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Pharmacogenetics in neuropsychopharmacology: from clinical associations to intermediate phenotypes of drug response

THESIS

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ABBREVIATIONS

Aβ42 – 42 amino acid β-amyloid peptide

ABC – ATP binding cassette transporter

AβMTL - β-amyloidosis in the medial temporal lobe

AD – Alzheimer's disease

APOE – apolipoprotein E

APP - Amyloid Precursor Protein

CSF – cerebrospinal fluid

CTF – C-terminal fragment

CYP - cytochrome P450

DNA – deoxyribonucleic acid

DRD4 – dopamine receptor D4

EM – extensive metabolizer

EOAD – early-onset Alzheimer's disease

FAD – familial Alzheimer's disease

FKBP5 – FK506 binding protein 5

HDRS - Hamilton depression rating scale

5-HIAA - 5-hydroxyindole acetic acid

5-HIES - 5-hydroxyindole acetic acid

HPA - hypothalamic-pituitary-adrenal axis

HPLC - High-performance liquid chromatography

5-HT - 5-hydroxy-tryptamine

5-HT2A receptor – serotonin receptor

5-HTT – serotonin transporter

HVA - homovanillinic acid

HWP – Hardy-Weinberg p-value

LD – linkage disequilibrium

LOAD – late-onset Alzheimer's disease

LOD – logarithm of the odds

L-Trp – L-tryptophan

MADRS - Montgomery-Asberg Depression Rating Scale

MINI - Mini International Neuropsychiatric Interview

MMSE – Mini Mental State Examination

NTF – N-terminal fragment

PCR – polymerase chain reaction

PM – poor metabolizer

PSEN1 – presenilin 1

PSEN2 – presenilin 2

SNP – single nucleotide polymorphism

TM - transmembrane

TPMT – thiopurine methyltransferase

VKORC1 – vitamin K epoxide reductase complex 1

UM – ultrarapid metabolizer

UTR – untranslated region

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1 INTRODUCTION

1.1 Introduction to genetic variability: polymorphisms

The interest to the variations in DNA sequence across the population was considerably arisen during the last decades when it was revealed how common some these variations are. Some of these variations may build up to a high frequency in a population and may represent a hidden source of variation important for the evolution. Polymorphisms are important markers in many studies that link sequence variations to phenotypic changes; such studies are expected to advance the understanding of human physiology and elucidate the molecular bases of diseases. Thus, polymorphisms are common enough to be considered a normal variation in the DNA and they are responsible for normal phenotypes such as eye colour, hair colour or blood type.

Each person's genetic material contains a unique polymorphism pattern that is made up of many different genetic variations. Although many polymorphisms have no negative effects on a person's health, some of these variations may cause a disease, determine susceptibility/resistance to a disease or the severity of progression. Also polymorphisms may be associated with the absorbance and clearance of therapeutic agents and, in such a way, influence individual response to the therapeutic agents, for example, cause an adverse drug reaction (ADR). Currently, there is no simple way to predict how a patient will respond to a particular medication - a treatment proven effective in one individual may be ineffective in others. Nowadays pharmaceutical companies are limited to developing agents to which the "average" patient will respond. As a result, many drugs that might benefit a small number of patients never appear on the market (Wilkinson 2005).

The polymorphisms appear to be useful tools in helping to understand why individuals differ in their abilities to absorb or clear certain drugs, as well as to determine why an individual may experience an adverse side effect to a particular drug. Majority of the polymorphisms observed are single nucleotide polymorphisms (SNPs), short tandem repeats and insertion-deletion polymorphisms (indels). The presence of these polymorphisms in the coding or the regulatory promoter regions of genes might lead to alterations of the protein function or the abolishment of transcription and as a consequence to the variation in the therapy response or the disease occurrence among individuals.

Even though the coding sequence variations are of particular interest, most polymorphisms are found outside of exonic regions. Intronic variation may affect enhancer/silencer

sequences and certain polymorphisms may alter architectural transcription factor binding elements (Kruger, Schroppel et al. 2002). Also non-coding region polymorphisms may serve as biological markers for pinpointing a disease on the human genome map. Therefore, the recent discovery of polymorphisms promises to facilitate the process of disease detection and to introduce the practice of personalized medicine.

Many common diseases in humans are not caused by a genetic variation within a single gene but are influenced by complex interactions among multiple genes as well as epigenetics. Although both environmental and lifestyle factors contribute to the disease development and progression, it is currently difficult to measure and evaluate their overall effect on a disease process. Therefore, scientists refer mainly to a person's genetic predisposition, or the potential of an individual to develop a disease based on genes and hereditary factors. Defining and understanding the role of genetic factors in disease will also allow evaluating the role non-genetic factors — such as diet, lifestyle, and physical activity.

Polymorphisms can also provide information about the molecular basis of disease. The finding of association between certain variation and a particular disease suggests that gene containing this variation may play a role in the development of the disease. In such a way new disease-relevant genes and new targets for drugs can be discovered.

1.2 Haplotypes and Hapmap project

The human genome has a mosaic structure of blocks inherited as one unit with the low rate of recombination within the block due to the linkage disequilibrium (LD). LD implies the existence of the non-random associations of alleles at two or more polymorphic loci within the block. Another word, in the case of LD the frequency in the population of the certain allele combinations is different from that would be expected in the case of random association. Such allele combinations are called haplotypes. Allele associations mean that in many chromosome regions there are only a few haplotypes which account for most of the variation among people in those regions (The international HM project).

The strong associations between polymorphisms in a region have a practical value: genotyping only a few, carefully chosen polymorphisms in the region will provide enough information to predict much of the information about the remaining common polymorphisms in that region. As a result, only few so called tagging polymorphisms are required to identify each of the common haplotypes in a region (Figure 1). Thus, a

substantial reduction in the amount of genotyping can be obtained with little loss of information, by using knowledge of the LD present in the genome. This approach uses information from a relatively small set of variants that capture most of the common patterns of variation in the genome, so that any region or gene can be tested for association with a particular disease, with a high likelihood that such an association will be detectable if it exists. In such a way polymorphisms of the human genome can serve as genetic markers to detect association between a particular genomic region and the disease, whether or not the markers themselves had functional effects (Collins, Guyer et al. 1997). If a polymorphism found to be in association with disease risk or ADR is not a causative one, the subsequent search for the causative variant can be limited to the set of polymorphisms which are in LD with the discovered marker. Afterwards, genetic variants showing positive associations with disease or disease traits that appear to be causal should be examined in functional studies in knock-out animals or cell lines in gene expression or enzyme activity studies, as appropriate.

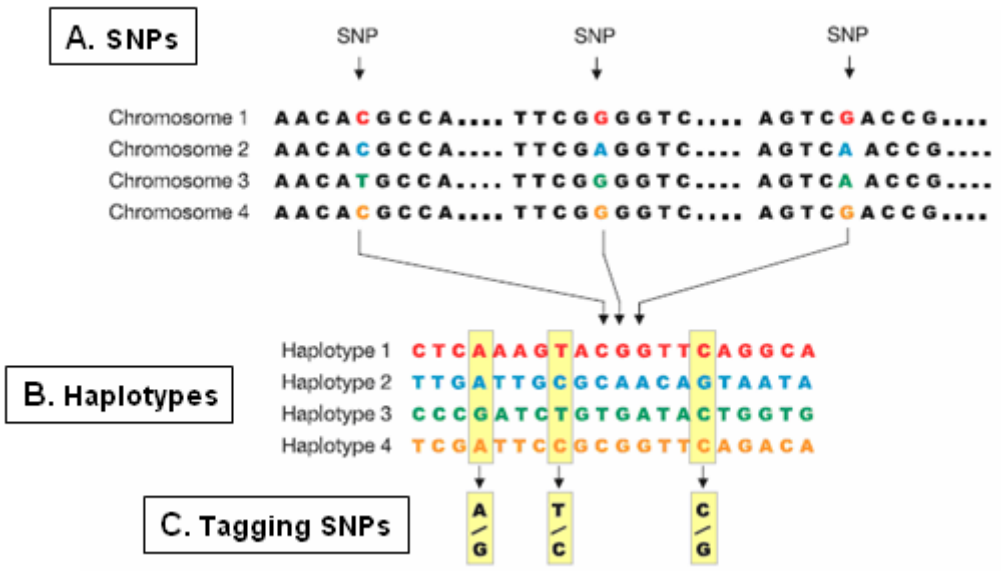


Figure 1. SNPs, haplotypes and tagging SNPs. A. SNPs. Shown is a short stretch of DNA from four versions of the same chromosome region in different people. Most of the DNA sequence is identical in these chromosomes, but three bases are shown where variation occurs. Each SNP has two possible alleles; the first SNP in panel **A** has the alleles C and T. ***B. Haplotypes.*** A haplotype is made up of a particular combination of alleles at nearby SNPs. Shown here are the observed genotypes for 20

SNPs that extend across 6,000 bases of DNA. Only the variable bases are shown, including the three SNPs that are shown in the panel A. For this region, most of the chromosomes in a population survey turn out to have haplotypes 1–4. ***C. Tagging SNPs.*** Genotyping just the three tagging SNPs out of the 20 SNPs is sufficient to identify these four haplotypes uniquely. For instance, if a particular chromosome has the pattern A–T–C at these three tagging SNPs, this pattern matches the pattern determined for haplotype 1. Note that many chromosomes carry the common haplotypes in the population.
The figure is adopted from "The International HapMap Project" (2003).

The aim of the International HapMap Project is to determine the common patterns of DNA sequence variation in the human genome by characterizing sequence variants, their frequencies, and correlations between them in DNA samples from populations with ancestry from parts of Africa, Asia and Europe. This project provides a haplotype map that includes the tagging polymorphisms selected to capture the most information of the human genome. It is a necessary tool to apply the knowledge about LD for fine mapping of complex disease genes and in genome wide association studies.

1.3 Pharmacogenetics

Interindividual variability in response to drug therapy observed for almost all medications. This variability found in a number of processes, including drug transport, drug metabolism, cellular targets, signalling pathways (e.g. G-protein-coupled receptors) and cellular response pathways (e.g. apoptosis, cell cycle control).

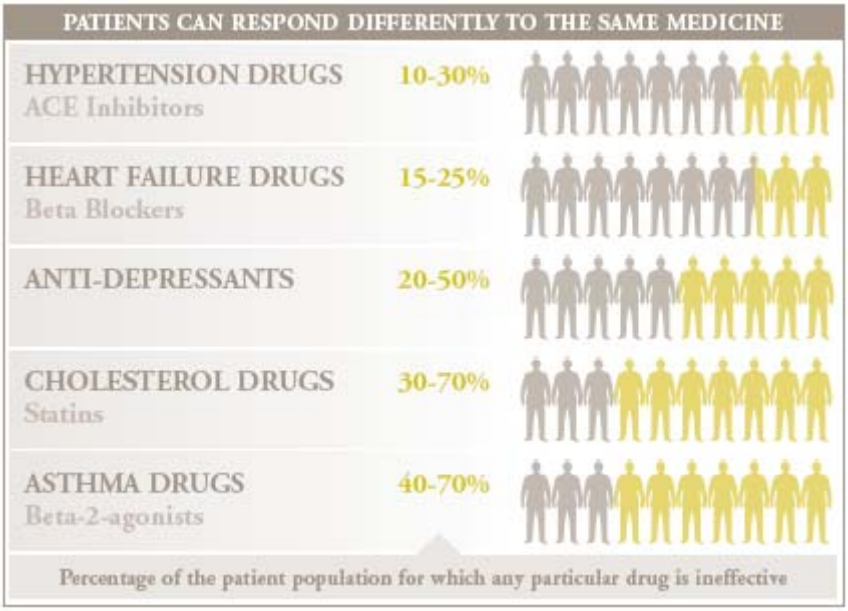


Figure 2. Difference in treatment response – the case for the personalized medicine. Adopted from www.personalizedmedicinecoalition.org

Although a vast majority of polymorphisms have no effects on a person's health, some of these variations may be important in relation to medical practice. Such small genetic differences may cause the different response to the therapy or affect the likelihood of ADR occurrence due to the alterations of the function of enzymes participating on the different stages of drug metabolism such as absorption, break down and elimination from the organism. Therapeutic failure can be caused by the variations in the target molecules as well. Understanding of how individual genetic make-up plays role in the efficacy of the medication and the magnitude of the side effects is the main purpose of pharmacogenetics.

In case when genetics modifies a drug response it's necessary to perform a pre-treatment genetic screening of patients in order to apply this knowledge in clinical practice. This "personalized medicine" approach will help doctors to prescribe the most effective medication with the most accurate dosage based on the individual's genetic profile instead of the body weight and age.

Variability in drug action may be pharmacokinetic or pharmacodynamic. Pharmacokinetic variability refers to variability in the delivery of drug, key molecular sites of action that mediate efficacy or toxicity, and elimination. The molecules involved in these processes include both drug-metabolizing enzymes (such as members of the cytochrome P450, or CYP superfamily) and drug transport molecules that mediate drug uptake into, and efflux from intracellular sites. Pharmacodynamic variability refers to variable drug effects despite

equivalent drug delivery to molecular sites of action. This may reflect variability in the function of the molecule that a drug targets to achieve its effects or in the broad pathophysiological context in which any drug interacts with its molecular target.
In the future, the most appropriate drug for an individual could be determined in advance of treatment by analyzing a patient's polymorphism profile. The ability to target a drug to the individuals who are most likely to benefit from it, would allow pharmaceutical companies to bring more drugs to market and allow physicians to prescribe individualized therapies specific to a patient's needs.

1.3.1 Pharmacogenetics influencing pharmacokinetics

Understanding interindividual differences in response to various drugs, including ADRs, is the main objective of pharmacogenomics (Pirmohamed and Park 2001). Genetic variation, although it is not the sole cause of differences in drug response, is thought to be one of the strongest factors in ADRs. Variants of genes encoding drug metabolizing enzymes or drug targets have been studied in association with personal drug responses. As introduced here, SNPs are popular molecular markers in such pharmacogenomic studies.
Metabolism is a critical component of the final clinical effect of a drug. At the metabolism stage therapeutic agents are converted to more water soluble compounds that are more easily excreted in bile or urine. In addition, in some cases, a drug must be metabolized to become therapeutically active. There are two phases of metabolism of xenobiotics in the organism. Phase I metabolic reactions are carried out in the liver and small intestine by CYP enzymes. It comprises oxidation/reduction and hydrolysis reactions. About 50% of all drugs are oxidised by CYP3A4, 20% by CYP2D6, 15% by CYP2C9 and remaining 15% by CYP2C19.
The phase II of metabolism comprises the attachment of an ionized group such as glutathione, methyl or acetyl groups to the metabolite of the phase I to create water soluble complexes that can be excreted.

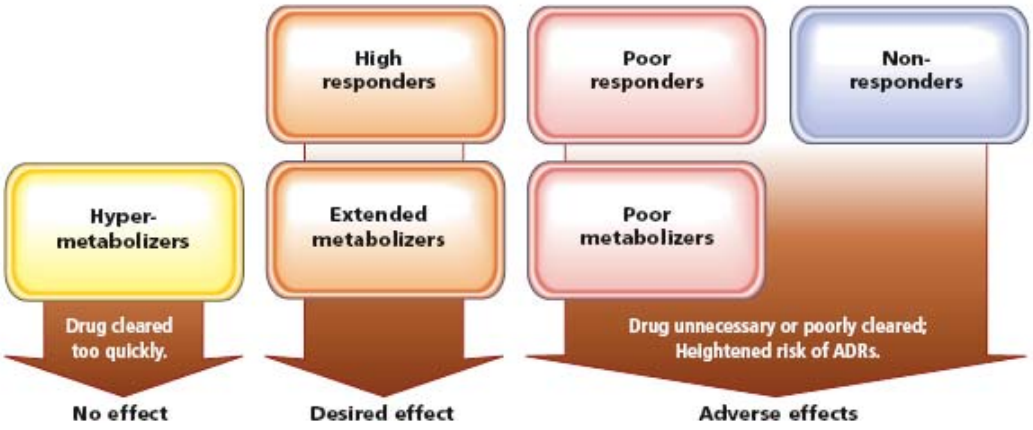


Figure 3. Factors determining an individual's drug reaction. Adopted from www.personalizedmedicinecoalition.org

Dependent on the genetic make-up individuals can be referred to the high responders, those who demonstrate high-drug efficacy; poor responders, those who demonstrate incomplete drug-efficacy; and non-responders, those who demonstrate no drug response (Figure 3).

An example for the genotype-dependent phenotype case is CYP2D6 that is involved in the metabolism of many drugs including neuroleptics, antiarrythmics, antidepressants, and selective serotonin reuptake inhibitors and blockers (Eichelbaum and Gross 1990). There is a large interindividual and ethnic variability in the metabolism of drugs by CYP2D6 that can be explained largely by genetic polymorphisms affecting the enzyme's function and expression (Weinshilboum 2003). The typical CYP2D6 phenotype is usually classified into three groups: poor metabolizers (PMs), extensive metabolizers (EMs) and ultrarapid metabolizers (UMs). Thus, the homozygous carriers of CYP2D6 null allele experience little or no analgesic effect.

For metoprolol, one of the most often prescribed beta-blockers, the role of CYP2D6 genetic polymorphisms in its pharmacokinetics seems to be well established. CYP2D6 catalyzes O-demethylation and even more specifically α-hydroxylation of the drug (Lennard, Silas et al. 1982; Fux, Morike et al. 2005; Bijl, Visser et al. 2009). Not only metoprolol plasma concentrations but also effects on heart rate correlated significantly with CYP2D6 metabolic phenotype (Lennard, Silas et al. 1982). In UMs carrying the CYP2D6 gene duplication, total clearance of metoprolol was about 100% higher compared with the EMs as the reference group. These pharmacokinetic differences were reflected in pharmacodynamics so that the reduction of exercise induced heart rate by metoprolol in the UM group was only about half of that observed in the EMs.

On the basis of the considerable impact of CYP2D6 polymorphism on the disposition of CYP2D6 substrates, it has often been suggested that CYP2D6 PMs are more susceptible to adverse effects than EMs at standard doses of metoprolol as well as antidepressive and antipsychotic drugs (Wuttke, Rau et al. 2002). For instance in a retrospective study of Wuttke et al the CYP2D6 PM genotype was overrepresented among individuals with severe metoprolol associated ADR.

Thiopurine metabolism is another example of a genetic polymorphism that causes a pharmacokinetic interaction that is currently monitored clinically. Genetic polymorphisms that affect the metabolism of mercaptopurine and azathioprine by thiopurine methyltransferase (TPMT) are identified. These polymorphisms are believed to be present in approximately 10% of the white and black populations and, if not identified prior to treatment, can result in severe, life-threatening myelosuppression. Patients with this polymorphism can receive a thiopurine, although the dose must be decreased. Interestingly, the pharmacogenomics test for TPMT activity was one of the first pharmacogenomics tests used clinically.

Membrane transporters are also of great pharmacological importance as they provide the target for many commonly used prescription medications and are a major determinant of the absorption, distribution, and elimination of a large number of clinically used drugs.

There are two transporter protein super families that play a critical role in the absorption and distribution of drugs. The family of solute carrier proteins transports molecules into the cell and the ATP binding cassette (ABC) transporter protein family functions as efflux pumps to remove excess of drugs and toxic materials (Leslie, Deeley et al. 2005), so these transporters work in concert to regulate systemic and intracellular drug levels. It is necessary to disclose the changes in the transport system functioning due to the presence of genetic variations in the transporters' genes and evaluate these changes referring to the drug response.

1.3.2 Pharmacogenetics influencing pharmacodynamics

The efficacy of the drug-target interaction may be influenced by the polymorphisms in the gene that codes for the target molecule, such as receptor or signal transduction modulator. One of the examples of the influence of pharmacogenetics on pharmacodynamics deals with overexpression of the HER2/neu oncogene. Women with metastatic breast cancer and

overexpression of the HER2/neu oncogene appear to derive the greatest benefit from trastuzumab (Herceptin) administration.

Warfarin is a mainstay of therapy for conditions associated with an increased risk of thromboembolic events. However, the use of this common agent is fraught with complications and little is known regarding inter-individual variation in warfarin response (Li, Lange et al. 2006). Following initiation of warfarin therapy, major bleeding episodes occur in approximately 12% of patients and death results in as many as 2% of patients (Levine, Raskob et al. 2001). Although computer aids, nomograms, and flexible protocols result in improved control of anticoagulation, determining a patient's optimal dose remains highly problematic.

Anticoagulants of the coumarin type are effective drugs for the treatment and prevention of thromboembolic diseases. However, these drugs have a narrow therapeutic range and show a large interindividual and intraindividual variability in dose requirement, which necessitates frequent monitoring of the anticoagulant effect and dosage adjustments (Schalekamp, Brasse et al. 2007). Known factors contributing to this variability are age, drug–drug interactions, ingestion of varying quantities of vitamin K, heart failure, infections, impairment of liver function (Hirsh, Dalen et al. 2001; Glasheen, Fugit et al. 2005) and polymorphisms of the CYP2C9 gene, which encodes for the main metabolizing enzyme of the coumarins (Sleeper, Bond et al. 2000; Daly and King 2003; Kirchheiner and Brockmoller 2005).

The determination of safe and effective doses of warfarin for individual patients is one of the most promising clinical applications of pharmacogenetics. There are large variations in warfarin dose from patient to patient and significant clinical consequences of doses that produce insufficient or excessive pharmacologic effects. Thus, reducing uncertainty in establishing the therapeutic dose in individual patients could improve quality of care as well as expand the range of patients who could be treated. In white patients, genetic factors are more strongly correlated with stabilized warfarin dose than all other known patient-related factors. Warfarin pharmacokinetics are affected by functional polymorphisms (*2, Arg144Cys; *3, Ile359Leu) in cytochrome P450 2C9 (CYP2C9). In addition, warfarin's effects are modulated by polymorphisms in the vitamin K epoxide reductase complex 1 (VKORC1) enzyme, a critical component of the vitamin K cycle discovered in part because of its contribution to bleeding disorders and warfarin resistance. Both VKORC1 and CYP2C9 polymorphisms independently correlate with warfarin dose and other clinical outcomes such as time to stabilized dose, bleeding events, and time within the target

therapeutic range. Combined polymorphisms in *VKORC1* and *CYP2C9* explain approximately 30% (20%-25% for *VKORC1*; 5%-10% for *CYP2C9*) of the variance in the stabilized warfarin dose distribution. The importance of these strong genetic effects was recognized by recent relabeling of warfarin by the FDA to raise awareness in the clinical community. However, it is important to note that patient demographics, clinical factors, and genetic variants combined explain only 45% to 55% of the total dose variance.
Both *VKORC1* and *CYP2C9* were identified to be important as a result of their functional relationship to warfarin pharmacology.

1.3.3 Psychopharmacology and pharmacogenetics

Considerable heterogeneity exists in the response to the treatment by psychotropic drugs. Interindividual variability in psychotropic drug response might result in lengthy drug trials, more hospital days, drug side effects, unnecessary drug exposure, and unremitting symptoms that could result in suicide. Thus, ready availability of accurate methods of predicting the therapeutic response to psychiatric medications is important—more so nowadays than before, given the wide range of psychotropic medications that have been introduced since the early 1950s.
The key phenotypes that have been observed in psychiatric pharmacogenetic studies are antipsychotic drug efficacy, antidepressant drug efficacy, and adverse reactions to psychotropic drugs.
Most pharmacogenetic studies of antipsychotic drugs have been based on the atypical antipsychotic clozapine because of its unique efficacy in treatment-resistant schizophrenia. The early studies were carried out in patients enrolled in clinical trials or were based on retrospective data on patients who were treated with clozapine. These studies suffered from the effects of selection bias based on age, ethnicity, and diagnostic heterogeneity. The prime targets of clozapine studies were candidate polymorphisms of serotonin and dopamine receptor genes (Kane and Freeman 1994). Arranz and colleagues examined the T102C polymorphism in the coding region of the serotonin 5-HT2A receptor on chromosome 13. These investigators found that a significant proportion of clozapine nonresponders were homozygous for the 102C allele (Arranz, Collier et al. 1995). Other research groups were unable to replicate these findings in small clozapine studies (Lin, Tsai et al. 1999). One of the studies showed a significant association between clozapine response and a functional promoter polymorphism G1438A in the 5HT2A gene (Arranz,

Munro et al. 1998). The other study was a meta-analysis of the 5HT2A gene in 373 clozapine responders and 360 nonresponders and showed a clear association between the response and the presence of a T102C polymorphism (Arranz, Munro et al. 1998).

Most studies have reported no significant relationship between clozapine response and common polymorphic markers in the genes of other serotonin receptor subtypes (5-HT2C, 5-HT3A, 5-HT3B, 5-HT6, 5-HT7) or for the serotonin transporter (SERT) (Malhotra, Goldman et al. 1996; Masellis, Basile et al. 2001; Gutierrez, Arranz et al. 2002).

Mixed results have been reported for dopamine receptor polymorphisms and the therapeutic response to antipsychotic drugs. For example, despite the ability of clozapine to prevent the high-affinity binding of dopamine with its D4 receptor (DRD4), clozapine studies have failed to demonstrate a role of the DRD4 polymorphisms in treatment response. In one preliminary report, a novel repeat polymorphism in the first intron of DRD4 did predict the clozapine efficacy; however, the reliability of this finding is limited by the small sample size used in this study (Ozdemir, Kalow et al. 2001).

Relatively few pharmacogenetic studies have focused on the D2 receptor (DRD2), even though several antipsychotic drugs bind with this receptor and neuroimaging reports suggest that DRD2 affinity might be related to the efficacy of these drugs. Schafer and colleagues have studied the DRD2 Taq I polymorphism and short-term response to haloperidol (Haldol), a strong DRD2 antagonist. They found that homozygosity for the A2 alleles predicted a poorer response (Schafer, Rujescu et al. 2001). The association of poorer response with high-affinity DRD2 antagonists and the A2/A2 genotype was strengthened in a risperidone (Risperdol) study that showed similar results. In addition, a 141C-insertion/deletion polymorphism in the DRD2 promoter region was linked to response to treatment with bromperidol (Impromen, Bromidol) or nemonapride (Sepan) (Suzuki, Kondo et al. 2001), but not clozapine (Arranz, Li et al. 1998). These data need to be verified by studies using larger sample sizes.

1.4 Project 1: Testing the influence of PSEN2 haplotypes on β -amyloid 42 level in CSF as a phenotype of PSEN function

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by progressive cognitive deficit and is the most common cause of dementia among elderly population (Ho, Saville et al. 2001). The prevalence of Alzheimer's disease in the general population increases from 1% in persons younger than 65 years to approximately 40% at 90 years old

(Evans, Nation et al. 1989). All AD cases can be subdivided into two groups: familial early-onset AD cases (EOAD) that account for about 10% of all AD cases and sporadic late-onset AD cases (LOAD) that are more common. Histopathologically these both types of AD are characterized by the presence of amyloid plaques in the brain consisting of the 42 amino acid β-amyloid peptide (Aβ42). The aberrant aggregation of 42-amino acid β-amyloid peptides (Aβ42) on the outside surface of neurons results in synaptic loss and death of neurons and, as a consequence, in dementia (St George-Hyslop 2000). This pathological form of β-amyloid peptide is derived through the proteolitic processing of the neuron membrane protein Amyloid Precursor Protein (APP) by beta- and subsequently gamma-secretases (Selkoe 1994).

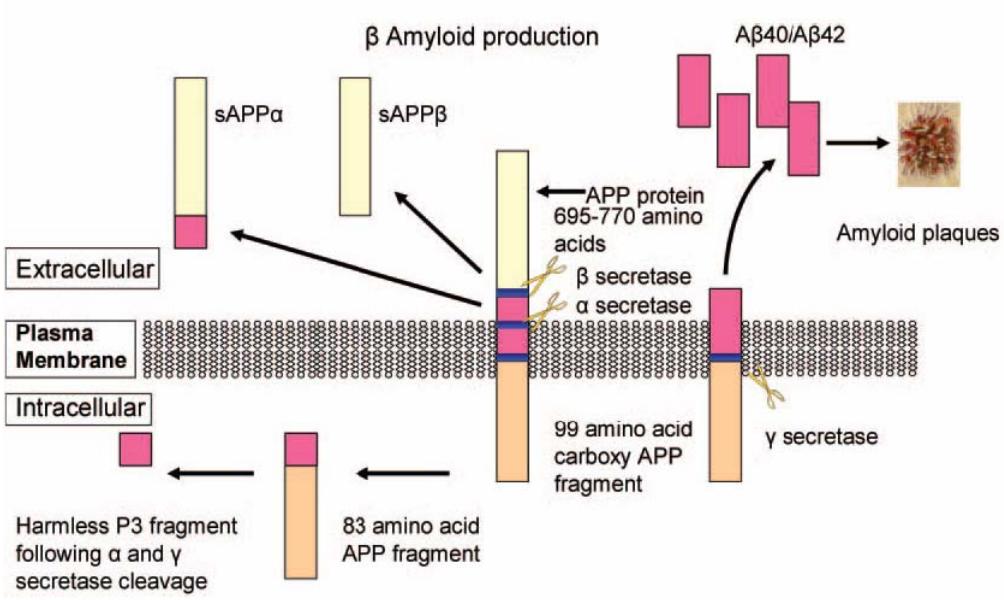


Figure 5. β-amyloid production. APP is a membrane protein producing a number of isoforms which range in size from 695–770 amino acids. Proteolysis of the APP protein involves α, β and γ secretases. APP cleavage by α secretase releases sAPPα from the membrane leaving an 83 amino acid APP fragment. Cleavage of the APP protein by β secretase releases sAPPβ from the membrane and leaves behind a 99 amino acid fragment which can be further cleaved by γ secretase to produce Aβ40/42 fragments extracellularly. Adopted from (Thomas and Fenech 2007).

Presenilin (PSEN) proteins have been shown to be involved in APP processing as a part of gamma-secretase complex (Wolfe, Xia et al. 1999). PSEN1 and PSEN2 are highly homologous polytopic transmembrane proteins with the amino acid sequence identity about 62%.

The transmembrane (TM) topology of PSEN1 and PSEN2 proteins is still being debated, nevertheless, an eight TM topology model with N- and C-termini, and a hydrophilic loop domain between TM 6 and 7 facing the cytosol, has been widely accepted (Doan, Thinakaran et al. 1996). Endoproteolytic processing of PSEN within this hydrophilic loop domain is highly conserved and, perhaps, is a critical event that regulates the stability of PSEN and possibly the biological activity of the proteins. While full length PSEN1 is relatively short-lived with a half-life of 1–2 h, endoproteolytically processed derivatives NTF and CTF have a half-life of 24 h (Podlisny, Citron et al. 1997; Ratovitski, Slunt et al. 1997). After the endoproteolysis the mature PSEN1 asembles with other γ-secretase subunits nicastrin, APH-1, and PEN-2 (Iwatsubo 2004).

A large number of non-synonymous mutations are found in APP, PSEN1 and PSEN2 genes which have been shown to be associated with familial AD (FAD) cases, most of those are early-onset cases with fast disease progression (De Strooper, Saftig et al. 1998; Goater and Pethig 1998). So far 167 PSEN1 non-synonymous mutations in 364 families and 10 PSEN2 mutations in 18 families have been identified (http://www.molgen.ua.ac.be/ADMutations/ or http://www.alzgene.org).

A major clue to the role of presenilins in the pathogenesis of FAD was the discovery that disease-causing mutations increase production of the highly fibrillogenic Aβ42 in transfected cells and transgenic mice brain expressing mutant PS1 and PS2 (Duff, Eckman et al. 1996; Citron, Westaway et al. 1997; Tomita, Maruyama et al. 1997; Steinhagen-Thiessen and Borchelt 1999; Chou, Yan et al. 2000). Under normal circumstances γ-secretase activity resulting in APP and Notch cleavage is believed to be associated with PSEN1 rather than PSEN2 complexes, and knockout of PSEN1 leads to an embryonic lethal mouse phenotype and dramatically reduced Aβ levels (Palacino, Berechid et al. 2000) whereas knockout of PSEN2 in a mouse produces no specific phenotype and essentially normal levels of Aβ.

Presenilin mutations might be causing FAD through a gain of deleterious function that alters the specificity of γ -secretase processing of APP and elevates the amount of extraneuronal Aβ42 (Duff, Eckman et al. 1996; Citron, Westaway et al. 1997; Tomita, Maruyama et al. 1997; Steinhagen-Thiessen and Borchelt 1999; Chou, Yan et al. 2000).

Despite the familiar associations with AD, there is a majority of sporadic cases showing a wide range in age of onset which has not been explained so far with certain single mutation or set of mutations in these genes (Bird, Levy-Lahad et al. 1996).

Another gene associated with AD is apolipoprotein E (APOE). Three alleles of the APOE have been described on the basis of two amino acid changes at positions 112 and 158. The APOE E2 allele is characterised by cysteine at both positions, E3 allele by cysteine in position 112 and arginine at position 158, and the E4 allele by arginine in both positions. In Caucasian populations APOE E4 heterozygous individuals have a threefold increased risk and homozygous persons an approximately eightfold increased risk for developing AD compared to E3 heterozygous individuals. This association was also confirmed in several subsequent studies and was described as s risk factor for sporadic AD. APOE is a primary cholesterol transporter in the brain and is a component of amyloid plaques and neurofibrillary tangles, even though the mechanism for the effects of APOE isoforms on dementia is unclear. The APOE gene does not cause AD, but acts as a marker altering individual risk based on possession of allelic combinations of the APOE E4 allele (Corder, Saunders et al. 1994).

Despite the relatively large effects of these variants, the use of APOE genotype information in disease prediction remains limited, since its diagnostic sensitivity is only 0.65 and specificity 0.68 compared with clinical diagnosis, which has reported sensitivity of 0.93 and specificity of 0.55 (Mayeux, Saunders et al. 1998).

The amyloid cascade theory was proposed to explain the aetiology and progression of the disease (Hardy and Allsop 1991; Hotopf, Hardy et al. 1997). However in recent years it has been found that certain observations cannot be explained by the hypothesis in its current form (Schonheit, Zarski et al. 2004). The cascade theory currently states that elevated deposits of the Aβ42 occur as a result of missense mutations or a failure of clearance mechanisms that invariably lead to the production of neuritic plaques. These plaques precede intracellular accumulation of tau and eventually result in neuronal cell death (Hardy and Allsop 1991; Hotopf, Hardy et al. 1997). The hypothesis also suggests that the plaques produce hyperphosphorylated tangles through the abnormal regulation of kinases and phosphatases as the disease progresses (Schellenberg 1995).

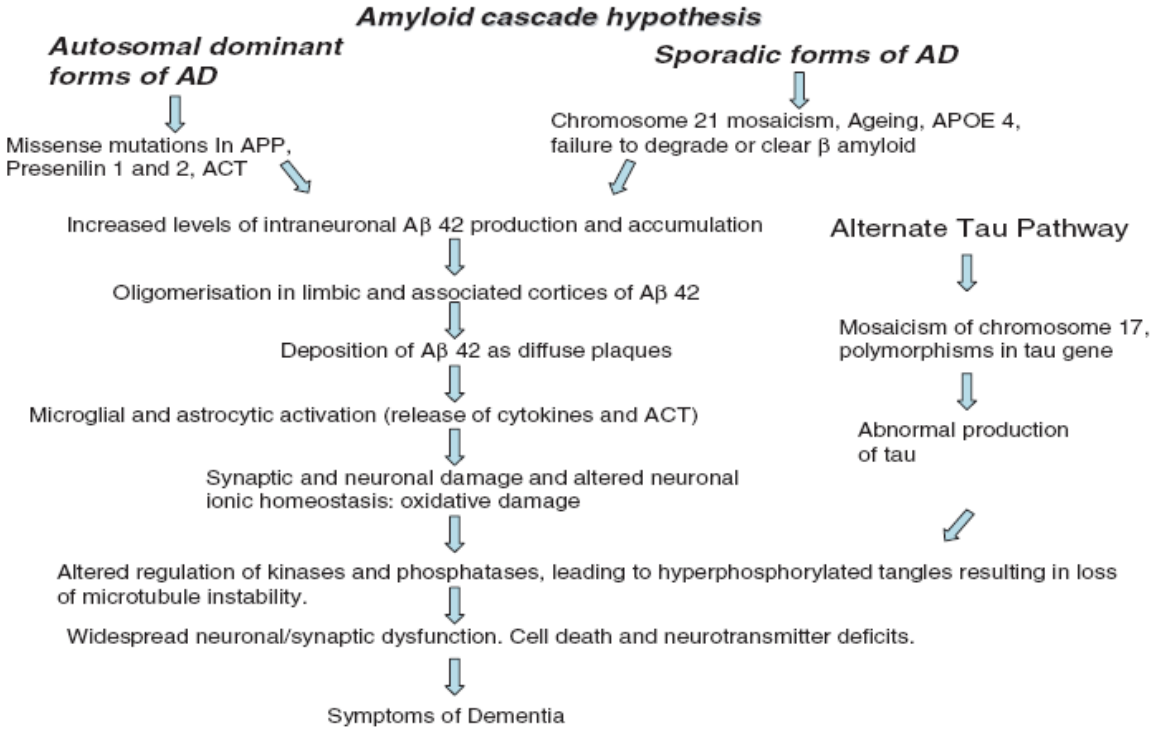


Figure 6. Amyloid cascade hypothesis showing pathways for β-amyloid and tau production and accumulation leading to Alzheimer pathology. Adopted from (Thomas and Fenech 2007).

Since AD is primarily a diagnosis of exclusion, an objective diagnostic test is sorely needed. The ideal biomarker should detect a fundamental neuropathologic feature of AD early in the course of the disease and should be highly sensitive and specific. Aβ42 and tau-protein levels in CSF are biomarkers that appear to be useful in diagnosing brain pathology characteristic of AD. Specifically, the combination of abnormally low CSF Aβ42 level and abnormally high CSF tau level was highly accurate in predicting the presence of pathologic features of AD.

Mechanisms leading to decreased concentrations of Aβ42 in CSF of patients with AD are not clarified so far. Accumulation of the peptide in the plaques is suggested by some investigators, however, this hypothesis cannot fully explain the picture since a selective decrease of the concentration of Aβ42 in the CSF of patients with Creutzfeldt-Jakob disease as well as in patients with bacterial meningitis has been shown without development of any amyloid plaques at all (Sjogren, Gisslen et al. 2001; Wiltfang, Esselmann et al. 2003). Nevertheless, taken as a diagnostic biomarker for AD, cerebrospinal Aβ42 discriminates AD from elderly controls at quite high sensitivity and

specificity (sensitivity between 78-85% and specificity between 81-84%) (Galasko, Chang et al. 1998; Hulstaert, Blennow et al. 1999; Blennow, Vanmechelen et al. 2001; Lewczuk, Esselmann et al. 2004; Wiltfang, Lewczuk et al. 2005) and has been shown to correlate with Aβ deposition in the brain (Fagan, Kagan et al. 2006). It is currently unknown whether genetic polymorphisms in PSEN1 and PSEN2 contribute to incidence and clinical course of sporadic AD. It is also not known whether PSEN polymorphisms are correlated with Aβ42 concentrations in CSF.

According to the variations of Aβ42-peptid levels in different laboratoris (Lewczuk, Esselmann et al. 2004) in our laboratory a cut-off 600 pg/ml for the differential diagnostic question (AD vs non-demented control) was established. By defining an "Aβ42 CSF-positive" phenotype (cut-off below 600 pg/ml), we aimed at identifying novel genetic modifiers for AD. As Aβ42 levels predominantly rely upon the activity of the active sites of γ-secretase, presenilin 1 and 2, we therefore tested the hypothesis whether rare genetic variants or frequent genetic haplotypes in PSEN1 and 2 are associated with decreased Aβ42 concentrations in CSF of AD patients compared to controls.

1.5 Project 2: FKBP5 genetic variants and antidepressant treatment response

The identification of pharmacogenetic markers predicting the individual drug response is one of the major goals of molecular medicine. Most findings on pharmacogenetic response predictors resulted from clinically characterized cohorts correlating response to drugs with one or several genetic polymorphisms. Non-replication of many of the primary findings in independent cohorts is a major problem hampering the clinical value of such findings. Hyperactivity of the hypothalamic-pituitary-adrenal (HPA) axis may be a relevant pathogenic factor in depressive disorders (Holsboer 2000; Ising, Horstmann et al. 2007). An impaired negative feedback regulation leading to partial glucocorticoid resistance, seems to cause this hyperactivity (Pariante and Miller 2001). In depressed patients this is reflected by basal hypercortisolemia and cortisol escape from dexamethasone suppression.

In search for the genetic and functional mechanisms underlying the HPA-dysfunction in depression, recent research focused on genes involved in HPA-axis regulation. Polymorphisms in the genes for corticotropin-releasing hormone receptor, the glucocorticoid receptor, the arginine vasopressin receptor, and angiotensin converting enzyme have shown at least in part influence on both, the HPA-activity and treatment

outcome during antidepressant treatments (van Rossum, Binder et al. 2006) (Keck and Holsboer 2001; Baghai, Binder et al. 2006). In addition cochaperones of the glucocorticoid receptor which might influence glucocorticoid receptor activity, have been shown to modulate HPA-activity (Pratt and Toft 1997). Stabilization of the HPA axis is implicated as a prerequisite to clinical response of antidepressant drugs (Holsboer and Barden 1996) and one mechanism of action of antidepressant drugs may be to restore negative HPA-axis feedback through the glucocorticoid receptor (Pariante and Miller 2001).

One of the cochaperones of the glucocorticoid receptor, FKBP5 gained growing interest when a genetic polymorphism was described leading to faster response to antidepressant drugs of various drug classes (Binder, Salyakina et al. 2004). The mechanism might be that individuals who are carriers of the polymorphism have a faster stabilization of their HPA axis thereby leading to faster onset of antidepressant drug response. The FKBP5 variants were not associated with susceptibility to affective disorders as primarily reported by Binder et al. 2004 (Binder, Salyakina et al. 2004) and later confirmed by a case-control study (Gawlik, Moller-Ehrlich et al. 2006). This was, however not confirmed in a recent study in 125 Taiwanese depressed patients treated with 20 mg fluoxetine (Tsai, Hong et al. 2007). In children, an association between FKBP5 polymorphisms and incidence of peritraumatic dissociation disorder as an acute stress response following trauma has been reported (Koenen, Saxe et al. 2005) but the finding has not yet been replicated by others.

In our study, FKBP5 seemed to be a suitable pharmacogenetic target since especially the early response to antidepressant drugs during the first 3 weeks has been documented. Therefore, we intended to replicate the findings of Binder et al. (Binder, Salyakina et al. 2004) on the FKBP5 SNPs rs1360780 and rs3800373 in an independent but clinically and ethnically comparable cohort of depressed patients.

1.6 Project 3: Serotonin transporter genotypes and monoamine plasma concentrations during acute tryptophan depletion as an intermediate phenotype of depression

Dysfunction of the central serotoninergic system plays an important role in several aspects of alcohol addiction including reward, craving and relapse. While alcohol, administered acutely, increases the release of serotonin within the brain, chronic alcohol consumption tends to decrease the amount of serotonin stored in the central nervous system (Lappalainen, Zhang et al. 1995). About 30 % reduction of brainstem serotonin

transporters in type I alcohol dependent individuals has been observed suggesting that chronic alcohol intoxication reduces central serotonin transporter density (Anklam, Gadani et al. 2002).

The serotonin transporter (5-HTT) has an important role in the high affinity re-uptake of 5-HT from the synapse, returning it into the presynaptic neuron where it can be degraded or retained for future release. The 5-HTT gene in the promoter region has been reported to have a functionally relevant 44-bp insertion (l) / deletion (s) polymorphism which significantly influences the transporter reuptake capacity (s allele carriers having lower capacity than l allele carriers) und the presynaptic number of transporters (Lesch, Bengel et al. 1996). It has been observed that homozygous carriers of the long allele of 5-HTT promoters (5-HTTLPR) are selectively more vulnerable to the neurotoxic effects of long-term excessive alcohol consumption than ss carriers (Anklam, Gadani et al. 2002).

Higher compulsive alcohol craving at the beginning of alcohol withdrawal was observed in patients with the long allele of the 5-HTTLPR compared to the short allele (Bleich, Bonsch et al. 2007).

Depressive symptoms are associated with alcohol dependence and may become a relevant problem in alcohol withdrawal. These problems appear to be related to disturbances in the serotoninergic system (Kranzler and Anton 1994; LeMarquand, Pihl et al. 1994; Charney 1998). One method to assess the role of serotonin in vivo is by rapid tryptophan depletion which is achieved by oral administration of a mixture of all essential amino acids but devoid of tryptophan (Young, Smith et al. 1985). It has been shown that acute tryptophan depletion (ATD) reduces brain concentrations of tryptophan and of serotonin (5-HT) (Young and Leyton 2002).

Several studies have shown that this so-called *5-HT transporter linked polymorphic region* (5-HTTLPR) genotype can influence depressive symptoms in response to acute tryptophan depletion (Lenzinger, Neumeister et al. 1999; Neumeister, Konstantinidis et al. 2002; Bilous, Dowsett et al. 2003; Pierucci-Lagha, Feinn et al. 2004; Neumeister, Hu et al. 2006). In one study of acute tryptophan depletion in non-drinking alcoholics with major depression, higher depressive symptoms emerged in homozygous carriers of the insertion genotype (ll) compared with heterozygotes (ls) and homozygous deletion genotype carriers (ss) during tryptophan depletion, but similar scores during the sham session (Pierucci-Lagha, Feinn et al. 2004).

The aim of this study was to assess 5-HTTLPR genotype related differences in serum monoamine concentrations in alcoholic men during alcohol withdrawal, and to study the

effect of modulation of tryptophan intake on development of depressive symptoms or craving in in dependence of the 5-HTTLPR genotype.

2 MATERIALS AND METHODS

2.1 Materials

2.1.1 Working solutions and equipment for PCR

10x PCR Buffer	Invitrogen, Germany
dNTPs set (100mM)	Invitrogen, Germany
$MgCl_2$ (50 mM)	Invitrogen, Germany
Primers	Biomers, Germany
Taq DNA Polymerase (5 U/ µl), recombinant	Invitrogen, Germany
96 Multiply® PCR plate natural	Sarstedt, Germany
Multiply® lid chains	Sarstedt, Germany
PTC-225 Peltier Thermal Cycler	MJ-Research, USA
Milli-Q plus	Millipore, Germany

2.1.2 Reagents and equipment for electrophoresis

Agarose	Sigma, USA
Boric Acid	J.T. Baker, Holland
Tris	USB Corporation, USA
EDTA	Merk, Germany
6x Loading Dye Solution	Fermentas, St. Leon-Rot
GeneRuler™ DNA Ladder Plus (50 bp, 100 bp, 1 kb)	Fermantas, Canada
Ethidiumbromid	Fluka, Switzerland
Precision Laboratory Balance	Sartorius, Germany
UV-Transilluminator UVT-20 ME	Herolab, Germany
CCD camera type E.A.S.Y. 429K	Herolab, Germany
Magnetic Mixer MR 2000	Heidolph, Germany
Electrophoresis Power Supply EPS 600	Pharmacia Biotech, USA
Gel casting tray and comb	Scientific Support, USA

2.1.3 Working solutions and equipment for Real-Time PCR

TaqMan® Universal PCR Master Mix, No AmpErase® UNG	Applied Biosystems, USA
Real-Time PCR Genotyping Assay	Applied Biosystems, USA
Real-Time PCR Expression Assay	Applied Biosystems, USA
7300 Real Time PCR System	Applied Biosystems, USA
MicroAmp® Optical 96-Well Reaction Plate	Applied Biosystems, USA
MicroAmp® Optical Adhesive Film	Applied Biosystems, USA

2.1.4 Kits for DNA/RNA isolation

QIAamp DNA Blood Mini Kit:

ATL tissue lysis buffer
AL lysis buffer
Ethanol 96-100%
QIAamp Mini spin columns — Qiagen, Germany
AW1 wash buffer
AW2 wash buffer
AE elution buffer
QIAGEN Protease

RNase-Free DNase Set:

DNase I, RNase-Free (lyophilized)
Buffer RDD — Qiagen, Germany
RNase-Free Water

PAXgene™ Blood RNA Kit:

PAXgene Blood RNA Tubes
BR1 resuspension buffer
BR2 binding buffer

Proteinase K	PreAnalytix, Germany
PAXgene Shredder spin column	
PAXgene RNA spin column	
BR3 wash buffer	
BR4 wash buffer	
BR5 elution buffer	
Vortex-genie 2	Scientific Industries, Canada
GeneQuantpro RNA/DNA Calculator®	Biochrom, Germany
Thermomixer compact	Eppendorf, Germany
Centrifuge 1-15K	Sigma, USA

2.1.5 Regents and equipment for sequencing

QIAquick PCR Purification Kit:

QIAquick columns	
PBI binding buffer	Qiagen, Germany
PE washing buffer	
EB elution buffer	
5x Sequencing Buffer	Applied Biosystems, USA
BigDye® Terminator v3.1 Cycle Sequencing Kit	Applied Biosystems, USA
Sephadex® G-50, for molecular biology, DNA Grade	Sigma-Aldrich, Germany
MultiScreen®-HV plate	Millipore, France
MultiScreen® 45 µl column loader	
MicroAmp® Optical 96-Well Reaction Plate	Applied Biosystems, USA
Megafuge 1.0R	Heraeus, Germany
3100-Avant Genetic Analyser	Applied Biosystems, USA
3100 POP-6™ Performance Optimized Polymer	Applied Biosystems, USA
3730 Buffer (10x) with EDTA	Applied Biosystems, USA

3130 & 3100-Avant Capillary array 50 cm	Applied Biosystems, USA

2.1.6 Reagents for RT-PCR

SuperScript^{TM} II Reverse Trnscriptase (200 U/µl)	Invitrogen, Germany
5x First-Strand Buffer	Invitrogen, Germany
DDT (0.1M)	Invitrogen, Germany
Oligo dT_{12-18} (0.5 µg/µl)	Invitrogen, Germany
RNase Inhibitor (20U/µl)	Applied Biosystems, USA
dNTP mix (2.5 mM each)	Applied Biosystems, USA
RNase-free water	Qiagen, Germany

2.1.7 Reagents for restriction

HhaI enzyme (20 U/µl)	New England Biolabs, USA
1xNEBuffer 4	New England Biolabs, USA
100xBSA	New England Biolabs, USA
Water bath 1083	GFL, Germany

2.1.8 Preparation of solutions

QIAGEN Protease working solution:

QIAGEN Protease (lyophilized)	7.5 AU
Milli-Q water	7 ml

Buffer AW1 working solution:

AW1 buffer concentrate	95 ml
ethanol absolute	125 ml

Buffer AW2 working solution:

AW2 buffer concentrate	66 ml
ethanol absolute	160 ml

Buffer BR4 working solution:

BR4 buffer concentrate	11 ml

ethanol absolute	44 ml

1xTBE:

Tris	54 g
Boric acid	27.5 g
0.5M EDTA (pH=8.0)	20 ml
H_2O	till 1 L

0.5M EDTA (pH=8.0):

EDTA	93.06 g (MG=372.2)
H_2O	500 ml
NaOH	till pH=8.0

EtBr gel staining solution:

H_2O	98 ml
50xTAE	2 ml
EtBr	10 µl

50xTAE:

Tris	242 g
glacial acetic acid	57.1 ml
0.5M EDTA	100 ml
H_2O	till 1 L

2.1.9 Software and internet sources

7300 System SDS Software	Applied Biosystems, USA
Primer Express 3.0	Applied Biosystems, USA
BLAST NCBI	Bethesda, USA
3100-Avant Data Collection Software 1.0	Applied Biosystems, USA
Sequencing Analysis 3.7 Software	Applied Biosystems, USA
E.A.S.Y® Win32 Basic Software Module A and camera control software	Herolab, Germany
Haploview 3.32	http://www.broad.mit.edu/mpg/haploview

HapMap Project web page	http://www.hapmap.org/
Protein Structure Analysis Program	http://www.uniprot.org/
SPSS 12.0	SPSS Inc, USA

2.2 Methods

2.2.1 Project1

2.2.1.1 Patient samples

All patients who were diagnosed with probable AD from 1999 to 2008 at the Memory Clinic of the Neurology University Hospital of Ulm participated in the study. Diagnosis was verified according to National Institute of Neurological and Communication Disorders Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) criteria and the Diagnostic and Statistical Manual of Mental Disorders, fourth edition (DSM-IV) (McKhann, Drachman et al. 1984).

The patients gave written informed consent to participate in the study and to have their clinical data documented together with results from genetic analyses in genes potentially relevant to AD. The study was approved by the university ethical review board (No. 2001/67).

Before diagnosis, subjects underwent a comprehensive clinical examination including their medical history, family history, a neurological and psychiatric examination, a routine blood analysis, a lumbar puncture with CSF analysis for Aβ42 and tau-protein level, morphological imaging (MRI or CT), APOE genotyping, and a comprehensive neuropsychological examination, including Alzheimer's Disease Assessment Scale–cognitive subscale (ADAS-cog). The degree of dementia was evaluated by the Mini-Mental State Examination (MMSE).

Subjects with AD diagnosis were included in the AD group if the CSF Aβ42 concentration was below the level of 600 pg/ml. As a control group, individuals who were admitted to the Memory Clinic but were subsequently diagnosed with something other than dementia were chosen if their CSF Aβ42 level was higher than 600 pg/ml.

CSF collection and preanalytical processing were performed using a standardized protocol in a clinical routine setting as previously described (Brettschneider, Petzold et al. 2006). Aβ42 and tau-protein measurements were performed using a commercially available sandwich ELISA according to the manufacturer's instructions.

As an independent cohort for the polymorphisms genotyping 109 samples of human brain tissue from patients who underwent an autopsy after death. Retrospective analysis of clinical data as well as neuropathological analysis was performed as reported previously

(Thal, Rub et al. 2002). Histological characterization for presence and localization of β-amyloid plaques in the medial temporal lobe of the respective brain samples had been performed in all samples as described earlier (Thal, Ghebremedhin et al. 2002; Thal, Capetillo-Zarate et al. 2006).

2.2.1.2 DNA isolation from blood

DNA extraction from 191 whole blood samples of AD group and 123 blood samples of control group was performed using QIAamp DNA Blood Mini Kit. QIAGEN Protease (20 µl) was mixed with whole blood (200 µl) and lysis buffer AL (200 µl), the whole mixture was vortexed thoroughly for 15 s to yield a homogeneous solution in order to ensure efficient lysis and incubated for 10 min at 56°C. Then 96-100% ethanol (200 µl) was added to the lysate, and vortexed for 15 s. The whole mixture was applied to the QIAamp Mini spin column and centrifuged at 6000xg (8000 rpm) for 1 min to let DNA bind to the membrane of the spin column. Two subsequent membrane washing included the application on the membrane of buffer AW1 (500 µl) and centrifugation at 6000xg (8000 rpm) for 1 min and, afterwards, application of buffer AW2 (500 µl) and centrifugation at full speed (20 000xg; 14000 rpm) for 3 min. To elute DNA from the membrane of the spin column the elution buffer AE (200 µl) was applied to the membrane, followed by incubation at room temperature for 1 min and centrifugation at 6000xg (8000 rpm) for 1 min.

2.2.1.3 DNA isolation from the brain tissue

DNA extraction from 109 samples from human autopsies was performed using QIAamp DNA Blood Mini Kit. ATL buffer (100 µl) and proteinase K (20 µl) were added to the 25 mg of the brain tissue cutted up into small pieces, the mixture was thoroughly vortexed to disperse the sample and incubated over night in the thermomixer at 56°C and 400 rpm shaking speed to let the tissue get completely lysed. After the incubation, buffer AL (200 µl) was added to the lysate, the mixture was vortexed for 15 s and incubated for 10 min at 70°C. Then absolute ethanol (200 µl) was added, the mixture was vortexed for 15 s, applied to the QIAamp Mini spin column and subsequently centrifuged for 1 min at 6000xg (8000 rpm) to let DNA bind the membrane of the column. Two subsequent membrane washing included the application on the membrane of buffer AW1 (500 µl) and centrifugation at 6000xg (8000 rpm) for 1 min and, afterwards, application of buffer AW2

(500 µl) and centrifugation at full speed (20 000xg; 14000 rpm) for 3 min. To elute DNA from the membrane of the spin column the elution buffer AE (200 µl) was applied to the membrane, followed by incubation at room temperature for 1 min and centrifugation at 6000xg (8000 rpm) for 1 min.

2.2.1.4 DNA quantification

DNA yields were determined from the concentration of DNA in the eluate, measured by absorbance at 260 nm on GeneQuantpro RNA/DNA Calculator®. Purity was determined using the value of A260/A280 ratio that has to lie in the range of 1.7–1.9 in the case of pure DNA.

Before the concentration measurement the DNA samples were diluted in water so that absorbance readings at 260 nm lie between 0.1 and 1.0 to be accurate. Water was used for the calibration of the spectrophotometer.

2.2.1.5 APOE genotyping

2.2.1.5.1 PCR

APOE genotypes of all the individuals and brain samples were determined by the restriction of PCR product with *HhaI* enzyme described earlier (Wenham, Price et al. 1991).

Extracted DNA was amplified by PCR in a PTC-225 Peltier Thermal Cycler using oligonucleotide primers Pr1 (5'-ACAGAATTCGCCCCGGCCTGGTACAC-3') and Pr2 (5'-TAAGCTTGGCACGGCTGTCCAAGGA-3').

The amplification reaction with the final volume of 25 µl contained:

10x PCR Buffer	2.5 µl
$MgCl_2$ (50 mM)	1.0 µl
dNTPs (2 mM)	1.0 µl
Pr1 (10 µM)	0.5 µl
Pr2 (10 µM)	0.5 µl
Taq DNA Polymerase (5 U/µl)	0.2 µl
H_2O Milli-Q	18.3 µl
DNA	1.0 µl

The PCR thermocycler program contained following steps:

Denaturation	94°C – 5 min	
Denaturation	94°C – 1 min	35 cycles
Annealing	60°C – 1 min	
Elongation	72°C – 2 min	
Final elongation	72°C – 7 min	

2.2.1.5.2 Restriction with HhaI

After the amplification, 5 units of *HhaI* enzyme were added directly to the reaction mixture for restriction of PCR-product. The mixture was incubated for 3 hours at 37°C.

2.2.1.5.3 PAAG

After the restriction each reaction mixture was loaded onto an 8% polyacrylamide nondenaturing gel (1.5 mm thick x 25 cm long) and electrophoresed for 3 h under constant current 45 mA. After electrophoresis, the gel was treated with ethidium bromide solution for 10 min and DNA fragments were visualized by UV illumination. The sizes of *Hhu*I fragments were estimated by comparison with the marker MspI-digested pUC18 DNA.
Figure 7 demonstrates the electrophoretic separation of fragments after the restriction by *HhaI*.

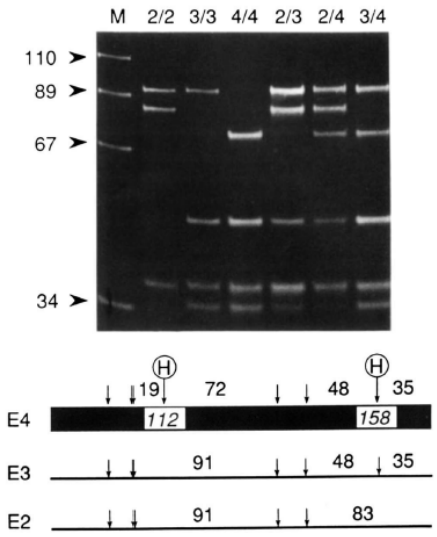


Figure 7. HhaI cleavage maps and electrophoretic separation of HhaI fragments. On the gel there are shown fragments from an E2E2 homozygote (lane marked 2/2), E3E3 homozygote (3/3), E4E4 homozygote (4/4), E2E3 heterozygote (2/3), E2E4 heterozygote (2/4), and E3E4 heterozygote (3/4). Lane M - Mspl-digested pUC18 marker. Below the gel, *HhaI* cleavage maps (downward arrows show sites) are given for amplified sequences. E4 is shown as a filled box containing codons 112 and 158, E3 and E2 maps are shown below E4. The distances in base pairs between polymorphic *HhaI* sites (circled H) that distinguish isoforms are shown for each cleavage map. Adopted from (Hixson and Vernier 1990).

2.2.1.6 Sequencing of the PSEN1 and PSEN2 exonic regions in EOAD group

2.2.1.6.1 PCR

DNA sequence analyses of all exonic regions of PSEN1 and PSEN2 as well as exons 16 and 17 of APP were performed in the subgroup of 37 patients with early-onset AD defined as age of onset <65 years according to previously published methods (Cruts, van Duijn et al. 1998; Wakutani, Watanabe et al. 2004).

About 80 ng DNA were amplified in a 25 µl reaction mixture containing.

10x PCR Buffer	2.5 µl
$MgCl_2$ (50 mM)	1.5 µl
dNTPs (2 mM)	1.0 µl
Pr-F (10 µM)	0.5 µl
Pr-R (10 µM)	0.5 µl
Taq DNA Polymerase (5 U/ µl)	0.2 µl
H_2O Milli-Q	17.8 µl
DNA	1.0 µl

The PCR thermocycler program contained following steps.

Denaturation	94°C – 2 min	
Denaturation	94°C – 20 s	38 cycles
Annealing	Tm°C – 10 s	
Elongation	72°C – 10 s	
Final elongation	72°C – 7 min	

Primers sequences for PSEN1 and PSEN2 genes are adopted from (Cruts, van Duijn et al. 1998).

Table 1. Primers for PSEN1 exons.

SNPs PSEN1	Primers	Annealing temperature, °C	Product length, bp
Exon1A	F: TTCTCCCCGCAATCGTTTCTCCAG R: GCCCATGTCCGCGGTGCCTTCC	71	297
Exon1B	F: AGGAGGGGCGGCCCGTTTCTCG R: AGCCTCTGCCACCACCGAGGATC	66	523
Exon2	F: TGGATGACCTGGTGAAATCCTATT R: CAGAAAACAAAGCCTCTTGAGGTT	64	223
Exon3	F: ACAAAGTTCTGTTTTTCTTTCCC	60	247

	R: CAGCATTTCTCAGAGGTGAGG		
Exon4	F: CGTTACCTTGATTCTGCTGA R: GACATGCTGTAAAGAAAAGCC	56	371
Exon5	F: GATTGGTGAGTTGGGGAAAAGTG R: ATACCCAACCATAAGAAGAACAGG	62	335
Exon6	F: GGTTGTGGGACCTGTTAATT R: TTAATTCTGAAAGACAGAC	52	149
Exon7	F: GGAGCCATCACATTATTCTAAA R: AACAAATTATCAGTCTTGGGTTT	65	326
Exon8	F: TTACAAGTTTAGCCCATACATTTT R: TCAAGTTCCCGATAAATTCTAC	52	215
Exon9	F: TGTGTGTCCAGTGCTTACCTG R: TGTTAGCTTATAACAGTGACCCTG	65	188
Exon10	F: CCAGCTAGTTACAATGACAGC R: TCAAAAAGGTTGATAATGTAGCT	59	345
Exon11	F: GGTTGAGTAGGGCAGTGATA R: TTAAAGGGACTGTGTAATCAAAG	63	275
Exon12	F: GTCTTTCCCATCTTCTCCAC R: GGGATTCTAACCGCAAATAT	54	199

Table 2: Primers for PSEN2 exons

SNPs PSEN2	Primers	Annealing temperature, °C	Product length, bp
Exon1	F: ACGTGGGTCTCTGAGGCGTGTAGC R: TGAGGTCTGCTCGGAGGGATGGA	70	447
Exon2	F: CAGGGCCAGGGGGAGGAA R: AAAAGCAGGTTGGGAGTCAC	68	303
Exon3	F: GTCCTCCACTGCCTTTGTCTCAC R: CTTCCCTTCTCCCTCCCGCATCAG	68	328
Exon4	F: GTCCTCCACTGCCTTTGTCTCAC R: CTTCCCTTCTCCCTCCCGCATCAG	68	328

Exon5	F: AGCCTCGAGGAGCAGTCAG R: GCAGACGGAGAGAAGCGT	64	241
Exon6	F: GGTATCAGTCTCAGGATCCTGGGG R: TGGGGAAGACTGGAGCTCGATG	61	265
Exon7	F: GTAAAGAGGGCCAGGTTGGG R: GTGCAGCACTGGGGACGATTT	72	387
Exon8	F: GGGCAGGCTCTTCTTCAGGG R: GAAAGCCACGGCCAGGAAG	68	251
Exon9	F: ACCGCCTGAGACGTGAACCTT R: TCCCTCTGCCCCTCCTGAACT	68	235
Exon10	F: CTCTGACCAGCTGTTGTTTC R: AGCCTCCACCCTCTGTCT	68	249
Exon11	F: TTCCATTCTGTGCACGCCTC R: ACCTGCCCCCACCACAATG	61	244
Exon12	F: ACACCAGGGATCACCACGCTCAC R: TGCCTCCTCCTCACCAAGTAAACA	59	344

Table 3. Primers for APP exons

APP	Primers	Annealing temperature, °C	Product length, bp
Exon 16	F: TAGAAAGAAGTTTTGGGTAGGCTTT R: AGAGTTAATAGGTCATTTGGCAAGACA	60	284
Exon 17	F: AATGAAATTCTTCTAATTGCGTTT R: TTCTCTCATAGTCTTAATTCCCACTT	61	343

2.2.1.6.2 Agarose gel electrophoresis

Agarose gel electrophoresis was used to verify the efficacy and specificity of PCR. Gel (2%) was prepared by dissolving of 2.4 g of agarose powder in the 1xTBE buffer (120 ml) at boiling temperature, and subsequent casting in the tray. PCR-product (8 µl) was mixed with the 6x Loading Dye Solution (2 µl) and loaded into each well. A DNA molecular

weight marker was loaded in one lane. Electrophoresis was carried out in a horizontal chamber containing 1xTBE buffer at 100 V for 60 minutes. Afterwards the gel was stained in the ethidium bromide staining bath (0.5 ug/mL) for 10-15 minutes and the bands were visualized using UV-transillumitanor and E.A.S.Y® Win32 Basic Software.

2.2.1.6.3 PCR-product purification

QIAquick PCR Purification Kit was used to remove primers, nucleotides, enzymes and salts from the reaction mixture after amplification and to prepare the PCR-product for the sequencing. The mixture of 1x volume of PCR-product and 5x volumes of PB buffer was applied on the QIAquick spin column and centrifuged for 1 min s to let PCR-product bind to the membrane of the spin column. For the washing of the membrane PE buffer (0.75 ml) was added to the QIAquick column and centrifuged for 1 min, afterwards the flow-through was discarded and the column centrifuged for an additional 1 min. To elute PCR-product EB buffer (50 µl) was added to the center of the membrane and centrifuged for 1 min. All the centrifuging steps should be carried out at lower than 15 000xg (13 000 rpm) velocity. The purified PCR-product was stored at -20°C.

2.2.1.6.4 Dye terminator cycle sequencing reaction

BigDye® Terminator v3.1 Cycle Sequencing Kit containing a set of ddNTP terminators labelled with fluorescent dyes was used for the cycle sequencing reaction with the purified PCR amplification product as template (2.5 ml). For the cycle sequencing reactions it was used one primer out of the pair (see tables 1, 2, 3 underlined primers).
Reaction mixture contained.

5x Sequencing Buffer	1.0 µl
BigDye® Terminator v3.1 Cycle Sequencing Kit	1.0 µl
Purified PCR-product	2.5 µl
Primer	0.5 µl

The dye terminator cycle sequencing reaction was performed using the following program.

Denaturation	80°C – 4 min	
Denaturation	96°C – 10 s	25 cycles
Annealing	50°C – 5 s	
Elongation	60°C – 4 min	

2.2.1.6.5 Post-Cycle Sequencing Reaction purification

Dry Sephadex® G-50 was loaded into a MultiScreen®-HV plate using the MultiScreen® 45 µl column loader as described in the instructions from Millipore (Bedford, MA), after which 300 µl of MilliQ-water was added to each well. The resin was allowed to swell at room temperature for 3 hours. Then the MultiScreen®-HV plate was placed on top of a 96-well plate. To pack the columns, the entire setup was centrifuged at 530xg (1800 rpm) for 5 minutes in a Megafuge 1.0R centrifuge. The columns were washed once with 150 µl of MilliQ-water and again centrifuged at 530xg (1800 rpm) for 5 minutes. Subsequently, 35 µl of MilliQ-water was added to the 5 µl cycle sequencing reactions, mixed and transferred onto individual wells of the prepared MultiScreen®-HV plates. The MultiScreen®-HV plate was placed on top of a MicroAmp® Optical 96-Well Reaction Plate. The entire microplate setup was centrifuged at 530xg (1800 rpm) for 5 minutes, thus 40 µl eluate containing purified dye labelled extension products of the cycle sequencing reaction was collected in the well of the MicroAmp® Optical 96-Well Reaction Plate.

2.2.1.6.6 Sequencing

After the post-cycle sequencing reaction purification step the MicroAmp® Optical 96-Well Reaction Plate containing the purified product was loaded into the 3100-Avant Genetic Analyser instrument containing 3130 & 3100-Avant Capillary array 50 cm (4 capillars). For the collection of the data the following settings in the3100-Avant Data Collection Software were chosen: Run Module – RapidSeq36_POP6Default Module, Analysis Module – BC-3100APOP6 RR_SeqoffFtoff.saz. After the run was completed the collected data could be observed in the Sequencing Analysis 3.7 Software window. Sequences of all exons were aligned and compared between 37 individuals of the EOAD group as well as the comparison with the Entrez Gene available genomic sequencies NT_026437.11 for PSEN1 and NT_004559 for PSEN2 was performed.

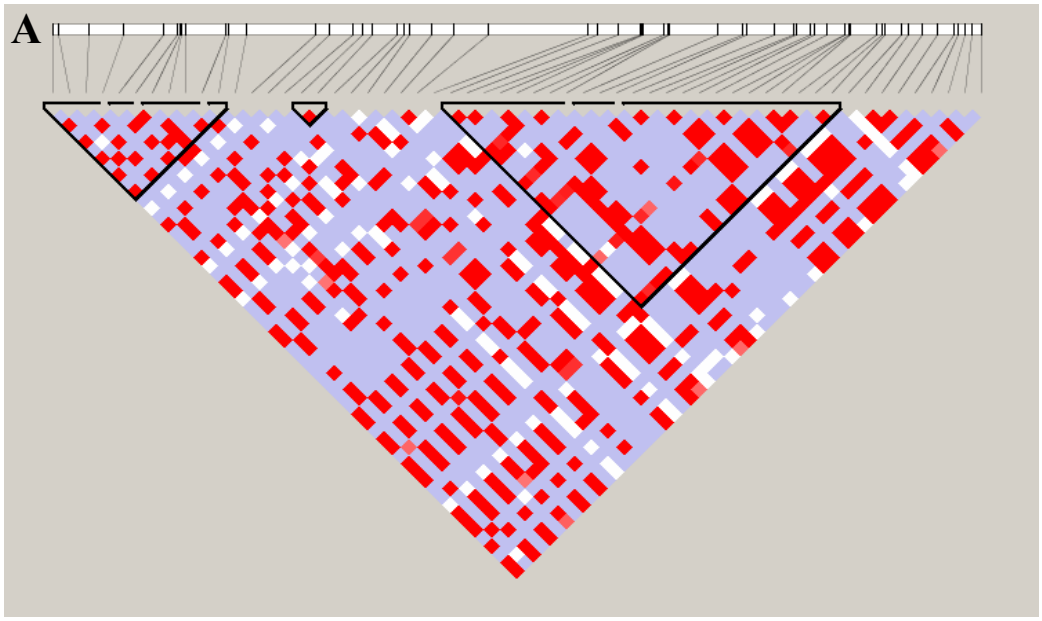
2.2.1.6.7 Description of PSEN1 and PSEN2 linkage disequilibrium plots

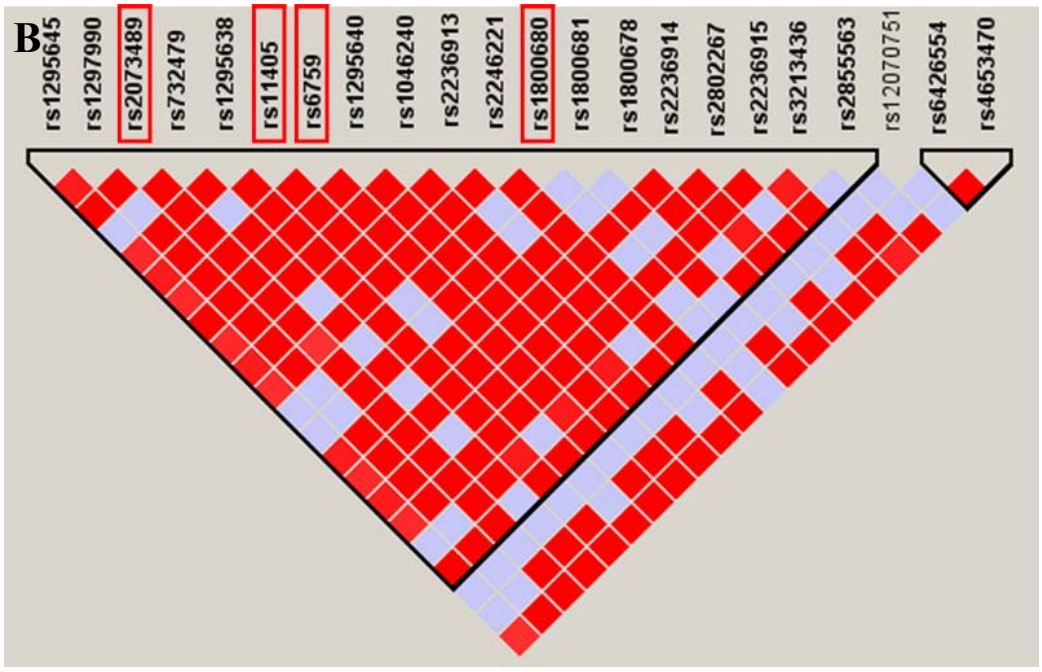
Haplotype analyses for PSEN1 and PSEN2 was performed in order to discriminate potentially functional haplotypes. One hundred thirty-three characterized polymorphisms for the PSEN1 gene and 30 polymorphisms for the PSEN2 gene for the CEPH population (90 Utah residents (30 trios) with ancestry from northern and western Europe, CEU) were downloaded from the HapMap project website (http://www.hapmap.org, release 23 by March 2008). The Haploview 3.32 software was used to generate the linkage disequilibrium (LD) plots from the downloaded data for the PSEN1 and PSEN2 genes (Haploview, http://www.broad.mit.edu/mpg/haploview) (Figure 8).

In the Figure 8 LD plots for PSEN1 and PSEN2 genes are represented as D'/LOD scheme of coloured squares. Each diagonal of the plot represents a definite SNP and each square indicates the pairwise magnitude of LD between two SNPs corresponding to the crossing diagonals.

The normalized disequilibrium coefficient D' and logarithm of the odds (LOD) are the parameters used to assess the probability of two loci to be inherited together and the level of recombination between them. The maximum value of D' equals to 1 that indicates the complete LD between two loci. LOD value reflects the statistical significance for LD, such as LOD value equals to 2 indicates that odds are 100 to 1 in favour of linkage. Red and pink squares on the D'/LOD scheme correspond to statistically significant LD between the pair of SNPs (LOD>2) with the maximum pairwise D' value for the red squares (D'=1). White squares indicate pairwise D' values lower than 1 with no statistically significant evidence of LD (LOD<2). Blue squares signify pairwise D' values of 1 but without statistical significance (LOD<2). Haplotype block is depicted as a black triangular comprising SNPs inherited together. Only SNPs with the minor allele frequency higher than 5% are depicted on the LD plot.

The PSEN1 LD plot in the Figure 8A built of 57 SNPs didn't contain expanded regions of LD including mainly the blue squares with D'=1 and LOD<2 thus demonstrating the low evidence for haplotype blocks existence, whereas PSEN2 LD plot in the Figure 8B built of 19 SNPs contained a spread region of red squares with D'=1 and LOD>2 that gives argument for the construction of haplotype block within this region.





D'/LOD-scheme	
White	D'<0, LOD<2
Blue	D'=1, LOD<2
Pink	D'<0, LOD>2
Red	D'=1, LOD>2

Figure 8. LD plots of PSEN1 (A) and PSEN2 (B) genes represented as D'/LOD-schemes (tagging SNPsmarked in red rectangular)

Two haploblocks of PSEN2 gene were created using Haploview 3.32 software. The major haploblock containing 19 SNPs covered the region 23kb. The second block consisting of 2 SNPs had size less than 1kb.

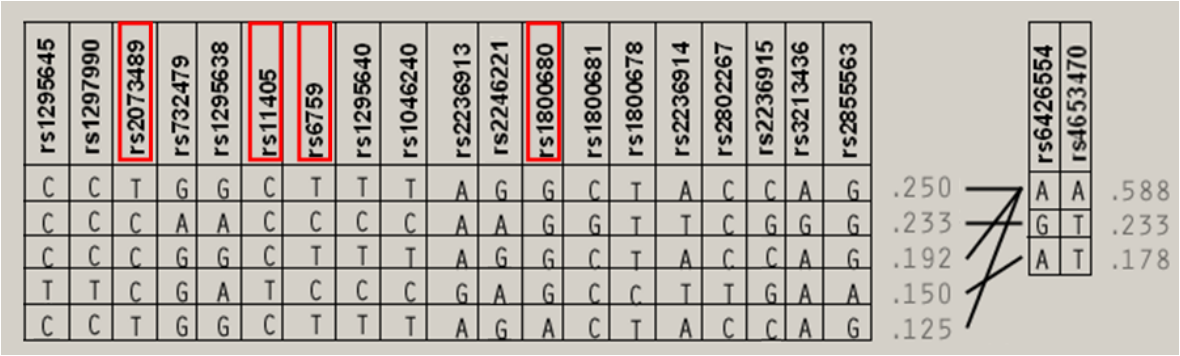


Figure 9. Haplotype frequencies of PSEN2 gene

Figure 9 represents five the most common haplotypes of major 23kb haploblock that comprise 98% of all possible combinations of SNPs and three haplotypes generated for the second haploblock. All three haplotypes of the minor block are linked with haplotypes of the major block, therefore the major haploblock was chosen for the selection of tagging SNPs.

2.2.1.6.8 Selection of tagging SNPs for PSEN2 gene

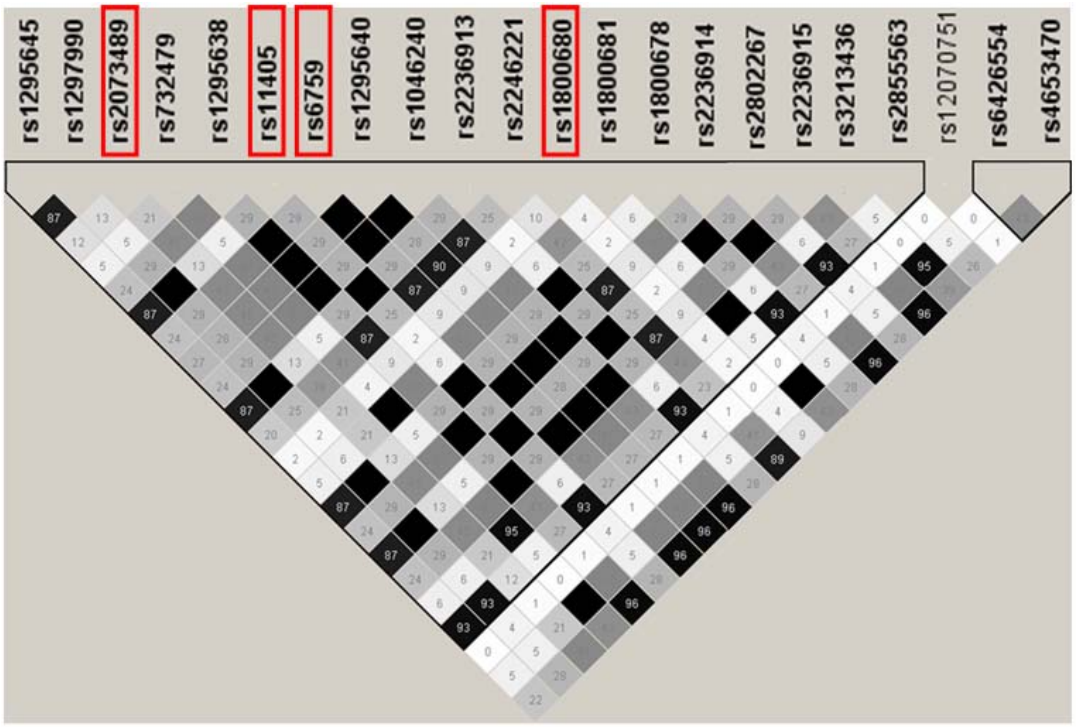


Figure 10. r^2- scheme of PSEN2 gene

The squared correlation coefficient r^2 is a measure of statistical association between two loci indicating the power to detect an effect at one locus by typing another one, whereas D' value provides the information about the history of recombination. Therefore r^2 coefficient is used for tagging SNPs selection. Figure 10 represents the r^2- scheme for PSEN2 gene with the pairwise values of r^2 inside each square. The maximum pairwise correlation value 1.0 signifies that genotyping anyone of the pair of SNPs will provide the same information.

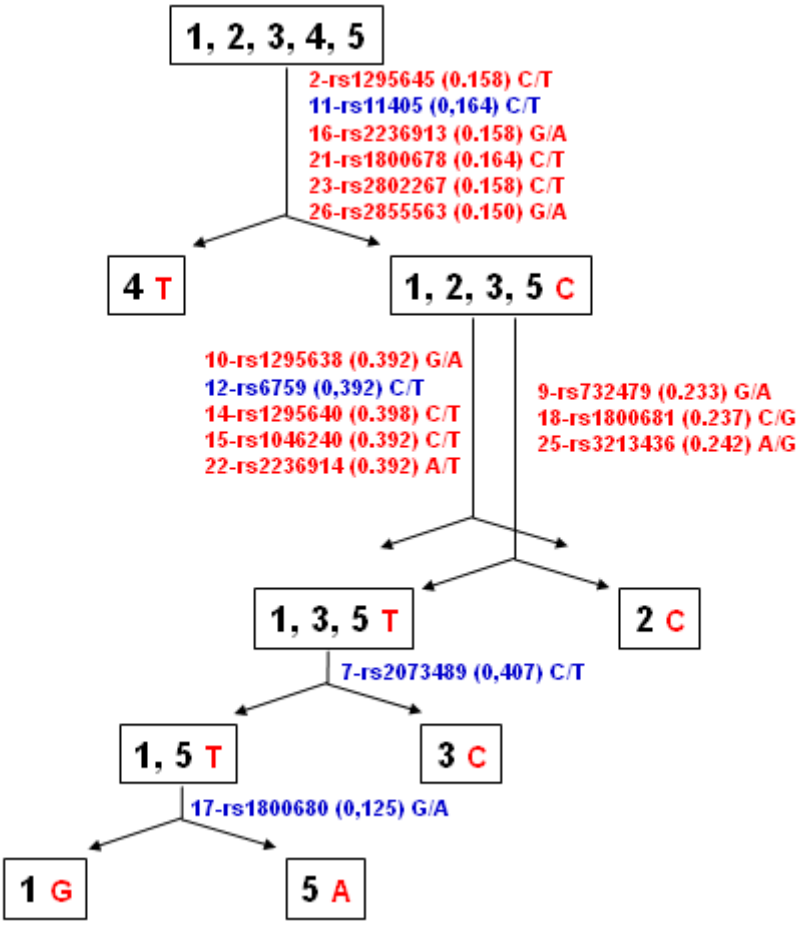


Figure 11. The scheme of selection of tagging SNPs

Figure 11 represents the strategy of tagging SNPs selection. The strategy is based on the step-by-step distinction of each haplotype from the rest ones by sets of SNPs with pairwise r^2 cut-off value 0.8 within the set.

Numbers 1 to 5 in the rectangular symbolize haplotypes that have to be distinguished. At the first step haplotype 4 can be distinguished from the rest of haplotypes by any of 6 SNPs included in the first set: rs1295645, rs11405, rs2236913, rs1800678, rs2802267 and rs2855563. The minor allele frequency for each SNP is shown in the parenthesis. These SNPs have the same distribution of alleles among the haplotypes (Figure 9) and pairwise r^2 coefficient is higher than 0.8 for all SNP pairs inside this set (Figure 10). The selection of tagging polymorphism in this set was based on the choice of that with the highest minor allele frequency and exonic location. Thus rs11405 was chosen as first tagging SNP.

At the second step haplotype 2 can be discriminated by any of two represented sets. The first set contains rs1295638, rs6759, rs1295640, rs1046240 and rs2236914. The second set contains rs732479, rs1800681 and 3213436. The pairwise r^2 coefficient value is higher

than 0.8 for SNP pairs within each set, but for any two SNPs from the different subsets the pairwise value ranges from 43 to 0.47 (figure 10). SNPs included in the same set have the same distribution of alleles among the haplotypes (Figure 9). Since usage of any of these two sets provides the same outcome, the selection of tagging polymorphism from one of them was based on the choice of SNP with the highest minor allele frequency, exonic location and the highest number of linked SNPs. Thus rs6759 was chosen as a tagging SNP. Subsequently the haplotypes 3 and 5 can be differentiated by rs2073489 and rs1800680 respectively. In such a way 4 polymorphisms rs2073489, rs11405, rs6759 and rs1800680 were defined as tagging SNPs distinguishing 5 haplotypes of the major PSEN2 haploblock. Rs11405 and rs6759 lead to synonymous amino acid substitutions Ala23Ala and Asn43Asn in the exon3 of PSEN2 gene. Rs2073489 is an intron 2 C to T exchange; rs1800680 G to A substitution is located in the intron 6.

2.2.1.6.9 Genotyping of PSEN2 tagging SNPs with TaqMan Real-Time PCR

Predefined and validated assays for TaqMan Real-Time PCR were used for the genotyping of tagging SNPs of the PSEN2 gene: rs2073489 (C_340454_1_), rs11405 (C_340278_20), rs6759 (C_9633596_20) and rs1800680 (C_9633614)). The genotyping assay contains sequence-specific forward and reverse primers to amplify the polymorphic sequence of interest and two TaqMan® MGB probes. One probe labeled with VIC® dye that detects the Allele 1 sequence and another probe labeled with FAM™ dye that detects the Allele 2 sequence.

Each reaction consisted of a 20 ng DNA template, 6 µl 2xTaqMan Universal PCR Master Mix, No AmpErase UNG®, and 0.3 µl 40xTaqMan SNP Genotyping Assay.

The reaction was performed under the following conditions:

pre-read run	allelic discrimination	60ºC – 1 min	
amplification run	absolute quantification	95ºC – 10 min 92ºC - 15 s 60ºC - 1 min	40 cycles
post-read run	allelic discrimination	60ºC – 1min	

To carry out the allelic discrimination it's necessary to perform the pre-read run to collect the fluorescent signal baseline data, then amplification run and afterwards the post-read run that collects the fluorescent signal after the amplification and subtracts it from the signal of the pre-read. Then the magnitudes of signals from different fluorophores are compared to each other and to the non-template control by the software and obtained data are presented as it is shown on the Figure 12.

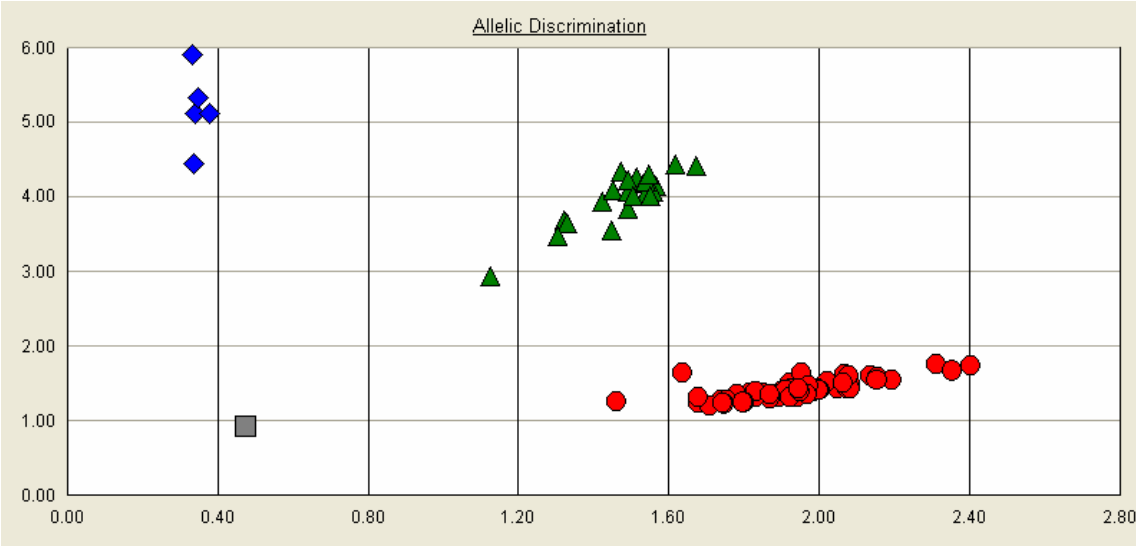


Figure 12. Allelic discrimination using TaqMan Real-Time PCR

2.2.1.7 Statistical analysis

Statistical analysis was performed using the SPSS statistical package Version 12.0 (SPSS Inc., Chicago, USA). The fit of genotype frequencies to the Hardy-Weinberg equilibrium was tested by a chi-square test. Differences in genotype frequencies in different subgroups were examined using a Pearson chi-square test. For assessing the effects of genetic variants on Aβ42 levels, the nonparametric Jonckheere Terpstra trend test was used to look for predefined trends in the direction of wildtype > heterozygous > homozygous carriers of the respective genotype.

Linear regression analysis was used to evaluate the strength of associations of Aβ42 concentrations with haplotype 1 in the EOAD and haplotype 2 in the LOAD, APOE E4 allele, age at disease onset (three age groups), MMSE. Aβ42 concentrations between the haplotypes were examined for significant differences using a general linear model analysis

and adjusting for age at disease onset, gender and APOE allele E4 number of copies. A value of $P<0.05$ was chosen as the cut-off level for statistical significance.

2.2.2 Project2

2.2.2.1 Study population

An unselected cohort of 304 in-patients suffering from a depressive episode of either unipolar or bipolar depression was collected from three psychiatric hospitals in the central area of Berlin, Germany. Patients were followed until 3 weeks of antidepressant drug treatment, and the 179 patients characterized for drug response were genotyped for FKBP5. Details of the study design have been described earlier (Kirchheiner, Nickchen et al. 2007). Briefly, diagnosis of major depression was confirmed by the Mini International Neuropsychiatric Interview (M.I.N.I.), and severity of depression was rated by the 21-item Hamilton depression rating scale (HDRS). Exclusion criteria were age younger than 18 years, Non-Caucasian ethnicity (determined by asking for birth place of parents and grandparents), inability to give informed consent, other diagnoses as first diagnoses such as drug or alcohol addiction, personality disorders as reason for depressive symptoms, or organic mental disorder.

For assessment of response, the sum of HDRS at day 21 was compared to the sum at day 1 of the study. Response was measured as percent decrease in HDRS. A minimum HDRS score of 8 was used as inclusion criteria.

All patients had to receive at least one antidepressant drug during the three weeks of the observation period. If more than one antidepressant drug was prescribed, antidepressant drugs were regarded to contribute to response if medication was started at least one week prior to response assessment (HDRS21) on day 21 and if it was not stopped before 3 days prior to severity assessment (HDRS1). Thus, all antidepressant drugs taken during the time frame of 3 days prior to first visit and 14 days after first visit were regarded to potentially contribute to drug response assessed on day 21.

The study was approved by the ethics committee of the Charité University Medical Centre of the Humboldt University and all patients gave their written informed consent to participate in the study.

2.2.2.2 Genotyping

Genomic DNA was obtained from whole blood using automatized DNA-extraction procedures based upon binding to Magna Pure® magnetic particles. Genotyping for the rs1360780 and rs3800373 variants in the *FKBP5* gene was performed with commercially available TaqMan allele discrimination assays (C___8852038_10 for rs1360780, and C__27489960_10 for rs3800373) according to the protocol supplied by the manufacturer. Amplification reactions were carried out in a 7300 Real Time PCR System with an initial hold step at 95°C for 10 min and 40 cycles of a two-step PCR with denaturation at 92°C for 15 s, annealing and elongation at 60°C for 1 min. TaqMan assays were performed in a reaction volume of 12.5 µl comprising 20 ng genomic DNA, 1 x SNP genotyping assay and 1 x Universal Master Mix, No AmpErase UNG. All analyses were carried out using internal controls for each genotype and at least 10% of the samples were done in duplicate.

2.2.2.3 Statistics

Antidepressant drug response was analyzed by calculating the percent of reduction in HDRS during 3 weeks of treatment: $(HDRS_1\text{-}HDRS_{21})/HDRS_1*100$ with $HDRS_1$ and $HDRS_{21}$ being the total score of the 21-item Hamilton depression rating scale at day 1 (baseline) and day 21, respectively. Similar as defined by others (Licinio, O'Kirwan et al. 2004), patients were referred to as responders if a more than 50% decrease in HDRS of was observed.

Statistical testing for comparison of frequencies of responders in each genotype group was performed using the Chi-square test or the exact Fisher test if sample size in the subgroups was below 5. Hardy-Weinberg equilibrium was tested with the Chi-square test. The effect size was calculated as Odds Ratio. Statistical testing for differences in mean values between groups was done using univariate analysis of variance (ANOVA) or t-test for two group comparisions.

To explore joint effect, confounding or effect modification between the different clinical predictors of response, a binary logistic regression analysis was performed. The dependent variable was responder or non-responder. As covariates, gender, number of previous depressive episodes (categorized into none, between 1 and 3, and more than 3), number of manic episodes, baseline severity of the depressive episode (categorized into HDRS score < 18, and >18), the hospitals (3 centers), and the FKBP5 genotype was included. All

statistical tests were done using the SPSS statistical software version 11 (SPSS Inc, Chicago, USA).

2.2.3 Project3

2.2.3.1 Study population

In a controlled double-blind crossover design, tryptophan depletion was tested in 24 men with alcohol dependence during inpatient withdrawal treatment at minimum 7-21 days after the last drink of alcohol. Patients with diagnosis of alcohol dependence according to ICD 10 (F10.2) or DSM IV were included if they had no concurrent diagnosis of major depression or other psychiatric disorders such as misuse of other drugs than alcohol beside tobacco smoking, psychotic disorder, dementia or any brain organic disorder; actual panic disorder. All patients were hospitalized for withdrawal treatment, and a minimum period of 7 days between study inclusion and the last drink of alcohol was prerequisite. The patients had to be free of vegetative, delirium or predelirium withdrawal symptoms and not taking any drugs fro relief of withdrawal symptoms. The patients were not included if they were taking any serotonergic acting drug (such as selective serotonin reuptake inhibitors) within the last 14 days before trial.

The participants ingested either a tryptophan-free (depletion) or tryptophan-containing (placebo) amino acid drink (Hood, Bell et al. 2005). The amino acid drink was prepared by the hospital pharmacy and contained the following amounts of amino acids: 5.5g of L-alanine, 4.9g of L-arginine, 2.7g of L-cystine, 3.2g of glycine, 3.2g of L-histidine, 8.0g of L-isoleucine, 13.5g of L-leucine, 11.0g L-lysine-HCL, 3.0g of L-methionine, 5.7g of L-phenylalanine, 12.2g of L-proline, 6.9g of L-serine, 6.9g of L-threonine, 6.9g of L-tyrosine, 8.9 L-valine. For the tryptophan-containing drink, L-tryptophan 2,3g was added. The amino acid mixture was dissolved in 0.5 L tap water.

Patients were included after a minimum period of 7 days after the last alcohol drink and free of any withdrawal symptoms. Participants were instructed to refrain from tryptophan containing food 24 hours prior to taking the amino acid drinks. For this purpose, any consumption of beef, chicken, or other poultry, milk products, eggs, nuts, peas, potatoes, chocolate, or tropical fruits was not allowed. The participants were not allowed to eat or drink anything apart from tap water during the observation time of 5 h after the amino acid drink. The order of the two study

days (placebo or depletion) was randomized and the exploratory analysis did not reveal any significant sequence effects.

Clinical symptoms scales for depressive symptoms (MADRS, Montgomery-Asberg Depression Rating Scale (Montgomery and Asberg 1979)) anxiety state (STAI, State Anxiety Inventory (Spielberger 1970), HAMA, Hamilton Anxiety Scale (Hamilton 1959) and a visual analogue scale for craving symptoms (Huskisson 1974; Nakama, Chang et al. 2008) was performed prior and 5h after the amino acid drink. Blood sampling for genotyping, mRNA analyses and monoamine metabolite analysis was performed prior to the tryptophan depletion drink and at 5 h after the tryptophan depleted or tryptophan supplemented amino acid drink. All volunteers have given their written informed consent and the study has been approved by the ethics committee of the University Medicine Göttingen.

2.2.3.2 Monoamine metabolite analysis in serum

L-tryptophan (L-Trp), 5-hydroxy-tryptamine (5-HT), 5-hydroxyindole acetic acid (5-HIAA), and homovanillinic acid (HVA) concentrations were measured in serum directly before and at 5 hours after intake of a tryptophan-free amino acid drink (depletion) or tryptophan containing amino acid drink (control) with HPLC and fluorescence detection according to the methods described earlier (Heuther, Hajak et al. 1992). 300 µl serum was filtered through a micromembrane before injection into the HPLC (cutoff 3 kD; VIVASPIN 500®; Sartorius AG, Göttingen, Germany). After ultrafiltration, the sample was diluted with equal volume of HPLC buffer, and 10 µl was injected into the column. The major tryptophan metabolites were measured by HPLC with online fluorescence and electrochemical detection according to the methods described (Heuther, Hajak et al. 1992). The limit of quantification was 0,8 ng/ml for serotonin (5-HT), 0,5 ng/ml for 5-hydroxyindole acetic acid (5-HIES), 1,6 ng/ml for für homovanillinic acid (HVA), and 10 ng/ml for free L-tryptophan.

2.2.3.3 Genetic analyses

The insertion/deletion polymorphism in the SLC6A4 gene coding for the high affinity serotonin transporter (5-HTT) was genotyped as described earlier using as forward primer 5'-GGCGTT-GCCGCTCTGAATGC-3' and as reverse primer 5'-GAGGGACTGAGCTGGACAACCAC-3' (Kaiser, Tremblay et al. 2002).

Total RNA was extracted from 2.5 ml whole blood with the PAXgene™ Blood RNA Kit (QIAGEN Inc., Valencia, CA, USA). Total RNA concentration was determined by absorbance readings at 260 nm with a spectrophotometer. About 1 µg of total RNA was transcribed into cDNA (single stranded complementary DNA) in a 30 µL reaction volume using oligo-dT primers (Invitrogen). Real-time PCR assays were performed by the TaqMan technology on an 7300 Real Time PCR System. Each PCR reaction contained 6 µL cDNA template (corresponding to approximately 120ng of the total extracted RNA), 10 µl 2×TaqMan Universal PCR Master Mix, No AmpErase® UNG (Applied Biosystems), 1 µl of SLC6A4 Hs00169010_m1 20xAssay supplemented with water to a final volume of 20µL, according to the manufacturer's instructions. As standard, human TATA box binding protein 20xAssay Hs00427620_m1 was used. Both samples and standards were performed in triplet. A non-template control was included in every assay. Samples were heated for 10 min at 95°C and then subjected to 40 cycles of denaturation at 95°C for 15 s and annealing and elongation at 60°C for 1min.

The insertion/deletion polymorphisms in the gene coding high affinity serotonin transporter (SERT) (SLC6) was genotyped as described earlier using as forward primer 5'-GGCGTTGCCGCTCTGAATGC-3' and as reverse primer 5'-GAGGGACTGAGC-TGGACAACCAC-3' (Kaiser, Tremblay et al. 2002).

The reaction was performed in the total volume of 25 µl with the following content.

10xBuffer	2.5 µl
dNTPs-GTP (2mM)	2.5 µl
deaza GTPs (1 mM)	1.25 µl
$MgCl_2$ (50mM)	0.75 µl
DMSO (3%)	0.75 µl
Pr_forw (10µM)	0.5 µl
Pr_rev (10µM)	0.5 µl
Taq Polymerase	0.15 µl
H_2O	13.85 µl
DNA	1 µl

The following PCR profile was used.

Denaturation	94°C – 2 min	
Denaturation	94°C – 30 s	38 cycles
Annealing	64°C – 30 s	
Elongation	72°C – 1 min	
Final elongation	72°C – 7 min	

8 µl of PCR product was mixed with 2 µl of the loading buffer. The mixture was applied to the 2% agarose gel and run for 90 min at 100V. 100 bp marker was used for the determination of the product length. The gel was stained in the ethidium bromide solution and visualized in the transiluminator under UV-light.

The genotype of the samples was determined according to the presence of the following bands:

l/l	l/s	s/s
528	528	-
-	484	484

2.2.3.4 Statistical analyses

Analyses for differences between genotype groups were performed by repeated measurements analysis of variance with the 5-HTTLPR genotype as between-subject factor and depletion/placebo as within subject factors. If differences between post and pre values were tested, baseline values were included as covariate in order to correct for influences of the amount of baseline values.

3 RESULTS

3.1 Project1

3.1.1 Clinical characteristics of EOAD, LOAD and control groups

The AD group included 191 individuals (115 female and 76 male) with a mean age of 71 ranging from 30 to 87 years. Thirty-seven (23 female and 14 male) out of 191 patients were younger than 65 at onset of the disease and were referred to the early-onset AD group (EOAD). Family history was available for 32 individuals with EOAD - 19% had positive AD family history; 22% had relatives with cognitive decline symptoms, and 6% had other non-neurological diseases such as cardiovascular diseases, diabetes and alcoholism. The mean age of onset in this subgroup was 58 (between a range of 30 – 64). One hundred fifty four patients (92 female and 62 male) had an age of onset above 65 and were referred to the late-onset AD group (LOAD) with a mean onset age of 74 (between a range of 66 – 87). In the LOAD group, family history was available for 120 individuals. Of those, 5% had positive AD family history, 18% had relatives with cognitive decline symptoms and 5% had other non-neurological diseases.

The clinical characteristics of the studied AD and control groups are summarized in Table 4.

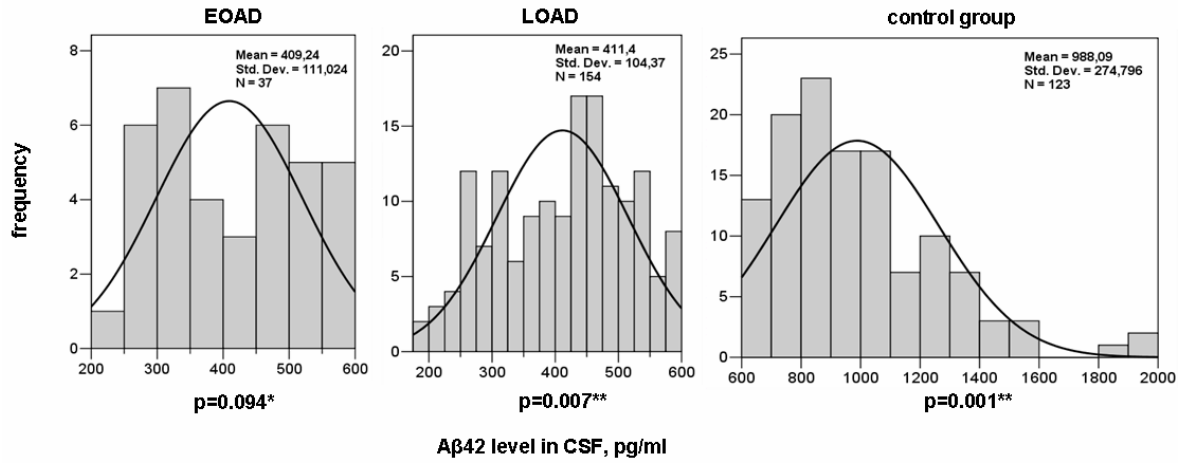
Table 4. Clinical characteristics (gender, age, Aβ42 and tau-protein level in CSF, MMSE value and APOE genotype) for EOAD, LOAD, AD and control groups.

		EOAD	LOAD	AD group	Control group
N		**37**	**154**	**191**	**123**
Gender	**female**	23	92	115	54
	male	14	62	76	69
Age at onset, y.o.		57.86±6.93	74.25±5.23	71.07±8.56	62.18±10.29
CSF Aβ42 level, pg/ml		409.24±111.02	411.40±104.37	410.98±105.40	988.09±274.81
CSF tau-protein level, pg/ml		580.49±381.15	560.27±348.01	564.21±353.74	249.89±120.13
MMSE		20.97±7.26	22.88±4.65	22.48±5.33	28.01±2.29
Positive family		13 (35%)	25 (16%)	38 (20%)	28 (23%)

history					
APOE genotypes	**E2/E2**	0	0	0	0
	E2/E3	2	3	5	11
	E2/E4	0	5	5	0
	E3/E3	13	38	51	68
	E3/E4	10	64	74	20
	E4/E4	8	21	29	0
APOE alleles	**E2**	2	8	10	11
	E3	38	143	181	167
	E4	26	111	137	20

EOAD: Early-onset Alzheimer's disease; LOAD: Late-onset Alzheimer's disease; CSF: cerebrospinal fluid; MMSE: mini-mental state examination score; APOE: apolipoprotein E alleles: x = any other than APOE E4 allele. A chi-square test was used to test for significant differences in genotype frequencies between EOAD and control groups: *: p=0.037. **: p=0.026

The non-demented disease controls comprised 123 individuals (54 female and 69 male) whose short term slight cognitive decline was caused by other factors than dementia. None of these patients displayed clinical features of neurodegenerative disease. The mean age of the control group was 62 years (35 – 82 years). MMSE. score was 28.01±2.29. In the control group family history was available for 122 individuals, 5% had positive AD family history, 25% had relatives with cognitive decline symptoms and 9% - with non-neurological diseases. The range of Aβ42 concentrations in the CSF in the AD group was 176 to 600 pg/ml and the range in the control group was 606 to 1966 pg/ml.
The distribution of the Aβ42 levels in CSF that is a key parameter for the sampling of AD and control groups is depicted in Figure 13.



* - Shapiro-Wilk (N<50)

**- Kolmogorov-Smirnov (N>50)

Figure 13. The distribution of Aβ42 in CSF concentrations in EOAD, LOAD and control groups.

3.1.2 Analyses of correlations of clinical parameters

Analysis of correlation of Aβ42 concentration in CSF with other clinical parameters was performed to assess the possible influence of different variables such as gender, MMSE value, age at disease onset on the Aβ42 level as the main independent variable of our study.

Each of the EOAD, LOAD and control groups was stratified by gender and analysed by Jonkheere-Terpstra non-parametric test to test the relationship of Aβ42 or tau-protein CSF concentrations and gender. Figure 14 demonstrates the absence of significant difference of CSF biomarker concentrations between gender subgroups. By the analysis of the whole AD group there was not observed any influence of gender on CSF biomarkers (not depicted on the Figure 14 ; p=0.112 for Aβ42; p=0.206 for tau-protein), although for the female subgroups of EOAD, LOAD, whole AD and control groups the Aβ42 mean concentrations were slightly higher.

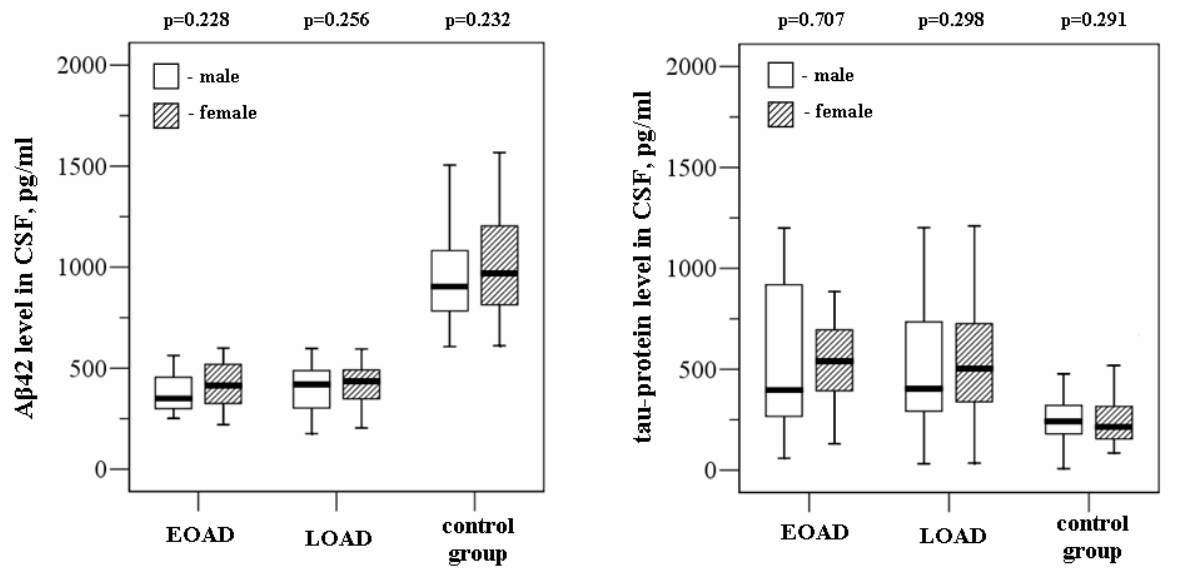


Figure 14. Comparison of Aβ42 and tau-protein CSF levels in male and female subgroups

Analysis of correlations between Aβ42 concentration and age at disease onset performed by Spearman's rank correlation test didn't reveal any statistically significant relationship neither in LOAD (R^2=0.021, p=0.064) nor in the control group (R^2=0.001, p=0.693) (Figure 15). EOAD was not analysed for correlation due to the small sample size. Analysis of correlation between Aβ42 concentration and age at lumbar puncture also didn't show any statistically significant correlation: LOAD (R^2=0.022, p=0.061), control group (R^2=3,575x$10^{(-5)}$, p=0.901).

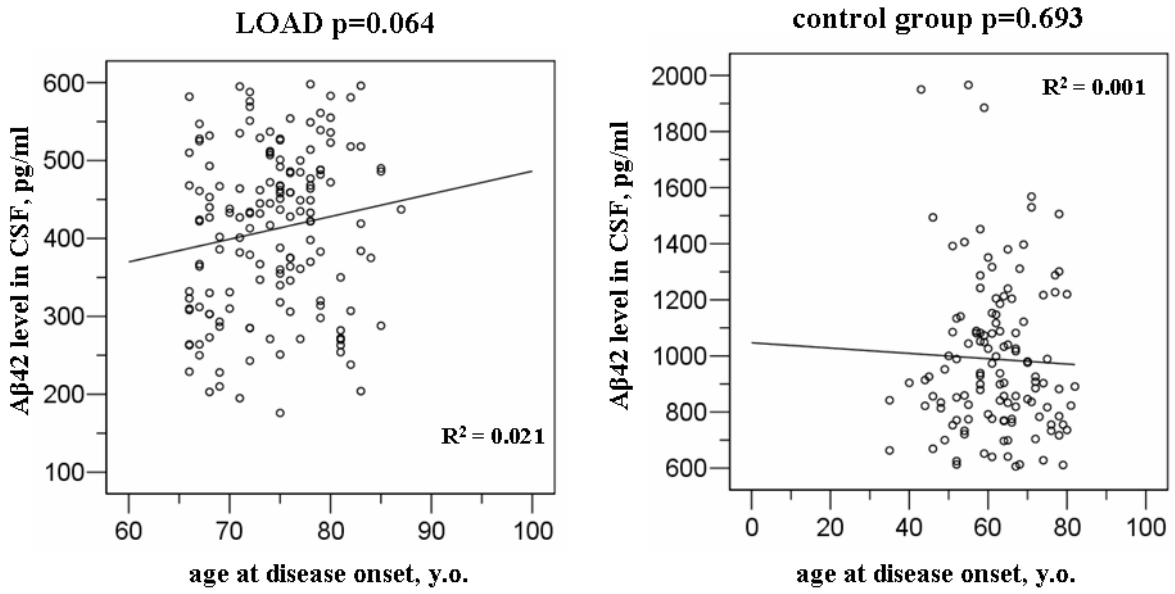


Figure 15. Correlation of Aβ42 level in CSF with the age at lumbar puncture in LOAD and control groups.

Even though it was found a correlation between CSF levels of Aβ42 and tau-protein in the control group (r = 0.191, p=0.034) the same was not observed neither in the whole AD group (r = -0.023, p=0.755) nor in the LOAD (r = -0.014, p=0.860 thus negating the potential effects of colinearity between biomarkers.

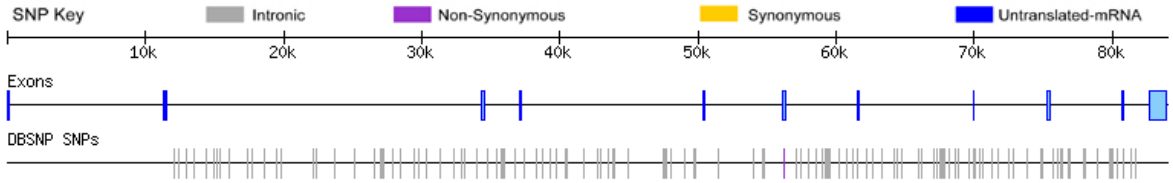
There was observed significant positive correlation between Aβ42 CSF concentrations and MMSE value in the whole AD group (r=0.173, p=0.032), but not in the LOAD (r=0.169, p=0.063) or control group (r=0.099, p=0.305).

It was found no correlation of tau-protein with MMSE value in AD group (r = -0.151, p=0.064), LOAD group (r = -0.110, p=0.232) or in the control group (r = -0.033, p=0.735). For the LOAD the correlation was not observed as well (r=-0.110, p=0.232). There was no correlation observed between MMSE value and the age of onset in the AD group (r = -0.028, p = 0.729).

The additional nominal variable intended to describe presence or absence of positive family history was introduced to the analysis. The presence of positive family history didn't influence the Aβ42 level in CSF.

3.1.3 Sequencing of exonic regions of PSEN1 and PSEN2 in EOAD

PSEN1



PSEN2

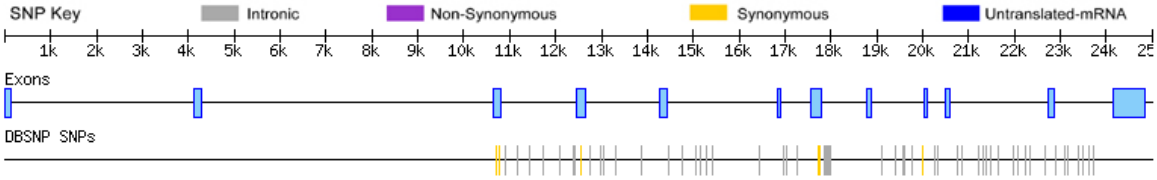


Figure 16. The schemes of PSEN1and PSEN2 genes

Table 5. Boarders and length of PSEN1 and PSEN2 exonic regions that were sequenced in the 37 EOAD individuals

PSEN1 exon number	PSEN1 contig sequence NT_026437.11	Length, bp	PSEN2 contig sequence NT_004559.13	Length, bp
Exon1 (UTR)	54602932-54603044	112	3261117-3261259	142
Exon2 (UTR)	54614256-54614337	81	3265220-3265405	185
Exon3	54614428-54614567	139	3271743-3271903	160
Exon4	54637258-54637508	250	3273560-3273774	214
Exon5	54640027-54640168	141	3275393-3275534	141
Exon6	54653314-54653381	67	3277946-3278013	67
Exon7	54659105-54659325	220	3278684-3278904	220
Exon8	54664492-54664590	98	3279890-3279988	98
Exon9	54672847-54672933	86	3281133-3281216	83
Exon10	54678230-54678403	173	3281598-3281699	101
Exon11	54683587-54683705	118	3283862-3283980	118
Exon12	54685595-54686862	1267	3285279-3285953	674

All exons of both PSEN1 and PSEN2 genes (Figure 16, Table5) as well as exons 16 and 17 of APP were sequenced in 37 EOAD patients. Heterozygous genotypes coding amino acid changes were found in three patients. In one patient, the Met146Leu substitution in exon 5 of PSEN1 (cont54640124, NT_026437.11) was detected, a mutation which was described earlier as being correlated with common AD (Sherrington, Rogaev et al. 1995). The patient had an Aβ42 level of 334 pg/ml and a MMSE value of 18 at the time of the first examination. The age of onset of the dementia was 38.

Two amino acid substitutions, which have not been described thus far, are Gln223Arg (cont 54659224) in exon 7 of PSEN1 and Val150Met (cont 3275484, NT_004559.13) in exon 5 of PSEN2. These substitutions were identified in two other patients. The patients had an Aβ42 level of 292 pg/ml and 503 pg/ml and a MMSE value of 25 and 30 respectively at the time of the first examination. The age of onset of the dementia was dated as 30 and 52. All three patients with the exonic mutations had a positive family history of AD. The three individuals were subsequently were excluded from the analyses on correlations between Aβ42and haplotypes of PSEN2. There were no mutations found in the exons 16 and 17 of the APP gene.

Moreover, in this study we found several novel mutations in PSEN1 and PSEN2 noncoding regions. In one patient with 553 pg/ml Aβ42 concentration there were found two mutations: G to A exchange in the 5'-UTR of PSEN1 (cont 54602870) and G to C exchange in intron2 of PSEN2 (cont 3265889). The last mutation also was found in another patient with 512 pg/ml Aβ42 level.

Another nucleotide changes A to G in intron6 (cont 3278214) and C to T in intron 9 (cont 3281580) of PSEN2 were determined in two different patients. One of the patients has Aβ42 CSF level of 339 pg/ml and the second has 585 pg/ml Aβ42 level and positive familial history of AD.

Figure 17 represents all the mutations found in the EOAD patients in the exons of PSEN1 and PSEN2 genes and flanking intronic regions and the Aβ42 level for the individuals carrying exonic exchanges. Figure 18 shows the protein structures of PSEN1 and PSEN2 with the pointed sites of amino acid exchanges.

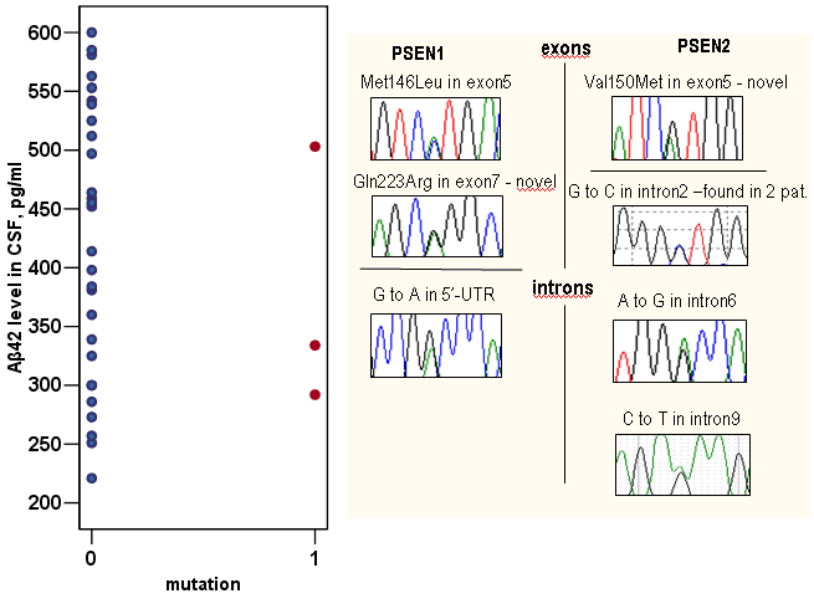


Figure 17. Mutations found in EOAD group in PSEN1 and PSEN2 genes

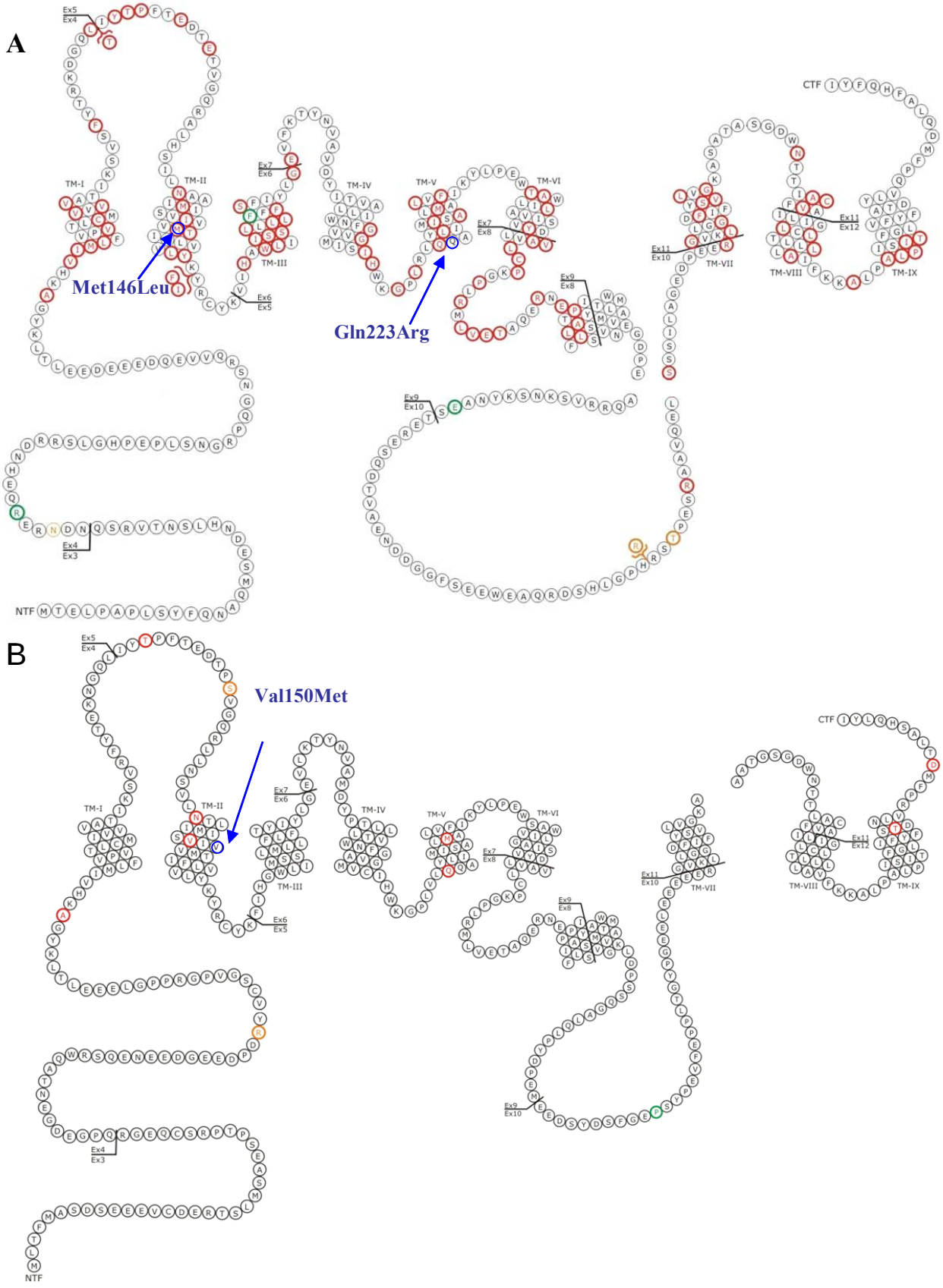


Figure 18. PSEN1 (A) and PSEN2 (B) protein structures

3.1.4 APOE-genotype frequencies in EOAD, LOAD and control groups and correlation of amyloid and tau-protein with APOE genotype

In order to understand the impact of possession of APOE E4 allele on age at onset of disease and Aβ42 concentration in CSF we have investigated this relationship in EOAD, LOAD and control groups. Genotyping for APOE alleles E2, E3 and E4 was performed in 33 EOAD, 131 LOAD and 99 control individuals.

Table 6. Comparison of obtained APOE allele and genotype frequencies in EOAD, LOAD and control group with published data (Davidson, Gibbons et al. 2007).

APOE	EOAD N=33	Davidson et al. EOAD N=357	LOAD N=131	Davidson et al. LOAD N=273	Control group N=99	Davidson et al. Healthy Pop. N=756
2/2	0 0.0%	1 0.3%	0 0.0%	1 0.4%	0 0.0%	2 0.3%
2/3	2 6.1%	22 6.1%	3 2.3%	10 4.5%	11 11.1%	108 14.3%
2/4	0 0.0%	2 0.5%	5 3.8%	4 1.5%	0 0.0%	15 2.0%
3/3	13 39.4%	133 37.3%	38 29.0%	71 26.0%	68 68.7%	445 58.9%
3/4	10 30.3%	147 41.1%	64 48.9%	138 50.5%	20 20.2%	174 23.0
4/4	8 24.2%	52 14.6%	21 16.0%	49 17.9%	0 0.0%	12 1.6%
E2	2 3.0%	26 3.6%	8 3.0%	16 2.9%	11 5.6%	127 8.0%
E3	38 57.6%	435 60.9%	143 54.6%	290 53.1%	167 84.3%	1172 78.0%
E4	26 39.4%	253 35.4%	111 42.4%	240 44.0%	20 10.1%	213 14.0%
Total	33	357	131	273	99	756
P-value	N.S.		N.S.		N.S.	

APOE allele and genotype frequencies for EOAD and LOAD groups as well as for the control group are given in the Table 6. Obtained APOE allele and genotype frequencies data were compared with published data by chi-square test (Davidson, Gibbons et al. 2007). There was no difference observed for the APOE genotype frequencies between the obtained data and published data.

As expected, the APOE E4 allele frequency in the LOAD group (111/262 alleles, 42.4%) was significantly higher ($p<0.05$) than that in the control group (20/198 alleles, 10.1%; Table 6). In the LOAD group, 52.7% of patients bore one APOE E4 allele and 16.0% were homozygous for APOE E4 allele, whereas in the control group 20.2% of patients bore one APOE E4 allele and no homozygous carriers of APOE E4 allele were found. There were no significant differences in APOE genotype or APOE E4 allele frequencies when AD patients were stratified into males and females (data not shown). Our data confirmed other studies that have shown the same distribution of APOE allele and genotype frequencies in EOAD, LOAD and control groups (Davidson, Gibbons et al. 2007).

Table 7. Stratification of EOAD, LOAD and control groups by APOE genotype.

		EOAD	**LOAD**	**Control group**
2/3	**Cases**	**2**	**3**	**11**
	Aβ42, pg/ml	375.50±144.957	365.33±128.255	943.82±155.262
	Tau-protein, pg/ml	832.50±122.329	474.50±113.844	202.82±102.427
	MMSE	20.00±2.828	25.00±4.583	27.20±2.150
	Age at onset	59.5±0.7	72.3±5.8	61.0±10.0
2/4	**Cases**	**0**	**5**	**0**
	Aβ42, pg/ml	-	434.00±89.808	-
	Tau-protein, pg/ml	-	566.20±419.230	-
	MMSE	-	21.50±5.447	-
	Age at onset	-	74.8±3.4	-
3/3	**Cases**	**13**	**38**	**68**
	Aβ42, pg/ml	414.46±123.074	411.43±119.593	1019.93±299.472
	Tau-protein, pg/ml	658.08±506.604	579.96±366.461	244.15±109.573

	MMSE	22.83±6.913	23.11±4.163	28.21±2.339
	Age at onset	53.2±9.9	75.8±5.9	62.1±10.2
3/4	**Cases**	**10**	**64**	**20**
	Aβ42, pg/ml	433.30±110.560	430.95±88.578	939.55±258.469
	Tau-protein, pg/ml	611.90±385.896	581.43±355.538	314.35±144.669
	MMSE	19.78±10.084	23.10±4.595	27.84±2.566
	Age at onset	59.4±2.5	73.8±4.6	62.9±12.0
4/4	**Cases**	**8**	**21**	**0**
	Aβ42, pg/ml	401.13±177.349	344.24±91.995	-
	Tau-protein, pg/ml	457.75±143.664	545.43±390.600	-
	MMSE	19.57±4.429	22.39±4.937	-
	Age at onset	61.9±1.6	71.4±4.1	-

Table 7 demonstrates the mean values for the Aβ42, tau-protein, MMSE and age at disease onset for the different APOE genotype subgroups of the EOAD, LOAD and control groups.

We found that in LOAD group the mean Aβ42 concentration in CSF in the APOE E4/E4 genotype subgroup is significantly lower compared to other genotype groups ($p=0.015$, ANOVA). The lower Aβ42 level was observed in the LOAD group subdivided into APOE E4 homozygous, one allele and non-carriers ($p=0.003$) (Figure 19).

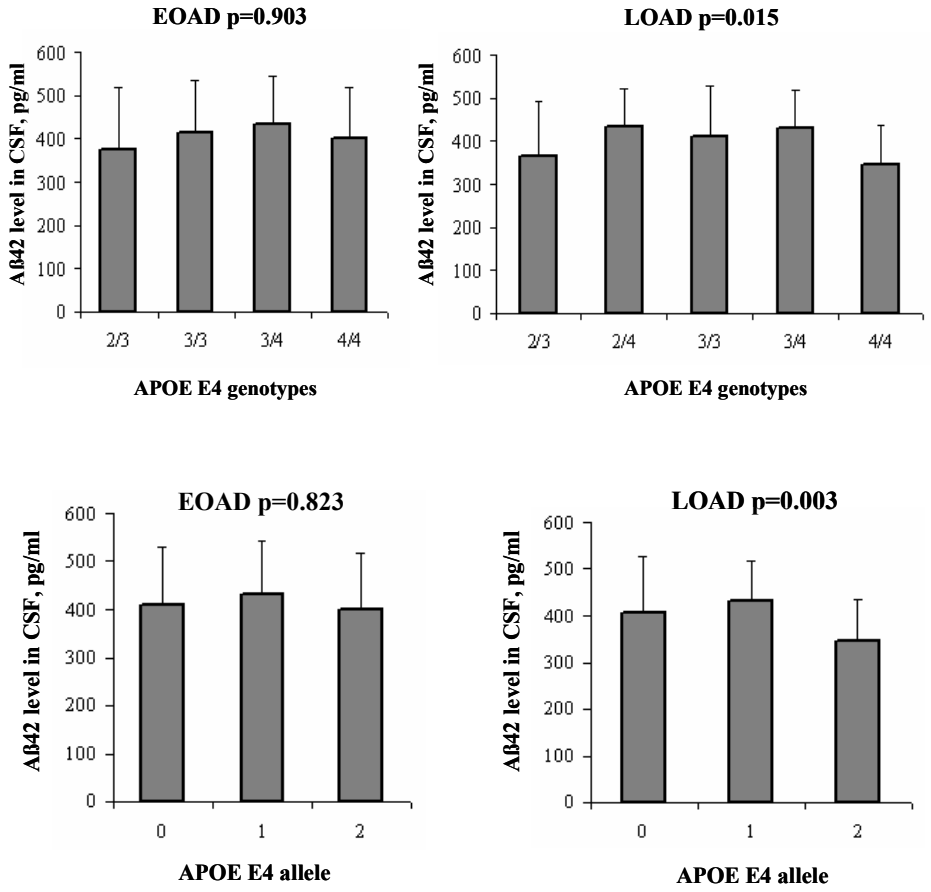


Figure 19. Aβ42 level in CSF dependent on APOE genotype frequencies analysed with ANOVA.

In the EOAD group we found that the mean age at onset in APOE E4/E4 genotype carriers (61.9±1.6 years) was significantly later (p=0.030, ANOVA) than in other genotypes carriers, whereas in LOAD there was a significant trend (p=0.030, ANOVA) towards earlier age at disease onset in cases possessing E4/E4 genotype (71.4±4.1 years) than in other genotypes carriers. These observations are consistent with previous studies (Corder, Saunders et al. 1994; Davidson, Gibbons et al. 2007). The same trends were observed in case of stratification of EOAD (p=0.024) and LOAD (p=0.010) groups by homozygous, one allele-carriers, non-carriers of APOE E4 allele (Figure 20).

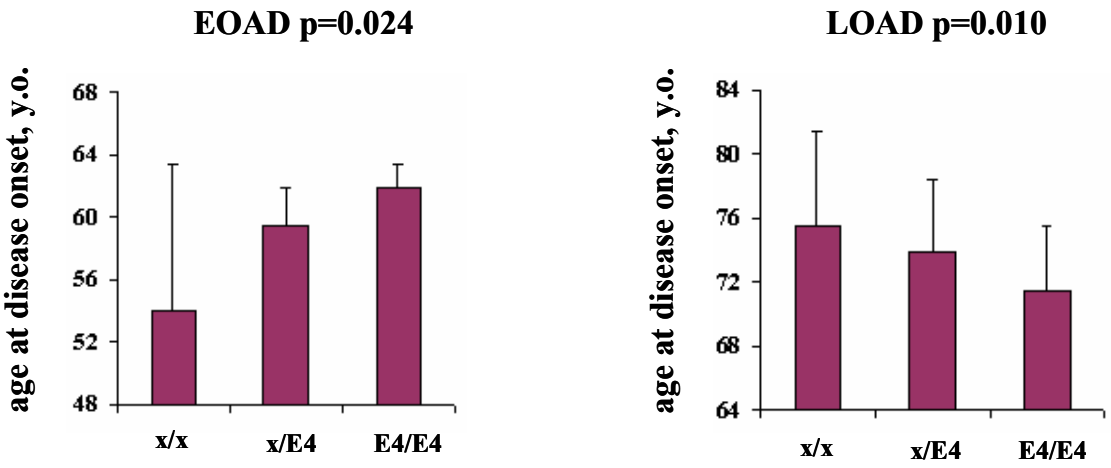


Figure 20. Mean age at disease onset values the LOAD group for x/x-non-carriers of E4 allele; x/E4 – heterozygous carriers of E4 allele; E4/E4 – homozygous carriers of E4 allele.

Since the presence of E4/E4 genotype was associated with earlier age at onset in LOAD group, we analysed the distribution of E4 allele homozygous, one allele and non-carriers in 65-74 and >74 years age subgroups (Figure 21). As it was expected the frequency of E4/E4 carriers was significantly higher in the 65-74 years subgroup compared to the >74 years one (p=0.011, chi-square).

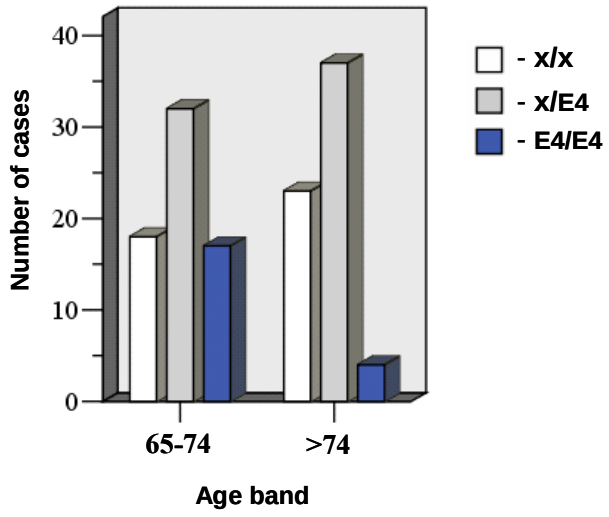


Figure 21. Distribution of APOE E4 allele frequencies in two age subgroups of the LOAD group. x/x-non-carriers of E4 allele; x/E4 – heterozygous carriers of E4 allele; E4/E4 – homozygous carriers of E4 allele.

APOE allele E4 number of copies did not influence CSF tau-protein levels neither in EOAD (p=0,439, ANOVA) nor in LOAD (p=0.928, ANOVA) groups, that was observed in earlier studies (Lasser, Dukoff et al. 1998). As well the analysis has shown the absence of significant difference of MMSE mean values between APOE E4 carriers and non-carrier in EOAD (p=0.604) and LOAD (p=0.798) (Monastero, Mariani et al. 2006).

3.1.5 Haplotype analysis of PSEN1 and PSEN2

The haplotype analysis of PSEN1 and PSEN2 genes revealed negligible LD spanning the whole PSEN1 gene region and two haploblocks of the PSEN2 gene. The major haploblock of PSEN2 forming 5 common haplotypes and covering 23kb region was chosen for the analysis. Four tagging SNPs rs2073489, rs11405, rs6759 and rs1800680 have been identified for the haplotypes determination and subsequent assessment of correlation between the defined haplotypes and the Aβ42 level in the CSF.

3.1.5.1 Genotype and allele frequencies of PSEN2 tagging SNPs

The genotype and allele frequencies for four selected tagging polymorphisms rs2073489, rs11405, rs6759 and rs1800680 in EOAD, LOAD, control group and brain samples cohort groups are given in the Table 8. The Hardy-Weinberg equilibrium was tested for each tagging SNP in all the groups. Obtained genotype frequencies were compared to the HapMap-CEU population (European ancestry) data available at http://www.ncbi.nlm.nih.gov/.

		Genotype frequency			**Allele frequency**		**HWP**
		MM	**Mm**	**mm**	**M**	**m**	
rs2073489	**EOAD N=37**	13 *0.351*	20 *0.541*	4 *0.108*	0.622	0.378	0.365
	LOAD N=154	47 *0..305*	76 *0.494*	31 *0.201*	0.552	0.448	0.978
	Control group N=123	44 *0.358*	56 *0.455*	23 *0.187*	0.585	0.415	0.491
	Brain samples N=109	36 *0.330*	62 *0.569*	11 *0.101*	0.615	0.385	0.036
rs11405	**EOAD N=37**	17 *0.459*	19 *0.514*	1 *0.027*	0.716	0.284	0.109
	LOAD N=154	95 *0.617*	58 *0.377*	1 *0.006*	0.805	0.195	0.013

	Control group N=123	77 *0.626*	40 *0.325*	6 *0.049*	0.789	0.211	0.785
	Brain samples N=109	63 *0.578*	41 *0.376*	5 *0.046*	0.766	0.234	0.606
rs6759	**EOAD N=37**	6 *0.162*	23 0.622	8 *0.216*	0.473	0.527	0.133
	LOAD N=154	53 *0.344*	73 0.474	28 *0.182*	0.581	0.419	0.744
	Control group N=123	37 *0.301*	58 *0.472*	28 *0.227*	0.537	0.463	0.565
	Brain samples N=109	22 *0.202*	62 *0.569*	25 *0.229*	0.486	0.514	0.148
rs1800680	**EOAD N=37**	26 *0.703*	11 *0.297*	0 *0.00*	0.851	0.149	0.288
	LOAD N=154	99 *0.643*	50 *0.325*	5 *0.032*	0.805	0.195	0.655
	Control group N=123	84 *0.683*	29 *0.236*	10 *0.081*	0.801	0.199	0.003
	Brain samples N=109	78 *0.716*	29 *0.266*	2 *0.018*	0.849	0.151	0.711

Table 8. Genotype and allele frequencies of tagging SNPs in EOAD, LOAD, control group and brain samples cohort. Genotype frequencies are given in number of individuals possessing corresponding genotype and in percentage below. M-major allele, m-minor allele; HWP – Hardy-Weinberg P-value

In the LOAD group there was observed a deviation from Hardy-Weinberg equilibrium of rs11405 polymorphism (HWP=0.013), opposite to the Hapmap population (HWP=0.670). Still there was no significant difference observed for rs11405 genotype frequencies in LOAD and Hapmap group (p=0.102 chi-square test).

In the control group there we detected the deviation from Hardy-Weinberg equilibrium of rs1800680 (HWP=0.003), even though the genotype frequencies were found to be close to HapMap frequencies by chi-square test (p=0.075).

In the brain samples cohort rs2073489 polymorphism was not in the equilibrium (HWP=0.036), although this polymorphism is deviated from the equilibrium in the HapMap population (HWP=0.010).

3.1.5.2 Genotype frequencies of PSEN2 tagging SNPs in Alzheimer's disease groups EOAD and LOAD compared to the control group

By the comparison of the tagging SNPs genotype frequencies in EOAD and LOAD in relation to the control group it was observed a statistically significant difference for the rs11405 CT genotype (p=0.037) between EOAD and control groups and for TT genotype (p=0.026) between LOAD and control groups (Figure 22). These differences are observed due to the overrepresentation of the rs11405 CT genotype in EOAD (19/37, 51.4%) compared to the control group (40/123, 32.5%) and underrepresentation of the rs11405 TT genotype in the LOAD group (1/154, 0.6%) compared to the control group (5/123, 4.9%). The misbalance between heterozygous CT and minor homozygous TT genotypes of rs11405 in the EOAD and LOAD groups was also visible with significant deviation from the Hardy-Weinberg equilibrium in the LOAD group (see 3.1.4.1). Randomly chosen from the AD group, 30 samples were resequenced for rs11405 but all sequencing results were the same as genotyped with Real-Time PCR, before.

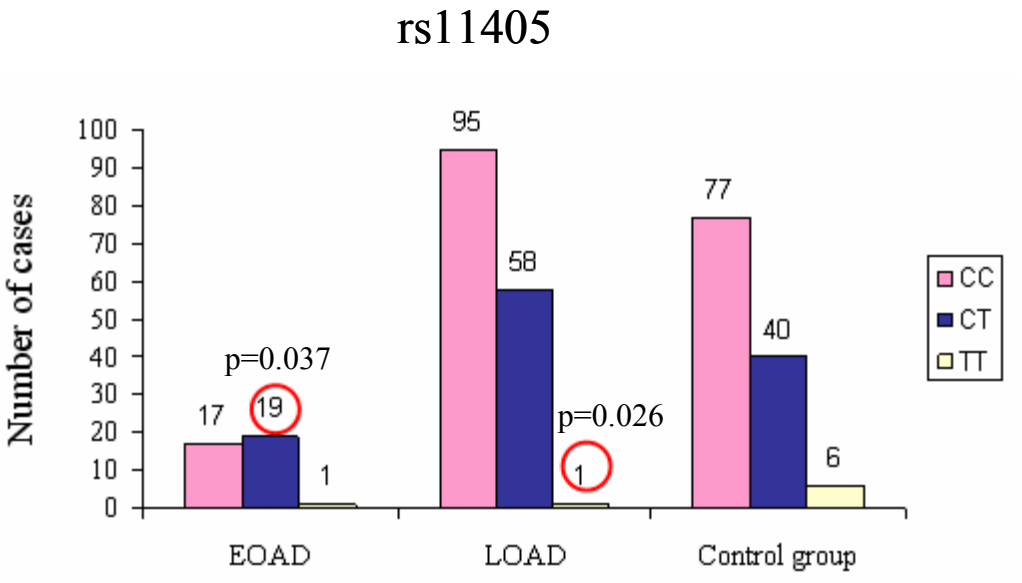


Figure 22. Rs11405 genotype frequencies in EOAD and LOAD in comparison with the control group. Genotype frequencies are represented as number of cases. The genotype frequency differed significantly from that of the control group is marked with red circle, corresponding p-values for the chi-square test are adduced.

No other significant differences of genotype frequencies of tagging SNPs between EOAD/LOAD and control group were observed.

3.1.5.3 Genotype frequencies of PSEN2 tagging SNPs in the control group compared to the brain samples group

By the comparison of the tagging SNPs genotype frequencies in the control group and in the brain samples cohort it was observed a statistically significant difference for the rs1800680 AA genotype (p=0.031) (Figure 23). This difference is observed due to the overrepresentation of the rs1800680 AA genotype in the control group (10/123, 8.1%) compared to the brain samples group (2/109, 1.8%) that might explain the deviation of this polymorphism from the HW equilibrium in the control group.

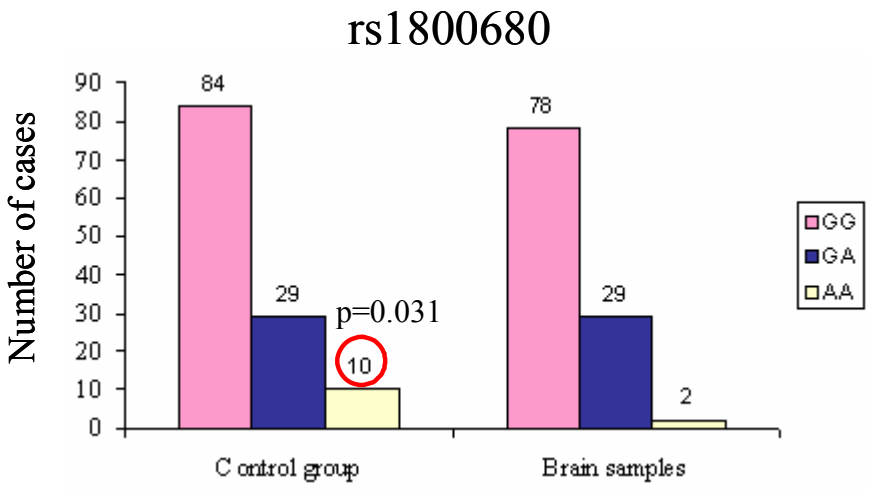


Figure 23. Rs1800680 genotype frequencies in control group in comparison with the brain samples cohort. Genotype frequencies are represented as number of cases. The genotype frequency differed significantly from that of the control group is marked with red circle, corresponding p-value for the chi-square test is adduced.

3.1.5.4 Frequencies of PSEN2 haplotypes

Table 9 demonstrates the frequencies of the haplotype combinations in EOAD, LOAD, control group and brain samples cohort. As it was expected it was observed a significant difference for the haplotype 4/4 frequency (p=0.026) between LOAD and the control group due the underrepresentation of the rs11405 minor genotype in the LOAD group.

Table 9. Number of PSEN2 haplotype cases (in parenthesis values in percentage) for EOAD, LOAD, control group and brain samples

Haplotype	EOAD	Chi-square	LOAD	Chi-square	Control group	Brain samples
1/1	3 (8.11%)	0.455	10 (6.49%)	0.567	6 (4.88%)	4 (0.04)
1/2	5 (13.51%)	0.725	14 (9.09%)	0.530	14 (11.38%)	17 (0.16)
1/3	1 (2.70%)	0.322	10 (6.49%)	0.788	9 (7.32%)	6 (0.06)
1/4	4 (10.81%)	0.733	19 (12.34%)	0.366	11 (8.94%)	14 (0.13)
1/5	1 (2.70%)	0.465	16 (10.39%)	0.159	7 (5.69%)	5 (0.05)
2/2	3 (8.11%)	0.875	8 (5.19%)	0.220	11 (8.94%)	8 (0.07)
2/3	2 (5.41%)	0.687	10 (6.49%)	0.788	9 (7.32%)	5 (0.05)
2/4	4 (10.81%)	0.733	18 (11.69%)	0.458	11 (8.94%)	12 (0.11)
2/5	1 (2.70%)	0.517	10 (6.49%)	0.567	6 (4.88%)	9 (0.08)
3/3	0 (0.00%)	-	6 (3.90%)	0.104	1 (0.81%)	2 (0.02)
3/4	3 (8.11%)	0.455	3 (1.95%)	0.172	6 (4.88%)	4 (0.04)
3/5	1 (2.70%)	0.866	6 (3.90%)	0.775	4 (3.25%)	3 (0.03)
4/4	1 (2.70%)	0.571	1 (0.65%)	0.026	6 (4.88%)	5 (0.05)
4/5	8 (21.62%)	0.056	18 (11.69%)	0.607	12 (9.76%)	11 (0.10)
5/5	0 (0.00%)	-	5 (3.25%)	0.074	10 (8.13%)	2 (0.02)
Total	37		154		123	107

3.1.5.5 Analysis of influence of PSEN2 haplotypes on Aβ42 concentrations in CSF

We analysed the Aβ42 CSF concentrations in homozygous, heterozygous and non-carriers of each PSEN2 haplotype (Table 10). We observed a significant trend towards higher Aβ42 concentrations of CSF with the increase of copy numbers of haplotype 1 (p=0.006) in the EOAD group but not in the LOAD or control group. Haplotype 2 showed a similar trend towards higher Aβ42 concentrations within the LOAD group with mean levels of 399 pg/ml, 418 pg/ml and 514 pg/ml in carriers of none, one or two haplotype 2 (p=0.033). Haplotype 5 correlated with lower Aβ42 concentrations in heterozygous carriers compared to wildtype (343 pg/ml vs. 440 pg/ml, p=0.002 in LOAD, 326 pg/ml vs. 459pg/ml,

p=0.013 in EOAD) within both groups. The 5 homozygous carriers of haplotype 5 found in the LOAD group had however, had similar concentrations as wildtype carriers.

The analysis has shown no effect of haplotypes 3, 4 and of the tagging SNPs per se on the Aβ42 level in CSF.

We analysed whether age had any influence on the effect observed for haplotype 1, 2 and 5 on Aβ42 CSF concentrations (Figure 24). For this, we grouped the LOAD patients into two age groups of individuals between 65-74, and those >74 years old. Haplotype 2 showed a significant effect on Aβ42 concentrations only within the >74 age group with mean levels of 405 pg/ml, 436 pg/ml and 516 pg/ml in carriers of none, one or two haplotype 2 (p=0.049).

Haplotype 5 correlated with lower Aβ42 concentrations in heterozygous carriers compared to wildtype within both 65-74 and >74 groups (p=0.009 and p=0.049 respectively), but homozygous carriers had similar concentrations as wildtype carriers.

Linear regression analysis has shown a linear association of Aβ42 concentrations with haplotype 1 number of copies in the EOAD (R^2=0.382, p=0.018) and haplotype 2 in the LOAD (R^2=0.045, p=0.009), and in the EOAD group with a significant effect of age of onset (p=0.038) while the other factors (age at onset in LOAD, APOE E4 allele, MMSE value) did not show any significant association with Aβ42.

In order to compare putative clinical and genetic influence factors on Aβ42 concentrations, we performed a general linear model analysis for the adjustment of observed significant findings for the PSEN2 haplotypes for gender, age at disease onset and the APOE E4 allele copy number. The trends observed earlier for the haplotype 1 in EOAD (p=0.006), haplotype 2 in LOAD (p=0.004) and haplotype 5 in EOAD (p=0.001) and LOAD (p=0.007) remained significant after the adjustment.

Haplotype		Number of haplotype copies	Number of cases	EOAD* Median Aβ42 pg/ml (range)	P-value	Number of cases	LOAD Median Aβ42 pg/ml (range)	P-value	Number of cases	Control Median Aβ42 pg/ml (range)	P-value
Haplotype 1	TCTG	0	21	360 (221-581)	0.006	85	422 (195-598)	0.787	80	920 (606-1966)	0.396
		1	10	510 (273-600)		59	427 (176-196)		37	930 (613-1950)	
		2	3	525 (512-539)		10	456 (271-569)		6	862 (611-1317)	
Haplotype 2	CCCG	0	21	384 (221-600)	0.781	94	426 (176-596)	0.033	72	9350 (611-1568)	0.724
		1	10	401 (257-563)		52	425 (229-581)		40	891 (613-1966)	
		2	3	414 (414-459)		8	519 (422-598)		11	891 (606-1392)	
Haplotype 3	CCTG	0	28	409 (221-600)	0.603	119	428 (203-598)	0.450	95	908 (606-1966)	0.475
		1	6	408 (300-585)		29	449 (176-582)		27	938 (640-1885)	
		2	0	-		6	384 (195-583)		1	1242	
Haplotype	CTCG	0	14	457 (273-585)	0.083	95	434 (176-598)	0.347	77	904 (606-	0.697

4										1966)	
(rs11405)		1	19	339 (221-600)		58	392 (203-595)		40	989 (625-1506)	
		2	1	497		1	384		6	848 (613-1494)	
Haplotype 5	TCTA	0	25	459 (257-600)	0.013	99	440 (176-598)	0.002	79	904 (606-1950)	0.815
(rs1800680)		1	9	326 (221-452)		50	343 (203-596)		34	933 (700-1966)	
		2	0	-		5	437 (270-472)		10	928 (641-1568)	
Rs2073489	CC	0	12	414 (257-581)	0.336	47	440 (195-598)	0.047	44	904 (606-1885)	0.729
	CT	1	19	384 (221-600)		76	402 (176-595)		56	971 (613-1966)	
	TT	2	3	525 (512-539)		31	434 (204-596)		23	842 (611-1568)	
Rs6759	TT	0	5	525 (325-585)	0.168	53	437 (176-596)	0.192	37	930 (611-1568)	0.908
	TC	1	23	384 (221-600)		73	382 (203-595)		58	932 (613-1966)	

	CC	2	8	414 (257-497)	28	463 (272-598)	28	897 (606-1506)

***For the EOAD group analysis, the three individuals with amino acid exchanges in PSEN1 or 2 were taken out of the analysis.**

Table 10. PSEN2 haplotypes and correlation with Aβ42 CSF level in the AD group and control group (P- values are from Jonckheere-Terpstra test)

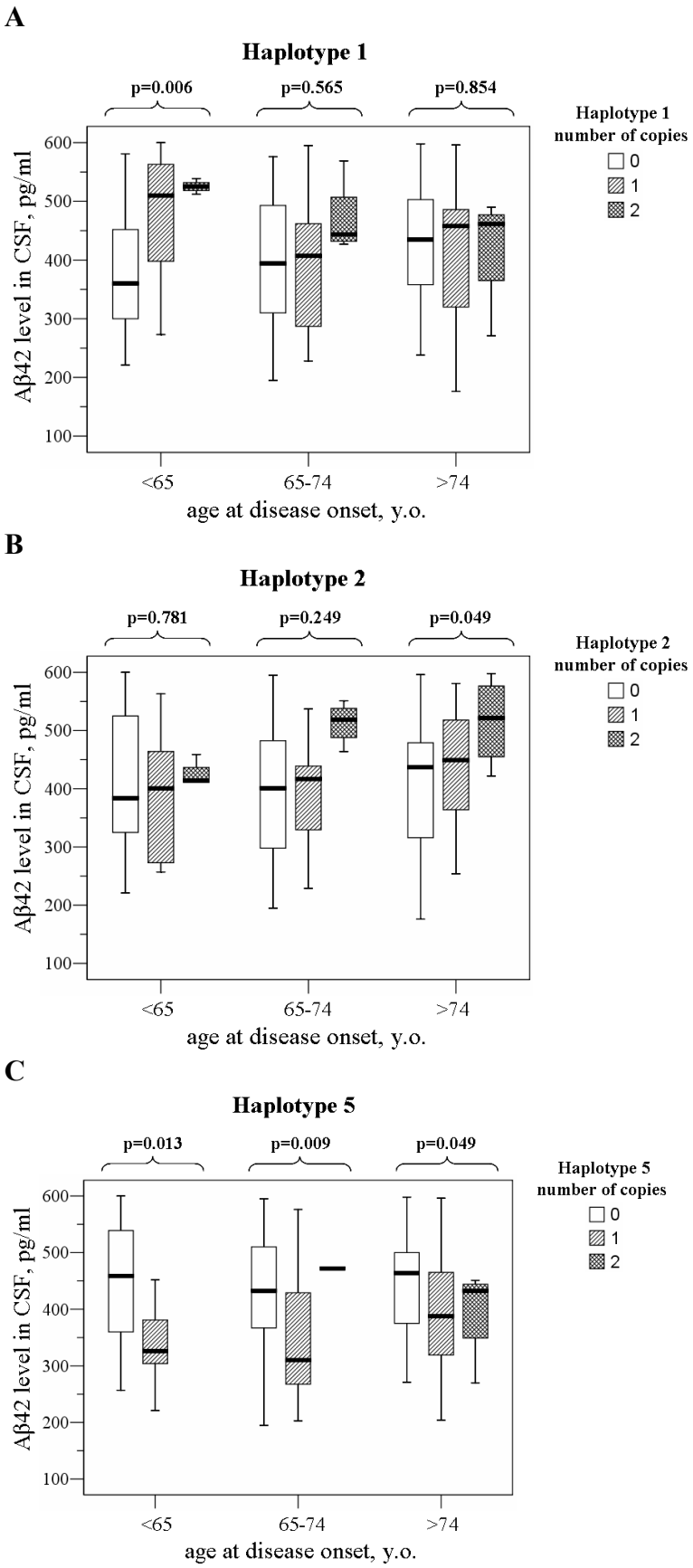


Figure 24. A boxplot graph showing the median (black), the 25th and 75th percentile (boxes) and the data range (antennas) of the Aβ42 values in CSF of

patients according to age. Figure A depicts Aβ42 in heterozygous and homozygous carriers of haplotype 1; figure B depicts the same for haplotype 2, and figure C for haplotype 5

3.1.5.6 AβMTL phase and PSEN2 haplotypes in the brain samples

As an independent group for comparison, 109 (54 females and 55 males) human brain samples were genotyped for the PSEN2 haplotypes, and the frequency of haplotypes was correlated with β-amyloidosis in the medial temporal lobe (AβMTL) which was graded from 0 to 4. The mean age at death was 74±9.08 years. Eighty-one individuals (40 female and 41 male) had had no clinical diagnosis of dementia, and 28 patients had a known diagnosis of dementia of different subtypes.

β-Amyloidosis in the medial temporal lobe (AβMTL) with AD-related Aβ pathology was observed in 74 cases (49 non-demented cases and 25 cases with dementia) as described earlier (Thal, Rub et al. 2002).

Table 11 presents the distribution of patients of each PSEN2 haplotype according to the β-amyloidosis severity grade. The homozygous haplotype 4 (rs11405) was detected in 5 brain samples. There was an underrepresentation of the homozygous haplotype 4 within the samples with β-amyloidosis (only one AβMTL phase 1 case,) while in the samples without β-amyloid plaques (phase 0), 4 individuals were carriers of the haplotype 4 (p=0.057, Chi-square test, exact two-sided). All haplotype 4 samples had no dementia.

Table 11. The AβMTL phase and PSEN2 haplotypes in the brain samples.

AβMTL phase		**0**	**1**	**2**	**3**	**4**	**Total**	**p-value**
Hap1	**0**	23 37%	12	9	10	8	62	ns
	1	10 23%	7	14	8	4	43	
	2	2 50%	0	0	1	1	4	
Hap2	**0**	17 30%	8	16	8	7	56	ns
	1	16 35%	9	6	10	4	45	
	2	2 25%	2	1	1	2	8	
Hap3	**0**	29 32%	16	20	15	9	89	ns
	1	5 27%	3	3	3	4	18	
	2	1 50%	0	0	1	0	2	

Hap4	**0**	19 30%	11	9	14	10	63	0.057
	1	12 29%	7	14	5	3	41	
	2	4 80%	1	0	0	0	5	
Hap5	**0**	26 33%	13	17	14	8	78	ns
	1	9 31%	6	5	4	5	29	
	2	0 0%	0	1	1	0	2	

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3.2 Project 2

The total patient sample comprised 179 patients who had been genotyped for FKBP5 and were available for rating of 3-weeks drug response. Genotype frequencies of the studied polymorphisms (FKBP5 rs3800373, FKBP5 rs1360780) are given in Table 12. The FKBP5 variants rs3800373 and rs1360780 were highly linked (with 87% heterozygous individuals of rs360780 being also heterozygous for rs3800373, and 93% of the homozygous individuals being homozygous on both positions). Both variants were in Hardy-Weinberg equilibrium, and did not differ significantly from the allele frequencies reported by Binder et al. No differences in baseline severity of depression were detected (Table 12). There was a higher percentage of patients diagnosed with bipolar affective disorder (9%) in the subgroup of carriers of the AA genotype of rs3800373 compared to the AC and CC carriers (p=0.04, Fisher's exact test).

Table 12. Clinical parameters. * p<0.05, we grouped homozygous and heterozygous carriers of rs1360780 allele T or rs3800373 allele C in one group and compared with those patients without these alleles

FKBP5	**rs3800373**			**rs1360780**		
	CC (n=15)	**CA (n=73)**	**AA (n=91)**	**TT (n=15)**	**TC (n=83)**	**CC (n=80)**
Mean age	45 ± 9	46 ± 12	46 ± 12	46 ± 10	47 ± 12	46 ± 12
Percentage of female patients	67%	71%	61%	67%	71%	60%
Baseline HDRS	29.7 ± 7.2	31.6 ± 6.8	30.2 ± 8.1	29.7 ± 7.2	31.5 ± 7.2	30.1 ± 7.8
No depressive episodes	1.5	3.6	3.9	1.5	3.6	3.9
Percent bipolar	0	1%	9%*	3%		6%
Number of hospitalisation	1.5	2.0	2.5	1.9		2.5
Mean percent decline in HDRS ± SD	40 ± 26	49 ± 29	41 ± 30	40 ± 26	49 ± 29	41 ± 30
Frequency of responder	40%	53%	39%*	40%	53%	38%*

Heterozygous or homozygous carriers of the rs3800373 or rs1360780 variant showed a trend towards a higher chance to respond to the therapy (p=0.04, Chi-square test for carriers versus non-carriers of both SNPs, odds ratio for both: 1.8 (95% confidence interval 0.98-3.3)) (table 12, Figure 25).

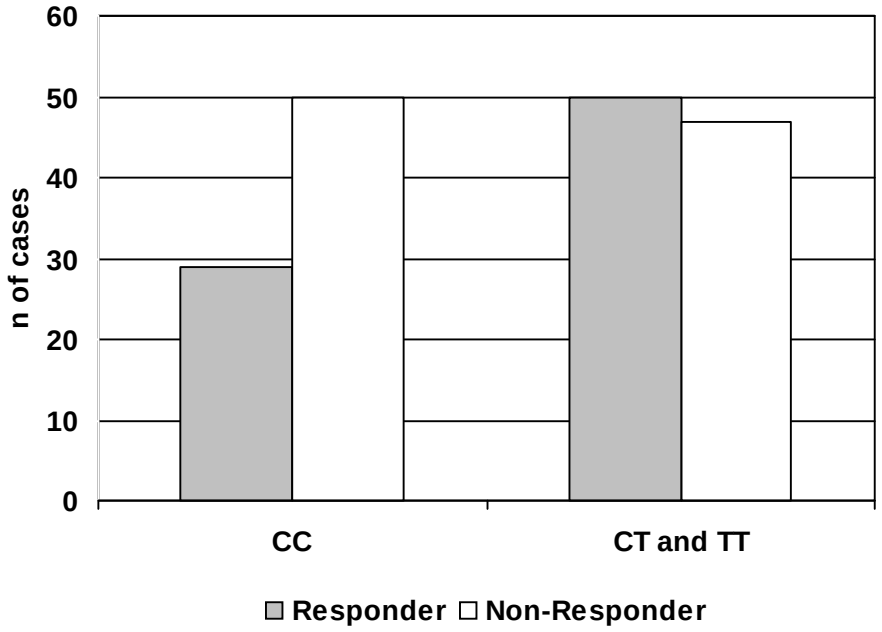

Figure 25. Number of responders to antidepressant drug treatment in the difference genotype groups as defined by rs3800373 of FKBP5. Carriers of the variant had a better chance to respond than non-carriers (p=0.04)

In a binary logistic regression model we analysed the influence of the covariates, gender, number of previous depressive or manic episodes (categorized into none, between 1 and 3, and more than 3), initial severity of the depressive episode (categorized into HDRS score < 18, and >18), the different three hospitals, and the effect of the *FKBP5* rs1360780 genotype on the binary outcome "responder" or "non-responder". A significant influence was observed for the factor hospital (p=0.002; in one site, 20 of the 25 included patients were responders), none versus more than 2 precedent depressive episodes (p=0.02, OR 2, 95%confidence interval :0.86-4.8), male sex (p=0.03; OR 1.8 for men, 95% CI: 0.98-3.3), and *FKBP5* genotype rs3800373 (p=0.02; OR:1.8; 95% CI:0.98-3.3) but not for initial severity of depression or the number of manic episodes.

Antidepressant drug intake was completely recorded during the observation period in all 179 patients. 151 patients were treated with antidepressant monotherapy during the

observation interval. 28 patients (16%) were treated with either combinations of two antidepressants or with two antidepressants given subsequently during the observation period. The majority of patients were treated with either an SSRI (28%), mirtazapine (26%), venlafaxine (16%) or with a tricyclic antidepressant drug (11%). 3% of the patients were treated with other antidepressants. For a more detailed analysis of the impact of the *FKBP5* rs1360780 polymorphism on the early outcome of therapy we grouped the patients into these five classes. Individuals receiving a combination of antidepressants from more than one class of antidepressants were included in a separate group. When analyzing response within the different drug classes, the effect was mostly due to differences in patients treated with several antidepressants (combination group) during the observation period (82% of the heterozygous carriers of FKBP5 rs3800373 were responders versus 18% of the wild-type carriers, p=0.008, Fishers exact test) and within the subgroup of patients treated with venlafaxine (n=29, 58% heterozygous carriers of FKBP5 rs3800373 responded and 42% wild-type carriers, p=0.04). The drug-class specific analyses of the FKBP5 rs3800373 variant is shown in Figure 26.

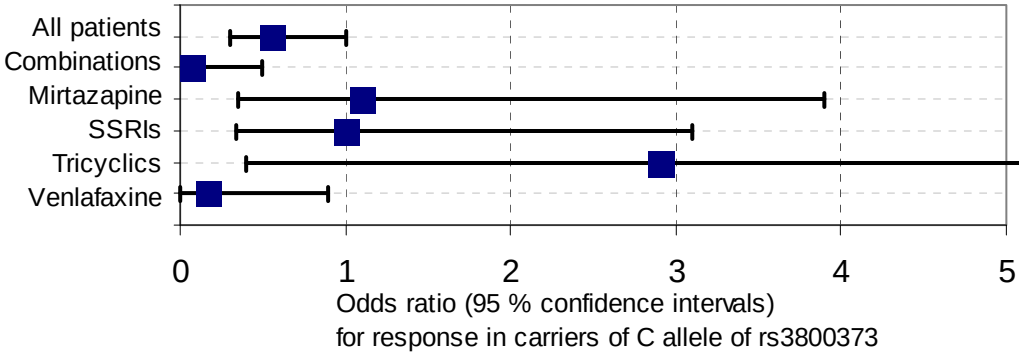

Figure 26. Odds ratios and 95% confidence intervals in subgroups of different antidepressants for carriers of any C allele of the rs3800373 variant. The effect in the whole group mostly derives from the patient group treated with several antidepressants during the observation time (combination group) or with venlafaxine.

3.3 Project3

3.3.1 L-Trp, 5-HT, HIAA, and HVA serum concentrations

Baseline concentrations of L-tryptophan, serotonin and the serotonin metabolites HIAA and HVA were measured in the morning of both study days and showed a high inter-occasion correlation. The baseline 5-HT concentrations were 69.8, 56.8 and 26.1 ng/ml (differences n.s.) in carriers of the ll, ls, and ss genotypes and the mean concentrations were almost identical at the second study day (Table 13).

Table 13. Serum concentrations (mean and standard deviations) of monoamine metabolites in serum of the participants before and after tryptophan/placebo. *Differences in baseline values (pre-placebo or pre-depletion) were analysed with ANOVA with depletion/placebo and HTTPLR genotype as fixed variables. **Influence of 5-HTTPLR genotype on differences (post – pre) for depletion/placebo was tested using ANOVA with depletion/placebo and HTTPLR genotype as fixed variables and baseline values (pre) as covariate.

	5-HTTLPR	**5-HT [ng/ml]**	**Free L-Trp [ng/ml]**	**Total L-Trp [µg/mL]**	**5-HIAA [ng/ml]**	**HVA [ng/ml]**
Pre-tryptophan	ll (n=8)	69.8 (±44.5)	843 (±255)	8.9 (±1.7)	5.5 (±1.8)	5.4 (±2.3)
	ls (n=15)	56.8 (±30.1)	1391 (±498)	11.1 (±1.2)	5.4 (±1.2)	7.0 (±1.7)
	ss (n=1)	26.1	711	7.3	5.9	26.8
Percent increase	ll	41% (±43%)	281% (±263%)	304% (±100%)	5% (±17%)	16% (±35%)
	ls	21% (±42%)	146% (±100%)	227% (±71%)	7% (±14%)	14% (±26%)
	ss	57%	744%	621%	-15%	-65%

Pre-placebo	ll	68.3 (36.4)	865 (222)	9.5 (±2.5)	6.6 (3.1)	6.2 (2.5)
	ls	56.5 (33.2)	1450 (580)	12.2 (±2.8)	6.1 (1.3)	8.2 (3.6)
	ss	18	184	3.9	6.4	25.2
Percent increase	ll	48% (±44)	-80% ±13%	-76% (±11%)	-32% (±20)	24% (±51%)
	ls	12% (±24)	-82%±8%	-74% (±13%)	-35% (±21)	1% (±25%)
	ss	27%	-53%	-63%	-43%	-24%
Significance	Baseline*	n.s.	0.01	0.008	n.s.	< 0.001
	Increase**	0.02	0.04	n.s.	n.s.	n.s.

Except for hydroxyindolic acid, there was a high constancy in the basal serum concentrations of the serotonin related biogenic amines (Figure 27). The percent coefficients of determination (squares of the linear correlation coefficients) were 74.0%, 69.6%, 39.9%, and 81.9% for free L-Trp, 5-HT, 5-HIAA, and HVA, respectively.

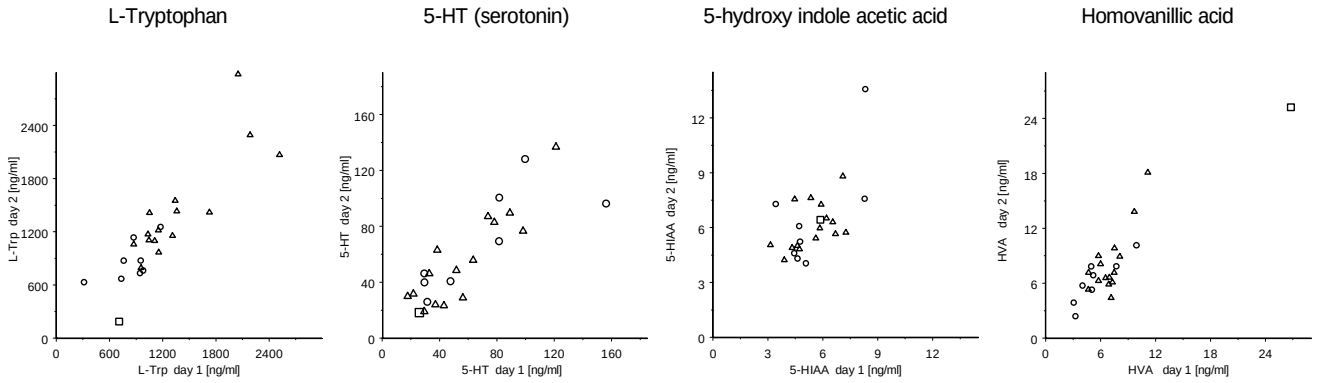

Figure 27. Intraindividual (between day) constancy of tryptophan, serotonin and its catabolites in serum. The figure shows the between-day correlation of the measured biogenic amines. Circles represent carriers of the SLC4A6 ll genotype, triangles those with the ls genotype and the squares stand for the ss genotype

This data may indicate that as much as 70% (L-tryptophan, serotonin) or 80% (homovanillic acid) of individual variation might be explained by individually constant factors. Multiple regression analysis confirmed that the 5-HTT genotype may play a certain role as individual predictor of the concentrations in blood for serotonin ($p=0.07$, $r^2=6.7\%$) and for HVA ($p<0.001$, $r^2=69.9\%$).

Baseline serum concentrations of 5-HIAA did not differ between the 5-HTTLPR genotypes. However, the concentrations of HVA, a known substrate of the 5-HTT, did differ in a gene-dose-dependent manner with concentrations of 5.4, 7.0, and 26.8 ng/ml at placebo baseline ($p<0.001$ for linear trend) and 6.2, 8.2, and 25.2 ng/ml at baseline prior to depletion ($p<0.001$ for linear trend).

The baseline serum concentrations of L-tryptophan, both, free and total, differed significantly between the subgroups of the 5-HTTLPR genotypes with lower mean free L-tryptophan in ll-carriers, compared to sl (865 ± 222 vs. 1450 ± 580, $p=0.01$, table 13). The only ss-individual showed an inconsistent picture with very low baseline L-tryptophan on the day of depletion (184 ng/ml) which dropped to 86 ng/ml after depletion, while at the placebo day, the baseline L-tryptophan was 711 ng/ml and increased to 6000 ng/ml during the placebo condition.

3.3.2 Tryptophan depletion/supplementation effects on Trp, 5-HT, HIAA, and HVA serum concentrations

There was a wide variation in the relative changes of serum L-tryptophan after tryptophan depletion or supplementation. After tryptophan depletion, the absolute free L-tryptophan values decreased slightly more in the ls group (82%±8% decrease) compared to ll (80%±13% decrease, $p=0.04$, ANOVA with depletion/placebo and HTTPLR genotype as fixed variables and baseline values (pre) as covariate). After placebo depletion there was an increase in L-tryptophan concentrations in all individuals with the highest absolute value (6000ng/ml) in the only ss-carrier.

Serum 5-HT showed an increase after placebo and after the depletion drink. Relative increase was 41% (±43), 21% (±42), and 57% in the ll, ls, and ss genotypes after supplementation and 48% (±44), 12% (±24), and 27% in the ll, ls, and ss genotypes after depletion ($p=0.02$, ANOVA with depletion/placebo and HTTPLR genotype as fixed variables and baseline values (pre) as covariate increase after depletion).

Serum concentrations of the serotonin metabolite 5-HIAA increased after supplementation to a minor extent, and decreased after depletion (Table 13). Relative increase was 5% (±17), 7%

(±14), and -15% in the ll, ls, and ss genotypes after supplementation and -32% (±20), -35% (±21), and -43% in the ll, ls, and ss genotypes after depletion.

Serum concentrations of HVA did not significantly change after supplementation or depletion but the baseline and post depletion/placebo values differed significantly in dependence of the 5-HTT genotypes (p=0.027 baseline before placebo) with HVA levels of 7.0ng/ml in ls carriers versus 5.4ng/ml in ll carriers and even 26.8 ng/ml in one ss-carrier at baseline.

3.3.3 Baseline psychiatric scores and depletion/supplementation effects

There were significantly higher scores of baseline MADRS in ll-carriers compared to ls and ss (p=0.04) indicating a higher prevalence of depressive symptoms in this subgroup. This genotype effect remained constant throughout the study. Similar, baseline VAS for craving was higher in the 5-HTT ll genotype groups (p=0.003), and remained higher in this genotype group during tryptophan depletion and supplementation.

The State-trait-Anxiety inventory (STAI) did not differ at baseline between the genotypes. However, after the tryptophan supplemented amino acid mix, the decrease in STAI was more pronounced in carriers of the ll-genotype than in carriers of the ls-genotype (delta STAI state items 1.7 (3.6) in ll vs. 1.0 (4.0) in ls carriers, p=0.048).

Table 14. Psychiatric scores (mean and standard deviations) dependent on tryptophan depletion status and HTTLPR genotypes.

	5-HTTLPR	MADRS	VAS on craving	STAI (median)
Pre-placebo	ll (n=8)	16 (11)	27 (26)	75 (61-124)
	ls (n=15)	7 (6)	7 (8)	84 (41-104)
	ss (n=1)	9	0	40
Post-placebo	ll	16 (12)	21 (21)	64 (46-127)
	ls	7 (6)	7 (8)	75 (41-106)
	ss	9	0	40
	ll	18 (13)	21 (23)	86 (48-128)

Pre-depletion	ls	7 (6)	9 (8)	73 (51-117)
	ss	11	0	43
	ll	18 (13)	21 (22)	88.5 (45-127)
Post-depletion	ls	8 (6)	9 (7)	77 (45-111)
	ss	11	0	44
Significance	Baseline*	0.04	0.003	n.s.
	Delta**	n.s.	n.s.	n.s.

3.3.4 SLC6A4 expression in PBMCs

For 11 individuals, mRNA was quantified from blood samples taken at the same time point as the monoamine metabolite samples. Delta CT values were analysed normalized to a housekeeping gene known to be stably expressed in lymphocytes (human TATA box binding protein). Delta CT values were significantly higher during the study in carriers of the ls genotype (mean Delta CT 4.7 (SD 0.6)) indicating lower amounts of mRNA of the serotonin transporter in this subgroup compared to the ll-carriers (4.1 (0.6), p=0.01) (Figure 28). However, there was no significant change in SLC6A4 mRNA at any of the time points (pre or post depletion, tryptophan versus placebo).

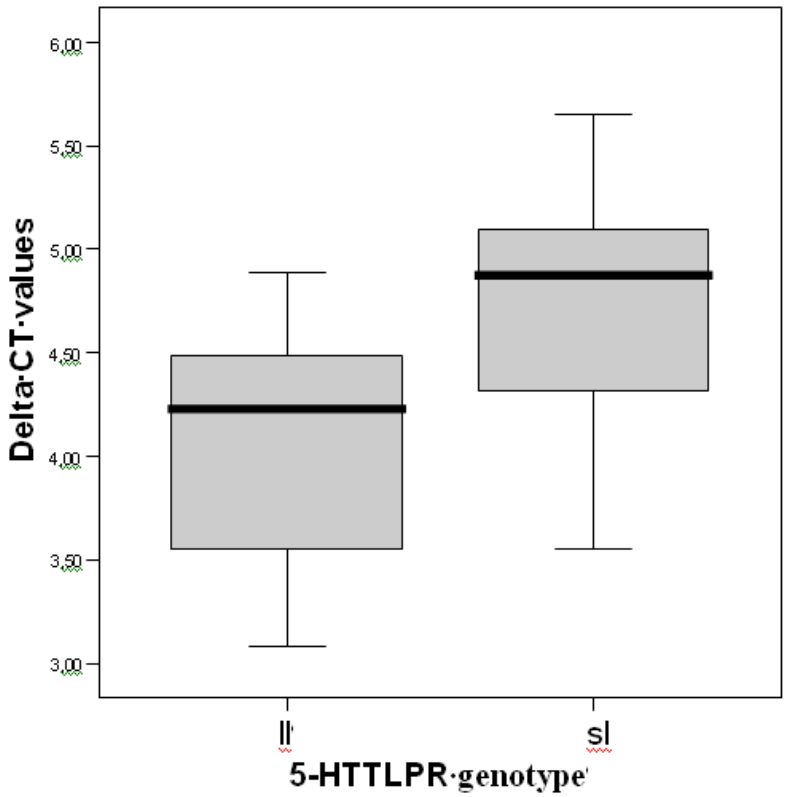

Figure 28. Median values of Delta CT for mRNA quantification of 5-HTTLPR in dependence of the genotypes in human blood cells. RNA was extracted from whole blood. RNA quantification could be performed only in 11 individuals but was performed at all 4 time points (pre and post depletion/placebo)

4 DISCUSSION

4.1 Project1

In patients with early-onset AD, three point mutations were detected, which might account for the disease and the low Aβ42 concentrations in the CSF. One of the amino acid substitutions, the Met146Leu mutation in exon 5 of PSEN1, had been described before causing familial AD (Sherrington, Rogaev et al. 1995). It is located in the exon 5 that forms the transmembrane domain TM II of PSEN1. The age of onset of the Met146Leu carrier was 38 years which corresponds to the early age of onset of 40 years described in 13 families with phenotypes of mixed AD and Pick's disease (http://www.molgen.ua.ac.be/ADMutations/). The patient with Gln223Arg mutation in the PSEN1 gene had a disease onset at 30 years old. The third patient with an amino acid substitution was a female with a Val150Met exchange in PSEN2 with an age of onset at 52 years. The probable harmful effect of the PSEN2 mutation located in the transmembrane domain TM V might be a disruption of the alpha-helical structure and this structural change in PSEN2 may result in differences in the patterns of proteolysis. Although similar effects have been reported for some PSEN1 missense mutations, reduced proteolysis of presenilin proteins does not seem to be a general mechanism in AD pathology (Cruts, van Duijn et al. 1998). The absence of X-ray crystallographic data and 3-D protein structure for PSEN proteins makes it difficult to explain the exact mechanism of mutations effect on the protein function (http://www.rcsb.org/pdb/home/home.do). Both amino acid exchanges had not been described in previous studies. All three patients had no APOE 4 allele, but the APOE 3/3 genotype, and had positive family history for AD. Thus, for these three individuals, the point mutations causing amino acid exchanges in PSEN1 and 2 might have accounted for the severe disease with extreme early age of onset and Aβ42 concentration far below 600 pg/ml. It has been reported that CSF Aβ42 levels in presymptomatic subjects with pathogenic mutations in the PSEN1 gene are significantly lower than in an age-matched control group (Moonis, Swearer et al. 2005), which is in line with already decreased Aβ42 levels in a patient with a MMSE of 30 and a PSEN2 exonic mutation.
However, the Aβ42 concentrations of these three patients did not differ significantly from the Aβ42 level of other early-onset AD patients whose genetic analysis did not reveal any

nucleotide exchanges in the coding region. Therefore we conclude that CSF analysis does not help distinguish early familial from non-familial AD cases.

In the next step, a haplotype analysis of PSEN1 and 2 was performed based on the data of HapMap project, and a distinct haplotype block covering the coding region of PSEN2 was assessed using 4 tagging SNPs resulting in 5 frequent haplotypes. Although the confidence intervals algorithm implemented in the Haploview software proposed three haplotype blocks in the PSEN1 gene, we did not find this selection of blocks persuasive since there are no distinct regions of high LD within the 100 kb of the gene and a large amount of tagging SNPs would have been necessary to capture all the variations. Nevertheless in a recent study investigating the association between PSEN1 polymorphisms and the risk for AD development, the whole gene was treated as a single haplotype block. Seven the most frequent haplotypes that occurred at a minor allele frequency of more than 5% were determined by 8 tagging SNPs. This study has revealed a marginally significant protective effect of the intronic rs165932 G allele (p=0.03) (Belbin, Beaumont et al. 2008). Even though such approach for tagging SNPs selection may cover most of the gene variations, it misses the evidence of existence of LD block.

The APOE gene E4 allele is another marker increasing the individual risk for AD (Corder, Saunders et al. 1994). Several studies show an association of the APOE E4 allele with reduced levels of Aβ42 in CSF in sporadic AD patients (Lasser, Dukoff et al. 1998; Prince, Zetterberg et al. 2004; Davidson, Gibbons et al. 2007). In our sample, we confirmed these findings with lower Aβ42 concentrations in CSF in the homozygous allele E4 carriers in the LOAD group (p=0.003), but not in the EOAD group.

We studied the correlation between PSEN2 haplotypes and Aβ42 concentrations in CSF since low level of Aβ42, but not Aβ40 and high level of tau-protein is used as a biomarker for AD (Skoog, Davidsson et al. 2003) and presenilin-dependent γ-cleavage is the last step in Aβ-production. A correlation between lower Aβ42 concentration in CSF and greater cognitive decline as well as higher abundance of amyloid plaques in the brain has been shown (Fagan, Roe et al. 2007; Gustafson, Skoog et al. 2007; Stomrud, Hansson et al. 2007). A significant difference of mean Aβ42 concentration between early-onset and late-onset groups has also been described before, as well as a certain stability of this marker with no consistent change of the CSF Aβ42 level of patients during 10 month and 20 month indicating that measuring Aβ42 might serve as a kind of biological marker for Alzheimer disease (Andreasen, Hesse et al. 1999). We therefore used Aβ42 for

endophenotyping AD patients, and preselected patients according to a cut-off of Aβ42 < 600 pg/ml.

For haplotype 4 we observed a significant underrepresentation of homozygous carriers in the patient groups which was replicated in the group of autopsy brain samples with an underrepresentation within the group of amyloid plaque positive samples. For all samples with homozygous haplotype 4, genotyping has been replicated using DNA sequencing in order to rule out technical issues of genotyping. We next addressed the question of the functional consequences of this haplotype. To do so, we performed quantification of mRNA and protein levels (semiquantitative Western Blot) of the brain samples with this haplotype and compared it to wildtype brain samples. We did not detect a significant difference in gene expression neither on RNA nor on protein level (data not shown). Further studies are necessary to elucidate whether or not these haplotypes have a functional role in APP processing and/or degradation. As underrepresentation of the homozygous haplotype 4 was detected in the AD group with higher Aβ42 levels, this haplotype might also influence Aβ42 production or secretion. Since AD patients were included only with Aβ42 concentrations < 600 pg/ml, this inclusion criteria might have biased the distribution of genotypes in the patient group. The heterozygous haplotype 4 genotype however, does not seem to be correlated to high Aβ42 concentrations, and there was no underrepresentation in the AD patient group. Apparently, there was no effect of 4/4 haplotype on the Aβ42 concentration in CSF, but this result can not be considered as valuable since among all AD cases there were only 2 patients carrying the 4/4 haplotype.

Within haplotype 4, there is one variant rs1295645 located in the promoter region of PSEN2 gene which is in 100% linkage with rs11405. Analysis of transcription factor binding sites in the promoter region of PSEN2 resulted in a loss of a recognition site for the MZF1 (Myeloid Zink Finger 1) transcription factor (http://molsun1.cbrc.aist.go.jp/research/db/TFSEARCH.html). Thus, haplotype 4 might lead to altered regulation of PSEN2 gene expression due to a loss of binding sites for MZF1 transcription factors.

In conclusion, in this study we assessed the relationship between PSEN2 genetic variability and the CSF Aβ42 concentrations in AD patients compared to controls. We detected three amino acid exchanges in PSEN1 and 2 explaining only few of early onset AD cases. Two of these were new and add to the number of PSEN mutations found so far, accounting for 8% of early-onset AD cases. This is lower than findings from previous research (Lleo, Blesa et al. 2002), reporting a frequency of 20% for APP and PSEN mutations in EOAD.

In addition to rare missense mutations in the early-onset form of AD, which may be transmitted as an autosomal dominant trait, more common polymorphisms may contribute to increased risk for AD. Thus the haplotype analysis of polymorphisms in PSEN2 gene was performed.

Haplotype analysis revealed a distinct haplotype block in PSEN2 which could partly be correlated with Aβ42 concentrations as biological intermediate phenotype of AD. We therefore propose that a "phenotyping" for Aβ-related mechanisms by biomarkers might contribute to identifying novel genetic risk factors for sporadic Alzheimer disease. Although the role of PSEN1 is currently better understood than that of PSEN2, the data on the rare Val150Met mutation in PSEN2 in one EOAD patient, the data pointing to lower susceptibility to AD in homozygous carriers of haplotype 4, and the data on haplotype 1 and 2 correlating with Aβ42 expression underlined an important role of PSEN2 in sporadic AD. Functional in vitro analyses are necessary now to elucidate the role of the PSEN2 haplotypes on APP metabolism.

4.2 Project 2

Replication of pharmacogenetic association studies in independent samples is necessary to estimate the true impact of genetic variants on outcome of drug therapies. We intended to study the effect of polymorphisms in the glucocorticoid receptor regulating cochaperone FKBP5 which have been reported to influence antidepressant dug response. Similar to the study of Binder et al. the patients of our naturalistic study were treated with antidepressant drugs of the doctors' choice. Assessment of antidepressant dug response was done relatively early in our cohort (already after 3 weeks) but a good correlation between early response and late response or remission has been reported (Nierenberg, Farabaugh et al. 2000; Binder, Salyakina et al. 2004; Nierenberg, Quitkin et al. 2004).

The findings of Binder et al., 2004 could partially be replicated with individuals being carriers of the C allele of rs3800373 or the T allele of rs1360780 having a nearly twofold better chance to respond than non-carriers. However, Binder et al. detected a more than 5-fold better response in homozygous carriers of the T allele of the rs1360780 variant whereas the heterozygous carriers did not significantly differ from the non-carrier group (Binder, Salyakina et al. 2004). In our study, the small group of homozygous carriers (only 8%) did not show a better chance to respond but there was a significant better response within the heterozygous carriers group versus the non-carrier group. Binder et al., also report a twice as high number of previously experienced depressive episodes in the homozygous TT carriers of rs1360780. We did not detect any genotype influence on baseline severity of depression or on the number of precedent depressive episodes. Especially there was no higher number of precedent episodes in carriers of the TT genotype of the rs1360780 as observed by Binder et al.

The major limitations of our study are the broad inclusion criteria, especially, the inclusion of patients with mild depression (HAMD > 8) and the liberal definition of the required antidepressant treatment period (only one week). These factors might have contributed to the low effect size. Since the choice of the antidepressant drug was not prescribed by the study protocol, the distribution of the antidepressant medication reflects the current prescription practice of the respective hospital sites in Germany. In our study, effect FKBP5 effect was strongest within the subgroup of patients treated with antidepressant drug combinations or with venlafaxine. This observation might indicate that the effect might be stronger if no selective acting antidepressant is given such as in antidepressant

drug combinations or in dual acting antidepressants but this hypothesis warrants further investigation.

The findings of this study support the role of the FKBP5 variants for antidepressant drug response but the effect in this cohort was much less pronounced than in the original cohort. Meanwhile, at least two other studies failed to detect any influence of the FKBP5 variants on antidepressant drug response (Papiol, Arias et al. 2007; Tsai, Hong et al. 2007). In the study of Tsai et al., interethnic differences might have contributed to the discrepancy: The rs1360780 polymorphism may be in linkage disequilibrium with a functional variant, and the extent of this linkage disequilibrium being not the same for all ethnic populations. The study by Papiol et al., could not replicate the FKBP5 findings but found several associations with other variants within the HPA axis proteins. This was not a naturalistic study but a prospective study on citalopram. The authors also discuss interethnic differences to be responsible for differences in linkage disequilibrium and haplotype structure (Papiol, Arias et al. 2007). Meanwhile, the role of the two SNPs analysed here has also been assessed in the Star*D study (Lekman, Laje et al. 2008), and no correlation with antidepressant drug response has been detected. Since our study is also based on the German population, tagging SNPs within FKBP5 might be the same as in the study population used in the work by Binder et al., and indeed might possess a certain potential to predict response within this specific population. The question remains if these results can be generalized to other populations.

The present analysis is only an exploratory approach to reproduce (or not) the results pointing to FKBP5 being an important gene for antidepressant drug response. The power for detecting these effects is low in our sample (around 40%) but still it is important to report on replication of this effect in independent samples.

In conclusion, we replicated the findings on FKBP5 variants on antidepressant drug response in another German cohort of depressed patients but the effect was much weaker than in the first observation.

4.3 Project 3

Chronic alcohol consumption tends to decrease the amount of serotonin stored in the central nervous system and leads to reduction of brainstem serotonin transporters. Genotypic variation in the serotonin transporter has been shown to influence in vivo availability of central serotonin transporters in alcoholic subjects (Anklam, Gadani et al. 2002). An increased risk of subsequent alcoholism after first alcohol challenge during lifetime (Schuckit, Mazzanti et al. 1999; Hinckers, Laucht et al. 2006), and higher compulsive alcohol craving during acute withdrawal (Bleich, Bonsch et al. 2007) support the thesis of higher vulnerability in l-allele carriers to neurotoxic effects of chronic alcohol consumption.

In our study, higher mRNA levels and lower serum concentrations of the 5-HTT transporter substrate HVA in ll-genotype carriers are consistent with in vitro studies showing higher serotonin transporter expression in human lymphocytes (Lesch, Gross et al. 1995).

Low in-between-subject variability versus high between-subject variation in serum concentrations of L-tryptophan, serotonin and its metabolites indicated a strong genetic background behind the interindividual variation in serotoninergic system (Masellis, Basile et al. 1998). The results on HVA concentrations in serum confirm earlier findings in cerebrospinal fluid and support the role of the peripherally expressed serotonin transporters in the uptake transport of HVA (Kishida, Kawanishi et al. 2003). This illustrates that the 5-HTTLPR may exhibit biological effects not only via its serotonin transport activity but also via transport of other metabolites.

Higher L-tryptophan concentrations in s-allele carriers have been observed in our study in patients after acute withdrawal at both baselines, before depletion and before placebo depletion. In alcoholic patients, an increase in total and free L-tryptophan serum concentrations has been described directly before onset of acute withdrawal (Badawy, Rommelspacher et al. 1998) while low serum tryptophan levels have been reported during acute alcohol withdrawal which returned into normal range on day six after stop drinking (Majumdar, Shaw et al. 1983). At 3 weeks or more post withdrawal, and after decline of acute withdrawal symptoms, higher free L-tryptophan levels in alcoholics than in controls have been reported (Farren and Dinan 1996).

Higher L-tryptophan serum concentrations as observed in s-allele carriers compared to ll-carriers might lead to higher availability of serotonin in the brain during withdrawal and protect them from craving symptoms. Indeed, in our study, scores for depression and craving were found to be significantly higher in carriers of the l/l genotype than in the l/s genotype during the whole experiment which confirms earlier data on craving symptoms at the beginning of alcohol withdrawal in ll carriers (Bleich, Bonsch et al. 2007).

After the tryptophan depletion drink, carriers of the s-allele had a more pronounced decrease in serum L-tryptophan than ll-carriers. Development of a higher depression score in response to tryptophan depletion has been observed in depressed patients who were carriers of the l/l-genotype versus l/s and s/s (Bilous, Dowsett et al. 2003). The underlying mechanism was hypothesized to be the rapid uptake of 5-HT in ll subjects combined with decreased brain 5-HT availability during tryptophan depletion causing a greater decrease in 5-HT neurotransmission. Similar, non-drinking alcoholics with major depression, showed slightly higher BDI scores in l/l subjects than l/s and s/s subjects during tryptophan depletion, but similar scores during the sham session (Pierucci-Lagha, Feinn et al. 2004). In our patient group, none of the patients fulfilled the criteria for major depression or anxiety disorder, but higher scores of depressive and anxiety symptoms in carriers of the l/l genotype support the theory of higher susceptibility to alcohol induced neurotoxicity in these individuals (Anklam, Gadani et al. 2002).

In conclusion, in this cohort of alcoholic patients after acute alcohol withdrawal, a clinically higher baseline craving and depression score in 5-HTTLPR l/l genotype carriers with alcohol dependence was detected and correlated with lower baseline L- tryptophan and lower homovanillic acid concentrations. According to concepts expressed by Kalow et al (Masellis, Basile et al. 1998), the high intraindividual constancy of their serum concentrations indicates a strong genetic background in L-tryptophan and the serotoninergic neurotransmitters which clearly may stimulate further functional genomic research.

5 REFERENCES

(2003). "The International HapMap Project." Nature **426**(6968): 789-96.

Andreasen, N., C. Hesse, et al. (1999). "Cerebrospinal fluid beta-amyloid(1-42) in Alzheimer disease: differences between early- and late-onset Alzheimer disease and stability during the course of disease." Arch Neurol **56**(6): 673-80.

Anklam, E., F. Gadani, et al. (2002). "Analytical methods for detection and determination of genetically modified organisms in agricultural crops and plant- derived food products." European Food Research and Technology **214**(1): 3-26.

Arranz, M., D. Collier, et al. (1995). "Association between clozapine response and allelic variation in 5-HT2A receptor gene." Lancet **346**(8970): 281-2.

Arranz, M. J., T. Li, et al. (1998). "Lack of association between a polymorphism in the promoter region of the dopamine-2 receptor gene and clozapine response." Pharmacogenetics **8**(6): 481-4.

Arranz, M. J., J. Munro, et al. (1998). "Evidence for association between polymorphisms in the promoter and coding regions of the 5-HT2A receptor gene and response to clozapine." Mol Psychiatry **3**(1): 61-6.

Arranz, M. J., J. Munro, et al. (1998). "Meta-analysis of studies on genetic variation in 5-HT2A receptors and clozapine response." Schizophr Res **32**(2): 93-9.

Badawy, A. A., H. Rommelspacher, et al. (1998). "Tryptophan metabolism in alcoholism. Tryptophan but not excitatory amino acid availability to the brain is increased before the appearance of the alcohol-withdrawal syndrome in men." Alcohol Alcohol **33**(6): 616-25.

Baghai, T. C., E. B. Binder, et al. (2006). "Polymorphisms in the angiotensin-converting enzyme gene are associated with unipolar depression, ACE activity and hypercortisolism." Mol Psychiatry **11**(11): 1003-15.

Belbin, O., H. Beaumont, et al. (2008). "PSEN1 polymorphisms alter the rate of cognitive decline in sporadic Alzheimer's disease patients." Neurobiol Aging.

Bijl, M. J., L. E. Visser, et al. (2009). "Genetic variation in the CYP2D6 gene is associated with a lower heart rate and blood pressure in beta-blocker users." Clin Pharmacol Ther **85**(1): 45-50.

Bilous, M., M. Dowsett, et al. (2003). "Current Perspectives on HER2 Testing: A Review of National Testing Guidelines." Mod Pathol **16**(2): 173-82.

Binder, E. B., D. Salyakina, et al. (2004). "Polymorphisms in FKBP5 are associated with increased recurrence of depressive episodes and rapid response to antidepressant treatment." Nat Genet **36**(12): 1319-25.

Bird, T. D., E. Levy-Lahad, et al. (1996). "Wide range in age of onset for chromosome 1--related familial Alzheimer's disease." Ann Neurol **40**(6): 932-6.

Bleich, S., D. Bonsch, et al. (2007). "Association of the long allele of the 5-HTTLPR polymorphism with compulsive craving in alcohol dependence." Alcohol Alcohol **42**(6): 509-12.

Blennow, K., E. Vanmechelen, et al. (2001). "CSF total tau, Abeta42 and phosphorylated tau protein as biomarkers for Alzheimer's disease." Mol Neurobiol **24**(1-3): 87-97.

Brettschneider, J., A. Petzold, et al. (2006). "The neurofilament heavy chain (NfH) in the cerebrospinal fluid diagnosis of Alzheimer's disease." Dement Geriatr Cogn Disord **21**(5-6): 291-5.

Charney, D. S. (1998). "Monoamine dysfunction and the pathophysiology and treatment of depression." J Clin Psychiatry **59 Suppl 14**: 11-4.

Chou, W. H., F. X. Yan, et al. (2000). "Extension of a pilot study: impact from the cytochrome P450 2D6 polymorphism on outcome and costs associated with severe mental illness." J Clin Psychopharmacol **20**(2): 246-51.
Citron, M., D. Westaway, et al. (1997). "Mutant presenilins of Alzheimer's disease increase production of 42-residue amyloid beta-protein in both transfected cells and transgenic mice." Nat Med **3**(1): 67-72.
Collins, F. S., M. S. Guyer, et al. (1997). "Variations on a theme: cataloging human DNA sequence variation." Science **278**(5343): 1580-1.
Corder, E. H., A. M. Saunders, et al. (1994). "Protective effect of apolipoprotein E type 2 allele for late onset Alzheimer disease." Nat Genet **7**(2): 180-4.
Cruts, M., C. M. van Duijn, et al. (1998). "Estimation of the genetic contribution of presenilin-1 and -2 mutations in a population-based study of presenile Alzheimer disease." Hum Mol Genet **7**(1): 43-51.
Daly, A. K. and B. P. King (2003). "Pharmacogenetics of oral anticoagulants." Pharmacogenetics **13**(5): 247-52.
Davidson, Y., L. Gibbons, et al. (2007). "Apolipoprotein E epsilon4 allele frequency and age at onset of Alzheimer's disease." Dement Geriatr Cogn Disord **23**(1): 60-6.
De Strooper, B., P. Saftig, et al. (1998). "Deficiency of presenilin-1 inhibits the normal cleavage of amyloid precursor protein." Nature **391**(6665): 387-90.
Doan, A., G. Thinakaran, et al. (1996). "Protein topology of presenilin 1." Neuron **17**(5): 1023-30.
Duff, K., C. Eckman, et al. (1996). "Increased amyloid-beta42(43) in brains of mice expressing mutant presenilin 1." Nature **383**(6602): 710-3.
Eichelbaum, M. and A. S. Gross (1990). "The genetic polymorphism of debrisoquine/sparteine metabolism--clinical aspects." Pharmacol Ther **46**(3): 377-94.
Evans, A. M., R. L. Nation, et al. (1989). "Lack of effect of cimetidine on the pharmacokinetics of R(-)- and S(+)- ibuprofen." Br J Clin Pharmacol **28**(2): 143-9.
Fagan, A. M., C. M. Roe, et al. (2007). "Cerebrospinal fluid tau/beta-amyloid(42) ratio as a prediction of cognitive decline in nondemented older adults." Arch Neurol **64**(3): 343-9.
Fagan, T., B. Kagan, et al. (2006). "Alzheimer Research Forum Live Discussion: Now you see them, now you don't: The amyloid channel hypothesis." J Alzheimers Dis **9**(2): 219-24.
Farren, C. K. and T. G. Dinan (1996). "Elevated tryptophan levels in post-withdrawal alcoholics." Acta Psychiatr Scand **94**(6): 465-70.
Fux, R., K. Morike, et al. (2005). "Impact of CYP2D6 genotype on adverse effects during treatment with metoprolol: a prospective clinical study." Clin Pharmacol Ther **78**(4): 378-87.
Galasko, D., L. Chang, et al. (1998). "High cerebrospinal fluid tau and low amyloid beta42 levels in the clinical diagnosis of Alzheimer disease and relation to apolipoprotein E genotype." Arch Neurol **55**(7): 937-45.
Gawlik, M., K. Moller-Ehrlich, et al. (2006). "Is FKBP5 a genetic marker of affective psychosis? A case control study and analysis of disease related traits." BMC Psychiatry **6**: 52.
Glasheen, J. J., R. V. Fugit, et al. (2005). "The risk of overanticoagulation with antibiotic use in outpatients on stable warfarin regimens." J Gen Intern Med **20**(7): 653-6.
Goater, A. D. and R. Pethig (1998). "Electrorotation and dielectrophoresis." Parasitology **117 Suppl**: S177-89.

Gustafson, D. R., I. Skoog, et al. (2007). "Cerebrospinal fluid beta-amyloid 1-42 concentration may predict cognitive decline in older women." J Neurol Neurosurg Psychiatry **78**(5): 461-4.
Gutierrez, B., M. J. Arranz, et al. (2002). "Novel mutations in 5-HT3A and 5-HT3B receptor genes not associated with clozapine response." Schizophr Res **58**(1): 93-7.
Hamilton, M. (1959). "The Assessment of Anxiety States by Rating." British Journal of Medical Psychology **32**: 50-55.
Hardy, J. and D. Allsop (1991). "Amyloid deposition as the central event in the aetiology of Alzheimer's disease." Trends Pharmacol Sci **12**(10): 383-8.
Heuther, G., G. Hajak, et al. (1992). "The metabolic fate of infused L-tryptophan in men: possible clinical implications of the accumulation of circulating tryptophan and tryptophan metabolites." Psychopharmacology (Berl) **109**(4): 422-32.
Hinckers, A. S., M. Laucht, et al. (2006). "Low level of response to alcohol as associated with serotonin transporter genotype and high alcohol intake in adolescents." Biol Psychiatry **60**(3): 282-7.
Hirsh, J., J. Dalen, et al. (2001). "Oral anticoagulants: mechanism of action, clinical effectiveness, and optimal therapeutic range." Chest **119**(1 Suppl): 8S-21S.
Hixson, J. E. and D. T. Vernier (1990). "Restriction isotyping of human apolipoprotein E by gene amplification and cleavage with HhaI." J Lipid Res **31**(3): 545-8.
Ho, P. C., D. J. Saville, et al. (2001). "Inhibition of human CYP3A4 activity by grapefruit flavonoids, furanocoumarins and related compounds." J Pharm Pharm Sci **4**(3): 217-27.
Holsboer, F. (2000). "The corticosteroid receptor hypothesis of depression." Neuropsychopharmacology **23**(5): 477-501.
Holsboer, F. and N. Barden (1996). "Antidepressants and hypothalamic-pituitary-adrenocortical regulation." Endocr Rev **17**(2): 187-205.
Hood, S. D., C. J. Bell, et al. (2005). "Acute tryptophan depletion. Part I: rationale and methodology." Aust N Z J Psychiatry **39**(7): 558-64.
Hotopf, M., R. Hardy, et al. (1997). "Discontinuation rates of SSRIs and tricyclic antidepressants: a meta-analysis and investigation of heterogeneity." Br J Psychiatry **170**: 120-7.
Hulstaert, F., K. Blennow, et al. (1999). "Improved discrimination of AD patients using beta-amyloid(1-42) and tau levels in CSF." Neurology **52**(8): 1555-62.
Huskisson, E. C. (1974). "Measurement of pain." Lancet **2**(7889): 1127-31.
Ising, M., S. Horstmann, et al. (2007). "Combined dexamethasone/corticotropin releasing hormone test predicts treatment response in major depression - a potential biomarker?" Biol Psychiatry **62**(1): 47-54.
Iwatsubo, T. (2004). "The gamma-secretase complex: machinery for intramembrane proteolysis." Curr Opin Neurobiol **14**(3): 379-83.
Kaiser, R., P. B. Tremblay, et al. (2002). "Relationship between adverse effects of antipsychotic treatment and dopamine D(2) receptor polymorphisms in patients with schizophrenia." Mol Psychiatry **7**(7): 695-705.
Kane, J. M. and H. L. Freeman (1994). "Towards more effective antipsychotic treatment." Br J Psychiatry Suppl(25): 22-31.
Keck, M. E. and F. Holsboer (2001). "Hyperactivity of CRH neuronal circuits as a target for therapeutic interventions in affective disorders." Peptides **22**(5): 835-44.
Kirchheiner, J. and J. Brockmoller (2005). "Clinical consequences of cytochrome P450 2C9 polymorphisms." Clin Pharmacol Ther **77**(1): 1-16.

Kirchheiner, J., K. Nickchen, et al. (2007). "A 40-basepair VNTR polymorphism in the dopamine transporter (DAT1) gene and the rapid response to antidepressant treatment." Pharmacogenomics J **7**(1): 48-55.
Kishida, I., C. Kawanishi, et al. (2003). "Lack of association in Japanese patients between neuroleptic malignant syndrome and the TaqI A polymorphism of the dopamine D2 receptor gene." Psychiatr Genet **13**(1): 55-7.
Koenen, K. C., G. Saxe, et al. (2005). "Polymorphisms in FKBP5 are associated with peritraumatic dissociation in medically injured children." Mol Psychiatry **10**(12): 1058-9.
Kranzler, H. R. and R. F. Anton (1994). "Implications of recent neuropsychopharmacologic research for understanding the etiology and development of alcoholism." J Consult Clin Psychol **62**(6): 1116-26.
Kruger, B., B. Schroppel, et al. (2002). "A Monocyte chemoattractant protein-1 (MCP-1) polymorphism and outcome after renal transplantation." J Am Soc Nephrol **13**(10): 2585-9.
Lappalainen, J., L. Zhang, et al. (1995). "Identification, expression, and pharmacology of a Cys23-Ser23 substitution in the human 5-HT2c receptor gene (HTR2C)." Genomics **27**(2): 274-9.
Lasser, R. A., R. Dukoff, et al. (1998). "Apolipoprotein E epsilon 4 allele in association with global cognitive performance and CSF markers in Alzheimer's disease." Int J Geriatr Psychiatry **13**(11): 767-74.
Lekman, M., G. Laje, et al. (2008). "The FKBP5-gene in depression and treatment response--an association study in the Sequenced Treatment Alternatives to Relieve Depression (STAR*D) Cohort." Biol Psychiatry **63**(12): 1103-10.
LeMarquand, D., R. O. Pihl, et al. (1994). "Serotonin and alcohol intake, abuse, and dependence: clinical evidence." Biol Psychiatry **36**(5): 326-37.
Lennard, M. S., J. H. Silas, et al. (1982). "Oxidation phenotype--a major determinant of metoprolol metabolism and response." N Engl J Med **307**(25): 1558-60.
Lennard, M. S., J. H. Silas, et al. (1982). "Defective metabolism of metoprolol in poor hydroxylators of debrisoquine." Br J Clin Pharmacol **14**(2): 301-3.
Lenzinger, E., A. Neumeister, et al. (1999). "Behavioral effects of tryptophan depletion in seasonal affective disorder associated with the serotonin transporter gene?" Psychiatry Res **85**(3): 241-6.
Lesch, K. P., D. Bengel, et al. (1996). "Association of anxiety-related traits with a polymorphism in the serotonin transporter gene regulatory region." Science **274**(5292): 1527-31.
Lesch, K. P., J. Gross, et al. (1995). "Primary structure of the serotonin transporter in unipolar depression and bipolar disorder." Biol Psychiatry **37**(4): 215-23.
Leslie, E. M., R. G. Deeley, et al. (2005). "Multidrug resistance proteins: role of P-glycoprotein, MRP1, MRP2, and BCRP (ABCG2) in tissue defense." Toxicol Appl Pharmacol **204**(3): 216-37.
Levine, M. N., G. Raskob, et al. (2001). "Hemorrhagic complications of anticoagulant treatment." Chest **119**(1 Suppl): 108S-121S.
Lewczuk, P., H. Esselmann, et al. (2004). "Neurochemical diagnosis of Alzheimer's dementia by CSF Abeta42, Abeta42/Abeta40 ratio and total tau." Neurobiol Aging **25**(3): 273-81.
Li, T., L. A. Lange, et al. (2006). "Polymorphisms in the VKORC1 gene are strongly associated with warfarin dosage requirements in patients receiving anticoagulation." J Med Genet **43**(9): 740-4.

Licinio, J., F. O'Kirwan, et al. (2004). "Association of a corticotropin-releasing hormone receptor 1 haplotype and antidepressant treatment response in Mexican-Americans." Mol Psychiatry **9**(12): 1075-82.
Lin, C. H., S. J. Tsai, et al. (1999). "No evidence for association of serotonin-2A receptor variant (102T/C) with schizophrenia or clozapine response in a Chinese population." Neuroreport **10**(1): 57-60.
Lleo, A., R. Blesa, et al. (2002). "Frequency of mutations in the presenilin and amyloid precursor protein genes in early-onset Alzheimer disease in Spain." Arch Neurol **59**(11): 1759-63.
Majumdar, S. K., G. K. Shaw, et al. (1983). "Changes in plasma amino acid patterns in chronic alcoholic patients during ethanol withdrawal syndrome: their clinical implications." Med Hypotheses **12**(3): 239-51.
Malhotra, A. K., D. Goldman, et al. (1996). "Clozapine response and the 5HT2C Cys23Ser polymorphism." Neuroreport **7**(13): 2100-2.
Masellis, M., V. Basile, et al. (1998). "Serotonin subtype 2 receptor genes and clinical response to clozapine in schizophrenia patients." Neuropsychopharmacology **19**(2): 123-32.
Masellis, M., V. S. Basile, et al. (2001). "Lack of association between the T-->C 267 serotonin 5-HT6 receptor gene (HTR6) polymorphism and prediction of response to clozapine in schizophrenia." Schizophr Res **47**(1): 49-58.
Mayeux, R., A. M. Saunders, et al. (1998). "Utility of the apolipoprotein E genotype in the diagnosis of Alzheimer's disease. Alzheimer's Disease Centers Consortium on Apolipoprotein E and Alzheimer's Disease." N Engl J Med **338**(8): 506-11.
McKhann, G., D. Drachman, et al. (1984). "Clinical diagnosis of Alzheimer's disease: report of the NINCDS-ADRDA Work Group under the auspices of Department of Health and Human Services Task Force on Alzheimer's Disease." Neurology **34**(7): 939-44.
Monastero, R., E. Mariani, et al. (2006). "Association between apolipoprotein E epsilon4 allele and apathy in probable Alzheimer's disease." Acta Psychiatr Scand **113**(1): 59-63.
Montgomery, S. A. and M. Asberg (1979). "A new depression scale designed to be sensitive to change." Br J Psychiatry **134**: 382-9.
Moonis, M., J. M. Swearer, et al. (2005). "Familial Alzheimer disease: decreases in CSF Abeta42 levels precede cognitive decline." Neurology **65**(2): 323-5.
Nakama, H., L. Chang, et al. (2008). "Association between psychiatric symptoms and craving in methamphetamine users." Am J Addict **17**(5): 441-6.
Neumeister, A., X. Z. Hu, et al. (2006). "Differential effects of 5-HTTLPR genotypes on the behavioral and neural responses to tryptophan depletion in patients with major depression and controls." Arch Gen Psychiatry **63**(9): 978-86.
Neumeister, A., A. Konstantinidis, et al. (2002). "Association between serotonin transporter gene promoter polymorphism (5HTTLPR) and behavioral responses to tryptophan depletion in healthy women with and without family history of depression." Arch Gen Psychiatry **59**(7): 613-20.
Nierenberg, A. A., A. H. Farabaugh, et al. (2000). "Timing of onset of antidepressant response with fluoxetine treatment." Am J Psychiatry **157**(9): 1423-8.
Nierenberg, A. A., F. M. Quitkin, et al. (2004). "Placebo-controlled continuation treatment with mirtazapine: acute pattern of response predicts relapse." Neuropsychopharmacology **29**(5): 1012-8.
Ozdemir, V., W. Kalow, et al. (2001). "Treatment-resistance to clozapine in association with ultrarapid CYP1A2 activity and the C-->A polymorphism in intron 1 of the

CYP1A2 gene: effect of grapefruit juice and low-dose fluvoxamine." J Clin Psychopharmacol **21**(6): 603-7.
Palacino, J. J., B. E. Berechid, et al. (2000). "Regulation of amyloid precursor protein processing by presenilin 1 (PS1) and PS2 in PS1 knockout cells." J Biol Chem **275**(1): 215-22.
Papiol, S., B. Arias, et al. (2007). "Genetic variability at HPA axis in major depression and clinical response to antidepressant treatment." J Affect Disord **104**(1-3): 83-90.
Pariante, C. M. and A. H. Miller (2001). "Glucocorticoid receptors in major depression: relevance to pathophysiology and treatment." Biol Psychiatry **49**(5): 391-404.
Pierucci-Lagha, A., R. Feinn, et al. (2004). "Effects of rapid tryptophan depletion on mood and urge to drink in patients with co-morbid major depression and alcohol dependence." Psychopharmacology (Berl) **171**(3): 340-8.
Pirmohamed, M. and B. K. Park (2001). "Genetic susceptibility to adverse drug reactions." Trends Pharmacol Sci **22**(6): 298-305.
Podlisny, M. B., M. Citron, et al. (1997). "Presenilin proteins undergo heterogeneous endoproteolysis between Thr291 and Ala299 and occur as stable N- and C-terminal fragments in normal and Alzheimer brain tissue." Neurobiol Dis **3**(4): 325-37.
Pratt, W. B. and D. O. Toft (1997). "Steroid receptor interactions with heat shock protein and immunophilin chaperones." Endocr Rev **18**(3): 306-60.
Prince, J. A., H. Zetterberg, et al. (2004). "APOE epsilon4 allele is associated with reduced cerebrospinal fluid levels of Abeta42." Neurology **62**(11): 2116-8.
Ratovitski, T., H. H. Slunt, et al. (1997). "Endoproteolytic processing and stabilization of wild-type and mutant presenilin." J Biol Chem **272**(39): 24536-41.
Schafer, M., D. Rujescu, et al. (2001). "Association of short-term response to haloperidol treatment with a polymorphism in the dopamine D(2) receptor gene." Am J Psychiatry **158**(5): 802-4.
Schalekamp, T., B. P. Brasse, et al. (2007). "VKORC1 and CYP2C9 genotypes and phenprocoumon anticoagulation status: interaction between both genotypes affects dose requirement." Clin Pharmacol Ther **81**(2): 185-93.
Schellenberg, G. D. (1995). "Genetic dissection of Alzheimer disease, a heterogeneous disorder." Proc Natl Acad Sci U S A **92**(19): 8552-9.
Schonheit, B., R. Zarski, et al. (2004). "Spatial and temporal relationships between plaques and tangles in Alzheimer-pathology." Neurobiol Aging **25**(6): 697-711.
Schuckit, M. A., C. Mazzanti, et al. (1999). "Selective genotyping for the role of 5-HT2A, 5-HT2C, and GABA alpha 6 receptors and the serotonin transporter in the level of response to alcohol: a pilot study." Biol Psychiatry **45**(5): 647-51.
Selkoe, D. J. (1994). "Cell biology of the amyloid beta-protein precursor and the mechanism of Alzheimer's disease." Annu Rev Cell Biol **10**: 373-403.
Sherrington, R., E. I. Rogaev, et al. (1995). "Cloning of a gene bearing missense mutations in early-onset familial Alzheimer's disease." Nature **375**(6534): 754-60.
Sjogren, M., M. Gisslen, et al. (2001). "Low cerebrospinal fluid beta-amyloid 42 in patients with acute bacterial meningitis and normalization after treatment." Neurosci Lett **314**(1-2): 33-6.
Skoog, I., P. Davidsson, et al. (2003). "Cerebrospinal fluid beta-amyloid 42 is reduced before the onset of sporadic dementia: a population-based study in 85-year-olds." Dement Geriatr Cogn Disord **15**(3): 169-76.
Sleeper, R., C. A. Bond, et al. (2000). "Psychotropic drugs and falls: new evidence pertaining to serotonin reuptake inhibitors." Pharmacotherapy **20**(3): 308-17.
Spielberger, C. (1970). "Manual for the state-trait anxiety inventory. ." Consulting Psychologists Press **Palo Alto**.

St George-Hyslop, P. H. (2000). "Genetic factors in the genesis of Alzheimer's disease." Ann N Y Acad Sci **924**: 1-7.
Steinhagen-Thiessen, E. and M. Borchelt (1999). Morbidität, Medikation und Funktionalität im Alter. Die Berliner Altersstudie. K. U. Mayer and P. B. Baltes. Berlin, Berlin-Brandenburgische Akademie der Wissenschaften**:** 151-183.
Stomrud, E., O. Hansson, et al. (2007). "Cerebrospinal fluid biomarkers predict decline in subjective cognitive function over 3 years in healthy elderly." Dement Geriatr Cogn Disord **24**(2): 118-24.
Suzuki, A., T. Kondo, et al. (2001). "The -141C Ins/Del polymorphism in the dopamine D2 receptor gene promoter region is associated with anxiolytic and antidepressive effects during treatment with dopamine antagonists in schizophrenic patients." Pharmacogenetics **11**(6): 545-50.
Thal, D. R., E. Capetillo-Zarate, et al. (2006). "The development of amyloid beta protein deposits in the aged brain." Sci Aging Knowledge Environ **2006**(6): re1.
Thal, D. R., E. Ghebremedhin, et al. (2002). "Two types of sporadic cerebral amyloid angiopathy." J Neuropathol Exp Neurol **61**(3): 282-93.
Thal, D. R., U. Rub, et al. (2002). "Phases of A beta-deposition in the human brain and its relevance for the development of AD." Neurology **58**(12): 1791-800.
Thomas, P. and M. Fenech (2007). "A review of genome mutation and Alzheimer's disease." Mutagenesis **22**(1): 15-33.
Tomita, T., K. Maruyama, et al. (1997). "The presenilin 2 mutation (N141I) linked to familial Alzheimer disease (Volga German families) increases the secretion of amyloid beta protein ending at the 42nd (or 43rd) residue." Proc Natl Acad Sci U S A **94**(5): 2025-30.
Tsai, S. J., C. J. Hong, et al. (2007). "Lack of supporting evidence for a genetic association of the FKBP5 polymorphism and response to antidepressant treatment." Am J Med Genet B Neuropsychiatr Genet **144B**(8): 1097-1098.
van Rossum, E. F., E. B. Binder, et al. (2006). "Polymorphisms of the glucocorticoid receptor gene and major depression." Biol Psychiatry **59**(8): 681-8.
Wakutani, Y., K. Watanabe, et al. (2004). "Novel amyloid precursor protein gene missense mutation (D678N) in probable familial Alzheimer's disease." J Neurol Neurosurg Psychiatry **75**(7): 1039-42.
Weinshilboum, R. (2003). "Inheritance and drug response." N Engl J Med **348**: 529-537.
Wenham, P. R., W. H. Price, et al. (1991). "Apolipoprotein E genotyping by one-stage PCR." Lancet **337**(8750): 1158-9.
Wilkinson, G. R. (2005). "Drug metabolism and variability among patients in drug response." N Engl J Med **352**(21): 2211-21.
Wiltfang, J., H. Esselmann, et al. (2003). "Beta-amyloid peptides in cerebrospinal fluid of patients with Creutzfeldt-Jakob disease." Ann Neurol **54**(2): 263-7.
Wiltfang, J., P. Lewczuk, et al. (2005). "Consensus paper of the WFSBP Task Force on Biological Markers of Dementia: the role of CSF and blood analysis in the early and differential diagnosis of dementia." World J Biol Psychiatry **6**(2): 69-84.
Wolfe, M. S., W. Xia, et al. (1999). "Two transmembrane aspartates in presenilin-1 required for presenilin endoproteolysis and gamma-secretase activity." Nature **398**(6727): 513-7.
Wuttke, H., T. Rau, et al. (2002). "Increased frequency of cytochrome P450 2D6 poor metabolizers among patients with metoprolol-associated adverse effects." Clin Pharmacol Ther **72**(4): 429-37.

Young, S. N. and M. Leyton (2002). "The role of serotonin in human mood and social interaction. Insight from altered tryptophan levels." Pharmacol Biochem Behav **71**(4): 857-65.
Young, S. N., S. E. Smith, et al. (1985). "Tryptophan depletion causes a rapid lowering of mood in normal males." Psychopharmacology (Berl) **87**(2): 173-7.

6 CURRICULUM VITAE

PERSONAL DATA

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July, 2006- 2009	Ulm University, Germany PhD
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July, 2006- present	PhD student Department of Pharmacology of Natural Products and Clinical Pharmacology Ulm University, Germany
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7 PUBLICATIONS

ORIGINAL PUBLICATIONS

- Kirchheiner J, Herchenhein T, **Lebedeva E**, Wedekind D, Seeringer A, Pilz J, Engel K, Brockmöller J, Falkai P, Havemann-Reinecke U. Serum monoamines during standardized modulation of tryptophan intake in relation to the serotonin transporter polymorphism in alcoholic patients. Journal of Clinical Psychopharmacology, 2009 (submitted).
- **Lebedeva E**, Kirchheiner J, Thal DR, Ghebremedhin E, Strauss J, Özer E, von Einem B, Tumani H, Otto M, Riepe MW, Ludolph AC, von Arnim CAF. Presenilin gene variants and correlation to cerebrospinal β-amyloid 42 in Alzheimer disease, 2009 (in preparation).
- Kirchheiner J, Lorch R, **Lebedeva E**, Seeringer A, Roots I, Sasse J, Brockmöller J. Genetic variants in FKBP5 affecting response to antidepressant drug treatment. Pharmacogenomics, 2008, 9(7):841-6.
- Kirchheiner J, Meineke I, Fuhr U, Rodriguez-Antona C, **Lebedeva E**, Brockmoeller J. Impact of genetic polymorphisms in cytochrome P450 enzymes and rosiglitazone intake on the formation of epoxyeicosatrienic acids. Pharmacogenomics, 2008, 9(3):277-88.
- Vasileva SV, **Krasnousova (Lebedeva) EE**, Donina AA, Abramova TV, Zhdanova LG, Kovalenko SP, and Silnikov VN. Synthesis of fluorescein-labeled oligonucleotides bearing a tag in position 2´ of modified adenosine and arabinoadenosine. Russian Chemical Bulletin, 2006, 9:1677-1683.

CONFERENCES ATTENDED

- XXVI CINP Congress (poster presentation). 2008. Munich, Germany
- 3rd International Russell Ross Symposium. 2007. Ulm University, Germany

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Erklärung

Hiermit erkläre ich, dass ich die beigefügte Dissertation selbständig verfasst und keine anderen als die angegebenen Hilfsmittel genutzt habe. Alle wörtlich oder inhaltlich übernommenen Stellen habe ich als solche gekennzeichnet.

Ich versichere außerdem, dass ich die beigefügte Dissertation nur in diesem und keinem anderen Promotionsverfahren eingereicht habe und, dass diesem Promotionsverfahren keine endgültig gescheiterten Promotionsverfahren vorausgegangen sind.

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