 29% for all other groups, $p = 0.064$).

These results confirm that there is an effect of the highly inducible CYP1A2*1F genotype in combination with CYP1A2 inducers on olanzapine metabolism. The genotype-dose relationship in the whole study population reflects the genotype-serum concentration relationship observed in the olanzapine serum subset. Of course, the former is less definite than the latter due to further influence factors especially in a heterogeneous patient population. Thus, genotyping of the CYP1A2*1F polymorphism - with a prevalence of 53% for the A/A genotype in the study population - could be useful as additional predictor of CYP1A2 activity and might help to foretell an appropriate initial dose in patients taking olanzapine.

4.4.1.3 CYP2C19

Nine (2.5%) of all patients were CYP2C19 poor metabolizers with two non-functional CYP2C19*2, *3, and *4 alleles, 27.7% (101) intermediate metabolizers with one non-functional and one functional allele and 69.9% (255) were extensive metabolizers with two functional CYP2C19*1 alleles.

Ninety-eight (27%) of the study subjects received CYP2C19 substrates (citalopram or the tricyclic antidepressants amitriptyline, clomipramine, imipramine, doxepin and trimipramine). No significant results were observed for the evaluation of (es)citalopram or all CYP2C19 substrates together. This may have several reasons. First of all, non-functional CYP2C19 alleles have a low prevalence in the population making statistical significant results difficult. Secondly, (es)citalopram has a broad therapeutic window, thus higher levels may not necessarily cause more side effects [337,351].

Entirely different, the therapeutic window of tricyclic antidepressants (TCAs) is narrow [337,351]. Some trends were visible despite the small number of patients (n = 41). More PMs and IMs than EMs received TCAs (22%, 14% vs. 10%) whereas the frequency of marked/moderate side effects was significantly higher in EMs than for IMs and PMs (10/22 = 46% for EMs vs. 1/13 = 8% for IMs and PMs, $p = 0.027$). In contrast, side effects were similar under non-CYP2C19 dependent medication (2/7 = 29% PMs, 24/81 = 30% IMs and 58/205 = 28% EMs, $p > 0.9$).

This seems to be an inversion of the result expected. Studies on the impact of CYP2C19 on the metabolism of TCAs are rare [417,425] and mainly show that the metabolism to the pharmacological active desmethyl-metabolite is lower in PMs than EMs [87]. TCAs (such as amitriptyline) are

metabolized by CYP2C19 to the pharmacological active desmethyl-metabolite (such as nortriptyline). The desmethyl-metabolite is then metabolized by CYP2D6 to an inactive metabolite. Thus, extensive CYP2C19 metabolism (especially in combination with poor CYP2D6 metabolism) leads to a higher serum concentration of the metabolite. As reported previously [426], the concentration of active desmethyl-metabolite of amitriptyline (nortriptyline) correlated with the side effects under amitriptyline therapy. This may explain the higher side effects frequency for CYP2C19 EMs in the study population. Additionally, EMs received on average (non-significant) higher doses than PMs and IMs (on average 106% vs. 50%/80% of WHO defined daily dose).

4.4.1.4 MDR1 Polymorphisms

The highly polymorphic multidrug resistant (MDR1) gene encodes the P-glycoprotein, which functions as an efflux transporter in the blood-brain barrier. Several polymorphisms of the MDR1 gene (C3435T, G2677TA and C1236T) have been associated (via higher bioavailability of P-gp substrates in the CNS) with better treatment response or more side effects [427]. One study reported better response for 1236TT under risperidone therapy but no association with the two other polymorphisms [98]. Other studies reported significant associations with better olanzapine treatment response for either 3435T or 2677T [99,100]. In a Japanese study, the wild type variant 2677G was associated with poor paroxetine treatment response [102]. These results were supported by a recent report on the 3435T-2677T-1236T haplotype variant which was shown to have a markedly different expression and function compared to the wild type [97].

Known P-glycoprotein substrates as risperidone, [91,92], olanzapine, amitriptyline, citalopram, trimipramine, doxepin, venlafaxine, and paroxetine [93-95] were evaluated but no effect of the single MDR1 polymorphisms or the MDR1 3435T-2677T-1236T haplotype [97] on clinical outcome (such as prescription frequencies, administered daily doses, frequency of adverse effects, improvement of symptoms, response onset or length of hospitalization) was observed in the study population.

The associations found in studies so far, however, were partly contradictory and modest in size. Moreover, not all studies found significant associations between MDR1 polymorphisms and clinical outcome [90,103-105] e.g., no effect of G2677T/A was found in a similar study population treated with amitriptyline [106]. The problems are evident. Genotyping based on individual SNPs may not be sufficient to predict functional consequences given the large size of the highly polymorphic MDR1 gene [429]. Moreover, the causative polymorphism(s) responsible for altered activity of MDR1 - if there are any - are not known to date [3,90,103,427,428].

4.4.2 Pharmacodynamic Polymorphisms and Clinical Outcome

It is now becoming increasingly evident that polymorphisms of pharmacological targets (pharmacodynamic polymorphisms) may in fact be more important and clinically relevant than polymorphisms of drug disposition (pharmacokinetic polymorphisms). As seen above in the olanzapine serum subset, pharmacodynamic polymorphisms - in the target structure of the drug (= site-of-action) or in the structures in the subsequent cellular and physiological pathways mediating long-term adaptive response - may be more important for the clinical outcome than the pharmacokinetic polymorphisms in the cytochrome P450 system. In the following chapters, the results for polymorphisms in the serotonergic, dopaminergic, (nor)adrenergic and other signal transduction pathways are discussed (cf. also § 1.3.3 and § 1.3.4).

4.4.2.1 Serotonergic Polymorphisms

4.4.2.1.1 5-HTT Polymorphisms and Response/Side Effects under SSRI Therapy

The serotonin transporter (5-hydroxytryptamine transporter, 5-HTT) is the primary site of action of the selective serotonin reuptake inhibitors (SSRIs) and plays also a role in therapeutic mechanism of venlafaxine and tricyclic antidepressants such as amitriptyline. Thus, it represents a logical site at which genetic variation could influence clinical outcome.

The presence of a 44-base-pair region in the promoter region of the 5-HTT - termed long or L-allele - has been associated with increased transcription of the gene and higher biological activity [108]. Thus, the high expression variant may lead to better and faster response and less side effects for antidepressant medications that target the transporter by increasing serotonin transporter levels in brain and other tissues. However, the results for this length polymorphism (5-HTTLPR) were inconsistent in several association studies with SSRIs [109]. A recent meta-analysis on 15 studies, with data from 1435 subjects including studies in non-Caucasian populations showed significant evidence of association of the L-allele with better treatment response [110]. This was not found in a large clinical sample with 1914 subjects [111]. Moreover, two previous studies reported an association between the unfavorable short (S)-allele and more side effects [430,431]. Recently, a SNP in the 5-HTT promoter (rs25531 A/G) nearby the 5-HTTLPR was reported to have additional influence on transcription activity [112-114]. In a recent study, the high expression variant L_A (i.e., 5-HTTLPR L-allele in combination with rs25531 A-allele) was associated with less adverse effects in out-patients with citalopram monotherapy [115].

Accordingly, patients in the study population who did not have the favorable high expression allele L_A and who were treated only with SSRIs as antidepressant therapy suffered from more side effects typical for SSRIs such as sweating, gastrointestinal side effects and dizziness ($n = 66$ available, $p < 0.001$ for gastrointestinal side effects). Moreover, their illness was rated as more severe after 4 treatment weeks by the treating psychiatrist ($n = 60$ available, $p = 0.04$). These patients stayed longer in hospital than the patients with the favorable high expression variant L_A (on average 110 vs. 60 days, $p = 0.01$). Side effects are probably one underlying reason for the significant longer hospitalization. The difference for the onset of treatment response was less pronounced (after 74 vs. 32 days, $p = 0.12$).

Severity of illness may be rated higher in patients experiencing side effects as side effects may mimic the psychiatric illness itself [400]. But there may be also an independent negative effect of the low expression alleles (S or L_G) on severity of illness compared with the favorable L_A-allele. An association of the low expression alleles with depression severity and stressful life events was observed in a previous study independently of medication in depressed patients [304]. Moreover, the high expression variant L_A was reported to be associated with better and faster treatment response to SSRIs in patients with depression [117,432] and with generalized social anxiety disorder [116].

These disadvantages in clinical outcome were not observed in those study patients who did have unfavorable low expression genotypes but did not receive SSRIs as antidepressant monotherapy. No effect was seen e.g., in patients receiving antidepressants with dual mechanism (serotonin and noradrenaline reuptake inhibitors) such as venlafaxine and tricyclic antidepressants. These patients did not suffer from elevated side effects typical for SSRIs ($p = 0.004$) or more severe illness

(p = 0.074). They stayed on average for only 53 days (p = 0.003) and responded after only 29 days (p = 0.067). This is in accordance with a previous study, which reported an influence of the 5-HTTLPR polymorphism on paroxetine, but not mirtazapine response [433].

4.4.2.1.2 5-HTR2A Polymorphisms and Side Effects/Length of Hospitalization under SSRI or Olanzapine Therapy

The serotonin receptor 5-HTR2A is one of the two major 5-HTR2 receptor subtypes in the human brain and also widely distributed in the periphery e.g., on smooth muscle cells in the gut and vasculature. Atypical antipsychotics such as olanzapine target - in addition to dopamine receptors - also serotonin receptors (particularly 5-HTR2A, 5-HTR2C and 5-HTR1A) [61]. Moreover, serotonin reuptake inhibitors (SSRIs) increase the availability of serotonin in the synaptic cleft. The efficacy of SSRIs is partly mediated by the effect of the released serotonin on the postsynaptic 5-HTR2A receptors. This may be a possible mechanism for side effects, although other receptors such as 5-HTR3 may also be involved [107,126]. Thus, the 5-HTR2A gene is a candidate gene for clinical outcome with regard to treatment with atypical antipsychotics and antidepressants, especially SSRIs.

Several 5-HTR2A polymorphisms were examined for associations with clinical outcome under antidepressant or antipsychotic therapy. Genetic association studies report conflicting and generally negative results. Only few results could be replicated at all [127]. Some significant associations were reported for the silent 5-HTR2A T102C polymorphism and/or the A-1438G, which are almost in complete linkage. Most replicated data suggested the C-allele of the 5-HTR2A T102C as risk factor for antipsychotic response, but a number of non-replicating studies did not allow to draw a definite conclusion [131-135]. Similarly, the association between the 5-HTR2A C-allele and better treatment response/more side effects to SSRIs could not be replicated in all studies [107,109,128-130]. The effect of the 5-HTR2A T102C polymorphism on clinical outcome - if any effect was found at all - was reported to be modest and may be not large enough to be of clinical relevance. Accordingly, no association was detected for the 5-HTR2A T102C polymorphism and clinical outcome under antipsychotic or SSRI therapy in the study population.

In a recent study with 1953 depressive patients, who were treated with the SSRI citalopram, a highly significant and reproducible association between treatment outcome and a marker in intron 2 of 5-HTR2A (rs7997012) was found. Participants that were A/A homozygous had an 18% reduction in absolute risk of having no response to treatment, compared with those homozygous for the other allele [126].

In the SSRI subpopulation, no effect of the A/A genotype on treatment response was observed, even though patients comedicated with antipsychotics or mirtazapine were excluded from evaluation due to the possible influence of these drugs on the target gene. Unlike the SSRIs, mirtazapine blocks the 5-HTR2A receptor, preventing interaction with serotonin. By blocking the receptor, mirtazapine could negate the effects of any 5-HTR2A functional variation [107]. Interestingly, the patients with A/A genotype suffered from more side effects which was significant despite the small sample size. Only 5% (1/19) of G-allele carriers, but 75% (3/4) of the patients with A/A genotype had moderate/marked side effects (Fisher-Exact-test, p = 0.009, Odds Ratio OR of 54 with a 95% confidence interval of 2.6 – 1117). There are many types of post-synaptic serotonin receptors. Some were reported to be responsible for increases in mood and decreases in anxiety (e.g., 5-HTR1A), whereas others have been shown to be related to SSRI side effects (e.g., 5-HTR2A receptors). 5-HTR2A antagonists have been shown to reverse the SSRI-associated sexual side effects in some studies [129]. Keeping this in mind, the association between 5-HTR2A and side effects observed in the study population seems plausible.

Furthermore, the same association with side effects was observed not only for SSRIs, but also for the atypical antipsychotic olanzapine that also target the 5-HTR2A receptor. The effect of the 5-HTR2A intron 2 A/A genotype on side effects was more pronounced in the subgroup without mirtazapine co-medication. Only 25% (11/44) of G-allele carriers, but 83% (5/6) of the patients with A/A genotype had moderate/marked side effects (n = 50, Fisher-Exact-test, p = 0.0098, Odds Ratio of 15 with a 95% confidence interval of 1.6 – 143).

4.4.2.1.3 No Influence of 5-HTR1A C-1019G or 5-HTR6 T267C

Serotonin or noradrenaline reuptake inhibitors desensitize the 5-HTR1A autoreceptors by increased synaptic 5-HT levels, which may contribute to their antidepressant efficacy [118,119]. Less treatment response to SSRIs and TCAs was reported for the putative non-functional 5-HTR1A -1019G-allele in some previous studies [119-125]. Antipsychotics, especially atypical ones, also target serotonin receptors (5-HTR1-7) in addition to dopamine receptors. Many antipsychotics have demonstrated high affinity for the 5-HTR6 receptor [148]. In addition, this receptor exhibits high affinity for a number of tricyclic antidepressant drugs like amitriptyline [149]. However, studies on the silent 5-HTR6 T267C polymorphism and treatment response to atypical antipsychotics (clozapine, risperidone) or antidepressants have yielded conflicting results or suggest only a minor role in clinical outcome [150-154]. Moreover, the role of this receptor in the clinical action remains unclear [146,147]. Accordingly, neither response nor side effects were significantly associated with the 5-HTR6 T267C or 5-HTR1A C-1019G genotypes in any of the different medication subgroups in the study population.

4.4.2.2 Dopaminergic Polymorphisms

4.4.2.2.1 DRD2 -141C ins/del or DRD2 TaqI A1/A2 and Response to Antipsychotics

All currently available antipsychotic drugs - typical or atypical - act on central dopamine receptors. The occupancy of the dopamine DRD2 receptor plays a major role in the antipsychotic mechanism of action [133]. Polymorphisms within these receptors may have an impact on treatment response. An increased mRNA expression was reported for the -141C ins-allele of the DRD2 -141C ins/del polymorphism [434] as well as a higher striatal dopamine DRD2 receptor density [435]. Despite contradictory results, a trend towards an association with favorable antipsychotic response seems to exist for the DRD2 -141C ins-allele and the DRD2 Taq1A A1-allele [155,156].

Accordingly, DRD2 -141C ins/ins carriers and DRD2 TaqI A1 carriers responded better after 4 weeks of antipsychotic treatment (CGI(2) rating available for 271 patients, p = 0.038 and 0.050). The two examined polymorphisms were in linkage (Pearson Chi-Square p = 0.002). The DRD2 -141C ins/del polymorphism is probably the underlying functional polymorphism as it lies in the 5'-flanking promoter region of the DRD2 gene, whereas the DRD2 Taq1A polymorphism lies 10 kbp 3'-downstream of DRD2. However, this variant causes an amino acid substitution within the ANKK1 (ankyrin repeat and kinase domain containing 1) kinase, which may affect substrate-binding specificity and changes in ANKK1 activity. The kinase gene is member of an extensive family of proteins involved in signal transduction pathways. This may provide an alternative explanation for previously described associations between the DRD2 Taq1A polymorphism and neuropsychiatric disorders [436].

4.4.2.2.2 DRD3 Ser9Gly and Side Effects under Antipsychotic Therapy

All currently available antipsychotic drugs act on central dopamine receptors including the DRD3 receptor. The DRD3 Ser9->Gly polymorphism results in an increased binding activity for dopamine and DRD3 selective ligands [437]. The examination of the DRD3 Ser9Gly polymorphism in pharmacogenetic studies so far yielded contradictory results. The Ser9-allele seems to be associated with better response to typical antipsychotics, and the 9Gly-allele with response to atypical antipsychotics [155,156]. The 9Gly-allele was also reported to be connected with side effects typical for antipsychotic therapy (akathisia and tardive dyskinesia) [157]. Accordingly, carriers of the DRD3 9Ser/9Ser wildtype treated with antipsychotics suffered from less side effects compared to carriers of the DRD3 9Gly-allele (p = 0.024; side effects score available for 263 patients).

4.4.2.2.3 DAT1 VNTR and Response to Antidepressants

As dopamine pathways play an important role in psychiatric diseases [160], the high affinity dopamine transporter DAT1 is another promising candidate gene which could influence clinical outcome. As the DAT1 VNTR is localized in the 3'-non-coding region, allelic variants are not likely to cause differences in DAT1 function [167]. Some groups, however, reported higher - *in vitro* as well as *in vivo* - DAT1

expression in carriers of the 10-repeat allele compared with carriers of the 9-repeat allele whilst others did not [158-162]. The reason for these differences might be an incomplete linkage of the VNTR with transcriptionally relevant polymorphisms [161]. Correspondingly, studies on extrapyramidal-motoric side effects [158,163] and response [164] under antipsychotic therapy mostly led to negative results. Accordingly, the DAT1 VNTR did not show any significant associations with response or side effects to antipsychotic drugs in the study population.

Dopamine may also play a role in depression as well as in the response to antidepressant drugs [167]. Lower dopamine concentrations have been measured in the cerebrospinal fluid (CSF) of depressed patients [165]. Dopamine transporter (DAT1) activities were found to be modulated during treatment with the SSRI citalopram. Reduced extracellular dopamine concentrations have been measured in striatum during medication with SSRIs [166,167]. In patients with a major depressive episode, a significantly lower number of rapid responders (i.e., within three weeks) was seen among homozygous carriers of the DAT1 9-repeat allele compared to heterozygous and homozygous carriers of the 10-repeat allele. The effect was independently from the type of antidepressive medication (SSRIs, tricyclic antidepressants, mirtazapine, venlafaxine) [168]. The results of the corresponding study subpopulation i.e., patients with a major depressive episode receiving any kind of antidepressant therapy were quite in the opposite direction. The PDS-D score (% improvement after 4 weeks) increased from 14% for 10/10-repeat carriers to 32% for heterozygote 9/10-repeat carriers and to 60% for the 9/9-repeat carriers with similar baseline PDS-D scores for all three genotypes. The reason for this might be an incomplete linkage as discussed above [161].

4.4.2.3 (Nor)Adrenergic Polymorphisms

4.4.2.3.1 NET C-182T and Response to NRI Therapy

Antidepressant efficacy is mediated not only by serotonin reuptake inhibition but also by norepinephrine (= noradrenaline) reuptake inhibition. The norepinephrine transporter (NET) modulates noradrenergic signaling by reuptake of norepinephrine from the synaptic cleft. Norepinephrine reuptake inhibitors (NRIs) are e.g., some TCAs such as nortriptyline, desipramine and maprotiline, the SNRIs milnacipram and reboxetine as well as the SSNRI venlafaxine.

A Japanese study on antidepressant response to the SNRI milnacipram revealed an influence of the NET T-182C and NET G1287A polymorphisms with superior response in carriers of the -182T-allele and slower response in carriers of the 1287A/A genotype [169]. A further study in Koreans associated the NET 1287G/G genotype with better response to nortriptyline treatment [170]. Neither of the NET polymorphisms examined so far alter the amino acid sequence. The NET T-182C variant, however, is localized within the NET promoter. The NET G1287A variant is a silent mutation in exon 9 [167], but higher cerebrospinal fluid (CSF) concentrations of 3-methoxy-4-hydrophenylglycol, a major norepinephrine metabolite [171], were reported for 1287G/G homozygote patients indicating a more active reuptake of norepinephrine. It was hypothesized that the effect of reuptake inhibition might occur more slowly in patients with the A/A genotype [169].

Deviating from these findings, the NET -182C-allele was associated with better response in the study subpopulation treated with NRIs. No associations were observed for NET G-1287A. First of all, the sample size was with 31 patients very small which may have led to false positive results. This finding, however, reminds of a similar deviation observed for the 5-HTTLPR, where Asians showed better response to the S-allele in some studies in deviation to the results in Caucasians with better results for the L-allele [438,439]. This may be due to different allele distribution in Asians and Caucasians or - as the polymorphisms examined may not be the underlying functional ones - to different linkage with the functional polymorphism that is responsible for the differences in treatment response. Correspondingly, one study found an excess of NET-182 T-allele in depressive Japanese patients [440], whereas no association between the NET T-128C polymorphism and susceptibility to major depressive disorder was observed in a German population [280].

4.4.2.4 Signal Transduction and Neurotransmitter Metabolism

Novel candidate genes more indirectly involved in drug action downstream of primary monoaminergic activation were examined in addition to the primary drug targets such as the dopaminergic, serotonergic and noradrenergic transporters and receptors.

4.4.2.4.1 No Influence of Polymorphisms in Neurotransmitter Metabolism (ACE ins/del, TPH1 A218C, COMT Val158Met, MAO-A uVNTR) on Clinical Outcome

No associations with clinical outcome were observed for polymorphisms examined in the neurotransmitter synthesis (TPH1 - responsible for serotonin synthesis), and neurotransmitter metabolism (COMT - responsible for degradation of dopamine and norepinephrine; ACE - responsible for degradation of brain neuropeptides including substance P that is involved in antidepressant efficacy; MAO-A - involved in serotonin metabolism and DBH - catalyses the conversion of dopamine to norepinephrine).

Only one interesting finding was observed. In patients with mood disorders (F3 diagnosis), higher age of onset was significantly associated with the COMT high (Val) genotype ($p = 0.002$). It has been shown that the high activity genotype (Val/Val) catabolizes dopamine up to four times faster than the low activity COMT genotype (Met/Met). This results in a significant reduction of synaptic dopamine after neurotransmitter release and a significant reduction of dopaminergic stimulation of the post-synaptic neuron [212]. The low activity COMT genotype (Met/Met) with its increase of dopamine concentration in the whole brain could be a limiting factor for an antidepressant like effect [215]. Increased dopaminergic stimulation may be a risk factor for developing mood disorders, but this is mere speculation. Similar considerations for susceptibility to schizophrenia yielded contradictory results [441-443].

4.4.2.4.2 No Influence of Novel Candidates in Signal Transduction (FKBP5 rs1360780, BDNF Val66Met) on Clinical Outcome

No associations with clinical outcome were also observed for the BDNF polymorphism examined. BDNF plays an important role in the survival, differentiation, and outgrowth of neurons during development and in adulthood and is thus involved in the neurodevelopment of neurotransmitter-related systems. There are several lines of evidence that the expression of BDNF is the downstream target of a variety of antidepressants that were reported to increase serum BDNF levels [237-240]. The effect - if any - is probably too small to be relevant in a clinical setting.

The FKBP5 polymorphism examined was associated with increased intracellular FKBP5 protein expression, which triggers adaptive changes in glucocorticoid receptors and, thereby, hypothalamic-pituitary-adrenal (HPA) axis regulation. HPA axis activity was associated with causality as well as with the treatment of depression [233]. Patients with a current depressive episode that carried the T-allele of the FKBP5 polymorphism, especially heterozygotes, had a significantly longer duration of illness in anamnesis and a trend to more previous episodes in the study population evaluated herein. Moreover the CGI(1) severity rating at baseline was lower for T-carriers. As reported previously, the FKBP5 variant-dependent alterations in HPA axis regulation could led to a faster improvement of illness [233] and may be more a marker of greater cyclicity rather than specific drug response [234].

4.4.2.4.3 GNB C825T and Antipsychotic Response Onset

As most monoaminergic receptors belong to the class of G-protein-coupled receptors, G-protein subunits (here b3 subunit) are candidate genes for the pharmacogenetics of antidepressant and antipsychotic drugs [179]. No association with antidepressant outcome was observed in the study population. Accordingly, the genotype-response associations observed in some of the previous studies [225-228] could not be replicated in other studies [129,229]. Interestingly, study patients treated with olanzapine that carried the GNB 825T/T genotype had a significantly faster onset of response. The 825 T-allele has been associated with better olanzapine response previously [230].

4.4.2.5 Weight Gain during Therapy

Weight gain, especially during therapy with atypical antipsychotics, is a severe problem. First of all, it may interfere with treatment compliance and may increase the risk of treatment interruption by the patient. Secondly, weight gain leads in the worst case to obesity-related diseases such as diabetes and cardiovascular morbidities [63,444,445]. Of all antipsychotics, clozapine and olanzapine induce particularly profound weight gain [343]. Most weight gain occurs during the first 6 - 8 weeks of olanzapine therapy and reaches a plateau by the end of the first year of treatment [392]. Moreover, tricyclic antidepressants, mirtazapine and risperidone lead to marked or intermediate weight gain [329,362].

The exact underlying pharmacodynamic mechanisms involved in drug-induced weight gain have not been fully elucidated. Body weight and energy homeostasis is regulated by a complex system of neurotransmitters, neuropeptides, hormones and immune related factors and involves the hypothalamus, the solitary tract and cortical structures. Drugs that have weight gain inducing properties may disrupt associated pathways at any of these levels, although it remains unclear what the mechanisms of action might be [446]. Serotonergic, histaminic, and adrenergic affinities have been implicated along with other metabolic mechanisms [65,66,392]. Candidate gene studies have produced significant findings in the 5-HTR2C and ADR2A receptor genes as well as in the leptin and GNB3 genes for antipsychotic-induced weight gain [66,133,140,177,178,230,231,344,447]. Less is known about the genetic basis of mirtazapine-induced weight gain.

4.4.2.5.1 5-HTR2C C-759T and Atypical Antipsychotics

The 5-HTR2C receptor gene is found on the X-chromosome and codes for one of the two major 5-HTR2 receptor subtypes that are targeted by antipsychotics. Promoter polymorphisms near the major transcription initiation site of the 5-HTR2C receptor at -703 were associated with obesity and diabetes in Japanese [139]. The -759C-allele showed less transcriptional activity than the T-allele [137,138] and was associated with higher weight gain under olanzapine and clozapine treatment [140-144,393,448] except for one study [145].

The results in the study patients receiving clozapine or olanzapine as antipsychotic treatment were in accordance with these findings. Patients homo- or hemizygous for the -759C-allele had on average a weight gain of 3.8% after 4 weeks compared to only 1.4% for T-allele carriers ($p = 0.007$) and compared to approx. 2% for all other patients without olanzapine/clozapine treatment. Thus, the C-allele seems to be a risk factor for weight gain.

In addition to genetic factors, a vast number of potentially relevant factors has been associated with antipsychotic-induced weight gain: environmental factors independent of therapy (gender, age, ethnicity, smoking, baseline weight, social factors) and treatment dependent factors (length of exposure, dose, treatment duration) [382,383]. In a naturalistic setting, co-medication has to be considered: Lithium, valproic acid and tricyclic antidepressants are known to lead to marked weight gain; mirtazapine and risperidone to intermediate weight gain [329]. Data from short-term studies identified better clinical outcome and low baseline BMI as predictive factors of acute weight gain [388,389].

Multiple regression analysis was performed for the study subpopulation to take possible additional influence factors into account, as given above (baseline weight or BMI, age, gender, diagnosis, dose, smoking status, response, treatment naïveness/first episode, co-medication with antidepressants or lithium). 27% of the weight variation can be explained by 5-HTR2C C-759T genotype, CGI(2) response and baseline BMI. The ADR2A C-1291G genotype (see below, $p = 0.072$) missed inclusion in the model.

4.4.2.5.2 ADR2A C-1291G and Atypical Antipsychotics/Mirtazapine

Most new antipsychotic drugs such as clozapine, risperidone, olanzapine, quetiapine and sertindole include adrenergic antagonism amongst their mechanism of action. ADR1A and 2A have been implicated in mediating antipsychotic response [172,173]. Recently, clozapine and olanzapine-induced weight gain was related to the -1291G-allele [177,178]. The adrenergic system plays a key role in regulating energy balance through the stimulation of both thermogenesis and lipid mobilization in adipose tissue [174,175]. The exact mechanisms involved in weight gain under treatment with olanzapine and other second-generation antipsychotics are, however, still unclear.

The results for study patients receiving clozapine or olanzapine as antipsychotic treatment showed the same trend as the previous findings. Patients with the rare ADR2A -1291G/G had on average the highest weight gain (n = 9, 5.6%), then C/G carriers (n = 33, 3.3%) and the C/C carriers (n = 37, 2.2%; p = 0.093) compared to approx. 2% for all other patients without olanzapine/clozapine treatment. This trend was not seen in first episode patients; all ADR2A genotypes suffered from weight gain (on average 3.9%). Weight gain in drug-naïve patients may be more pronounced than in patients with multiple past medication [338]. In the other patients the C/C genotype seems to be protective against weight gain (24 C/C, 1.0%) compared to G-allele carriers (18 C/G, 3.8% and 1 G/G, 7.6%, p = 0.087).

Mirtazapine shows a high affinity to ADR2A receptors, therefore the C-1291G may play a role in weight gain under mirtazapine therapy. In study patients receiving only mirtazapine as treatment, the C/C genotype seems also to be protective against weight gain (10 C/C, 0.5%) compared to G-allele carriers (7 C/G, 4.0% and 2 G/G, 3.8%). The observation was significant (p = 0.008) despite the small sample size but needs to be verified in larger study populations.

Interestingly, the same polymorphism seems to play a role in weight gain under mirtazapine and olanzapine/clozapine therapy. The underlying mechanisms by which the antipsychotics cause weight gain via this polymorphism are still unclear. Increased levels of catecholamines inhibiting subcutaneous lypolysis through ADRA2-receptors were discussed as link in two previous studies [177,178]. The connection between the ADR2A polymorphism and mirtazapine, which pharmacological activity is mediated by blocking the ADR2 and 5-HTR2 receptors, is plausible. Mirtazapine, however, blocks the receptor, which seems to be in contradiction to the explanation given for the antipsychotics above. The underlying mechanism has to be examined in further studies.

4.5 Conclusion

4.5.1 Pharmacokinetic Polymorphisms and Clinical Outcome

Citalopram and CYP2C19

Significantly higher serum concentrations of both racemic citalopram and escitalopram in the clinical cohort evaluated herein show a significantly reduced clearance and less effective drug metabolism for CYP2C19 intermediate metabolizers (with one defective allele) compared to extensive metabolizers. Thus, not only poor metabolism (two defective alleles) but also one non-functional allele seems to be of relevance. Results obtained were robust against the "background noise" of a naturalistic clinical setting despite the relatively small sample size and might be clinically relevant.

Moreover, a correlation between escitalopram serum concentration and side effects seems to exist and shows the need of genotype-adjusted dose recommendations. The dose reduction should be discussed not only for the relatively small number of CYP2C19 poor metabolizers as done before. Only about 2 - 6% of individuals of European origin (Caucasians), 15 - 20% of Asian origin, and 10 - 20% of African origin are CYP2C19 poor metabolizers, but CYP2C19 intermediate metabolizers have a much higher prevalence in all populations. In central Europe not only the approx. 5% poor metabolizers would benefit but approx. 25% of the population (poor and intermediate metabolizers).

A preliminary proposal for genotype-adjusted dose recommendation suggested a reduction to 60% of dose for CYP2C19 poor metabolizers and 80% for intermediate metabolizers. It was, however, limited by the fact that only data for the administration of racemic citalopram in 10 CYP2C19 extensive metabolizers and 6 poor metabolizers was taken into account [87]. The results presented herein for intermediate vs. extensive metabolizers and racemic citalopram as well as escitalopram, however, suggest that a greater dose reduction might be necessary, especially in consideration of the enantioselective metabolism of the pharmacological active escitalopram. These results and conclusions should be replicated in a larger study population and under stereoselective determination including metabolites.

Mirtazapine and CYP2D6

In this study, the interindividual pharmacokinetic variation in psychiatric in-patients treated with mirtazapine has been investigated. The study was a good reflection of natural practice including patients of all ages (>18 years), with different kinds of comorbidities and concomitant medications. Due to the mixed influence of different cytochrome P450 enzymes and stereoselective metabolism, no significant influence of CYP2D6 or CYP1A2 genotypes on (racemic) mirtazapine serum concentrations was seen in the study cohort. Other known influence factors such as female gender, old age and co-medication with carbamazepine, however, caused significantly higher serum concentrations. Confirming the results of previous studies, the racemic serum concentrations of mirtazapine alone do not seem to be of high predictive value for response or for the incidence of side effects. This may be due to the stereoselective metabolism and transport of mirtazapine.

Interestingly, CYP2D6 poor metabolizer status - as a measure of stereoselective metabolism - was connected with a significantly higher incidence of side effects. The results should be interpreted with caution due to the low number of poor metabolizers (n = 11 of 110) and should be replicated in a larger study population. Nevertheless genotyping may be useful to complement the normal clinical evaluation process.

Olanzapine and CYP21A2*1F

A significant influence of the CYP1A2*1F A/A genotype alone on olanzapine serum levels was observed (on average -22%), similar to that of inducing agents (tobacco smoke, carbamazepine, on average -28%). Consistently, the combined effect of CYP1A2*1F A/A genotype and inducers was almost twice as high (on average -42%). Due to the standard diet and controlled environment during hospitalization, an extraordinary exposure to other inducing agents (e.g. alimentary inducers) is unlikely and cannot explain the influence of the CYP1A2*1F A/A genotype alone. Studies with larger

patient groups are needed to confirm the independent effect of this polymorphism on olanzapine metabolism. Genotyping of the CYP1A2*1F could be useful as additional predictor of CYP1A2 activity and may provide important additional information beyond the known influence factors on olanzapine clearance such as co-medication, smoking, age and gender.

Despite the naturalistic setting with a heterogeneous patient pool and different kinds of co-medications, the curvilinear olanzapine concentration - response relationship found previously could be confirmed for the study cohort examined herein. This again emphasizes the need to monitor olanzapine serum concentrations and possible influence factors such as the CYP1A*1F genotype among others as given above.

For antipsychotic induced weight gain within a clinical setting, pharmacodynamic polymorphisms in the DRD2 and 5-HTR2C receptors as well as better clinical outcome and low baseline BMI seem to be more relevant than olanzapine serum concentrations and pharmacokinetic polymorphisms in the metabolizing enzyme CYP1A2 though a lower threshold of 20 ng/mL for olanzapine-induced weight gain seems to exist.

4.5.2 Pharmacokinetic Polymorphisms and Clinical Outcome

Length of hospitalization and response onset are prolonged for patients treated with CYP2D6 dependent drugs. On the one hand, poor metabolizers (PMs, gene dose 0) suffered from more side effects compared to patients with extensive or ultrarapid metabolism (gene dose 1.5 - 2 or > 2, respectively) with CYP2D6 medication and compared to PMs without CYP2D6 medication. But also intermediate metabolizers (IMs, gene dose 0.5 or 1) treated with higher doses of CYP2D6 dependent drugs suffered from more side effects than EMs with CYP2D6 medication and compared to IMs without CYP2D6 medication. Probably as a consequence of more side effects, the treatment response was lower under CYP2D6 medication than under non-CYP2D6 medication for IMs, but not EMs. On the other hand, UMs tended to have more switches due to non-response in medical history. If UMs and EMs were treated with higher doses of CYP2D6 medication, the responder rate tended to be higher. Thus, not only the relatively small number of PMs (5 - 10% in central Europe) and UMs (2 - 5%) may benefit from CYP2D6 genotyping as a therapeutic tool. Identification not only of PM, but also IM status (38% in the study population) might help to avoid adverse effects by starting treatment with *a priori* lower doses for CYP2D6 drugs and keeping doses low throughout the treatment. Moreover, special diligence is necessary in case of non-response: switching to another drug might be a better option than increasing the dose for IMs. Increasing the dose, however, would be very well an option for EMs and UMs. Ultimately, pre-treatment identification of CYP2D6 genotype might help to shorten the time until response onset, and to shorten the length (and costs) of hospitalization.

The genotype-serum concentration relationship found in the olanzapine serum subset was confirmed by a genotype-dose relationship found in the whole study population for the combination of the highly inducible CYP1A2*1F genotype in combination with CYP1A2 inducers. Thus, genotyping of the CYP1A2*1F may be useful in routine treatment with olanzapine, which is known for its strong concentration-response relationship that was also found in the serum cohort. Studies with larger patient groups are needed to confirm the effect of this polymorphism on olanzapine metabolism in addition to the known influence factors on olanzapine clearance such as co-medication, smoking, age and gender.

A significant genotype-side effects relationship was found - despite the low prevalence of CYP2C19 polymorphisms - for tricyclic antidepressants, but not (es)citalopram. This may be due to the fact that tricyclic antidepressants (TCAs) have a narrow therapeutic window. Due to the two-stage metabolism of the examined TCAs (CYP2C19 to an active metabolite and then CYP2D6 to an inactive metabolite), genotyping of both CYP2D6 and CYP2C19 might be helpful for predicting the risk of elevated concentrations and side effects.

It appears that the MDR1 polymorphisms examined so far have no major impact on clinical outcome of P-glycoprotein substrates. Minor effects, however, may have been missed due to the heterogeneous patient pool with possibly interfering confounders but do not seem to be of relevance in a clinical setting.

4.5.3 Pharmacodynamic Polymorphisms and Clinical Outcome

Polymorphisms in the serotonin transporter (5-HTTLPR and rs25531) significantly influenced side effects only in patients treated with selective serotonin reuptake inhibitors (SSRI). No effect was seen in patients treated with unselective serotonin reuptake inhibitors or other antidepressants such as mirtazapine. Similarly, the influence of a marker in intron 2 of 5-HTR2A (rs7997012) on side effects was evident only for antidepressants and antipsychotics that intensely target this receptor i.e., SSRIs and the atypical antipsychotic olanzapine. Accordingly, the norepinephrine transporter polymorphism (NET T-182C) only seems to have an effect on norepinephrine reuptake inhibitors, but not on SSRIs or mirtazapine.

Antipsychotics target primarily dopamine receptors. Polymorphisms in the dopamine receptor DRD2 (-141C ins/del and TaqA1/A2) influenced response to antipsychotic treatment, whereas the DRD3 polymorphism (Ser9Gly) was more related to side effects. The polymorphism in the dopamine transporter (DAT1 VNTR) did not exhibit an observable effect on the clinical outcome.

In opposite to the 5-HTT and NET polymorphisms where associations with clinical outcome were only observed for the patients receiving intensely targeting drugs, dopaminergic mechanisms seem to be involved in depression and efficacy of all kinds of antidepressant treatment in a more general way. Higher activity of COMT (COMT VAl158Met) leading to less dopaminergic stimulation in the CNS was associated with higher age of illness onset in all patients diagnosed with mood disorders. Less antidepressant response for all different kinds of antidepressants was seen for the dopamine transporter DAT1 VNTR 10-repeat allele in patients with a major depressive episode.

Two candidate genes - downstream of primary monoaminergic activation and only indirectly involved in drug action - showed an effect on course of disease (more rapid cyclicity/faster response onset). The FKBP5 polymorphism (rs1360780) was associated with longer duration of illness in anamnesis and a trend to more previous episodes; the GNB C825T polymorphism with faster response onset.

Further influence of polymorphisms in candidate genes other than the primary drug targets on response and side effects were not observed in the naturalistic setting examined herein. This is in accordance with previous findings. Studies for such polymorphisms that are not direct targets of antidepressants/antipsychotics are much rarer and the positive results found in some studies could not be replicated in others. This may be due to the fact that these polymorphisms have indeed no influence on the therapeutic outcome and the observed results were false positive. Another possibility is that the existing effect could not be detected in the study population as it is too small to be detectable in the heterogeneous study population examined herein. The effect of such polymorphisms may be found only in specific diagnosis subtypes. Moreover, an influence may exist only for specific drugs that were not common in the study population and that were not examined separately herein. Or, as shown for the COMT, FKBP5 and GNB polymorphisms examined herein, the polymorphisms rather play a role in etiopathogenesis or course of disease than in response to treatment. For that reason their effect may be very indirect and difficult to measure. In any case, these polymorphisms do not seem to be very relevant for clinical practice.

Especially the observed effects of 5-HTT and 5-HTR2A polymorphisms on severity and frequency of side effects might be relevant in clinical practice. First of all, they were found in a heterogeneous patient pool with different kinds of comorbidities and co-medications as examined herein. Secondly and even more important, severe side effects were accompanied by a significant longer hospitalization. Thus, pre-treatment identification of these genotypes might help to shorten the length (and costs) of hospitalization.

Two polymorphisms in 5-HTR2C (C-759T) and ADR2A (C-1291G), known to play a role in weight gain under olanzapine/clozapine therapy, showed an association in the study population examined herein, though for the ADR2A polymorphism the trend did not reach statistical significance. This may be due to only a minor, more indirect effect or due to the short observation period of only four weeks. Moreover, the underlying mechanisms by which the antipsychotics cause weight gain via this polymorphism are still unclear. Interestingly, the same polymorphism seems to play a - so far unknown - role in weight gain under treatment with mirtazapine that blocks ADR2 receptors.

4.5.4 Overall Conclusion and Future Prospects for Genotyping

Genotyping for relevant polymorphisms could be useful to improve therapy with existing drugs, to maximize clinical outcome and minimalize risks [18]. Genotyping has to be accomplished only once in a lifetime and the costs of currently available tests are often less than those of the drugs themselves. The dose could be optimized at the beginning, therapy duration and costs would be lower [1]. This line of argument is supported by observations for several polymorphisms in the study herein. Length of hospitalization and response onset were prolonged in study patients treated with CYP2D6 dependent drugs (on average 7 days). Similarly, unfavorable variants in the 5-HTT promoter and 5-HTR2A gene were also associated with prolonged hospitalization in this study.

Prior to the establishment of routine genotyping, the relevant polymorphisms have to be identified. This is difficult in psychopharmacology. Problems in pharmacogenetic studies range from incomplete knowledge of disease mechanisms to difficulties in defining proper determinants of drug response. Many published gene-trait associations could not be replicated as can be seen also in this study. Many of the not replicated associations are likely to represent type I error (false positive).

Moreover, the genetics underlying the medication response and side effects are complex and multifactorial. As a result, the response/side effects are likely the product of multiple effects, each modest on its own. With so many genes involved, it is difficult to identify and characterize the most relevant ones. Cautious reviews have suggested that perhaps 10 to 15% of drugs are influenced by a small number of high-penetrance genes, while 35 to 40% are polygenetic. Small effects are more difficult to detect which may be another reason for the contradictory results reported on some polymorphisms so far.

Even when the data is detailed enough to guide clinical practice such as in the case of the cytochrome P450 enzymes, the translation into clinical medicine is difficult. A wide range of environmental factors (e.g., certain foods, smoking, comorbid illnesses, drug-drug interactions, age, gender, non-compliance) also influences rates of drug metabolism [449]. As a result of many non-genetic influences on drug response and side effects and due to population/ethnic differences with regard to frequencies of gene variants, it is likely that pharmacogenetic testing will be neither completely predictive nor universally applicable [1].

Thus, one reason for slow adoption of pharmacogenetic testing is that genotyping of the individual pharmacogenetic status is very complex and that it is still - with some exceptions - under discussion which polymorphisms are clinically relevant. Another reason is that - even for the indisputable polymorphisms - data on cost-effectiveness is limited. This is obviously a complex issue that relates not only to the cost of genotyping itself but also to competing costs, such as those of caring for patients with adverse drug reactions. Moreover, prospective clinical trials demonstrating that such testing can improve the benefit/risk ratio of drug therapy are still lacking [3]. In a recent clinical study, however, higher response rates were observed for the patient group where pre-treatment genotyping (5-HTTLPR) was performed to decide which class of antidepressant should be used [450].

5 Summary

It is one of the major future goals in clinical medicine to individualize treatment through molecular pharmacogenetics. Pharmacogenetic screening of the relevant polymorphisms could be used to guide pharmacotherapy and to improve therapy with existing drugs i.e., to maximize the clinical outcome and minimalize side effects and risks by choosing the optimal dose and drug.

Variability of treatment response is a crucial problem in psychopharmacology, where therapy is time- and cost-intensive. In the two psychiatric diseases with the highest prevalence in population (depression and schizophrenia), 30 - 50% of all patients do not sufficiently respond to the initial treatment - which is only seen after weeks due to the delayed effect onset of antidepressants and antipsychotics - or suffer from substantial adverse effects making switches to other drugs necessary [2]. It was estimated that 60 - 90% of interindividual variability in treatment response of antidepressants is due to heritable traits [21]. Similar is true for response and side effects of antipsychotics [22-26].

Identification of the relevant polymorphisms is the first step towards routine genotyping. The intention of this study was to evaluate which polymorphisms have such an impact on outcome in a clinical setting that an examination maybe useful as assistance for future treatment decisions. Fast, sensitive and reliable methods suitable for routine testing in clinical practice were developed for polymorphisms in 19 candidate genes. The steady-state serum concentrations of the most frequently prescribed drugs (olanzapine, mirtazapine, citalopram) were measured and the associations between polymorphisms, serum concentrations and clinical response/side effects were examined.

Changes in the pharmacokinetics, i.e. in serum concentrations, are the result of polymorphisms in drug metabolism like in key enzymes such as the cytochrome P450 system. CYP2C19, CYP2D6 and CYP1A2 belong to the most important cytochrome P450 enzymes in the metabolism of antidepressants and antipsychotics. Impaired CYP2C19 metabolism (due to one non-functional allele) had significant influence on serum concentrations of the CYP2C19 substrate (es)citalopram. A direct genotype-side effects relationship, however, was only seen for tricyclic antidepressants, but not for (es)citalopram, which has a wider therapeutic window. Moreover, it was shown that the CYP1A2*1F polymorphism influences olanzapine concentrations beyond the effect of common CYP1A2 inducers. CYP2D6 was - due to stereoselective metabolism and influence of CYP3A4 - not a good predictor of mirtazapine concentrations, but for adverse effects of predominantly metabolized substrates. In addition to the well-known impact on the relatively rare poor metabolizers (9% of study population), an impact on the more frequent (38%) intermediate metabolizers receiving high doses of CYP2D6 substrates could be demonstrated for the first time in a clinical setting. Moreover, the response rate was lower in intermediate metabolizers receiving CYP2D6 drugs compared to those receiving other medication.

Pharmacodynamic polymorphisms in the direct drug target structures showed the best associations with clinical outcome. Well-known polymorphisms in the serotonin transporter (5-HTTLPR [110] und rs25531 [115]) significantly influenced side effects and length of hospitalization in patients treated with selective serotonin reuptake inhibitors. Similarly, the influence of a marker in intron 2 of the serotonin receptor 5-HTR2A (rs7997012) on side effects was evident only for drugs that are effective via this receptor as could be shown for selective serotonin reuptake inhibitors. Additionally, a novel effect of this polymorphism on side effects under therapy with the atypical antipsychotic olanzapine was found. All antipsychotics target dopamine receptors and thus an influence of polymorphisms in the dopamine receptors on response or side effects was seen for the whole group of antipsychotics.

Indirect targets (i.e., candidate genes downstream of primary monoaminergic activation and only indirectly involved in drug action) seem more to modulate the course of disease (more rapid cyclicity/ faster response onset) than to influence the extent of response or side effects. A polymorphism in the beta-3 subunit of the G-protein (GNB) was associated with faster response onset under olanzapine therapy and a polymorphism in the FK506 binding protein 51 (FKBP5) with longer duration of illness in anamnesis and a trend to more previous episodes. All other examined polymorphisms showed no associations with clinical outcome or course of disease in the naturalistic setting examined herein. This may be due to actual non-existence of the associations reported previously. Some existing but small effects may have been missed due to the heterogeneity and limited number of the patients examined. In any case these indirect involved polymorphisms do not seem to be very relevant for clinical outcome. But they may be responsible for particular side effects such as weight gain. Weight

gain is a severe problem in psychiatric treatment as it may interfere with treatment compliance. Weight gain under olanzapine/clozapine therapy was influenced by two polymorphisms in the serotonin receptor 5-HTR2C and the alpha 2-adrenergic receptor ADR2A. The ADR2A polymorphism also plays a so far unknown role in weight gain under treatment with mirtazapine which blocks adrenoceptors.

To summarize, the results of the prospective diagnostic study herein could confirm some previous findings from controlled, monotherapeutic studies in a naturalistic setting. Moreover, novel associations were found especially for side effects. This was an explorative study demonstrating the proof of principle. Especially the novel results and conclusions should be replicated and confirmed in a larger study population including more restrictive statistical testing (e.g., power analysis, which was not performed herein as the effect sizes of the examined variants was not known *a priori*). Yet this study was a good reflection of natural practice. Results observed - especially the impact of CYP2D6, 5-HTT and 5-HTR2A polymorphisms on side effects (and length of hospitalization) - were robust against the “background noise” of a heterogeneous patient pool and might therefore be clinically relevant. The study adds another part to pharmacogenetic puzzle and forms the basis for further research which will hopefully make an optimized and individual psychopharmacotherapy in future possible.

6 Zusammenfassung

Die medizinische Behandlung mit Hilfe von molekulargenetischen Methoden maßgerecht auf den einzelnen Patienten zuzuschneiden ist eines der großen zukünftigen Ziele in der klinischen Medizin. Das Testen der relevanten Polymorphismen würde es erlauben, die Pharmakotherapie zu steuern und die Therapie mit bereits vorhandenen Arzneistoffen zu verbessern d.h. durch Wahl des optimalen Arzneistoffes und der optimalen Dosis den Therapieerfolg zu maximieren und die Nebenwirkungen und Risiken zu minimieren.

Variables Ansprechen auf die Therapie ist ein entscheidendes Problem in der Psychopharmakologie, besonders da die Therapie zeit- und kostenintensiv ist. Dreißig bis fünfzig Prozent der Patienten, die unter Depression oder Schizophrenie leiden, den beiden psychiatrischen Krankheiten mit der höchsten Prävalenz in der Bevölkerung, sprechen nicht genügend auf die anfängliche Therapie an (was wegen des verzögerten Wirkeintritts von Antidepressiva und Antipsychotika erst nach Wochen sichtbar wird) oder leiden unter beträchtlichen Nebenwirkungen, die einen Wechsel zu anderen Arzneistoffen nötig machen [2]. Schätzungsweise 60 - 90% des unterschiedlichen Ansprechens auf Antidepressiva ist auf Vererbung zurückzuführen [21]; ähnliches gilt für Antipsychotika [22-26].

Die Identifizierung der relevanten Polymorphismen ist der erste Schritt hin zu einer routinemäßigen Genotypisierung. Es war das Ziel dieser Arbeit herauszufinden, welche Polymorphismen einen Einfluss auf den Therapieerfolg im klinischen Rahmen haben, so dass eine Untersuchung als Hilfe bei Behandlungsentscheidungen in Zukunft nützlich sein könnte. Dazu wurden schnelle, sensitive und zuverlässige Genotypisierungsmethoden für Polymorphismen in 19 Kandidatengenen entwickelt, die für den Routineeinsatz in der klinischen Praxis geeignet sind. Die „steady-state“ Serumkonzentrationen der 3 häufigsten Arzneistoffe (Olanzapin, Mirtazapin, Citalopram) wurden gemessen und die Assoziationen zwischen Polymorphismen, Serumkonzentrationen und Therapieansprechen sowie Nebenwirkungen untersucht.

Pharmakokinetische Veränderungen, d.h. in den Serumkonzentrationen, sind das Ergebnis von Polymorphismen in Schlüsselenzymen wie dem Cytochrom P450 System. Die wichtigsten Cytochrom P450 Enzyme für den Metabolismus von Antidepressiva und Antipsychotika sind CYP2C19, CYP2D6 and CYP1A2. Ein beeinträchtigter CYP2C19 Metabolismus (aufgrund eines nicht funktionellen Alleles) hatte signifikanten Einfluss auf die Serumkonzentrationen des CYP2C19 Substrates (Es)Citalopram. Eine direkte Beziehung zwischen Genotyp und Nebenwirkungen war für trizyklische Antidepressiva vorhanden, nicht jedoch für (Es)Citalopram, das ein größeres therapeutisches Fenster hat. Darüber hinaus wurde zum ersten Mal in einer klinischen Studie gezeigt, dass der CYP1A2*1F Polymorphismus die Olanzapinkonzentrationen auch unabhängig von CYP1A2 Induktoren beeinflusst. CYP2D6 zeigte - aufgrund von stereoselektivem Metabolismus und Einfluss von CYP3A4 - keine große Auswirkung auf Mirtazapinkonzentrationen, aber auf Nebenwirkungen von Arzneistoffen, deren Metabolismus zum großen Teil über CYP2D6 läuft. Zusätzlich zu dem bekannten Effekt auf die relativ seltenen „Poor Metabolizers“ (langsame Metabolisierer, 9% der Studienpopulation), konnte ein Effekt auf die häufigeren „Intermediate Metabolizers“ (teilweise beeinträchtigte Metabolisierer, 38%) gezeigt werden, die hohe Dosen von CYP2D6 Substraten enthalten hatten. Überdies war die Ansprechrate von den „Intermediate Metabolizers“ unter CYP2D6-Medikation niedriger als unter anderen Medikamenten.

Pharmakodynamische Polymorphismen in den direkten Zielstrukturen von Antidepressiva oder Antipsychotika zeigten die besten Assoziationen mit dem klinischen Ergebnis. Wohlbekannte Polymorphismen im Serotonintransporter (5-HTTLPR [110] und rs25531 [115]) beeinflussten signifikant Nebenwirkungen und Länge des Krankenhausaufenthaltes von Patienten, die mit selektiven Serotonin-Wiederaufnahmehemmern behandelt wurden. In ähnlicher Weise war der Einfluss eines neuen Markers im Intron 2 des Serotoninrezeptor 5-HTR2A (rs7997012) auf Nebenwirkungen nur für Arzneistoffe nachzuweisen, deren Wirkung über diesen Rezeptor vermittelt wird, wie für selektive Serotonin-Wiederaufnahmehemmer gezeigt werden konnte. Zusätzlich konnte erstmalig eine Auswirkung dieses Polymorphismus auf Nebenwirkungen unter Therapie mit dem atypischen Antipsychotikum Olanzapin gezeigt werden. Die Wirkung aller Antipsychotika ist vornehmlich dopaminerg vermittelt und entsprechend wurde ein Einfluss von Polymorphismen in Dopaminrezeptoren auf Ansprechen oder Nebenwirkungen für die gesamte Gruppe der Antipsychotika gesehen.

Kandidatengene, deren Wirkmechanismus der primären monoaminergen (serotonergen oder dopaminergen) Aktivierung durch Antidepressiva und Antipsychotika nachgeschaltet ist und die daher nur indirekt von der Wirkung dieser Arzneistoffe betroffen sind, scheinen mehr den Verlauf der Krankheit zu modulieren (wie schnellere Zyklizität/schnelleres Ansprechen) als das Ausmaß der Wirkung und der Nebenwirkungen zu beeinflussen. Ein Polymorphismus in der G-protein beta-3 Untereinheit war mit schnellerem Ansprechen auf Olanzapintherapie und ein Polymorphismus im FK506 bindenden Protein 51 (FKBP5) mit längerer Krankheitsdauer in der Vorgeschichte und einem Trend zu mehr vorherigen Krankheitsepisoden verbunden. Die weiteren untersuchten Polymorphismen zeigten keinen Einfluss auf den Therapieerfolg oder -verlauf. Dies mag entweder darauf zurückzuführen sein, dass Assoziationen, die berichteten wurden, in Wirklichkeit nicht existieren oder aber darauf, dass vorhandene, aber geringfügige Effekte aufgrund der Heterogenität und begrenzten Anzahl der untersuchten Patienten nicht nachgewiesen werden konnten. In jeden Fall scheinen diese nur indirekt beteiligten Polymorphismen nicht sehr relevant für den Therapieerfolg im klinischen Alltag zu sein, können jedoch für spezielle Nebenwirkungen wie z.B. Gewichtzunahme verantwortlich sein. Diese stellt für bestimmte Antidepressiva und Antipsychotika ein ernstzunehmendes Problem dar und kann zu Problemen mit der Compliance (Therapiebefolgung) bis hin zum Therapieabbruch durch den Patienten führen. Gewichtszunahme unter Olanzapin- oder Clozapintherapie wurde durch zwei Polymorphismen im Serotoninrezeptor 5-HTR2C und im Adrenozeptor ADR2A beeinflusst. Der ADR2A Polymorphismus spielte auch eine bisher unbekannte Rolle bei der Gewichtzunahme unter Behandlung mit Mirtazapin, welches u.a. auch Adrenozeptoren blockiert.

Insgesamt konnte die hier vorliegende prospektive Diagnostikstudie einige Ergebnisse aus kontrollierten, monotherapeutischen Studien in einem klinischen Rahmen bestätigen. Andererseits konnten einige neue, noch nicht beschriebene signifikante Assoziationen zwischen Polymorphismen und v.a. Nebenwirkungen gezeigt werden. Diese explorativ gewonnenen Erkenntnisse sollten in größerem Maßstab wiederholt und unter Verwendung von stringenteren statistischen Methoden bestätigt werden (z.B. Poweranalyse, die hier nicht verwendet werden konnte, da die Größe der beobachteten Effekte nicht von vornherein bekannt war). Die Studie spiegelte die tatsächliche klinische Praxis jedoch recht gut wieder. Die Ergebnisse – v.a. der Einfluss von CYP2D6, 5-HTT und 5-HTR2A Polymorphismen auf Nebenwirkungen – wurden vor dem Hintergrund einer heterogenen Patientenpopulation und trotz Komedikation gewonnen und waren somit robust genug, um im klinischen Alltag relevant zu sein. Die Studie trägt somit ein weiteres Stück zum pharmakogenetischen Gesamtbild bei, das in Zukunft hoffentlich eine optimierte und individuell zugeschnittene Psychopharmakotherapie ermöglicht.

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Publications, Congress Contributions and Awards

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Laika B, Steimer W. Fluorescent Hybridisation Probe Detection of the C-1019G Promoter Polymorphism in the 5-HT1A Gene. *Ther Drug Monitor* 2007; **29** (4): 546.

IATDMCT– Oral Presentation:

The abstract was selected by the International Scientific Committee at the 10th International Congress of Therapeutic Drug-Monitoring and Clinical Toxicology 2007 in Nice for oral presentation.

Laika B, Leucht S, Heres S, Steimer W. HTR1A and Serotonin Transporter Gene Polymorphisms Influence Therapeutic Response. *Ther Drug Monitor* 2007; **29** (4): 486.

Laika B, Leucht S, Heres S, Steimer W. Detection of a new HTR2A Polymorphism with Fluorescent Hybridization Probes using the LightCycler and its Association with Therapeutic Outcome in Antidepressant Therapy. *Clin Chem* 2007; **53** (S6): A157 C-172.

Laika B, Leucht S, Heres S, Steimer W. Influence of Cyp2C19 polymorphisms on citalopram serum concentrations and clinical outcome. *Clin Chem* 2008, **54** (S6): D-54.

NACB Distinguished Abstract Award – Oral Presentation

The abstract was awarded by the National Academy of Clinical Biochemistry at the AACC (American Association of Clinical Chemistry) Annual Meeting 2008 in Washington DC as one of 26 out of 725 accepted abstracts and chosen as one out of 10 for oral presentation.

Laika B, Leucht S, Heres S, Steimer W. Influence of CyplA2*1F polymorphism on olanzapine serum concentrations and clinical outcome. *Clin Chem* 2008, **54** (S6): D-52.

NACB Distinguished Abstract Award

The abstract was awarded by the National Academy of Clinical Biochemistry at the AACC Annual Meeting 2008 in Washington DC as one of the 26 out of 725 accepted abstracts.

Laika B, Leucht S, Heres S, Steimer W. CYP2D6 Intermediate Metabolizer Status Influences Therapeutic Outcome in Antidepressant and Antipsychotic Drug Therapy. *Clin Chem* 2009 (in press).

TDM/Toxicology Division Abstract Award - The abstract was awarded at the AACC Annual Meeting 2009 in Washington DC.

Tables

Figures

A Appendix

A1 Baseline Data

Table 62: Study medication - antidepressants

Medication	N of prescriptions	N of treated patients	% of study population
antidepressants/antipsychotics	**767**	**365**	**100.0**
	1 drug(s) in 114 patient(s)		
	2 drug(s) in 140 patient(s)		
	3 drug(s) in 80 patient(s)		
	4 drug(s) in 24 patient(s)		
	5 drug(s) in 5 patient(s)		
	6 drug(s) in 2 patient(s)		
antidepressants	307	**234**	**64.1**
	1 drug(s) in 171 patient(s)		
	2 drug(s) in 53 patient(s)		
	3 drug(s) in 10 patient(s)		
mirtazapine		122	33.4
venlafaxine		17	4.7
reboxetine		8	2.2
tranylcypromine		1	0.3
moclobemide		1	0.3
SSRI	116	**114**	**31.2**
citalopram	1 drug(s) in 112 patient(s)	44	12.1
escitalopram	2 drug(s) in 2 patient(s)	28	7.7
sertraline		18	4.9
paroxetine		16	4.4
fluoxetine		10	2.7
TCA	42	**41**	**11.2**
doxepin	1 drug(s) in 40 patient(s)	17	4.7
trimipramine	2 drug(s) in 1 patient(s)	16	4.4
amitriptyline		7	1.9
clomipramine		1	0.3
imipramine		1	0.3

Note: medication of one patient was changed from escitalopram to citalopram

Table 63: Study medication - antipsychotics

Medication	N of prescriptions	N of treated patients	% of study population
antipsychotics	461	**292**	**80.0**
atypical antipsychotics	330 1 drug(s) in 189 patient(s) 2 drug(s) in 57 patient(s) 3 drug(s) in 9 patient(s)	**255**	**69.9**
olanzapine		130	35.6
risperidone		61	16.7
amisulpride		49	13.4
quetiapine		46	12.6
clozapine		26	7.1
ziprasidone		13	3.6
aripiprazole		3	0.8
zotepine		1	0.3
sertindole		1	0.3
typical antipsychotics	131 1 drug(s) in 81 patient(s) 2 drug(s) in 22 patient(s) 3 drug(s) in 2 patient(s)	**105**	**28.8**
haloperidol		25	6.8
flupentixol		12	3.3
fluspirilene		2	0.5
pimozide		1	0.3
zuclopentixol		5	1.4
chlorpromazine		11	3.0
melperone		2	0.5
perazine		19	5.2
pipamperone		35	9.6
prothipendyl		3	0.8
levomepromazine		6	1.6
promethazine		8	2.2
sulpiride		2	0.5

Table 64: Concomitant study medication

Concomitant medication	N of prescriptions	N of treated patients	% of study population
mood stabilizers	100	**92**	**25.2**
lithium	1 drug in 84 patients	40	11.0
carbamazepine	2 drugs in 8 patients	29	7.9
valproic acid		20	5.5
lamotrgine		7	1.9
oxcarbazepine		3	0.8
gabapentin		1	0.3
biperiden (17 only if required)	52	**52**	**14.2**
metergoline	3	**3**	**0.8**
benzodiazepines	234	**196**	**53.7**
lorazepam	1 drug in 159 patients	157	43.0
lormetazepam	2 drugs in 36 patients	37	10.1
diazepam	3 drugs in 1 patient	22	6.0
flunitrazepam		7	1.9
tetrazepam		3	0.8
alprazolam		3	0.8
oxazepam		2	0.5
bromazepam		2	0.5
clorazepate		1	0.3
other sedatives or anxiolytics			
zopiclon		23	6.3
zolpidem		20	5.5
chloralhydrate		1	0.3
barbexaclone		1	0.3
buspirone		2	0.5
opipramole		3	0.8
thyroid drugs	63	**63**	**17.3**
thyroxine	1 drug in 45 patients	42	11.5
iodine	2 drugs in 9 patients	17	4.7
carbimazole		3	0.8
perchlorate		1	0.3

Table 64 (ctd): Concomitant study medication

Concomitant medication	N of prescriptions	N of treated patients	% of study population
proton pump inhibitors	31	**31**	**8.5**
omeprazole		23	6.3
pantoprazole		8	2.2
antihypertensives	155	**84**	**23.0**
bisoprolol	1 drug in 44 patients	37	10.1
hydrochlorothiazide	2 drugs in 21 patients	20	5.5
metoprolol	3 drugs in 10 patients	15	4.1
enalapril	4 drugs in 6 patients	13	3.6
ramipril	5 drugs in 3 patients	9	2.5
candesartan		8	2.2
propanolol		8	2.2
losartan		7	1.9
piretanide		5	1.4
amlodipine		5	1.4
tamsulosin		4	1.1
triamteren		4	1.1
verapamil		3	0.8
felodipin		2	0.5
lisinopril		2	0.5
furosemide		2	0.5
valsartan		1	0.3
captopril		1	0.3
peridopril		1	0.3
nifedipine		1	0.3
nitrendipine		1	0.3
torasemide		1	0.3
isosorbide mononitrate		1	0.3
monoxidine		1	0.3
nebivolol		1	0.3
carvediol		1	0.3
spironolacton		1	0.3

Table 64 (ctd): Concomitant study medication

Concomitant medication	N of prescriptions	N of treated patients	% of study population
lipid-lowering drugs	17	**17**	**4.7**
atorvastin		8	2.2
simvastatin		5	1.4
pravastatin		1	0.3
lovastatin		1	0.3
befibrate		1	0.3
fenofibrate		1	0.3
antidiabetic drugs	26	**17**	**4.7**
metformin	1 drug in 10 patients	9	2.5
insulin	2 drugs in 5 patients	9	2.5
glimepride	3 drugs in 2 patients	4	1.1
miglitol		1	0.3
pioglitazone		1	0.3
repaglinide		1	0.3
acarbose		1	0.3
anticoagulants	20	**20**	**5.5**
acetylsalicylic acid		8	2.2
phenprocoumon		7	1.9
clopidogrel		5	1.4
analgetics	11	**11**	**3.0**
mesalazine, ibuprofene, diclofenac (n = 3), tramadol, metamizole, rofecoxib (n = 4)			
other	39	**34**	**9.3**
allopurinol (n = 4), tibolon, tolterodin (n = 2), trospiumchloride, metoclopramide (n = 3), simeticon, pirenzepine (n = 2), clemastine, alendronate sodium, ginko, beta-acetyldigoxine, prednisone (n = 2), azathioprine, clomethiazole, terbinafine, acetylcysteine, betahistidine, pentoxifylline, etilefrine, finasteride, mebeverine, tamoxifen/leuprorelinacetate, estrogen/progesterone (n = 6)			

A2 Allele Frequencies

A2.1. Polymorphisms in CYP1A2, CYP2C19 and CYP2D6

CYP1A2*1F	C/C	C/A	A/A	C	A
N	37	136	192	210	520
%	10.1	37.3	52.6	28.8	71.2
Sachse et al. (1999) [79] :					
Healthy Caucasians (n=236), %	10.2	44.1	45.8	32	68
HWE	p=0.082645				

CYP2C19	N	%	%
*1*1	255	69.9	75.0*
*1*2	99	27.1	20.0*
*1*4	2	0.5	-
*2*2	8	2.2	5.0*
*2*3	1	0.3	-
*1	611	83.7	85.0*
*2 (rs4244285)	116	15.9	15.0*
*3 (rs4986893)	1	0.1	0.0*
*4 (rs28399504)	2	0.3	0.0*
*17	nd	nd	18.0**
PM	9	2.5	
IM	101	27.7	
EM	255	69.9	
UM	nd	nd	

* http://snp500cancer.nci.nih.gov; HapMap Controls Caucasian (n=60)

** Sim et al. (2006): 314 Swedes [85]

nd = not determined

CYP2D6	N	%	
*1*1	58	15.9	
*1*10	9	2.5	
*1*1xN	1	0.3	
*1*2	51	14.0	
*1*2xN	2	0.5	
*1*3	1	0.3	
*1*4	60	16.4	
*1*41	18	4.9	
*1*4xN	1	0.3	
*1*5	12	3.3	
*1*6	5	1.4	
*1*9	9	2.5	
*10*41	3	0.8	
*1xN*2	3	0.8	
*1xN*3	2	0.5	
*1xN*4	4	1.1	
*1xN*41	1	0.3	
*1xN*9	1	0.3	
*2*10	1	0.3	
*2*2	12	3.3	
*2*2xN	1	0.3	
*2*3	4	1.1	
*2*4	24	6.6	
*2*41	16	4.4	
*2*5	2	0.5	
*2*6	2	0.5	
*2*9	2	0.5	
*2xN*2	2	0.5	
*2xN*3	1	0.3	
*2xN*4	1	0.3	
*3*10	2	0.5	
*3*3	1	0.3	
*3*4	4	1.1	
*3*41	1	0.3	
*3*5	2	0.5	
*4*10	1	0.3	
*4*4	19	5.2	

CYP2D6	N	%	%
*4*5	4	1.1	
*4*6	1	0.3	
*4*9	2	0.5	
*41*41	3	0.8	
*5*9	1	0.3	
*9*41	1	0.3	
*1	285	39.0	33.4 - 36.4*, **
*2	132	18.1	17.5**
*3	19	2.6	1.0 -2.0*
*4	153	21.0	18.9 - 20.7*
*5	21	2.9	2.0 - 7.3*
*6	8	1.1	0.9 - 1.4*
*9	16	2.2	1.8 - 2.6*, **
*10	16	2.2	1.4 - 2.0*, **
*41 (G2988A)	60	8.2	8.4**
*1xN	12	1.6	0 - 0.5*
*2xN	7	1.0	1.1 - 1.6*, **
*4xN	1	0.1	0.1*
duplication	20	2.7	1.6 - 2.1*
gene dose			
0.0	31	8.5	
0.5	20	5.5	
1.0	118	32.3	
1.5	56	15.3	
2.0	129	35.3	
2.5	2	0.5	
3.0	9	2.5	
2988A present in *2(-1584C)	60/64	93.8	92.9**

* Bradford 2002 [72]: Review of Marez 1997 [451], Sachse 1997 [88] and Griese 1998 [452] (n = 672/589/195)

**Raimundo 2004 [89] (n = 308)

A2.2. Polymorphisms in the MDR1 Gene

MDR1 G2677TA rs2032582	G/G	G/T	T/T	G/A	T/A	G	T	A
N	118	177	53	8	9	421	292	17
%	32.3	48.5	14.5	2.2	2.5	57.7	40.0	2.3
http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs=2032582								
P1 (global, 2n=202), %	37.3	42.2	12.7	4.9	2.9	60.9	35.2	3.9
Cascorbi, Gerloff et al. 2001 [453]								
German (n=461), %	30.9	49.2	16.1	2.0	1.8	56.5	41.6	1.9
HWE	p=0.1314							

MDR1 C3435T rs1045642	C/C	C/T	T/T	C	T
N	85	192	88	362	368
%	23.3	52.6	24.1	49.6	50.4
http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs=1045642					
HapMap-CEU (2n=120), %	15.0	61.7	23.3	45.8	54.2
Cascorbi, Gerloff et al. 2001 [453]					
German (n=461), %	20.8	50.5	28.6	44.1	53.9
HWE	p=0.319317				

MDR1 C1263T rs1128503	C/C	C/T	T/T	C	T
N	127	178	60	432	298
%	34.8	48.8	16.4	59.2	40.8
http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs=1128503					
HapMap-CEU (2n=120), %	35	51.7	13.3	60.8	39.2
Cascorbi, Gerloff et al. 2001 [453]					
German (n=461), %	34.4	49.2	16.4	59.0	41.0
HWE	p=0.858190				

A2.3. Polymorphisms in the Serotonergic System

5-HTTLPR rs4795541/rs2553	L_AL_A	L_AL_G	L_GL_G	SL_G	SL_A	SS	L_A	L_G	S
N	108	24	0	13	144	76	384	37	309
%	29.6	6.6	0.0	3.6	39.5	20.8	52.6	5.1	42.3
Hu 2007 [115]: Finnish (n=771), %	24.0	9.0	1.0	8.0	44.0	15.0	50.5	8.5	41.0
Zalsman 2006 [304]: Caucasians									
Patients (n=191), %	28.8	5.2	0.0	5.8	42.4	17.8	52.6	5.5	41.9
Controls (n=125), %	26.4	5.6	0.8	6.4	39.5	21.6	49.0	6.0	44.6

Note: no data available at http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs=4795541 and http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs=2553

5-HTR1A C-1019G rs6295	C/C	C/G	G/G	C	G
N	87	186	92	360	370
%	23.8	51.0	25.2	49.3	50.7
Lemonde et al. 2004 [119]: 95% Caucasian					
Depressive patients (n=118), %	23.7	50.0	26.3	48.7	51.3
HWE	p=0.711344				

Note: no data for Caucasians available at *http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs=6295*

5-HTR2A intron 2 rs7997012	G/G	G/A	A/A	G	A
N	119	179	67	417	313
%	32.6	49.0	18.4	57.1	42.9
http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs=7997012					
HapMap-CEU (n=120)	33.3	55.0	11.7	60.8	39.2
HWE	p=0.982600				

Note:no frequency data in literature

5-HTR2A T102C rs6313	T/T	C/T	C/C	T	C
N	70	167	128	307	423
%	19.2	45.8	35.1	42.1	57.9
http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs=6313					
HapMap-CEU (n=120)	21.7	46.7	31.7	45.0	55.0
Arranz et al. 1998 Metaanalysis [131]					
Schizophrenic patients (n=733), %	18.0	47.6	34.4	41.8	58.2
HWE	p=0.242106				

5-HTR2C C-759T rs3813929	C/C	C/T	T/T	C	T
Females (N)	132	64	7	328	78
Females (%)	65.0	31.5	3.4	80.8	19.2
Males (N)	-	-	-	134	28
Male (%)	-	-	-	82.7	17.3
http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs=3813929					
HapMap-CEU (n=46)	73.9	17.4	8.7	82.6	17.4
Templeman et al. 2005: Caucasians					
Schizophrenics (n=73), %	-	-	-	76.7	23.3
HWE	p=0.823721 (females)				

5-HTR6 T267T rs1805054	C/C	C/T	T/T	C	T
N	259	94	12	612	118
%	71.0	25.8	3.3	83.8	16.2
http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs=1805054					
HapMap-CEU (2n=112), %	83.9	16.1	0	92.0	8.0
Masellis et al. 2001: North Americans [151]					
Schizophrenics (n=173), %	74	23	3	85.0	15.0
HWE	p=0.341435				

A2.4. Polymorphisms in the Dopaminergic System

DAT1 VNTR rs28363170	99	910	1010	911	1011	9	10	11
N	35	137	187	1	5	208	516	6
%	9.6	37.5	51.2	0.3	1.4	28.5	70.7	0.8
http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs=28363170								
PDR90-Global (2n=122), %	2	23	75	0	0	13	87	0
Sander 1997: German								
Alcoholics (n=293), %	6.5	36.2	56.0	0.3	1.0	24.7	74.6	0.7
Controls (n=93), %	5.4	25.8	64.5	1.1	3.2	18.8	79.0	2.2
HWE	p=0.1355							

DRD2 -141C ins/del rs1799732	ins/ins	ins/del	del/del	ins	del
N	302	57	6	661	69
%	82.7	15.6	1.6	90.5	9.5
http://alfred.med.yale.edu/alfred/SiteTable1A_working.asp?siteuid=SI000135J					
Danes (2n=180)	-	-	-	92.8	7.2
HWE	p=0.093910				

DRD2/ANKK1 TaqI rs1800497	A1/A1	A1/A2	A2/A2	A1	A2
N	13	115	237	141	589
%	3.6	31.5	64.9	19.3	80.7
http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs=rs1800497					
HapMap-CEU (2n=120)	8.3	28.3	63.3	22.5	77.5
HWE	p=0.835799				

DRD3 Ser9Gly rs6280	A/A SerSer	A/G SerCys	G/G CysCys	A Ser	G Cys
N	182	148	35	512	218
%	49.9	40.6	9.6	70.1	29.9
http://alfred.med.yale.edu/alfred/SiteTable1A_working.asp?siteuid=SI000672P					
Europeans (2n=180)	-	-	-	68.1	31.9
HWE	p=0.540476				

A2.5. Polymorphisms in the Noradrenergic System

NET C-182T rs2242446	C/C	C/T	T/T	C	T
N	32	139	194	203	527
%	8.8	38.1	53.2	27.8	72.2
http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs=2242446					
HapMap-CEU (2n=120), %	3.3	46.7	50.0	26.7	73.3
Zill 2002: German					
Patients (n=193), %	11	40	49	31	69
Controls (n=136), %	11	39	50	32	68
HWE	p=0.325032				

NET G1287A rs5569	G/G	G/A	A/A	G	A
N	148	181	36	477	253
%	40.5	49.6	9.9	65.3	34.7
http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs=5569					
HapMap-CEU (2n=120), %	33.3	53.3	13.3	60.0	40.0
Zill 2002: German					
Patients (n=193), %	53	36	11	70	30
Controls (n=136), %	44	44	12	66	34
HWE	p=0.069911				

ADR2A C-1291G rs1800544	C/C	C/G	G/G	C	G
N	204	130	31	538	192
%	55.9	35.6	8.5	73.7	26.3
http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs=1800544					
PGA_CEPH-PANELEu (2n=44), %	59.1	40.9	0.0	79.5	20.5
Bolonna 2000: British/Caucasians					
Patients (n=254), %	54.0	39.0	7.0	74.0	26.0
Controls (n=122), %	59.0	36.0	5.0	77.0	23.0
HWE	p=0.120454				

A2.6. Polymorphisms in the Signal Transduction and Neurotransmitter Metabolism

BDNF Val66Met (G196A) rs6265	G/G	G/A	A/A	G	A
N	229	116	20	574	156
%	62.7	31.8	5.5	78.6	21.4
http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs=6265					
HapMap-CEU (2n=120), %	68.3	28.3	3.3	82.5	17.5
Anttila 2005: Finnish					
Schizophrenic patients (n=94), %	69.1	27.7	3.2	83.0	17.1
Controls (n=98), %	74.5	20.4	5.1	84.7	15.3
HWE	p=0.299374				

COMT Val158Met (G472A) rs4680	G/G	G/A	A/A	G	A
	Val/Val	Val/Met	Met/Met		
N	78	191	96	347	383
%	21.4	52.3	26.3	47.5	52.5
http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs=4680					
HapMap-CEU (2n=120), %	21.7	53.3	25.0	48.3	51.7
Arias 2005: Italian, Spanish					
Depressive patients (n=346), %	29.8	47.4	22.8	53.5	46.5
Controls (n=164), %	29.9	49.4	20.7	54.6	45.4
HWE	p=0.347954				

FKBP5 rs1360780	C/C	C/T	T/T	C	T
N	180	155	30	515	215
%	49.0	42.5	8.2	70.5	29.5
http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs=1360780					
HapMap-CEU (2n=120), %	53.3	45	1.7	75.8	24.2
Binder 2004: German					
Depressive patients (n=318), %	51.3	39.6	9.1	71.1	28.9
HWE	p=0.675640				

GNB C825T rs5443	C/C	C/T	T/T	G	A
N	174	159	32	507	223
%	47.7	43.6	8.8	69.5	30.5
http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs=5443					
HapMap-CEU (2n=116), %	32.8	56.9	10.3	61.2	38.8
Serretti 2003: Italian					
Depressive patients (n=490), %	45.7	44.1	10.2	67.8	32.3
HWE	p=0.611132				

ACE rs4646994	ins/ins	ins/del	del/del	ins	del
N	79	184	102	342	388
%	21.6	50.4	27.9	46.8	53.2
http://alfred.med.yale.edu/alfred/recordinfo.asp?condition=(populations.pop_name='French')					
French (2n=1716), %	-	-	-	43.3	56.7
Baghai 2001					
German (n=99)	21.2	48.5	30.3	45.5	54.6
Bondy 2005					
Patients (n=273), %	17.6	52.7	29.7	44.0	56.0
HWE	p=0.815128				

Note: no data available at *http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs=4646994*

MAO-A VNTR (repeats)	2/2	2/4	3/3	3/3.5	3/4	3/5	4/4	4/5	2	3	3.5	4	5
Females (N)	-	1	32	3	78	1	85	3	1	146	3	252	4
Females (%)	-	0.5	15.8	1.5	38.4	0.5	41.9	1.5	0.2	36.0	0.7	62.1	1.0
Males (N)	-	-	-	-	-	-	-	-	1	56	0	105	0
Male (%)	-	-	-	-	-	-	-	-	0.6	34.6	0.0	64.8	0
Cusin 2002: 441 Italian depressives													
Female	-	-	11.5	-	41.4	-	47.1	-	-	32.2	-	67.8	-
Male	-	-	-	-	-	-	-	-	-	34.1	-	65.9	-

DBH ins/del	ins/ins	ins/del	del/del	ins	del
N	110	169	86	389	341
%	30.1	46.3	23.6	53.3	46.7
Yamamoto 2003: Canadians					
Schizophrenics (n = 106)	27.4	45.3	27.4	50.0	50.0
HWE	p=0.181411				

TPH1 A218C rs1800532	A/A	A/C	C/C	A	C
N	69	183	113	321	409
%	18.9	50.1	31.0	44.0	56.0
http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs=1800532					
HapMap-CEU (2n=118), %	18.6	42.4	39	39.8	60.2
Serretti 2004: Italian					
Depressed patients (n=221), %	19.5	43.4	37.1	41.2	58.8
HWE	p=0.737747				

A3 Primers and Probes - Sequence and Location

Table 65: Primers and probes - sequence and location (1)

Gene	Polymorphism	Sequence (5`→ 3`)		Primer length (bp)	T_m	PCR product (bp)	GC (%)	Reference Sequence (GenBank No.)
CYP 2D6	C100T *10 rs1065852 (allele-specific method)*	2D6*10 F 2D6*10 Ji wt 2D6*10 Ji mut	GAGCCCATTTGGTAGTGAG GGGGGCCTGGTG**G** GGGGGCCTGGTG**A**	19 13 13	61.7 61.8 60.2	137 bp	52.6 84.6 76.9	1595 - 1613 1731 - 1719 1731 - 1719 M33388 C1719T
CYP 2D6	C100T *10 rs1065852 (LightCycler method)	LC 2D6*10 F LC 2D6*10 R 2D6*10 anc 2D6*10 sen	CTGGCCTGACTCTGCCAC CACATGCAGCAGGTTGCC GGCAGTGGCAGGGGGCC-fluorescein LCRed640-GTG**A**GTAGCGTGCAGCC-Ph.	18 18 17 17	66.7 66.3 71.3 63.2	346 bp	66.7 61.1 82.4 64.7	1421 - 1438 1766 - 1749 1725 - 1741 1706 - 1722 M33388 C1719T
CYP 2D6	G2988A *41 (LightCycler method)	2D6*41 F 2D6*41 R 2D6*41 anc 2D6*41 sen	TTCTCTGCCGGGATGG CCTGGTCAAGCCTGTG LCRed640-CTGCACTGTTTCCCAGATGGGC-Ph. CCCTT**C**CTCCCTCGGC-fluorescein	16 16 22 16	60.9 60.9 68.9 63.7	186 bp	62.5 62.5 59.1 75.0	4490 - 4505 4675 - 4660 4594 - 4573 4612 - 4597 M33388 G4607A
ABCB1 (MDR1)	rs2032582 G2677TA (RFLP method)*	MDR 2677 F MDR 2677 G-R MDR 2677 T-R MDR 2677 A-R	TACCCATCATTGCAATAGCAG TTTAGTTTGACTCACCTTGCTAG TTTAGTTTGACTCACCTTTCTAG TTTAGTTTGACTCACCTTCCC	21 23 23 21	62.3 62.5 56.6 61.7	107 bp	42.9 39.1 34.8 42.9	65324 - 65304 65218 - 65240 65218 - 65240 65218 - 65238 AC005068 C65241AT
ABCB1 (MDR1)	rs2032582 G2677TA (LightCycler method)	MDR 2677 F MDR 2677 R MDR 2677 anc MDR 2677 sen	GTCCAAGAACTGGCTTTG TGGCAACTAACACTGTTAC LCRed640-CTTTCTTATCTTTCAGTGCTTG TCCAGAC- Ph. ACCTTCCCAG**A**ACCTTCTAG-fluorescein	18 19 29 20	60.1 59.2 66.4 61.7	309 bp	50.0 42.1 41.4 50.0	65047 - 65064 65355 - 65337 65253 - 65281 65231 - 65250 AC005068 C65241AT

Note: Ph = phosphate * reference / control assay underlined and bold = SNP underlined = sequence changes e.g., in trick primers framed = change of published primer

Table 66: Primers and probes - sequence and location (2)

Gene	Polymorphism	Sequence (5`→ 3`)		Primer length (bp)	T_m	PCR product (bp)	GC (%)	Reference Sequence (GenBank No.)
ABCB1 (MDR1)	rs1045642 C3435T (Multiplex LightCycler method)	MDR 3435 F	AAGGCATGTATGTTGGCCTC	20	64.6	197 bp	50.0	43233 - 43252
		MDR 3435 R	TGTTTTCAGCTGCTTGATGG	20	61.7		45.0	43429 - 43410
		MDR 3435 S3'	GACAACAGCCGGGTGGTGTCA-fluorescein	21	69.5		61.9	43300 - 43280
		MDR 3435 S5'	LCRed640-GGAAGAGAT**C**GTGAGGGCAG-Ph	20	64.5		60.0	43277 - 43258 AC005068 A43268G
ABCB1 (MDR1)	rs1128503 C1236T (Multiplex LightCycler method + RFLP control)	MDR 1236 F3	GAATGAAGAGTTTCTGATGTTTTCTTG	27	62.5	286 bp	33.3	84369 - 84343
		MDR 1236 R3	GGTCTAGCTCGCATGGG	17	62.5		64.7	84084 - 84100
		MDR 1236 anc	LCRed705-GTGCAGAGTGGGCAGACG-Ph	18	65.2		66.7	84211 - 84194
		MDR 1236 sen	AGGG**C**CTGAACCTGA-fluorescein	15	59.2		60.0	84228 - 84214 AC005068 A84224G
SLC6A4 (5-HTT, SERT)	rs4795541= HTTLPR (l/s), rs25531 (A/G) (TaqMan LightCycler method + RFLP control)	HTT L_AL_G F	GCAACCTCCCAGCAACTCCCTGTA	24	72.6	181 bp/ 138 bp	58.3	26150 - 26173
		HTT L_AL_G R	GAGGTGCAGGGGGATGCTGGAA	22	73.0		63.6	26330 - 26309
		HTT A:	FAM-CCCCCCTGCACCCCC**A**GCATCCC-BHQ1	23	78.4		78.3	26232 - 26254
		HTT G:	LCRed610-CCCCTGCACCCCC**G**GCATCCCC-BHQ2	22	78.9		81.8	26234 - 26255 AC104984 26234-26276 43 bp del and A26247G
5-HTR_{1A}	rs6295 C-1019G (RFLP method)*	HT_{1A} -1019 F	TGGAAGAAGACCGAGTGTGTCT**A**C	24	67.7	182 bp	50.0	2 - 25
		HT_{1A} -1019 R	TTCTCCCTGAGGGAGTAAGGCTGG	24	71.2		58.3	183 - 160 Z11168 C159G
5-HTR_{1A}	rs6295 C-1019G (LightCycler method)	HT_{1A}-1019 F LC	CGTTTTGTTGTTGTTGTCG	19	60.2	188 bp	42.1	50 - 68
		HT_{1A}-1019 R LC	CCTGAATGGGAAGGTG	16	57.4		56.3	237 - 222
		HT_{1A}-1019 anc	CGCGAGAACGGAGGTAGCTTTT-fluorescein	22	68.0		54.5	131 - 152
		HT_{1A}-1019 sen	LCRed640-AAAA**C**GAAGACACACTCGGTC- Ph	21	63.7		47.6	155 - 175 Z11168 C159G

Note: Ph = phosphate * reference / control assay underlined and bold = SNP underlined = sequence changes e.g., in trick primers framed = change of published primer

Table 67: Primers and probes - sequence and location (3)

Gene	Polymorphism	Sequence (5`→ 3`)		Primer length (bp)	T_m	PCR product (bp)	GC (%)	Reference Sequence (GenBank No.)
5-HTR_{2A}	rs7997012 intron 2 (LightCycler method + RFLP control)	HT_{2A} in2 F	GTCACCTCACATTGGC	16	58.6	194 bp	56.3	93318 - 93333
		HT_{2A} in2 R	AGTGTCCTTATGAACAGC	18	58.1		44.4	93511 - 93494
		HT_{2A} in2 anc	LCRed640-TTTGTCACTTGCCTATGCAAGCCC-Ph	24	69.0		50.0	93431 - 93454
		HT_{2A} in2 sen	GCCATTATCTTCAAAGACTTAATT**A**ACAA-fluorescein	29	62.1		27.6	93400 - 93428 AL136958 G93424A
5-HTR_{2A}	rs6313 T102C (adapted RFLP method)	HT_{2A} T102C F	TCTGCTACAAGTTCTGGCTT	20	63.2	342 bp	45.0	111 - 130
		HT_{2A} T102C R	CTGCAGCTTTTTCTCTAGGG	20	62.4		50.0	452 - 433 NM000621 T236C
5-HTR_{2C}	rs3813929 C-759T (LightCycler method + RFLP control)	HT_{2C} -759 F	ATCTCCACCATGGGTCTCGC	20	66.8	252 bp	60.0	249 - 268
		HT_{2C} -759 R	CAATCTAGCCGCTCCAAAGG	20	63.7		55.0	500 - 481
		HT_{2C} -759 anc	GCACCACGCTCTTGGGCCA-fluorescein	19	70.2		68.4	399 - 381
		HT_{2C} -759 sen	LCRed640-AGC**A**GGATGAGGGGAGG- Ph	17	62.9		67.7	378 - 362 U49648 C375T
5-HTR_6	rs1805054 T267C (adapted RFLP method)	HT_6 T267C F	TGCTGATCGCGCTCATCTGCACTC	24	73.4	578 bp	58.3	604 - 627
		HT_6 T267C R	CTGCAGCGTCTCCGAGGCCTGACTG	25	76.6		68.0	1181 - 1157 NM000871 T734C
SLC6A3	rs28363170 DAT1 VNTR (adapted method)	DAT1 F	TG**C**GGTGTAGGGAACGGCCTGAGA	24	75.7	483 bp	62.5	1384123 - 1384100
		DAT1 R	CTTCCTGGAGGTCACGGCTCAAGG	24	72.8		62.5	1383641 - 1383664 NT006576 (10repeats)

Note: Ph = phosphate * control assay underlined and bold = SNP underlined = sequence changes e.g., in trick primers framed = change of published primer

Table 68: Primers and probes - sequence and location (4)

Gene	Polymorphism	Sequence (5`→ 3`)		Primer length (bp)	T_m	PCR product (bp)	GC (%)	Reference Sequence (GenBank No.)
NET	rs5569 G1287A (RFLP method)*	NET G1287A F NET G1287A R	TCCAGGGAGACCCTAATTCC TTGACTTTATTGAAATGCGGC	20 21	64.4 61.9	241bp	55.0 38.1	9345959 - 9345978 9346199 - 9346179 NT010498 G9346034A
NET	rs5569 G1287A (LightCycler TaqMan method)	NET G1287A F2 NET G1287A R2 NET Taq G1287 NET Taq 1287A	AGTTTCCGGTGTCGCTTCAG CCAGATGGGAGGCATGGA FAM-CAGGCC**C**GTGATGA-BHQ1 HEX-AGGCC**T**GTGATGACA-BHQ1	20 18 14 15	66.9 65.2 56.5 55.8	75 bp	55.0 61.1 64.3 53.3	9346078 - 9346059 9346004 - 9346021 9346040 - 9346027 9346039 - 9346025 NT010498 G9346034A
NET	rs2242446 T-182C (LightCycler method + RFLP control)	NET T182C F NET T182C R NET T182C anc CCCTGCGTCCGC NET T182C sen	GAACGAGGAAAAGTGCTGC CGAGGCTCTGCTTGGATAAA LCRed640- TCAGCGCGCGCTCATCCC-Ph GACGCGCGC**T**CTTTTCTGGGA-fluorescein	19 20 30 21	63.6 64.0 84.6 70.7	257 bp	52.6 50.0 76.7 61.9	9304505 - 9304523 9304761 - 9304742 9304637 - 9304666 9304615 - 9304635 NT010498 C9304624T
ADR_{2A}	rs1800544 C-1291G (reference RFLP method)	ADR A_{2A} F ADR A_{2A} R	ACACCGGAGGTTACTT GGACGAGCCCTTTGGA	16 16	57.9 62.4	236 bp	50.0 62.5	194148 - 194163 194383 - 194368 AL158163 C194273G
ADR_{2A}	rs1800544 C-1291G (TaqMan LightCycler method)	A_{2A}-1291 F A_{2A}-1291 R A_{2A}-1291C A_{2A}-1291G	GTGCCCGTTGCGTTCTG TGGGAGTTGGCCATGCA FAM-CCGTCGGCCC**C**GAG-BHQ1 HEX-CCGTCGGCCC**G**GAG-BHQ1	17 17 14 14	66.0 65.8 64.1 64.1	51 bp	64.7 58.8 85.7 85.7	194244 - 194260 194294 - 194278 194263 - 194276 194263 - 194276 AL158163 C194273G

Note: Ph = phosphate * reference / control assay underlined and bold = SNP underlined = sequence changes e.g., in trick primers framed = change of published primer

Table 69: Primers and probes - sequence and location (5)

Gene	Polymorphism	Sequence (5`→ 3`)		Primer length (bp)	T_m	PCR product (bp)	GC (%)	Reference Sequence (GenBank No.)
DBH	5´ins/del 3 kbp upstream start codon (adapted method)	DBH F DBH R	GCAAAAATCAGGCACATGCAC CCTCCAATAATTTGGCCTCAATC	21 23	65.6 63.8	167 bp 148 bp	47.6 43.5	43198 - 43218 43364 - 43342 AC002101 (ins) 6221 - 6241 6368 - 6346 AL365494 (del)
MAO-A	uVNTR in promoter (adapted method)	MAO-A F MAO-A R	AACAGCCTGACCGTGGAGAAG CACTCAGAACGGACGCTCCA	21 20	68.6 68.5		57.1 60.0	46552 - 46572 46882 - 46863 AL109855 (3-allele)
COMT	rs4680 Val158Met G472A (LightCycler method + RFLP control)	COMT F COMT R COMTanc3 COMTsen3	GGGCCTACTGTGGCTACTCAGC GCCCTTTTTCCAGGTCTGACAAC LCRed640-TCCGCTGGGTGATGGCGGC- Ph TCA**C**GCCAGCGAAATCCAC-fluorescein	22 23 19 19	70.8 68.1 73.0 66.1	177 bp	63.6 52.2 73.7 57.9	3103296 - 3103317 3103472 - 3103450 3103403 - 3103385 3103424 - 3103406 NT011519 C3103421T
TPH1	rs1800532 A218C (adapted RFLP method)	TPH 218 F TPH 218 R	TTCAGATCCCTTCTATACCCCAGA GGACATGACCTAAGAGTTCATGGCA	24 25	66.0 68.5	847 bp	45.8 48.0	16835304- 16835281 16834458- 16834482 NT009237 A16835057C
TPH2	G1463A (adapted allele-specific method)	TPH2 hOut F TPH2 hOut R TPH2 1463 G TPH2 1463 A	ATGTGTGAAAGCCTTTGACCCAAAGACA TGCGTTATATGACATTGACTGAACTGCT TAGGGATTGAAGTATACTGAGAAGG[T]A**C** TAGGGATTGAAGTATACTGAGAAGG[T]A**T**	28 28 28 28	71.2 68.8 65.2 63.4	492 bp (control) 294 bp	42.9 39.3 39.3 35.7	122372 - 122399 122863 - 122836 122665 - 122638 122665 - 122638 AC090109 G122638A

Note: Ph = phosphate * reference / control assay underlined and bold = SNP underlined = sequence changes e.g., in trick primers framed = change of published primer

Table 70: Primers and probes - sequence and location (6)

Gene	Polymorphism	Sequence (5`→ 3`)	Primer length (bp)	T_m	PCR product (bp)	GC (%)	Reference Sequence (GenBank No.)
ACE	rs4646994 ins/del (adapted method and control assay)	ACE1 F CACTCCCATCCTTTCTCCCATTT ACE2 R GTGGCCATCACATTCGTCAGAT ACE3 F GCCCGCCACTACGCCCGGCTAATTT ACE4 R TCGCCAGCCCTCCCATGCCCATAA	23 22 25 24	66.5 67.1 77.3 76.3	180 bp (del) / 469 bp (ins) 323 bp (ins) / - (del)	47.8 50.0 64.0 62.5	13772 - 13794 13951 - 13930 - 13997 - 13974 AY436326 (Deletion) 14052 - 14074 14520 - 14499 14244 - 14268 14566 - 14543 AF118569 (Insertion)
BDNF	rs6265 Val66Met G196A (reference RFLP + LightCycler method)	BDNF F CTCTGGAGAGCGTGAAT BDNF R GCACTTGACTACTGAGCA BDNF anc LCRed640- GATGAGGACCAGAAAGTTCGGCC-Ph BDNF sen AACAC**A**TGATAGAAGAGCTGT-fluorescein	17 18 23 21	59.7 60.8 68.2 59.6	177bp	52.9 50.0 56.5 38.1	26467228 - 26467210 26467052 - 26467069 26467139 - 26467117 26467162 - 26467142 NT009237 G26467157A
FKBP5	rs1360780 C143831T (reference RFLP + LightCycler method)	FKBP5 F2 TATAGCTGCAAGTCCC FKBP5 R2 CCTGAAAAGATTATCTGATG FKBP5 anc2 AGATCCAGGCACAGAAGG-fluorescein FKBP5 sen2 LCRed640- TTCACATAAGCAAAGTTA**C**ACAAA-Ph	16 20 18 24	54.8 54.5 62.4 59.0	197 bp	50.0 35.0 55.6 29.2	143731 - 143746 143927 - 143908 143793 - 143810 143813 - 143836 AL033519 C143831T
GNB3	rs5443 C825T (adapted RFLP method)	G$_\beta$ C825T F CTCAGTTCTTCCCCAATG G$_\beta$ C825T R CACACGCTCAGACTTCAT	18 18	58.8 61.2	221 bp	50.0 50.0	5371 - 5388 5591 - 5574 NC000012 C5501T

Note: Ph = phosphate * reference / control assay underlined and bold = SNP underlined = sequence changes e.g., in trick primers framed = change of published primer